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Effects of Embryonic Arsenic Exposure on Killifish (Fundulus heteroclitus) Growth, Feeding Behavior, and Intestinal Morphology and Cell Types

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EFFECTS OF EMBRYONIC ARSENIC EXPOSURE ON KILLIFISH *(FUNDULUS HETEROCLITUS)* GROWTH, FEEDING BEHAVIOR, AND INTESTINAL MORPHOLOGY AND CELL TYPES.

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ABSTRACT

Arsenic is found as a contaminant of drinking water, rice, and other crops. Epidemiological studies have shown that embryonic exposure to arsenic can cause changes in behavior and reductions in growth, but the mechanisms for these effects are not well understood. So, we were interested in examining potential mechanisms by which arsenic could be affecting growth. Additionally, while many studies have looked at higher levels of arsenic exposure, we wanted to focus on environmentally-relevant levels to see if these concentrations could have lasting consequences on growth, even after the exposure had ended.

Killifish (*Fundulus heteroclitus*) were used as the model organism for this investigation for two reasons. First, they produce a large number of eggs, which can increase statistical power when observing affects over multiple time points. Second, earlier studies have shown effects on developmental processes at arsenic levels similar to human exposures. In rodent or zebrafish models, investigators typically need to use arsenic concentrations that are 100X higher to see similar effects. Thus, killifish were exposed to 0, 10, 50, and 200ppb arsenic (as sodium arsenite) as embryos, and after hatching were reared in clean water until adulthood at 28 weeks. The study was designed to represent a full embryonic/fetal arsenic exposure *in utero*, and then to examine whether effects persisted, worsened, or resolved into early adulthood. We found that growth, assessed by condition factor (weight/length^3), was significantly reduced by 24% in the 200 ppb embryonic exposure groups at 8 weeks, with a dose dependent decrease in the 10 and 50 ppb groups. These trends persisted up to 28 weeks, although variability was much

higher. As we had seen similar reductions in growth in a previous embryonic arsenic exposure study that used higher arsenic concentrations, we therefore investigated three potential mechanisms responsible for the growth reduction.

First, we analyzed feeding behavior, as it has been found to correlate to amount of nutrient intake. Embryonic arsenic exposure did indeed reduce the percentage of fish initially responding to food and increased the amount of time it took for the fish to start their response, particularly at the 28 week time period. So, one possibility is that arsenic reduces activity or alters olfaction, thus reducing their response to food. The second mechanism examined was whether embryonic arsenic exposure altered the morphology of the intestine, or altered several specific cell types needed for nutrient uptake. There was a slight, but statistically significant reduction in intestinal villus height at 16 weeks, this change did not persist. Intestinal enterocytes and Goblet cell number, as measured by immunohistochemistry, did not change with arsenic concentration or time. However, the number of PCNA-positive intestinal cells, indicating cell proliferation, was reduced in a dose-response manner at all sampling time points. This may indicate that embryonic arsenic exposure permanently altered the ability of intestinal stem cells to proliferate.

The third possibility we examined was whether embryonic arsenic exposure altered the expression of skeletal and hepatic insulin like growth factor (IGF-1), its receptor (IGFR-1) on skeletal muscle cells, and its associated binding proteins (IGFBP-1 & -5) in the muscle and liver. We hypothesized that changes in their levels might alter growth and muscle body weight, since epidemiological studies have found an inverse relationship between arsenic and IGF-1 in plasma levels, which correlate to reductions in

birth weight. Reductions of hepatic IGF1 and IGFBP-1 are highly correlated with condition factor reductions in the 8 week old fish. However, by 28 weeks, hepatic IGF-1 and IGFBP-1 still remain tightly correlated, but are actually increased in a statistically significant, dose-response manner. This might be a compensatory response to potentially making up for any growth deficits seen in earlier stages.

Overall, the results from this study show that embryonic-only arsenic exposure can alter growth factor expression, such as hepatic IGF-1, which correlates with a reduction in condition factor during an essential growth period such as the juvenile stage. As the fish reach sexual maturity, it appears that by increasing levels of IGF-1 and restoring a consistent intestinal environment, they are able to compensate for early growth deficits after embryonic exposure to lower levels of arsenic.

DEDICATION

This thesis is dedicated to my wonderful and supportive parents my dad, Phil Sims, for putting the light of scientific discovery and a thirst for knowledge in my heart as a scientist himself and to my mother, Kathy Brown, for always lending an ear or shoulder to cry on when it felt like I couldn't make it. In addition I want to dedicate this to my late beautiful cousin Amanda Ashworth for teaching me how to smile and keep my chin up in rough times, you are dearly missed. I want to also dedicate this to my undergraduate professors at the University of North Georgia for believing in me and guiding me towards the path of science.

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CHAPTER ONE

LITERATURE REVIEW

Purpose of the study

In this study, our main objective is to determine whether arsenic exposure (10, 50, and 200ppb) during early life stages reduces growth and alters feeding behavior long after the exposure has ended, using killifish (*Fundulus heteroclitus*) as a model organism.

Arsenic species and occurrence in the environment

Arsenic (As) is a naturally occurring element found all over the world that has known toxic effects on animals[1]. The degree of toxicity can depend on the As species, since studies have shown that inorganic iAs (arsenite As^{III} & arsenate As^{V}) as well as GSH-mediated methyl organic metabolites (methylarsonate and dimethlyarsinate)[2] can be toxic [3,4]. Arsenite and arsenate are most commonly found in aqueous environments due to the weathering of rocks with arsenic sulfides, mining activity, combustion of fossil fuels, which then interconvert from As^{III} & As^{V} in an aqueous environment depending on pH and redox conditions[3,5]. Since arsenite predominates in reducing anaerobic environments, it is the form commonly found in groundwater[6]. Other organic arsenic species include arsenobetanine, arsenosugars, and arsenocholine. Exposures to them are typically through the diet, as they are commonly found in fish, shellfish, and poultry growth feed additives (Rocxarsone)[4,7,8]. These forms have been found to be generally nontoxic[2]. In addition, arsenic can form mono-, di-, and trimethylated metabolites. The different oxidation states and chemical structure of arsenic species can correlate to its

cytotoxicity[9]. For example, one study found that some human cells (hepatocytes, keratinocytes, and bronchial epithelial cells) are more sensitive to cytotoxic effects of MMA^{III}[10] compared to inorganic As^{III} [11]. Another study found that DNA damage in human hepatocytes and urotheial cells is induced primarily induced by MMA^{III} and DMA^{III} [12].

Geographic arsenic water exposure

Throughout history and in the present day, arsenic has been used for industrial and agricultural purposes such as the manufacturing of car batteries, alloyed semiconductor materials, and as a pesticide[13], as well as being, used as an effective drug for treatment of acute promyelocytic leukemia (APL) [9]. However, the major source of human exposure to arsenic is through drinking water. In the past 10 years, China alone has experienced at least 4 major arsenic water contamination accidents through ill-managed industrial waste discharge in the following water sources: 620 µg/L in Xinqiang River, 570 µg/L in Duliu Creek, 180 µg/L in Yangzonghai Lake and 530 µg/L in Dashahe River[3]. While the U.S. EPA drinking water standard is 10ppb (10µg/L), higher levels can be found in private wells, which are often unregulated. These higher levels of arsenic raise concern for roughly 13 million U.S households[14]. It was estimated that 42.7 % of aquifers in the southwestern part of the U.S had greater than 10µg/L[15]. Other parts of the U.S also have elevated arsenic levels. For example, 8% of wells measured in Pennsylvania had concentrations > 10 µg/L [16], New England found that 30% of wells had > 10 µg/L [17], North Carolina found around 1,436 wells out of 63,000 with

levels higher than 10µg/L (maximum was 806µg/L) [15] and well water in Arizona can be up to 61µg/L[18]. Additionally, arsenic pollution of water has been reported in epidemiological studies in other countries including Chile (up to 110 µg/L As) [19], Bangladesh (0.1-864 µg/L As) [20], Taiwan (<0.1-347.43 µg/L As) [21], and Hungary (0-50< µg/L As)[22] to list a few.

Dietary arsenic exposure

Not only are people exposed through their water supply, but arsenic has been found in dietary sources. According to the European Food Safety Authority, the main competent to the dietary exposure to iAs comes from grain-based processed products such as wheat bread and rolls in adults, while infant and toddler exposures were from rice, milk, and dairy products[23]. The mean dietary exposure in infants and toddlers ranged from 0.20-2.09 µg/kg body weight/day, while adults ranged from 0.09-0.64 µg/kg body weight/day[21]. In fish and seafood, there are usually greater amount of arsenobetaine and arsenocholine, both of which have a lower potential for toxicity to humans[23]. Arsenic is easily taken up into major food sources such as rice, which has been shown to accumulate inorganic arsenic at a 10-fold high rate than other grains [24]. One study examining arsenic concentrations in various types of rice found levels of 200 µg /kg in brown rice, 130 µg /kg in white rice, and 70 µg /kg in other color rice [25,26]. Another study conducted a product test investigating arsenic content in 9 different rice-based infant snacks found ranges from 36.5-568 ng/g total As[27].

A report in the U.S looking at dietary consumption patterns found that in some infant formulas, infants could be exposed on average to 9.4-14 µg/day iAs when consuming formulas using brown rice syrup as a healthy alternative to sugar. This study also found adults consuming rice could be exposed to an average to 11 µg/day iAs[28]. Based on epidemiology studies, arsenic exposure between 0.3 and 8 µg/kg body weight/day was estimated to result in a 1% increased risk of lung skin and bladder tumors in humans[23]. A recent European study looking at the need for arsenic risk reduction deduced that based on a daily consumption of 2L of water a day at 10 µg/L arsenic by a person weighing 70 kg equals 0.3µg/kg body weight/day which is in the 1% benchmark does range for carcinogenesis[23]. Additionally, the U.S. Agency for Toxic Substances and Disease Registry's (ATSDR) Minimal Risk Levels (MRLs) for acute oral consumption at 5µg/kg/day and chronic oral consumption at 0.3µg /kg/day arsenic as safe doses for infants[28]. In order to reduce the risk of cancer later in life for infants' minimizing and regulating the amount of arsenic exposure through diet and water is essential.

Naturally-occurring and anthropogenically-introduced arsenic sources continue to raise the need for further investigation not only for arsenic remediation efforts but also the mechanisms behind its toxic effects on animals. Furthermore, since studies have shown that arsenic exposure in animals can contribute to cancer, behavior and cognitive dysfunction, as well as impaired growth and development, it is extremely important to investigate arsenic's effects at early life stages[29]. It is also essential to understand the

metabolism of arsenic and what species could potentially increase harmful developmental effects.

Arsenic biotransformation in animals

In animals, arsenic (iAs^{V} and iAs^{III}) is absorbed from the intestine and is typically metabolized through a specific biotransformation pathway usually occurring in the liver. It can enter a cell via aquaporins and is later exported, in part, through membrane bound efflux transporters (MRPs) [5]. The metabolism of iAs happens through a series of oxidative methylation and reduction reactions that promotes As excretion in urine[5]. For example, inorganic arsenic As^{III} goes through oxidative methylation along with conjugation of GSH to convert it into monomethyl arsenic or MMA^{v}. MMA^{v} is reduced to MMA^{III} which undergoes oxidative methylation to form dimethylated arsenic or DMA^{v} by an S-adenosylmethionine -dependent enzyme termed arsenic methyltransferase (As3MT) [4,30,31]. The reduction of pentavalent As to trivalent As is usually mediated by an arsenate reductase enzyme with the addition of a methyl group[5]. Figure A. shows a general arsenic biotransformation pathway.

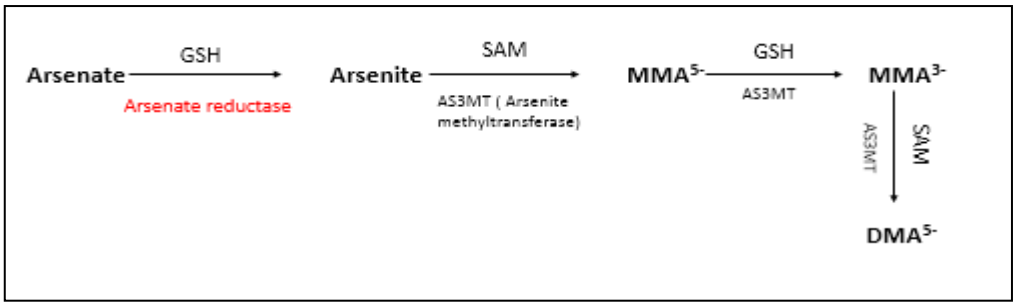

Figure A. General arsenic biotransformation pathway. Modified from Casarett and Doull's Toxicology 8th edition.

All forms of arsenic - As^{v}, As^{III}, MMA, and DMA can be detected in urine, with DMA being the most commonly detected species[5,32]. These methylation pathways have been seen in aquatic animals such as fish with metabolism usually occurring in the gills and liver, but they have a greater tendency to accumulate arsenobetanine and arsenocholine which are thought to be less toxic forms[5]. While methylation of iAs is an attempt at detoxification, it can also be a bioactivating pathway that leads to greater toxicity as studies have found that the intermediate trivalent methylated arsenicals (MMA^{III} and DMA^{III}) can be more effective inhibitors of enzymes, and cause more cytotoxicity and genotoxicity than iAs[5,33].

Growth and intestinal development in mammals

As previously mentioned, arsenic and its metabolites can impair growth and development. In order to understand these effects, it is first important to understand normal animal development. In mammals after sperm entry into the egg, the first cleavage begins about a day later and the two nuclei produced by this cleavage are the first to contain the entire genome. During cleavage events, the zygotic genome is activated at different times depending on the species; human zygotic genes, genes transcribed by the zygote's DNA, are activated at the 8- cell stage, whereas mouse and goat zygotic genes can be activated in late zygote stage and continue through 2-cell stage. In order for zygotic genes to be activated, parental chromatin undergoes changes and gamete-specific methyl groups on DNA are removed unless imprinted, and new DNA methylation patterns are established. By the 8-cell stage, the genome of each cell is

hypomethylated and appear to be pluripotent which sets the stage for cell differentiation to occur[34].

The blastocyst contains trophoectoderm cells which will give rise to extraembryonic tissues like the chorion around day 10-12 in humans, and an inner cell mass with pluripotent cells which will give rise to the embryo. The inner cell mass then delaminates into the epiblast, which becomes the three germ cell layers during gastrulation. Around day 12-15, the primitive streak is formed, which goes on to form embryonic mesoderm (giving rise to muscles, skeleton, connective tissue, reproductive organs, kidneys, and circulatory structures) and endoderm (gives rise to the gastrointestinal tract, lining of respiratory tract, and organs like the liver and pancreas) while the embryonic epiblast forms the embryonic ectoderm (gives rise to the nervous system)[34]. After the completion of gastrulation, organogenesis begins around 5 weeks in humans.

As previous studies in our lab have noticed a reduction in growth of killifish exposed to arsenic as embryos, we wanted to determine if arsenic might have an effect on intestinal development, potentially leading to a reduction in nutrient uptake and growth. The endodermal function in the embryo is to construct the linings of the digestive tube, which is signaled by the *Sox17* transcription factor that is necessary for gut endoderm morphogenesis[35]. The digestive tube constricts to form the esophagus followed by the stomach, small intestine, and large intestine, and then puts out branches that become the

thyroid, thymus, pancreas, and liver[36]. Gut tissue forms by reciprocal interactions between endoderm and mesoderm. Signals playing a role in specification of the gut tube are typically in gradients and Wnt is thought to be important in this process[36]. Wnt signals, which are instructed by RA (retinoic acid) and FGF gradients from posterior mesoderm, induce the posteriorizing transcription factors Cdx1, Cdx2 as well as paracrine factor Indian hedgehog that play a role in intestine formation. At high concentrations, Cdx1 and 2 induce the formation of the large intestine, and at low concentrations, they induce the formation of the small intestine[36]. In the anterior region of the gut tube, which forms thymus, pancreas, stomach, and liver, Wnt signaling is blocked. In the stomach forming area, the gut tube mesenchyme lining expresses transcription factor Barx1 which turns on Frzb-like Wnt-blocking proteins. In a study observing intestinal development in mice showed that villi form around embryonic day 15 and the crypts, where stem cells reside, were set up shortly after birth due to the expression of Hedgehog. Growth of the villus is promoted by BMP2 and 4[37]. The morphogenesis of villi and crypts occur by folding of the endodermal epithelium and depends on the decreased expression of ephrin B1 in villi and increased ephrin B2 and 3 in the crypts where β-catenin plays a role in repression and up regulation of these proteins, respectively[37]. Additional studies on mice have found that cells expressing adult intestinal stem cell markers such as Lgr5, Ascl2, and Olfm4 appear around embryonic day 15 and reside in the inter-villus region, suggesting that Wnt-dependent progenitors of adult intestinal stem cells are established prior to birth[38]. In this study, they found that Lgr5 progenies give rise to all differentiated intestinal epithelial cell types including;

enterocytes, enteroendocrine, goblet, and Paneth cells[38]. The enterocytes are the most abundant intestinal epithelial cells, making up to about 80% of all epithelia cells. They function in increasing the absorptive surface, and have hydrolytic and absorptive functions to take up and degrade nutrients[39]. In mice, the turnover rate for enterocytes is estimated to be around 3 days. Goblet cells represent about 5% of the epithelial cells and contain mucus that constitutes a barrier against the intestinal contents. Turnover for goblet cells is around 3 days as well. Enteroendocrine cells make up a much smaller percentage of the intestinal epithelial cells and they produce hormones that help in regulating gastrointestinal motility. Paneth cells function mostly in antimicrobial defense of the intestine, roughly 10 Paneth cells are present per crypt, and they and have a slower turnover rate of about 20 days[39].

The rapid cell division in crypts is dependent upon Wnt/β-catenin signaling[37]. The stem cells divide rapidly into progenitor cells in the intestine then take on their fate based on signals from genes such as Math1 (aka Atoh1)[40], which promotes the fate of secretory (goblet and endocrine) cells along with a decrease in Notch[41]. Whereas the Notch ligand Delta turns on transcription factors such as Hes-1 to specify enterocyte fate[41]. Below is a general diagram of these signals and the fate of the stem cell in Figure B.

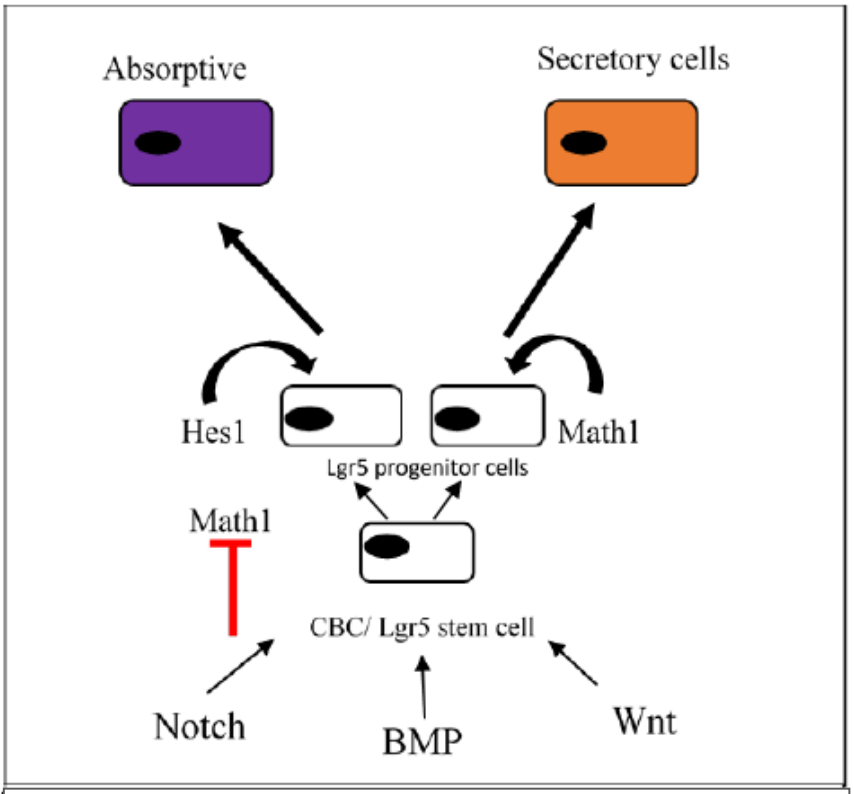

Figure B. Signals involved in the intestinal crypt based columnar cell (CBC) and Lgr5 marked stem cell fate. Modified image from (Boaz et al., 2014)

As embryonic development is complete and the fetus is growing, *in utero* signaling pathways such as insulin-like growth factor (IGF-1) is extremely important for growth. In humans serum IGF-1 concentration from 15-37 weeks of gestational age there is a positive correlation with IGF-1 concentration and fetal weight and bone length[42]. Similarly, umbilical cord IGF-1 levels reflect fetal IGF-1 levels at birth and decreased IGF-1 levels correlate with a decrease birth weight in humans. There has also been evidence that IGF-1 is detectable in fetal tissue in the first trimester suggesting IGF-1 might play a role in early development[42]. Similar to mammals, IGF-1 signaling plays a

role in juvenile fish development, providing evidence of its significance for normal growth[43]. A general diagram of IGF-1 signaling pathway located in Figure C.

Growth and development in fish

My work uses killifish *(Fundulus heteroclitus*) as model organisms. Fish eggs are telolecithal meaning the majority of the egg cell cytoplasm is occupied by yolk[44]. Cleavage can only occur in a thin region of the yolk free cytoplasm called the blastodisc. The first cell divisions after fertilization have a reproducible pattern of meridional and equatorial cleavage. Fish embryos undergo mid-blastula transition, which is when zygotic gene transcription begins around the 10^{th} cell division. At this time three distinct cell populations develop: the yolk syncytial layer (role in directing cell movements of gastrulation), the enveloping layer which is a protective covering that allows the embryo to develop in hypotonic solutions, and the deep cells which give rise to the embryo proper[44].

During gastrulation, all three layers undergo epiboly, the movement of epithelial layers that spread to enclose the deeper layers of the embryo, and progress through it until the entire yolk cell is covered by the blastoderm[44]. Once the blastoderm has covered about half the yolk cell, a thickening occurs referred to as the germ ring which is made of the epiblast (later to be the ectoderm) and the hypoblast (later to be the endoderm and mesoderm). The endoderm expresses *casanova (Sox 32)* and *spiel-ohne-grenzen (Oct 4)* genes controlled by nodal which will become intestine, liver, and pharynx. Mesoderm expresses genes like *notail (brachyury)* and *spadetail (Tbx6 and VegT)* which will

become heart, muscle, head, blood, and fins[45]. Ectoderm expresses *BMPs* and certain *Wnt* proteins which will become epidermis, brain, nose, eye, and spinal cord. Once organogenesis is complete and the fish continues to grow to adulthood, signaling pathways such as IGF play a role in growth by promoting proliferation and differentiation of growing tissue, similar to fetal growth in humans. As shown in Figure 2, IGF binds to specific receptors on the cell surface of growing tissue to mediate proliferation. It can also bind to IGF binding proteins (IGFBPs) that have various functions, depending on the tissue type and the health status of the organism[46]. The binding proteins can aid in the control of IGF distribution between extracellular environments and cell surface binding sites, and may also regulate IGF bioactivity by modulating its interaction with the receptor[46]. For example, like growth factor binding protein-1 (IGFBP-1) is found predominantly in the liver and inhibits the bioavailability of IGF typically under stressful conditions[47]. In one study in zebrafish, they found that under times of starvation, IGFBP-1 was significantly increased[47]. In contrast, IGFBP-5 located primarily in the muscle is considered pro-myogenic and increases the bioavailability of IGF during myoblast differentiation playing an important role in fish muscle growth[48]. Additionally, other factors related to the gastrointestinal systems play a role in fish growth such as peptide transporters located on enterocytes and part of the brush border membrane. They play a major role in animal

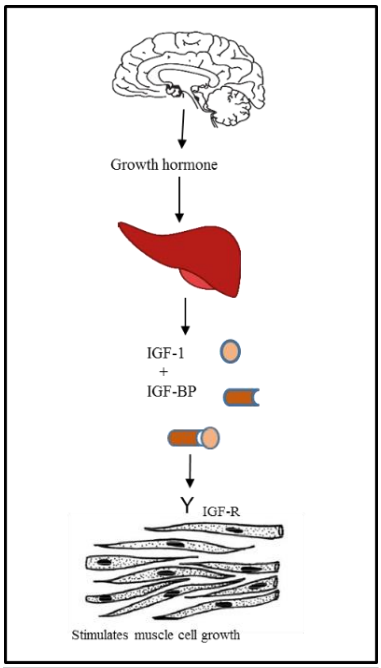

Figure C. IGF-1 Signaling pathway to promote cell proliferation.

growth through amino acid transport and availability. Peptide transporter 1 (PEPT1) is primarily involved in the uptake of dietary protein degradation products and fish growth[49]. In studies using PEPT1 knockout mice, body weight was reduced and microvilli were shortened[50].

Intestinal development and stem cell signals in fish

Intestinal development in fish is like that of mammals. While not much investigation has been done on killifish intestinal development, its development in zebrafish has been thoroughly investigated. One study found that the intestinal tract has undergone extensive remodeling around the fourth day of development, along with compartmentalization into three main segments: the intestinal bulb, mid-intestine, and posterior intestine, which will be composed of cells that will perform specialized functions[51]. These specialized cells consist of enteroendocrine cells, which are hormone secreting cells observed 52-76 hours post fertilization, goblet cells, which secrete mucous, and enterocytes, which are absorptive cells, both observed 76-126 hours post fertilization. After 5 days of development, epithelial folding is more extensive in the intestinal bulb while mid- and posterior intestine remain unfolded and the presence of goblet cells and enterocytes is restricted to the mid-intestine. The folding processes proceeds rostrocaudally, as it does in mammals, with folding in the mid-intestine appearing around 8 days and folding in the posterior intestine appearing around 12 days[51]. The morphological characteristics of the intestinal bulb represent tall fold (plicae) or villus like extensions, which become progressively shorter through the mid to posterior

intestine. Although zebrafish and other minnows lack crypts where stem cells reside, they do have proliferative compartments at the base of the villus-like folds [51].

Differentiation of intestine stem cells in zebrafish is dependent on the same pathways and signals seen in mammals. Delta, the Notch ligand, accumulates in secretory cells of zebrafish gut and activates the Notch cascade in neighboring cells, which turns on transcription factors (Hes-1) specifying enterocyte fate, in part due to the repression of Ascl1a. Ascl1a is a gene that plays a role in specification of goblet and enteroendocrine cells[52]. Like mammals, epithelial cells in zebrafish migrate from the base of the intestinal folds to the tip of the fold within 5-7 days in the anterior intestine and 7-10 days in the mid intestine. Mechanisms driving differentiation of epithelial cells towards the secretory lineage (goblet cells or enteroendocrine) seems to be highly conserved in vertebrates and dependent on Delta-Notch signaling[41, 53, 54]. Interestingly, previous studies in our lab looking at arsenic's effects on neuronal cell differentiation found that arsenic exposure decreased Ascl1 expression by 2.5-, 7-, and 4-fold on days 5, 7 and 9 of stem cell differentiation. An increase in Ascl1 expression has been shown to promote neuronal differentiation, so decreased expression could be a mechanism by which arsenic inhibits stem cell differentiation[55]. As stated above, Ascl1a also plays role in intestinal stem cell differentiation, which suggests that it may also be reduced in the intestine of embryonically exposed killifish, and therefore reduce intestinal cell development. Therefore, arsenic exposure during development is a major concern.

Arsenic's effect on growth and development

It is known that arsenic can easily cross the placental barrier [29]. A study in Bangladesh investigated levels of arsenic in maternal and cord blood of pregnant women who were drinking water with of 90.5µg As/L which is much higher than the 10µg/L standard set by the WHO[56]. They found that arsenic levels in the maternal blood (11.7µg/L) and cord blood (15.7µg/L total arsenic) were similar, indicating that the fetus was also readily exposed to arsenic levels that could interfere with growth and development[56]. A mass poisoning event occurred in 1955 in Japan where bottle-fed infants were exposed to about 500µg arsenic/kg body weight through their formula (Morinaga milk powder) and led to more than 100 infant deaths. Infants less than 12 months of age had symptoms of anorexia, skin pigmentation, diarrhea, vomiting, fever, and abdominal distention which was first misdiagnosed as bronchitis or pneumonia until the link between patient's dried milk brand and arsenic content was made[57,58]. This event raised a need to investigate arsenic's long term effects following an early life-stage exposure. Another study followed the arsenic exposed infants 50 years later, and found among 50 individuals their average height was 6.5 cm below the control group indicating there might be potential for abnormalities in proper skeletal growth due to early life arsenic exposure[59]. Another study looked at neurological issues in these patients and found that the group exposed as infants to arsenic exhibited neuropsychological deficits such that they showed an average performance of at least 1.2 standard deviations below

the average for the control unexposed group in memory tests[57], had drastically reduced IQs, and increases in central nervous disorders like epilepsy and mental retardation[58] Other studies have found reductions in birth weight and gain following in infants exposed *in utero* to arsenic. For example, a study in Bangladesh examining low level arsenic exposure (<100ppb) during pregnancy found a significant decrease in birth weight, with each 1µg/L increase in urinary being associated with a 1.68 g reduction in birth weight[60]. A European study looking at prenatal exposure to chemical mixtures found that arsenic was the only chemical in the mixture to have a significant inverse correlation to babies' birth weight, declining 90g as arsenic increased in cord blood samples[61]. Additionally, a study in Bangladesh found that with increasing arsenic levels, IGF-1 plasma levels were significantly decreased and correlated with reduced birth weight[62]. Long term, *in utero* arsenic exposure has been associated with reductions in weight gain. For example, a study in Bangladesh followed pregnant woman exposed to arsenic and cadmium. They found that the children at 5 years of age had a reduction in weight gain and height with 41% of the children being underweight and 33% were stunted, and that these effects were most apparent in girls[63].

Similar findings have been seen in animal models. In a study looking at the effects of dietary arsenic exposure in juvenile rainbow trout found that there was a significant 15 and 34% decrease in weight gain in fish that fed on oligochaetes exposed to 4.5mg/L and 8.2mg/L As respectively. The uptake of arsenic into these oligochaetes ranged from 35- 58µg As/g dry weight [64]. A study looking at mice found that drinking

water arsenic exposure (10 ppb & 42.5 ppm) from gestational day 10 until birth was correlated with significantly greater body weight gain, fat content, and glucose intolerance through young adulthood[65]. Moreover, a study looking at rats exposed to 100 ppb arsenic during development plus a western style diet found an increase in body weight beginning at week 5 until the end of the study at week 15 [66]. Not only are studies finding a reduction in weight at birth but they are also finding an increase in body weight later in life which some studies think this could be a result of diet[66]. As a handful of studies have shown that arsenic was correlated with a reduction in weight gain, it is crucial to begin to discover some of the underlying mechanisms involved. Arsenic has been analyzed in studies of weight gain and IGF-1 which could be a potential pathway of concern as IGF-1 is a major factor in growth and cell proliferation but it would also be interesting to investigate arsenics effects on the intestine as it is the primary organ of nutrient uptake which is essential for proper growth.

Arsenic exposure and behavior

Arsenic has also been shown to influence animal behavior, and alterations in feeding and the ability to detect and recall food in the environment could lead to changes in nutritional uptake, and decrease growth and survival. A study discovered that weanling rats exposed to arsenic displayed an impairment in spatial memory after putting them through hidden platform acquisition tests and visible platform trials[67]. In this study, both adult and infant rats exposed to arsenic were tested using a variety of operant condition tests. The study found learning deficits and time required to learn a new task

was increased in rats exposed as infants compared the unexposed controls [67]. Another study looking at photo motor response as a behavioral assay in zebrafish embryos exposed to 900-1,000µM As found that the arsenic exposed embryos exhibited hypoactivity[68]. Additionally, a study reported that As at 0.75 mM impaired long term memory in avoidance training behavioral tasks, providing more evidence that arsenic could be affecting normal brain development and function as a neurotoxicant[69].

For fish, olfaction is crucial to behaviors such as finding food, selecting mates, migratory routes, and evaluating the risk of predation. An olfaction study in rainbow trout showed that metals, such as cadmium and copper, reduced the ability of rainbow trout olfactory epithelium to L-alanine and taurocholic acid[70]. L-alanine mimic's food detection and taurocholic acid should elicit a social response via olfaction[70]. This study demonstrates that exposure to metals can cause impairments of the olfactory response in fish, which could result in a variety of behavioral deficits such as food detection. We hypothesize that changes in feeding behavior in fish exposed to arsenic could be another potential mechanism for weight gain reductions.

In humans, behavior related studies have focused more on arsenics effects on memory, IQ, learning, anxiety and depression. A meta-analysis study focused on arsenic's effects on intelligence in children (5-15 years old) found that increases in urinary arsenic reduced IQ[71, 72]. Additionally, a study in India looking at children (5-15

yrs) with an average lifetime arsenic exposure of 147µg/L found a 12-20% decrease in vocabulary, object assembly, and picture completion test score[73].

Hypothesis and rationale

I hypothesize that arsenic exposure during embryogenesis results in a reduction in weight gain in our model species, killifish. The potential mechanisms that I will be examining are: 1) changes in feeding behavior 2) alterations in IGF signaling pathway expression, and 3) changes in intestinal morphology possibly interfering with nutrient uptake. I hope the results from these data could help elucidate if the arsenic standard drinking water limit (10 ppb) is protective of proper embryo growth and development, and if there are any long-term consequences to exposures of environmentally relevant arsenic levels (50 and 200ppb).

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CHAPTER TWO

EMBRYONIC ARSENIC EXPOSURE REDUCES GROWTH, ALTERS FEEDING BEHAVIOR, INTESTINAL STRUCTURE, AND IGF SIGNALING PATHWAYS

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Abstract

Arsenic is found as a contaminant of drinking water, rice, and other crops. Epidemiological studies have shown that embryonic exposure to arsenic can cause changes in behavior and reductions in growth, but the mechanisms for these effects are not well understood. Thus, killifish were exposed to 0, 10, 50, and 200ppb As^{III} as embryos, and after hatching, were reared in clean water for up to 28 weeks. Growth, assessed by condition factor (weight/length^3), was significantly reduced in the 200 ppb groups at 8 weeks by 24% with a dose dependent increase in 10 and 50 ppb. Significant behavioral changes were also noticed at 16 and 28 weeks, including reduced response to food. Additionally, there was a reduction in intestinal villus area and absorptive intestinal surface area at 16 weeks. A decrease in liver IGF1 and IGFBP-1 transcript levels were observed, particularly a decrease at 8 weeks when there was a reduction in condition factor as well and an increase in muscle IGF1 and IGFBP-5. By 16 and 28 weeks, there is a shift in that IGF-1 liver and IGFBP-1 increase with arsenic exposure, and are highly correlated to one another. We hypothesize that embryonic exposure to arsenic impairs fish's ability to maximize nutrient intake due to a decreased feeding response and reduced numbers of intestinal enterocytes, ultimately causing compensatory changes in key proteins of the IGF signaling pathway.

1. Introduction

Arsenic (As) is a naturally occurring element found in water, sediments, and soils, with the main route of exposure being drinking water. The most commonly found arsenic species in aqueous environments are the inorganic forms, arsenite (As^{III}) and arsenate (As^{V}) with arsenite being the predominate species in groundwater[1-3]. Currently, the World Health Organization (WHO) has set the standard for arsenic in drinking water at 10 ppb, although higher levels of arsenic are found all over the world in countries like China, Bangladesh, Hungary, Chile, and Japan[2,4-8]. Even in the U.S., arsenic levels above 10ppb can be found in unregulated wells, exposing roughly 13 million households to high arsenic concentrations in their drinking water[9]. The 10 ppb standard was set to help protect people from chronic exposures that can cause cancer and cardiovascular diseases, as well as potential developmental problems[10,11]. Not only is water a concern, but arsenic has been found in dietary sources such as wheat bread, rice, milk and dairy products[12]. Arsenic levels in rice, a staple crop for many people, pose a particular problem, as a study found levels of 200 µg/kg in brown rice, 130 µg/kg in white rice, and 70 µg/kg in other color rice[13, 14]. Another study investigated arsenic content in 9 different rice-based infant snacks and found ranges from 36.5-568 ng/g total as[15]. These high levels of arsenic content in rice and other snacks is alarming considering families that rely on rice a staple crop can consume 0.4 kg of rice per day per person and dietary arsenic exposure between 0.3 and 8 µg/kg body weight/day was estimated to result in a 1% increased risk of lung skin and bladder tumors in humans[12].

Studies have often focused on arsenic as a carcinogen, but less is known about embryonic arsenic exposure and its effects on development and growth. It is known that arsenic can cross the placental barrier exposing a developing fetus to this toxicant[16], as studies have found levels of arsenic in maternal (11.7μg/L) and fetal (15.7μg/L total arsenic) blood to be similar[17]. A Bangladesh study found that exposure to arsenic (<100 ppb) during pregnancy was associated with a significant decrease in birth weight with each 1μg/L increase in urinary As concentration being associated with a 1.68 g reduction in birth weight[18]. A European study looking at prenatal exposure to chemical mixtures found that arsenic was the only chemical in the mixture to have a significant inverse correlation to babies' birth weight, declining 90g as arsenic increased in cord blood samples[19]. Additionally, a study in Bangladesh found that with increasing arsenic levels, IGF-1 plasma levels were significantly decreased and correlated with reduced birth weight[20]. In an animal model, mice exposed to 10 ppb As *in utero* and postnatally weighed 18-23% less than the controls up to 42 days of age in female mice[21].

In addition to changes in weight, other studies have found correlations between arsenic exposure and behavioral changes, particularly in children. In Bangladesh, a study looking at two cohorts of children at age 6 and age 10 found reduced intellectual function and performance, and reduced processing speed with arsenic exposures between 5-50 μg/L during development[22]. Even studies on adults in India found that 19% of patients aged 18-65 years old who were exposed to 25-900 μg/L arsenic exhibited depression and/or anxiety-like behavior[23]. Animal models have also supported the trend in behavioral alterations. A study found that both adult and juvenile rats exposed to

arsenic had increased learning deficits and time required to learn a new task compared the unexposed controls[24]. Additionally, a study looking at photomotor response as a behavioral assay in zebrafish embryos exposed to 900-1,000µM As found that the arsenic-exposed embryos exhibited hypoactivity[25]. Changes in behavior could potentially reduce feeding activity, and therefore reduce growth.

While arsenic's effects on growth and behavior have been investigated, very little is known about its effects on the intestine, a key organ involved in the uptake of arsenic from drinking water and food. One study using the HT-29 human intestinal epithelial cell line did find that arsenic increased superoxide levels, causing dysregulation of barrier functions in epithelial cells[26]. Additionally other metals, such as copper, induce oxidative stress in fish intestinal enterocytes after *in vivo* and *in vitro* exposures[27].

Intestinal development is similar in mammals and fish, although most fish lack true crypts and instead have proliferative compartments at the base of the villus[28]. The morphogenesis of villi and crypts occur by folding of the endodermal epithelium, and stem cell markers such as Lgr5 and Ascl1 start to appear in the intervillus region[29]. The Wnt/β-catenin and Notch signaling pathways are important in the differentiation of the intestinal stem cells [29, 30]. Like mammals, epithelial cells in zebrafish migrate from the base to the tip of the intestinal fold within 5-7 days. Mechanisms driving intestinal cell differentiation appear to be highly conserved in vertebrates, and produce the same main cell types: enterocytes, Goblet cells, and enteroendocrine cells[31-33]. Cell division in crypts is dependent upon Wnt/β-catenin signaling, in which the Lgr5+ stem cells differentiate into progenitor cells. From the progenitor cells, the Notch ligand Delta

turns on transcription factors such as Hes-1 to specify enterocyte fate[31, 34] while the Ascl1 transcription factor plays a role in specification of goblet and enteroendocrine cells[35].

Interestingly, a previous study in our lab found that arsenic exposure decreased Ascl1 expression by ~ 4-fold during embryonic stem cell differentiation *in vitro,* thereby reducing the formation of sensory neurons[36, 37]. Since Ascl1a also plays a crucial role in intestinal stem cell differentiation, we hypothesize that arsenic may also impact intestinal development *in vivo*. Thus, the goal of the current study is to determine the mechanisms by which arsenic exposure during embryogenesis permanently impairs growth using killifish (*Fundulus heteroclitus*) as the animal model. Potential mechanisms that we will examine include changes in intestinal stem cell differentiation and structure, changes in feeding behavior, and changes in growth factors needed for skeletal muscle proliferation.

2. Methods

2.1 Killifish collection

Adult killifish (*Fundulus heteroclitus*) were collected from the National Estuarine Research Reserve (NERR) site in Georgetown, SC using baited minnow traps, and housed at Clemson University for the collection of eggs and milt. All fish throughout this study were kept on a 16:8 light/dark cycle at 26^{o}C in 18ppt salt water (CoraLife). Adult killifish were fed Ziegler Zebrafish food twice a day and maintained until egg and milt collection.

2.2 Exposure of killifish embryos to arsenic

Eggs were stripped from 3-4 females and milt collected from 1-2 males to fertilize ~300 eggs. Fertilized eggs were randomly placed in one of 28 Petri dishes, and the process of fertilization continued until each Petri dish contained 80 eggs. The fertilized eggs were continuously exposed to 0, 10, 50, and 200ppb arsenic, as sodium arsenite, with 7 replicate petri dishes per concentration. Water changes were done every other day, and embryos monitored for viability and hatching daily. After hatching, the fry were placed in 10 gallon tanks with arsenite-free 18 ppt salt water within 24hr of hatching, and fed 5% of their estimated body weight per day. Juveniles were fed brine shrimp until ~4 weeks of age, then were transitioned to pellet food (Zeigler Brothers). Sampling time points were at 8, 16, and 28 weeks of age.

2.3 Assessing arsenic metabolite formation

Using the process described above, additional eggs were fertilized and juveniles grown up in clean water for 16 weeks to assess arsenic uptake and metabolism. Fish were exposed to 0, 5, or 10ppm arsenic as sodium arsenite (n= 6 tanks per exposure level; each tank had 2-3 killifish) for 5 days in a static renewal system. Fish were fed twice per day, and water changes were conducted once a day. Fish were euthanized in buffered MS-222, rinsed in clean water, patted dry, and frozen whole at -80^{o}C. In order to have enough tissue mass for speciation, fish from 3 tanks were combined per exposure group, giving n=2 replicates per concentration. The arsenic species in our adult fish food (Ziegler Brothers zebrafish pellets) was also determined.

All processing and arsenic analyses were conducted by Brooks Applied Labs (Bothell, WA). Fish and food samples were homogenized and digested in nitric acid (modified EPA Method 3050B), and total recoverable arsenic performed by inductively coupled plasma triple quadrupole mass spectrometry (ICP-QQQ-MS). Arsenic species examined include arsenite, arsenate, monomethylarsonic acid (MMAs), dimethylarsinic acid (DMAs), trimethyl arsine oxide, arsenocholine, and arsenobetaine. Analyses were conducted by ion chromatography coupled to an inductively coupled plasma collision reaction cell mass spectrometer (IC-ICP-CRC-MS). Results are presented as the amount of each arsenical species per whole body kg wet weight.

2.4 Killifish feeding behavior and growth measurements

Sampling time points were at 8, 16, and 28 weeks of age. Two weeks prior to the sampling points, feeding behavior was assessed by videotaping fish movement on two separate days. A grid with 5cm rows was placed in the back of each home tank one week prior to the start of the test. Shielding was used to hide the observer from the fish, and fish were not fed for 15 hrs prior to the test. On the day of the test, the normal position of fish within the tank was recorded for 15 seconds using a video camera mounted to a tripod. To start the test, zebrafish food pellets (Ziegler) were gently placed in the top of the water column, and response of the fish to the food was videoed for 60 seconds. Using the recordings, the following parameters were quantified: 1) time for 10% of the fish to start moving in response to the stimuli and 2) percentage of fish within each tank responding. The trials were conducted on two separate days, and averaged per tank.

At each sampling time point (8, 16, and 28 weeks of age), 5-6 fish per tank were euthanized in 1g/L buffered MS-222. Fish were patted dry, and weight, body length and intestinal length measured. Condition factor (weight/ length^3) for each fish was calculated, and averaged per tank. At the 28 week sampling point, when we were able to visually determine sex, 3 males and 3 females were sampled per tank, tissue was collected, labeled with sex, and kept separate. Muscle, liver, and anterior intestine were collected from 3 of the 6 fish per tank. Tissue samples were stored in RNAlater at -80^{o}C. Additional anterior intestine sections taken from the other 3 fish per tank for histology were fixed overnight in 10% buffered formalin, dehydrated in graded ethanol, embedded in paraffin, cut into 7 µm transverse sections, and placed on slides.

2.5 Intestinal morphology

Morphological changes in the intestines of embryonically exposed fish were examined by hematoxylin and eosin (H&E) staining (n=4 fish per exposure per sampling time point). Once gender could be visually confirmed at 28 weeks of age, 2 males and 2 females per exposure was collected for histology. Sections were stained with H&E and imaged using a Leica ICC50 HD light microscope. ImageJ software (National Institute of Health) was used to determine height and width (mm) of 5 villi per field of view.

2.6 Immunohistochemistry

Paraffin embedded intestinal sections (7µm) were examined for markers of absorptive enterocytes (gut absorptive cell epitope antibody; Biorbyt, # orb324078), while the number of proliferating cells were examined by the expression of proliferating cellular

nuclear antigen (PCNA) (Abcam, #ab29). Sections underwent antigen retrieval in Tris-EDTA buffer, pH9, and were then quenched with 3% hydrogen peroxide for 10 minutes. Blocking was in avidin- biotin (Vector Labs, Burlingame, CA) for 30 minutes and then 10% horse serum (Vector ABC Elite Kit) for 17 minutes. After washes, the appropriate dilution of primary antibody (PCNA 1:750; absorptive cells 1:400) was added and incubated overnight at 4^{o}C. Tissues sections were then washed and biotinylated secondary antibody was added for 30 minutes. Then Vectastain ABC reagent (Biotinylated horseradish peroxidase + Avidin DH) was applied to the sections. Antibody labeling was detected with Nova Red, and sections were counter-stained with hematoxylin if the primary was not nuclear. Using these antibodies, Goblet cells can be clearly identified based upon morphology. PCNA positive cells were counted per 2 intervillus regions within field of view at 40X and averaged per villus. Absorptive surface area was quantified by outlining 4 villi per field of view at 10X for absorptive surface and determining the percentage of stained cells using ImageJ. The colors were converted to a black and white image so only cytoplasmic staining of the enterocytes would be determined. Goblet cells were counted from each of the 4 villi and averaged per villus. All sections were averaged within the group, and statistical significance was determined by ANOVA followed by Tukey's.

2.7 qPCR

RNA was extracted from liver, muscle, and intestines using TRIZol (Sigma-Aldrich, St. Louis, MO). Purity and RNA concentration were determined using a NanoDrop Lite ,and 2µg RNA was reverse-transcribed to cDNA using MMLV-RT. To

conduct qPCR, 40ng cDNA, RT^2 SYBR Green mix (Qiagen, Alameda, CA), and gene-specific primers (Table 1) were run in triplicate on a iQ5 thermocycler (BioRad). For 8 and 16 weeks, 5 fish per exposure group were randomly selected to run on one plate per gene. At 28 weeks, 5 fish per gender were examined. A 5-point standard curve (10^{-3} to 10^{-7} ng) for each gene was used to determine the efficiency and linearity of each reaction. In muscle, levels of insulin-like growth factor-1 (IGF-1), insulin-like growth factor-1 receptor (IGF-1R), and insulin-like growth factor binding protein 5 (IGFBP-5) were examined. In the liver, expression of insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein 1 (IGFBP-1) were examined. In the intestine, levels of the solute carrier SCL15A1b were examined. Samples were run in triplicate and gene expression data was normalized with 18S rRNA as the housekeeper using the comparative threshold (Ct) method[38]. All comparative threshold averages were compared to the control, which was set at a value of 1.

Primer	Forward	Reverse	ºC
18s	5'-TTT CTC GAT TCT GTG GGT GGT GGT-3'	5'-TAG TTA GCA TGC CGG AGT CTC GTT-3'	60
IGF-1	5'- AAA CAG ATA AAC CAACAG GCT ATG-3'	5'-GCA GCT CAC AAC TCT GGA A-3'	54
IGF-1R	5'-CGT CTT TGA CCA CAC CCT T-3'	5'-CGC AGA AAT GTA CGT ACC AGA-3'	55
IGFBP-5	5'GAA GGA CAC TTC TCG GGT TAT G-3'	5' TTG CAC TGT TTG CGC TTG-3'	56
IGFBP-1	5' CAT GGC TCT GTG CAC TAC AT-3'	5' ATC GCG TTA ACT CTG GCT TT-3'	59
SLC15Alb	5'-CTC CAC AAC CAT CTA CCA CAC-3'	5'-CTC ATG GCT CGG AAA GTT CA-3'	52

Table 1. Killifish qPCR primers

2.8 Statistical analysis

To analyze differences in arsenic metabolites, condition factor, feeding behavior, intestinal morphology, SCL15A1b gene expression, number of $PCNA^+$ cells, and the number of goblet cells, an ANOVA followed by a Tukey's (JMP) was used to compare differences between exposure groups. In all cases, a fit model showed there was no effect of day, tank, or sex. For transcript expression in the liver and skeletal muscle, multivariate ANOVA was used to test equality of the two variables across the doses. If MANOVA suggested differences in the four doses, multivariate contrasts were used to determine significances of the two variable between specific sets of doses. This allowed us to determine the exact nature of the dose impact of the two variables being analyzed at a given time point. Data was consider significant if $p<0.05$.

3. Results

3.1 DMA and MMA make up a high percentage of arsenic species in killifish

First, we wanted to determine whether killifish could metabolize arsenic into mono- and dimethylated metabolites in a manner similar to humans. Sixteen-week old killifish were exposed to 0, 5, or 10ppm arsenite, and levels of As (III), As (V), monomethylarsonic acid (MMA),

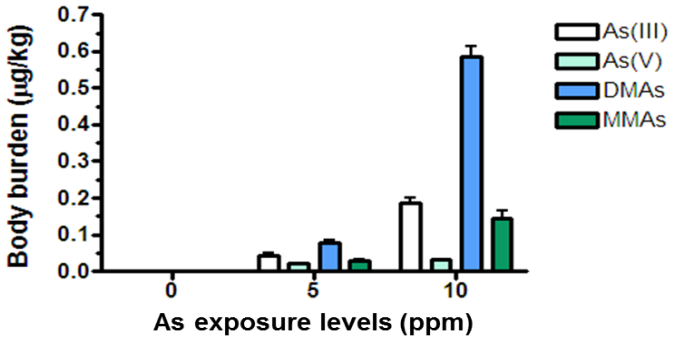

Figure 1. Killifish can readily metabolize arsenic to monomethylated (MMA) and dimethylated (DMA) species. Sixteen week-old killifish were exposed to 5 or 10ppm arsenite for 96 hours (n=2 replicates; each replicate contained 15-20 fish). Speciation was performed by Brooks Applied Labs using IC-ICP-CRC-MS.

dimethylarsinic acid (DMA), and arsenobetaine (AsB) quantified. AsB was the only arsenical species present in the control fish, and its levels did not change due to arsenic exposure (average = 417±62μg/kg; data not shown). DMA, MMA, As (III), and As (V) were below the detection limits in control fish, and their values increased in a dose-responsive manner in the 5ppm and 10ppm exposure groups (Figure 1) with DMA accounting for 45-61% and MMA accounting for 15-17% of the total arsenicals. These percentages are similar to paired maternal and newborn blood samples exposed to 90ppb arsenic in their water, in which 43±9% of total arsenic was as DMA and 31±6% was as MMA[17]. Note that in (Figure 1) we are looking at whole body levels, rather than blood levels, which accounts for some of the variation in MMA and DMA percentages. The adult fish food contains arsenical species, but >98% of them are arsenobetaine and arsenocholine, with As (III) levels below the detection limit. Arsenite in our water is also below detection limits (data not shown). These data indicate that killifish readily metabolize arsenic, and can be used as models for human exposures.

3.2 Embryonic arsenic exposure reduces condition factors in 8 week old killifish

To assess whether embryonic-only arsenic exposure altered growth in juvenile and adult killifish, condition factors (CF; weight/length^3) were determined. Killifish were exposed as embryos, and after hatching, were grown out in clean water. No differences in hatchling success or survival up to 28 weeks of age were seen (data not shown). At 8 weeks of age, condition factor was significantly reduced at 200ppb by 20% compared to the control (Figure 2A). No difference was found at 16 or 28 weeks among

any of the exposure groups, but there was a slight trend in condition factor reduction at 16 weeks (Figure 2A). At 28 weeks, when male and females were combined, there were no differences in condition factor (Figure 2C), although decreases can be seen in the females when separated from the males.

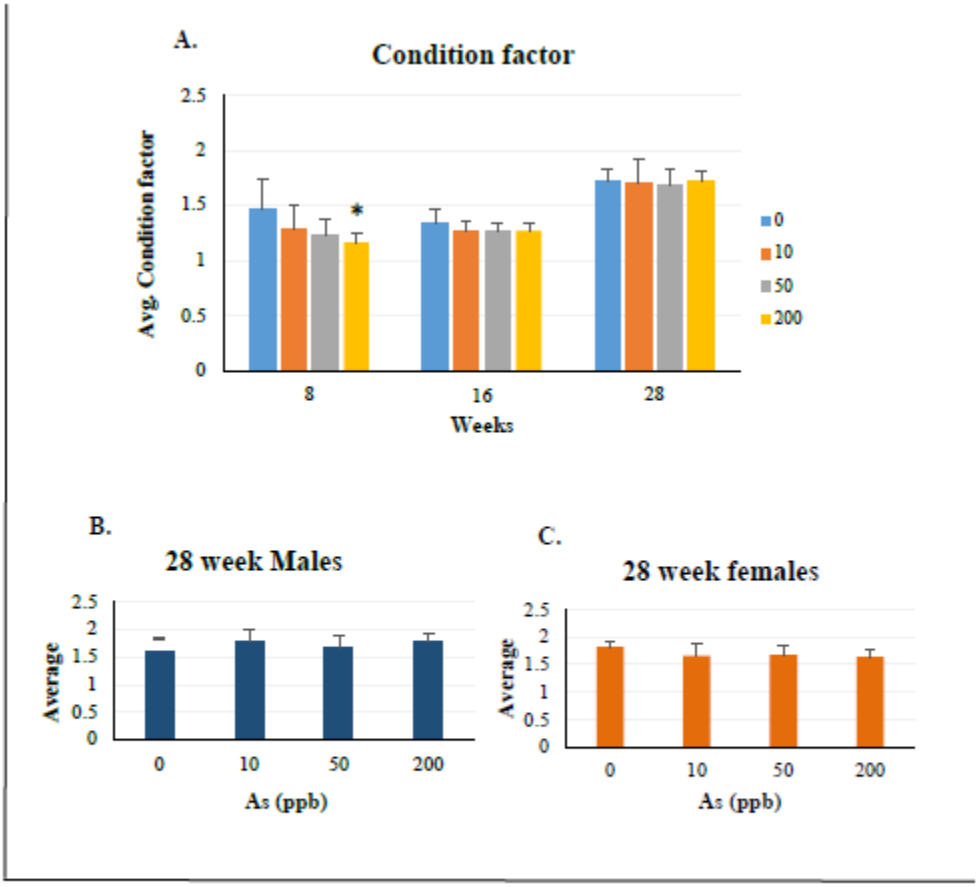

Figure 2. Embryonic arsenic exposure slightly reduces condition factors. At each given sampling time point, weight and length were measured for 6-8 fish per tank for each exposure (0, 10, 50, and 200 ppb arsenic) and used to calculate condition factor. These values was averaged for each tank (n=7), and statistical differences (*) were determined using one-way ANOVA followed by Tukey's post hoc ($p<0.05$). Included are the condition factors at each time point, (A), condition factors for 28 weeks males (B), and females (C).

3.3 Embryonic arsenic exposure alters feeding behavior

Since reductions and trends in condition factors were seen after embryonic arsenic exposure, similar to previous studies[39, 40], we wanted to examine potential mechanisms for the lack of growth. Since one possibility is that the arsenic-exposed embryos were not actively seeking enough food, we assessed differences in feeding behavior. At 8 weeks of age, the arsenic-exposed fish appear to take longer to respond to food, with the control fish taking an average of 23 seconds to respond to food placed in their tanks and the arsenic exposed fish taking an average of 33 seconds, but there were no significant differences compared to the control (Figure 3). At 16 weeks of age, there was a significant 1.7-fold increase in the average time to start a response in the 10 ppb embryonically-exposed fish (Figure 3). While the 50 and 200ppb exposure groups also had start time increases of 1.4- to 1.6-fold, there were no

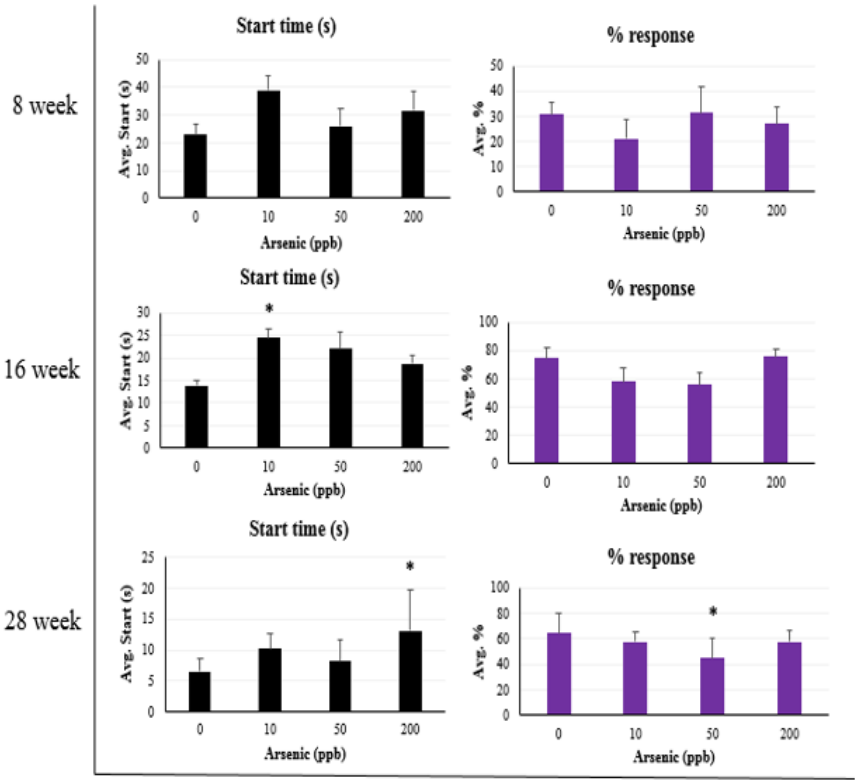

Figure 3. Feeding behavior is significantly changed at 16 and 28 weeks. Time to start a response and number of fish responding per tank were analyzed in recorded videos and each parameter was averaged per tank (n=7 tanks per exposure). Averages were compared using a one-way ANOVA followed by a Tukey's post-hoc, and significance (*) is at $p<0.05$.

significant differences (Figure 3). At 28 weeks of age, there was a significant 2-fold increase in the start time for 200 ppb group, and increases of 1.2- to 1.6-fold time to start for the other groups. Similarly, the percentage of fish responding to food within the first minute at 28 weeks of age was decreased in the arsenic exposed groups, ranging from a 7% to a 20%t decrease (Figure 3). These data indicate that embryonic arsenic exposure can inhibit responsiveness to feeding which could therefore be a potential reason for reductions in growth.

3.4 Embryonic arsenic exposure reduces intestinal villus height

Another possible mechanism for the reductions in growth was that embryonic arsenic exposure reduced the number of enterocytes or otherwise altered the ability of the intestine to absorb nutrients. To first examine morphometric changes, hematoxylin and eosin (H&E) staining was used to quantify average intestinal villus area by multiplying height and width (Figure 4A). No significant differences were found at 8 weeks of age. At 16 weeks of age, there is some reduction in villus area, but these differences do not persist at 28 weeks of age (Figure 4B). The reduction in villus area at 16 weeks does follow similar trends with reduced SCL15A1b peptide transporter expression (Figure S1. supplemental data), which was examined since other studies in mice have seen correlations between reduced SCL15A1b peptide transporter expression, reduced villus height, and reduced weight gain[2]

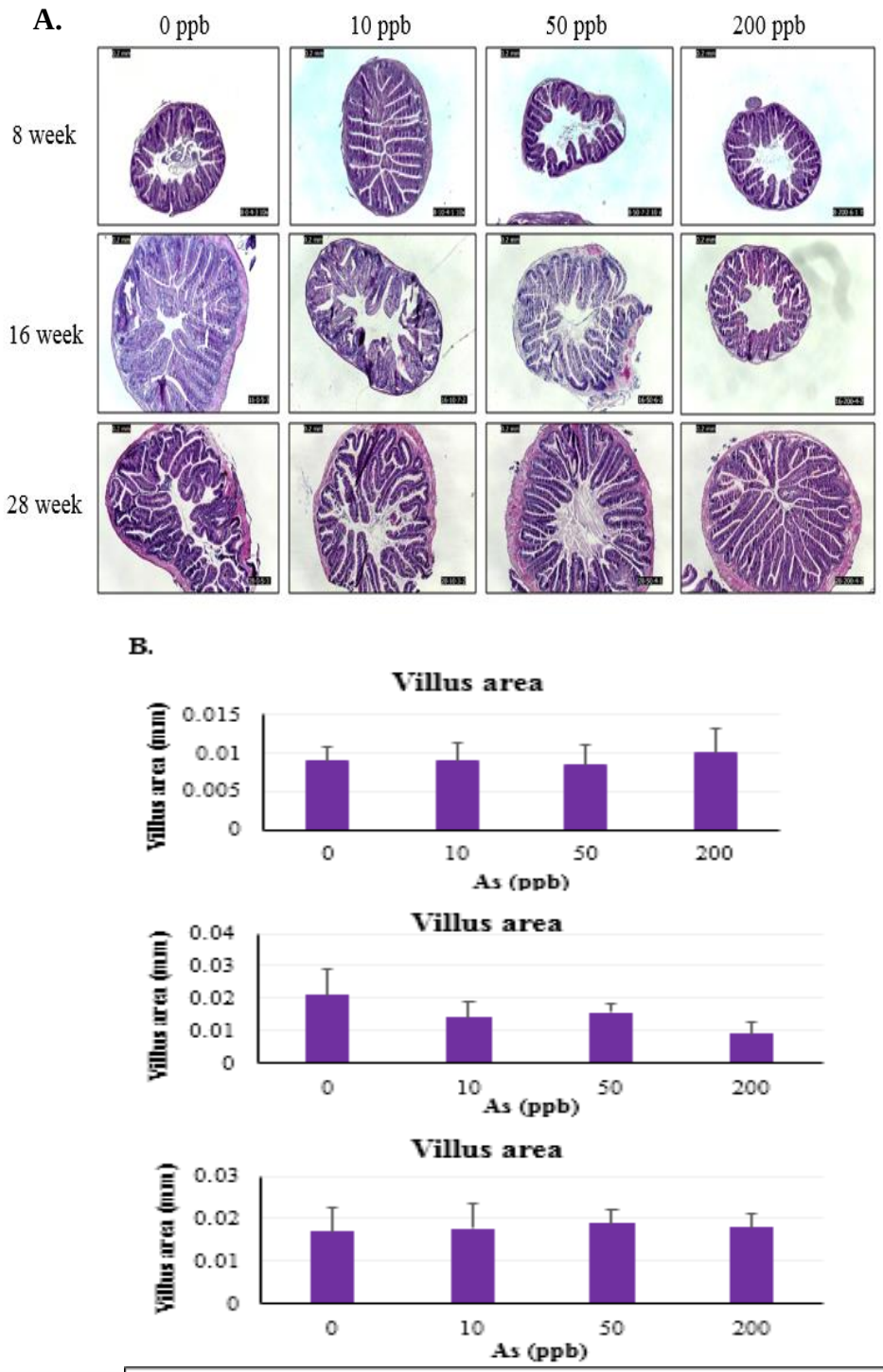

Figure 4. H&E staining of anterior transverse sections indicates no morphometric differences following embryonic arsenic exposure. H&E images (10x) of the anterior intestine at 8, 16, and 28 weeks (A) (n=4 per exposure group per time point). Images were used to quantify villus area (width * height) using ImageJ (B). Averages were compared using a one-way ANOVA followed by a Tukey's post-hoc, and significance (*) is at $p<0.05$.

3.5 Embryonic-only arsenic exposure reduces number of PCNA (+) cells and absorptive surface

Immunohistochemistry was used to examine the number of proliferating cells (Figure 5A), enterocytes, and goblet cells (Figure 5B). Since a fit model indicated there was no significant effect of sex, all data were combined. The data show that embryonic arsenic exposure appears to reduce the number of proliferating cells up to 28 weeks after exposure, but only a trend was found (Figure 6). Similarly, the percentage of cells labeled as enterocytes, were reduced, although this was only statistically significant in the 50ppb exposure group at 28 weeks (Figure 6B). There were no differences in the numbers of Goblet cells, which averaged 3.5-5 cells per villus (Figure 6C).

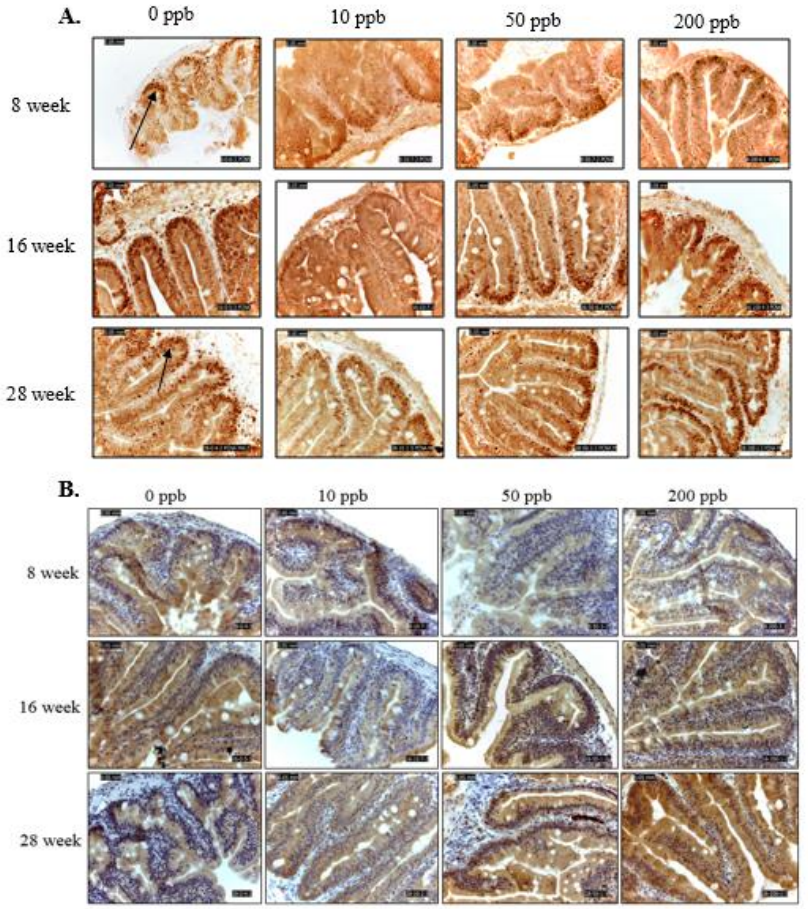

Figure 5. Immunohistochemistry to assess proliferating cells, enterocytes, and Goblet cells. Images of 8, 16, and 28 week PCNA positive cells (A) in the intervillus region, enterocytes and Goblet cells (B). Images are at 40x. Black arrows in control (0ppb) point to PCNA cells (A). Positive cells were counted in 2 intervillus regions then averaged per sample (n=4 per exposure group per time point). For quantification of enterocytes (B), ImagesJ was used to quantify absorptive surface area at 10X, which was averaged for 4 villi per field. Number of goblet cells (B) were counted in those 4 villi and averaged (n=4 per exposure per time point).

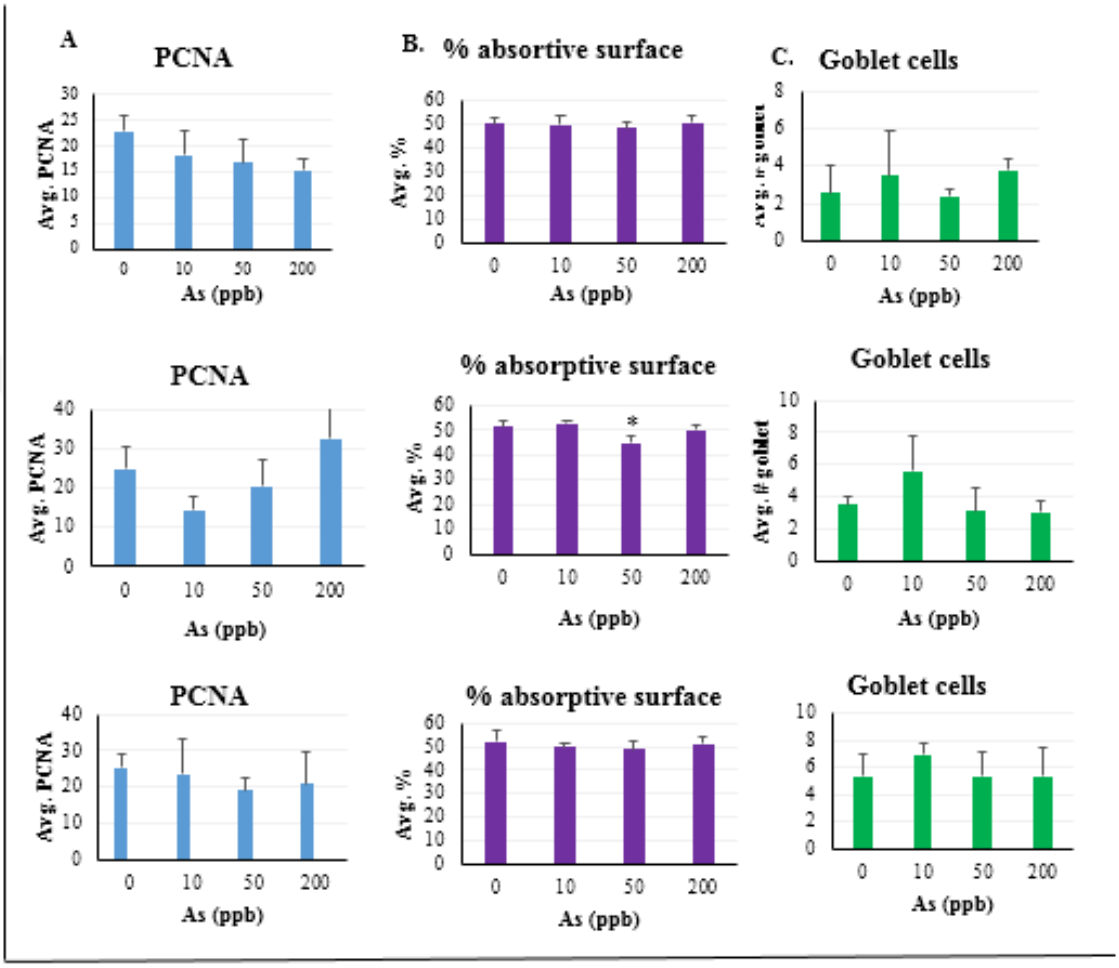

Figure 6. Intestinal cell numbers did not change. Average numbers of PCNA (+) cells per intervillus region (A), percent absorptive surface area (B), and number of Goblet cells (C) were quantified. Significant differences (*) were analyzed by a one-way ANOVA followed by Tukeys post-hoc (n=4 per exposure per time point; $p<0.05$).

3.6 Embryonic arsenic exposure alters skeletal muscle IGF-1 and IGFBP-5 transcript expression

Since few changes were seen in intestinal cells types or morphology, we wanted to see if there were changes in genes involved in the IGF-1 pathway as it is a major promotor of growth, especially during the juvenile stage. In a previous study, we had seen that embryonic arsenic exposure at higher concentrations and later time periods resulted in increased expression of muscle IGF-1 and IGF-1R[39]. We wanted to expand upon this earlier study by examining a more comprehensive set of genes, and assess whether lower arsenic concentrations also resulted in the same effect. Thus, in addition to examining muscle IGF-1 mRNA levels, we also assessed levels of the pro-myogenic retention protein IGF binding protein -5 (IGFBP-5)[41]. At 8 and 16 weeks of grow-out in clean water, muscle IGFBP-5 and

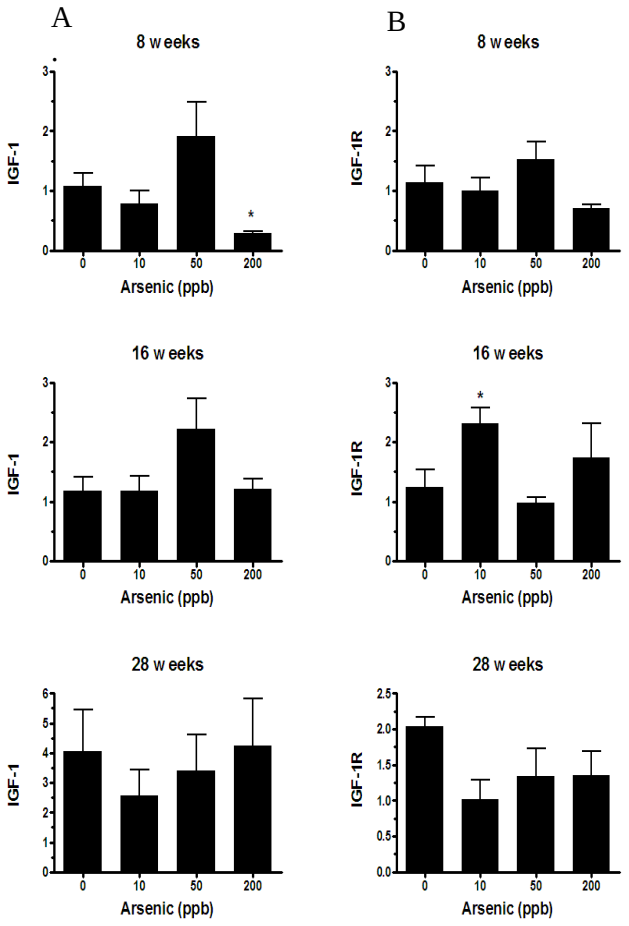

Figure 7. Muscle IGF-1 and IGFBP-5 increase at 8 and 16 weeks then are reduced by 28 weeks. At each time point muscle IGF-1 and IGFBP-5 were quantified using the comparative threshold method (2^ddCt). Averages were analyzed for significant difference ($p<0.05$) indicated by (*) using the one-way ANOVA method (n=5 per exposure per time point).

IGF-1 levels increased in the 50 ppb group compared to the control (Figure 7). Additionally there was a notable increase in IGFBP-5 at 16 weeks in the 10 ppb. However, by 28 weeks, there is a decrease in IGFBP-5 expression in the 10 ppb group, along with a trend such that muscle IGF and IGFBP-5 levels are reduced in all exposures (Figure 7). No real trends were seen at any time points with IGF-R (data not shown).

3.7 Arsenic exposure alters the correlation between hepatic IGF-1 and IGFBP-1

Since the liver is the primary organ for IGF-1 synthesis, we analyzed hepatic IGF-1 levels as well as the IGFBP-1 binding protein, which sequesters IGF-1 under stress making it less bioavailable[42]. When the relationship between hepatic IGF and IGFBP-1 was compared, there is a significant correlation between the two at the 8 and 28 week time points (r^2=0.93 and 0.98, respectively). At 16 weeks, the correlation is still apparent, but is not statistically significant (r^2=0.80). At 8 weeks, there is a significant reduction in IGF-1 and its binding protein at all

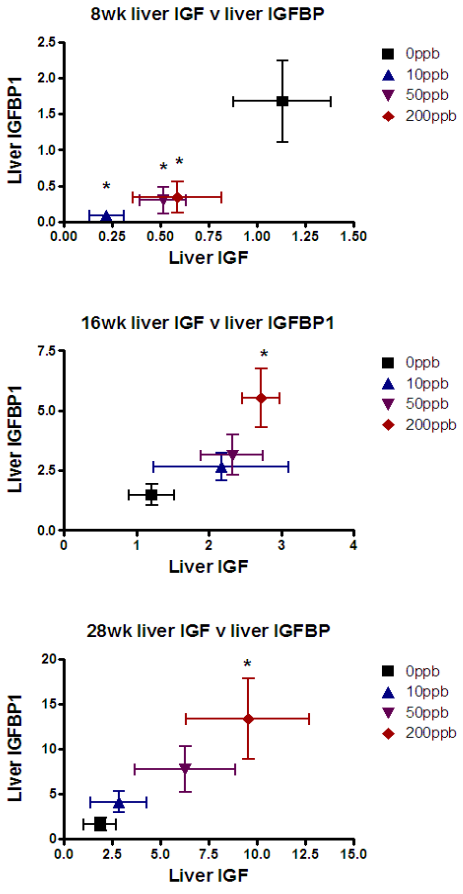

Figure 8. Liver IGF-1 and IGFBP-1 decrease at 8 weeks and then increase at 16 and 28 weeks. At each time point liver IGF-1 and IGFBP-1 were quantified using the comparative threshold method (2^ddCt). Averages were analyzed for significant difference ($p<0.05$) indicated by (*) using the MANOVA and multivariate contrast.

arsenic exposure groups compared to the control (Figure 8). At 16 and 28 weeks, the comparisons have changed such that hepatic IGF-1 and IGFBP-1 are significantly increased at the 200ppb group, and a trend towards increasing expression exists at 10 and 50ppb at both time points (Figure 8). The pattern in decreasing liver IGF-1 correlates with a decreasing condition factor at 8 weeks of age therefore, indicating embryonic arsenic exposure could reduce liver IGF-1 in correlation with a reduction in growth (Figure 9).

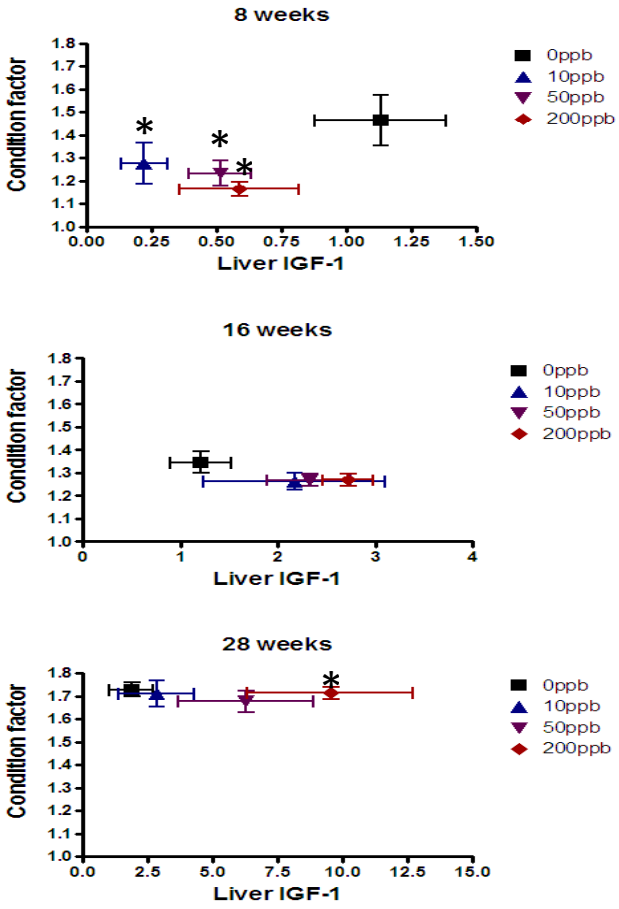

Figure 9. Liver IGF-1 and condition factor decrease at 8 At each time point liver IGF-1 quantified using the comparative threshold method (2^ddCt). Condition factor and IGF-1 liver Averages were analyzed for significant difference ($p<0.05$) indicated by (*) using the MANOVA and multivariate contrast method (n=5 per exposure per

4. Discussion

The results of this study indicate that embryonic arsenic exposure reduces growth of killifish early on during juvenile stages. Reductions in feeding behavior and changes in IGF signaling pathway transcript levels might be underlying mechanisms as to why arsenic can reduce growth long after the exposure has ended.

4.1 Embryonic arsenic exposure reduces growth at early time points

A previous study examining embryonic-only arsenic exposure and long term growth found that at 16 and 28 weeks, condition factor was reduced in the arsenic-exposed groups (50 , 200, and 800ppb) and the reduction persisted up to 52 weeks[39]. Additionally, a study looking at earlier time points, but at much higher exposure concentrations, found that 800-5000ppb embryonic arsenic exposure reduced weight in 8 and 16 week old juvenile fish[40]. The current study was conducted to assess the mechanisms responsible for these changes in growth at more environmentally relevant embryonic arsenic exposures. After analyzing condition factor at 8 weeks, there were dose-dependent reductions in all exposure groups, but a significant reduction (24%) was only found in the 200 ppb embryonic exposure group. At 16 weeks, there is a dose-dependent trend for reduced condition factor. These findings were expected and indicate that embryonic exposure to arsenic can reduce growth and weight gain during the juvenile period. However, by 28 weeks, the females show a reduction in condition factor in a dose-dependent manner, but the males actually had an increase in condition factor in the exposure groups.

These findings are not entirely consistent with our previous study. However, when the food from the previous study was analyzed for specific arsenic species, we found it contained small amounts of trivalent arsenic such that a fish at 16 weeks of age weighing an average of 0.3g would have received a continuous arsenic exposure equivalent to 2.25ppb arsenic, while a 28 week old fish weighing ~1g would have received an exposure equivalent to 7.5ppb continuously. The zebrafish food used in our current study had trivalent arsenic levels below the detection limit. Thus, the earlier study may have had more pronounced growth reductions because of continuous exposure to arsenite through the diet.

In addition, studies have shown that gender differences can impact the toxicity of arsenic[43, 44]. A recent study looked at gene expression differences in males and females *in utero* exposed to 0.36 µg/L arsenic on average through household tap water. Aquaporin 9 (AQP9), a known transporter of trivalent arsenic, was positively correlated with maternal urinary arsenic (U-As) levels in female offspring, but not in male offspring[45]. Additionally, this study examined expression of developmental genes involved in the Wnt, Notch, and stem cell regulator pathways and found that genes such as LGR5, HES1, GLI3 were negatively associated and IGFBP6 was positively associated with AQP9 levels in females. This study suggests that AQP9 transport of inorganic arsenic could be a mediator of arsenic's sex-specific birth weight reductions in females, and the investigators hypothesized that estrogen might play a role in facilitating transcription of AQP9 in liver cells [45]. Another study found a reduction in birth weight in females by 1.5 g per 1µg/L increase in urinary arsenic concentrations, but only a

reduction of 0.15 g birth weight in males. Only female birth weight was positively associated with GLI3 and LGR5 and negatively associated with urinary arsenic levels[46]. These finding indicate that exposure to arsenic during early developmental time points can have sex- specific outcomes that relate to reductions in birth weight. The reduction in condition factor in females in our study is similar to an epidemiology study that looked at children exposed *in utero* to arsenic, and found that 5 years later, there was a significant reduction in weight gain and height with 41% of the children being underweight and 33% were stunted, and that these effects were most apparent in girls[47]. Therefore, arsenic might inhibit proper growth in females more than it does in males.

As growth was reduced at early time points in embryonic-arsenic exposed killifish, it was interesting to observe the reduced feeding behavior. In our study, we noticed an increase in time to feed and a decrease in the number of fish actively feeding, particularly at 16 and 28 weeks of age. An increase in the time it takes to start feeding could potentially indicate a lack of interest in feeding, decreased olfactory ability to detect the food, or hypoactivity. Previous studies have shown that embryonic arsenic exposure reduced feeding behavior in killifish at 28 and 40 weeks of age following an embryonic exposure of 200 and 800 ppb asrenic[39], which is in line with our findings. Similarly, a study in rainbow trout found that exposure to cadmium and copper reduced the ability of the fish to detect L-alanine, an amino acid that mimics food[48], while exposure to copper induced apoptosis in salmon olfactory sensory neurons, thereby inhibiting behaviors critical to salmon survival such as food detection[49]. Indeed, a number of investigations have determined that the olfactory epithelium is a target of

metal toxicity. Additionally, a study found that zebrafish embryos exposed to arsenic exhibit hypoactivity during embryonic tail flexions from a photomotor response[25]. While we did not quantify normal activity in the embryonically-exposed killifish, we hypothesize that arsenic exposure either alters their ability to detect food or reduces their normal swimming activity. These changes in behavior as a result of exposure to toxicants can affect the fish's capacity to feed[25, 50], and therefore reduce growth.

In addition to feeding behavior changes, intestinal morphology and numbers of specific cell types might play a role in the ability to uptake nutrients. Indeed, we found a reduction in intestinal area in all exposure groups at 16 weeks of age. Intestinal villus height was a part of the area calculation and height plays an important role in increasing absorptive surface area, which could help increase growth[51]. Furthermore, proliferating cells are essential in the intestine as the cell turnover is typically 5-7 days, at least in zebrafish[32]. In this study, we found a decreasing trend in the number of PCNA cells in a dose dependent manner at 8, 16, and 28 weeks. A decreasing trend in PCNA could indicate a decreased ability for intestinal cellular proliferation, which is needed to maintain the different cell types in the intestine.

One type of cell is the enterocyte, which make up ~80% of the intestinal epithelial cells, and have absorptive functions to take up nutrients[53]. In our study, we found a significant decrease in absorptive surface which is primarily made up of enterocytes, at 16 weeks of age, but not any other time points. This could be associated with the reduction in villus area at 16 weeks as well as a reduction in PCNA cells. If the number of cells is decreased from 8-16 weeks, this could indicate that intestinal cells are not

proliferating at a sufficient rate, therefore decreasing the size of the villus and subsequently the absorptive surface area of enterocytes. Arsenic has been found to cause oxidative damage leading to necrosis in intestinal epithelial tissue in rats exposed to 600 ppb in the drinking water[54]. Overall, the trend in decrease villus height and decrease in absorptive surface at 8 and 16 weeks could be a mechanism involved in reducing condition factor.

4.3 Arsenic exposure alters insulin-like growth factor (IGF) pathway transcript levels

IGF-1 is an important signaling pathway involved in growth of mammals as well as fish that can act in an autocrine, paracrine, and endocrine manner[56, 57]. IGF-1 mRNA expression is modulated by nutrition in a number of fish, and there are strong arguments for IGF-1 as a biomarker of growth in fish[58]. In several fish studies, IGF-1 mRNA expression is directly related to plasma IGF-1 levels[59]. Production of IGF-1 protein is stimulated by growth hormone (GH) produced by the anterior pituitary gland, which then binds to its receptor in the liver to signal hepatic cells to synthesize IGF-1 through the JAK/STAT pathway[59,60]. Then IGF-1 is distributed to tissue types with IGF-1 receptors (IGF-1R) that activate the PI3K-AKT-TOR signaling pathway in the target tissue to increase the expression of genes such as myogenic regulator factors, PCNA, and myostatin[61]. Additionally, *in vitro* or *in vivo* treatment of tilapia with estradiol decreased IGF-1mRNA in the liver, which could help explain why there might be sex differences in growth in the presence of steroid hormones[62]. IGF-1 is bound by binding proteins

(IGFBP) that either transport IGF-1 to its receptors on target tissue, or bind and sequester IGF-1 under stressful conditions such as malnutrition[41, 42, 63]. The interesting correlation in this study is that at 8 weeks, when there is a dose-dependent decrease in condition factor, there is also a significant decrease in liver IGF-1. Another study found that pre-pubertal mice exposed to arsenic had reduced circulating levels of IGF-1 [64]. While hepatic IGF-1 levels follow growth patterns at 8 weeks, when we analyzed 16 and 28 weeks, there was a shift in the hepatic IGF-1 and IGFBP-1 levels where it was upregulated in a dose dependent manner, potentially as a compensatory mechanism to catch up in normal growth.

Our previous study examining embryonically-exposed killifish at 52 weeks of age noticed an increase in muscle IGF-1 levels[39]. We hypothesized that arsenic had reduced liver IGF-1 levels and the fish were compensating by producing more IGF-1 in the skeletal muscle. While the liver is the primary organ of IGF-1 synthesis, other tissues, such as skeletal muscle, can produce IGF-1 if under stress or ablation of the primary hepatic source of IGF-1[65]. For muscle IGFBP-5, the promyogenic binding protein in muscle that increases IGF bioavailability by greatly increasing IGF-1 half life[41, 66], there were increases in its transcript levels at 8 weeks in the fish embryonically exposed to arsenic. While growth was reduced at 8 week and liver IGF-1 levels were decreased, the killifish could have been increasing IGFBP-5 as a compensatory growth mechanism. By 28 weeks of age, we noticed a complete shift in that all exposure groups had reduced muscle IGF-1 and IGFBP-5 levels and increased liver IGF-1 and IGFBP-1 levels. Therefore, this supports the idea that when the main organ of IGF production, the liver, is

not synthesizing or producing IGF in adequate amounts, the other tissue trying to grow such as muscle will produce it in order to compensate for growth deficits[67]. Studies have shown that in *utero* arsenic exposure can decrease birth weight which correlates to reduced IGF levels[20]. While we see reduction in growth and hepatic IGF-1 levels early on, it appears that embryonic arsenic effects are remediated potentially by compensating through increasing local production of muscle IGF until normal hepatic IGF levels are restored.

5. Conclusion

Embryonic only arsenic exposure can produce reductions in growth during juvenile life stages. Reductions in growth might be due to alterations in feeding behavior, reductions in intestinal villus height and absorptive surface, and alterations in IGF-1, IGFBP-1, and IGFBP-5 in liver and skeletal muscle at early time points. This could indicate that while arsenic can impair growth early on, the fish may be able to overcome its effects at these lower levels of arsenic exposure by increasing the production of IGF-1 and IGFBP-5 in skeletal muscle as a growth compensatory mechanism.

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CHAPTER THREE

CONCLUSION

While there is a good amount of literature on arsenic's effects on growth, there is still a need for research on arsenic's effects during the embryonic and fetal period, along with its long term consequences. It is already known that arsenic exposure in *utero* via mother's ingestion of contaminated water is correlated with reductions in IGF-1 plasma levels and weight gain even 4.5 years after exposure [1, 2]. Reductions in plasma IGF-1 have even been found in other animal models where female rats were exposed to 10 mg/kg As^{III}, which resulted in an average daily intake of 575µg/day per female rat which be the equivalent to human daily intake of 57.5 µg based on a 10 fold difference due to differences in metabolism of arsenic between humans and rodents [3]. This study found that arsenic exposure suppressed circulating levels of IGF-1 and authors hypothesized that arsenic exposure reduced hepatocyte viability[3]. As IGF-1 regulates growth during fetal and postnatal periods, if the liver is damaged through oxidative stress, the secretion of hepatic IGF-1 might be impaired[3]. Studies have shown that arsenic exposure increased levels of MDA in hepatic tissue in brown trout indicating lipid peroxidation from oxidative damage[4].

Since arsenic is found all over the world in groundwater at levels that exceed the WHO safe drinking water standard of 10 ppb[8,9] and can easily cross the placental barrier[10] exposure to arsenic is of great concern[11-14]. A previous study found that arsenic levels in the maternal blood (11.7µg/L) and cord blood (15.7µg/L total arsenic) were similar when pregnant mothers were exposed to drinking water containing 90.5µg As/L, which indicates that the fetus might be readily exposed to arsenic levels that could

interfere with growth and development[15]. Similar metabolism and methylation pathways of arsenic occur in aquatic animals and mammals, but fish tend to accumulate higher levels of arsenobetanine and arsenocholine[5]. Our body burden study removed arsenobetanine, which is non-toxic[16], and found that when 16 week-old killifish were exposed to 5 and 10 ppm arsenic, they accumulate 1429 and 2832 μg/kg respectively. Using linear regression, exposures of 10, 50, and 200ppb would result in body burdens ranging from 48 – 101.8 μg/kg. While these levels are of whole body accumulation instead of plasma or urine, it still indicates a fairly high body burden. Typically, a 10-fold safety factor is used in risk assessment when extrapolating from a surrogate species to humans, and another 10-fold safety factor added to account for variability in a population. So, if adverse effects are seen at a 10ppb exposure, and safety factor of 100-fold is included, the body burden levels which are considered to be protective are in the range of 0.4μg/kg. Using the maternal-fetal study above, an exposure of 90μg As/L results in 12 μg/L arsenic in the blood, or that approximately 13% of the ingested arsenic is accumulated. Then you can assume if an adult consumes about 3L of 10ppb arsenic contaminated water a day, this might yield levels of 4 -5μg/L per kg in both the mother and fetus. Studies have estimated that arsenic exposure between 0.3 and 8 μg/kg body weight/day results in a 1% increased risk of lung, skin, and bladder tumors in humans[17]. Additionally, the U.S. Agency for Toxic Substances and Disease Registry's (ATSDR) Minimal Risk Levels (MRLs) for acute oral consumption at 5μg/kg/day and chronic oral consumption at 0.3μg /kg/day arsenic as safe doses for infants[18]. However, our data

suggests that these values might not really be protective enough and that drinking water standards for arsenic may need to be lowered.

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APPENDIX A

Supplementary Figure 1. Arsenics increases SCL15A1b at 8 weeks, a peptide transporter in the intestine, after 10ppb embryonic exposure.

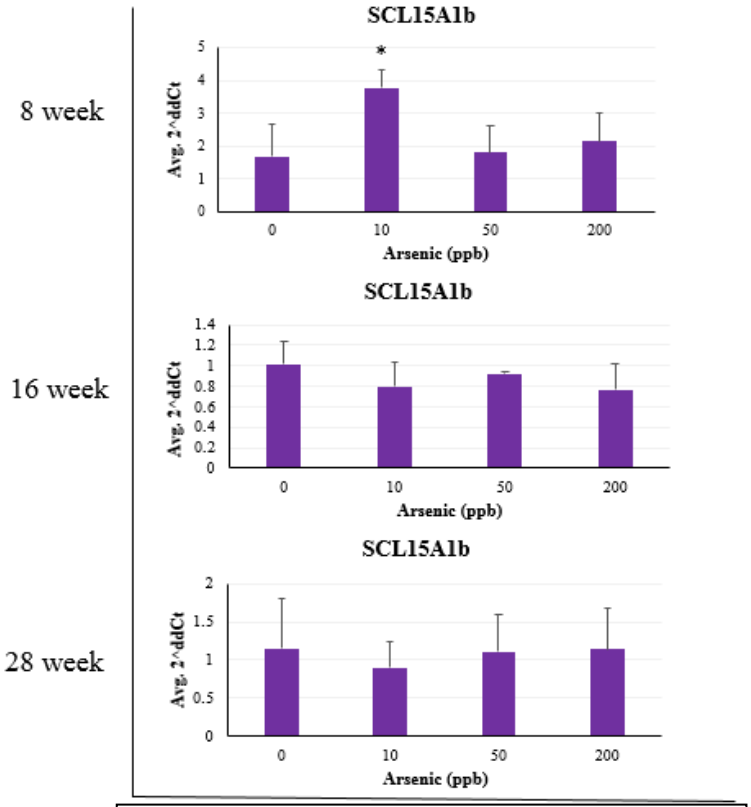

Figure S1. Average SCL15A1b 2^ddCt is compared at 8, 16, and 28 weeks. 8 week time point show significant increase in the 10 ppb when on-way ANOVA was run * $p<0.05$ compared to control. (n=5 per exposure for each time point)