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 800ppb As^{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 no changes in hepatic 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 800ppb 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.

Keywords:

Arsenic; killifish; embryo; IGF-1; IGF-1R; skeletal muscle

1. 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^{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^{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.

2. Methods

*2.1 Killifish (*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.

2.2 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 10.67 μ M; 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.

2.3 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 was 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 recorded 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.

2.4 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.

2.5 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 7 μ m 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 (mm²) to calculate density (fibers/mm²). Average densities for all fish and males/females were determined for each tank.

2.6 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 (2 μ g) 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:

AAACAGATAAACCAACAGGCTATG; IGF-1 Reverse: GCAGCTCACAACCTCTGGAA; IGF-1R Forward: CGCAGAAATGTACGTACCAGA; IGF-1R Reverse: CGTCTTTGACCACACCCTT; 18S Forward: TTTCTCGATTCTGTGGGTGGTGGT; 18S Reverse: TAGTTAGCATGCCGGAGTCTCGTT). 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.

2.7. 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 then used to test the factors, followed by Tukey's ($p < 0.05$). Differences in hatching success and percent survival were analyzed by one-way ANOVA, followed by Tukey's ($p < 0.05$) in GraphPad Prism 4. Differences in condition factors, muscle fiber densities, IGF-1R, and IGF-1 levels were assessed by two-way ANOVA, followed by Bonferroni correction ($p < 0.05$), using GraphPad Prism 4.

3. Results

3.1 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 (data not shown). 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; $\text{weight}/\text{length}^3$) 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.

3.2 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).

3.3 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).

3.4. 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). There were statistical differences in IGF-1R levels between males and females, but these were only significant in the 200ppb exposure group at 28 and 52 weeks (Fig. 6).

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. There are sex differences in the 52 week fish, with males have significantly higher levels of IGF-1 in the 0, 200, and 800ppb exposure groups. We hypothesize that the 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.

4. 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.

4.1. 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, control fish condition factors were actually reduced while the arsenic exposed fish continued to grow, but this was not statistically significant. 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 ($>10\mu\text{m}$) 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).

5. 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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Figure Legend

Fig. 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 $\pm$ SD with $n = 7$ tanks for each exposure group. Statistical significance was determined using one-way ANOVA and Tukey's post-hoc test ($p < 0.05$).

Fig. 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/length³). 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 between exposures ($a = 50\text{ppb}$, $b = 200\text{ppb}$, $c = 800\text{ppb}$) and gender ($\#$) were determined using two-way ANOVA followed by Bonferroni correction ($p < 0.05$).

Fig. 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 a two-way ANOVA and Tukey's post-hoc test through a JMP statistical model ($p < 0.05$).

Fig. 4. Representative images of killifish trunk muscle cross sections. Tissues were formalin fixed, paraffin embedded and stained with hematoxylin and eosin.

Fig. 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 between control and exposures (*) and gender (#) were determined using a two-way ANOVA followed by Bonferroni correction ($p<0.05$).

Fig. 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 between control and exposures (*) and gender (#) were determined using a two-way ANOVA followed by Bonferroni correction ($p<0.05$).

Fig. 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 between control and exposures (*) and gender (#) were determined using a two-way ANOVA followed by Bonferroni correction ($p < 0.05$).

Figure 1.

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