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Embryonic Arsenic Exposure Reduces Intestinal Cell Proliferation and Alters Hepatic IGF mRNA Expression in Killifish (*Fundulus heteroclitus*)

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Abstract

Arsenic (As) is a toxicant found in food and water throughout the world, and studies suggested that exposure early in life reduces growth. Thus, the goal of this study was to examine mechanisms by which As impacted organismal growth. Killifish (*Fundulus heteroclitus*) were exposed to 0, 10, 50 or 200ppb As as embryos, and after hatching, were reared in clean water for up to 40 weeks. Metabolism studies revealed that killifish biotransform As such that monomethylated and dimethylated arsenicals account for 15–17% and 45–61%, respectively, of the total metal. Growth, as measured by condition factor (CF), was significantly and dose-dependently reduced at 8 weeks of age, but was similar to controls by 40 weeks. To determine mechanisms underlying the observed initial decrease, intestinal proliferation and morphology were examined. Arsenic-exposed fish exhibited significant 1.3- to 1.5-fold reduction in intestinal villus height, and 1.4- to 1.6-fold decrease in proliferating cell nuclear antigen (PCNA+) intestinal cells at all weeks examined. In addition, there were significant correlations between CF, PCNA+ cells, and intestinal villus height. Upon examining whether fish might compensate for the intestinal changes, it was found that hepatic mRNA expression of insulin-like growth factor 1 (IGF-1) and its binding protein (IGFBP-1) were dose-dependently increased. These results indicate that embryonic exposure initially diminished growth, and while intestinal cell proliferation remained reduced, fish appear to compensate by enhancing transcript levels of hepatic IGF-1 and IGFBP-1.

Keywords

arsenic; intestine; IGF-1; monomethylarsonic acid (MMA); stem cell; killifish

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Conflicts of interest

The authors declare that no conflicts of interest exist.

Data Accessibility

Data are available upon request (lbain@clemson.edu)

Introduction

Arsenic (As) is a naturally occurring element found in water, sediments, and soils, with the main route of exposure being through drinking water (NRC 2014; Singh et al. 2015). The most commonly found species in aqueous environments are the inorganic forms, arsenite (As^{III}) and arsenate (As^{V}), with arsenite as the predominate species in groundwater (Ventura-Lima et al. 2011; Jha et al. 2017). Currently, the World Health Organization (WHO) has set the standard for As in drinking water at 10 ppb, although higher levels of metal are found all over the world (Naujokas et al. 2013). Even in the U.S., As levels above 10 ppb may be found in wells, exposing roughly 13 million households to high metal concentrations in their drinking water (Chappells et al. 2014; Frederick et al. 2016). In addition to water, As has also been found in food (Nacano et al. 2014; Gundert-Remy et al. 2015). Levels of 200 $\mu\text{g}/\text{kg}$ As were detected in brown rice, 130 $\mu\text{g}/\text{kg}$ in white rice, and 70 $\mu\text{g}/\text{kg}$ in other color rice (Zavala and Duxbury 2008; Diaz et al. 2015). Rice-based infant snacks can range from 36.5–568 ng/g total As (Naujokas et al. 2013; Karagas et al. 2016).

Investigators often focused on As as a carcinogen (Barbosa 2017) but less is known regarding its effects on development and growth (Golub et al. 1998). It is known that As crosses the placental barrier and exposes the developing fetus (Vahter 2009), as evidenced by similar As levels in maternal (11.7 $\mu\text{g}/\text{L}$) and fetal (15.7 $\mu\text{g}/\text{L}$) blood (Hall et al. 2007). Exposure to As (<100 ppb) during pregnancy was associated with a 1.68 g reduction in birth weight for each 1 $\mu\text{g}/\text{L}$ rise in urinary metal concentration (Rahman et al. 2009). A European study examining prenatal exposure to chemical mixtures noted that As was the only chemical in the mixture to exhibit a significant inverse correlation to birth weight (Campbell et al. 2016). In addition, increasing As levels in infants were associated with decreased IGF-1 plasma levels and reduced birth weight (Ahmed et al. 2013). Similarly, female offspring of mice exposed to 10 ppb As both *in utero* and postnatally weighed 18–23% less than controls at 42 days of age (Kozul-Horvath et al. 2012). In killifish, embryonic exposure to 50–800 ppb As reduced growth up to 40 weeks of age, and altered skeletal muscle satellite cell proliferation during both normal development and in response to an injury (Szymkiewicz et al. 2017; 2018).

While As-mediated effects on skeletal muscle development have been investigated, less is known regarding metallic actions on the intestine, a key organ involved in the uptake of As from drinking water and food. It is known that As is readily taken up into intestinal cells *in vitro* (Calatayud et al. 2010), and that this exposure generates superoxide radicals, which reduce the levels of claudins and impair tight junction functioning (Jeong et al. 2017). In addition, As and its methylated metabolites increase expression of tumor necrosis factor alpha, IL-6, and IL-8 in intestinal Caco-2 cells (Calatayud et al. 2014). Arsenic exposure is also known to alter the gut microbiome at both the community and functional level (Lu et al. 2014).

Intestinal morphogenesis and development is similar in mammals and fish, although most fish lack true crypts and have instead proliferative compartments at the base of the villus (Ng et al. 2005; Chin et al. 2017). 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 (Olden et al. 2008; Takashima et al. 2013; Brugman 2016). As in mammals, epithelial cells in zebrafish migrate from the base to the tip of the intestinal fold within 5–7 days (Ng et al. 2005). Cell division in crypts is dependent upon Wnt/ β -catenin signaling, in which the Lgr5+ stem cells differentiate into progenitor or transit amplifying (TA) cells (Pinto et al. 2003). From the progenitor cells, the Notch ligand Delta turns on transcription factors such as Hes1 to specify enterocyte fate (Takashima et al. 2013; Merker et al. 2016) while the Ascl and Atoh transcription factors play roles in specification of goblet and enteroendocrine cells (Roach et al. 2013).

Previous investigators demonstrated that As exposure during cellular development and differentiation decreased the levels and/or functioning of many transcription factors, including Ascl, β -catenin, and Hes (Liu and Bain 2014; Bain et al. 2016). Since these same transcription factors are also important in intestinal development, it was postulated that As exposure might impair intestinal cell turnover. Thus, the current study used killifish (*Fundulus heteroclitus*) to assess whether embryonic As exposure produced changes in intestinal morphology and/or cellular differentiation, thereby leading to diminished organismal growth.

Materials and methods

Exposure of killifish embryos to arsenic

Adult killifish (*Fundulus heteroclitus*) were collected from the North Inlet National Estuarine Research Reserve site in Georgetown, SC, a relatively uncontaminated area, 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 hr light/dark cycle at 26°C in 18 ppt salt water (CoraLife). Adult killifish were fed Ziegler Brothers adult zebrafish food (Gardners, PA) twice per day. Eggs were stripped from three to 4 females and milt collected from two males to fertilize approximately 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 or 200 ppb As, as sodium arsenite, with 7 replicate petri dishes per metal concentration. Water changes were done every other day, and embryos monitored daily for viability and 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 approximately 4 weeks of age, and subsequently transitioned to Zeigler adult zebrafish food. Sampling time points were at 8, 16, and 40 weeks of age. All procedures were approved by Clemson's Institutional Animal Care and Use Committee (IACUC).

Assessing arsenic metabolite formation

Additional eggs were fertilized and juveniles grown in control water for 16 weeks. Fish were exposed to 0, 5, or 10 ppm As as sodium arsenite (n= 6 tanks per exposure level; each tank had 2–3 killifish) for 5 days in a static renewal system to assess metallic uptake and metabolism. Higher arsenic levels were used to increase the ability to detect multiple arsenical species. Fish were fed twice per day, and water changes conducted once a day. Fish were euthanized in buffered MS-222, rinsed in clean water, patted dry, and frozen whole at

–80°C. In order to have sufficient tissue mass for speciation, fish from three tanks were combined per exposure group, yielding two replicates per concentration. The As concentration and speciation in our adult fish food (Ziegler Brothers zebrafish pellets) was also determined.

All processing and metallic 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 As 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.

Killifish growth measurements

At each sampling time point (8, 16, and 40 weeks of age), 5–6 fish per tank were randomly sampled and euthanized in 1 g/L buffered MS-222. These times were selected to examine growth from the juvenile to sexually mature adult stages, and enable comparisons to other studies (D'Amico et al. 2014; Szymkowicz et al. 2017). Fish were patted dry, and weight, body length and intestinal length measured. As a measurement of changes in body size as the fish aged, condition factor ($\text{weight}/\text{length}^3$) for each fish was calculated, and averaged per tank. At the 40 week sampling point, when it was possible to visually determine sex, three males and three females were sampled per tank. Anterior intestinal sections were fixed overnight in 10% buffered formalin, dehydrated in graded ethanol, embedded in paraffin, cut into 7 μm transverse sections, and placed on slides. Livers were collected and placed in RNAlater, snap-frozen in liquid nitrogen, and stored at –80 °C until further processing.

Intestinal morphology

Morphological changes in the intestines of embryonically exposed fish were examined by hematoxylin and eosin (H&E) staining ($n=6$ fish per exposure per sampling time point). 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.

Immunohistochemistry

Paraffin embedded intestinal sections (7 μm) were examined for markers of absorptive enterocytes (gut absorptive cell epitope antibody; Biorbyt, # orb324078), while the number of proliferating cells was determined by expression of proliferating cell nuclear antigen (PCNA) (Abcam, #ab29). Sections underwent antigen retrieval in Tris-EDTA buffer, pH 9, and were then quenched with 3 % hydrogen peroxide for 10 min. Blocking was in avidin-biotin (Vector Labs, Burlingame, CA) for 30 min and then 10 % horse serum (Vector ABC Elite Kit) for 17 min. After washes, the appropriate dilution of primary antibody (PCNA 1:750; absorptive cells 1:400) was added and incubated overnight at 4 °C. Tissues sections were washed, biotinylated secondary antibody added, and Vectastain ABC reagent

(Biotinylated horseradish peroxidase + Avidin DH) 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 were 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 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.

Reverse transcription and quantitative PCR

RNA was extracted from liver using TRIzol (Sigma-Aldrich, St. Louis, MO) and treated with DNase I. Purity and RNA concentration were determined using a NanoDrop Lite and Quant-IT RNA Assay kit (Life Technologies, Carlsbad, CA). RNA (2 µg) was reverse-transcribed to cDNA using MMLV-RT and random hexamers. To conduct qPCR, 40 ng cDNA, RT² SYBR Green mix (Qiagen, Alameda, CA), and gene-specific primers were run in triplicate on a iQ5 thermocycler (BioRad). For 8 and 16 weeks, 5 fish per exposure group were randomly selected to run per gene. At 40 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. A no-template control and melt curve were also conducted to assess the specificity of the reaction. Levels of insulin-like growth factor-1 (IGF-1; forward: 5'-AAA CAG ATA AAC CAACAG GCT ATG-3'; reverse: 5'-GCA GCT CAC AAC TCT GGA A-3'; 54 °C) and insulin-like growth factor binding protein 1 (IGFBP-1; forward: 5'-CAT GGC TCT GTG CAC TAC AT-3'; reverse: 5'-ATC GCG TTA ACT CTG GCT TT-3'; 59 °C) were determined. Samples were run in triplicate and gene expression data was normalized with both 18S rRNA (forward: 5'-TTT CTC GAT TCT GTG GGT GGT GGT-3'; reverse: 5'-TAG TTA GCA TGC CGG AGT CTC GTT-3'; 60 °C) and β 2 microglobulin (forward: 5'-CAA AGA GAC TCC ACC CAA AGT-3; reverse: 5'-AGA GGA GGT CGA TGG TGA T-3'; 53°C). Additional details on the qPCR reactions are provided in Supplementary Table 1. Ct values from the two reference genes were averaged per sample and comparisons between treatment groups made using the comparative threshold (Ct) method (Livak and Schmittgen 2001). All comparative threshold averages were compared to control fish at each time point, which was set at a value of 1.

Statistical analysis

To analyze changes in condition factor, feeding behavior, intestinal morphology, number of PCNA⁺ cells, and 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 tank or sex, such that data from fish in each exposure group at a given time point were averaged together. For transcript expression in the liver, villus height, number of PCNA⁺ cells, and condition factor, multivariate ANOVA was used to test equality of the two variables across the doses. If MANOVA suggested differences in the 4 doses, multivariate contrasts were used to determine significances of the two variables between specific sets of

doses (JMP). This enabled us to determine the exact nature of the dose impact of the two variables being analyzed at a given time point. Data was considered significant if $p < 0.05$.

Results

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

To determine whether killifish metabolize As into mono- and dimethylated metabolites in a manner similar to humans, 16-week old killifish were exposed to 0, 5, or 10 ppm arsenite, and levels of As (III), As (V), monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenobetaine (AsB) quantified. AsB was the only arsenical species present in control fish, and levels did not change due to metallic exposure (average = 417 ± 62 $\mu\text{g/kg}$; data not shown). DMA, MMA, As (III), and As (V) were below limits of detection (LOD) in control fish, and values increased in a dose-responsive manner in the 5 and 10 ppm treatment groups (Figure 1) with DMA accounting for 45–61% and MMA accounting for 15–17% of total arsenicals. These % are similar to paired maternal and newborn blood samples exposed to 90 ppb As in their water, in which $43 \pm 9\%$ of total As was DMA and $31 \pm 6\%$ was MMA (Hall et al. 2007). The adult fish food contains arsenical species, but $> 98\%$ of them are arsenobetaine and arsenocholine, with As (III) levels below the LOD. Arsenite in our water is also below LOD (data not shown). These data indicate that killifish readily metabolize As, and thus may be used as models for human exposures.

Embryonic arsenic exposure reduces condition factors in 8 week old killifish

To assess whether embryonic-only As treatment altered growth in juvenile and adult killifish, condition factors (CF; weight/length^3) were determined. Killifish were exposed to environmentally-relevant concentrations of As as embryos, which included 10 ppb (the current drinking water standard), 50 ppb (the former drinking water standard), and 200 ppb (considered a high exposure, but still routinely found in many countries). After hatching, fish were transferred and grown in water without the addition of metal. No marked differences in hatchling success or survival up to 40 weeks of age were seen (data not shown). In past studies, no significant changes in weight, length, or CF are seen in < 24 hr old hatchling fish, even at As concentrations up to 25,000 ppb (Gaworecki et al. 2012; D'Amico et al. 2014). As the fish age, CF gradually rose. As a result of arsenic exposure, at 8 weeks of age, CF was significantly reduced by 20 % in the 200 ppb embryonic exposure group (Figure 2). There was no significant effect detected in CF at 16 and 40 weeks of age (Figure 2). An analysis separating out males and females was conducted, since sex was visually determined at this age, but no significant differences in CF were observed between male and female fish.

Embryonic arsenic exposure reduces villus height and number of PCNA+ cells in intestine

One possibility for the early growth impairment in As exposed fish is a reduction in the number and/or turnover of cells in the anterior intestine. First, hematoxylin and eosin (H&E) staining was used to quantify average intestinal villus height by measuring from the base to the tip of the villi (Figure 3A). Villus height gradually increased with age, such that the villi height in the 40 week old fish was nearly double that of the 8 week old fish. Embryonic As treatment resulted in significantly lower intestinal villus height by 1.3- to 1.5-fold in the 16

week and 40 week old fish (Figure 3B), while villus width did not change markedly over the course of the study (data not shown).

Next, intestinal cell proliferation was assessed by measuring the number of PCNA+ cells (Figure 4A). Data demonstrated that embryonic As exposure significantly decreased the number of PCNA+ proliferating cells by 1.4- to 1.6-fold at 8, 16, and 40 weeks after treatment (Figure 4C). However, there was no marked difference in the % of villi covered by enterocytes, which was typically approximately 50% (Figure 4D), and no significant alterations in number of goblet cells, which averaged 3.5–5 cells per villus (Figure 4E).

Experiments were also undertaken to examine whether reductions in the number of PCNA+ cells resulted in shorter intestinal villi. Indeed, at 8 weeks post-exposure, villus height and PCNA+ cells were significantly diminished in the 200 ppb group (Figure 5A). All treatment groups exhibited significant correlations between villus height and PCNA+ cells at 16 (Figure 5B) and 40 weeks of age (Figure 5C). These results suggest altered intestinal cell turnover leads to reduction in villus height. It was postulated that the decrease in intestinal cell proliferation might impair fish ability to grow at an appropriate rate. Indeed, reductions in the number of PCNA+ cells significantly correlate with decrease in CF in 8-week old fish, with an r^2 -value of 0.94 (Figure 5D). At 16 and 40 weeks of age, no such correlations existed even though the number of PCNA+ cells remains significantly lower (Figure 5E and 5F).

Arsenic exposure alters hepatic IGF-1 and IGFBP-1 transcripts

Since intestinal cell turnover was impaired throughout the life of the fish, yet growth in metal-exposed fish was similar controls, compensatory mechanisms were examined. In fish, IGF-1 directly controls larval (Kaneko et al. 2011) and adult fish growth rates (Duan 1998; Reindl and Sheridan 2012). Since liver is the primary organ for IGF-1 synthesis, hepatic IGF-1 and IGF-1 binding protein (IGFBP-1) transcript levels were determined. At 8 weeks, there are no differences between arsenic exposure groups and the control group (Figure 6A). By 16 weeks of age, hepatic IGF-1 and IGFBP-1 were significantly increased in the 50 and 200 ppb group (Figure 6B). At 40 weeks of age, hepatic IGF-1 and IGFBP-1 mRNA levels remain upregulated in the 200 ppb As-exposed fish (Figure 6C).

The relationships between hepatic IGF-1, IGFBP-1, and CF were also examined. At 8 weeks of age, the 200 ppb As-exposed group is significantly different from the controls with respect to hepatic IGF-1 transcripts and CF (Figure 7A). At 16 weeks, the livers of the exposed fish are transcribing large amounts of IGF-1 mRNA in both the 50 ppb and 200 ppb As-exposed groups (Figure 7B), resulting in nearly equivalent CF by 40 weeks of age (Figure 7C). The pattern was similar between IGFBP-1 and CF. At 8 weeks of age, IGFBP-1 mRNA levels were less in the fish treated with As as embryos (Figure 8A). At 16 weeks of age, exposed fish exhibited elevated IGFBP-1 mRNA levels in a dose-dependent manner (Figure 8B). Even as CF increased, hepatic IGFBP-1 levels continued to be significantly elevated in the 200 ppb group (Figure 8C).

Discussion

Embryonic arsenic exposure impairs intestinal cell proliferation

Data showed that intestinal villus height was reduced by 1.3- to 1.5-fold and remained decreased throughout the life of the fish. This reduction is similar to previous studies with other metals. High levels of dietary nickel administered to one day-old chickens for 42 days diminished intestinal villus height (Wu et al. 2013). Villus height is dependent upon the number of proliferating and differentiating cells arising from the base of the villus, and thus shortened villi may be attributed to reductions in stem cell proliferation. Indeed, at all weeks examined post-exposure, the number of dividing cells, as measured by PCNA expression, were also significantly decreased in As-exposed fish by 1.2- to 1.5-fold. There was also a significant correlation between PCNA+ cells and intestinal villus height, as well as a correlation between PCNA+ cells and reductions in CF.

It is known that diminished nutrient availability decreases intestinal stem cell numbers and reduces proliferation (Ross and Mayhew 1985). While it is not possible to definitively state that nutrient availability was altered in the growing fish, animal size was reduced early during the juvenile period. Arsenic treatment was found to decrease the expression of several transcription factors, such as β -catenin, Ascl1, and Hes1 and 5 (Hong and Bain 2012; Bain et al. 2016), which are needed to convert intestinal stem cells into absorptive and enterendocrine cells (Roach et al. 2013; Takashima et al. 2013; Merker et al. 2016). Although an attempt to examine the numbers of specific intestinal cell types was made, the only antibody that recognized killifish epitopes was for absorptive cells. While overall % relative to other cells remained constant at approximately 50%, given that the height of the villus was reduced following As exposure suggests that the total absorptive capacity of the fish was likely diminished.

This is one of the few studies to determine the influence of As on the intestine. It is known that As may be readily absorbed from drinking water and food, and crosses the cell membrane of Caco-2 intestinal cell lines (Calatayud et al. 2010; Sun et al. 2012). In Caco-2 and human colorectal HT-29 cells, As treatment reduced barrier functions (Laparra et al. 2005; Chiocchetti et al. 2018) by decreasing the expression of the tight junction proteins claudin-1 and claudin-5 (Jeong et al. 2017). *In vivo*, As was found to produce oxidative damage leading to necrosis in intestinal epithelial tissue of rats exposed to 600 ppb As (Acharyya et al. 2015).

Embryonic arsenic exposure alters hepatic IGF-1 and IGFBP-1 expression

IGF-1 is an important growth signal in both mammals and fish (Bower and Johnston 2010; Reindl et al. 2011). Hepatic IGF-1 protein production is stimulated by growth hormone via the JAK/STAT pathway (Vélez et al. 2018) and secreted into the blood stream where it binds IGF-1 receptors (IGF-1R) on cells such as skeletal myocytes. The binding activates the PI3K-AKT-TOR signaling pathway to enhance the expression of genes such as myogenin, PCNA, and myostatin, thereby increasing skeletal muscle proliferation and differentiation (Rechler and Clemmons 1998; Fuentes et al. 2013).

IGF-1 is postulated to be one of the most important modulators of growth in fish (Duan 1998; Kaneko et al. 2011; Vélez et al. 2018). Szymkowicz et al (2017) previously reported that skeletal muscle IGF-1 transcript levels were elevated in 16, 28, 40, and 52 week old killifish exposed to 200 and 800 ppb As as embryos. Significant 1.6- to 10-fold reductions in expression of hepatic IGF-1 and IGFBP-1 mRNA were seen at 8 weeks of age following an embryonic exposure of 10, 50, and 200 ppb metal. Epidemiological studies found that *in utero*, As exposure decreased birth weight, which correlates with reduced plasma IGF-1 levels (Ahmed et al. 2013). While the just-hatched fish in our study were not weighed, 8-week old killifish exhibited lowered CF that are significantly associated with decreased hepatic IGF-1 and IGFBP-1 transcript levels. Reilly et al (2014) showed that pre-pubertal mice exposed to As contained lower circulating levels of IGF-1 protein. IGF-1 and IGFBP-1 can be postrationally modified (Bach 2018), but it is known that IGF-1 protein levels in the blood are directly correlated to hepatic IGF-1 mRNA levels (Reindl and Sheridan 2012). Although protein levels of IGF-1 were not examined in the current study, measuring hepatic transcript levels of IGF-1 and IGFBP-1 appears to be a reasonable substitute.

The As-exposed fish as 8 week of age displayed reduced IGF-1 and IGFBP-1 transcripts, however, by 16 weeks of age, there was a complete shift such that all treatment groups showed increased hepatic IGF-1 and IGFBP-1 mRNA levels. It is conceivable that fish embryonically exposed to As need to compensate to enhance growth by first increasing production of skeletal muscle IGF-1 transcripts (Szymkowicz et al. 2017) followed by rising hepatic IGF-1 transcripts later on. In the killifish, hepatic IGF-1 and IGFBP-1 mRNA levels are correlated with each other. When examining the relationship between CF and IGF-1 or IGFBP-1, the 8 week old fish exhibited reductions in IGF-1 and IGFBP-1 levels that correlate with decreasing CF. As fish age, they begin to transcribe large amounts of both IGF-1 and IGFBP-1. Growth in the embryonically-exposed fish was similar to controls by 40 weeks of age; however, the level of IGFBP-1 transcripts remains significantly elevated in treated fish.

The tight positive correlation between IGF-1 and IGFBP-1 is somewhat unexpected. The expression of the two proteins are typically thought to oppose each other, since high amounts of IGFBP-1 often inhibit interaction of IGF-1 with its receptor (Jansson et al. 1998; Maures and Duan 2002; Lang et al. 2003; Wheatcroft and Kearney 2009). However, IGFBPs do stabilize and prolong circulating IGF in the blood stream, with unbound IGF having a half-life of ~10 minutes and bound IGF having a half-life of ~15 hours (Guler et al. 1989). IGFBP-1 may readily be induced during stress, injury, hypoxia, or malnutrition and is postulated to prevent IGF-1 signaling in order to shift energy away from growth and development (Maures and Duan 2002; Kajimura et al. 2005; Shimizu and Dickhoff 2017). However, with long-term fasting and re-feeding studies, IGFBP-1 often remains elevated during the re-feeding period. Peterson and Waldbieser (2009) found after a 30 day fasting period in catfish followed by 15 days of re-feeding that hepatic IGF-1 levels were restored to control levels while hepatic IGFBP-1 mRNA levels remained significantly elevated. In humans, caloric restriction for 8–12 weeks increases both total and bioactive serum IGF-1 and IGFBP-1 serum levels (Espelund et al. 2005; Arciero et al. 2008; Belobrajdic et al. 2010). Indeed, as the killifish aged in our study, similar trends in which both hepatic IGF-1 and IGFBP-1 transcripts remain elevated were noted. Although feeding was not restricted in

our study, it is possible that reductions in intestinal surface area and stem cell turnover in the killifish embryonically exposed to As may result in less absorption of nutrients.

The chronic consequences of elevated IGF-1 and IGFBP-1 in fish are not known, although studies in fish showed significant correlations between CF and IGFBP-1 expression (Kawaguchi et al. 2013; Taniyama et al. 2016). In humans, elevated serum IGFBP-1 is associated with reduced muscle mass (Stilling et al. 2017) and a higher risk of mortality in older adults (Kaplan et al. 2008; Nolte et al. 2015).

Interestingly, the IGF-1 receptor is expressed in intestinal stem cells and stimulation by IGF-1 enhanced proliferation (Van Landeghem et al. 2015). Conversely, decreases in IGF-1 results in reductions in intestinal growth in rats (Ballinger et al. 2000). Although it was not possible to accurately measure IGF-1R and IGF-1 levels in intestinal cells, it is conceivable that decreases in hepatic IGF-1 levels might be a mechanism by which intestinal proliferation is lowered. At 8 weeks of age, IGF-1 transcripts in the liver were lowered, which significantly correlates with a reduction in the number of proliferating, PCNA+ cells (data not shown). However, even as IGF-1 mRNA levels in hepatic tissue rose during later time periods, the number of PCNA+ cells was not significantly correlated. Perhaps the role of IGF-1 in intestinal stem cells is different in fish than mammals. It is known that in *Drosophila*, there is little response of intestinal stem cells to IGF during periods of starvation, but these proliferate in response to IGF-1 during refeeding (O'Brien et al. 2011). Whether this occurs in killifish remains to be determined.

Overall, the results of this study show that embryonic only As exposure reduced growth and intestinal cell proliferation during juvenile life stages. Fish growth in the exposed fish ultimately returned to control levels, in part due to increased expression of hepatic IGF-1 and IGFBP-1 transcripts. However, decreased intestinal villus height, lower intestinal cell turnover, and elevated levels of hepatic IGF-1 and IGFBP-1 remain long after the exposure has ended.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Arsenic metabolism to monomethylated (MMA) and dimethylated (DMA) species. Sixteen week-old killifish were exposed to 5 or 10ppm arsenite for 96 hr (n=2 replicates; each replicate had 15–20 fish). Speciation was performed by Brooks Applied Labs using IC-ICP-CRC-MS.

Figure 2.

Embryonic arsenic exposure on condition factor. . At each sample time point, weight and length were measured for 6–8 fish per tank, and used to calculate condition factor (CF) (weight/length³). Values were averaged for each tank (n = 7 per exposure group) and statistical differences (*) were determined using one-way ANOVA followed by Tukey's post-hoc ($p < 0.05$).

Figure 3.

Intestinal villi height following embryonic arsenic treatment. Anterior intestinal cross sections were stained with hematoxylin and eosin ($n = 6$ per exposure group per time point). Representative images (10X) are shown (A) and used to quantify the height (mm; indicated by orange bars) of 5 villi per field of view using ImageJ by measuring from the base to the tip of the villus (B). Statistical differences (*) were determined by ANOVA followed by Tukey's ($p < 0.05$).

Figure 4.

The number of proliferating cells in anterior intestine in embryonically-exposed killifish. Immunohistochemistry was used to examine the number of proliferating cells as measured by nuclear staining of PCNA (A; indicated by arrows), the number of goblet cells (B; indicated by arrows), and % enterocytes or absorptive surface (B) (representative images are shown of $n = 6$ fish per exposure group per time point). The number of PCNA+ cells were counted per 2 intervillus regions at a 40X field of view, and averaged per villus (C). Absorptive surface area was quantified by outlining 4 villi per field of view at 10X for absorptive surface and determining % stained cells using ImageJ (D). Goblet cells were counted from 4 villi per field of view at 10X, and averaged per villus (E). The colors were converted to a black and white image so only cytoplasmic staining of the enterocytes would be determined. All sections were averaged per group, and statistical significance (*) was determined by ANOVA followed by Tukey's ($p < 0.05$).

Figure 5.

Correlations between intestinal villus height, the number of PCNA+ cells, and condition factor (CF). Linear regression was used to correlate intestinal villus height (mm) and the number of PCNA+ cells, as well as CF and number of PCNA+ cells ($n = 6$ fish per exposure group per time point). Data are presented as the average $\pm$ S.D. deviation for each parameter and includes villus height versus PCNA+ cells (A = 8 weeks; B = 16 weeks; C = 40 weeks), and PCNA+ cells versus condition factor (D = 8 weeks; E = 16 weeks; F = 40 weeks).

MANOVA followed by multivariate contrasts (JMP) were used to assess whether arsenic exposure significantly altered the combinations in a dose-dependent manner (*; $p < 0.05$).

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Figure 6.

Arsenic exposure effect on hepatic IGF-1 and IGFBP-1 transcript levels. At 8 weeks (A), 16 weeks (B), and 40 weeks (C), hepatic IGF-1 and IGFBP-1 mRNA levels were determined by qPCR and quantified using the comparative threshold method ($2^{-\Delta\Delta C_t}$) using 18S rRNA and $\beta 2$ microglobulin as reference genes ($n = 6$ fish per exposure group per time point). Data are presented as the average $\pm$ S.D. deviation of each gene transcript. Then, linear regression, MANOVA, and multivariate contrasts (JMP) were used to assess whether arsenic exposure

significantly altered the combination of IGF-1 and IGFBP-1 in a dose-dependent manner (*; $p < 0.05$).

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Figure 7.

Arsenic-exposed fish transcribe large amounts of IGF-1 even after condition factors have leveled out.

The correlation between fold differences in IGF-1 mRNA levels and CF was assessed by linear regression ($n = 6$ fish per exposure group per time point). Data are presented as the average $\pm$ S.D. deviation for each parameter. Then, MANOVA followed by multivariate contrasts (JMP) were used to assess whether arsenic exposure significantly altered the

combination of IGF-1 and condition factor in a dose-dependent manner at 8 weeks (A), 16 weeks (B), or 40 weeks-post exposure (C) (*; $p < 0.05$).

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Figure 8.

Arsenic-exposed fish transcribe large amounts of IGFBP-1 even after condition factors have leveled out. The correlation between fold differences in IGFBP-1 mRNA levels and CF was assessed by linear regression ($n = 6$ fish per exposure group per time point). Data are presented as the average $\pm$ S.D. deviation for each parameter. Then, MANOVA followed by multivariate contrasts (JMP) were used to assess whether arsenic exposure significantly

altered the combination of IGF-1 and condition factor in a dose-dependent manner at 8 weeks (A), 16 weeks (B), or 40 weeks-post exposure (C) (*, $p < 0.05$).

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