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Arsenic Exposure to Killifish During Embryogenesis Alters Muscle Development

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Epidemiological studies have correlated arsenic exposure in drinking water with adverse developmental outcomes such as stillbirths, spontaneous abortions, neonatal mortality, low birth weight, delays in the use of musculature, and altered locomotor activity. Killifish (*Fundulus heteroclitus*) were used as a model to help to determine the mechanisms by which arsenic could impact development. Killifish embryos were exposed to three different sodium arsenite concentrations and were collected at 32 h post-fertilization (hpf), 42 hpf, 168 hpf, or < 24 h post-hatch. A killifish oligo microarray was developed and used to examine gene expression changes between control and 25-ppm arsenic-exposed hatchlings. With artificial neural network analysis of the transcriptomic data, accurate prediction of each group (control vs. arsenic-exposed embryos) was obtained using a small subset of only 332 genes. The genes differentially expressed include those involved in cell cycle, development, ubiquitination, and the musculature. Several of the genes involved in cell cycle regulation and muscle formation, such as fetuin B, cyclin D–binding protein 1, and CapZ, were differentially expressed in the embryos in a time- and dose-dependent manner. Examining muscle structure in the hatchlings showed that arsenic exposure during embryogenesis significantly reduces the average muscle fiber size, which is coupled with a significant 2.1- and 1.6-fold upregulation of skeletal myosin light and heavy chains, respectively. These findings collectively indicate that arsenic exposure during embryogenesis can initiate molecular changes that appear to lead to aberrant muscle formation.

Key Words: **arsenic; *Fundulus*; muscle; embryonic; CapZ; development; microarray.**

INTRODUCTION

Arsenic is found in water systems throughout the world, and although the Environmental Protection Agency and World Health Organization have lowered the acceptable limits of arsenic in drinking water to 10 ppb, there are still many areas of the world in which arsenic concentrations far exceed this limit (Mandal and Suzuki, 2002; Smedley and Kinniburgh, 2002). Chronic exposure to arsenic can result in adult-onset diseases, such as cancer, diabetes, skin lesions, and cardiovascular disease (Guha Mazumder, 2008; Mink *et al*., 2009; Platanias, 2009; Schuhmacher-Wolz *et al*., 2009; States *et al*., 2009). Additionally, arsenic can also impact the developing embryo (Concha *et al*., 1998; Jin *et al*., 2010; Xie *et al*., 2007). Most notably, *in utero* arsenic exposure results in increases in the rates of miscarriages, stillbirths, and neonatal mortalities (Cherry *et al*., 2008; Hopenhayn *et al*., 2003; Milton *et al*., 2005; Rahman *et al*., 2007, 2009; Vahter, 2009; von Ehrenstein *et al*., 2006; Watanabe *et al*., 2003). *In utero* arsenic exposure has been shown to decrease body weight in humans and rodents (Hill *et al*., 2008; Petrick *et al*., 2009; Rahman *et al*., 2009; Rodríguez *et al*., 2002; Xi *et al*., 2009). Embryonic arsenic has also decreased body length in zebrafish (Li *et al*., 2009), growth in tilapia (Tsai and Liao, 2005), and increased incidences of deformities in killifish (*Fundulus heteroclitus*) (Gonzalez *et al*., 2010).

Along with the reductions in birth weight, studies indicate that arsenic can have a negative effect on muscle development. Japanese infants who were accidentally fed arsenic-contaminated formula had delays in walking ability, which was assessed through follow-up evaluations of these patients during adolescence (Dakeishi *et al*., 2006). Epidemiological evidence also indicates that patients exposed to 100 ppb arsenic in drinking water displayed limb weakness or atrophy, which was confirmed by electromyography (Mukherjee *et al*., 2003).

The changes in muscle development seen in human epidemiological studies have also been confirmed in rodent and fish models. Arsenic exposure can inhibit muscle regeneration in mice (Yen *et al*., 2010) and alter muscle protein composition both in fish and in mice (Gonzalez *et al*., 2006; Lantz *et al*., 2008; Palaniappan and Vijayasundaram, 2008; Yen *et al*., 2010). Additionally, there is increased spontaneous locomotor activity and altered muscle contractile activity in rodents exposed to

arsenic in drinking water (Bardullas *et al*., 2009; Gur *et al*., 2005; Li *et al*., 2009; Rodríguez *et al*., 2001, 2002). In C2C12 mouse myoblast cells, exposure to arsenic resulted in delayed myoblast differentiation and reduced numbers of multinucleated myotubes caused by a decrease in the expression of myogenin, the transcription factor needed to convert myoblasts into myotubes (Steffens *et al*., 2010; Yen *et al*., 2010).

Although arsenic has been shown to alter muscle growth and development during embryogenesis, the mechanism behind this developmental toxicity is unclear. One of the few studies investigating the effects of arsenic on early cellular differentiation demonstrated downregulated genes indicative of ectoderm, mesoderm, and endoderm lineage in human embryoid bodies exposed to arsenic for 15 days (Flora and Mehta, 2009).

Killifish have been used as an embryonic and developmental model for many years as they produce large translucent eggs in which embryos develop over a period of 10–14 days. There are numerous studies that indicate that toxicant exposure to killifish alters reproduction and development, including increased embryonic mortality and reduced growth, developmental deformities, changes in reproductive success, steroid hormone levels, and steroid hormone-producing enzymes (reviewed in Burnett *et al*., 2007) in a manner similar to mammalian species. Thus, the goal of the present study was to examine the mechanisms by which the embryonic arsenic exposure can alter muscle development using killifish as a model.

METHODS

Fish culture. Adult killifish (*F. heteroclitus)* were collected from the North Inlet-Winyah Bay National Estuarine Research Reserve (Georgetown, SC) and transported to Clemson University where they were allowed to acclimatize for at least 2 weeks. Fish were maintained at 26°C with a 16:8 light:dark cycle in 18 ppt seawater and fed TetraMin flake food (Blacksburg, VA) twice daily. Water renewals (80%) were performed three times weekly.

Pilot study. In order to determine appropriate exposure concentrations, a pilot study was conducted using sodium arsenite (Fisher Scientific) to provide arsenic concentrations ranging from 0.3 to 100 ppm. Briefly, adult female killifish were stripped of eggs, whereas males were euthanized and their milt poured over the eggs for fertilization. Eggs were then divided into Petri dishes with 31–48 eggs ($n = 3$ per concentration) and monitored for survival and hatching success. Test water was renewed and eggs examined daily. There were no significant differences in hatching success up to 50 ppm (Fig. 1), so arsenic concentrations of 5, 15, and 25 ppm were chose for the full study.

Arsenic exposure. Eggs were collected and fertilized as described above. Fertilized eggs were divided into Petri dishes containing 40 eggs and cultured in 0, 5, 15, or 25 ppm arsenic as sodium arsenite ($n = 10$ replicate Petri dishes per concentration per time point). Test water was renewed and eggs examined daily. Embryos were collected at four different time points during development: 32 h post-fertilization (hpf) (7–10 somites), 42 hpf (20–26 somites), 168 hpf (mid-development), and < 24 h post-hatch. Hatchlings were euthanized in MS-222. Embryos and fish were then stored either in formalin solution at 4°C or were snap frozen and stored at −80°C. An additional set of eggs was used to determine arsenic body burdens. Fertilized eggs were placed into Petri dishes containing 31 ± 6 eggs each ($n = 5$ replicate Petri dishes per exposure concentration) and cultured in 0, 5, 15, or 25 ppm arsenic. Fish were collected within 2 h of hatching, rinsed in clean water, and euthanized in MS-222. Weights and lengths were measured, and fish were stored at −55°C. For each replicate, all hatched fish were pooled, microwave digested, and arsenic concentrations analyzed by inductively coupled plasma optical emission spectrometry with a detection limit of 10 ppb.

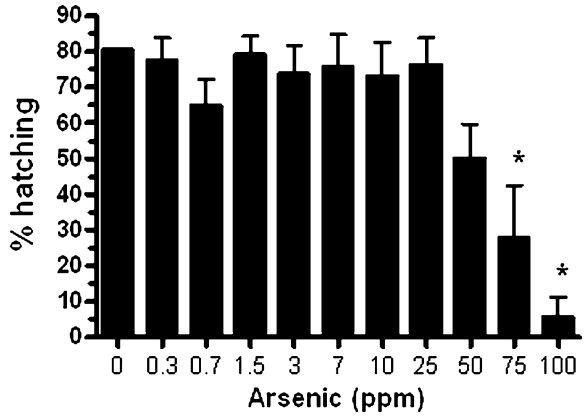

FIG. 1. Hatching success of killifish eggs exposed to arsenic. To determine the appropriate arsenic concentrations to use, a pilot study was conducted. After fertilization, embryos were exposed to 0–100 ppm arsenic as sodium arsenite ($n = 31$–48 eggs per dish, three dishes per concentration). The embryos were allowed to develop and hatch. Data are represented as average percent hatch per dish ± standard deviation. Statistical significances from control were determined using ANOVA followed by Dunnett's (*$p < 0.05$).

Microarrays. A 4 × 44-K long oligo microarray consisting of 21,821 unigenes (each gene printed twice) was manufactured by Agilent Technologies (Santa Clara, CA). The 60-bp oligonucleotide probes were designed from *F. heteroclitus* sequences available in GenBank using Agilent's eArray system. The standard Agilent control probes were also included in the array platform. Details on the array can be found on the Gene Expression Omnibus (GEO) website under platform GPL13522.

Hatchlings from the control and 25-ppm exposure groups were used for microarray analysis. Four to five hatchlings from a single Petri dish were pooled together to obtain RNA ($n = 10$ per exposure group). RNA was extracted using Qiagen's RNeasy Mini Elute kit with an on-column DNase treatment. The concentration and quality of the RNA were assessed using a 2100 Bioanalyzer (Agilent) and a NanoDrop ND-1000 Spectrometer (NanoDrop Technologies). Total RNA (700 ng) was amplified and labeled with Cy3 dye using the One-color Quick Amp Labeling kit following manufacturer's protocol (Agilent Technologies). The quality of the Cy3-labeled complementary RNA (cRNA) was assessed using the NanoDrop. Cy3-labeled cRNA (1.65 μg) hybridized to the microarray for 17 h at 65°C and then washed according to manufacturer's protocol (Agilent Technologies). The microarrays were scanned immediately following the last wash using an Agilent Array Scanner (Model # G2505B). The scanned images were extracted with Agilent Feature Extraction version 10.7.3. The data for this series of experiments can be accessed using GEO series accession number GSE29976.

Data analysis. The background subtracted intensity values from each scan was subjected to variance stabilized normalization (VSN) and the resulting VSN values for each gene were subjected to a *t*-test for differences between the treatment groups. The 5000 genes with the lowest *p* values from the *t*-test were selected for analysis via artificial neural networks (ANNs) following procedures previously published (Chapman *et al*., 2009). The ANN analysis was proceeded by randomly selecting 80% of the samples from control and experimental groups to train the ANNs and retained the remaining samples as a cross-validation (cv) set. Following 20 rounds of ANN training with random selection of samples for each session, the sensitivities of each gene were computed for all iterations. The genes with sensitivities in the top 250 for at least five models ($n = 332$) were then

selected for a further round of ANN training and cv with 20 iterations and the same random selection of samples for each iteration.

The 332 genes identified above were also subject to a reverse analysis in which the experimental conditions were mapped to individual genes. This is equivalent to ANOVA analysis. This mapping was done to generate the expected gene expression levels in control and arsenic-exposed samples, and the residuals from this analysis were subjected to modified modularity clustering (MMC) (Stone and Ayroles, 2009). This effort was designed to identify modules of coregulated genes. The gene pairs with $r^2 > 0.7$ from the MMC were then used in Cytoscape to construction coregulation networks (Smoot *et al.*, 2011).

Gene expression. Quantitative PCR (qPCR) was used to examine differential expression of several genes from embryos collected at 32, 42, and 168 hpf exposed to 0, 5, 15, or 25 ppm arsenic ($n = 6$ replicates per exposure and time period). qPCR was conducted using SYBR Green for detection (RT^2 SYBR Green; SABiosciences, Frederick, MD). Gene selection was based upon the MMC analysis for the hatchlings and is found in modules 1 and 15 (Supplementary table S-1). The selected genes are involved in cell cycle regulation and muscle development and include fetuin B (FetB; #CN955747; forward: 5′-TAGGAAGGACTGGAAGCAATGCGA-3′; reverse: 5′-TGAAAGTTGAGCAGTCGGGACAGA-3′), cyclin D-type–binding protein 1 (CDBP1; #EV460252, forward: 5′-GCCCACACGGTTTACATCTACCAA-3′; reverse: 5′-TTCTGCAGGAGGTTCAGGTGTTCA-3′), actin filament capping protein Z (CapZ; #CN983867; forward: 5′-TCCTCAGCAGATCGAGAAGAACCT-3′; reverse: 5′-CGTCGCGATTGTAATCACAGAGCA-3′), and skeletal troponin (Tnnc2; #EV464250, forward: 5′-ATGGAGACAAGAACAACGACGGCA-3′; reverse: 5′-TTTCACAGGAGGTGGGAGGAATGT-3′). *Fundulus* myosin light chain 2 (MLC2), which was previously shown to be increased following arsenic exposure (Gonzalez *et al.*, 2006), was examined (MLC2; #EV412164; forward: 5′-AGAACAGAGACGGCATCATCAGCA-3′; reverse: 5′-AGCTCCTCGTTCTTCACGTTCAGT-3′). Myosin heavy chain 2 (MHC2) was also examined, using primers designed after clustering sequences from *Siniperca chuasti* (#HQ829290), *Danio rerio* (#XM003200911), and *Oryzias latipes* (#AB258223) (MHC2; forward: 5′-GAAGAAAGCCCTCCAAGAGGC-3′; reverse: 5′-CTCTTGGCTCTCTCAAGGTCC-3′). 18S ribosomal RNA (rRNA) (#M91180; forward: 5′-TTTCTCGATTCTGTGGGTGGTGGT-3′; reverse: 5′-TAGTTAGCATGCCGGAGTCTCGTT-3′) was used as a housekeeping gene. Samples were run in triplicate, and relative gene expression was calculated using the comparative threshold (Ct) method (Livak and Schmittgen 2001). Messenger RNA (mRNA) levels of the selected genes in the 32-hpf samples were below the detection limit and are therefore not shown.

Histology. Hatchlings were fixed in 10% formalin, stepwise dehydrated in ethanol, and embedded in resin blocks using the Polysciences Immuno-Bed Kit. Blocks were sectioned at 5 μm and stained with azure II and basic fuchsin. Muscle cross-sections were taken from the post-anal trunk, and density and diameter were calculated using Image J Software (National Institutes of Health) from five to seven organisms per treatment. To calculate muscle density, individual muscle fibers were counted and this value was divided by measured skeletal muscle area, omitting skin, red muscle, centrum (neural tube and notochord), and neural and hemal arches. Muscle diameters were measured on two randomly selected muscle fibers per 0.7 μm^2 grid sections along one half of each tissue section. This resulted in measurement of an average of 140 ± 22 ($n = 100$–186) fibers per organism to determine size distribution.

RESULTS

Effects of Arsenic on Hatching Success and Growth

A pilot study was conducted to determine the appropriate arsenic concentrations for embryonic exposure. Exposure concentrations of 75 or 100 ppm significantly reduced embryonic survival. Although survival in the 50-ppm exposure group was not statistically different from controls, it was reduced by 38% compared with control (Fig. 1). Thus, the full study was conducted by exposing the embryos to 0, 5, 15, or 25 ppm arsenic as sodium arsenite. Hatching success of the embryos in the full study was not significantly different in arsenic-exposed groups compared with controls (Table 1), similar to that found in the pilot study. Condition factor of the organisms was also not significantly different, although there was a downward trend in the higher exposure groups (Table 1).

TABLE 1
Hatching Success and Condition Factor of Killifish Eggs Exposed to Arsenic

Arsenic concentration (ppm)	Hatching success (%)	Condition factor[a]
0	88.2 ± 11.1	0.65 ± 0.08
5	91.0 ± 11.6	0.62 ± 0.05
15	94.3 ± 5.3	0.62 ± 0.11
25	98.0 ± 4.9	0.61 ± 0.05

Note. Data are the average ± standard deviation ($n = 5$–10 replicate Petri dishes per concentration).

[a]Condition factor is 100,000 × weight in grams/cubic length in millimeter.

A second set of embryos was used to determine arsenic body burdens. Control fish had arsenic concentrations of 2.3 ± 2.5 μg/g wet weight, whereas the 5, 15, and 25 ppm groups contained 3.4-, 10.3-, and 18.7-fold higher arsenic body burdens, respectively (Fig. 2). These data indicate that arsenic can readily cross the chorion and enter into the embryo.

Differential Gene Expression After Embryonic Arsenic Exposure

Next, the genes differentially expressed in embryonically exposed killifish offspring were examined. The r^2 values from the initial ANN run mapping 5000 genes to control and arsenic-exposed groups are shown in Table 2. For the analysis

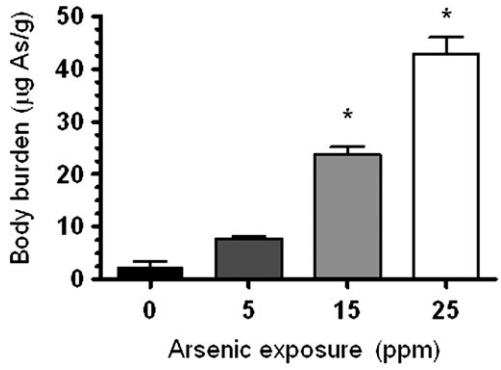

FIG. 2. Arsenic body burdens in hatchlings after embryonic arsenic exposure. Fish were collected within 2 h after hatch to determine arsenic body burdens via inductively coupled plasma ($n = 5$ replicates per concentration; each replicate had 9–33 hatchlings). Data are represented as average arsenic concentration (micrograms arsenic per gram wet weight) ± standard deviation. Statistical significances were determined using ANOVA followed by Dunnett's ($*p < 0.05$).

TABLE 2
Results of the ANN Analysis for the 5000 and 332 Gene Sets

	5000 genes			332 genes		
Run	Number of nodes	r^2 model	r^2 CV	Number of nodes	r^2 model	r^2 CV
1	5	1	0.255	5	1	1
2	11	1	0.795	5	1	1
3	8	1	0.866	5	1	1
4	8	1	0.874	6	1	1
5	8	1	0.838	14	1	1
6	5	1	0.757	7	1	1
7	11	1	0.911	10	1	1
8	8	1	0.888	9	1	1
9	8	1	0.795	5	1	1
10	8	1	0.835	4	1	1
11	5	1	0.719	10	1	1
12	5	1	0.109	7	1	1
13	5	1	0.849	10	1	1
14	11	1	0.923	9	1	1
15	5	1	0.795	7	1	1
16	8	1	0.814	5	1	1
17	5	1	0.995	5	1	1
18	12	1	0.030	5	1	1
19	8	1	0.802	6	1	1
20	8	1	0.850	14	1	1

of the 5000 gene set, the training data set was 100% accurate for all 20 models with cv $r^2 > 0.7$ in all but three cases. In the reduced (332) gene set, the model and cv $r^2 = 1$ for all 20 models, indicating that the ANN modeling generated extremely accurate and robust prediction of experimental treatment versus controls using a very small gene set.

To find coregulated genes in the 332 gene set, MMC was employed. The MMC analysis of residuals found 16 modules (Fig. 3) of highly correlated gene expression profiles. Many of these genes are involved in cell cycle control, protein ubiquitination and degradation, bone morphogenesis, axonal regeneration, and muscle regulation (Supplementary table S-1). Module 1 from the MMC only contains two genes, fetuin B and cyclin D–binding protein 1 (CDBP1), both of which are involved in cell cycle control. Module 2 contains a variety of genes that act as transcription factors or are involved in signal transduction pathways (retinoid X receptor α, Zn3h15, and Arts1) and those involved in protein ubiquitination and degradation (Fbp32, Rfp7, and Usp18) (Supplementary table S-1).

Modules 3–7 contain few genes whose functions can be assigned, except for the ribosomal proteins (protein synthesis) and cytochrome C oxidase (energy metabolism) which are typically downregulated by environmental stress (Chapman *et al.*, 2009). In modules 8–10, there is one significant muscle transcript, desmin, which is an intermediate filament associated with the Z-band of sarcomeres (Bär *et al.*, 2004). Modules 11 and 12 contain a number of genes associated with immune response, energy metabolism, and along with module 13, contain

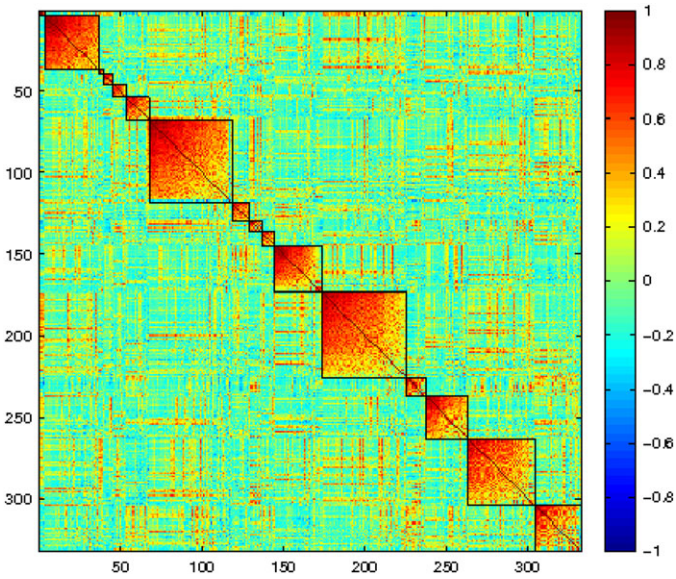

FIG. 3. Output of the MMC clustering showing the modules with highly correlated ($r > 0.7$) expression.

genes needed for cellular development and differentiation (β-catenin, BMP1b, and transmembrane 4l six family member 5).

Module 14 contains the Prox1 gene, which is required for transcriptional regulation of alpha-actinin, nebulin-related anchoring protein, and zyxin, which function to maintain the actin-alpha-actinin association of the sarcomere (Risebro *et al.*, 2009). Module 15 from the MMC indicated that genes involved in the musculature, such as troponin C2, ataxin 2, glycogen synthase kinase 3β, and CapZ were altered.

Because the genes differentially expressed after MMC appeared to be mainly involved in cell cycle control and the musculature, we wanted to determine (1) whether muscle structure was altered and (2) examine changes in gene expression in the embryos to determine when during development, the alterations first occurred and whether they persisted throughout embryogenesis. First, several genes from the arrays involved in cell cycle control were examined using RNA from embryos at 32, 42, and 168 hpf. Module 1 from the MMC only contains two genes, fetuin B and CDBP1, both of which are involved in cell cycle control. Expression of these two genes was not detectable in the 32 hpf fish (data not shown). qPCR indicated that CDBP1 was significantly upregulated in the 42 hpf samples by 27- and 51-fold in the 15- and 25-ppm exposure groups, respectively (Fig. 4). At 168 hpf, CDBP1 expression was not significantly different between the exposure groups. Expression of fetuin B was undetectable at 42 hpf but was significantly increased in the 15- and 25-ppm exposure groups by 43- and 72-fold, respectively, at 168 hpf (Fig. 4).

Arsenic Alters Muscle Gene Expression and Reduces Muscle Fiber Size

Module 15 from the MMC indicated that genes involved in the musculature, such as troponin C2 and CapZ were altered

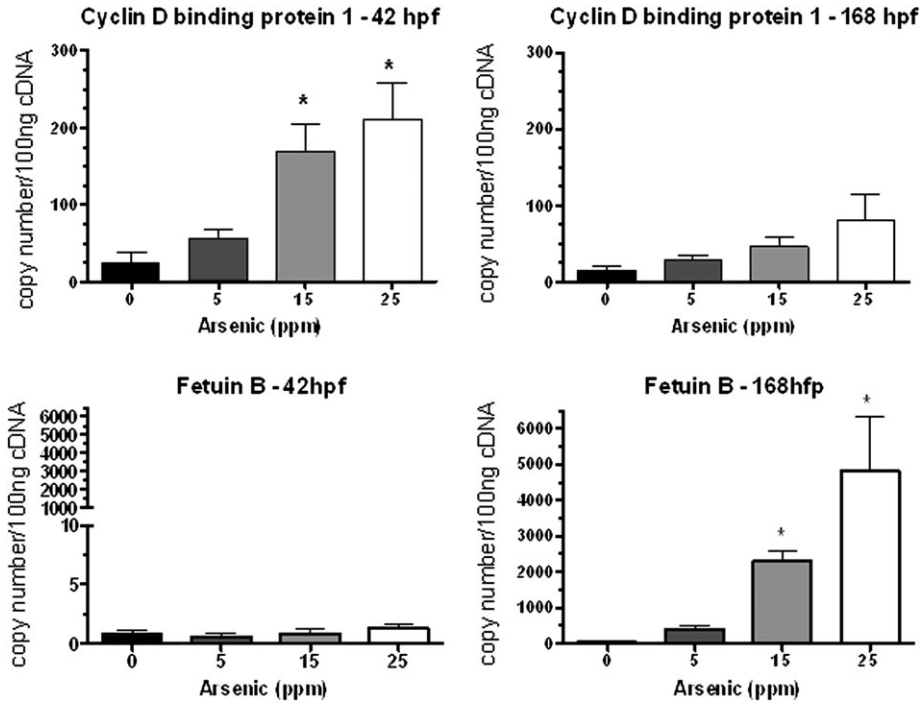

FIG. 4. Module 1 genes are induced in a time- and dose-dependent manner. The expression of CDBP1 and fetuin B (FetB), the only two genes in module 1, was examined at 42 h post-fertilization (hpf) and 168 hpf using qPCR. Gene expression was normalized to 18S rRNA and is expressed as the relative fold change ($n = 6$ replicates per exposure and time period). Each run was repeated twice. Statistical significance was determined using ANOVA followed by Dunnett's (*$p < 0.05$).

(Fig. 5A). Troponin is part of the component that regulates muscle contraction by control myogenin binding access to actin, whereas CapZ caps the barbed ends of actin in muscle cells and is located in the muscle Z-band. We examined changes in expression of troponin and CapZ in the 42 hpf embryos, the 168 hpf embryos, and the hatchlings by qPCR. Troponin expression was not significantly changed due to arsenic exposure (Fig. 5B). Although CapZ expression was undetectable at 42 hpf, it was significantly increased in the 25-ppm exposure groups by 92- and 5-fold, respectively, in 168 hpf embryos and in the hatchlings (Fig. 5B).

We then examined whether muscle fiber formation was altered in the offspring. Arsenic exposure during embryogenesis appeared to dramatically alter muscle fiber formation in a dose-responsive manner (Fig. 6A). As the concentration of arsenic increases, the muscle fibers in the embryo appear to be more loosely bundled together, suggesting a decrease in muscle tissue organization. The number of fibers in a given area does not differ between control and arsenic-exposed fish (Fig. 6B). However, arsenic exposure significantly reduces the size of muscle fibers (Fig. 6C). The control fish muscle fiber sizes are normally distributed, with most fibers ranging from 7 to 12 mm in diameter. Arsenic exposure skews the distribution so that most fibers range from 4 to 9 mm. Indeed, there is a significant fold increase in the number of small fibers in the arsenic-exposed embryos, whereas the percentage of large muscle fibers is reduced by 1.6- to 7.5-fold in the arsenic exposure group (Table 3). Finally, MLC2 and MHC2 expressions are significantly altered in the hatchlings, being induced by 2.1-fold and 1.6-fold, respectively (Fig. 6D).

DISCUSSION

The findings of this study indicate that embryonic arsenic exposure can alter genes important in the cell cycle, the ubiquitination pathway, and muscle formation and function. This resulted in a significant reduction in muscle fiber size and altered expression of MLC2, MHC2, and CapZ in the hatchlings embryonically exposed to arsenic. These findings may help to explain how arsenic lowers birth weight (Llanos and Ronco, 2009) and alters locomotor activity (Bardullas *et al*., 2009; Gur *et al*., 2005; Li *et al*., 2009; Rodríguez *et al*., 2001, 2002).

The microarray data indicated that the transcription of genes involved in muscle formation and function were among those altered in the hatchlings (Fig. 5; Supplementary table S-1). Additionally, glycogen synthase kinase 3β is downregulated in the arsenic-exposed hatchlings. This protein phosphorylates β-catenin (also downregulated, module 12, Supplementary table S-1) that, among other functions, anchors the actin cytoskeleton. Indeed, a significant, very large upregulation in CapZ was seen both in the 168 hpf embryos and in the hatchlings (Fig. 5). CapZ caps actin filaments at the barbed end

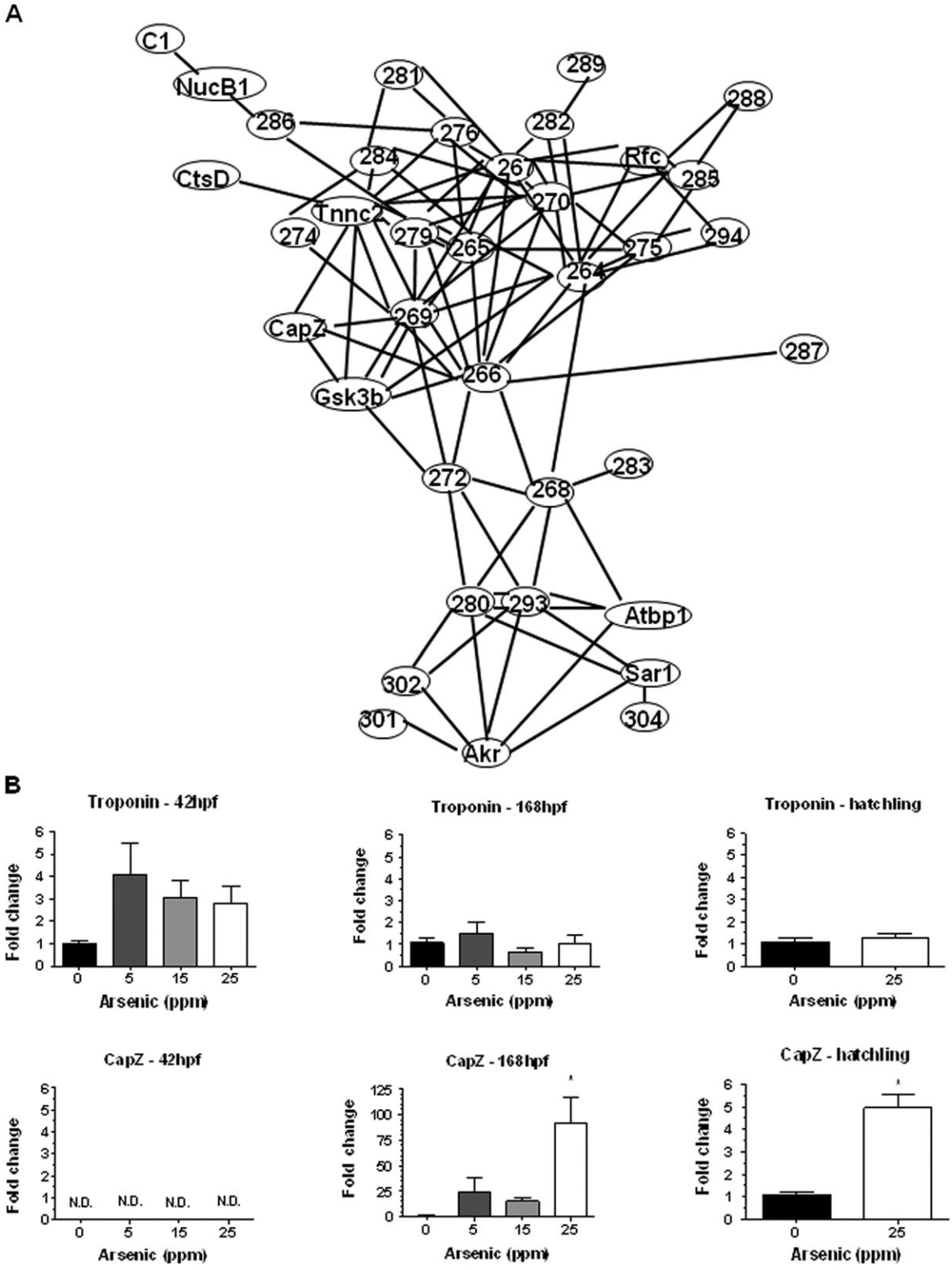

FIG. 5. Module 15 genes involved in musculature are altered. (A) The interconnectivity between module 15 genes is shown, with known gene products labeled. The numbers represent expressed sequence tags. Accession numbers, gene names, and fold changes are listed in Supplementary table S-1. (B) qPCR was used to examine the expression of troponin (Tnnc2) and actin capping protein Z (CapZ) in the 42 hpf embryos, 168 hpf embryos, and hatchlings. Data are the average fold change ± standard deviation ($n = 6$ embryos or 10 hatchlings per group). Statistical significances were determined using ANOVA followed by Dunnett's (42 and 168 hpf; $*p < 0.05$) or Student's *t*-test (hatchlings; $*p < 0.05$).

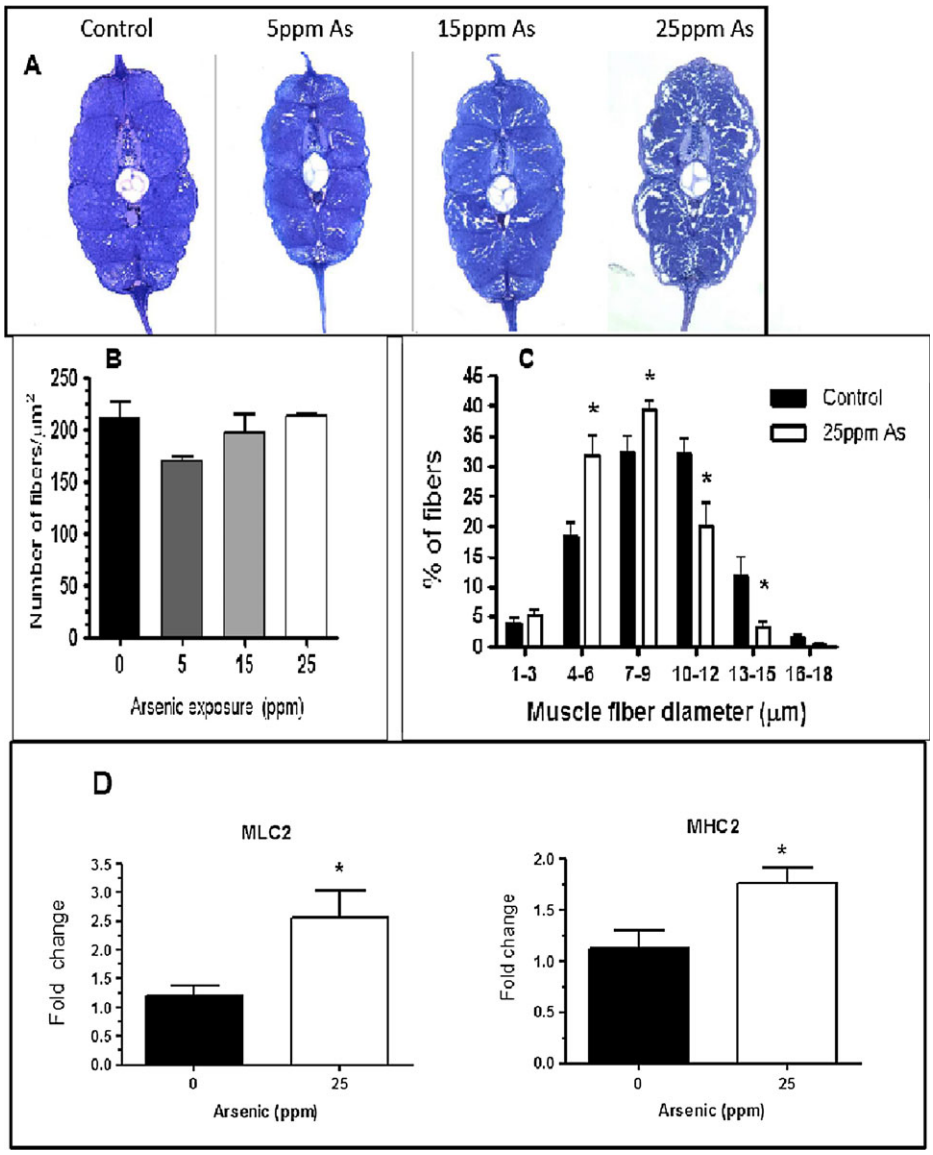

FIG. 6. Arsenic exposure alters muscle fiber size and myosin gene expression. Embryos were exposed to arsenic and, after hatch, were fixed, embedded, cut in 5-μm cross-section of the tail region, and stained using azure II and basic fuchsin. (A) Pictures were taken at ×10 resolution and representative examples for each exposure group are shown. Muscle fiber density and diameters were calculated using Image J software. Two areas were measured per 0.7 μm^2 along one longitudinal half of each tissue section. (B) Individual fibers were counted and divided by the muscle area. Data are the average ± standard deviation ($n = 5$–7 hatchlings) and statistical significance was determined using Kruskal-Wallace followed by Dunn's ($^*p < 0.05$). (C) Muscle diameter was calculated from 140 ± 22 ($n = 100$–186) fibers per organism to determine size distribution. Data are the average ± standard deviation ($n = 5$–7 hatchlings). Statistical significance was determined using Student's *t*-test ($^*p < 0.05$). (D) qPCR was used to examine the expression of MLC2 and MHC2. Data are the average fold change ± standard deviation ($n = 10$ hatchlings). Statistical significance was determined using Student's *t*-test ($^*p < 0.05$).

and at the Z line and thereby controls filament length, stiffness of actin filament networks, and normal thin filament assembly (Schafer *et al.*, 1995; Xu *et al.*, 1999). In the sarcomere, myosin filaments are set up after actin, although the interactions between myosin and filaments are needed to prevent altered myofibril dimensions (Soeno *et al.*, 1999).

Our laboratory has previously shown that killifish offspring whose parents were exposed to arsenic had upregulated expression of proteins involved in the musculature, such as MLC2 and tropomyosin (Gonzalez *et al.*, 2006). It is worth noting that the two studies are not entirely comparable because in the current study, killifish were exposed as embryos and the examined offspring were < 24 h old, whereas in the previous study, the parents (gametes) were exposed and changes in gene expression examined in the offspring at 6 weeks post-hatching. However, our data in the current study also show an increased expression of MLC2 and MHC2 in the arsenic-exposed hatchlings (Fig. 6). Similarly, in chicken atrioventricular canal explants undergoing an epithelial-mesenchymal transition, arsenic exposure upregulated several myosin heavy chain

TABLE 3
Muscle Fiber Diameter Changes in Arsenic-Exposed Killifish

Fiber size (μm)	Fold change in arsenic-exposed fish
1–3	1.4
4–6	1.7*
7–9	1.2*
10–12	1.6*
13–15	3.8*
16–18	7.5

Note. Data are the fold change in the percentage of fibers at a particular class size.

*Statistically different using Student's *t*-test ($p \leq 0.05$).

isoforms and myozenin 2, which is expressed in cardiac and slow twitch skeletal muscle (Lencinas *et al*., 2010), whereas *in utero* arsenic exposure and examination of 18-day-old rat fetal lungs indicate that skeletal slow troponin expression changes as well (Petrick *et al*., 2009). Thus, muscle-related gene expression appears to be a common pathway changed during embryonic arsenic exposure, and the alteration of these genes would be expected to negatively impact muscle formation.

Arsenic's effects on muscle formation have been seen in previous *in vitro* and rodent studies. For example, exposure of mouse skeletal myoblast cells to sodium arsenite delays or prevents their formation into myotubes (Steffens *et al*., 2010; Yen *et al*., 2010). Arsenic exposure alters or prevents muscle regeneration after injury (Yen *et al*., 2010). The mechanism for this delay in muscle formation is, in part, due to a reduction in myogenin (Steffens *et al*., 2010; Yen *et al*., 2010), the transcription factor that controls differentiation from myoblasts to myotubes.

Additionally, muscle fiber sizes were altered in the hatchlings, such that arsenic reduced the number of large muscle fibers while not altering the overall density of muscle fibers (Fig. 6). In cultured 3T3 cells, arsenic also resulted in a loss of thick cables of actin filaments, and those retained thin filaments were spatially disorganized (Li and Chou, 1992). One of the questions that remain to be answered is what a reduction in fiber size means for the organism. Mice with a conditional knockout of myogenin, such that myogenin expression is turned off just prior to birth, have smaller muscle fiber diameters than wild-type mice (Meadows *et al*., 2008). The conditional myogenin knockouts also are 30% smaller than wild-type mice (Knapp *et al*., 2006). Indeed, other studies have shown a greater percentage of small muscle fibers in animals with low birth weights (Handel and Stickland, 1987). Therefore, reduced muscle development and smaller muscle fiber diameter from embryonic arsenic exposure may lead to low birth weight. In our study, we saw a dose-dependent downward trend in weight at hatch, but this was not statistically significant. Human epidemiological studies have reported birth weight reductions in babies whose mothers were exposed to arsenic in drinking water (Hopenhayn *et al*., 2003; Rahman *et al*., 2009). Whether the low birth weight in humans is due to only to species differences or is from changes due to maternal exposure prior to fertilization and gestation is unknown. Additional studies will be needed to determine whether the altered muscle development persists and what the resultant consequences are.

Only a few other studies have examined transcriptional changes after embryonic exposures to arsenic and nearly all have looked at the liver rather than the whole embryo. For example, Mattingly *et al*. (2009) exposed zebrafish embryos to 0.1 ppm sodium arsenite and found no genes differentially expressed using traditional parametric or nonparametric tests. However, by relaxing their p value, they could discern that a cluster of transcripts involved in the proinflammatory response was altered in the embryos exposed to arsenic. An earlier study exposed zebrafish to 15 ppm arsenic as arsenate to also examine gene expression changes in liver. Their data, categorized via gene ontology, indicated that genes involved in stress response, such as heat-shock proteins, genes involved in protein biosynthesis and protein ubiquitination, and the cytoskeleton were all upregulated (Lam *et al*., 2006). Ubiquitin is one gene that had previously been shown to be upregulated following embryonic arsenic exposure in both killifish and zebrafish (Gonzalez *et al*., 2006; Mattingly *et al*., 2009). In the present study, the probe for ubiquitin itself was not on the array; however, a number of genes involved in the ubiquitination process, such as Rfp7, ubiquitin-specific protease 18 (Usp18), F box protein 32 (Fbp32), Fbp25, and proteasome beta type 10 (Psmb10) were changed (Supplementary table S-1). Likewise, other studies examining gene expression in livers of zebrafish exposed to arsenic have also seen ubiquitin-related genes upregulated (Lam *et al*., 2006).

In two similar studies examining transplacental arsenic exposure of 85 ppm sodium arsenite, one examined livers of 18-day-old fetuses (Liu *et al*., 2007), whereas the other examined livers in newborn pups (Xie *et al*., 2007). In the newborns, genes involved in glutathione metabolism were upregulated, whereas P450 genes and genes in the insulin-like growth factor (Igf) signaling pathway were downregulated. In the fetal livers, proteasomal genes were induced and Igf-binding protein genes and P450 genes involved in steroid metabolism were repressed (Liu *et al*., 2007). Other than a single HSP 70 transcript, we did not find heat shock, inflammatory, or P450 transcripts altered in our study but that may be due to both species differences and differences associated with using a whole organism rather than a particular tissue, such as the liver. However, we deem it more likely that the analytical tools employed relegated these genes to less important roles compared with the genes selected for the final analysis. In other words, genes typically associated with stress were less important to the discrimination of arsenic exposure that the genes selected for analysis. Using the whole organism, we would likely detect only the genes that were highly expressed in one or a number of tissues or genes with large fold changes in expression between control and arsenic exposure.

However, an interesting finding in the transplacental rodent studies is the consistent downregulation of Igf signaling proteins. One of the functions of Igf in an organism is to increase muscle mass. Indeed, deletion of Igf-1 or the Igf-1 receptor reduces muscle mass (Liu *et al*., 1993), whereas overexpression of Igf increases myoblast differentiation (Coleman *et al*., 1995). In the two transplacental rodent array studies described above and in a third study transplacental study, arsenic consistently suppresses the Igf signaling pathway (Liu *et al*., 2006). We would hypothesize that the Igf suppression along with the reduction in myogenin levels seen in previous studies (Steffens *et al*., 2010; Yen *et al*., 2010) are perhaps the mechanisms behind the reduction in muscle growth in the killifish embryos exposed to arsenic.

In summary, the present study indicates that embryonic arsenic exposure results in transcriptional changes in genes involved in the cell cycle, development, ubiquitination, and the musculature, which reduces the average muscle fiber size such that large diameter fiber formation is significantly reduced. These findings indicate that arsenic exposure during embryogenesis can initiate molecular changes that appear to lead to aberrant muscle formation, which may help explain the mechanisms by which arsenic reduces body weight and alters locomotion.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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