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Dose-Responsive Gene Expression Changes in Juvenile and Adult Mummichogs (*Fundulus heteroclitus*) After Arsenic Exposure

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Abstract

The present study investigated arsenic's effects on mummichogs (*Fundulus heteroclitus*), while also examining what role that gender or exposure age might play. Adult male and female mummichogs were exposed to 172ppb, 575ppb, or 1,720ppb arsenic as sodium arsenite for 10 days immediately prior to spawning. No differences were noted in the number or viability of eggs between the groups, but there was a significant increase in deformities in 1,720ppb arsenic exposure group. Total RNA from adult livers or 6-week old juveniles was used to probe custom macroarrays for changes in gene expression. In females, 3% of the genes were commonly differentially expressed in the 172 and 575ppb exposure groups compared to controls. In the males, between 1.1-3% of the differentially expressed genes were in common between the exposure groups. Several genes, including apolipoprotein and serum amyloid precursor were commonly expressed in either a dose-responsive manner or were dose-specific, but consistent across genders. These patterns of regulation were confirmed by QPCR. These findings will provide us with a better understanding of the effects of dose, gender, and exposure age on the response to arsenic.

Keywords

Arsenic; *Fundulus heteroclitus*; fish; apolipoprotein; serum amyloid precursor protein; arrays; gene expression

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1. Introduction

Arsenic is an element present in water bodies throughout the world, due to both natural and anthropogenic processes (Mandal and Suzuki, 2002; Smedley and Kinniburgh, 2002). Because of its widespread presence, both the U.S. Agency for Toxic Substances and Disease Registry and the World Health Organization have classified arsenic as the number one substance of health concern in the world (ATSDR, 2007; NRC, 1999). Long-term exposure to arsenic is toxic to organisms. For example, epidemiological studies have shown correlations between disease and human exposure to arsenic in drinking water in Taiwan, Chile, Mexico, Japan, Germany and Bangladesh (reviewed in Bates et al., 1992; Engel et al., 1994; Col et al., 1999; NRC, 1999; Tseng et al., 2000; Yoshida et al., 2004; Chen et al., 2007; Díaz-Villaseñor et al., 2007; Hays et al., 2008; NRC, 1999; Raqib et al., 2009). Disease outcomes reported include increases in hyperpigmentation, keratosis, cancer, diabetes, cardiovascular disease, respiratory illnesses, and developmental and reproductive problems (Bates et al., 1992). In light of the epidemiological findings, the U.S. Environmental Protection Agency has placed arsenic as one of the top pollutants of environmental concern, and implemented a reduction in the drinking water standard from 50ppb to 10ppb starting in 2006 (EPA, 2001). However, the effects of arsenic at these low exposure levels are not yet clearly understood (NRC, 1999).

In aquatic organisms, sublethal exposure to arsenic results in several deleterious effects, such as immune system suppression in zebrafish (*Danio rerio*) and in the Indian catfish (*Clarias batrachus*) (Datta et al., 2009), hepatocyte proliferation in the Indian catfish (Datta et al., 2007), altered ability of mummichogs (*Fundulus heteroclitus*) to adapt to their environment (Bears et al., 2006; Shaw et al., 2007a; Shaw et al., 2007b), and changes in antioxidant enzymes in zebrafish (Ventura-Lima et al., 2009). Arsenic exposure has also caused mutations in the p53 gene in salamanders (*Hynobius leechii*) (Chang et al., 2009).

Additionally, recent studies in fish link changes in reproduction and development to arsenic exposure. For example, exposure to arsenic resulted in a reduction in 11-ketotestosterone-induced spermatogenesis in catfish (*Pangasianodon hypophthalmus*) (Yamaguchi et al., 2007), while zebrafish fed metal-contaminated oligochaetes for 42-68 days had a significant reduction in the cumulative number of eggs, number of spawns, and percent of hatching success (Boyle et al., 2008). Zebrafish exposed to high concentrations of arsenic during development (64,950-259,800ppb) showed dorsal curvature, cardiac edema along with cardiac malformations, inappropriate apoptosis and methylation patterns, and altered development of the neuro-muscular system (Li et al., 2009). These findings corroborate studies in which offspring of mummichogs whose parents were exposed to 230ppb arsenic had a 2.8-fold increase in trunk curvatures. These abnormalities were correlated with differential expression of genes important in cellular and organismal structure, such as myosin light chain 2, tropomyosin, parvalbumin and type II keratin genes (Gonzalez et al., 2006). These effects are supported by the fact that arsenic has been shown to be transferred from the mother to the offspring in a variety of species (Concha et al., 1998; Kubota et al., 2005; Fängström et al., 2009). There also does appear to be gender-based differences in diseases caused by arsenic, although whether males or females are more sensitive seems to depend on the specific type of effect (reviewed in (Vahter et al., 2007).

Thus, there is a need to investigate arsenic's effects on aquatic organisms at low concentrations, while also examining what modifying role that gender or age of exposure may play. One way to do this is through the use of microarrays. Arsenic exposure can alter the expression of genes including those involved in stress response, proto-oncogenes, signaling molecules, transcription factors, chemokine receptors, and DNA repair enzymes in a variety of cell lines at concentrations ranging from 0.125 μM to 25μM (Chen et al., 2001; Zheng et al., 2003; Snow et al., 2005; Posey et al., 2008; Yamamoto et al., 2008). A number of studies have demonstrated

the utility of microarrays in environmental toxicogenomics. For example, a sheepshead minnow estrogen responsive array was developed to monitor the action of xenoestrogens in aquatic environments (Larkin et al., 2002). Arrays have also been developed for *Fundulus* to examine differential gene expression after chromium exposure to both adults and juveniles (Roling et al., 2006). Microarrays were used to identify biomarkers of cadmium exposure in the European flounder (*Platichthys flesus*) (Sheader et al., 2006), and in rainbow trout (Koskinen et al., 2004). Clusters of rainbow trout genes involved in energy metabolism, protein synthesis, and metal ion transport were upregulated at high exposures (0.5mg/L cadmium), while downregulated at medium or low exposures (0.25mg/L, 0.05mg/L respectively). In contrast, other genes involved in stress response, receptor signaling, G-protein coupled receptor, lipid biosynthesis and sulfur metabolism were upregulated at low doses and repressed at high doses (Koskinen et al., 2004).

Only a few studies have used microarrays to examine arsenic's effects on aquatic organisms. Using arrays, zebrafish exposed to 15ppm arsenic had decreased hepatic glycogen, increased incidence of cholestasis, and changes in overall hepatocyte morphology. Concurrent with these findings, the authors found genes involved in carbohydrate catabolism, DNA repair, and oxidant status-related proteins to be differentially expressed (Lam et al., 2006). Consequently, the present study used microarrays to investigate changes in the patterns of gene expression after adult and parentally-exposed juvenile mummichogs were exposed to three arsenic concentrations. These findings will provide us with a better understanding of dose-response relationships and the effects of gender and age on the response to arsenic.

2. Methods

2.1. Fish exposures

Mummichogs (2 males and 5 females) were randomly assigned to one of 10 different aquaria, 5 of which contained 20ppt saltwater (Coral Life, Burbank, CA) and 5 of which contained sodium arsenite in 20ppt saltwater. Three separate studies were conducted using the same set-up, but one exposure was with 172ppb (1.32μM), the second with 575ppb (4.43μM), and the third with 1,720ppb (13.2μM) arsenic, provided as sodium arsenite. Fish were exposed for a total of 10 days, maintained on a natural photoperiod, and fed daily with AquaTox flaked fish food (Zeigler Brothers, Gardners, PA) supplemented with freeze-dried brine shrimp. All studies were conducted using a static-renewal exposure, with 80% of the water being replaced every 48 hours. Although arsenic concentrations in the control aquaria water were below the detection limits, arsenic concentrations in the food (0.03ng/g) are similar to what others have reported (Shaw et al., 2007b).

Spawning substrates were placed in each tank on the three days surrounding the full moon to collect eggs. The total number of eggs produced was counted and their viability was determined. Eggs from each of the aquaria on each day were placed into petri dishes containing 20ppt saltwater to monitor their viability, development, and time to hatch. After completion of spawning, the parents were euthanized in 1g/L buffered MS-222 and two female fish from each group were stored at −80°C to determine arsenic body burdens. For the remaining adults, livers were removed, placed in TriReagent, and stored at −80°C. The hatchlings from each exposure group were transferred to an individual 1 gallon aquarium containing 20ppt seawater, maintained on a 16-h light/8-h dark photoperiod at 25°C, and fed recently hatched brine shrimp. The juveniles were observed over the next six weeks to determine whether additional morphological changes, developmental delays, or death would occur, but no additional phenotypes were noticed. Six weeks after hatching, the juveniles were euthanized with an overdose of buffered MS-222 and were stored at −80°C.

2.2. Chemical residue analysis

One intact adult female fish carcass from each aquaria in 1,720ppb experiment was freeze-dried (Labconco, Kansas City, MO), microwave digested (CEM, Mathews, NC) following the USEPA 3051 method (Kingston and Jassie, 1988) and arsenic levels analyzed using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP/OES) Optima 4300 DV (Perkin-Elmer Instruments, Edgewood, NM). Arsenic was detected at 189nm, which has a detection limit of 3ppb. A standard curve for sodium arsenite was used to determine body burdens in the fish, and statistical differences between the groups were determined using Student's t-test.

2.3. Morphometric analyses of the vertebrae

Ten control and ten 575ppb parentally-exposed juveniles were examined for changes in vertebral and fin bone size and number. Briefly, the fish were fixed in 10% formalin for 7 days and placed in Alcian Blue dye in 80% ethanol/20% glacial acetic acid solution for 12 hours. After neutralization with potassium hydroxide, the specimens were bleached in 3% hydrogen peroxide. The fish were then digested in a sodium borate/trypsin solution until bones and cartilage were visible, and stored in glycerol (Taylor and Van Dyke, 1985). A confocal microscope (Carl Zeiss, Thornwood, NY) was used to quantify the area of each vertebra, which was normalized to the weight of each fish.

2.4. Gene Expression Changes using Macroarrays

Targeted mummichog cDNA arrays were constructed using genes from subtractive hybridization or differential display experiments from fish exposed in the laboratory or collected from field sites containing Cr(VI), Cr(III), anthracene, pyrene, and arsenic (Bain, 2002; Maples and Bain, 2004; Peterson and Bain, 2004; Roling et al., 2004; Roling et al., 2006; Gonzalez et al., 2006). Each membrane contained 270 duplicate clones, including 13 blanks or plasmid controls. Specific information on the array platform is available on the Gene Expression Omnibus website (GEO-GPL2535) (Roling et al., 2006).

For each experiment, five arrays were probed using RNA samples from control groups and five were probed with samples from the arsenic-exposed groups. Array experiments were conducted for the 172, 575, and 1,720ppb males and for the 172 and 575ppb females. RNA was extracted from a pool of 7 to 10 juveniles per aquarium using TriReagent (Sigma, St. Louis, MO) and treated with DNase I. The pools of RNA were obtained from the 172, 575, and 1,720ppb exposure groups, plus their respective controls. Probes were generated by reverse transcription using 2μg of RNA, 0.1μM oligo dT primer, 1.2mM dNTPs lacking dATP, [^{33}P] dATP (3000Ci/mmol, Perkin Elmer, Boston, MA) and 200U MMLV-RT at 37°C for 2 hours. Meanwhile, the array membranes were prehybridized for 4-6 hour in Express hybridization solution (Clontech, Palo Alto, CA) containing 100mg/ml denatured salmon sperm. Each probe was purified using Amersham's Probe Quant-50 columns (Piscataway, NJ), denatured, and incubated overnight with the array at 68°C. Then, the arrays were washed four times with 2X SSC/0.5%SDS at 64°C and four times with 0.5X SSC/0.5%SDS at 64°C. The membranes were wrapped in plastic, placed on a phosphorimaging screen, and developed using a Bio-Rad Molecular Imager FX (Hercules, CA). The intensity of each spot was quantified using the Invitrogen ResGen Pathways 4-Universal Microarray Analysis Software (Carlsbad, CA). The data for this series of experiments has been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and can be accessed using GEO series accession number GSE16772 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16772).

2.5. Microarray analysis

The CLEAR-test method was used to analyze the microarray data for identifying differentially expressed genes between normal state and different degrees of arsenic exposure for each fish group (Valls et al., 2008). This method combines the z-test, which focuses on large changes, with a x^2 test to evaluate variability, and has the advantage of not reporting genes with small changes and low variances as differentially expressed. For all the duplicate spots, the raw pixel intensity values were first averaged before running CLEAR-test. Average fold expression changes for each gene were obtained for each of the three arsenic concentrations in the male, female, and juvenile groups.

2.6. Changes in RNA abundance by real-time PCR

Total RNA from the liver of 5 adult fish of each gender per exposure group was isolated using TRI-Reagent (Sigma) and then treated with RNase-free DNase. Total RNA from a pooled sample of 3 whole juveniles was also isolated after homogenization (n=5 pools per group). To prepare cDNA, total RNA (2μg) was incubated with 50ng random heblxamers, RNasin, 10mM dNTP mix, and 200U Moloney murine leukemia virus (MMLV) reverse transcriptase at 37°C for 1 hour. Real-time PCR was performed in Bio-Rad's I-Cycler (Hercules, CA) using RT^2 SYBR Green/Fluorescein qPCR master mix (SABiosciences, Frederick, MD), along individual sets of primers for the EST 9-7 (#CO897482; forward primer 5′-GCCAACACCATGTGTGCAAGTTCT -3′; reverse primer 5′-TCTGGACACAGTCACACACAGACT-3′), apolipoprotein (8-56) (#DN474949; forward primer 5′-ATCTTGCAGCTGCTGTTCTGTTGC-3′; reverse primer 5′-GTGTCAAAGTGGGTCTTGGCCTTT-3′), and serum amyloid precursor (1-31) (#AY735160, forward primer 5′-AGGTTATCAGCGATGCCAGAGAGT-3′; reverse primer 5′- ATTTGCTTCCTGATCGGCCTCTGA-3′). 18S rRNA (#X00686; forward primer 5′-TTTCTCGATTCTGTGGGTGGTGGT-3′; reverse primer 5′-TAGTTAGCATGCCGGAGTCTCGTT-3′) was used as a housekeeping gene to normalize the total number of molecules in each sample. All PCR products had a denaturing step of 95 C for 15 seconds, an annealing/extension step at 57°C for 1 minute for a total of 40 cycles. The cycle threshold values obtained from the real-time PCR were converted into starting number of molecules per 100ng cDNA using known concentrations of the specific gene product, which was normalized to the number of 18S rRNA molecules (Gonzalez et al., 2006). The standards were prepared by RT-PCR and sequenced at Clemson University to confirm their identity.

3. Results

3.1. Arsenic body burdens

At the conclusion of spawning, female fish from each control and 1,720ppb arsenic aquaria were used to determine the body burdens of arsenic. In the adults, arsenic levels were 2.6-fold higher in the exposed fish than in the controls after 10 days of exposure (Figure 1). This level of uptake is consistent with other studies in *Fundulus*. For example, adult mummichogs exposed to 8,000ppb arsenic for 4 days had approximately 6 times more arsenic than controls while those fish exposed to 100ppb arsenic for 14 days had approximately 3 times more arsenic than the controls (Miller et al., 2007). Similarly, mummichogs exposed to 787 ppb for 14 days had 2.8-fold higher hepatic arsenic levels than controls (Bears et al., 2006).

3.2. Hepatic gene expression changes in adult Fundulus exposed to arsenic

Microarrays were used to determine both the dose-dependent patterns of gene expression changes in livers following arsenic exposure and whether the response to arsenic differed between genders. Replicated test array experiments were conducted for 2 different exposure concentrations in the females or 3 different exposure concentrations in the males. Of the 257

genes on the array, between 4.7-8.2% were differentially expressed after arsenic exposure amongst the 5 groups of experiments using the CLEAR test method. Analyzing the data for significantly differential changes in gene expression in a dose-responsive manner indicated that in the two female exposure groups, 172ppb and 575ppb arsenic, 16 and 19 hepatic genes were significantly changed compared to the controls, respectively. There were 7 common differentially expressed genes (Figure 2A). In comparing the livers of male fish exposed to arsenic with the controls, 21, 12, and 13 hepatic genes were differentially expressed in 172, 575, and 1,720ppb exposures. There was only 1 gene shared in common between all three male groups, which is an EST termed 10-15. Shared genes between two of the three male exposures ranged from 3 to 7 (Figure 2A). When examining the data from Table 1 for the number of common genes changed after arsenic exposure based upon gender, a total of 9 differentially expressed genes were common between males and females. The differential expression was more likely to occur in the 172ppb exposure group than the 575ppb group (Figure 2B).

Several genes were expressed in a dose-responsive manner. Only genes with statistically different expression are shown in Table 1. These included apolipoprotein, which was significantly downregulated in both female exposure groups and in two of the three male exposure groups. Apolipoprotein is represented by two partially overlapping clones on the array, 8-56 and Ab35, and both were changed in the same manner and the same magnitude (Table 1). Lysozyme precursor is also represented by two clones, 1-11 and 1-163, with both consistently demonstrating downregulation in the 172ppb males but upregulation in the 1,720ppb males exposed to arsenic (Table 1). Other clones show differences based upon gender, such as tributyltin (TBT)-binding protein, which is downregulated in females but upregulated in males (Table 1). A second overlapping clone for TBT-binding protein termed C3C61 (accession #BM084929) was also significantly downregulated (0.33-fold) in the 575ppb arsenic-exposed females (data not shown). Several other genes were differentially expressed in only one exposure group, but many of these genes are associated with cell stress, damage, and repair, such as heat shock proteins, glutathione S-transferases, transcription elongation factors, and ATP synthase (Table 2).

3.3. Reproductive effects, developmental effects, and gene expression changes in the juveniles

In addition to changes in hepatic gene expression, we were interested in investigating the effects of arsenic on embryonic development. After the exposure period, the fish were mated and eggs collected to determine changes in embryonic development and hatchling success. There were no differences in the number of eggs laid per tank between the control and the arsenic-exposed groups at any concentration, nor any differences in egg viability (Figure 3a). After hatching, any deformities in the embryos were recorded. In the offspring whose parents were exposed to 1,720ppb arsenic, there was a significant increase in deformities including embryos with abnormal trunk curvatures, and head and eye deformities (Figure 3b). Because of these abnormalities, it was hypothesized that arsenic could be altering the structure of bone or cartilage. However, no differences were observed in the number, orientation, or position of the vertebrae in parentally-exposed offspring compared to control offspring (Figure 3c).

Unlike the adult hepatic gene expression, we used RNA extracted from whole 6-week old juveniles. There were very few genes in common between the three different parental exposure groups, except for two ESTs, 2-80 and 9-7 (Table 1). There was only one additional gene, complement component C8, which was shared between the juveniles and the adults. However, this gene was only significantly differentially expressed in the highest juvenile exposure group.

3.4. Confirmation of gene expression patterns

To confirm the expression patterns of several of the genes, we used quantitative PCR (QPCR) to examine expression levels of several selected genes in the livers of the adult males and females. Expression levels of the EST shared in common with the adults, 9-7, was also examined in the juveniles (Figure 4). Apolipoprotein expression on the arrays was downregulated in all adult arsenic exposed groups, regardless of gender or concentration. This was validated in four of the five QPCR reactions. The exception was the 172ppb female group, which based upon QPCR expression, showed no change in expression (fold change=1.03). The EST termed 9-7 showed a bit more variability, but the arrays and QPCR validated one another in 5 of the 7 groups (Figure 4). The expression of 9-7 in the 172ppb female group was downregulated in the arrays but upregulated in the QPCR, while the 575ppb male group was upregulated in the arrays but downregulated based upon QPCR. The levels of 9-7 in two juvenile groups examined showed similar expression patterns, being upregulated in both the arrays and when using QPCR (Figure 4).

Serum amyloid precursor expression was downregulated in all of the male exposures and the 172ppb female exposure, but upregulated in the 575ppb female exposure group in the arrays. Again, the direction of change using QPCR matched in four of the five groups, but this time, the 172ppb male group showed no changes in expression based upon the QPCR data (fold change=1.07). For the higher exposure groups, both the directionality and magnitude of change were similar. The expression of serum amyloid precursor in the 575ppb males was downregulated 0.83-fold in the arrays and 0.67-fold in the QPCR, while the expression in the 1,720ppb males was downregulated 0.32-fold in the arrays and 0.28-fold in the QPCR. Interestingly, serum amyloid precursor expression was upregulated in the 575ppb female group, and the magnitude of change between the arrays and QPCR was also similar (2.5-fold in arrays; 3.7-fold in the QPCR). Overall, the QPCR data showed similar fold-changes and directionality, validating the array data.

4. Discussion

4.1. Arsenic-mediated changes in gene expression

The use of fish toxicogenomics is suited for studying contaminants such as arsenic, as fish can act as both models for human diseases as well as models for the health of the aquatic environment (Williams et al., 2003; Gorman and Breden, 2007). In the present study, adult mummichogs were exposed to three different arsenic concentrations, and the differential expression of genes was investigated by examining the effects of gender and maternal transfer to offspring. Several genes were differentially expressed as a result of the arsenic exposure, including apolipoprotein, serum amyloid precursor, tributyltin-binding protein, and cytochrome c oxidase. Overall, arsenic appears to perturb genes involved in energy utilization.

Apolipoprotein was one gene that was downregulated in the adult livers at all exposure concentrations tested. Using QPCR, the underexpression of apolipoprotein and the magnitude of downregulation were in concordance with the array data. Apolipoproteins are lipid binding proteins important in lipid secretion, lipolytic enzyme activation, and transport and binding of lipoproteins in cells (Atkinson, 1992). Several isoforms have been identified, which bind to and act as ligands for low density or very low density lipoprotein receptors (Atkinson, 1992). In our experiment, both clones of the apolipoprotein C-1 were down-regulated similarly in females as well as males. The reduction in apolipoprotein expression is in concordance with other arsenic exposure studies. For example, arsenic downregulated apolipoprotein in the lung fluid of male mice exposed to 50ppb sodium arsenite for four weeks (Lantz et al., 2008), and was differentially expressed in zebrafish embryos exposed to arsenic (Mattingly et al., 2009).

Another protein, serum amyloid precursor, was also downregulated in the adult males and in the 172ppb female exposure group. Its altered expression was examined by QPCR, which confirmed a dose-responsive reduction in the male 172, 575, and 1720ppb groups. Serum amyloid precursor proteins (APPs) are also a family of apolipoproteins (Zheng and Koo, 2006). Although most often associated with the development of Alzheimer's disease (reviewed in: (Esler and Wolfe, 2001; Jaeger and Pietrzik, 2008), APPs are thought to function to control cholesterol transport, ApoE metabolism, and help maintain cholesterol homeostasis (Yao and Papadopoulos, 2002; Liu et al., 2007). Additionally, cellular cholesterol can modify the both the processing and transcription of APP (Bodovitz and Klein, 1996; Fassbender et al., 2001; Kiyosawa et al., 2004). In general, the downregulation of APP in the adult mummichogs mimics the reduction in apolipoprotein expression.

Both apolipoprotein and APPs are involved in lipid homeostasis, and in other studies, exposure to arsenic does appear to alter lipid levels. Mice exposed to arsenic via drinking water from weaning to 1 year of age, or from 8 weeks to 1 year of age had significant reductions in serum triglycerides, total cholesterol, and HDL cholesterol compared to controls (Ahlborn et al., 2009). The authors hypothesized that the reduction in lipids could be due to alterations in fatty acid production, as there was a reduction in the mRNA expression of stearoylCoA desaturase 1, the rate limiting enzyme in monounsaturated fatty acid synthesis (Ahlborn et al., 2009). Interestingly, the mice in their study exposed to arsenic only *in utero* did not have alterations in serum lipids (Ahlborn et al., 2009). These findings are in concordance with our studies showing a marked reduction in both apolipoprotein and APP proteins in the adults, but not in the offspring. Thus, arsenic exposure may impair cholesterol homeostasis, which could have adverse physiological consequences for fish (Heath, 1995). Examining lipid levels in the livers and serum of arsenic-exposed fish, and how that may impact adult health and egg quality would be worthy of future investigation.

Indeed the present study, along with many others, shows that arsenic often exhibits a complex gene expression profile. For example, arsenic exposure in zebrafish embryos resulted in the differential expression of several immune-responsive genes following a 10μg/L exposure, but no change in expression at 100μg/L (Mattingly et al., 2009). In additional microarray studies using cell lines or rodent models, arsenic exposure results in very few commonly differentially expressed genes between the exposure groups (Ahlborn et al., 2008), or can alter the expression of genes in opposite directions at low versus high concentrations (Andrew et al., 2003; Lau et al., 2004; Snow et al., 2005; Andrew et al., 2007). This may indicate that differing transcriptional networks are activated at low arsenic levels versus high arsenic levels, resulting in differing biological processes being altered (Bodwell et al., 2004; Bodwell et al., 2006).

4.2. Gender differences in gene expression

We also found differing responses depending on the gender of the fish. For example, serum amyloid precursor was upregulated in the livers of the highest female exposure group, but downregulated in the livers of the males. In contrast, several ESTs were upregulated in the males by downregulated in the females. Gender-based differences in disease outcomes after arsenic exposure have been reported in the literature. For example, men have a higher incidence of pathological liver changes after arsenic exposure, while women are at greater risk of arsenic-related kidney, lung, and bladder cancers than men (Vahter et al., 2007; Lindberg et al., 2008b). The same increase in bladder lesions in females has also been reported in studies with rodents (Shen et al., 2006; Waalkes et al., 2006), while increases in liver tumors after arsenic exposure are seen in male rodents (Waalkes et al., 2003; Waalkes et al., 2004a; Waalkes et al., 2004b; Ahlborn et al., 2009). It is known that hepatic metabolism of contaminants in some organisms can vary by gender (reviewed in Burger et al., 2007) and it has been speculated that sex hormones affect in arsenic metabolism, altering its methylation, excretion, and thus its

pathology (Shen et al., 2006; Lindberg et al., 2008a; Lindberg et al., 2008b). As the fish in our study were reproductively active, hormonal changes may have influenced the effects of arsenic on gene expression.

4.3. Effect of arsenic on development

The second part of this study was to examine changes in development and gene expression in the offspring whose parents were exposed to arsenic. Viability was not different between the control and arsenic-exposed groups at any exposure concentration. However, parental exposure to arsenic increased the incidence of developmental deformities, such as spinal curvatures and smaller heads, by 17.4-fold at the highest concentration as compared to controls. In a previous experiment using a concentration of 230ppb arsenic, there was a 2.8-fold increase in the incidence of spinal deformities in the hatchlings of exposed parents (Gonzalez et al., 2006). A study with zebrafish revealed various similar morphological abnormalities including dorsal curvature, flattened head, and pericardial edema (Li et al., 2009), and mice exposed *in utero* via maternal oral treatment also exhibited skeletal abnormalities (Hill et al., 2008). In humans, a decreased head and chest size was noted in children exposed to arsenic *in utero* (Rahman et al., 2009).

It has been well documented that arsenic can cross the maternal blood supply and be detected in the fetus (Concha et al., 1998; Kubota et al., 2005; Fängström et al., 2009). A study looking at the maternal transfer of arsenic from organisms such as adult black-tailed gulls noted that 11.3% of the arsenic burden was transferred to eggs (Kubota et al., 2002), fetal porpoise arsenic burden was about 36% of that measured in the mothers (Kubota et al., 2005), and newborn mouse livers had 10% of the average arsenic concentration in maternal livers (Xie et al., 2007). Thus, even the low amounts of arsenic transferred to the offspring may impact its gene expression and subsequent development.

It is interesting to note that offspring exposed to arsenic *in utero* have very different pathologies based upon their gender. In mice exposed to arsenic *in utero*, males have increase in liver and adrenal tumors, while females have increases in urogenital tumors, but no changes in the incidence of liver or adrenal tumors (Waalkes et al., 2003; Waalkes et al., 2004a; Waalkes et al., 2004b; Ahlborn et al., 2009). We did not determine the sex of the juveniles in our study before using them in the microarray study. Given that in rodents, very different arsenic metabolite profiles and pathologies are seen, not sexing our juvenile fish might have contributed to the lack of gene expression changes and the high amount of variability associated with the offspring.

In summary, we observed changes in lipid responsive genes at environmentally-relevant concentrations, indicating that arsenic exposure may impair cholesterol homeostasis, which could have adverse effects on organismal health. These findings will provide us with a better understanding of the effects of dose, gender, and exposure age on the response to arsenic.

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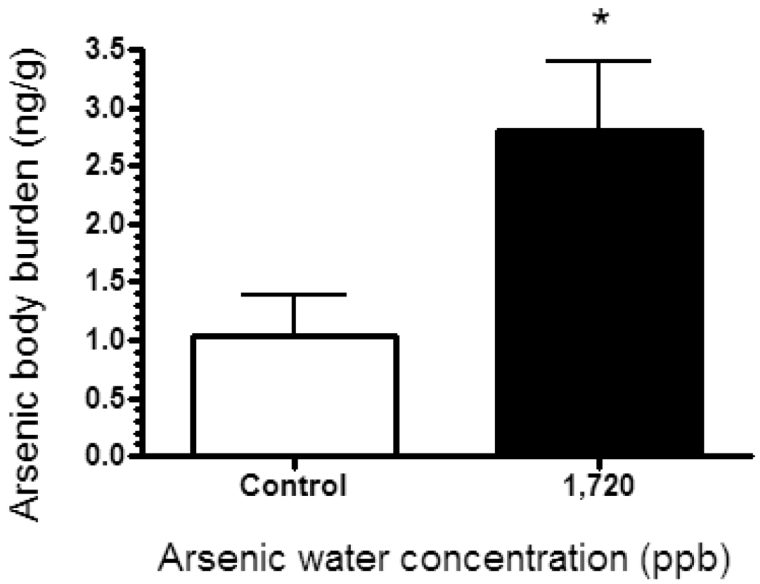

Fig. 1. Arsenic body burdens

Arsenic concentrations were measured in the adult females after the 10 day exposure. Data is the average ± standard deviation of 5 intact fish per group. Significant differences (*) were determined using Student's t-test ($p<0.05$).

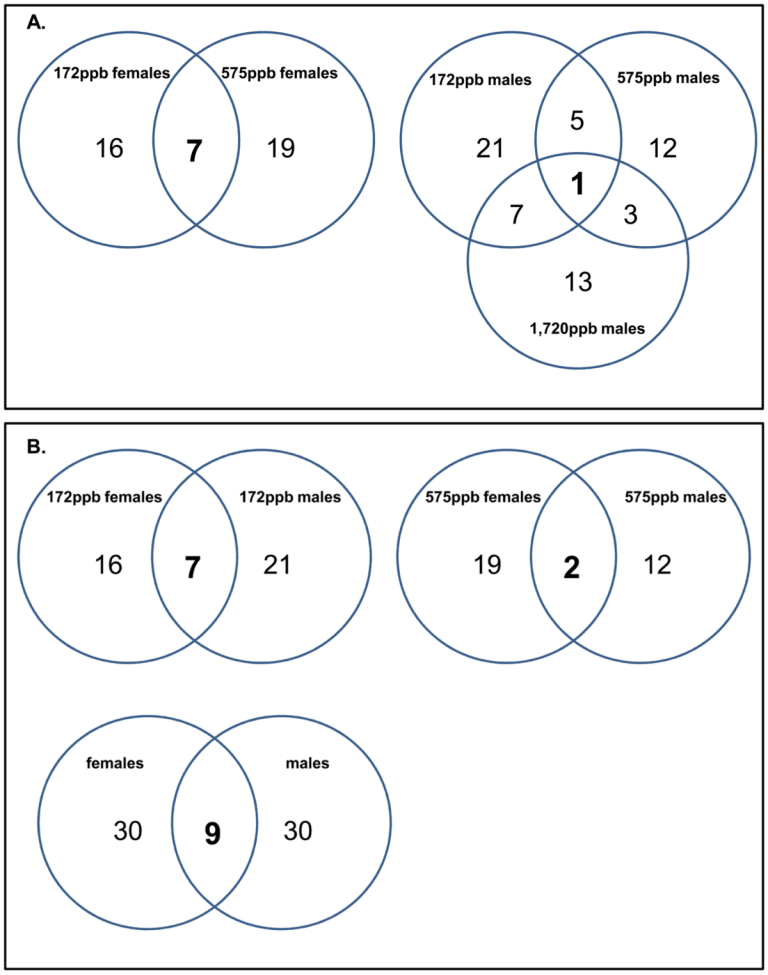

Fig. 2. Gene expression profiles for males and female *Fundulus* exposed to different concentrations of arsenic

Hepatic gene expression was examined using the *Fundulus* cDNA array (GEO-GPL2535) and analyzed for differential expression using the CLEAR Test Method. The numbers of genes with significantly different expression ($p \leq 0.05$) are indicated, and those changed by more than one exposure are found in the overlapping areas. **A.** Dose-responsive changes in hepatic gene expression in male and female fish **B.** Gender-responsive changes in hepatic gene expression in male and female fish.

	% viability	% deformities	Fold increase in deformities
Control	71.2 ± 21.4	0	
172ppb	82.5± 6.9	0	0
Control	91.0 ± 6.5	0	
575ppb	88.3 ± 8.3	0	0
Control	55.7 ± 32.6	0.7 ± 1.1	
1720ppb	77.7 + 25.2	12.2 + 6.0*	17.4*

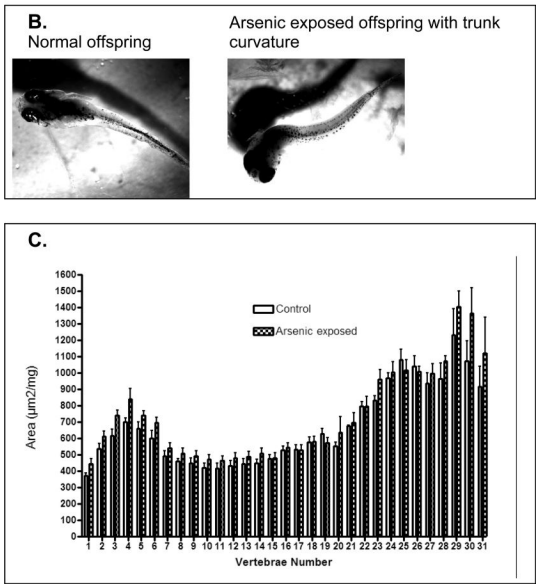

Fig. 3. Percentage viability and morphological abnormalities following arsenic exposure

Adults were exposed to 172, 575 or 1,720ppb arsenic for 10 days prior to spawning. Eggs were collected for three days following arsenic exposure, and their viability, along with any developmental abnormalities observed after 7 days, was recorded. **A.** Viability and deformity percentages are expressed as the average + standard deviation for offspring from 4 –5 tanks, with statistical differences (*)determined by Mann-Whitney ($p<0.05$). **B.** Representative photographs from normal and deformed offspring. **C.** Morphological analysis of the vertebrae. The total size (area) for individual vertebrae were quantified using the LSM5 Pascal software. Values are the average area in square micrometers divided by the weight of the 6-week old fish. Significant differences (*) were determined using Student's t-test ($p<0.05$).

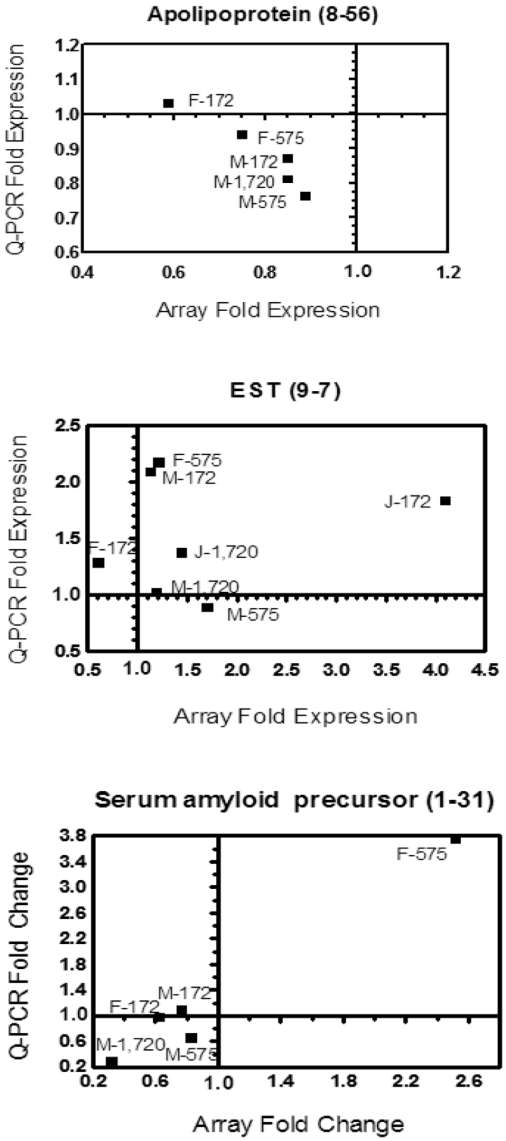

Fig. 4. Comparisons of gene expression changes between the arrays and quantitative real-time PCR (QPCR)

Expression of three genes from the arrays that were differentially expressed in almost all genders, ages, and exposure groups were verified by QPCR using specific primers (n=5 for each group). Array fold changes were plotted against QPCR fold changes to determine their directionality and magnitude. Genes examined were apolipoprotein, serum amyloid precursor, and an EST termed 9-7. The letter indicates females (F), males (M), or juveniles (J), while the number (172, 575, or 1,720) indicates the exposure group.

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Table 1

Gene Expression Changes in Fundulus Exposed to Arsenic. Fold-changes in gene expression were calculated and significant differences were determined using the CLEAR Test method ($p<0.05$). Only genes that are differentially expressed and shared between more than 1 exposure group are listed.

			Fold change (Arsenic/control)							
			Females		Males			Juveniles		
Clone ID	Gene Name	GenBank #	172ppb	575ppb	172ppb	575ppb	1,720ppb	172ppb	575ppb	1,720ppb
8--56	apolipoprotein	DN474949	0.59	0.75	0.85		0.85			
Ab35	apolipoprotein	CN992696	0.59	0.79	0.85		0.84			
9--7	EST	CO897482	0.61	1.22	1.14	1.71		4.1		1.45
9--12	TBT-binding protein	AY725225	0.56	0.6		1.95				
8-6-T7	EST	DN596277	5.71	0.45						
Bb56	EST	DN596273	0.67	1.79						
1--31	serum amyloid precursor	AY735160	0.63	2.52	0.77		0.32			
2--80	EST	DN474945	0.54		0.81	2.13			1.54	1.29
9--16	EST	CO897478	0.7		0.74	1.39				
pa 1--15	complement component C8	AY521664	0.7		0.82	1.93				1.58
Bb53	cytochrome c oxidase subunit II	CV821001		1.57	0.7					
8--40	cytochrome c oxidase subunit II	CN976180		0.8	0.6					
10--15	EST	CO897479			1.14	1.8	0.87			
10--57	EST	CO897481			1.41		0.55			
1--11	lysozyme precursor	AY735156			0.3		2.65			
1--163	lysozyme precursor	AY735156			0.29		2.85			
8--46	EST	DN474948				1.37	0.82			
8--8	EST	CO897478				1.68	0.82			

Table 2

Genes showing unique differential expression. Fold-changes in hepatic gene expression in the males and females were calculated and significant differences were determined using the CLEAR Test method ($p \leq 0.05$). Only genes that were differentially expressed in only 1 exposure group are listed. GenBank accession numbers are indicated in parentheses.

Clone Identity and GenBank Accession Number					
172ppb females	**575ppb females**		**172ppb males**	**575ppb males**	**1,720ppb males**
Small inducible cytokine A4 (AY735153)	EST (CX700381)		GLUT2 (AY521663)	Elongation factor 1α (AY735180)	EST (CO897478)
Myosin light chain 2 (CX700395)	Coagulation factor XIIIB precursor (CN988088)		Hemopexin precursor (AY735182)	70kDa HSP (AY735159)	Warp65 (AY735166)
EST (CX700386)	14-3-3 (AY725221)		EST (CO897478)	GSTμ (AY725220)	EST (DN596272)
Map kinase kinase (BI993599)	Deiodinase (U70869)		transferrin (AY735165)	EST (CO897478)	EST (CX700393)
EST (DN474942)	Vitellogenin (U07055)		Cytochrome b (AY725226)		
EST (CO897490)	ATP synthase F_o complex (AY735178)		fucolectin (AY735152)		
	EST (CO897485)		EST (CX700390)		