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Transcriptome Alterations Following Developmental Atrazine Exposure in Zebrafish Are Associated with Disruption of Neuroendocrine and Reproductive System Function, Cell Cycle, and Carcinogenesis

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Atrazine, a herbicide commonly applied to agricultural areas and a common contaminant of potable water supplies, is implicated as an endocrine-disrupting chemical (EDC) and potential carcinogen. Studies show that EDCs can cause irreversible changes in tissue formation, decreased reproductive potential, obesity, and cancer. The U.S. Environmental Protection Agency considers an atrazine concentration of ≤ 3 ppb in drinking water safe for consumption. The specific adverse human health effects associated with a developmental atrazine exposure and the underlying genetic mechanisms of these effects are not well defined. In this study, zebrafish embryos were exposed to a range of atrazine concentrations to establish toxicity. Morphological, transcriptomic, and protein alterations were then assessed at 72 h postfertilization following developmental atrazine exposure at 0, 0.3, 3, or 30 ppb. A significant increase in head length was observed in all three atrazine treatments. Transcriptomic profiles revealed 21, 62, and 64 genes with altered expression in the 0.3, 3, and 30 ppb atrazine treatments, respectively. Altered genes were associated with neuroendocrine and reproductive system development, function, and disease; cell cycle control; and carcinogenesis. There was a significant overlap (42 genes) between the 3 and 30 ppb differentially expressed gene lists, with two of these genes (*CYP17A1* and *SAMHD1*) present in all three atrazine treatments. Increased transcript levels were translated to significant upregulation in protein expression. Overall, this study identifies genetic and molecular targets altered in response to a developmental atrazine exposure to further define the biological pathways and mechanisms of toxicity.

Key Words: **atrazine; development; endocrine disruption; gene expression; transcriptomics; zebrafish.**

Recent research concerning the effects of endocrine-disrupting chemicals (EDCs) on human health has increased substantially and heightens the need for further research into the mechanistic effects of exposure to these compounds (Swedenborg *et al*., 2009). EDCs are diverse in structure and are present in many products such as plasticizers, pharmaceuticals, and pesticides, making human exposure to these potentially harmful chemicals a likely event. The likelihood of exposure to EDCs through multiple routes and sources results in heightened concerns as to the adverse health effects associated with exposure, especially in vulnerable populations including fetal and childhood exposures. In addition, there is now considerable research investigating the lasting impacts of a developmental exposure to EDCs throughout the lifespan of an individual, supporting the idea that several diseases and disorders may originate from a developmental exposure to these contaminants (Birnbaum and Fenton, 2003; Ma *et al*., 2010; Prins *et al*., 2007).

Atrazine is a commonly used herbicide predominately applied as a pre-emergent herbicide to control broadleaf and grassy weeds in a variety of crops and is implicated as an EDC and potential carcinogen (Cooper *et al*., 2000; Eldridge *et al*., 1999; Freeman *et al*., 2005, 2011; Hayes *et al*., 2010; Holloway *et al*., 2008; McElroy *et al*., 2007; Wetzel *et al*., 1994). Because of its widespread use in the United States, atrazine is commonly reported to contaminate potable water supplies and thereby exposing a substantial proportion of the population (Ochoa-Acuña *et al*., 2009b). A number of studies aimed at evaluating atrazine as an endocrine disruptor and identifying the adverse impacts of exposure are completed, but the mechanisms of atrazine toxicity and health risks associated with atrazine exposure, especially at exposure levels likely to be encountered in the environment, are not distinctly understood. Early reports indicated that exposure to atrazine at high doses alters the reproductive system in rats by disrupting the surge of luteinizing hormone (LH) from the pituitary gland and prolactin from the hypothalamus and increases incidence of mammary gland tumors in female rats (Cooper *et al*., 2000; Wetzel *et al*., 1994). These effects appear to be mediated through disruption of the hypothalamus-pituitary-gonadal

(HPG) axis (Cooper *et al*., 2007). Studies using *Xenopus laevis* tadpoles reported sublethal doses of atrazine to decrease the time to metamorphosis indicating that atrazine alters the thyroid axis (Freeman and Rayburn, 2005; Freeman *et al*., 2005). Moreover, low doses of atrazine are shown to alter development of reproductive organs and function in fish and amphibians, especially in males whereby "demasculinization" of the testes occurs resulting in reduced spermatogenesis and fertility (Hayes *et al*., 2002, 2010). One endpoint that is attributed to this observed effect is the stimulation of cytochrome P450 aromatase activity, a key enzyme in the conversion of androgens to estrogen. Indeed, several reports cite increases in aromatase activity upon exposure to atrazine that lead to an increase in intracellular cAMP levels (Holloway *et al*., 2008; Suzawa and Ingraham, 2008). Although a body of literature implicates atrazine exposure with negative effects on neuroendocrine function, the main mode of action is not fully understood.

Furthermore, most of these studies evaluated the effects of atrazine exposure in tissue culture, juvenile, or adult organisms. Only a few studies investigated the mechanisms of toxicity of atrazine exposure during embryogenesis, and recent epidemiologic studies indicate that prenatal atrazine exposure is linked with various adverse birth outcomes. Studies report that atrazine exposure is associated with an increased prevalence of small for gestational age (SGA) and impairments in fetal growth (Chevrier *et al*., 2011; Ochoa-Acuña *et al*., 2009b; Villanueva *et al*., 2005). Moreover, elevated concentrations of atrazine in water from April to July coincided with a higher risk of birth defects in live births by months of last menstrual period from April to July (Winchester *et al*., 2009). In addition, limb birth defects increased in relation to proximity of cornfields but not of soybean fields, indicating that risk factors may be spatially associated with corn but not with soybeans (Ochoa-Acuña *et al*., 2009a). One such factor is environmental agrochemical exposure with atrazine applied exclusively to cornfields and not soybean fields. These findings conclude that further studies are needed to define these adverse effects and to determine the underlying mechanisms of toxicity.

The European Union recently banned the use of atrazine due to continued contamination of potable water sources (Sass and Colangelo, 2006). Additionally, the United States Environmental Protection Agency is currently reviewing the registration decision on atrazine and the safety risk of the current maximum contaminant level (MCL) of 3 ppb in drinking water. Thus, in this study, we assessed morphological alterations and defined transcriptomic profiles immediately following a developmental atrazine exposure using the zebrafish vertebrate model system at 0.3, 3, or 30 ppb, concentrations very likely to occur in environmental exposures to the general population including pregnant mothers and their fetuses (the exposure regimen our study design targets). There are considerable strengths in utilizing the zebrafish to address questions pertaining to developmental toxicity including rapid *ex utero* development, the ability to produce a large number of embryos thereby increasing sample size and number of replicates, and a near-transparent chorion that allows for ease in observation when assessing the toxicity of a given chemical. As many of the genetic and molecular pathways are conserved across vertebrate species and with a recently completed sequenced genome, the zebrafish provides a powerful model organism for investigating alterations in biological processes during early development as a result of atrazine exposure.

MATERIALS AND METHODS

Zebrafish husbandry. Zebrafish (*Danio rerio*, wild-type AB strain) were housed in a Z-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 h light:dark cycle and maintained at 28ºC with a pH of 7.2 and conductivity range of 470–520 µS. Adult zebrafish were bred in cages and embryos were collected, staged, and rinsed with system water as described previously for experimental use (Peterson *et al*., 2011).

Atrazine acute toxicity test. A stock solution of technical grade atrazine (CAS 1912–24–9; Chem Service, West Chester, PA) at a concentration of 10 parts per million (ppm; 10 mg/l) was prepared near the solubility limit in water as previously described (Freeman *et al*., 2005) and thus no solvent was needed. An acute toxicity assay was conducted as previously described using point dilutions of 0.3, 3, 30, 300, 3000, and 10,000 ppb atrazine from 1 to 120 h postfertilization (hpf) (Peterson *et al*., 2011). Three biological replicates (each started from a different clutch) consisting of 50 embryos each (considered as subsamples) were included in this experiment, and an ANOVA was performed with SAS software (SAS Institute Inc., Cary, NC) by developmental time point. Means were compared using the least significant difference test (LSD) at $\alpha = 0.05$ when a significant ANOVA was observed. Stock and diluted sample concentrations were verified using an U.S. EPA–approved immunoassay kit for atrazine (Abraxis Atrazine ELISA kit) as previously described (Freeman *et al*., 2005).

Morphological assessment following developmental atrazine exposure. An atrazine stock solution and test dilutions were prepared, and atrazine concentration was verified as described above. Fertilized embryos were collected as described above and exposed to 0, 0.3, 3, or 30 ppb atrazine (representing a range 0.1× to 10×, the current U.S. EPA MCL) from 1 to 72 hpf. Upon completion of exposure at 72 hpf, zebrafish larvae were rinsed with system water and collected for analysis of developmental effects. Twenty larvae from each treatment (considered as subsamples) were analyzed with light microscopy using a Nikon SMZ1500 dissecting microscope with NIS Elements imaging software (Melville, NY) to attain eye diameter, head length, and total larval length (measured snout to tail). Three biological replicates were completed ($n = 3$). An ANOVA was used to analyze differences among treatments, and a LSD test at $\alpha = 0.05$ was used when a significant ANOVA was observed.

Global gene expression analysis following developmental atrazine exposure. Fifty embryos (considered as subsamples) were exposed to 0, 0.3, 3, or 30 ppb atrazine from 1 to 72 hpf as described above. At 72 hpf, embryos were pooled, homogenized in TRIzol (Life Technologies, Carlsbad, CA), and flash frozen in liquid nitrogen. Samples were stored at −80°C until RNA isolation was performed. Three biological replicates were completed ($n = 3$). Water samples were collected and analyzed for atrazine concentration as described above. Total RNA was isolated and converted to cDNA following established protocols (Peterson *et al*., 2011). Genomic microarray analysis was conducted to compare gene expression profiles among the atrazine treatments with the zebrafish 385K expression platform (Roche NimbleGen, Madison, WI), and array image data were analyzed similarly as described in Peterson *et al.* (2011). A robust and reproducible list of differentially expressed genes for each atrazine treatment using recommendations from the Microarray Quality Consortium (Guo *et al*., 2006; Shi *et al*., 2006) was first determined by genes consistently

expressed using an ANOVA in SAS software ($p < 0.1$) and substantially altered with a mean absolute $\log_2$ expression ratio of at least 0.585 (50% increase or decrease in expression). Each gene list was imported into Ingenuity Pathway Analysis for gene ontology and molecular pathway analysis following similar parameters as described previously (Peterson *et al*., 2011). Genes referred to in the Results and Discussion sections are the human homologues of the genes identified to be altered by the microarrays.

Quantitative PCR comparison. Quantitative PCR (qPCR) was performed on a subset of selected genes altered in the microarray analysis (*AVP*, *CYP17A1*, *FAM84B*, *GLO1*, *LHb*, *MHC1UFA*, *PIM1*, *SIK2*, *TPD52L1*, and *si:ch73-41e3.7*.) using the BioRad iQ SYBR Green Supermix kit according to the manufacturer's recommendations. In addition, *CYP19A1* was also included to confirm a lack of expression change. Probes specific to target genes were designed using the Primer3 Web site (Table 1). qPCR was performed following similar methods as described previously (Peterson *et al*., 2011; Zhang *et al*., 2011). Gene expression was normalized to β-actin. Three biological replicates ($n = 3$) were analyzed and compared between the control and 30-ppb-treated samples to confirm gene expression alterations detected in the microarray analysis. An evaluation of linear correlation was performed and statistical significance of the correlation determined with a Pearson's correlation coefficient test using SAS software ($p < 0.05$). For the correlation analysis, data input was the $\log_2$ expression value for the microarray data and ratio of relative expression against β-actin levels for the qPCR data.

Protein expression analysis. Western blot analysis was performed to determine whether gene expression alterations were translated to changes in protein expression following similar parameters as previously described (Peterson *et al*., 2011). Twenty embryos (considered as subsamples) were exposed to control conditions or 30 ppb atrazine from 1 to 72 hpf as described above. Antibodies for CYP17A1 (ab64886), GLO1 (ab85420), SIK2 (ab53423), and TPD52L1 (ab14822) were obtained from Abcam (Cambridge, MA). Image Lab Software (Bio-rad, Hercules, CA) was used to calculate relative quantification of three biological replicates ($n = 3$) after normalizing proteins of interest to ACTIN (A2066; Sigma-Aldrich, St Louis, MO). A paired Student's *t*-test was used to determine significant alterations in protein expression using SAS software ($p < 0.05$).

RESULTS

Developmental Effects of Low-Dose Atrazine Exposure

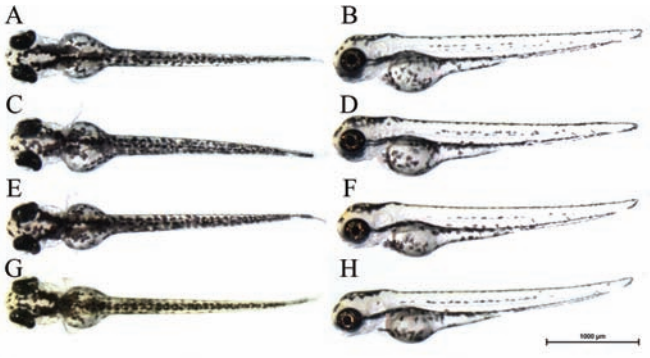
An acute toxicity assay was initially conducted to determine the toxicity profile of atrazine in the zebrafish vertebrate model system. Developmental atrazine exposure from 1 to 120 hpf to concentrations up to 10 ppm (near the solubility limit of atrazine in water) did not alter survival rates (Supplementary fig. 1A) or hatching rates (Supplementary fig. 1B) in any of the concentrations tested during the experiment. We then tested whether a developmental exposure to environmentally relevant concentrations of atrazine (0, 0.3, 3, or 30 ppb) resulted in altered physiological processes. For this purpose, embryos were exposed to the atrazine treatments from 1 hpf through the end of embryonic development (72 hpf) in three biological replicates and imaged, and specific developmental endpoints, including eye, head, and total larvae length, were assessed in each atrazine treatment. There was no increase in gross malformations in larvae in the atrazine treatments compared with those in the control treatment; however, larvae in all atrazine treatments had a significant increase in head length compared with the control ($p = 0.0005$; Fig. 1). Moreover, a significant head-to-body ratio was observed in the 30-ppb-exposed larvae ($p = 0.029$).

Transcriptome Profiles of Embryonic Zebrafish Exposed to Atrazine

Global gene expression analysis with zebrafish collected at 72 hpf was then performed to elucidate the transcriptomic response as a consequence of developmental exposure to atrazine. Results from the microarrays revealed probes

TABLE 1
qPCR Primers for Comparative Analysis and Associated Biological Processes

SEQID	Gene symbol	Primer sequence	Biological process
NM_178293	*AVP*	CCCATCAGACAGTGTATGTCGT GACAGCTGCTCCTCTTCCAT	Cell cycle/carcinogenesis
ENSDART00000016862	*CYP17A1*	GTGTGTTTCCATACGAGACCAA ATCAGCACGTGATCCTCTGTAA	Reproduction/carcinogenesis
OTTDART00000015937	*CYP19A1*	CAGGGCATCATATTCAACTCAA AGGTGGTGCAGATCTCCATAGT	Reproduction
OTTDART00000028564	*FAM84B*	CTAAACTCCGGCAAACAATTTC TTCAAAAACGCAACACGTAATC	Protein binding/carcinogenesis
OTTDART00000023474	*GLO1*	GCGCCATTTCATCATATACTCC GGTCGGTCATTTTTAGGTGTGT	Carcinogenesis
NM_205622	*LHb*	TCTGTGCACCATAAACACTTCC AATAATGCTGAGCTCGACTGTG	Reproduction
NM_194403	*MHC1UFA*	TCACACTTTCCAGTTCATGGTC GTTTCCGGTATTCATTCTGAGC	Immune system
TC262595	*PIM1*	TTGATTGGTCAAGGATCAACAG AGTCATCAAACCGACCTCTTGT	Cell cycle/carcinogenesis
ENSDART00000089953	*SIK2*	GAGGATGATCATACCCACGTTT TGACCCATGCTGAACAGTTTAC	Cell signaling/carcinogenesis
OTTDART00000018768	*TPD52L1*	GCTAATATGGAGCCCAGACAAC ACTCATTCTCCATTTCCTCTCG	Cell cycle/carcinogenesis
BM035007	*si:ch73-41e3.7*	TGATGCAGATGGTGCTTTATTC GTCTGCATGGAGTCTGTGTTTC	Reproduction



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Treatment	Whole Larvae (μm)	Head (μm)	Eye (μm)
Control	3147±379.14	550±68.73	293±37.35
0.3 ppb	3260±256.15	576±49.56*	295±27.14
3 ppb	3258±299.81	576±54.92*	294±29.28
30 ppb	3276±193.88	596±37.81*	294±21.89

FIG. 1. Morphological analysis at 72 hpf. (A–H) Representative images of zebrafish larvae at 72 hpf exposed to control treatment (A and B), 0.3 ppb (C and D), 3 ppb (E and F), or 30 ppb (G and H) atrazine treatment. (I) Summary of measurements obtained for whole larval length, head size, and eye diameter. A significant increase in head size was observed in all atrazine treatments in comparison to the control treatment (*$p < 0.05$). Data are representative of three independent replicates of 20 subsamples each ± standard deviation.

differentially expressed in all exposures consisting of 61, 202, and 198 unique probes representing 21, 62, and 64 genes in the 0.3, 3, and 30 ppb treatments, respectively (Fig. 2). There was a mixture of up (57%) and downregulated (43%) probes representing 12 and 9 genes, respectively, in the 0.3 ppb treatment (Supplementary table 1). However, a majority of the probes (89 and 97%) significantly altered in the 3 and 30 ppb exposures showed an increase in transcript expression, representing 55 and 61 genes, respectively (Supplementary tables 2 and 3). Furthermore, 66–68% of the differentially expressed genetic targets (132 probes representing 42 genes) were found in both the 3 and 30 ppb gene lists with two of these genes (*CYP17A1, SAMHD1*) present in all three atrazine treatments (Table 2).

Gene ontology analysis showed enrichment for genes corresponding to genetic disorders and developmental processes including organ and reproductive system development in the 0.3 ppb treatment (data not shown). Examples of functions with altered genes included development of testis, maturation of oocytes, and congenital adrenal hyperplasia. Analysis from the 3 ppb gene list showed enrichment for genes associated with carcinogenesis and tumorigenesis including prostate, pancreatic, and renal cancers. This analysis also showed several genes enriched for cell cycle functions such as cell division and cell cycle progression and neurological and reproductive

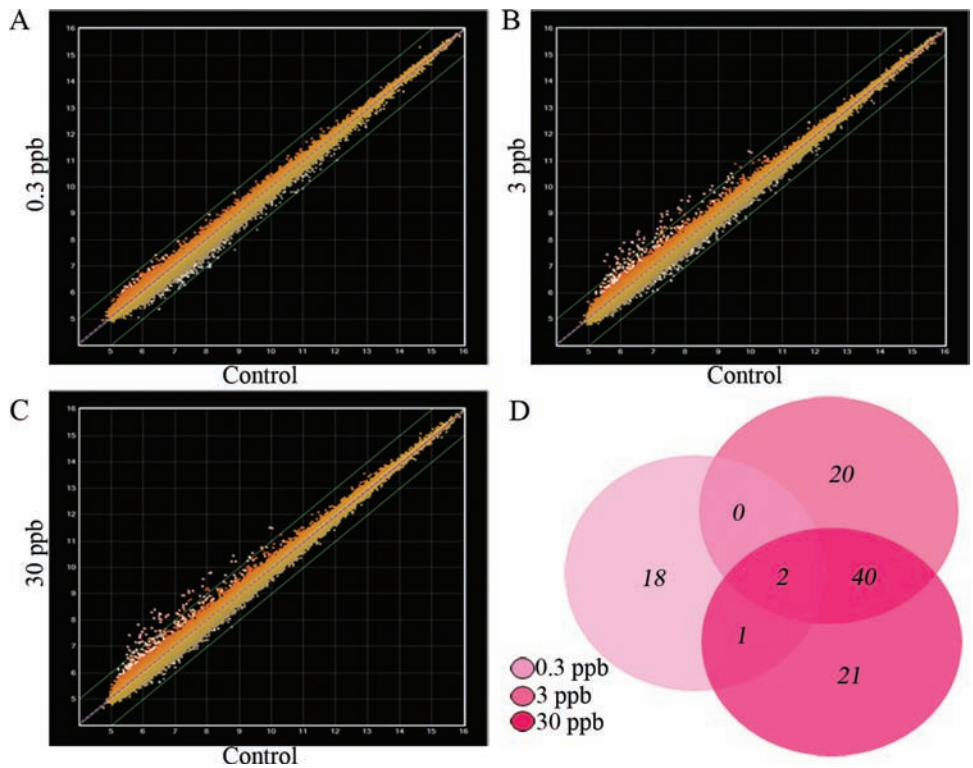

FIG. 2. Global gene expression analysis following a developmental atrazine exposure. (A–C) Mean intensity values for each probe are represented by white dots with atrazine-treated embryos plotted against controls. Using set criteria, 61 probes were found to be altered in the 0.3 ppb treatment (A), 202 probes in the 3 ppb treatment (B), and 198 probes in the 30 ppb treatment (C). Diagonal white lines represent $\log_2$ expression ratio of 1 or −1 between treated and controls (200 or 50% alteration in expression). (D) A Venn diagram indicating the unique and common genes (after gene ontology analysis) among treatments. This figure can be viewed in color online.

TABLE 2
Genes Differentially Expressed in Both the 3 and 30 ppb Concentrations Following Developmental Atrazine Treatment From 1 to 72 hpf in the Zebrafish

Gene symbol[a]	Biological process	Direction of change in expression
ADAM23	Cell signaling and development/carcinogenesis	Up
ANTXR2	Cell morphology/carcinogenesis	Up
ATPBD4	ATP binding	Up
AVP	Cell cycle/carcinogenesis	Up
BRCA2	Cell cycle/carcinogenesis	Up
CD209	Cell growth and proliferation	Up
CDH13	Neural differentiation/carcinogenesis	Up
CHRNB3	Carcinogenesis	Up
CLCN2	Cell cycle/carcinogenesis	Up
COX18	Cell protein transport/carcinogenesis	Up
CYP17A1[b]	Reproduction/carcinogenesis	Up
DDX39	RNA regulation/carcinogenesis	Up
DVL3	Cell signaling	Up
EFHB	Cell signaling	Up
FAM179B	Unknown	Up
FAM84B	Protein binding/carcinogenesis	Up
FUT7	Cell signaling/carcinogenesis	Up
GLO1	Carcinogenesis	Up
GYG1	Metabolic processes	Up
HNRNPA0	Cell growth	Up
KIAA1456	Methylation	Up
KIF5A	Cell movement	Up
LH	Reproduction	Up
LRP2	Endocrine system/carcinogenesis	Up
MAD2L2	Cell cycle	Up
MCM7	Cell cycle/carcinogenesis	Up
MHC1UFA	Immune system	Up
MUC-16	Cell adhesion/carcinogenesis	Up
PDE1A	Cell signaling	Up
PIM1	Cell cycle/carcinogenesis	Up
PRCP	Intrinsic processes	Up
RBM4B	RNA regulation/carcinogenesis	Down
RNF14	Cell growth and proliferation/carcinogenesis	Up
RSL24D1	Cellular development	Up
SAMHD1[b]	Immune function	Up
SIK2	Cell signaling	Up
SLC12A6	Cell growth and proliferation/carcinogenesis	Up
SLC15A2	Cellular transport	Up
SNCG	Cell growth and proliferation/carcinogenesis	Up
TDRD6	Cell growth and development	Up
TPD52L1	Cell cycle/carcinogenesis	Up
TTC3	Cell differentiation/carcinogenesis	Up

Notes. [a]Expression ratios and *p* values specific to each treatment are listed in Supplementary tables 2 and 3 for each gene.
[b]*CYP17A1* and *SAMHD1* were found to be altered in all three atrazine concentrations tested.

system disease (Table 3). The differentially expressed gene list from the 30 ppb treatment was similar to the 3 ppb treatment with enrichment of genes with established roles in biological processes such as cell division, growth, proliferation, and development (Table 4). In addition, altered genes were also associated with carcinogenesis including ovarian, pancreatic, and renal cancers and reproductive system disease. Of the genes common to both the 3 and 30 ppb differentially expressed gene lists, the most notable biological functions were associated with reproductive disease, cell cycle functions, and cancer (Table 2).

Affirmation of Microarray Data

Ten genes with differential expression in the microarray analysis and one gene not altered in the microarray analysis but indicated in previous studies to be altered by atrazine exposure (*CYP19A1*) were confirmed independently by qPCR. The data were statistically significant with a positive correlation between the microarray and qPCR data ($R = 0.769$, $p = 0.006$; Fig. 3).

Translational Effects of Developmental Atrazine Exposure

To investigate the translation of our observed altered gene expression targets, we performed Western blot analysis on

TABLE 3
Gene Enrichment at 3 ppb Atrazine Exposure

Biological function	*p* Value[a]	Number of genes[b]
Cancer		
Tumorigenesis	2.26E–04 to 4.13E–02	24
Prostate cancer	4.36E–03	7
Renal cancer	5.73E–03	4
Pancreatic cancer	4.09E–02	3
Neurological disease		
Major depression	7.26E–04 to 1.29E–02	5
Epilepsy	7.32E–04	4
Neuropathy	1.42E–02	10
Neurodegeneration	2.24E–02 to 3.44E–02	2
Reproductive system disease		
Reproductive system disorder	1.41E–02	7
Cell cycle		
Cell cycle progression	1.36E–03 to 4.13E–02	4
Cell division process	7.07E–04 to 3.98E–02	10
Cell stage	3.61E–04 to 4.75E–02	8

Notes. [a]Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.
[b]Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

TABLE 4
Gene Enrichment at 30 ppb Atrazine Exposure

Biological function	*p* Value[a]	Number of genes[b]
Cancer		
Ovarian cancer	1.47E–02	5
Pancreatic cancer	7.10E–03	4
Renal cancer	5.73E–03	4
Tumorigenesis	7.48E–05	26
Carcinoma	3.60E–05	16
Reproductive system disease		
Reproductive system disorder	1.41E–02	7
Genital tumor	4.85E–04	8
Cell cycle		
Mitosis	7.84E–04 to 1.99E–02	3
Cell division process	8.53E–03 to 2.64E–02	11
Cell stage	5.60E–03 to 1.77E–02	9
Cellular growth and proliferation		
Proliferation	3.74E–04 to 1.93E–02	6
Growth	4.18E–03 to 2.01E–02	14
Stimulation	9.68E–03 to 1.68E–02	3

Notes. [a]Derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.
[b]Classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

several gene targets associated with reproductive system development and function, cell cycle regulation, and carcinogenesis. There were significant changes in protein expression in atrazine-treated samples for each of the targets tested ($p = 0.022$ for GLO1, $p = 0.033$ for TPD52L1, $p = 0.020$ for CYP17A1, $p = 0.039$ for SIK2; Fig. 4). The change in direction of protein expression was the same as the levels of transcript identified in the microarrays.

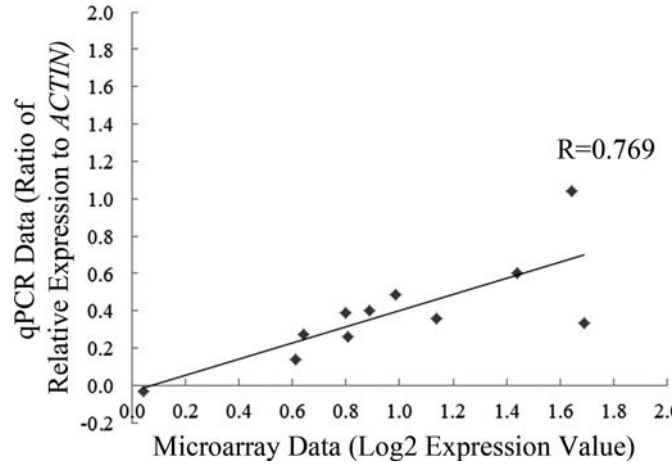

FIG. 3. qPCR comparative analysis of microarray data. qPCR analysis was performed to compare gene expression changes detected on the microarray on 11 gene targets. All targeted genes were significantly altered on the microarray except for *CYP19A1*. A linear correlation was completed and statistical significance of the correlation determined using one-way ANOVA. The data were statistically significant with a positive correlation between the microarray and the qPCR data ($R = 0.769$, $p = 0.006$). The values for the microarray data are $\log_2$ expression values and for qPCR are the ratio of relative expression against *ACTIN*.

DISCUSSION

The potential health risks associated with exposure to the herbicide atrazine is the subject of much debate in the field of endocrine disruption. One subject of this debate surrounds the concentration at which adverse effects are observed with low and high doses of atrazine reported to disrupt reproductive function and to be carcinogenic (Eldridge *et al*., 1994; Hayes *et al*., 2002; Wetzel *et al*., 1994). In this study, we first exposed zebrafish embryos immediately following fertilization to atrazine concentrations ranging from 0 to 10 ppm (near the solubility limit of the compound) and evaluated mortality and hatching rates. Results showed no substantial mortality in any of the concentrations nor were there any significant changes observed in hatching rates. Thus, even at a concentration of 10 ppm, atrazine does not appear to be overtly toxic to zebrafish, which is in agreement with results previously reported (Wiegand *et al*., 2000, 2001).

Recent studies report a link between *in utero* atrazine exposure and prevalence of SGA and impairments to fetal growth in human births (Chevrier *et al*., 2011; Munger *et al*., 1997; Ochoa-Acuña *et al*., 2009b; Villanueva *et al*., 2005). In addition, studies evaluating *in utero* agrochemical exposure indicate an increased risk of birth defects with atrazine exposure (Ochoa-Acuña *et al*., 2009a; Winchester *et al*., 2009). Although no increase in gross malformations was observed in our study, subtle morphological alterations including a significant increase in head length and head-to-body ratio was observed. These results indicate that low levels of atrazine can stimulate development and alter the physiology of exposed individuals.

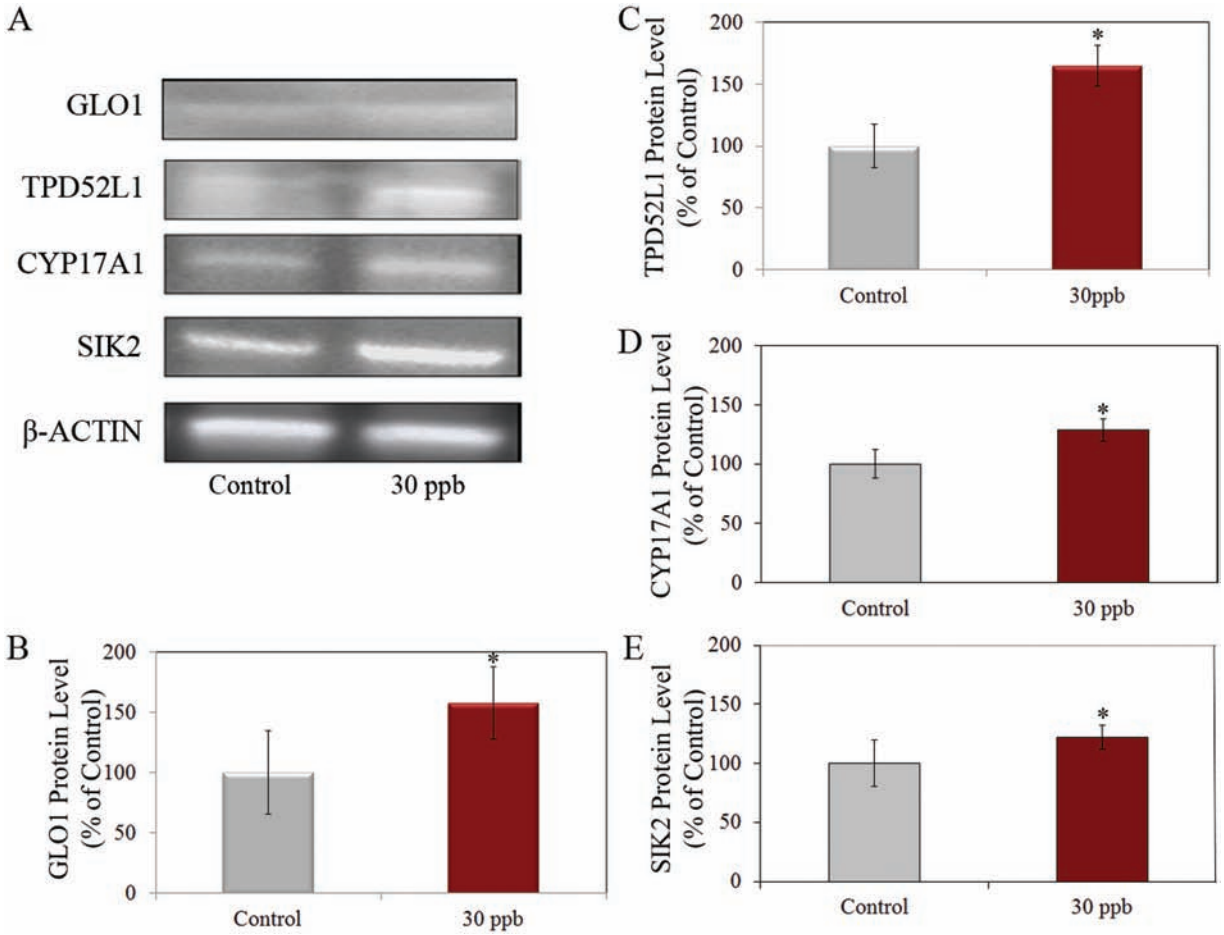

FIG. 4. Translation of gene expression alterations to the protein level. (A) Western blot analysis was conducted for specific molecular targets associated with carcinogenesis identified to be altered on the microarray to investigate translation of gene expression alterations to the protein level. (B–E) The protein levels of GLO1 (B), TPD52L1 (C), CYP17A1 (D), and SIK2 (E) were increased in the atrazine 30 ppb treatment compared with the control treatment in agreement with the microarray data. *$p < 0.05$.

Studies performed in rodent and *Xenopus* models suggest that atrazine targets the HPG axis by altering levels of LH and prolactin and by increasing the activity of aromatase (CYP19) (Cooper *et al.*, 2007; Hayes *et al.*, 2010). However, some of these findings are questioned because results are not able to be recapitulated in the hands of other investigators (Eldridge *et al.*, 2008; Solomon *et al.*, 2008). As many of the genetic and molecular pathways are conserved across vertebrate species, we used the zebrafish as a vertebrate model to identify and investigate alterations in biological processes during early development as a result of atrazine exposure. Microarrays revealed enrichment in gene sets related to previously reported functional impacts associated with atrazine exposure including cell cycle function; reproductive system development, function, and disease; and cancer (Cooper *et al.*, 2000; Eldridge *et al.*, 1994; Freeman *et al.*, 2005; Hayes *et al.*, 2002; Powell *et al.*, 2011; Wetzel *et al.*, 1994). Western blot analysis on a subset of identified targets showed alterations in the transcriptome were translated to the protein level. Previous studies showed that atrazine affects the function of the reproductive system in multiple vertebrate organisms, but a limited number of genetic and molecular targets have been associated with these morphological and functional impacts (Hayes *et al.*, 2011). In our current study, the *LH* gene was altered in both the 3 and 30 ppb treatments, and its release from the pituitary was shown to be disrupted in previous work (Cooper *et al.*, 2000). In addition, *CYP17A1*, which has established roles in the biosynthesis of steroid hormones and works upstream of *CYP19*, a key enzyme in the conversion of androgens to estrogens and a reported target of atrazine toxicity, was altered in all three concentrations tested in this study (although *CYP19* was not found to be altered) (Hayes *et al.*, 2002, 2010; Holloway *et al.*, 2008; Pogrmic-Majkic *et al.*, 2010; Suzawa and Ingraham, 2008).

Alteration of normal cell cycle progression is reported to occur after acute exposure to atrazine, resulting in an accumulation of cells in S-phase *in vitro* assessed by flow cytometry analysis (Freeman and Rayburn, 2006; Powell *et al.*, 2011). Furthermore, it was shown that the expression level of cyclin B and E proteins

was downregulated, indicating a stall in cell cycle progression at G1/S transition. Several of the genes with altered expression in the 3 and 30 ppb atrazine treatments have established roles in cell cycle progression and proliferation. Notably, *AVP*, *BRCA2*, *MAD2L2*, *MCM7*, *PIM1*, and *TPD52L1* were all significantly altered with *BRCA2*, *MCM7*, and *PIM1* having known functions relating to the progression of the cell cycle from late G1 through S-phase. However, although previous reports indicate that atrazine may cause cell cycle arrest in S-phase, our results show an increase in expression levels of the previously identified genes and a decrease in *CDK6* expression in the 3 ppb exposure. This suggests atrazine may accelerate cell cycle progression. Regardless of the interpretation, these data indicate that atrazine alters the cell cycle at the level of transcription, and as a result, it disrupts the normal orchestration of cell cycle progression.

There is much speculation surrounding the issue as to whether atrazine may indeed be a carcinogen. It was previously reported that atrazine leads to an increase in mammary tumor development when administered at extremely high doses over an extended period of time (Eldridge *et al*., 1994). The authors concluded that the high doses of atrazine created a disruption in normal estrous cycling and that an increase in estrogen secretion led to an accelerated reproductive aging process and an overall increase in levels of estrogen in the animals, which could explain the observed increase in mammary tumors. Although the evidence in support of a carcinogenic potential for atrazine is not definitive and has only been shown in rodent models (Wetzel *et al*., 1994), our study identified a number of genes associated with carcinogenesis. Several of the identified gene targets with altered expression were found in both the 3 and 30 ppb treatments, including the proto-oncogenes *GLO1*, *PIM1*, and *TPD52L1* and the tumor suppressor gene *BRCA2*. These results suggest that atrazine at environmentally relevant concentrations could potentially cause or promote carcinogenesis, and it is intriguing that the strong number of cancer-associated genes has established regulatory functions in cell cycle progression and genomic stability. In addition, a previous study identified copy number variable regions in rhabdomyosarcoma, T-cell acute lymphoblastic leukemia, and melanoma transgenic zebrafish cancer models (Freeman *et al*., 2009), and there are similarities with genes located within these copy number variable regions in these transgenic cancer models and the current study. For example, *SAMHD1* is located within an identified copy number variable region in the rhabdomyosarcoma transgenic zebrafish cancer model and had altered expression in all three atrazine treatments in the current study. In addition, *PIM3* and *TPD52L2* are located within copy number variable regions identified in these transgenic zebrafish cancer models. Although we did not directly observe altered expression of *PIM3* and *TPD52L2* in our current study, we did identify the closely related family members *PIM1* and *TPD52L1*, respectively. Furthermore, we observed that GLO1 and TPD52L1 protein levels were significantly increased in response to atrazine exposure. These results demonstrate that alterations to the transcriptome due to atrazine exposure are being translated and thereby lead to alterations of biological pathways in which these genes are implicated. Thus, if atrazine does initiate and/or promote carcinogenesis it may be at the level of transcription and specifically by altering the normal progression of the cell cycle leading to an accelerated and uncontrolled growth of cells, a hallmark in the development of a plethora of cancers (Schwartz and Shah, 2005).

In summary, atrazine is reported to influence adverse human birth outcomes and to alter the reproductive system by acting on the endocrine system. Most of the evidence to date points to a disruption in the release of LH and stimulation of aromatase activity, but the precise mode of action of atrazine is still unclear. In this study, we sought to take a different approach and analyze transcriptome changes that occur as a result of atrazine exposure. Multiple genes associated with neuroendocrine and reproductive function, cell cycle regulation, and cancer were identified to be altered after acute developmental atrazine exposure at 0.3, 3, and 30 ppb. In order to establish a mechanism through which atrazine acts, it is imperative that the key targets in the pathway(s) are identified to piece together the atrazine puzzle. This study serves the initial steps in establishing a genetic profile that can be anchored to physiological changes and responses that are a result of a developmental atrazine exposure and lay the foundation for future studies to evaluate the later life consequences of this developmental atrazine exposure.

SUPPLEMENTARY DATA

Supplementary data are available on-line at http://toxsci.oxfordjournals.org.

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