Journal of Proteomics 186 (2018) 71–82

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures

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ARTICLE INFO

Keywords:
Atrazine
Developmental toxicity
Gene expression
Behavior
Proteomics
Zebrafish

ABSTRACT

Atrazine (ATZ), the second most commonly used herbicide in the United States, is an endocrine disrupting chemical linked to cancer and a common drinking water contaminant. This study further investigates ATZ-related developmental toxicity by testing the following hypotheses in zebrafish: the effects of embryonic ATZ exposure are dependent on timing of exposure; embryonic ATZ exposure alters brain development and function; and embryonic ATZ exposure changes protein abundance in carcinogenesis-related pathways. After exposing embryos to 0, 0.3, 3, or 30 parts per billion (ppb) ATZ, we monitored the expression of cytochrome P450 family 17 subfamily A member 1 (*cyp17a1),* glyoxalase I (*glo1*), ring finger protein 14 (*rnf14*), salt inducible kinase 2 (*sik2*), tetratricopeptide domain 3 (*ttc3*), and tumor protein D52 like 1 (*tpd52l1*) at multiple embryonic time points to determine normal expression and if ATZ exposure altered expression. Only *cyp17a1* had normal dynamic expression, but *ttc3* and *tpd52l1* had ATZ-related expression changes before 72 h. Larvae exposed to 0.3 ppb ATZ had increased brain length, while larvae exposed to 30 ppb ATZ were hypoactive. Proteomic analysis identified altered protein abundance in pathways related to cellular function, neurodevelopment, and genital-tract cancer. The results indicate embryonic ATZ toxicity involves interactions of multiple pathways.

Significance: This is the first report of proteomic alterations following embryonic exposure to atrazine, an environmentally persistent pesticide and common water contaminant. Although the transcriptomic alterations in larval zebrafish with embryonic atrazine exposure have been reported, neither the time at which gene expression changes occur nor the resulting proteomic changes have been investigated. This study seeks to address these knowledge gaps by evaluating atrazine's effect on gene expression through multiple time points during embryogenesis, and correlating changes in gene expression to pathological alterations in brain length and functional changes in behavior. Finally, pathway analysis of the proteomic alterations identifies connections between the molecular changes and functional outcomes associated with embryonic atrazine exposure.

1. Introduction

Exposure to environmental stressors, including environmental toxicants, during the developmental period can cause immediate and long lasting health effects [1]. Multiple characteristics of developing organisms, including limited biotransformation of xenobiotics, lack of a blood-brain-barrier, immature immune system, and increased metabolic rate may contribute to greater toxicity during development [2]. As a consequence, toxic effects appear at much lower exposure concentrations in developing organisms compared to the concentration of toxicant required to cause adverse effects in adults. In addition, developmental plasticity is thought to be significant and critical, as

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https://doi.org/10.1016/j.jprot.2018.07.006
Received 29 March 2018; Received in revised form 29 June 2018; Accepted 2 July 2018
Available online 20 July 2018
1874-3919/

perturbations in physiologic pathways during development can result in non- or maladaptive phenotypes of disease [3]. Certain time points in the embryonic period represent critical windows for gene-environment interactions and heightened susceptibility to extrinsic and intrinsic stressors that result in phenotypic alterations [4].

Endocrine disrupting chemicals (EDCs) represent a broad class of chemicals that interfere with the action of hormones. Exposure to EDCs can disrupt normal physiology and homeostasis throughout development and the life course of an organism [5, 6], though organisms appear to have the greatest sensitivity to EDCs during the developmental period [7]. Hormones are critical for the normal growth and development of many organs and tissues, from reproductive organs to the brain. Any disruptions of the hormonal milieu has the potential to cause irrevocable changes in tissue and organ structure or function [8]. In addition to reproductive dysfunction [9], developmental EDC exposure is associated with cancer [10], alterations in innate immune function [11], obesity [12], and altered cognition, including learning and memory [13]. Furthermore, low-dose exposure to EDCs can have significant health implications, as EDCs often have nonmonotonic, U-shaped, inverted U-shaped, or other non-traditional dose response curves [8, 14]. EDCs include chemicals found in plastics and resins, plasticizers, pharmaceuticals, and pesticides [6, 15].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; ATZ), is a triazine herbicide used to control broadleaf and select grassy weeds. As of 2012, it was estimated that between 64 and 74 million pounds was used annually in the United States, making ATZ the second most commonly used agricultural pesticide [16]. Post-application rainfall causes ATZ to leach from fields into ground and surface water where it can persist in the environment [17–20]. The heavy use combined with the estimated 146 day half-life of ATZ in groundwater results in ATZ being the most common pesticide detected in agricultural stream water and both agricultural and urban groundwater sources [21]. Due to the presence of ATZ in public water sources, the US Environmental Protection Agency (EPA) regulates the concentration of ATZ in drinking water with a Maximum Contaminant Level of 3 parts per billion (ppb; μg/L) [22]; however, in 2003, the European Union effectively banned ATZ due to concerns over groundwater contamination and environmental persistence [23].

ATZ is a significant environmental toxicant because it is linked to endocrine disruption, cancer, reproductive disorders, birth defects, and altered nervous system function [24]. Epidemiological studies link ATZ exposure to decreased semen quality in Midwestern men [25], increased risk of breast cancer for women living in areas with medium or high exposure [26]; menstrual cycle irregularities [27], and increased prevalence of small-for-gestational-age infants [28]. ATZ exposure in amphibians is associated with abnormal metamorphosis and feminization [29–32], and in rodents, ATZ disrupts the hypothalamic-pituitary-gonadal axis [33–37]. The central nervous system is also a target of ATZ, with ATZ exposure altering dopaminergic and serotonergic neurotransmission as well as neurobehavior [38–43]. Although evidence suggests ATZ is an EDC, the mechanism of action is still under investigation. ATZ does not appear to have intrinsic estrogenic activity and does not bind to the estrogen receptor [44]. Instead, ATZ seems to alter intracellular signaling through the inhibition of type 4 cyclic nucleotide phosphodiesterases (PDE4), resulting in an increase in cyclic adenosine monophosphate (cAMP) and decreased expression of steroidogenic proteins [45–48]. Additionally, ATZ appears to modify the epigenome. ATZ alters microRNA levels [49] and inhibits the activity and expression of DNA methyltransferases, resulting in decreased global DNA methylation [50]. These epigenetic modifications further provide a mechanism of altered gene expression.

The zebrafish (*Danio rerio*) biomedical model has many advantages in toxicological research, including small size, large clutches, easy husbandry, well-characterized and rapid ex vivo development, short generational interval, a sequenced genome, and conserved metabolic pathways [51–55]. A previous study from our laboratory characterized the effects of embryonic, environmentally relevant, low-dose ATZ exposure on the growth and development and the transcriptome of larval zebrafish and found alterations in head length and disruptions of gene pathways associated with neuroendocrine system development and function, reproductive system development and function, and carcinogenesis [56]. This study further investigates the effects of embryonic ATZ exposure by evaluating how gene expression normally changes over a developmental time course and how ATZ exposure alters gene expression at specific developmental time points, how ATZ effects brain development and behavior, and how ATZ changes the proteome in larval zebrafish. We expect that embryonic ATZ exposure dynamically alters the expression of select genes during critical windows for toxicity. To test this hypothesis, six genes were chosen from the previous study that had altered gene expression at 72 h post fertilization (hpf; the end of embryogenesis) as a result of embryonic ATZ exposure [56]. The genes are cytochrome P450 family 17, subfamily A, member 1 (*cyp17a1),* glyoxalase I (*glo1*), ring finger protein 14 (*rnf14*), salt inducible kinase 2 (*sik2*), tetratricopeptide domain 3 (*ttc3*), and tumor protein D52 like 1 (*tpd52l1*). Each of the genes had altered expression in at least two of the ATZ treatments (3 ppb and 30 ppb) and are associated with cancer (*cyp17a1*, *glo1, rnf14, sik2, ttc3* and *tpd52l1*), the central nervous system (*cyp17a1*, *glo1*, *sik2*, and *ttc3*), and/or the endocrine system (*cyp17a1, glo1, rnf14, sik2, ttc3,* and *tpd52l1*) [56]. The normal expression of these genes was monitored throughout embryogenesis and the effects of embryonic ATZ exposure evaluated at each developmental time point (24, 36, 48, 60, and 72 hpf). We also hypothesize that embryonic ATZ exposure alters the neurodevelopment of larval zebrafish. We measured brain length and behavioral responses to a visual motor response test to evaluate brain morphology and function. Finally, we performed a proteomic analysis to identify differences in protein levels resulting from embryonic ATZ exposure. We hypothesized that pathways associated with cancer, neurological disease, reproductive system disease, and cell cycle and proliferation, which were previously altered in transcriptomic analysis, would also have altered protein levels. By performing a proteomic analysis we aim to link changes in protein levels to behavioral alterations, changes in brain morphology, and changes in gene expression throughout development.

2. Materials and methods

2.1. Zebrafish husbandry and treatment

Embryos were obtained from a breeding colony of wild-type AB strain laboratory zebrafish (*Danio rerio*). Adult zebrafish are maintained in a *Z*-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle. Water is maintained at 28 °C, the pH at 7.0–7.3, and salinity at 470–550 μS conductivity. Fish and aquaria are monitored twice daily and fed a mixture of brine shrimp (*Artemia franciscana*; Artemia International LLC., Fairview, Texas), Golden Pearls 500–800 μm (Artemia International LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, PA). Adult zebrafish were bred in spawning tanks according to established protocols [57, 58] and embryos collected immediately after the breeding interval, approximately at the 4–8 cell stage of embryonic development. The embryos were rinsed, randomly sorted into treatment groups, exposed to 0 (filtered aquaria water), 0.3, 3, or 30 ppb (μg/L) ATZ and incubated at 28.5 °C. The ATZ solutions were prepared from aliquots of a stock solution of technical grade atrazine (98.1% purity) (CAS 1912–24-9; Chem Service, West Chester, PA) as previously described [43, 56]. ATZ treatment concentrations were confirmed with an US EPA approved immunoassay kit (Abraxis Atrazine ELISA Kit, Warminster, PA) as previously described [59, 60]. Unless collected beforehand, larvae were rinsed at 72 h post fertilization (hpf) with aquaria water to end ATZ exposure and then maintained in clean aquaria water until collected. All protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely with regard to prevention

and alleviation of suffering.

2.2. Transcript analysis

Genes previously identified as being altered by ATZ exposure at 72 hpf were evaluated for normal changes in expression through development and time specific alterations in gene expression resulting from ATZ exposure. For the developmental time course, each replicate consisted of 5 petri dishes containing 50 embryos treated with filtered aquaria water. One of the 5 petri dishes was then collected at 24, 36, 48, 60, and 72 hpf. For the atrazine exposure, 50 embryos per petri dish were exposed to 0, 0.3, 3, or 30 ppb ATZ. A replicate consisted of a petri dish of embryos from each treatment collected at each of the 24, 36, 48, and 60 hpf time points. For both the time course and atrazine exposures a total of 6 biological replicates (n = 6) were collected, with each biological replicate representing embryos from a different breeding clutch. During collection, embryos were euthanized via anesthetic overdose with 0.4 mg/mL tricaine-S (ethyl m-amino benzoate methanesulfonate; Western Chemical Inc., Ferndale, WA), homogenized in Trizol (Life Technologies, Carlsbad, CA), flash frozen in liquid nitrogen, and stored at −80 °C until RNA isolation. RNA was isolated with the RNEasy Mini Kit (Qiagen, Germantown, MD) and cDNA was synthesized using the Superscript First Strand Synthesis Kit (Life Technologies, Carlsbad, CA) following established protocols [61]. The relative expression of *cyp17a1, glo1, rnf14, sik2, ttc3,* and *tpd52l1* was determined via quantitative PCR (qPCR) following similar methods as described previously [56, 57, 62, 63] and MIQE guidelines [64]. Forward and reverse primers for the target genes (Integrated DNA Technologies, Coralville, IA) were designed using the Primer3 Website (Table 1) and checked using NCBI Primer-BLAST [65]. As in previous studies [43, 56] *β-actin* was chosen as a reference gene due to consistent expression that did not vary across atrazine exposures. The expression of *β-actin* was not altered between 24, 36, 48, and 60 hpf (data not shown). qPCR analysis was performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with a standard protocol [63]. Melting and standard dilution curves and no template controls were evaluated to ensure appropriate efficiency (100 ± 10%) and specificity. Experimental samples were run in triplicate to provide technical replicates. Gene expression was normalized to *β-actin* (gene of interest/*β-actin*). A one-way ANOVA on SAS 94 software (SAS Institute Inc., Cary, NC) was used to evaluate for differences between groups. When the outcome was statistically significant, a Fisher's Least Significant Difference (LSD) post hoc test at $\alpha = 0.05$ was performed to determine groups significantly different from the control (72 hpf time point for the time course and 0 ppb treatment for ATZ exposures).

2.3. Brain measurement analysis

To determine if brain development was altered by ATZ exposure, 50 embryos per treatment were exposed to 0, 0.3, 3, or 30 ppb ATZ. At 72 hpf, larvae were euthanized via anesthetic overdose and the brain lengths, defined as the distance from the most rostral aspect of the brain to the brainstem spinal cord junction [66], of 20 randomly selected larvae (considered subsamples) were measured per treatment per replicate. A total of six biological replicates (n = 6) were imaged via light microscopy using a Nikon SMZ1500 dissecting microscope with a Nikon Digital Sight DS-fil camera and NIS Elements imaging software (Nikon Instruments Inc., Melville, NY). A one-way ANOVA was used to analyze differences among treatments, and a Fisher's LSD test at $\alpha = 0.05$ was used when a significant ANOVA was observed.

2.4. Larval visual motor response test

To evaluate if developmental ATZ exposure was associated with behavioral alterations, larval zebrafish behavior during a visual motor response (VMR) test was monitored at 120 hpf. This is the earliest time in which zebrafish have robust, testable, locomotion when reared at 28.5 °C [67]. A total of 5 biological replicates (n = 5) were completed. For each replicate, 50 embryos per treatment were exposed to 0, 0.3, 3, or 30 ppb ATZ and 24 larvae from each treatment (considered subsamples) were placed into separate wells in a 96-well plate with 0.5 mL of filtered aquaria water. Grossly malformed or dead larvae were excluded. Treatments were balanced between columns to reduce location based test artifact. The loaded 96-well plate was incubated at 28 °C for 10 min before being transferred to the Noldus DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands). The Noldus Temperature Control Unit maintained the observation chamber water temperature at 28 °C throughout the course of the experiment. Following a 10 min dark acclimation period, the Noldus White Light Routine was used to test the VMR by exposing the larvae to series of 10 min alternating dark and light periods for a total of 50 min [68]. All behavioral experiments were performed 11 am-1 pm to minimize circadian variability in movement. The infrared movement traces were recorded at a rate of 25 frames per second with a Basler GenICam acA 1300-60 g camera and analyzed with the Noldus EthoVision 11.5 software. Tracks were smoothed via a minimum distanced moved profile set to > 0.2 mm and data for movement endpoints such as total distance moved, velocity, time spent moving, turn-angle, angular velocity, meander, and turning direction were collected. An ANOVA was used to analyze differences among treatments and a Fisher's LSD test at $\alpha = 0.05$ was used when a significant ANOVA was observed.

Table 1
Primers used in qPCR analysis.

Seq ID	Gene symbol	Primer sequences[a]	Biological function
NM_212806.3	*cyp17a1*	gtgtgtttccatacgagaccaa atcagcacgtgatcctctgtaa	Steroid biosynthesis
NM_213151.1	*glo1*	gcgccatttcatcatatactcc ggtcggtcatttttaggtgtgt	Formation of s-lactoyl-glutathione
NM_001002087.1	*rnf14*	gtttgcgggatgagtatctttc ttctgtatattggtgccacagc	Binds to androgen receptor and coactivator of target gene expression
ENSDART00000089953	*sik2*	gaggatgatcatacccacgttt tgacccatgctgaacagtttac	Regulation of insulin receptor signaling pathway
XM_009305043.1	*ttc3*	acccctacactgatgaggaaga ctcaccactgttgttctcgaag	Negative regulator of cell differentiation
NM_001020734.1	*tpd52l1*	gctaatatggagcccagacaac actcattctccatttcctctcg	Cell proliferation and calcium signaling
NM_181601	*β-actin*	ctaaaaactggaacggtgaagg aggcaaataagtttcggaacaa	Cell motility, structure, and integrity (Reference gene)

[a] Primer sequences for cyp17a1, glo1, sik2, and tpd52l1 originally appeared in Weber et al. (2013).

2.5. Proteomics

To determine if embryonic ATZ exposure results in altered protein levels, 50 embryos per treatment were exposed to 0, 0.3, 3, or 30 ppb ATZ. At 120 hpf, larvae were euthanized via hypothermic shock and 30 randomly selected larvae from each treatment per replicate were pooled in a 1.5 mL microcentrifuge tube, rinsed in phosphate buffered saline (PBS), flash frozen in liquid nitrogen, and stored at −80 °C until further processing. A total of 6 biological replicates were collected. Samples were collected at 120 hpf to minimize the presence of yolk proteins. For proteomic analysis, 100 µL of 100 mM ammonium bicarbonate (ABC) was added to the zebrafish before transferring the samples into reinforced 2 mL tubes containing 2.8 mm ceramic (zirconium oxide) beads (Cayman Chemical, Ann Arbor, MI). The tubes were loaded into a Precellys 24 homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France), and samples were homogenized at 6500 rpm in 3 cycles of 20 s each. Protein concentration was determined using a bicinchoninic acid (BCA) assay, and 50 µg of protein was isolated for digestion using an acetone precipitation. After removing acetone, samples were reduced and alkylated, and sequence grade Lys-C/Trypsin (Promega, Madison, WI) was used to enzymatically digest the extracted protein. All digestions were carried out in the Barocycler NEP2320 at 50 °C under 20,000 psi for 1 h. Digested samples were cleaned over C18 spin columns (Nest Group, Southborough, MA) and dried in a vacuum centrifuge. Resulting pellets were resuspended in 97% purified H2O/3% acetonitrile (ACN)/0.1% formic acid (FA).

For liquid chromatography/mass spectrometry (LC/MS), samples were analyzed using the Dionex UltiMate 3000 RSLC Nano System coupled to the Q Exactive™ HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA). Peptides were loaded onto a trap column (20 µm × 350 mm) and washed using a flow rate of 5 µL/min with 98% purified water/2% ACN/0.01% FA. The trap column was switched in-line with the analytical column after 5 min, and peptides were separated using a reverse phase Acclaim PepMap RSLC C18 (75 µm × 15 cm) analytical column using a 120 min method at a flow rate of 300 nl/min. Mobile phase A consisted of 0.01% FA in water while mobile phase B consisted of 0.01% FA in 80% ACN. The linear gradient started at 5% B and reached 30% B in 80 min, 45% B in 91 min, and 100% B in 93 min. The column was held at 100% B for the next 5 min before being brought back to 5% B and held for 20 min. Sample was injected into the QE HF through the Nanospray Flex™ Ion Source fitted with an emission tip from Thermo Scientific. Data acquisition was performed monitoring the top 20 precursors at 120,000 resolution with an injection time of 100 milliseconds. For quality assurance and quality control, instrument evaluations and calibrations are run weekly and a standard *E. coli* digest (Waters, Milford, MA) is used routinely to check instrument performance.

The files from the MS were processed using the MaxQuant computational proteomics platform version 1.5.5.1 (Max-Planck-Gesellschaft, München, Germany) [69]. The peak list generated was searched against the *Danio rerio* sequences from UNIPROT retrieved on 10/25/2016 and a common contaminants database (MaxQuant, Max-Planck-Gesellschaft, München, Germany). The following settings were used for MaxQuant: default Orbitrap parameters, minimum peptides length of seven amino-acid, data was analyzed with 'Label-free quantification' (LFQ) checked and the 'Match between runs' interval set to 1 min, protein FDR was set to 1%, enzyme trypsin and LysC allowing for two missed cleavage and three modifications per peptide, fixed modifications were Iodoethanol (C), variable modifications were set to Acetyl (Protein N-term) and Oxidation (M). Additional information on peptide and protein results are in the supplementary data.

An in-house script was used to perform the following steps on the MaxQuant results: removed all the common contaminant proteins, log transformed [log2(x)] the LFQ intensity values, input the missing values using the average values of the other two samples when just one sample was missing and use half of the lowest intensity when all three samples were missing in one group and present in all three samples in the other group. The statistical analyses were performed in R® (www.cran.r-project.org). An ANOVA was performed on the LFQ intensities and only proteins with p-value < 0.05 were used in further analyses. Tukey's post hoc test identified differences between treatment groups. The list of proteins was imported into Ingenuity Pathway Analysis (IPA; Qiagen, Germantown, MD) and matched to the human orthologs of the zebrafish proteins for gene ontology and molecular pathway analysis.

3. Results

3.1. Gene expression through development and after ATZ exposure

The normal expression of target genes *cyp17a1, glo1, rnf14, sik2, ttc3,* and *tpd52l1* was determined throughout a developmental time course (24, 36, 48, 60, and 72 hpf). These genes were chosen based on identification of altered expression at 72 hpf in a previous study [56] and their association with cancer, the central nervous system, and/or the endocrine system. The effect of ATZ exposure during embryogenesis was evaluated by comparing gene expression across treatments at 24, 36, 48, and 60 hpf.

The developmental time course for *cyp17a1* showed significant alterations in expression, with significantly decreased relative expression of *cyp17a1* at 24 hpf and 60 hpf (p = 0.0001; Fig. 1A) compared to 72 hpf. However, following atrazine exposure, there were no significant changes at 24, 36, 48, or 60 hpf (p = 0.3892, p = 0.9207, p = 0.6050, and p = 0.2709, respectively; Fig. S1 A-D).

There were no significant differences in the relative expression of

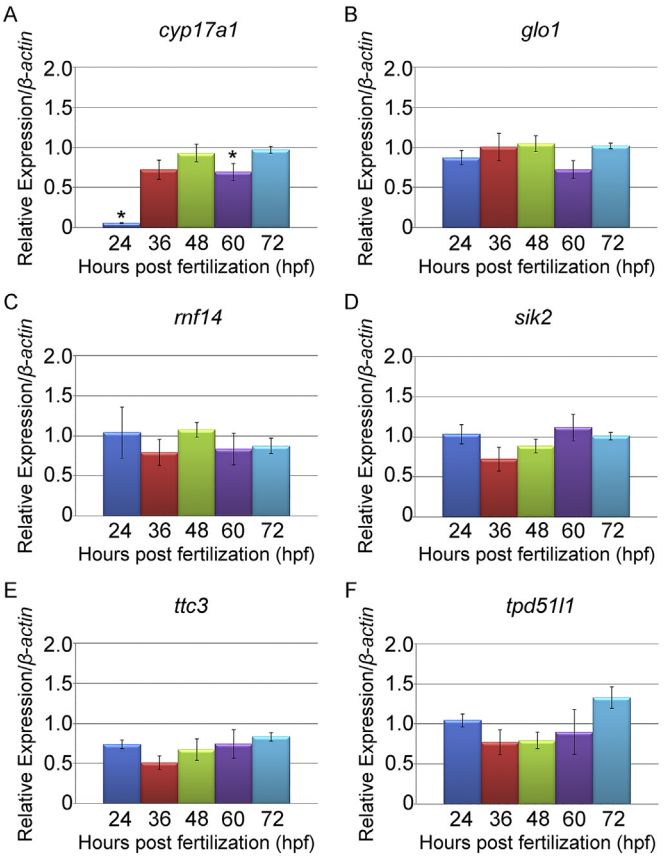

Fig. 1. The relative expression of select genes throughout the time course of development. The relative expression of *cyp17a1* was significantly decreased at 24 and 60 hpf compared to the expression at 72 hpf (A). The relative expression of *glo1* (B), *rnf14* (C), *sik2* (D), *ttc3* (E), and *tpd52l1* (F) was not significantly different from the expression at 72 hpf at any time point. n = 6, qPCR run in triplicate, error bars represent standard deviation, * = p < 0.05.

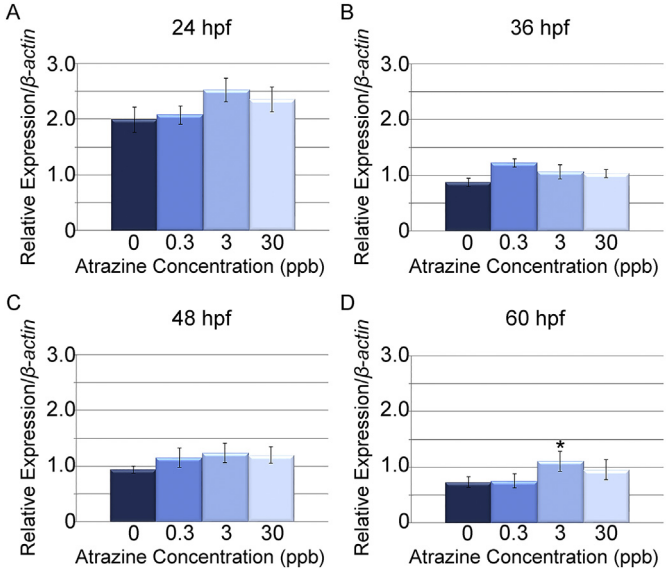

Fig. 2. The time course of *ttc3* relative expression after developmental ATZ exposure. No significant differences in relative expression were observed between treatment groups at 24 hpf (A), 36 hpf (B), or at 48 hpf (C). At 60 hpf, the 3 ppb treatment had increased relative expression as compared to the controls (D). n = 6, qPCR run in triplicate, error bars represent standard deviation, * = $p < 0.05$. (hpf: hours post fertilization).

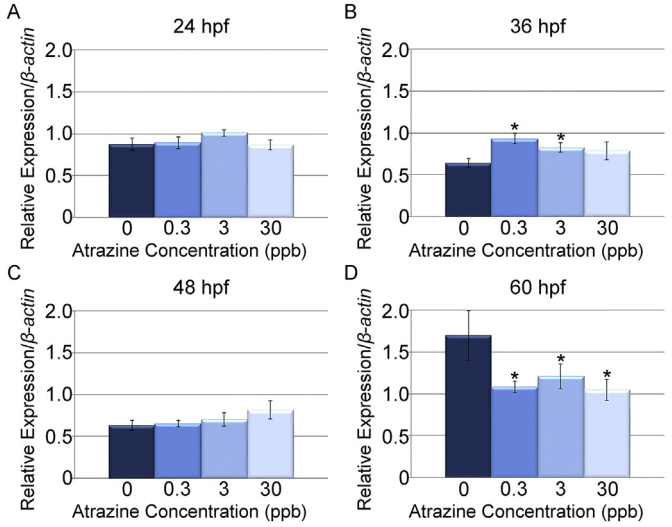

Fig. 3. The time course of *tpd52l1* relative expression after developmental ATZ exposure. No significant differences in relative expression were observed between treatment groups at 24 hpf (A). At 36 hpf, the 0.3 and 3 ppb treatment groups had a significant increase in relative gene expression compared to the controls (B), but there was no significant differences between treatments again at 48 hpf (C). At 60 hpf, the 0.3, 3, and 30 ppb treatments all had a significant decrease in relative expression compared to the controls (D). n = 6, qPCR run in triplicate, error bars represent standard deviation, * = $p < 0.05$. (hpf: hours post fertilization).

glo1 during the developmental time course ($p = 0.2491$; Fig. 1B). Exposure to ATZ did not significantly alter relative gene expression at 24, 36, 48, or 60 hpf ($p = 0.4299$, $p = 0.3210$, $p = 0.3952$, and $p = 0.6788$, respectively; Fig. S2A-D).

During the developmental time course, no significant differences in the relative expression of *rnf14* were observed ($p = 0.2431$; Fig. 1C). Similarly, ATZ exposure did not elicit significant changes in relative gene expression at 24, 36, 48, or 60 hpf ($p = 0.4726$, $p = 0.3905$, $p = 0.8840$, and $p = 0.1657$, respectively; Fig. S3A-D).

The relative expression of *sik2* was not significantly altered during the developmental time course ($p = 0.0711$; Fig. 1D). There were no statistically significant changes in the relative expression of *sik2* following ATZ exposure at 24, 36, 48, and 60 hpf ($p = 0.0596$, $p = 0.4104$, $p = 0.2148$, and $p = 0.2822$, respectively; Fig. S4A-D).

The relative expression of *ttc3* did not significantly change throughout the developmental time course ($p = 0.3893$; Fig. 1E). Although no significant differences in relative gene expression were observed at 24, 36, or 48 hpf ($p = 0.2589$, $p = 0.2369$, and $p = 0.2949$; Fig. 2A–C), at 60 hpf, the relative expression of *ttc3* was increased in the 3 ppb treatment group as compared to the 0 ppb controls ($p = 0.0099$; Fig. 2D).

Throughout the developmental time course, relative *tpd52l1*expression did not significantly change ($p = 0.0714$; Fig. 1F). In the ATZ treated groups, there were no significant differences in relative gene expression at 24 and 48 hpf ($p = 0.2360$, $p = 0.3040$; Fig. 3A,C). However, ATZ exposure did alter the relative expression of *tpd52l1* at 36 and 60 hpf. At 36 hpf the 0.3 and 3 ppb treatment groups had a significant increase in relative gene expression compared to the control ($p = 0.0253$; Fig. 3B). At 60 hpf, there was a decrease in relative expression for all ATZ treatments (0.3, 3, and 30 ppb) compared to the controls ($p = 0.0246$; Fig. 3D).

3.2. Brain morphology

Brain length measurements were taken at 72 hpf. The 0.3 ppb treatment group had a significantly longer brain as compared to the 0 ppb controls ($p = 0.0027$, Fig. 4A–B). The 3 ppb and 30 ppb brain lengths were not significantly different from the controls.

3.3. Larval behavior

In the VMR test, the 30 ppb treatment group was significantly different from the 0 ppb controls with respect to distance moved ($p = 0.0199$), velocity ($p = 0.0197$), and time spent moving ($p = 0.0123$) (Fig. 5A–C). The 30 ppb treatment had decreased distance moved, decreased velocity, and spent less time moving compared to the controls. There was no difference in other locomotor parameters including heading, turn angle, angular velocity, meander, or rotational direction ($p = 0.567$, $p = 0.8384$, p = 0.8384, $p = 0.8568$, $p = 0.4418$, and $p = 0.1798$; Fig. S5).

3.4. Proteomics

Out of a total of 1690 proteins identified, 28 proteins had significant ($p < 0.05$) LFQ intensity values. Of the 28 proteins, 21 had significantly altered abundance in at least one treatment group compared to the controls according to Tukey post hoc comparisons (Table 2). Three proteins had significant differences in abundance between the 0 ppb and 0.3 ppb treatment groups, 4 proteins had significant differences in abundance between the 0 ppb and 3 ppb groups, and 16 proteins had significant differences in abundance between the 0 ppb and 30 ppb treatments. Comparing the 0.3 ppb treatment to the controls, 67% of altered proteins were upregulated and 33% were downregulated, with PSMB4 having the largest $\log_2$ fold change of 0.5755. In the 3 ppb treatment group, 50% of proteins were upregulated and 50% downregulated compared to the controls, with HP1BP3 having the largest $\log_2$ fold change of −1.4097. In the 30 ppb treatment, 50% of proteins were upregulated and 50% downregulated compared to controls, with MYH7B having the largest $\log_2$ fold change increase of 0.9816 and ATP5MD having the largest $\log_2$ fold change decrease of −1.2348. FTMT and PFN2 abundance was altered in both the 3 ppb and 30 ppb treatments compared to controls.

Pathway analysis was performed on all 21 identified proteins to elucidate pathways altered by ATZ exposure. It should be noted that IPA creates pathways based on human orthologs of zebrafish proteins. The list of proteins was enriched for transforming growth factor beta 1

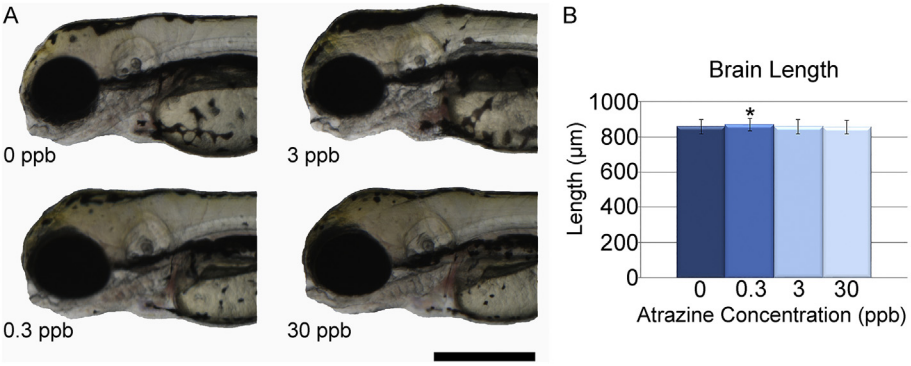

Fig. 4. Brain length in 72 hpf larvae with developmental ATZ exposure. Representative images of larval zebrafish measured at 72 hpf after developmental exposure to ATZ (A). The 0.3 ppb treatment group had a significantly longer brain compared to the controls (B). n = 6, 20 subsamples per replicate, error bars represent standard deviation, scale bar = 500 µm, * = $p < 0.05$.

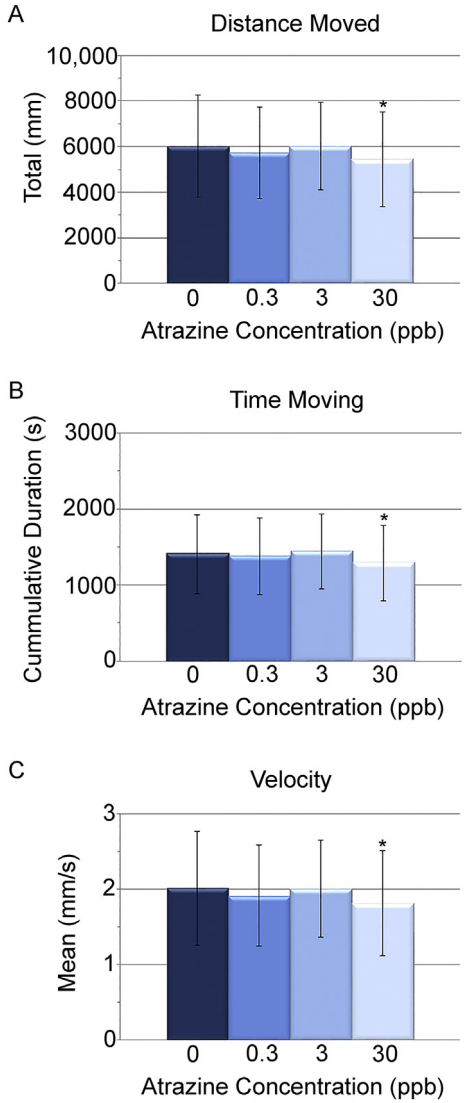

Fig. 5. Behavioral alterations after developmental ATZ exposure. A VMR test performed at 120 hpf found that the 30 ppb treatment group had decreased total distance moved (A), decreased velocity (B), and spent less time moving (C) than the controls. n = 5, 24 subsamples per replicate, error bars represent standard deviation, * = $p < 0.05$.

(TGFB1), peoniflorin, gonadotropin-releasing hormone (GnRH) analog, L-3,4-dihydroxyphenylalanine (levodopa; L-Dopa), and prostaglandin J2 as top upstream regulators (Table 3). The top diseases and biological functions associated with the protein list included cardiovascular disease, organismal injury and abnormalities, and cancer (Table 4). The most enriched physiological system development and function pathways included cardiovascular system development and function, embryonic development, and nervous system development and function (Table 5). Fifteen proteins from the list were associated with urogenital and genital tract cancers, including prostate cancer (Fig. 6). Networks associated with cellular compromise, cell death and survival, and neurological disease were also enriched (Fig. S6).

4. Discussion

During the embryonic period, the expression of certain genes is dynamically regulated to control normal development and differentiation [70]. Of the 6 selected genes with expression changes at 72 hpf, only *cyp17a1* was dynamically expressed earlier during the developmental time course under normal conditions, with lowered expression at 24 and 60 hpf. *cyp17a1* is a critical enzyme in steroid hormone biosynthesis, having both 17α-hydroxylase and 17,20-lyase activities. When *cyp17a1* favors 17α-hydroxylase activity, the steroidogenic pathway favors the production of glucocorticoids; however, when the 17,20-lyase activity predominates the pathway favors the creation of androgens and estrogens [71, 72]. Glucocorticoids and sex hormones are both important for regulating development, with altered sex hormone levels causing reproductive abnormalities and altered cortisol levels causing impaired growth, altered somitogenesis, and embryonic malformations [73–75]. Although we identified *cyp17a1* as a dynamically regulated gene during zebrafish development, embryonic exposure to environmentally relevant levels of the herbicide ATZ did not alter the relative gene expression of *cyp17a1* at 24, 36, 48, or 60 hpf. In a previous study, *cyp17a1* expression was altered in all three ATZ exposure at 72 hpf, suggesting 72 hpf is a critical time point in ATZ toxicity [56].

Developmental exposure to ATZ did, however, selectively disrupt the relative gene expression of *ttc3* and *tpd52l1* before 72 hpf. *ttc3* codes for an E3 ubiquitin-protein ligase involved in the negative regulation of cell proliferation through a mechanism of ubiquitination and increased proteasomal degradation of the serine-threonine protein kinase, AKT1 [76, 77]. *ttc3* is also linked to Down Syndrome [78], and increased expression is associated with inhibition of neuronal differentiation and decreased neurite formation associated with abnormal actin polymerization and altered Golgi organization [79, 80]. In our study, the relative expression of *ttc3* was increased compared to controls in the 3 ppb ATZ exposure group at 60 hpf. At 72 hpf, the expression of *ttc3* was upregulated at both 3 and 30 ppb [56], indicating that developmental ATZ exposure has nonmonotonic, but stimulatory effects on the relative expression of *ttc3* at 60–72 hpf. *tpd52l1* codes for a protein with a coiled-coil domain that was first identified associated with human breast carcinoma [81] and is implicated in cell proliferation [82] and calcium signaling [83], as well as in the regulation of apoptosis signal-regulating kinase 1 (ASK1) induced apoptosis [84]. In our study, the relative expression of *tpd52l1* was altered by developmental exposure to ATZ. The upregulation at 36 hpf followed by down relation at 60 hpf

Table 2
List of Altered Proteins.

Protein ID	Protein name	Gene symbol	Human ortholog	Biological function	ANOVA *p*-value	Fold Change[a]
Proteins altered in 0.3 ppb exposure compared to control treatment						
A0A0R4IML0	Capping protein (actin filament), gelsolin-like b	*capgb*	CAPG	Barbed-end actin filament capping	0.0188	−0.3859
Q568F3	Proteasome subunit beta type (EC 3.4.25.1)	*psmb4*	PSMB4	Proteasome core complex	0.0280	0.5755
F1RBR6	Phosphotriesterase-related protein	*pter*	PTER	Zinc ion binding/catabolic process	0.0231	0.5627
Proteins altered in 3 ppb exposure compared to control treatment						
Q6DHT8	Ferritin	*zgc:92066*	FTMT	Cellular iron ion homeostasis/transport	0.0206	0.6707
A0A0A0MPG3	Gamma-crystallin N-B	*crygn2*	–	–	0.0361	0.6599
A0A140LGU1	Heterochromatin protein 1 binding protein 3	*hp 1 bp3*	HP1BP3	Heterochromatin organization	0.0123	−1.4097
Q7ZVJ0	Profilin	*pfn2l*	PFN2	Actin cytoskeleton organization	0.0139	−0.4252
Proteins altered in 30 ppb exposure compared to control treatment						
F1R6L1	Adducin 3 (gamma) a	*add3a*	ADD3	Cytoskeleton	0.0085	0.7959
H0WES8	ATP synthase membrane subunit DAPIT	*atp5md*	ATP5MD	Mitochondrial proton-transporting ATP synthase	0.0323	−1.2348
B8JK21	Caspase 3	*casp3a*	CASP3	Execution of apoptosis	0.0320	−0.5241
Q90X19	Creatine kinase; muscle a	*ckma ckm*	CKM	ATP binding/kinase activity	0.0172	0.3759
Q6P2V1	Cathepsin C	*ctsc*	CTSC	Cysteine-type peptidase activity	0.0083	−0.6671
Q6DHT8	Ferritin	*zgc:92066*	FTMT	Cellular iron ion homeostasis/transport	0.0206	0.6662
Q08BL9	Kelch-like family member 24a	*klhl24a klhl24 zgc:153342*	KLHL24	Protein ubiquitination	0.0240	−0.3795
Q6DHB6	Keratin 17/91	*krt17 krt91 zgc:92533*	KRT17	Intermediate filament	0.0283	0.9372
E7F5L1	Myosin, heavy chain 7B, cardiac muscle, βb	*myh7bb*	MYH7B	Myosin complex/ATP binding	0.0118	0.9816
Q6NUY8	Ndufa9 protein	*ndufa9a ndufa9*	NDUFA9	Response to hypoxia	0.0418	0.8526
Q7ZVJ0	Profilin	*pfn2l*	PFN2	Actin cytoskeleton organization	0.0139	−0.4353
Q6IQL7	Peptidylprolyl isomerase D	*ppid*	PPID	Protein folding	< 0.0001	−0.7583
M5BFV8	Collagen type XXII alpha 1 chain	*prp col22a1*	COL22A1	Extracellular matrix/blood vessel morphogenesis	< 0.0001	9.3083
Q9PUS1	Proteasome subunit beta type	*psmb*	PSMB7	Proteasome core complex	0.0420	−0.7667
U3JB26	Serine/arginine-rich splicing factor 2a	*srsf2a*	SRSF2	Nucleic acid/nucleotide binding	0.0456	0.9047
Q6IQD7	Tropomyosin 2	*tpm2 zgc:86810*	TPM1	Striated muscle thin filament	0.0480	−0.3891

[a] Log_2 transformation.

Table 3
Top upstream regulators.

Name	Biological function	p-value of overlap[a]
TGFB1	Cell proliferation, differentiation, and other functions	1.06E-04
Peoniflorin	Stimulates aromatase activity	1.54E-04
GnRH analog	Regulates follicle-stimulating hormone and luteinizing hormone release	2.21E-04
L-Dopa	Precursor of dopamine	3.10E-04
Prostaglandin J2	Neuroinflammation	3.74E-04

[a] Derived from the likelihood of observing the degree of enrichment in a protein set of a given size by chance alone.

Table 4
Top enriched diseases and disorders.

Name	p-value[a]	# Molecules[b]
Cardiovascular Disease	4.29E-02 - 5.06E-04	7
Organismal Injury and Abnormalities	4.91E-02 - 5.06E-04	17
Cancer	4.91E-02 - 9.11E-04	17
Connective Tissue Disorders	3.50E-02 - 9.11E-04	7

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

Table 5
Top enriched physiological system development and function pathways.

Name	p-value[a]	# Molecules[b]
Cardiovascular System Development and Function	4.37E-02 - 9.11E-04	5
Connective Tissue Development and Function	4.55E-02 - 9.11E-04	5
Embryonic Development	4.98E-02 - 9.11E-04	6
Nervous System Development and Function	4.37E-02 - 9.11E-04	6

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

suggests that the effect of ATZ on *tpd52l1* expression is time sensitive. Interestingly, at 72 hpf, *tpd52l1* was again upregulated in the 3 and 30 ppb ATZ treatment groups [56]. The nonmonotonic changes in relative expression of *ttc3* and *tpd52l1* combined with the dynamic up- and downregulation suggests compensation in expression to maintain normal homeostatic conditions.

The relative expression of the remaining genes (*glo1*, *rnf14*, and *sik2*) was not altered either in the developmental time course or after developmental ATZ exposure at the time points evaluated. However, in our previous study *glo1*, *rnf14*, and *sik2* were upregulated in the 3 and 30 ppb ATZ groups at 72 hpf [56]. The lack of earlier alterations in gene expression suggests that 72 hpf represents a critical time point for these genes in developmental ATZ toxicity. It is also possible that other overlapping pathways are disrupted at specific time points, leading to an adaptive response through up- and downregulation of associated pathways that is observable at 72 hpf [85].

Developmental ATZ toxicity is a result of the dynamic changes in the global transcriptome. Therefore, it is important to note that although we chose to investigate the effect of ATZ on the relative

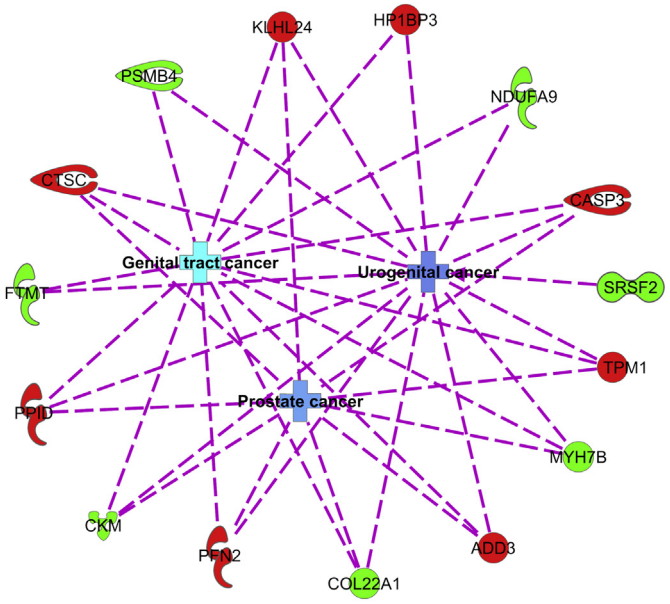

Fig. 6. Genital Cancer Network. Based on network analysis, 15 out of 21 altered proteins were associated with genital tract cancers, prostate cancer, or urogenital cancer in general. Red indicates proteins with increased abundance; green indicates proteins with decreased abundance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of 6 genes (*cyp17a1, glo1, rnf14, sik2, ttc3,* and *tpd52l1*) throughout a developmental time course, in reality, developmental ATZ exposure also effects the expression of hundreds of other genes as previously identified via transcriptomic analysis [56]. Thus, by evaluating the relative expression of a subset of genes previously linked to cancer, the central nervous system, and the endocrine system, we did not seek to establish a mechanism of toxicity, but rather to characterize normal expression through a developmental time course and identify the critical time points for developmental ATZ toxicity. Moreover, the ATZ related disruption of epigenetic mechanisms [49, 50] provides a basis to explain why ATZ seems not to consistently target a single processes within a cell [86], as transcriptomic and epigenetic alterations can compound after developmental exposure to toxicants [87, 88].

We also sought to characterize the effects of altered gene expression on the nervous system and hypothesized that embryonic ATZ exposure would alter normal neurodevelopment. This hypothesis is based on evidence suggesting developmental ATZ exposure alters transcriptomic pathways associated with neuroendocrine system function and neurological disease in zebrafish larvae, and on the previous finding of altered head lengths in 72 hpf larvae with embryonic exposure to ATZ. In Weber et al. (2013), larvae exposed to 0.3, 3, and 30 ppb ATZ had significantly increased head lengths compared to control larvae. To evaluate neurodevelopment we measured the brain length, as brain length has been shown to be altered in postnatal mice with prenatal nicotine exposure [89], in zebrafish embryos with early exposure to cyclosporine [90], and as a result of altered wingless-related integration site (*wnt)* signaling [91], suggesting altered brain length as an endpoint in evaluating developmental neurotoxicity. In our study, only larvae with exposure to 0.3 ppb ATZ had significantly larger brains when compared to controls at 72 hpf. The lack of significant brain length changes in the 3 and 30 ppb ATZ treatment groups could suggest that either the brain is proportionally smaller than head, which would indicate impaired brain development or suggest activation of compensatory pathways at higher exposures [14]. We did not measure head lengths in our larvae and are thus unable to determine if the brains of the 3 and 30 ppb groups are proportionally smaller. However, the increased brain size of the 0.3 ppb group does suggest that low dose ATZ exposure during embryogenesis perturbs normal neurodevelopment.

We further hypothesized that developmental ATZ exposure would alter neurologic function. We used the VMR behavioral assay to determine if changes in gene expression and altered brain morphology could be linked to behavioral alterations. The VMR is a broad test of neurologic and locomotor function and behavioral differences in larvae can be a result of altered neurological signaling or structural defects [92, 93]. It is therefore possible that both alterations in gene expression and brain development contributed to the altered VMR test results in our study. Hypoactivity, characterized by decreased distance moved, decreased velocity, and decreased time spent moving, was observed in the 30 ppb treatment group, our highest developmental exposure. Interestingly, Liu et al. (2016) performed a similar experiment with developmental ATZ exposures of 30, 100, and 300 ppb ATZ, and in a similar locomotor assay found decreased free swimming distance in the 100 and 300 ppb treatments but not the 30 ppb treatment [94]. Experimental conditions were different between our study and Liu et al (2016). In their study, ATZ concentrations were made in 0.01% dimethyl sulfoxide (DMSO) while our ATZ was prepared in filtered aquaria water. Similarly, the length of the experiment and light settings were different between experiments, and our sample size was larger, with 120 total individual subsamples (n = 5, 24 subsamples per treatment per replicate) compared to 24 (n = 4 with 6 subsamples per treatment per replicate). It is possible that the increased power associated with a larger sample size and the differences in experimental protocols account for the differences in results. Behavioral alterations have also been observed in C57BL/6 mice with gestational and lactational exposure to ATZ. Juvenile offspring of dams exposed to 1.4 mg/kg/day ATZ had altered performance on forced swim and marble burying tests and were hyperactive in an open field test [41]. Although the exposures are difficult to compare, these results provide additional support for ATZ related behavioral changes.

The proteome after developmental ATZ exposure was also evaluated to determine if changes in gene expression were translated into altered protein abundance. The pathways identified by IPA are centered around organism and organ development, cardiovascular disease, connective tissue disorders, and cancer. It should be cautioned that small data sets, such as in this study, are often limited to and biased towards broad, categorical results based on assigned gene ontology data [95]. Additionally, although there are no overlap between genes found altered at 72 hpf in our previous study [56] and in the current list of altered proteins, the pathways identified are complementary. The lack of overlap may be due in part to the difference in sample collection time, with transcriptomic evaluation occurring at 72 hpf, but proteomic evaluation occurring at 120 hpf to avoid the overwhelming presence of yolk proteins. It is likely that proteomic evaluation at 120 hpf is catching downstream changes in protein abundance that result from transcriptomic alterations at 72 hpf and are related to adverse health outcomes previously associated with atrazine toxicity in studies by our laboratory and others [29, 37, 40, 41, 43, 50, 56, 60, 96–99].

The pathways altered on proteomic analysis are related to development and cellular function and maintenance. Proteins that were altered after ATZ exposure include those associated with ATP binding, the proteasome complex, heterochromatin and nucleic acid binding, regulation of apoptosis, and neurodevelopment. Many of these processes are associated with cell cycle progression and thus potentially carcinogenesis. The human orthologs of zebrafish proteins Myh7bb and Ckma both bind to ATP in skeletal muscle and therefore have roles in both energy management and musculoskeletal development [100, 101]. Creatine kinase (CK) normally converts ATP to phosphocreatine in a reversible reaction. Altered CK abundance has been linked to cancer as dysregulated cellular ATP handling can lead to abnormal regulation of mitosis and cell division [102]. Atp5md, an ATP synthase membrane subunit, is associated with mitochondrial ATP synthase and

thus is also associated with energy production and management [103]. Psmb4 and Psmb7 are both proteosomal subunits and are involved in the removal of unwanted proteins. Proteasomes are highly conserved, multi-subunit complexes that traditionally have a role in the degradation of ubiquitin-tagged proteins [104]. Proteasomes have also been non-canonically implicated in the regulation of chromatin structure and function, in that proteasomes can act as canonical proteases but also as protein chaperones to alter the initiation of transcription and the modification of chromatin [105]. Hp1bp3 and Srsf2a have roles in heterochromatin and nucleic acid biding respectively. Hp1bp3 is a binding protein of heterochromatin protein 1 (Hp1) and is related to the linker histone H1 family, a group of proteins that bind to nucleosomes and interact with nucleosomal DNA [106]. Srsf2a has been provisionally identified in zebrafish, but human orthologs for the *srsf2a* gene have yet to be identified. In general, serine/arginine-rich splicing factors are involved in both alternative and constitutive splicing of pre-mRNAs [107]. Altered abundance of these proteins suggests a possible epigenetic component to developmental ATZ exposure, as altered expression of genes that regulate DNA accessibility or post-transcriptionally modify RNA could have far reaching effects on gene expression. Another protein of interest is Casp3a. Casp3a is an executioner caspase involved in apoptosis; however, caspase 3 is also implicated in tissue differentiation and neurodevelopment in mammals [108]. In addition to Casp3a, Ckm, Myh7bb, and Pfn2l are also associated with neuritogenesis and Ckm, Myh7bb, and Pfn2l are linked to dendritic growth and neuron morphology [109–111]. Finally, FTMT, an enzyme with ferroxidase activity and a role in iron metabolism, is also associated with Friedreich ataxia, restless leg syndrome, and Alzheimer's disease [112]. Alterations in these proteins could suggest a possible mechanism for altered neurodevelopment or later-life neurodegeneration.

Although there are no previous reports of global proteomic alterations in zebrafish after developmental ATZ exposure, livers from adult female zebrafish with 14 day exposure to either 10 or 1000 ppb ATZ had 7 proteins with altered abundance after ATZ exposure and the proteins were associated with cellular stress, oncogenesis, lipid metabolism, and protein transport [113]. This suggests that acute and developmental ATZ exposures are associated with carcinogenesis and disruptions of cellular signaling pathways. Our results are also in accordance with proteomic changes in other species observed after ATZ exposure. For example, gonads from *Xenopus laevis* tadpoles exposed to 100 ppb ATZ had altered protein pathways associated with p53 signaling, apoptosis, tight junctions, and amino acid metabolism, and lipid metabolism [114]. Similarly, the liver proteome in 1-year-old largemouth bass (*Micropterus salmoides*) had altered protein abundance associated with energy production, lipid oxidation, and protein folding and catabolism after an acute, 96 h exposure to a 3 μg/g total body burden exposure [115]. Finally, human MCF-7 breast cancer cells exposed to 100 ppb of ATZ for 24 h had altered abundance of proteins involved in oxidative stress, cell morphology, and ubiquitination [116], while exposure to 200 ppb of ATZ for 24 hpf altered the abundance of membrane proteins [86]. Thus, ATZ seems to consistently affect molecular and cellular networks associated with carcinogenesis, protein processing, and cellular signaling. Although only 28 proteins were identified as having significant LFQ values in our study, this is in line with other zebrafish embryo toxicity studies [117] and the total number of detected proteins (1690) in our study is higher than 2DE [117–120] but equivalent to SDS-PAGE [121] gel methods used in other zebrafish embryo studies.

5. Conclusions

The developmental period is characterized by marked plasticity, with dynamic changes in the expression of genes important for growth and development. Perturbations during this period can disrupt normal gene expression in multiple, compensatory pathways, as an organism seeks to maintain homeostatic conditions. We have identified genes, such as *cyp17a1* that are dynamically expressed during development as well as genes such as *ttc3* and *tpd52l1* that have critical windows before 72 hpf where exposure to ATZ will disrupt normal gene expression. Developmental exposure to ATZ has physical outcomes of increased brain size and functional changes observed through decreased activity and locomotion. Proteomic evaluation at 120 hpf indicates that ATZ alters the abundance of proteins associated with organism and organ system development, cellular processes, neurodevelopment, and cancer. Our results highlight the utility of pathways analysis in evaluating the effects of ATZ exposure and link transcriptional and proteomic alterations to physiological outcomes. Changes in these cellular processes may have broad effects that could activate compensatory signaling pathways or other processes. Thus, the effects of developmental ATZ exposure are broad, time sensitive, and involve the interaction of many pathways.

Acknowledgements

The authors acknowledge the use of the facilities of the Bindley Bioscience Center, a core facility of the NIH-funded Indiana Clinical and Translational Sciences Institute.

Funding

This work was supported by the National Institutes of Health, National Institute of Environmental Health Sciences (R15 ES019137), the National Institute for Occupational Safety and Health (T42/OH008672), the Indiana Clinical and Translational Sciences Institute (funded in part by grant # UL1TR001108 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award), the Purdue Cancer Prevention Internship Program (CPIP), and the Purdue Center for Cancer Research Summer Undergraduate Research Program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.07.006.

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