

Anxiety-related behavior and associated brain transcriptome and epigenome alterations in adult female zebrafish exposed to atrazine during embryogenesis

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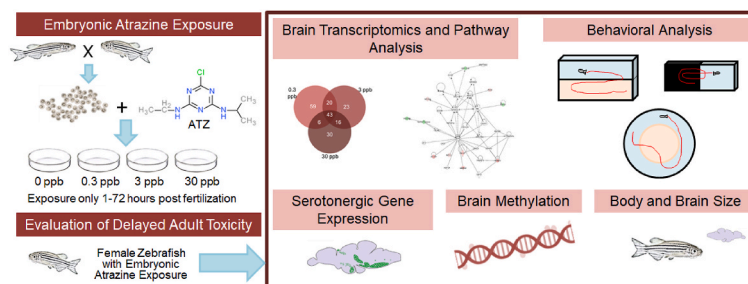
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HIGHLIGHTS

- Adult female zebrafish have anxious phenotypes after embryonic atrazine exposure.
- Embryonic atrazine exposure alters cancer and organismal injury pathway expression.
- Beta-estradiol and the estrogen receptor were top upstream regulators.
- Brain methylation is increased in females with 0.3 ppb embryonic atrazine exposure.
- Body and brain size were altered in adult females with embryonic ATZ exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

Atrazine often contaminates drinking water sources, exceeding the maximum contaminant level established by the US Environmental Protection Agency at 3 parts per billion (ppb; $\mu\text{g/L}$). Atrazine is linked to endocrine disruption, neurotoxicity, and cancer, with delayed health effects observed after developmental exposure in line with the developmental origins of health and disease (DOHaD) hypothesis. To test the hypothesis that embryonic atrazine exposure induces delayed neurotoxicity in adult female zebrafish (*Danio rerio*), embryos were exposed to 0, 0.3, 3, or 30 ppb atrazine during embryogenesis (1–72 h post fertilization (hpf)) and raised to adults with no additional atrazine exposure. Behavioral outcomes were tested through a novel tank test, light-dark box, and open field test and indicated female zebrafish had more anxious phenotypes at 9 months post fertilization (mpf). Female brain transcriptomic analysis at 9 mpf found altered gene expression pathways related to organismal injury and cancer with beta-estradiol and estrogen receptor as top upstream regulators. These results were

Abbreviations: 5mC, 5-methylcytosine; ATZ, atrazine; DOHaD, developmental origins of health and disease; dpf, days post fertilization; EDC, endocrine disrupting chemical; ELISA, enzyme-linked immunosorbent assay; EPA, Environmental Protection Agency; hpf, hours post fertilization; IPA, Ingenuity Pathway Analysis; LDB, light-dark box; LH, luteinizing hormone; MCL, maximum contaminant level; mpf, months post fertilization; NOAEL, no observed adverse effects level; NTT, novel tank test; OFT, open field test; ppb, parts per billion; qPCR, quantitative polymerase chain reaction.

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compared to 9 mpf male and 6 mpf female groups with the same atrazine embryonic exposures and showed differences in specific genes that were altered, but similarities in top molecular pathways. Molecular pathways associated with behavior were observed only in the 6 mpf transcriptomic profiles, suggesting prediction of observed behavioral outcomes at 9 mpf. The expression of genes associated with serotonin neurotransmission was also evaluated at 14 mpf to determine persistence; however, no significant changes were observed. Brain global methylation in 12 mpf zebrafish observed an increased percent 5 mC in females with embryonic 0.3 ppb atrazine exposure. Finally, the body length, body weight, and brain weight were determined at 14 mpf and were altered in all treatment groups. These results indicate that embryonic atrazine exposure does cause delayed neurotoxicity within the DOHaD framework, which is significant given atrazine's presence and persistence in the environment.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) was first registered in 1958 for control of broadleaf and select grassy weeds and is now the second most frequently used agricultural herbicide in the United States (U.S. Environmental Protection Agency, 2006). Atrazine is of regulatory concern because it is water soluble, leaches after application into nearby surface and ground water, and persists in the environment (Dao et al., 1979; Thurman et al., 1991; Schwab et al., 2006; Vonberg et al., 2014a). Atrazine is the most frequent pesticide detected in ground and surface waters in the US (Gilliom et al., 2006) with streams in the Midwest having up to 224 parts per billion (ppb; $\mu\text{g/L}$) (Battaglin et al., 2000). The US Environmental Protection Agency's (EPA) maximum contaminant level (MCL) in drinking water is 3 ppb (U.S. Environmental Protection Agency, 2006); however, this level is often exceeded seasonally in Midwestern community water systems (Almberg et al., 2018). ATZ is also used heavily in China, with ATZ detected in 100% of ground water samples and 99.5% of tap water samples (Wang et al., 2020) at concentrations up to 1.441 $\mu\text{g/L}$ in tap water. In contrast, atrazine was banned in the European Union (EU) in 2003 due to concerns over persistence in groundwater sources (European Commission, 2003). Despite being banned by the EU in 2003 and individual member countries prior to the EU ban, ATZ remains a ubiquitous contaminant in waters in Italy and Germany (Guzzella et al., 2006; Vonberg et al., 2014b).

The environmental and drinking water contamination is a significant concern because atrazine is linked to adverse health outcomes including reproductive toxicity, neurotoxicity, developmental toxicity, and cancer primarily through endocrine disruption pathways (Mostafalou and Abdollahi, 2017; Ahkin Chin Tai and Freeman, 2021). Altered dopaminergic and serotonergic neurotransmission after atrazine exposure is reported in rodent and zebrafish models (Coban and Filipov, 2007; Bardullas et al., 2011; Rodriguez et al., 2013; Li et al., 2014; Lin et al., 2014; Wirbisky et al., 2015); feminization and abnormal metamorphosis associated with atrazine is described in frogs (Hayes et al., 2002; Freeman et al., 2005; Brodeur et al., 2013); and disruptions of the hypothalamic-pituitary-gonadal axis are reported in rodents and zebrafish (Cooper et al., 1996; Friedmann, 2002; Victor-Costa et al., 2010; Foradori et al., 2011, 2013; Song et al., 2014). Atrazine is also associated with increased risk of breast cancer in women (Kettles et al., 1997), menstrual cycle length irregularities (Cragin et al., 2011), enriched expression of genes associated with abortion (Harris et al., 2020), higher prevalence of small for gestational age infants (Ochoa-Acuna et al., 2009), and congenital defects in children of women living in atrazine use areas (Waller et al., 2010; Agopian et al., 2013). Additionally, developmental atrazine exposure is also associated with later life neurotoxicity (Belloni et al., 2011; Lin et al., 2013; Wirbisky et al., 2015, 2016b) and reproductive dysfunction (Wirbisky et al., 2016d). Overall, there is current agreement that the atrazine toxicity mode of action involves suppression of the luteinizing hormone (LH) surge resulting in altered neuroendocrine signaling. These neuroendocrine effects are the primary toxicity outcome of regulatory concern for the US EPA (Rickard et al., 2018), but the research literature is growing in regards to additional neurotoxicity outcomes at doses near or below

the current no observed adverse effects levels (NOAELs) (reviewed in (Stradtman and Freeman, 2021)).

Endocrine disrupting chemical (EDC) exposure during developmental life stages can significantly affect tissue organization, structure, and function and these effects have the potential to persist into or only become apparent during adulthood (Welshons et al., 2003; Diamanti-Kandarakis et al., 2009; Barouki et al., 2012; Gore et al., 2015). Although the developmental period has the highest sensitivity to EDCs (Bern, 1992), predicting the immediate and lasting effects of EDC exposure can be difficult and is complicated by the fact that EDCs are often associated with non-monotonic dose response curves (Vandenberg et al., 2012). The delayed consequences of developmental endocrine disruption are consistent with the Developmental Origins of Health and Disease (DOHaD) hypothesis, which contends that developmental exposure to stressors, including environmental chemical contaminants, can cause genetic, epigenetic, or physiologic alterations that are associated with later life disease (Haugen et al., 2015; Heindel et al., 2015). Although multiple mechanisms of action have been proposed for ATZ, including disruption of the luteinizing hormone surge (Foradori et al., 2011) and disruption of cAMP-specific phosphodiesterase-4 (PDE4) with lowered expression of some genes related to steroidogenesis (Roberge et al., 2004; Abarikwu et al., 2011; Kucka et al., 2012), the mechanism of atrazine endocrine disruption is not fully elucidated and additional epigenetic mechanisms are suggested. Atrazine is associated with changes in DNA methylation and microRNA activity (Wirbisky et al., 2016c; McBirney et al., 2017; Wirbisky-Hershberger et al., 2017; Sánchez et al., 2020; Xie et al., 2021), which is mechanistically important, considering epigenetic alterations are associated with many of the delayed health effects associated with EDCs (Dolinoy and Jirtle, 2008; Barouki et al., 2012).

Our laboratory has previously used the zebrafish (*Danio rerio*) model to study embryonic atrazine exposure and found immediate effects on development and larval behavior (Horzmann et al., 2018) and later in life impacts at 6 months post fertilization (mpf) related to sex-specific gene expression and neurotransmitter alterations (Wirbisky et al., 2015, 2016b; Horzmann et al., 2021). Most recently, we reported decreased locomotion and abnormal brain gene expression in male zebrafish at 9 mpf with embryonic atrazine exposure (Horzmann et al., 2021). The zebrafish is a useful model in toxicologic research due to the well described development, conserved metabolic pathways, sequenced genome, and defined behavioral catalog (Kimmel et al., 1995; Hill et al., 2005; de Esch et al., 2012; Bailey et al., 2013; Howe et al., 2013). Additionally, zebrafish metabolize atrazine similar to mammals after developmental exposure (Ahkin Chin Tai et al., 2021), suggesting zebrafish are well suited to model atrazine toxicity. This study further examines atrazine neurotoxicity by testing the hypothesis that embryonic atrazine exposure induces sex-specific outcomes related to delayed neurotoxicity in adult female zebrafish. Specific endpoints assessed included movement and anxiety-related behavior, brain gene expression and methylation, and body and brain morphology in adult female zebrafish at 9, 12, or 14 mpf. Results were then compared to our past studies in the adult male zebrafish aged 9 mpf and younger adult female zebrafish aged 6 mpf to determine sex- and age-specific outcomes.

2. Materials and methods

2.1. Chemical exposure solutions

A stock solution of 98.1% purity ATZ (CAS, 1912-24-9; Chem Service) was prepared as in (Weber et al., 2013; Wirbisky et al., 2015) at 10 parts per million (ppm; mg/L). The 10 ppm stock was diluted with filtered (0.20 μ m polystyrene vacuum filter) aquaria water to make exposure solutions of 0.3, 3, and 30 ppb (μ g/L). The negative control treatment consisted of filtered aquaria water (0 ppb). An US EPA approved enzyme-linked immunosorbent assay (ELISA) kit (Abraxis) confirmed the exposure concentrations (Freeman and Rayburn, 2005).

2.2. Zebrafish husbandry and embryo dosing

Zebrafish embryos were from a colony of AB wild-type zebrafish (*Danio rerio*). The breeding colony is housed in a Pentair Aquatics System with a 14:10 light-dark cycle and water temperature of 26–28 °C, pH 7.0–7.3, and conductivity of 470–550 μ S and is monitored twice daily. Fish are fed brine shrimp (*Artemia franciscana*; Artemia International) twice daily with a mixture of Golden Pearls 500–800 μ m (Artemia International) and Zeigler adult zebrafish food (Zeigler Brothers). Adults were bred in spawning tanks (Westerfield, 2007; Peterson et al., 2011). Embryos were collected immediately after breeding (4–8 cell stage), rinsed, sorted into groups with 50 embryos in a Petri dish per treatment, and exposed to 20 mL of 0, 0.3, 3, or 30 ppb atrazine. Embryos were immersed in atrazine exposure solutions at 28.5 °C until 72 hpf (the end of embryogenesis) when the atrazine exposure was ended by rinsing the eleuthero-embryos in filtered aquaria water. The fish were raised with general husbandry conditions until 9, 12, or 14 months post fertilization (mpf) (Horzmann et al., 2021). Larval zebrafish 5 days post fertilization (dpf) through 28 dpf had a stocking density of 50 larvae/L and juvenile zebrafish older than 28 dpf and adult zebrafish had a stocking density of 5–10 fish/L. The sex ratio in raised zebrafish was approximately 1:1 as observed in prior studies (Wirbisky et al., 2016d). A timeline of the study is provided in Fig. S1. Female zebrafish were chosen for this study due to the sex specific effects of atrazine in 6 mpf zebrafish observed in a previous study (Wirbisky et al., 2015) and that results from male zebrafish aged 9 mpf were previously reported (Horzmann et al., 2021). The Purdue University Animal Care and Use Committee approved all protocols (IACUC #1110000088) and all fish were treated humanely with care to prevent and alleviate suffering.

2.3. Zebrafish behavior at 9 mpf

The behavior at 9 mpf was evaluated in 10 female zebrafish (as subsamples) randomly chosen from 4 biological replicates ($n = 4$, with a total of 40 subsamples per treatment). Biological replicates are defined as cohorts of adults raised from clutches of embryos spawned on independent weeks and raised in separate tanks. Zebrafish were tested individually with three behavioral tests: the Novel Tank Test (NTT), the Light-Dark Box (LDB), and the Open Field Test (OFT) as previously described (Horzmann et al., 2021), with each test lasting 10 min. In brief, the NTT tests measures locomotion and anxiety-like behavior by recording the activity of a zebrafish placed into a novel tank with lower and upper detection zones. Anxious phenotypes prefer the lower zone (diving response). In the LDB test, a similar tank is half covered in black paper and half covered in white paper. The LDB is another test of anxiety-like behavior, specifically scototaxis or dark preference, and locomotion as zebrafish are placed in the tank and their movement and zonal preferences recorded. The OFT is a third test of locomotion and anxiety-like behavior, specifically thigmotaxis or wall-hugging, with the movement of fish recorded as they move through inner and outer zones. Zebrafish were placed in holding tanks maintained at 28 °C for 10 min prior to the first test and for 80–160 min between tests. The behavioral tests were completed over multiple testing days, and the while the

battery of tests were performed in the same order each day, the treatment order was varied to reduce bias (Kalueff et al., 2016). On each testing day 1–2 subsamples underwent the battery of tests. Videos of the tests were recorded at 5 frames per second with an Ikegami ICD-49 Super-Cube DSP Monochrome Camera and analyzed with Noldus EthoVision XT 12 software (Noldus Information Technology). Two observers (KH and BT) checked the accuracy of video recognition and tracking. Endpoints assessed included distance moved, time spent moving, time in zones, and frequency of zone entries.

2.4. Adult 9 mpf female brain microarray

The 9 mpf brain transcriptome was evaluated in one female zebrafish from each treatment group (0, 0.3, 3, or 30 ppb atrazine) from each biological replicate ($n = 4$). Zebrafish were euthanized in 0.4 mg/mL buffered tricaine-S (Western Chemical). Brains were dissected, homogenized in Trizol (Life Technologies), frozen in liquid nitrogen, and kept at –80 °C. The RNeasy MinElute Cleanup Kit (Qiagen) was used for RNA isolation. Treatment differences in the brain transcriptome were analyzed via a custom one-color multiplex zebrafish 4×180 K microarray platform (Agilent Technologies) (Wirbisky et al., 2016a, 2016d; Horzmann et al., 2021) that targets 36,000 known and predicted genes based on the UCSC Genome and Ensembl databases. The arrays were scanned on an Agilent Technologies SureScan Microarray Scanner (Agilent Technologies) and Agilent Feature Extraction Software 12.0 was used for data extraction. MIAME guidelines were followed for microarray analysis (Brazma et al., 2001). Gene ontology and molecular pathway analysis were determined with Ingenuity Pathway Analysis (IPA). IPA identifies human orthologs of zebrafish genes and the human orthologs were used for pathway analysis. Gene notation thus reflects the identified human orthologs. The results from the 9 mpf female brain transcriptome analysis were compared to previous results from 9 mpf male brain (Horzmann et al., 2021) and 6 mpf female brain (Wirbisky et al., 2015) transcriptome data to assess sex- and age-specificity.

2.5. Quantitative polymerase chain reaction (qPCR)

Microarray gene expression changes were confirmed by performing qPCR on a group of genes (*ace*, *decr1*, *krt8*, *nmrk2*, and *strip1*). The same samples as used for the microarray were used for cDNA synthesis ($n = 4$). cDNA synthesis was completed using the SuperScript First-strand synthesis system (Invitrogen) (Peterson and Freeman, 2009). Primers specific to the target genes (Integrated DNA Technologies) were created using Primer3 (<http://primer3.ut.ee/>) (Table S1). Primers were checked for genome location specificity with NCBI Primer-BLAST (Untergasser et al., 2012). The Bio-Rad Sso Advance SYBR Green Supermix was used for qPCR on a Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad) following MIQE guidelines (Bustin et al., 2009) and as described previously (Peterson et al., 2011; Zhang et al., 2011; Weber et al., 2013; Freeman et al., 2014). Similar to previous studies (Weber et al., 2013; Wirbisky et al., 2015; Horzmann et al., 2021), β -actin was used as a reference gene following confirmation of consistent expression that did not change with atrazine exposure. Technical triplicate replicates of the experimental samples were included for assessment of the mean. Gene expression of the test gene was normalized to the reference gene, β -actin, using the $2^{-\Delta\Delta C_q}$ method (Livak and Schmittgen, 2001). No template controls, along with melt curves and standard dilution curves, were included to assess reaction specificity and efficiency ($100 \pm 10\%$).

Expression of genes related to the serotonergic pathway (*hrt1a*, *hrt1ab*, *hrt2cl1*, *hrt4*, *sarta*, *serb*, *tp1a*, *tp1b*, *tp2*, *mao*) was evaluated in 14 mpf female zebrafish brain. Brains were removed from six zebrafish per treatment group. RNA isolation, cDNA synthesis, and qPCR analysis was performed similarly as to the qPCR confirmation of microarray results. Primers are listed in Table S2.

2.6. Evaluation of global brain methylation at 12 mpf

The brains of 6–8 zebrafish per treatment were removed at 12 mpf and genomic DNA extracted as previously described (Dobrynski et al., 2011; Wirbisky-Hersberger et al., 2017), but modified slightly for smaller brain samples (Horzmann et al., 2021). A commercial ELISA kit (5-mC DNA ELISA Kit, Zymo Research) quantified global 5-methylcytosine (5 mC). Each sample was tested in duplicate and the average of the two values used for comparison.

2.7. Body and brain size analysis at 14 mpf

Body and brain size was evaluated in 2–35 adult female zebrafish aged 14 mpf (subsamples) in each treatment in each biological replicate ($n = 3$; 14–69 total subsamples per treatment). Body mass and length (snout to tip of caudal fin) was first measured. Then the brain was dissected and weighed to attain brain mass (Horzmann et al., 2021).

2.8. Statistical analysis

Statistical significance was tested by a one-way analysis of variance (ANOVA) with SAS software (SAS Institute Inc.) for behavior, methylation, and body and brain size. A Fisher's Least Significant Difference (LSD) post hoc test was included to determine differences between treatments when the ANOVA was significant ($\alpha = 0.05$). Microarrays were analyzed using GeneSpring 14.9 (Agilent Technologies) with an ANOVA followed by a Tukey HSD post hoc test ($\alpha = 0.05$). In addition, a 1.5 fold change was incorporated to evaluate for biological significance (Dalman et al., 2012). qPCR comparison to microarray results was assessed by a Pearson's correlation using $\log_2$ fold change and $\log_2$ relative expression ($\alpha = 0.05$).

3. Results

3.1. Adult female behavior at age 9 mpf

The results of the behavioral battery of tests are outlined in Table 1. At 9 mpf behavioral assessments of female zebrafish exposed to atrazine only during embryogenesis was completed. Female zebrafish had no significant difference in movement related parameters such as distance moved ($p = 0.9277$) or time spent moving ($p = 0.6731$) in the NTT; however, the group exposed to 3 ppb ATZ during embryogenesis had a significant decrease ($p = 0.0254$) in the time spent in the upper zone compared to the controls, suggesting an increase in anxiety-like

behavior, although there was no difference in the frequency of upper zone entry ($p = 0.4177$; Fig. S2).

In the LDB test, no significant differences were observed for distance moved ($p = 0.8986$), time spent moving ($p = 0.5563$), frequency of light zone entry ($p = 0.7536$), or time spent in the light zone ($p = 0.2858$), suggesting embryonic ATZ exposure does not affect scototaxis (Fig. S3). Similarly, in the OFT no significant differences were seen for distance moved ($p = 0.1559$), time spent moving ($p = 0.1388$), or time spent in the inner zone ($p = 0.7686$), but the groups with 0.3 or 30 ppb embryonic ATZ exposure had a significantly increased frequency of inner zone entry compared to controls ($p = 0.0396$; Fig. S4), possibly suggesting a stress response or altered thigmotaxis.

3.2. Adult female zebrafish brain transcriptome at 9 mpf

Brain transcriptomic analysis was performed to identify genes in 9 mpf female zebrafish with differential expression after embryonic exposure to different levels of ATZ. Transcriptomic analysis identified 128 genes with altered expression that mapped to human orthologs in the 0.3 ppb exposure, 102 mapped genes with altered expression in the 3 ppb exposure, and 95 mapped genes with altered expression in the 30 ppb exposure. Of these genes, 43 had altered expression in all three exposure groups (GSE112503; Fig. 1; Table 2).

Gene ontology analyses with IPA identified enriched pathways associated with disease and biological functions in each treatment group. Females with embryonic exposure to 0.3 ppb ATZ had enrichment in pathways associated with reproductive system disease, RNA post-transcriptional modification, embryonic development, and cancer (Table S3) with 109 genes associated with abdominal neoplasms, 92 genes with gastrointestinal carcinoma, and 61 genes with genital tract cancer (Fig. S5). The females with embryonic exposure to 3 ppb ATZ had enrichment in pathways associated with cell morphology, endocrine system disorders, nervous system development and function, and cancer (Table S4) with 92 genes associated with solid tumors, 77 with gastrointestinal carcinoma, 54 with urogenital cancer, and 5 genes with axonogenesis. Females with 30 ppb ATZ embryonic exposure had enrichment in pathways associated with cell death and survival, organismal development, and cancer (Table S5) with 74 genes associated with gastrointestinal carcinoma and 49 genes with genitourinary carcinoma. Beta-estradiol was identified as a top upstream in the 3 and 30 ppb treatment groups, while the estrogen receptor was also identified in only the 30 ppb treatment group.

Genes that were altered in all three exposures were identified. Gene ontology of the 43 mapped genes in females identified enrichment of

Table 1
Summary of behavioral test results in 9 mpf female zebrafish with embryonic ATZ exposure.

Behavioral Test	Anxiety Measure	Relevant Zones	Interpretation		
Novel Tank Test	Diving Response	Upper vs Lower	Decreased time/entry in upper zone indicates increased anxiety-like behavior		
Light-Dark Box	Scototaxis	Dark vs Light	Decreased time/entry in light zone indicates increased anxiety-like behavior		
Open Field Test	Thigmotaxis	Inner vs Outer	Decrease time/entry in inner zone indicates increased anxiety-like behavior		
Behavioral Results of 9 mpf female zebrafish with embryonic ATZ exposure					
Test	Sex	Distance Moved	Time Spent Moving	Time Spent in Zone	Frequency of Zonal Entry
Novel Tank Test	Female	No change	No change	Decreased time in upper zone - 3 ppb	No change
Light-Dark Box	Female	No change	No change	No change	No change
Open Field Test	Female	No change	No change	No change	Increased entry to inner zone - 0.3 and 30 ppb
Behavioral Results of 9 mpf male zebrafish with embryonic ATZ exposure ^a					
Test	Sex	Distance Moved	Time Spent Moving	Time Spent in Zone	Frequency of Zonal Entry
Novel Tank Test	Male	No change	Decreased time - 3 and 30 ppb	No change	No change
Light-Dark Box	Male	Decreased distance - 30 ppb	Decreased time - 0.3 and 30 ppb	No change	No change
Open Field Test	Male	No change	No change	No change	No change

^a 9 mpf male data from Horzmann et al. (2021).

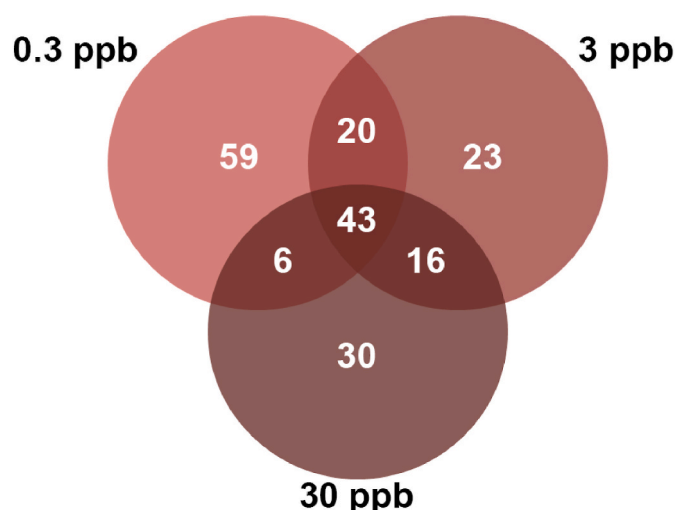


Fig. 1. Number of mapped genes with altered expression in each embryonic ATZ exposure group in female brain aged 9 months. $n = 4$ with significance determined via ANOVA with Tukey's HSD post hoc test.

pathways associated with cancer, cell-to-cell signaling and interaction, and embryonic development (Table 3). Of the common genes in females, 34 genes were associated with abdominal cancer and 25 with urogenital cancer. The top networks associated with the common gene pathways included cell morphology, cellular movement, and cell cycle (Fig. 2). This network is consistent with the previously identified pathways and general concepts of carcinogenesis. Notable genes in this network include *SP8* (sp8 transcription factor), *PRPF31* (pre-mRNA Processing Factor 31), *TP53* (tumor protein 53), and *RB1* (retinoblastoma gene 1), which regulate transcription or cell cycle control and are commonly altered in cancer (Sherr and McCormick, 2002).

3.3. qPCR comparison of microarray data

A group of genes with altered relative expression in all three ATZ treatments were evaluated via qPCR to confirm the microarray results. The relative expression of five genes (*ace*, *decr1*, *krt8*, *nrmk2*, and *strip1*) was determined in each of the female ATZ exposure groups and correlated to the $\log_2$ fold change determined on microarray analysis for that respective treatment group. There was a significant strong positive correlation between qPCR and microarray results for the 0.3 ppb ($R = 0.94046$, $p = 0.0173$) and the 30 ppb ($R = 0.88245$, $p = 0.0475$) exposures. The 3 ppb exposure had a strong correlation ($R = 0.86770$) with $p = 0.0566$ (Fig. S6).

3.4. Sex comparison of adult zebrafish brain transcriptome at 9 mpf

Brain transcriptomic alterations associated with embryonic ATZ exposure have previously been reported in male 9 mpf zebrafish (Horzmann et al., 2021). We compared the lists of genes with altered expression from the females in this study with the previous male data to determine if there were common gene targets between the sexes. Although there were no common genes that were altered in all three embryonic exposures for both sexes, there were 7 genes with altered expression in both the female and male 0.3 ppb ATZ exposure, 4 genes in common between females and males with 3 ppb embryonic ATZ exposure, and 4 genes with altered expression common to both females and males with embryonic exposure to 30 ppb ATZ (Table S6). Notably, the expression of *CDK5*, a gene associated with neuron development and synaptic vesicle exocytosis, was altered in both the 0.3 and 3 ppb treatment groups for both sexes.

This initial comparison indicated sex-specific differences in specific genes, but further investigation into the top pathways altered in both

Table 2

List of genes altered in female brain aged 9 mpf in all ATZ treatment groups.

Name	Gene Symbol ^a	Biological Function ^b
Angiotensin I converting enzyme	<i>ACE</i>	Regulation of angiotensin; brain development; embryo development; female pregnancy; regulation of neurogenesis
ATP/GTP binding protein like 2	<i>AGBL2</i>	Protein side chain deglutamylation; proteolysis
Ankyrin repeat and SOCS box containing 2	<i>ASB2</i>	Intracellular signal transduction; myoblast differentiation; post-translational protein modification; protein polyubiquitination
ATCAY, caytaxin	<i>ATCAY</i>	Mitochondrion distribution; regulation of glutamate metabolism; nervous system and neuron projection development
Cdk5 and Abl enzyme substrate 1	<i>CABLES1</i>	Cell cycle; cell division; G1/S transition of mitotic cell cycle; nervous system development; regulation of cell cycle
Calpain 7	<i>CAPN7</i>	Positive regulation of epithelial cell migration; proteolysis; self-proteolysis
Cadherin 1	<i>CDH1</i>	Embryonic development; regulation of canonical Wnt receptor signaling; pituitary development; regulation of neuron migration
Coatomer protein complex subunit zeta 2	<i>COPZ2</i>	ER to Golgi vesicle-mediated transport; intracellular protein transport; vesicle-mediated transport
CTP synthase 1	<i>CTPS1</i>	De novo CTP biosynthetic process; B cell proliferation; glutamine metabolic process; immune system process
2,4-dienoyl-CoA reductase 1	<i>DECRI</i>	Fatty acid beta-oxidation; fatty acid metabolic process; lipid metabolic process; oxidation-reduction process; protein homotetramerization
Filamin binding LIM protein 1	<i>FBLIM1</i>	Cell adhesion; cell junction assembly; mRNA processing; regulation of alternative nuclear mRNA splicing, via spliceosome
Guanylate binding protein 1	<i>GBP1</i>	Response to interferon- β and interferon- γ ; interferon- γ -mediated and calcium-mediated signaling; ERK1/ERK2 regulation
Iroquois homeobox 1	<i>IRX1</i>	Metanephros development; negative regulation of transcription from RNA polymerase II promoter
Keratin 17	<i>KRT17</i>	Cornification; epidermis development; hair follicle morphogenesis
Keratin 8	<i>KRT8</i>	Cell differentiation in placenta; cell morphogenesis in differentiation; extrinsic apoptotic signaling pathway; keratinization
Lipolysis stimulated lipoprotein receptor	<i>LSR</i>	Establishment of blood-brain barrier; lipoprotein transport; liver development; regulation of lipid metabolic process
Mediator complex subunit 11	<i>MED11</i>	Protein ubiquitination; regulation of transcription, DNA-dependent
Migration and invasion enhancer 1	<i>MIEN1</i>	197 Negative regulation of apoptotic process; positive regulation of cell migration; positive regulation of filopodium assembly
Matrix metalloproteinase 8	<i>MMP8</i>	Collagen catabolism; endodermal cell differentiation; extracellular matrix disassembly; neutrophil degranulation; ossification; proteolysis
Mannose-P-dolichol utilization defect 1	<i>MPDU1</i>	Dolichol-linked oligosaccharide biosynthetic process; protein folding; transport
Myosin X	<i>MYO10</i>	Cell motility; cytoskeleton-dependent intracellular transport; regulation of cell-cell adhesion, cell shape, and filopodium assembly
Myosin IE	<i>MYO1E</i>	Endocytosis; <i>in utero</i> embryonic development; kidney development; post-embryonic hemopoiesis; vasculogenesis

(continued on next page)

Table 2 (continued)

Name	Gene Symbol ^a	Biological Function ^b
NDUFA4, mitochondrial complex associated	<i>NDUFA4</i>	Mitochondrial electron transport; oxidation-reduction process
NADH:ubiquinone oxidoreductase subunit S6	<i>NDUFS6</i>	Cardiovascular system development; fatty acid metabolism; mitochondrial electron transport
Nicotinamide riboside kinase 2	<i>NMRK2</i>	NAD biosynthetic process; NAD metabolic process; regulation of myoblast differentiation; pyridine nucleotide biosynthetic process
NOP2 nucleolar protein	<i>NOP2</i>	Maturation of LSU-rRNA; positive regulation of cell proliferation; regulation of transcription; rRNA base methylation; rRNA processing
Otoancorin	<i>OTOA</i>	Cell-matrix adhesion; C-terminal protein lipidation; sensory perception of sound; transmission of nerve impulse
Prolyl 3-hydroxylase family member 4	<i>P3H4</i>	Bone remodeling; collagen biosynthesis; collagen fibril organization; peptidyl-lysine hydroxylation; synaptonemal complex assembly
Periplakin	<i>PPL</i>	Cornification; cytoskeleton organization; keratinization; response to mechanical stimulus
Protein phosphatase 1 regulatory subunit 3B	<i>PPP1R3B</i>	Glycogen biosynthesis and metabolic process; protein dephosphorylation; regulation of catalytic activity
Pre-mRNA processing factor 31	<i>PRPF31</i>	Assembly of spliceosomal tri-snRNP; mRNA processing; ribonucleoprotein complex localization; snoRNA localization
Protease, serine 48	<i>PRSS48</i>	Proteolysis
Retinoic acid receptor responder 3	<i>RARRES3</i>	Lipid catabolism; regulation of cell proliferation; phospholipid metabolism; phosphatidylethanolamine acyl-chain remodeling
Ras homolog family member V	<i>RHOV</i>	Regulation of small GTPase mediated signal transduction
Sciellin	<i>SCEL</i>	Embryo development; epidermis development; keratinocyte differentiation; regulation of canonical Wnt receptor signaling
Serologically defined colon cancer antigen 8	<i>SDCCAG8</i>	Establishment of cell polarity; G2/M transition; microtubule organizing center; neuron migration; tube formation
Selenium binding protein 1	<i>SELENBP1</i>	Brown fat cell differentiation; protein transport; transport
Sp8 transcription factor	<i>SP8</i>	Dorsal/ventral pattern formation; embryonic limb morphogenesis; regulation of transcription
Steroid 5 alpha-reductase 2	<i>SRD5A2</i>	Androgen and progesterone biosynthesis; cell differentiation; female genitalia development; hippocampus and hypothalamus development
Striatin interacting protein 1	<i>STRIP1</i>	Cortical actin cytoskeleton organization; regulation of cell morphogenesis
TSC complex subunit 2	<i>TSC2</i>	Aging; cell cycle arrest; regulation of axonogenesis; neural tube closure; regulation of dendritic spine development; social behavior

n = 4 with significance determined via ANOVA with Tukey's HSD post hoc test.

^a Human ortholog of zebrafish gene.

^b Determined via Ingenuity Pathway Analysis.

transcriptome data sets revealed more similarities. For example, top molecular pathways altered in the brain of both sexes identified associations with embryonic and organismal development; cellular movement, function, and maintenance; organismal injury and abnormalities; renal and urological system development and function; and developmental disorder. Pathways related to cancer in the males were limited to those fish exposed to 0.3 or 30 ppb atrazine, while these pathways were

Table 3

Top pathways altered in all three ATZ exposures.

Diseases and Disorders		
Name	p-value ^a	# Molecules ^b
Cancer	3.47E-02 - 3.16E-04	35
Gastrointestinal Disease	3.64E-02 - 3.16E-04	35
Organismal Injury and Abnormalities	3.64E-02 - 3.16E-04	36
Developmental Disorder	3.64E-02 - 5.08E-04	12
Renal and Urological Disease	3.64E-02 - 5.08E-04	7
Molecular and Cellular Functions		
Name	p-value ^a	# Molecules ^b
Cell-to-Cell Signaling and Interaction	3.13E-02 - 1.65E-04	10
Cellular Assembly and Organization	3.59E-02 - 1.65E-04	19
Cellular Function and Maintenance	3.59E-02 - 1.65E-04	15
Cell Morphology	3.59E-02 - 5.08E-04	15
Cellular Movement	3.64E-02 - 5.64E-04	15
Physiological System Development and Function		
Name	p-value ^a	# Molecules ^b
Embryonic Development	3.54E-02 - 2.25E-04	14
Hair and Skin Development	3.13E-02 - 2.25E-04	9
Organ Development	3.64E-02 - 2.25E-04	13
Organismal Development	3.58E-02 - 2.25E-04	18
Tissue Development	3.58E-02 - 2.25E-04	19

n = 4 with significance determined via ANOVA with Tukey's HSD post hoc test.

^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.

identified in all three atrazine treatment in the females. Furthermore, when looking by treatment group of each sex, there is almost complete agreement with top disease and disorder molecular pathways (i.e., reproductive system disease, organismal injury and abnormalities, cancer, and gastrointestinal disease) in fish in the 0.3 ppb treatment group. Interestingly, nervous system development and disease is only identified as a top physiological system development and function pathway in fish in the 3 ppb treatment group in both sexes.

3.5. Comparison of adult female zebrafish brain transcriptome at 6 and 9 mpf

Previously we evaluated the transcriptomic changes in the brain of 6 mpf female zebrafish with embryonic ATZ exposure (Wirbisky et al., 2015). The results of the 9 mpf transcriptomic analysis in this study were compared to the findings of the 6 mpf study to evaluate for persistent changes in gene expression. There was commonality for 27 genes in fish exposed to 0.3 ppb ATZ, 3 genes in those exposed to 3 ppb ATZ, and 7 genes in those exposed to 30 ppb ATZ during embryogenesis at both ages (Table S7). Eight of these genes had persistent upregulation and an additional 8 genes had persistent downregulation, with two genes with persistent upregulation in the 30 ppb females and the remaining in the 0.3 ppb females. Only two genes, *GBP1* (guanylate binding protein 1) and *ZNF729* (zinc finger protein 729), were altered in all three ATZ exposure groups at 6 and 9 mpf. *GBP1* is involved in cellular response to interferons, regulation of ERK1 and ERK2 cascade, and regulation of calcium signaling, while *ZNF729* assists in the regulation of transcription.

Overall, the comparison in the female zebrafish indicates age-specific brain transcriptome profiles with limited persistence in specific genes, but similar to the sex-specific analysis further assessment of the top molecular pathways was completed. Similarities in the top molecular pathways perturbed in all three treatment groups at both ages included cancer; gastrointestinal disease; organismal injury and abnormalities; cellular assembly, organization, and morphology; cell-to-cell signaling and interaction; and embryonic and organismal development. Additionally, reproductive system pathway was similar in the brain of female zebrafish exposed to 0.3 ppb during embryogenesis at both ages, while nervous system development and function and hair and skin

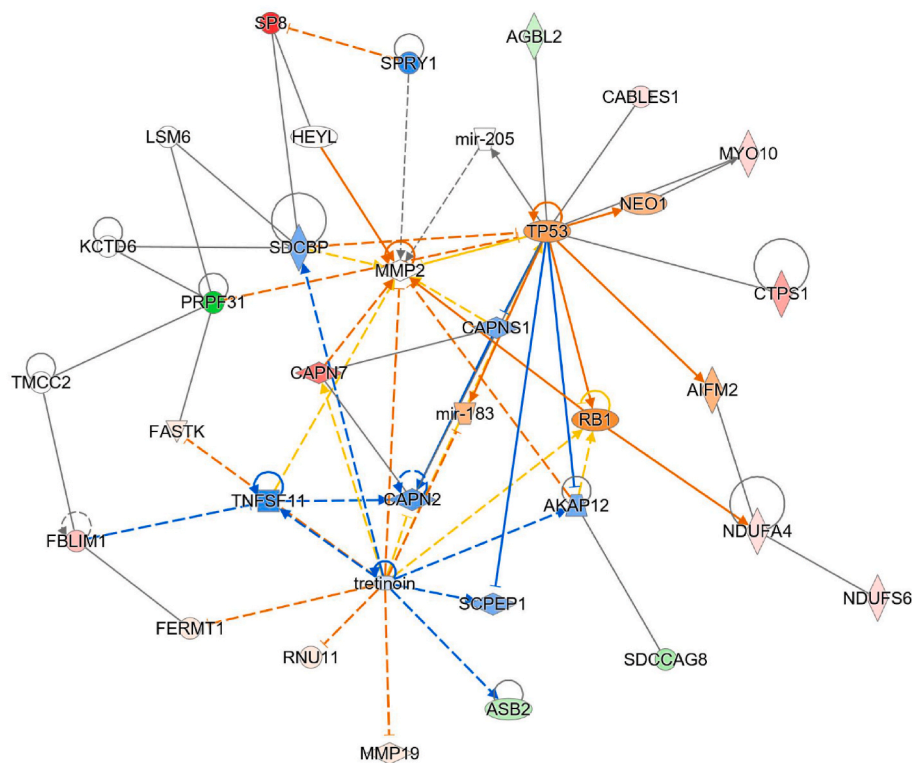


Fig. 2. Network analysis of genes altered in the brain of female zebrafish aged 9 mpf from all three ATZ exposure groups. Common altered expression of genes in a cell morphology, cellular movement, and cell cycle network were observed in the brain of females exposed to ATZ during embryogenesis. Red indicates increased measurement, green indicates decreased measurement, orange indicates predicted activation, and blue indicates predicted inhibition with depth of color related to magnitude of change. Large ovals are transcription regulators, small ovals are hormones, squares are cytokines, vertical diamonds are enzymes, horizontal diamonds are peptidases, inverted triangles are kinases, trapezoids are transporters, inverted trapezoids are microRNA, and circles are other molecules. Lines with arrows indicate activation, lines without arrows indicate interactions, solid lines indicate direct interaction, and dashed lines indicate indirect interaction. Orange lines indicate a predicted relationship that leads to activation, blue lines indicate a predicted relationship that leads to inhibition, yellow lines indicate findings that may be inconsistent with downstream molecules, and black lines indicate no predicted relationship. Gene symbols are human orthologs of zebrafish genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

development pathways were identified at both ages in the 3 ppb treatment group. Behavioral and neurological related pathways were more prevalent in all three treatment groups at the earlier life stage compared to the 9 mpf profiles, indicating predictive potential.

3.6. Expression of serotonin pathway genes in brain at age 14 mpf

The expression of genes related to serotonergic neurotransmission was evaluated in 14 mpf female zebrafish brain with an embryonic exposure to ATZ to see if the anxious phenotypes observed in the NTT and OFT were linked to persistent gene alterations in this pathway given altered gene expression in the 6 mpf female brain transcriptome profiles. No significant differences in gene expression between embryonic ATZ exposures of *htr1aa* ($p = 0.0583$), *htr1ab* ($p = 0.2715$), *htr2c1* ($p = 0.8602$), *htr4* ($p = 0.3907$), *serta* ($p = 0.4945$), *sertb* ($p = 0.7479$), *tp1a* ($p = 0.1666$), *tp1b* ($p = 0.1662$), *tp2* ($p = 0.3371$), or *mao* ($p = 0.6930$) in 14 mpf female brain was observed (Fig. S7).

3.7. Global brain methylation at age 12 mpf

Global methylation status was evaluated in 12 mpf zebrafish female brain with embryonic ATZ exposure. A significant difference in percent 5 mC between treatments was seen with the 0.3 ppb embryonic ATZ treatment having significantly increased percent 5 mC compared to controls ($p = 0.0267$; Fig. 3).

3.8. Body and brain size at age 14 mpf

At 14 mpf, body length, body weight, and brain weight to body weight ratio was assessed. Females exposed to 30 ppb ATZ during embryogenesis had a significantly increased body length ($p = 0.0030$, Fig. 4A). A significant difference in body weight between females with an embryonic ATZ exposure was also observed ($p < 0.0001$), with the 3 ppb exposed fish having decreased body weight and the 30 ppb exposed fish having increased body weight compared to controls (Fig. 4B). The females with exposure to 0.3 or 3 ppb ATZ had significantly ($p = 0.0025$)

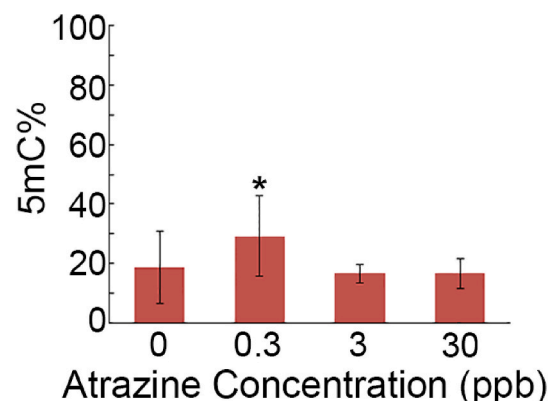


Fig. 3. Global brain methylation of female zebrafish with embryonic ATZ exposure. Females with 0.3 ppb embryonic ATZ exposure had a significant increase in percent 5 mC compared to controls. $n = 6-7$, technical measurements run in duplicate. Error bars represent standard deviation. Significance determined via ANOVA with Fisher's LSD post hoc test. * $p < 0.05$.

smaller brains compared to controls (Fig. 4C), but when the brain weight to body weight ratio was calculated, the 0.3 and 30 ppb treatment groups had a significant ($p = 0.0032$) smaller brain per body weight (Fig. 4D).

4. Discussion

The later life effects of embryonic ATZ exposure were investigated by using a battery of behavioral tests, transcriptomic analysis, targeted serotonin pathway gene expression analysis, evaluation of methylation, and body and brain measurements to determine if embryonic ATZ exposure causes neurotoxicity in adult female zebrafish. Our laboratory has previously found that embryonic ATZ exposure altered neurodevelopment, neurotransmission, and brain gene expression in

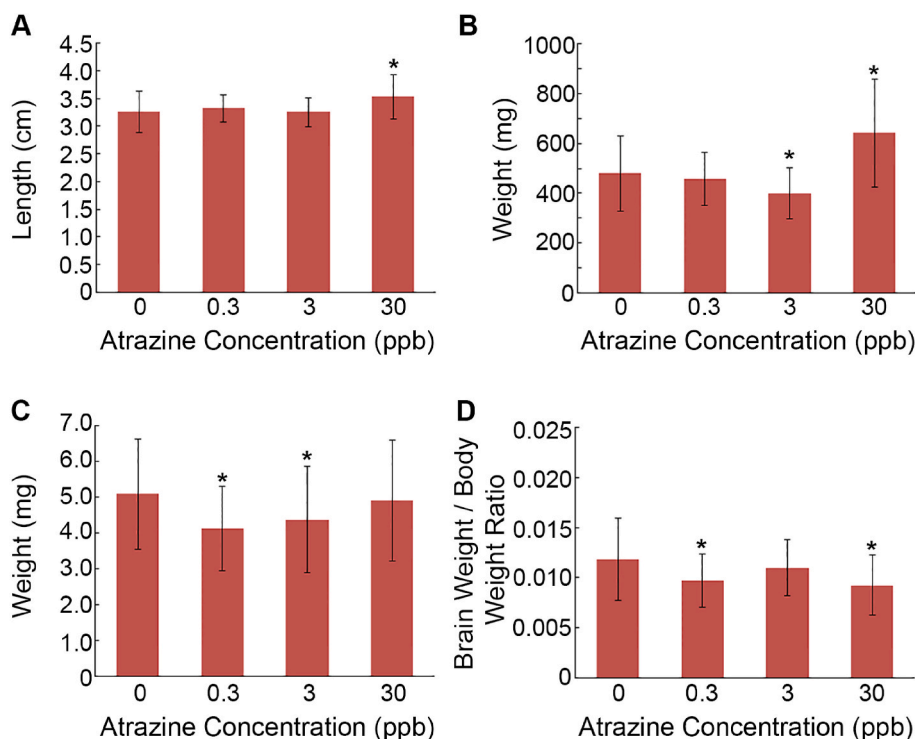


Fig. 4. Alterations in adult body length and weight, and brain weight in females aged 14 mpf with ATZ exposure during embryogenesis. Female zebrafish with embryonic exposure to 30 ppb ATZ had increased body length compared to controls (A). The female 3 ppb embryonic ATZ exposure had a decreased body weight, but the 30 ppb exposure group had an increased weight compared to controls (B). The brain weight was decreased in the 0.3 and 3 ppb exposures compared to the controls (C). The brain weight to body weight ratio was decreased in the females with 0.3 and 30 ppb embryonic exposures (D). $n = 3$, with 14–69 subsamples per treatment per replicate. Error bars represent standard deviation. Significance determined via ANOVA with Fisher's LSD post hoc test. * $p < 0.05$.

eleuthero-embryo, larval, and adult zebrafish (Weber et al., 2013; Wirbisky et al., 2015, 2016b; Horzmann et al., 2021). This study advances previous research by focusing on female zebrafish at older ages (9, 12, and 14 mpf) and exploring additional endpoints such as performance on behavioral tests and brain methylation.

Female zebrafish with embryonic ATZ exposure have more anxious phenotypes during behavioral testing in this study. The NTT, LDB, and OFT are well described tests used to evaluate different types of anxiety and behavior in zebrafish, namely the diving response, scototaxis, and thigmotaxis (Egan et al., 2009; Kalueff et al., 2014, 2016). The diving response refers to a zebrafish tendency to dive to the bottom in an unfamiliar or anxiety-inducing environment for safety, scototaxis refers to the dark preference exhibited by zebrafish, and thigmotaxis refers to “wall-hugging” behavior associated with anxiety-like behavior in multiple species (Egan et al., 2009; Blaser et al., 2010; Champagne et al., 2010). The zonal data collected in the NTT, LDB, and OFT allow measurement of these behaviors and can help elucidate the behavioral effects of embryonic ATZ exposure. Female zebrafish had non-monotonic alterations in anxiety related endpoints, such as having decreased time spent in the upper zone of the NTT. In a study evaluating behavior of 9 mpf male zebrafish with embryonic ATZ exposure, a hypoactive phenotype was observed (Horzmann et al., 2021). Males with increased embryonic ATZ exposure had decreased distance moved and velocity, but no alterations in endpoints related to anxiety, suggesting developmental ATZ exposure causes sex-specific alterations in neurobehavioral endpoints. The non-monotonic nature of some of the female results, specifically the 3 ppb group's time spent in the NTT upper zone and the 0.3 and 30 ppb groups' number of entries to the OFT inner zone, is consistent with the non-monotonic effects reported after low dose exposure to other EDCs (Vandenbergh et al., 2012).

ATZ has previously been linked to altered behavior in other zebrafish studies. Decreased free swimming speeds and hypo-locomotion has been observed in 120 hpf larval zebrafish with developmental ATZ exposure (Liu et al., 2016; Horzmann et al., 2018), with differential changes observed in light and dark phases (Ahkin Chin Tai et al., 2021). Although larval zebrafish behavioral tests are powerful in high throughput chemical toxicity screening (Truong et al., 2014; Reif et al.,

2016; Horzmann and Freeman, 2018), they may not be able to evaluate nuanced anxiety-related behavior observed in adult zebrafish, which was observed in this study. Adult zebrafish exposure to higher concentrations of ATZ has been associated with altered light dark preference (Steinberg et al., 1995) and shoaling behavior (Schmidel et al., 2014); however this study shows that embryonic ATZ exposure alters behavior at lower, environmentally relevant ATZ concentrations. Studies in other species have also shown behavioral changes later in life associated with a developmental ATZ exposure. CD-1 mice with gestational exposure to 1 $\mu\text{g/kg/day}$ ATZ were also observed to have altered behavior, with juvenile males having less masculine behavior and female mice having increased latency in a passive avoidance learning test (Belloni et al., 2011). Juvenile C57BL/6 mice with developmental exposure to 3000 ppb ATZ in maternal drinking water had altered activity, time swimming on a forced swim test, and marble burying behavior (Lin et al., 2013).

The brain transcriptome of female 9 mpf zebrafish with embryonic ATZ exposure was evaluated to identify genes with differential expression and to determine what significant pathways and networks are affected by embryonic ATZ exposure at this later adult life stage and to align with age of behavioral testing (Note: IPA identifies human orthologs of zebrafish genes identified via microarray and human gene notation is used to maintain consistency with the pathway analysis). The common genes that were altered in our 9 mpf female exposure groups were associated with cancer, cell signaling, and embryonic development pathways. Interestingly, 22 of the genes altered in the female brain after embryonic ATZ exposure are also altered in genital tract cancers; however, 7 of these genes, *ACE*, *CHD1*, *CTPS1*, *LSR*, *OTOA*, *SRD5A2*, and *TSC2*, also have functions associated with either neurodevelopment or neural function. Beta-estradiol was identified as a top upstream regulator in the 3 and 30 ppb treatment groups, while estradiol was also a top upstream regulator in the 30 ppb treatment group. Atrazine is a known endocrine disruptor that impacts the hypothalamus-pituitary-gonadal axis through suppression of luteinizing hormone surge. While atrazine is thought to not directly bind estrogen receptors, atrazine is reported to alter estrogen production in tissues of several species including decreased levels of a primary estradiol metabolite in the urine of women

in higher exposed atrazine groups (Coady et al., 2005; Cragin et al., 2011; Gojmerac et al., 1996; Holloway et al., 2008; Tennant et al., 1994).

The findings of the 9 mpf female brain transcriptomic analysis were compared to the results of our previously published analysis of the brain transcriptome from 9 mpf male zebrafish with embryonic ATZ exposure (Horzmann et al., 2021). Although there were no genes that were altered in all exposures in both sexes, there were common genes shared between sexes for each embryonic exposure. For example, *CDK5* expression was altered in both the female and male 0.3 and 3 ppb ATZ exposure groups. *CDK5* is a member of the cyclin-dependent kinases family with roles in learning and memory, neuronal survival, synaptic plasticity, and behavior. Dysregulation of *CDK5* is also associated with several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and Huntington's disease (Shah and Lahiri, 2014). *FA2H* was altered in both females and males with 0.3 ppb embryonic ATZ exposure. Mutations of *FA2H* are associated with a neurodegenerative disease characterized by abnormal myelin production with symptoms of a mixed movement disorder with spasticity (Gregory et al., 2018). While similarities in specific genes were limited among the two sexes, more agreement was observed when comparing the top molecular pathways that were altered, suggesting similarities in overall biological pathways that atrazine is targeting in this exposure paradigm in both sexes. These findings also hint at the strength of using transcriptome profiles for predicting molecular pathway alterations of a toxicant and application of the data to go beyond differentially expressed gene lists.

The results of the 9 mpf female zebrafish transcriptomic analysis were also compared to a previous transcriptomic analysis of 6 mpf female zebrafish brain with similar ATZ exposures (Wirbisky et al., 2015) to investigate persistence of ATZ induced differential gene expression. We found only two genes (*GBP1* and *ZNF729*) that were altered in all three exposures in females at both 6 and 9 mpf. *GBP1* modulates the cellular response to interferon- γ , regulates signaling cascade, participates in endosomal trafficking and autophagy, and can also contribute to the control of cancer or response to chemotherapy (Honkala et al., 2020). *ZNF729* is thought to regulate RNA polymerase II, but mutations in the gene have been linked to reproductive tract cancers (Prieske et al., 2018). Additional genes are shared for each exposure group. For example, females with 0.3 ppb embryonic ATZ exposure had 27 genes with persistent altered expression, with 10 of those genes having functions related to brain development or function (*ACE*, *ATCAY*, *CACNG8*, *CTHRC1*, *LSR*, *PHACTR4*, *POMC*, *PTPRU*, *SDCCAG8*, and *STMN1*). With only 3 genes persistently altered in the 3 ppb embryonic exposure and 7 with persistent expression changes in the 30 ppb exposure, it appears that exposure to 0.3 ppb ATZ during embryogenesis causes larger disruptions in gene expression at the specific aged time points and over time (Wirbisky et al., 2015). As in the sex-specific analysis, going beyond the differentially expressed gene list and comparing top molecular pathways of both data sets shows more similarities within genes found in all three treatment groups and within each treatment group. Overall, the 9 mpf female brain transcriptomic profiles showed little indication of behavioral pathways being altered, while these pathways were much more prevalent in the 6 mpf female brain transcriptomic profiles. These findings align with the predictive potential of the brain transcriptome profile from the earlier age for the observed anxiety-related behavior outcomes at 9 mpf.

Transcriptomic analysis of 72 hpf eleuthero-embryo zebrafish with embryonic ATZ exposure found differential expression of genes linked to the function of the reproductive and neuroendocrine systems and carcinogenesis (Weber et al., 2013). The transcriptomic analysis in this study identified common genes associated with cancer, which is consistent with the 72 hpf eleuthero-embryo data and proteomic analysis of 120 hpf larval zebrafish with embryonic ATZ exposure (Horzmann et al., 2018). In that study, 15 proteins related to urogenital and genital tract cancers were identified as differentially abundant in ATZ exposure groups. In the current study, 25 genes common to all three

female embryonic ATZ exposures were associated with urogenital cancer, suggesting developmental exposure to ATZ could cause persistent changes in pathways related to these cancers. Only one protein related to genital tract cancers from Horzmann et al. (2018) had altered gene expression in our current study. *KRT17* expression, which functions in keratinization, was downregulated in females with all three embryonic ATZ exposures. The genes from our study otherwise related to genital tract cancers have functions related to regulation of transcription and cellular metabolism.

Wirbisky et al. (2015) also found that 9 mpf female zebrafish with embryonic exposure to 0.3 or 3 ppb ATZ had decreased levels of 5-HIAA and decreased serotonin turnover. Based on these results, we expected to find persistent expression changes in genes associated with the brain serotonergic system and a more anxious phenotype on behavioral tests. We did not observe altered expression of genes directly related to serotonin in our female brain transcriptomic analysis at 9 mpf or in targeted qPCR analysis at 14 mpf. As transcriptomic evaluation only captures a snapshot of gene activity, it is possible that the gene expression changes later in life do not reflect previous alterations in gene expression that influence development and function. Again, the gene alterations at 6 mpf appear to better predict the functional outcomes in behavior observed at 9 mpf and the previous measured alterations in neurotransmitter levels (Wirbisky et al., 2015).

Global percent 5 mC was increased in the female zebrafish brain with embryonic exposure to 0.3 ppb ATZ. Changes in DNA methylation are associated with altered gene expression, with increased methylation associated with gene silencing (Siegfried and Simon, 2010). Non-linear increased methylation was also reported in human neuroblastoma SH-SY5Y cells exposed to similar concentrations of ATZ (Xie et al., 2021). In contrast, decreased global 5 mC was seen in zebrafish eleuthero-embryos (72 hpf) exposed to similar ATZ concentrations (Wirbisky-Hershberger et al., 2017) and decreased CpG methylation was seen in HEK293T cells with similar ATZ exposures (Sánchez et al., 2020). Global percent 5 mC was unchanged between treatments in 9 mpf male zebrafish brain with embryonic ATZ exposure (Horzmann et al., 2021), suggesting that the changes in percent 5 mC are sex-specific, which is consistent with ATZ acting as an EDC. It is possible that the early decreased percent 5 mC observed in the 72 hpf eleuthero-embryos causes an overcompensation in 5 mC levels in adult female zebrafish. One limitation is that only global DNA methylation was evaluated in this study, and it is unknown which specific genes are silenced or if differential methylation occurs at different exposures. The changes in methylation associated with ATZ exposure may be linked to reported multigenerational toxicity. Altered morphology was seen in progeny of zebrafish with embryonic ATZ exposure (Wirbisky et al., 2016d) and transgenerational toxicity, including reproductive abnormalities and altered behavior was reported in F3 generation Hsd:Sprague following an F0 generation ATZ exposure (McBirney et al., 2017). Additional research into patterns of methylation and the consequences of ATZ related epigenetic toxicity is needed.

Evaluation of the body and brain size of female 14 mpf zebrafish with embryonic ATZ exposure indicated that the females with embryonic exposure to 30 ppb ATZ had increased body size (length and weight), but proportionally smaller brains. The raw brain weight for the 30 ppb exposed females was not significantly different from controls, and it must be considered that the decrease in brain to body weight ratio is due to increased body weight rather than decreased brain weight. Females in the 0.3 ppb ATZ group had a significant decrease in brain to body weight ratio that cannot be explained by changes in body size, suggesting true decrease in brain size. In 72 hpf zebrafish, embryonic exposure to 0.3, 3, and 30 ppb ATZ was associated with increased head length (Weber et al., 2013) and a larger brain length was observed only in eleuthero-embryos exposed to 0.3 ppb ATZ (Horzmann et al., 2018). Although the relationship between early brain length increase and later life brain weight decrease is uncertain, it is possible that early disruption of neurodevelopment results in physical changes that persist through the life

course, or that the smaller brains later in life represent an age related pathologic atrophy of tissue consistent with degenerative neurological diseases (Romanowski and Wilkinson, 2011).

5. Conclusions

This study used female zebrafish to investigate later life neurotoxicity associated with developmental ATZ exposure. In a battery of behavioral tests, female zebrafish with embryonic ATZ exposure had sex-specific, non-monotonic alterations in anxiety-related behavior. Female zebrafish with embryonic ATZ exposure had sex- and age-specific gene expression changes when evaluating differentially expressed gene lists, but more similarities were observed in top molecular pathways altered in both sexes and at both ages. Although changes in serotonin neurotransmission was previously reported in the brain of adult female zebrafish at 6 mpf, the expression of serotonergic pathway genes was not altered at 9 or 14 mpf. Overall, the brain transcriptomic profiles indicated analysis at an earlier age to be predictive of observed behavioral phenotypes at older age. Brain DNA hypermethylation was observed at 12 mpf, which supports ATZ related epigenetic toxicity, but further analyses are needed to identify site-specific perturbations that could be related to altered transcriptome. Finally, alterations in body and brain size after embryonic ATZ exposure was observed, suggesting early ATZ exposure causes lasting physical effects. The assessment of ATZ later life neurotoxicity is important due to the heavy use of ATZ and possible human health implications, especially considering exposures around the US EPA regulatory limit in drinking water.

Author contributions

Katharine A Horzmann: Conceptualization, Methodology, Formal Analysis, Investigation, Writing – Original Draft, Visualization, Funding Acquisition
Li F Lin: Investigation
Boghos Taslakjian: Investigation
Chongli Yuan: Conceptualization, Supervision
Jennifer L Freeman: Conceptualization, Resources, Writing – Review and Editing, Supervision, Project Administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Transcriptomic data is available in the Gene Expression Omnibus as GSE112503.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2022.136431>.

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