DEFINING DEVELOPMENTAL TOXICITY OF THE AGRICULTURAL HERBICIDE ATRAZINE IN THE EXPOSED AND SUBSEQUENT GENERATIONS USING ZEBRAFISH

by

Janiel Ahkin Chin Tai

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

School of Health Sciences

West Lafayette, Indiana

August 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Jennifer Freeman, Chair
School of Health Sciences

Dr. Jason Cannon
School of Health Sciences

Dr. Ellen Wells
School of Health Sciences

Dr. Chongli Yuan
Department of Chemical Engineering

Approved by:
Dr. Jason R. Cannon
Dr. Aaron B. Bowman

I dedicate my thesis to my great granddaddy Rupert and great grandmother Ilene. Your hard work in the sugarcane fields, and vision for education in our family paved the way for my success. Our families lived experiences led to my pursuit of toxicology in seek of closure. Grandaddy always wanted to be a doctor but was a man defined by his skin color, and socioeconomic status. Rather than lament, he chose to work in hard, dangerous working conditions to ensure a better future for generations to come in our family. Mama also had a vision of access to a better life through education. Mama, as I write this it has been 10 years since you have been gone. Our family misses you dearly, but I want you and grandaddy to know that we have more people than ever finally getting a chance to go to school because of both of you. It's a couple generations late, but we finally have our first medical doctor in the family, Tashane, and now me, the first PhD. I want you both to rest easy knowing that you have paved the way for our family, and for me. There are not enough words in all the languages combined to portray how lucky I am to have such dedicated family, and how sad I am that you did not get the same opportunities as me. Every academic achievement I have ever achieved has been because you built the foundation. Thank you for everything, and I love you so much.

ACKNOWLEDGMENTS

I'd like to acknowledge a couple professors and programs who helped pave the way to graduate school for me. First, I'd like to thank Dr. Bryan Dewsbury who is now an associate professor at Florida International University. When I was an undergrad, Bryan was the first black graduate student and scientist I had seen. He was also from the Caribbean, like my family. He encouraged me to consider graduate school and the pursuit of a PhD as a route to success and a better life. You cannot be what you cannot see or do not know, so thank you. I promise to advocate for our community through mentorship. I'd next like to acknowledge my first research advisor, Philip Stoddard, professor at Florida International University. Phil, you were the first person outside of my community to believe in me. You understood that diversity isn't simply opening a space in a lab, but fostering a supportive environment so students like me could succeed and focus on learning. You worked with me to creatively problem solve any roadblocks that would have kept me out of the lab, as well as on my research project. I am especially appreciative of the time you spent to mentor and advocate for me, since you are a professor, dad, husband, and the literal mayor of South Miami. Working in your lab, was one of the most empowering and formative experiences of my life. You gave me the confidence to pursue research, even in spaces where I did not see myself reflected. It was a pleasure and a privilege to be your student, and I hope I made you proud. I'd additionally like to thank Quantifying Biology in the Classroom (QBIC) as well as MARC U STAR, which were undergraduate research programs that prepared me for my PhD at FIU.

I'd like to acknowledge my PhD thesis committee for offering scientific support and advice. Additionally I'd like to thank Dr. Johnathan Shannahan, who was on my preliminary committee for his encouragement and scientific advice. I thank the Freeman lab for providing the space and tools necessary to complete my degree as well as the freedom to pursue academic interests and career developmental paths. I'd additionally like to than AGEP as a safe space created for students form underrepresented backgrounds to pursue professional/career development and emotional support.

This work was supported by the National Institutes of Health, National Institute of Environmental Health Sciences (R03 ES030545), the Indiana Clinical and Translational Sciences Institute (funded in part by Award Number UL1TR002529 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences

Award), and as part of AgSEED Crossroads funding to support Indiana's Agriculture and Rural Development.

I also acknowledge the use of the Metabolite Profiling Facility and the Purdue Proteomics Facility, Bindley Bioscience Center, core facilities of the NIH-funded Indiana Clinical and Translational Sciences Institute. I especially thank Victoria E. Hedrick and Tiago J.P. Sobreira for their assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HIAA: 5-hydroxyindoleacetic

ACTH: adrenocorticotropic hormone

ANOVA: Analysis of Variance

ATZ: atrazine

CORT: corticosterone

CRH: corticotropin releasing Hormone

DACT: diaminochlorotriazine

DEA: desethyl-atrazine

DIA: deisopropyl-atrazine

DOPAC: 3,4-dihydroxyphenylacetic acid

EDC: endocrine disrupting chemical

EPA: environmental protection agency

GnRH: gonadotropin releasing hormones

HPA: hypothalamic-pituitary-adrenal

HPF: hours post fertilization

HPG: hypothalamic-pituitary-gonadal

HPT: hypothalamic-pituitary-thyroid

HVA: homovanillic acidd

Kiss1r: g protein coupled receptor 54

KNDy: kisspeptin-neurokininB-dynorphin

LH: luteinizing hormone

MCL: maximum contaminant level

PND: Postnatal day

ppb: parts per billion

$T_{3:}$ thyroxine

T_4: triiodothyronine

TSH: Thyroid stimulating hormone

ABSTRACT

Atrazine (ATZ) is an agricultural herbicide. The US Environmental Protection Agency has set the maximum contaminant level at 3 µg/l in potable water, though concentrations can greatly exceed this amount depending on the time of year. Epidemiological studies report associations with developmental health outcomes with potable water exposure. Studies in model organisms identify ATZ as a neurotoxicant and endocrine disrupting chemical. The zebrafish model system was used to test the hypothesis that developmental ATZ exposure has immediate health consequences as well as in the subsequent generation. It was first hypothesized that developmental ATZ exposure generates metabolites similar to those found in mammals and alters morphology and behavior in larvae. In the exposed generation, targeted metabolomic analysis found that zebrafish produce the same major ATZ metabolites as mammals. The visual motor response test at 120 hpf detected hyperactivity in larvae in the 0.3 ppb treatment group and hypoactivity in the 30 ppb treatment group. These findings suggest that developmental ATZ exposure generates metabolite profiles similar to mammals leading to behavioral alterations supporting ATZ as a neurodevelopmental toxicant. In the subsequent generation (F1), it was hypothesized that parental ATZ exposure altered protein expression leading to modifications in morphology and behavior in developing progeny. Proteomic analysis identified differential expression associated with neurological development and disease and organ and organismal morphology, specifically the skeletomuscular system. Head length and the ratio of head length to total length was significantly increased in the F1 in the 0.3 and 30 ppb ATZ treated groups. Craniofacial morphology was assessed based on molecular pathway analysis and revealed decreased cartilaginous structure size, decreased surface area and distance between saccular otoliths, and a more posteriorly positioned notochord, indicating delayed ossification. The visual motor response assay showed hyperactivity in the F1 of the 30 ppb treatment group for total distance and time spent moving in the F1 of the 0.3 and 30 ppb treatment groups for all phases. Collectively, these results demonstrate persistent ATZ developmental toxicity in this multigeneration study.

CHAPTER 1. INTRODUCTION[1]

1.1 Abstract

Atrazine (ATZ) is an agricultural herbicide. Due to persistence and contamination of drinking water sources, ATZ was banned by the European Union, but is still commonly used in the US and other global regions. The US Environmental Protection Agency has set the maximum contaminant level at 3 µg/l in potable water, though concentrations can greatly exceed this amount depending on the time of year. Alternatively, the World Health Organization drinking water guideline is 100 µg/l. Epidemiological studies report associations with developmental health outcomes and raise concerns about ATZ as a potential carcinogen. Studies in model organisms have identified ATZ as a neurotoxicant. Although there is evidence to support the site of action as the hypothalamus, further studies are needed to determine if this herbicide targets specific pathways within the hypothalamus or if it generally interferes with pathways that the hypothalamus regulates. This chapter focuses on ATZ developmental neurotoxicity with studies reporting disruption of the hypothalamus, neurotransmission, neuronal development, and behavioral processes.

1.2 Introduction to developmental neurotoxicity of the herbicide atrazine

Atrazine (ATZ) is a synthetic agricultural herbicide used to control broad leafy weeds. ATZ is commonly applied to corn and other crop fields, though it can be used on lawns as well (USGS 2016; British Crop Protection Council 1977; Li et al 2019; Russart and Rhen 2016). ATZ works by interfering with the electron transport chain to interrupt photosynthesis. Resistant plants can absorb and metabolize ATZ, but are not vulnerable to impeded photosynthesis (Cheremisinoff and Rosenfeld 2011; Eldridge et al 1999). ATZ was introduced in 1958 and is the second most

[1] Sections of this chapter were originally published in:
Ahkin Chin Tai JK, Freeman JL. 2020. Zebrafish as an integrative vertebrate model to identify miRNA mechanisms regulating toxicity. Toxicol Rep 7:559–570; doi:10.1016/j.toxrep.2020.03.010.

Ahkin Chin Tai, JK, Freeman, JL. Developmental Neurotoxicity of the Herbicide Atrazine. 2021. In: Diagnosis, Management and Modelling of Neurodevelopmental Disorders: The Neuroscience Of Development. Academic Press. 219-228; https://doi.org/10.1016/B978-0-12-817988-8.00019-1.

commonly applied herbicide in the US (Cheremisinoff and Rosenfeld 2011; USGS 2016; Ochoa-Acuña et al 2009). In 2003 the European Union banned its use due to contamination concerns (Bethsass and Colangelo 2006). The triazine herbicides are potential carcinogens and endocrine disrupting chemicals (EDC).

1.3 Toxicant profile

Triazine herbicides consist of nitrogenous heterocyclic rings. These herbicides work by binding to the Q_B protein in photosystem II of broad leafy weeds. This protein binding blocks the movement of electrons through the photosynthetic electron transport chain. Resistant crop plants have rapid metabolic detoxification through glutathione S-transferase and dealkylation through cytochrome P_{450} (LeBaron et al 2008). ATZ is classified as a triazine herbicide and is the most commonly used in its class. ATZ is composed of a triazine ring with an ethylamine, propylamine, and chlorine as its functional groups (Figure 1.1). ATZ is a white, odorless powder that is moderately soluble in water.

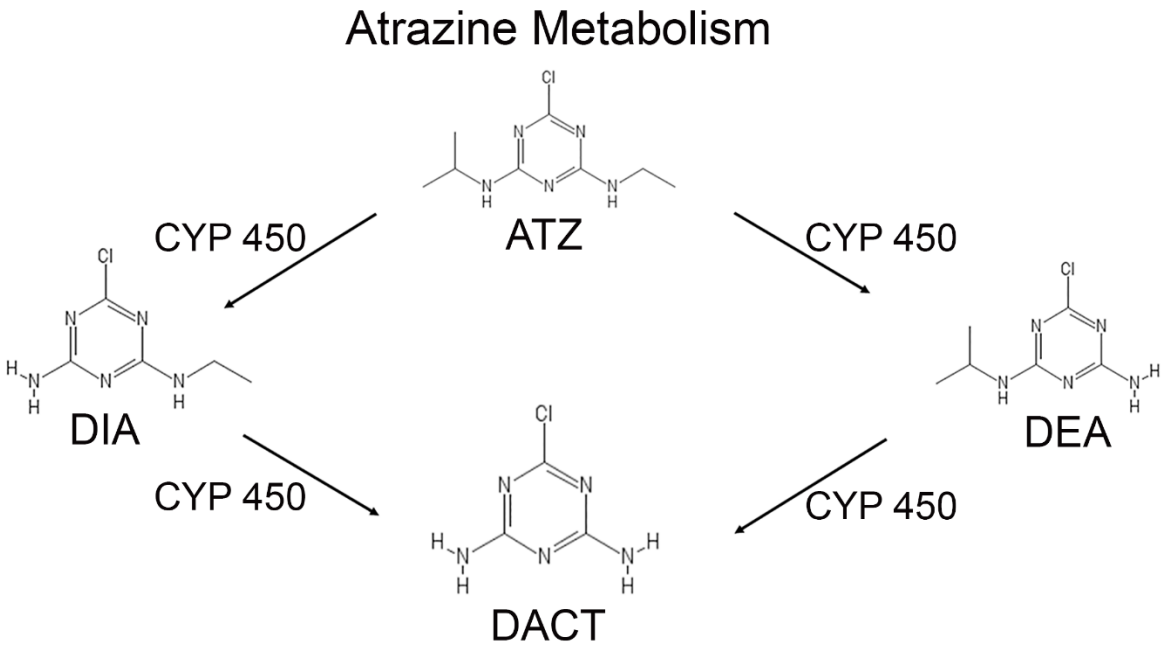


Figure 1-1. **Atrazine metabolism**. The parent chemical ATZ can break down into over 40 different metabolites, but many are not regulated or monitored. ATZ detection is most common, followed by DEA, DACT, and DIA in rural wells of the US, which are the primary ATZ metabolites.

1.4 Toxicokinetics

ATZ can breakdown into over 40 metabolites (Figure 1.1). ATZ metabolites appear to have toxicities comparable to or lower than ATZ. Syngenta, the manufacturer of ATZ, elected the metabolites diaminochlorotirazine (DACT), deethyl-atrazine (DEA) and deisopropyl-atrazine (DIA) for monitoring through the Syngenta Monitoring Program. These daughter compounds can be generated through degradation of ATZ in surface water and soil via photolysis of microorganisms and have been found in animal tissue. ATZ is the most common detected, followed by DEA, DACT, and DIA in rural wells in the US. Another metabolite, hydroxyatrazine, is more commonly found in groundwater. This metabolite is not water soluble, causing crystal formation and is considered a nephrotoxicant. Although the half-life of ATZ is 125 days, the half-life of its metabolites is around 20-50 days (WHO 2011).

ATZ and its metabolites can be absorbed gastrointestinally and minimally through the skin. ATZ is rapidly eliminated through urine and feces. Circulating in the system, ATZ and its metabolites have an affinity for protein, binding readily to erythrocytes, liver, spleen, and kidney. In humans, ATZ is primarily metabolized via dealkylation (Cheremisinoff and Rosenfeld 2010; Barr et al 2007; WHO 2011).

1.5 Occupational exposure

Chemical applicators and factory workers are the occupational population of concern. Although applicators may only get exposure seasonally when the herbicide is applied, factory workers who manufacture the chemical have the possibility of chronic exposure. A triazine factory in Switzerland in a matched cohort study found an association between increased incidence of gastritis and gastroenteritis (Ware 1987). In response to health concerns, the Occupational Safety and Health Administration set a limit of 5 g ATZ/m^3 of exposure in an 8-hour workday. ATZ is also registered as a restricted use pesticide to prevent uncertified applicators from applying (ATSDR 2003).

1.6 Environmental exposure

Exposure routes via atmosphere, food, and groundwater are reported; however, water contamination is the main route of environmental exposure for several reasons. Many areas of

heavy ATZ application have clay soils or other low filtration soils where the herbicide not absorbed by crops or weeds is only partially absorbed by the soil. ATZ also does not bind efficiently to soil particles. Combined with heavy application in the Midwestern US, these factors contribute to the herbicide being highly mobile in runoff. These factors and ATZ's persistence in the environment make chemical exposure a concern (Gammon et al 2005; WHO 2011; Meffe and de Bustamante 2014; Wang and Xie 2012). ATZ contaminates potable water, and although the US EPA has set the maximum contaminant level (MCL) at 3 µg/l, concentrations can greatly exceed reaching up to 30 µg/l or even 100 µg/l (WHO 2011; Blanchard and Lerch 2000). Since ATZ frequently contaminates potable water supplies, is applied heavily in the Midwestern US, and can be found at concentrations above the MCL, understanding health risks from either environmental or occupational exposures and mechanisms of toxicity is important.

1.7 Human Health Risks

Populations of concern are centered around occupational exposures and residents who live in areas near crop fields where ATZ is applied. Runoff from crop fields have the ability to infiltrate lakes, rivers, streams, and groundwater. Epidemiological studies link ATZ contaminated potable water to developmental toxicity, birth defects, and risks for cancer and neurodegenerative disease. Maternal exposure to ATZ during gestation is linked with increased risk of preterm birth as well as birth defects (Agopian et al 2013; Migeot et al 2013; Stayner et al 2017; Waller et al 2010). Although a clear association with ATZ exposure and cancer is still controversial, trends for increased risk of certain types of tumors and thyroid cancer have been suggested (Freeman et al 2007; Rusiecki et al 2004). In addition, a few studies suggest increased incidence in Parkinson's disease with pesticide exposure with ATZ as a potential pesticide of interest (Li et al 2014; Priyadarshi et al 2000; Semchuk et al 1992). Overall, these outcomes suggest concern for developmental exposure and neurological disorders. Although there is some controversy and mostly associative data in epidemiological studies, investigations have been conducted in various model organisms to further define toxicity outcomes.

1.8 Toxicity Studies

Studies in various model organisms at a range of concentrations have assessed health concerns related to ATZ exposure. Chronic exposure to ATZ at high doses caused earlier onset of mammary gland tumors in Sprague Dawley rats (Wetzel et al 1994). The mechanism of tumor induction is suggested to be related to ovarian function. Although this exposure level was above biologically relevant exposure levels, this study raised questions regarding atrazine's safety (WHO 2011; Ware 1987). This study also helped spur restricted personnel exposure levels. A follow up study noted a reduction of luteinizing hormone (LH), which led to disrupted estrous cycle with 100, 200, or 300 mg/kg body weight of ATZ/day (Cooper et al 2000). LH and prolactin levels are regulated by the hypothalamus, and there is speculation about ATZ disruption occurring at the level of the hypothalamus or the kisspeptin neurons above the hypothalamus. Tumor formation in Sprague Dawley rats occurs by altering catecholamine function and gonadotropin releasing hormone in the hypothalamus. This ultimately affects the HPG axis (WHO 2011). Studies in other model organisms also support ATZ's role as an EDC along the HPG axis.

As an EDC, ATZ does not necessarily act in a dose dependent manner, and several studies have displayed sex differences. A study exposing embryonic snapping turtles to 2 or 40 µg/l ATZ determined androgen receptors and kisspeptin 1 receptors were upregulated in both sexes at hatching, but female specific upregulation of aromatase was observed. The gene prodynorphin, which is associated with the HPA axis, was also upregulated (Russart and Rhen 2016). Six months later, at 2 µg/l, aromatase and prolactin releasing hormone mRNA expression was increased in both sexes. In vitro studies utilizing H295R adrenocortical carcinoma cell line had a 1.5-2 fold increase in aromatase mRNA activity with doses ranging from 0-30 µM ATZ, with a follow up study determining that DEA and DIA also upregulated aromatase in the same cell line, as well as breast and placental cells (Suzawa and Ingraham 2008; Ware 1987). Building on this study, Caron-Beaudoin et al (2016) found promoter regions PIII and I.3 transcription levels to be increased for aromatase.

1.9 Neuroendocrine system toxicity

Many studies support the hypothalamus as the site of action for ATZ (ATSDR 2003; WHO 2011). The HPG, HPA, and HPT axes in particular work together to regulate synthesis and sensitivity to stress, sex steroid hormones, growth, and reproductive function.

1.10 Hypothalamic-pituitary-gonadal axis

The specific site within the hypothalamus where regulation of the HPG axis starts is the infundibular nucleus in humans and arcuate nucleus in rats. To understand how ATZ disrupts hypothalamic function, it is important to understand how the HPG axis works (Figure 1.2). *Kiss1* gene produces kisspeptin, which is important for normal reproductive function as well as its receptor G-protein coupled receptor 54 (kiss1R). Kisspeptin and kiss1R are expressed in several places throughout the body, but hypothalamic kiss1 is responsible for regulating metabolic information to the HPG axis. This peptide hormone is also important for signaling GnRH neurons for the onset of puberty. Kisspeptin-neurokininB-dynorphin (KNDy) neurons coordinate pulsatile GnRH secretions in GnRH neurons via kiss1R receptors. GnRH is then released into the anterior pituitary gonadotropins. The anterior pituitary creates follicle-stimulating hormone (FSH) and LH, which is carried throughout the body in the bloodstream. LH secretion then stimulates theca cell development in the secondary follicle of the ovaries by binding to an LH receptor. Theca cells synthesize estradiol, which binds to KNDy neurons to stop pulsatile secretion of GnRH to regulate the system (Marques et al 2000; Karapanou and Papadimitriou 2010; Magoffin 2005).

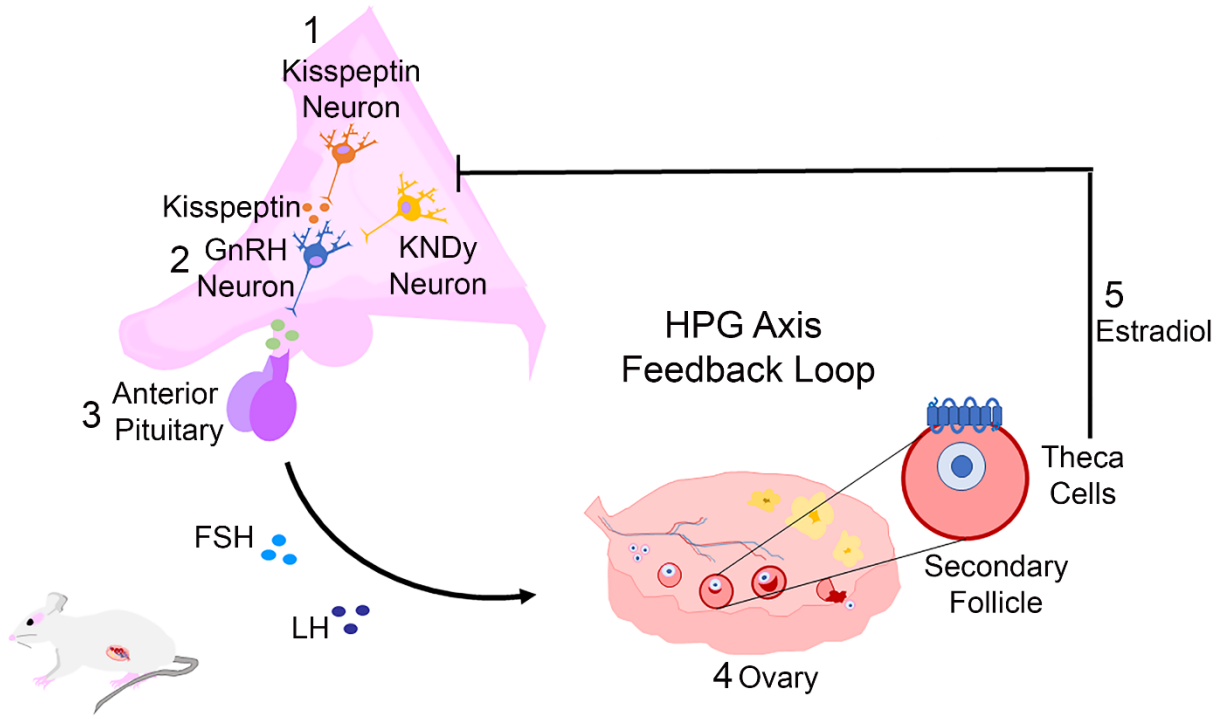


Figure 1-2. HPG axis in rats. Kisspeptin neurons secrete kisspeptin (1), which binds to receptors on the GnRH neuron (2). The GnRH neurons sends pulsatile secretion of GnRH to the anterior pituitary (3). GnRH binds to receptors on gonadotropes in the anterior pituitary releasing FSH and LH. LH stimulates thecal cell growth in the secondary follicle of the ovary (4). Theca cells generate estradiol, which acts on KNDy neurons to inhibit GnRH activation (5).

A handful of developmental studies delve into ATZ exposure and its effects on the HPG axis (Table 1.1). For example, an embryonic ATZ exposure in snapping turtles reported changes in genes associated with the HPG axis (Russart and Rhen 2016). Sex- and age-dependent changes in *Kiss1* and its receptor *Kiss1R* were found. Young female Japanese quails exposed developmentally to ATZ had decreases in hypothalamic *GnRH* and underdeveloped reproductive organs (Qin et al 2015). Many studies focus on adult ATZ exposure in various rat models along the HPG axis and report decreases in *Kiss1* expression, decreases in GnRH pulse frequency and amplitude, as well as a surge in LH (Figure 1.3). These effects have the potential to alter downstream gonadal development with several studies noting apoptosis in female reproductive cells and a decrease in uterine and ovarian weight (Goldman et al 2013; Kimura et al 2019; Marques et al 2000). Although no widely accepted conclusion is drawn on what reproductive cells are specifically targeted with ATZ exposure, there is a trend in decreased integrity in female

reproductive tissue that can ultimately affect reproductive function. Sex steroids generated from the HPG axis to promote reproductive function can also regulate the HPA axis.

Table 1-1. ATZ neurotoxicity along the HPG axis

Reference	Model organism	Results	ATZ concentration
Russart and Rhen 2016	Developing snapping turtles	Males had higher *kiss1* and *GnRH* mRNA, in embryos; *Kiss1R* higher in hatchling females	2, 40 µg/l during embryogenesis
Foradori et al 2009	Ovariectomized adult female Wistar rats	High dose of ATZ altered LH pulse, amplitude and suppressed GnRH	50, 100, 200 mg/kg
Qin et al 2015	Developing female quails	Underdeveloped ovaries/oviduct, decreased hypothalamic *GnRH* mRNA, LH in pituitary, and FSH in ovary; Upregulated FSH and prolactin in pituitary	50, 250, 500 mg/kg at 18 days for 45 days
Wilhelms et al 2006	Developing quails	At 14 days, hatchling and ovarian weight decreased as well as circulating progesterone at highest concentration	123, 246, 504 µg/kg *in ovo*
Goldman et al 2013	Adult Long Evans female rats	4 days of ATZ suppress LH surge, a single exposure elevated Increased *kiss1* mRNA expression	4 days of 100 mg/kg or a single exposure
Kimura et al 2019	Ovariectomized female rats	Increased LH surge and decrease in kiss1 mRNA in AVPV	100 mg/kg

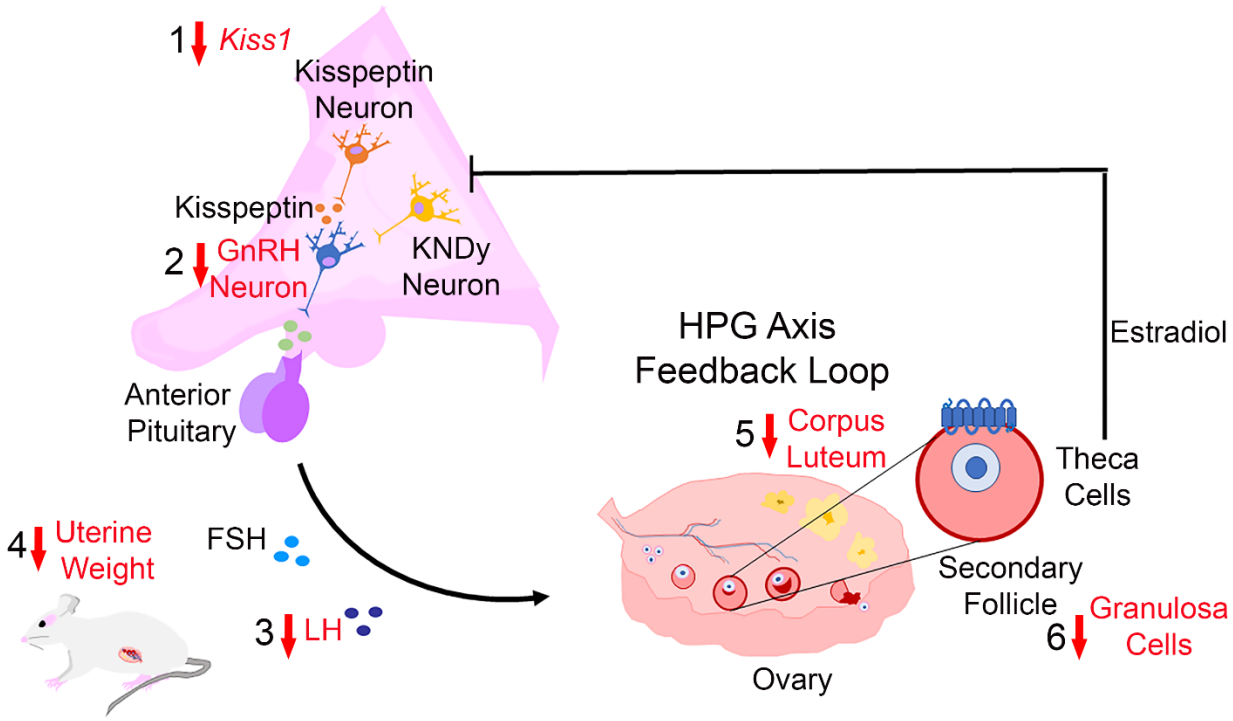


Figure 1-3. ATZ neurotoxicity along the HPG axis. ATZ is shown to decrease expression of Kiss1 (1) and decrease GnRH neurons (2) in the hypothalamus. A decrease in the LH surge is widely documented with ATZ exposure (3). ATZ has been associated with uterine weight loss (4), corpus luteum loss (5), and a decline in granulosa cell quality (6). Integrating the findings from multiple studies in rat models implicates ATZ acting through the neuroendocrine system to influence reproductive health.

1.11 Hypothalamic-pituitary-adrenal axis

The HPA axis regulates stress related hormones, particularly corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and corticosterone (CORT). CRH is synthesized in the paraventricular nucleus within the hypothalamus to help synthesize ACTH in the pituitary and is considered a neuropeptide mediator of stress and HPA activation. CRH releases ACTH to stimulate glucocorticoids (e.g., CORT) in response to stress in a negative feedback loop. Stress during proestrus or exposure to estradiol in females causes an increase in plasma levels of ACTH and generally higher levels of CRH, CORT, and progesterone. Conversely in males, testosterone decreases ACTH levels and gonadectomy increases ACTH. Although the HPA axis regulates stress hormones and is also regulated by sex hormones, it can also indirectly regulate the HPG axis by altering levels of GnRH, pituitary sensitivity to GnRH, and LH secretion (Fraites et al 2009; Toufexis et al 2014).

Few studies have addressed developmental ATZ exposure and changes in glucocorticoids or other endpoints for HPA activation from a neurotoxicity standpoint (Table 1.2). A study with embryogenic ATZ exposure in snapping turtles looked at immediate and later in life changes in genes associated with stress in the HPA pathway. The authors found upregulated proopiomelanocortin (POMC), a precursor to ACTH, at hatching and at 6 months in females. This implicates developmental ATZ exposure can cause changes in the HPA later in life. Many studies focus on various stress responses in adult rats. These studies support ATZ and some of its metabolites causing increases in blood plasma levels of CORT and ACTH (Foradori et al 2018; Foradori et al 2009; Riffle et al 2014). A study in adult female Sprague-Dawley rats found that pituitary removal prevented CORT synthesis, implicating the pituitary and specifically the CRH receptor is necessary for ATZ to modulate increases in CORT levels (Foradori et al 2018). More studies focusing on developmental ATZ exposure and its effect on the HPA axis will shed light on how ATZ neurotoxicity affects stress responses at the neuroendocrine level.

Table 1-2. ATZ neurotoxicity along the HPA axis

Reference	Model organism	Results	ATZ concentration
Foradori et al 2018	Adult female Sprague-Dawley rats	Changes in adrenal morphology and indirect adrenal stimulation to generate CORT (CRH receptor important for ATZ modulating corticosterone increase) ATZ, DIA, and DEA elevate plasma corticosterone concentrations	100 mg/kg 200 mg/kg
Russart and Rhen 2016	Developing snapping turtles	Increase in hypothalamic POMC in hatchlings and at 6 months in females	2, 40 µg/l during embryogenesis
Fraites et al 2009	Adult female Long Evans rats	Increase in ACTH, progesterone, and CORT; DACT had highest plasma levels, but didn't affect HPA hormone release	60.5, 70 mg/kg of ATZ, DIA, or DACT
Riffle et al 2014	Castrated male Wistar rats	Increase in adrenal proteins associated with stress; Increase in serum CORT and progesterone	5, 25, 75, 200 mg/kg
Lin et al 2014	C57BL/6 mouse dams	Increased anxiety-like behavior, decreased perirhinal cortex serotonin, and perirhinal cortex serotonin homeostasis	1.4 mg/kg/day

1.12 Serotonin and the HPA axis

Higher circulating levels of CORT and other stress related hormones and proteins in females not only modulate the sensitivity to sex steroids estrous cyclicity by altering GnRH and LH production through the HPG axis exposure, but also increase risk for developing psychological disorders (Fraites et al 2009; Toufexis et al 2014). The developmental origin of adult disease hypothesis suggests that toxicant exposure during development can increase the risk of certain diseases during adulthood, so developmental exposure could also increase risk for adult neurological disorders. The HPA axis is also important for regulation of psychological disorders like depression and anxiety, which are higher in human females. A study exposed zebrafish to 0, 0.3, 3, or 30 µg/l during embryogenesis and found a significant reduction in 5-hydroxyindoleacetic acid (5-HIAA), a major metabolite of serotonin in adult females. A subset of genes was down regulated throughout the serotonergic pathway (Wirbisky et al 2015). Another study in C57BL/6 mice used a combination of behavioral and neurochemical assays to determine ATZ effects on the brain and behavior. Offspring were exposed *in utero* from gestational day 6 to postnatal day (PND) 23 to 0, 0.03, or 30 mg/l. The authors found decreases in perirhinal cortex serotonin in adult females and increases in anxiety-like behavior. These results suggest that ATZ exposure during development causes sex-specific alterations in serotonergic systems that can ultimately affect behavior (Lin et al 2014).

1.13 Hypothalamus-pituitary-thyroid axis

The HPT axis is important for regulating metabolism, growth, and reproduction. The thyroid produces the hormones thyroxine (T_3) and triiodothyronine (T_4) that regulate activity of thyroid receptor signaling found on Sertoli cells across vertebrates. At the pituitary level, there is speculation that GnRH causes an increase in TSH and T_3 in some amphibians and fish (Castañeda Cortés et al 2014). Thyroid hormones can affect gonadal development and androgen levels, particularly in males. Effects of ATZ and its metabolites have been noted in thyroid histology and outcomes in gonadal pubertal development, but not many studies to date have linked endpoints in the HPT axis to ATZ neurotoxicity (Stoker 2002).

1.14 Dopamine

ATZ exposure has been shown to affect neurotransmission, particularly with dopamine (DA), which has implications for diseases such as Parkinson's Disease. A summary of developmental atrazine effects in the dopaminergic system is provided in table 1.3. DA is an important monaminergic transmitter responsible for facilitating motor movement with the main metabolites being 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) (Li et al 2014; Meiser et al 2013). In a study using 25 and 50 mg/kg of ATZ in pregnant mice with exposure from gestational day 0 to PND 1, offspring exposed *in utero* had decreased levels of DA, its precursors and transporters (DAT, TH), and decrease in genes and proteins necessary for mecenphalic DA neuron growth (Nurr1, VMAT2) 6 months after developmental exposure ceased in the substantia nigra. Follow up studies on ATZ exposure in pubertal rats around 20 days old was performed with 0, 50, 100, or 200 mg/kg with the same endpoints and found similar results at the highest ATZ exposure. In contrast, CB57BL/6 mice exposed *in utero* from gestational day 6 to PND 23 at 0, 0.03, 3, or 30 mg/L ATZ had increases in striatal DA and decreases in DA turnover as juveniles at PND 35. As adults, DA levels were not altered, but increases in metabolites methoxytyramine and 5-HT were detected in both sexes, and decreases in the metabolite DOPAC in females was found. Behavioral analysis revealed hyperactivity as juveniles in exposed animals and increases in anxiety-like behavior in females (Li et al 2014). These findings suggest that developmental exposure to ATZ can have lasting neurological impacts into adulthood.

Table 1-3. Summary of ATZ exposure and developmental DA effects

Reference	Model organism	Results	ATZ concentration
Ma et al 2015	SH-SY5Y (neuroblastoma) BV-2 cells(microglia)	Microglia increase ROS, inflammatory cytokines, and decrease cell viability	12.5, 25, 50 µM
Li et al 2014	Sprague Dawley rat pups exposed *in utero*	Decrease in DA, precursors, and genes for DA neurogenesis in substantia nigra	0, 25, 50 mg/kg gestational day 0-PND 1
Li et al 2015	Pubertal Sprague Dawley male rats	Decrease in DA and precursors and decrease in genes for mecenphalic DA neurogenesis in substantia nigra at highest ATZ concentration	0, 50, 100, 200 mg/kg from 27-54 days old
Li et al 2014	Pubertal Sprague Dawley rats	Decrease in DA, its precursors, and genes for mecenphalic DA neurogenesis in the substantia nigra	0, 25, 50 mg/kg from 22-62 days old
Bardullas et al 2011	Male juvenile Sprague Dawley rats	Impaired motor coordination after 10-12 month exposure; Striatal decrease of DA	10 mg/kg at 21 days until rat was 300 g

A study has also shown microglial activation with ATZ exposure, which signals inflammation and brain pathology (Ma et al 2015). A study using BV-2 microglia and SH-SY5Y neuroblastoma cells found dose-dependent relationships with SH-SY5Y cell viability, an increase in ROS in BV-2 microglial cells, and proteins associated with inflammation and microglial activation when exposed to 12.5, 25, or 50 μM ATZ. Results from this study showed that high levels of ATZ exposure during the stages of juvenile development affects the dopaminergic neurons during this period of growth (Li et al 2014; Xia et al 2016; Xia et al 2017). These results are particularly important because Nurr1 is important for regulating development of dopaminergic neurons.

1.15 Conclusion of ATZ developmental neurotoxicity

Although ATZ has been banned by the European Union, it is still a common herbicide used in the Midwestern US and other global regions. Sex differences with ATZ exposure have been shown, with sex-dependent changes in genes, proteins, and circulating hormone levels. Although some studies show ATZ acting in a dose-dependent manner, other studies have evidence to support ATZ as an EDC that does not act in a dose-dependent manner. Association with cancer is still controversial, but health concerns related to developmental outcomes and neurotoxicity have been documented in several model organisms. As an EDC, ATZ's site of action is proposed to be the neuroendocrine system and in particular the hypothalamus. The hypothalamus is an important site in the brain that modulates development, reproduction, and stress through multiple endocrine axes. Studies support disruption of the HPG axis with alterations in female reproduction and the HPA pathway related to stress with a developmental ATZ exposure. Furthermore, ATZ is reported to alter the dopaminergic system.

Although there is growing evidence to support the hypothalamus as ATZ's site of action, there are still gaps in the literature that need to be explored to further elucidate ATZ mediated changes and to what extent the HPG, HPA, and HPT axes are affected. In addition, more developmental studies exploring effects of ATZ on all three axes will provide a comprehensive understanding on how ATZ neurotoxicity impairs hypothalamic function. Moreover, there is a need to further our understanding on the influences of a developmental ATZ exposure on neurotransmission as studies to date have primarily focused on the dopaminergic system with a few studies on the serotonergic system.

1.16 Zebrafish as a model organism in toxicology

Zebrafish (*Danio rerio*) are an established model for developmental biology due to their physiology, genetics, and assaying potential. Embryos develop *ex vivo*, offering a non-invasive approach to study developmental events. Transparency of embryos and larvae in early developmental stages make it easy to visualize internal organs/structures and embryogenesis is complete in only 3 days (Figure 1.4). In the first few hours after fertilization, rapid cell division occurs in the zygote, cleavage, blastula, and gastrula periods. Between 24-48 hours post fertilization (hpf) the brain has 5 lobes, the embryo increases rapidly in length, the circulatory system forms, and the heart begins to beat. Between 48- 72 hpf, the craniofacial cartilage development allows the mouth to become distinct and rudimentary gills develop. By 72 hpf, most of morphogenesis is completed (Kimmel et al. 1995). From 72-120 hpf, zebrafish are referred to as eleutheroembryos. While in the process of hatching the embryo is not very active, but the eleutheroembryo stage is noted with gradual increased activity (swimming, jaw, eye, and fin movement). At 96-120 hpf it is common for behavioral assays for light/dark, acoustic stimuli, and toxicological responses to be initiated (Tegelenbosch et al. 2012). Behavioral assays, such as the photomotor response can be used to screen thousands of neuroactive drugs (Garcia et al. 2016; Kokel et al. 2010; Tegelenbosch et al. 2012). In addition, zebrafish have a fully sequenced genome with the capability of creating genetic models using multiple gene editing tools including CRISPR-Cas9 technology, making them ideal for mechanistic and discovery based studies on how genes impact physiology and behavior (Huang et al. 2011; Peng et al. 2014). Due to assaying potential and genetic strengths, zebrafish are a good model for human translation, because zebrafish have 70% gene homology which increases to 82% when considering genes related to human disease (Howe et al. 2013). Additionally, there is high conservation of metabolizing enzymes (Garcia et al. 2016; Goldstone et al. 2010).

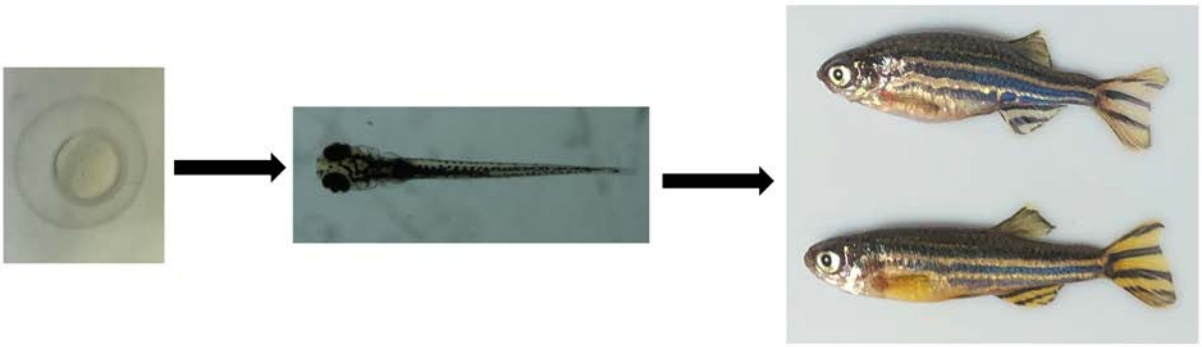


Figure 1-4. Zebrafish life cycle. Images are of an embryo at 1 hour post fertilization (hpf), an eleutheroembryo at 5 days post fertilization (dpf), and adult female (top) and male (bottom) zebrafish.

As noted above, the zebrafish model organism can be used in high-throughput assays for drug discovery and chemical toxicity screening (Cassar et al. 2020; Garcia et al. 2016; Hallare et al. 2006; Zon and Peterson 2005). Due to their developmental and biomedical strengths zebrafish are increasingly being used to define mechanisms of toxicity (Aluru et al. 2013; Bailey et al. 2013; Tegelenbosch et al. 2012) with recent reviews highlighting the strengths of the zebrafish as a model for toxicology (Garcia et al. 2016; Horzmann and Freeman 2018). Taking advantage of the developmental and biomedical strengths, the US FDA has used zebrafish toxicity assays for drug approval (Garcia et al. 2016; He et al. 2014). Many toxicological studies are also evaluating chemical effects on a protein, organ, and organismal level to provide a multi-level approach to understanding how a chemical affects health at different life stages and then determining human translation relevance (Horzmann et al. 2018). Since zebrafish have shorter life periods, there is also the advantage of looking at the effects of a developmental exposure throughout the lifespan in the developmental origins of health and disease (DOHaD) paradigm, and even across generations. These advantages have made the zebrafish the most common fish model to perform multi- and transgenerational studies evaluating epigenetic mechanisms of toxicity (Horzmann and Freeman 2018).

1.17 Central Hypothesis and Specific Aims of Thesis

To further define the health outcomes from developmental atrazine exposure, the zebrafish model was used to determine consequences of developmental ATZ exposure immediately and in

the subsequent generation in this thesis. To further characterize the health effects of developmental ATZ exposure in the zebrafish developmental model metabolites, proteomics, changes in morphology, and behavior were evaluated. The central hypothesis was that developmental ATZ exposure results in zebrafish producing similar major metabolites found in mammals, which will result in behavioral and morphological changes in the exposed generation (F0) and in the subsequent generation (F1). Specific aim 1 (Chapter 2), tested the hypothesis that developmentally exposed larvae would elicit similar metabolite profiles found in mammals leading to morphological and behavioral alterations (Figure 1.5). In specific aim 2 (Chapter 3), it was hypothesized that parental ATZ exposure (F0) altered protein expression leading to modifications in morphology and behavior in developing progeny (F1). (Figure 1.5).

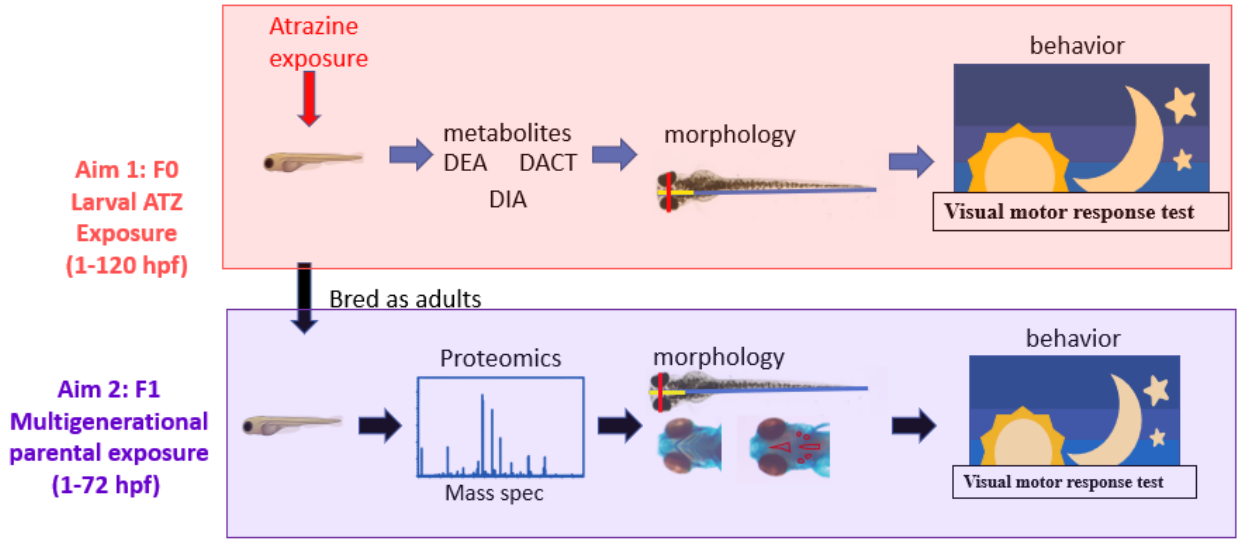


Figure 1-5. Project aims. Visual representation of Specific Aim 1, which is evaluating the consequences of ATZ exposure using targeted metabolomics, morphological changes, and behavior. Specific Aim 2 looks at consequences of parental ATZ exposure through proteomics, morphological assessments, and behavior.

1.18 References

1. Agency for Toxic Substances and Disease Registry. (2003) FAQ of Atrazine. *US Department of Health and Human Services.* Retrieved from https://www.atsdr.cdc.gov/tfacts153.pdf

2. Agopian, A. J., Lupo, P. J., Canfield, M. A., & Langlois, P. H. (2013). Case-Control Study of Maternal Residential Atrazine Exposure and Male Genital Malformations. *American Journal of Medical Genetics Part A, 161*(5), 977–982.

3. Barr, D. B., Panuwet, P., Nguyen, J. V., Udunka, S., & Needham, L. L. (2007). Assessing Exposure to Atrazine and Its Metabolites Using Biomonitoring. *Environmental Health Perspectives*, *115*(10), 1474–1478.

4. Bethsass, J., & Colangelo, A. (2006). European Union Bans Atrazine, While the United States Negotiates Continued Use. *International Journal of Occupational and Environmental Health*, *12*(3), 260–267.

5. Blanchard P.E. Lerch R.N. (2000) Watershed Vulnerability to Losses of Agricultural Chemicals: Interactions of Chemistry, Hydrology and Land Use. *Environ. Sci. Technol. 34*, 3315-3322.

6. British Crop Protection Council. (1977). *Pesticide manual: Basic information on the chemicals used as active components of pesticides* (5th ed. / edited by Hubert Martin, Charles R. Worthing). Droitwich, Eng.: The Council.

7. Caron-Beaudoin, É., Denison, M. S., & Sanderson, J. T. (2016). Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. *Toxicological Sciences*, *149*(1), 134–144.

8. Castañeda Cortés, D. C., Langlois, V. S., & Fernandino, J. I. (2014). Crossover of the Hypothalamic Pituitary-Adrenal/Interrenal, -Thyroid, and -Gonadal Axes in Testicular Development. *Frontiers in Endocrinology*, *5*, 139.

9. Cheremisinoff, N.P., & Rosenfeld, P.E. (2011). Handbook of Pollution Prevention and Cleaner Production, Vol.3. Best Practices in the Agrochemical Indsutry. (Chapter 3,4)

10. Cooper, R. L. (2000). Atrazine Disrupts the Hypothalamic Control of Pituitary-Ovarian Function. *Toxicological Sciences*, *53*(2), 297–307.

11. Eldridge, J. C., Wetzel, L. T., & Tyrey, L. (1999). Estrous cycle patterns of Sprague-Dawley rats during acute and chronic atrazine administration. *Reproductive Toxicology*, *13*(6), 491–499.

12. Foradori, C. D., Healy, J. E., Zimmerman, A. D., Kemppainen, R. J., Jones, M. A., Read, C. C., … Handa, R. J. (2018). Characterization of Activation of the Hypothalamic-Pituitary-Adrenal Axis by the Herbicide Atrazine in the Female Rat. *Endocrinology*, *159*(9), 3378–3388.

13. Foradori, C. D., Hinds, L. R., Hanneman, W. H., Legare, M. E., Clay, C. M., & Handa, R. J. (2009). Atrazine Inhibits Pulsatile Luteinizing Hormone Release Without Altering Pituitary Sensitivity to a Gonadotropin-Releasing Hormone Receptor Agonist in Female Wistar Rats1. *Biology of Reproduction*, *81*(1), 40–45.

14. Fraites, M. J. P., Cooper, R. L., Buckalew, A., Jayaraman, S., Mills, L., & Laws, S. C. (2009). Characterization of the Hypothalamic-Pituitary-Adrenal Axis Response to Atrazine and Metabolites in the Female Rat. *Toxicological Sciences*, *112*(1), 88–99.

15. Freeman, L. E. B., Rusiecki, J. A., Hoppin, J. A., Lubin, J. H., Koutros, S., Andreotti, G., … Blair, A. & Alavanja, M. C. R. (2011). Atrazine and Cancer Incidence Among Pesticide Applicators in the Agricultural Health Study (1994-2007). *Environmental Health Perspectives, 119*(9), 1253-1259.

16. Gammon, D. W., Aldous, C. N., Carr, W. C., Sanborn, J. R., & Pfeifer, K. F. (2005). A risk assessment of atrazine use in California: Human health and ecological aspects. *Pest Management Science, 61*(4), 331–355.

17. Goldman, J. M., Davis, L. K., Murr, A. S., & Cooper, R. L. (2013). Atrazine-induced elevation or attenuation of the LH surge in the ovariectomized, estrogen-primed female rat: Role of adrenal progesterone. *REPRODUCTION*, *146*(4), 305–314.

18. International Programme on Chemical Safety (Ed.). (1990). *Atrazine: Health and safety guide*. Geneva: World Health Organization.

19. Karapanou, O., & Papadimitriou, A. (2010). Determinants of menarche. *Reproductive Biology and Endocrinology*, *8*(1), 115.

20. Kimura, M., Ishii, M. N., Seki, N., Sakai, Y., Yamashita, T., Awatsuji, H., … Matsui, H. (2019). Reduction of *Kiss1* expression in the anteroventral periventricular nucleus is associated with atrazine-induced attenuation of the luteinizing hormone surge in female rats. *Biology of Reproduction*, *100*(1), 41–48.

21. LeBaron H.M., McFarland JE, Burnside OC. *The Triazine Herbicides*. (2008). Elseiver.

22. Li, J., Li, X., Bi, H., & Li, B. (2019). The MEK/ERK/CREB signaling pathway is involved in atrazine induced hippocampal neurotoxicity in Sprague Dawley rats. *Ecotoxicology and Environmental Safety*, *170*, 673–681.

23. Li, Y., Sun, Y., Yang, J., Wu, Y., Yu, J., & Li, B. (2014). Age-dependent dopaminergic dysfunction following fetal exposure to atrazine in SD rats. *Environmental Toxicology and Pharmacology*, *37*(3), 1275–1282.

24. Lin, Z., Dodd, C. A., Xiao, S., Krishna, S., Ye, X., & Filipov, N. M. (2014). Gestational and Lactational Exposure to Atrazine via the Drinking Water Causes Specific Behavioral Deficits and Selectively Alters Monoaminergic Systems in C57BL/6 Mouse Dams, Juvenile and Adult Offspring. *Toxicological Sciences, 141*(1), 90–102.

25. Ma, K., Wu, H.-Y., Zhang, B., He, X., & Li, B.-X. (2015). Neurotoxicity effects of atrazine-induced SH-SY5Y human dopaminergic neuroblastoma cells via microglial activation. *Molecular BioSystems*, *11*(11), 2915–2924.

26. Magoffin, D. A. (2005). Ovarian theca cell. *The International Journal of Biochemistry & Cell Biology*, *37*(7), 1344–1349.

27. Marques, P., Skorupskaite, K., George, J. T., & Anderson, R. A. (2000). Physiology of GNRH and Gonadotropin Secretion. In K. R. Feingold, B. Anawalt, A. Boyce, G. Chrousos, K. Dungan, A. Grossman, D. P. Wilson (Eds.), *Endotext*.

28. Meffe, R., & de Bustamante, I. (2014). Emerging organic contaminants in surface water and groundwater: A first overview of the situation in Italy. *Science of The Total Environment*, *481*, 280–295.

29. Meiser, J., Weindl, D., & Hiller, K. (2013). Complexity of dopamine metabolism. *Cell Communication and Signaling : CCS*, *11*, 34.

30. Migeot, V., Albouy-Llaty, M., Carles, C., Limousi, F., Strezlec, S., Dupuis, A., & Rabouan, S. (2013). Drinking-water exposure to a mixture of nitrate and low-dose atrazine metabolites and small-for-gestational age (SGA) babies: A historic cohort study. *Environmental Research*, *122*, 58–64.

31. Ochoa-Acuña, H., Frankenberger, J., Hahn, L., & Carbajo, C. (2009). Drinking-Water Herbicide Exposure in Indiana and Prevalence of Small-for-Gestational-Age and Preterm Delivery. *Environmental Health Perspectives; Research Triangle Park*, *117*(10), 1619–1624.

32. Priyadarshi, A., Khuder, S. A., Schaub, E. A., & Shrivastava, S. (2000). A meta-analysis of Parkinson's disease and exposure to pesticides. *Neurotoxicology*, *21*(4), 435–440.

33. Qin, L., Du, Z.-H., Zhu, S.-Y., Li, X.-N., Li, N., Guo, J.-A., … Zhang, Y. (2015). Atrazine triggers developmental abnormality of ovary and oviduct in quails (Coturnix Coturnix coturnix) via disruption of hypothalamo-pituitary-ovarian axis. *Environmental Pollution*, *207*, 299–307.

34. Riffle, B. W., Klinefelter, G. R., Cooper, R. L., Winnik, W. M., Swank, A., Jayaraman, S., … Laws, S. C. (2014). Novel molecular events associated with altered steroidogenesis induced by exposure to atrazine in the intact and castrate male rat. *Reproductive Toxicology*, *47*, 59–69.

35. Rusiecki, J. A., De Roos, A., Lee, W. J., Dosemeci, M., Lubin, J. H., Hoppin, J. A., … Alavanja, M. C. R. (2004). Cancer Incidence Among Pesticide Applicators Exposed to Atrazine in the Agricultural Health Study. *JNCI Journal of the National Cancer Institute*, *96*(18), 1375–1382.

36. Russart, K. L. G., & Rhen, T. (2016). Atrazine alters expression of reproductive and stress genes in the developing hypothalamus of the snapping turtle, Chelydra serpentina. *Toxicology*, *366–367*, 1–9.

37. Semchuk, M., Love, J., & Lee, G. (1992). Parkinson's disease and exposure to agricultural work and pesticide chemicals. *Neurology*, *42*(7), 1328–1335.

38. Stayner, L. T., Almberg, K., Jones, R., Graber, J., Pedersen, M., & Turyk, M. (2017). Atrazine and nitrate in drinking water and the risk of preterm delivery and low birth weight in four Midwestern states. *Environmental Research*, *152*, 294–303.

39. Stoker, T. E. (2002). The Effects of Atrazine Metabolites on Puberty and Thyroid Function in the Male Wistar Rat. *Toxicological Sciences*, *67*(2), 198–206.

40. Toufexis, D., Rivarola, M. A., Lara, H., & Viau, V. (2014). Stress and the Reproductive Axis. *Journal of Neuroendocrinology*, *26*(9), 573–586.

41. US Geological Survey. 2016 Pesticide Use Maps. Retrieved from https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2016&map=ATRAZINE&hilo=L

42. Waller, S. A., Paul, K., Peterson, S. E., & Hitti, J. E. (2010). Agricultural-related chemical exposures, season of conception, and risk of gastroschisis in Washington State. *American Journal of Obstetrics and Gynecology*, *202*(3), 241.e1-241.e6.

43. Wang, Q., & Xie, S. (2012). Isolation and characterization of a high-efficiency soil atrazine-degrading Arthrobacter sp. Strain. *International Biodeterioration & Biodegradation*, *71*, 61–66.

44. Ware, G. (1987) *Reviews of environmental contamination and toxicology.Vol 143.* New York: Springer-Verlag. (pp 47-57)

45. Wetzel, L. T., Luempert, L. G., Breckenridge, C. B., Tisdel, M. O., Stevens, J. T., Thakur, A. K., … Eldridge, J. C. (1994). Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats. *Journal of Toxicology and Environmental Health*, *43*(2), 169–182.

46. Wirbisky, S. E., Weber, G. J., Sepúlveda, M. S., Xiao, C., Cannon, J. R., & Freeman, J. L. (2015). Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis. *Toxicology*, *333*, 156–167.

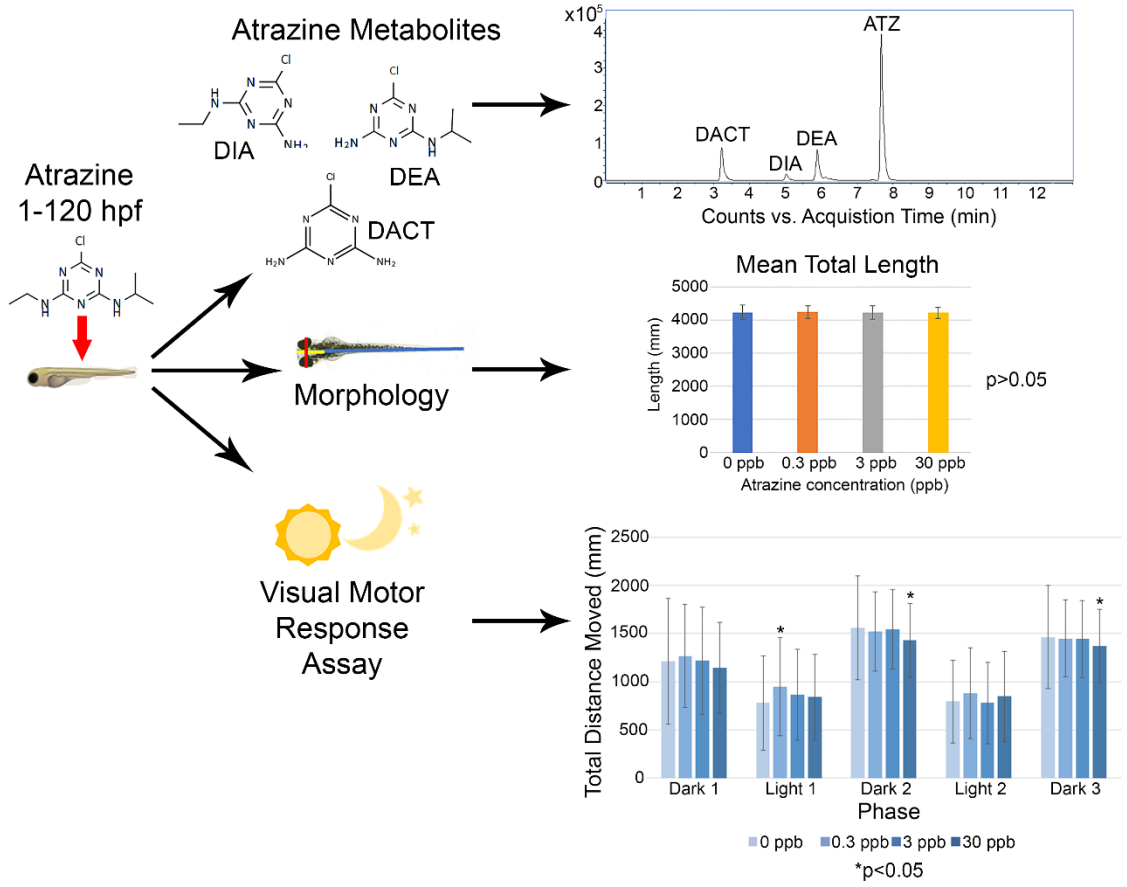
47. World Health Organization. (2011). Atrazine and its Metabolites in Drinking Water. Geneva.

48. Xia, J., Lin, J., Zhu, S.-Y., Du, Z.-H., Guo, J.-A., Han, Z.-X., … Zhang, Y. (2016). Lycopene protects against atrazine-induced hepatotoxicity through modifications of cytochrome P450 enzyme system in microsomes. *Experimental and Toxicologic Pathology*, *68*(4), 223–231.

49. Xia, J., Qin, L., Du, Z.-H., Lin, J., Li, X.-N., & Li, J.-L. (2017). Performance of a novel atrazine-induced cerebellar toxicity in quail (Coturnix C. coturnix): Activating PXR/CAR pathway responses and disrupting cytochrome P450 homeostasis. *Chemosphere*, *171*, 259–264.

CHAPTER 2. DEVELOPMENTAL ATRAZINE EXPOSURE IN ZEBRAFISH PRODUCES THE SAME MAJOR METABOLITES AS MAMMALS ALONG WITH ALTERED BEHAVIORAL OUTCOMES[2]

2.1 Abstract



Atrazine (ATZ) is the second most commonly applied agricultural herbicide in the United States. Due to contamination concerns, the U.S. EPA has set the maximum contaminant level in potable water sources at 3 parts per billion (ppb; µg/l). Depending on the time of year and sampling location, water sources often exceed this limit. ATZ is an endocrine disrupting chemical in

[2] This paper was published in:
Ahkin Chin Tai JK, Horzmann KA, Franco J, Jannasch AS, Cooper BR, Freeman JL. 2021. Developmental atrazine exposure in zebrafish produces the same major metabolites as mammals along with altered behavioral outcomes. Neurotoxicol Teratol 106971; doi:10.1016/j.ntt.2021.106971.

multiple species observed to target the neuroendocrine system. In this study the zebrafish vertebrate model was used to test the hypothesis that a developmental ATZ exposure generates metabolites similar to those found in mammals and alters morphology and behavior in developing larvae. Adult AB zebrafish were bred, embryos were collected, and exposed to 0, 0.3, 3, or 30 ppb ATZ from 1 to 120 hours post fertilization (hpf). Targeted metabolomic analysis found that zebrafish produce the same major ATZ metabolites as mammals: desethyl atrazine (DEA), deisopropyl atrazine (DIA), and diaminochloroatrazine (DACT). The visual motor response test at 120 hpf detected hyperactivity in larvae in the 0.3 ppb treatment group and hypoactivity in the 30 ppb treatment group ($p<0.05$). Further analysis into behavior during the dark and light phases showed zebrafish larvae exposed to 0.3 ppb ATZ had an increase in total distance moved in the first light phase and time spent moving in the first dark and light phase ($p<0.05$). Alternatively, a decrease in total distance moved was observed in the second and third dark phase in zebrafish exposed to 30 ppb ATZ ($p<0.05$). No significant differences were observed for any of the morphological measurements following ATZ exposure from 1-120 hpf ($p>0.05$). These findings suggest that a ATZ exposure during early development generates metabolite profiles similar to mammals and leads to behavioral alterations supporting ATZ as a neurodevelopmental toxicant.

2.2 Introduction

Atrazine (ATZ) is a triazine herbicide that is commonly used on crops in the Midwestern U.S. and globally (Bethsass and Colangelo 2006; Ochoa-Acuña et al. 2009). While ATZ is the second most common herbicide applied in the U.S. (Mitchell 2014), ATZ was banned in the European Union in 2003 due to widespread water contamination concerns (European Commission 2003). Atrazine has a half-life of about four months, and the half-life of metabolites range from about one to two months (WHO 2011). Furthermore, ATZ is highly mobile in soil and is detected in surface and groundwater in all regions where ATZ is applied. As such, the most common environmental exposure route to the general population is via contaminated drinking water. The U.S. Environmental Protection Agency (EPA) has set the maximum contaminant level in potable water at 3 parts per billion (ppb), but depending on the time of year, concentrations can exceed this limit (Blanchard 2000; Suhl et al. 2016; US EPA 2015). Due to this chemical persistence and contamination of drinking water, it is imperative to fully understand and identify adverse health risks of exposure, especially during development, a susceptible life stage to toxicant exposure.

Developmental ATZ exposure is reported to result in a variety of adverse health outcomes in several animal models. Neurodevelopmental outcomes include altered development of the nigrostriatal dopamine system at 50 mg/kg, 100 mg/kg, and 200 mg/kg (Li et al. 2015), ultrastructure of neurons in the dentate gyrus, and impaired spatial memory at 10 and 100 mg/kg (Li et al. 2019b) in rats exposed during puberty. In the zebrafish, an embryonic ATZ exposure resulted in altered serotonergic pathways and altered genes associated with neuronal growth and morphology in the brain of adult fish aged 6 months exposed to 0.3, 3, or 30 ppb ATZ (Wirbisky et al. 2015; 2016a). Developmental exposure caused increased head size in zebrafish larvae exposed to 0.3, 3, or 30 ppb ATZ (Weber et al. 2013), craniofacial alterations in cartilage at 35 ppm (Lenkowski and McLaughlin 2010) and bone in zebrafish (Walker et al. 2018), and altered muscle development in *Xenopus laevis* at 0.1 µM ATZ (Lenkowski and McLaughlin 2010). Epidemiological studies support altered developmental outcomes in humans including an increase in birth defects and in small for gestational age births with a maternal median exposure of 0.12 nmol/L ATZ (Chevrier et al. 2011), at greater than 0.1 µg/L ATZ (Ochoa-Acuña et al. 2009), and at up to 0.1 µg/L ATZ metabolites (Migeot et al. 2013). In addition, low birth weights were reported in children born to women with increased ATZ exposure (median ATZ concentration of 0.17 µg/L in drinking water) (Almberg et al. 2018).

ATZ is a known endocrine disrupting chemical (EDC) targeting the neuroendocrine system. ATZ exposure leads to suppression of luteinizing hormone (LH) via the gonadotropin-releasing hormone (GnRH) in rats through a hypothalamic mechanism yet to be clearly identified when exposed to a range of 100-300 mg/kg (Cooper et al. 2000; Marques et al. 2000). ATZ can also cause sex-specific adverse outcomes, such as attenuated LH surge in female rats at 100 mg/kg (Kimura et al. 2019) and upregulation of aromatase with an embryonic ATZ exposure at 2 or 40 ppb in female snapping turtles (Russart and Rhen 2016). In addition, an embryonic ATZ exposure (3 or 30 ppb) caused increased ovarian progesterone, follicular atresia, and transcriptomic ovarian alterations in adult female zebrafish (Wirbisky et al. 2016c).

ATZ and its metabolites desethyl atrazine (DEA), deisopropyl atrazine (DIA), and diaminochlorotriazine (DACT) are detected in surface and groundwater in regions where ATZ is applied due to environmental degradation pathways (WHO 2011). In addition, ATZ is metabolized to DEA or DIA and further to DACT in vivo in humans and other mammals by cytochrome P450s (Figure 2-1) (McMullin et al. 2007; Joo et al. 2010). ATZ and its metabolites can be detected in

urine of agricultural workers and in individuals living in regions where ATZ is applied (Barr et al. 2007; Catenacci et al. 1993; Mendaš et al. 2012; Migeot et al. 2013). In laboratory studies, ATZ and the same major metabolites are also detected in the urine, liver, and brain tissue of mice (Lin et al. 2011; Ross et al. 2009) and rats (Brandhonneur et al. 2020; McMullin et al. 2007). Some studies in humans (Barr et al. 2007; Catenacci et al. 1993) and mice (Brandhonneur et al. 2020; Ross et al. 2009) support DACT as the major metabolite detected in urine or tissues. For example, ATZ exposure in mice resulted in the highest detection of DACT, with a 10-fold reduction in plasma levels of DEA and DIA (Ross et al. 2009). Studies indicate CYP1A2, CYP2C19, and CYP3A4 to be among the major drivers of ATZ dealkylation in humans, but other cytochrome P450s have also been reported (ATSDR, 2003; Joo et al., 2010). In addition, studies in rodents indicate similarities and also differences in the specific isozymes in ATZ dealkylation indicating species conservation and species-specific metabolism (ATSDR, 2003). Furthermore, how ATZ is metabolized in other popular vertebrate animal models (e.g., the zebrafish) is even less understood.

The zebrafish is an established model for developmental and toxicological studies based on their ex vivo development and short developmental stages (Garcia et al. 2016; Horzmann and Freeman 2018). Zebrafish have 70% gene similarity to humans, and over 80% similarity for genes related to human disease (Howe et al. 2013). While zebrafish have been used in several studies evaluating ATZ toxicity, it is not yet known if zebrafish produce the same major metabolites as mammals following a developmental exposure. Thus, the first goal of this study was to determine if the same major ATZ metabolites (i.e., DEA, DIA, and DACT) would be present in zebrafish larval tissue following a developmental exposure. Second, zebrafish were exposed to concentrations ranging from 0.1 to 10 times the current U.S. EPA maximum contaminant level in drinking water (0, 0.3, 3, or 30 ppb) from 1-120 hpf to further assess ATZ developmental toxicity including behavior and morphological parameters. We hypothesized that a developmental ATZ exposure would elicit similar metabolite profiles found in mammals leading to behavioral and morphological alterations.

Currently, there are no publications assessing the major ATZ metabolites (i.e., DEA, DIA, and DACT) in zebrafish tissue. There is some evolutionary conservation in the functions of cytochrome P450s in the zebrafish, but there are also differences compared to mammals. Zebrafish have a total of 94 cytochrome P450 (CYP) genes that are in the same 18 gene families as humans and mammals, but these families vary in their respective gene numbers. For example, zebrafish

CYP genes in families 1-4 are different than those in humans, while those within CYP families 5-51 have 32 direct orthologs to human CYPs, suggesting conservation of enzyme activity and function for these specific enzymes (Goldstone et al. 2010). Furthermore, along with cytochrome P450s, the enzymes erythromycin N-demethylase (ERND), aminopyrine N-demethylase (APND), and NADPH-P450 reductase were activated in zebrafish exposed to ATZ in a sex-specific manner (Dong et al. 2009). Due to the similarities and differences between zebrafish and mammalian CYPs, it is important to determine whether the same metabolites found in mammals are also present in zebrafish tissue.

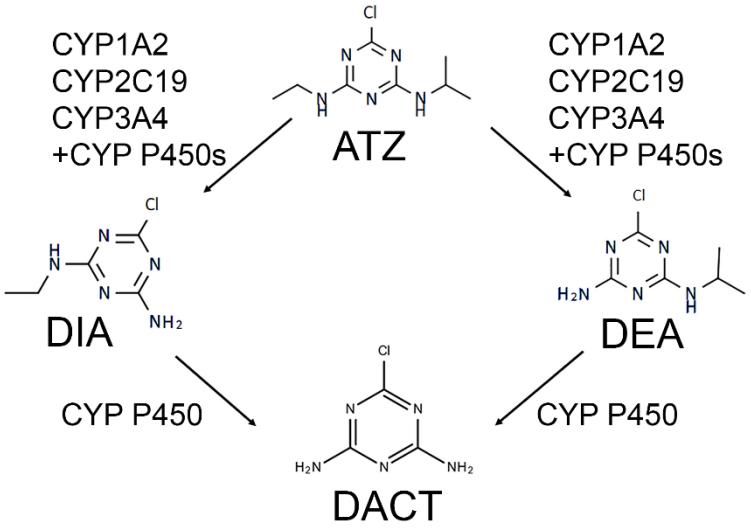


Figure 2-1. Major metabolites of atrazine. Atrazine is metabolized into desethyl atrazine (DEA), deisopropyl atrazine (DIA), and diaminochlorotriazine (DACT) via cytochrome P450 enzymes in mammals. Studies indicate CYP1A2, CYP2C19, and CYP3A4 to be major drivers of atrazine metabolism to DEA and DIA in humans, along with other cytochrome P450s. Cytochrome P450s also further metabolize DEA and DIA to DACT, but more studies are needed to better understand the specific enzymes. There are similarities but also differences in zebrafish cytochrome P450s compared to humans supporting the need to determine if zebrafish produce the same major atrazine metabolites following a developmental atrazine exposure.

2.3 Materials and methods

2.3.1 Zebrafish husbandry and ATZ exposure parameters

Wild-type AB strain adult zebrafish (*Danio rerio*) were housed on a Z-Mod System (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle. Water quality was maintained at a range of 28-29°C, pH at 7.0-7.3, and salinity at 470-550 µS conductivity. Adult fish were 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 fish were bred in breeding tanks according to previous protocols (Peterson et al. 2011) and embryos were collected 1 hour after fertilization with chorions intact, at approximately the 4-8 cell stage of embryonic development. Embryos were then rinsed, exposed to 0 (filtered aquaria water), 3, 30, 300, 1,000, 3,000, or 10,000 ppb (µg/L) ATZ in the targeted metabolite study or to 0, 0.3, 3, or 30 ppb ATZ in the behavior and morphology study. Chemical exposures were conducted similar to our past ATZ studies in a sterile polystyrene petri dish in groups of 50 and maintained in incubators set at 28.5°C through 120 hpf (Weber et al. 2013; Wirbisky et al. 2015; Horzmann et al. 2018, 2020).

ATZ stock solutions are prepared from technical grade atrazine (98.2% purity) (CAS 1912-24-9; Chem Service, West Chester, PA) according to previous protocols (Weber et al. 2013; Wirbisky et al. 2015a). ATZ concentrations in treatment solutions were confirmed with a US EPA approved immunoassay kit (Abraxis Atrazine ELISA Kit, Warminster, PA) as previously described (Freeman et al. 2005; Wirbisky et al. 2016a) and were found to be within expected concentration range ($\pm$ 0.1 ppb within test kit range). At 24 hours, survival was assessed, and groups with 80% survival or higher were kept to attain high quality of clutches. Similar to other studies, no difference in mortality was seen between all treatment groups (Weber et al. 2013). Larvae were rinsed at 120 hpf with aquaria water to end ATZ exposure. The protocols used for these experiments were approved by Purdue University Animal Care and Use Committee and all zebrafish were treated humanely for prevention and alleviation of suffering.

2.3.2 Targeted Metabolomics

At the end of the ATZ exposure, larvae were pooled from each petri dish to attain one biological replicate (~45-50 larvae per pool) for targeted metabolomics. A total of 7-12 replicates were collected (N=7-12). Sample preparation and quantitation were based on procedures by Brox et al. (2014). Samples were spiked with 10 ng of deuterated internal standards (d5-Atrazine, d7-DEA, and d5-DIA). Samples were homogenized in 2 mL vials with 1.4 mm ceramic (zirconium oxide) beads with 194 µL of methanol using Precellys 24 tissue homogenizer (Bertin Technologies, Rockville, MD, USA). The homogenate was transferred to a microcentrifuge tube and 200 µL of 100% acetonitrile was added. The mixture was vortexed, centrifuged at 18,600 x g for 10 minutes and the supernatant was transferred and evaporated. The extract was reconstituted in 50 µL of 50% methanol.

Quantitation used HPLC tandem mass spectrometry. A total of 8 µL of the reconstituted sample was delivered to the column through an Agilent 1290 Infinity II LC with multisampler (Agilent Technologies, San Jose, CA) into a 6470 triple quadrupole mass spectrometer (Agilent Technologies, San Jose, CA) equipped with Jet Stream ESI ion source. The HPLC column used was an Agilent Zorbax SB-Phenyl (4.6 x 150 mm, 5 µm; Agilent Technologies, San Jose, CA). The binary pump flow rate was set at 0.8 mL/min. Mobile phase A was 90:10 (v/v) of water at 50 mM ammonium acetate: acetonitrile, with 0.1% formic acid and mobile phase B was 90:10 (v/v) of acetonitrile: water at 50 mM ammonium acetate, with 0.1% formic acid. The HPLC column was initially set to 20% B for 0.5 minutes. The binary pump was set in a linear gradient to 100% B in 8.5 min and held for 1 min. It was then returned to 20% B in 0.5 min and re-equilibrated for 3 min.

The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Data processing utilized Agilent MassHunter (B.06.00). For ATZ, DIA, and DEA, concentrations in ng per sample were obtained by ratioing the endogenous compound peak area by the corresponding deuterated internal standard peak area, then multiplying by the amount of internal standard added (10 ng). Standard range was 2.5 to 25,000 ng per sample, with a quantitation limit of 2.5 ng per sample. For DACT, a calibration curve was generated, using d5-ATZ as the internal standard. Standard range was 10 to 100,000 ng per sample, with a quantitation limit of 10 ng per sample. The concentration of ATZ, DIA, DEA, and DACT in zebrafish tissue was calculated as ng/mg.

2.3.3 Behavior

Behavior was assessed at 120 hpf with the visual motor response routine on the Noldus Daniovision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands). Each treatment group within a biological replicate consisted of twenty-four larvae (subsamples) that were placed into separate wells of a 96 well plate with 500 µL of filtered water. This was repeated 8 times with 24 subsamples in each biological replicates to total 192 larvae total per treatment group being included in this analysis. The observation chamber water temperature was regulated at 28°C during the experiment. Following a 10 minute dark acclimation period, the Noldus white light routine was used to test visual motor response by exposing larvae to 10 minute intervals of alternating light and dark periods for 50 minutes as described in a previous study

(Horzmann et al. 2017). All behavioral experiments were performed between 11 am - 1 pm to reduce circadian variability in movement. Infrared movement traces were recorded at 25 frames per second with a Basler GenICam acA 1300-60g camera and analyzed with Noldus EthoVision 12.0 software. Tracks were smoothed with a minimum distance moved profile set at >0.2 mm and data were collected for endpoints such as distance moved, velocity, and time spent moving.

2.3.4 Morphology

Following ATZ exposure, larvae were euthanized via anesthetic overdose in 0.4 mg/mL buffered tricaine-S (Western Chemical Inc.). From each treatment group in each biological replicate, 9-16 larvae (subsamples) were imaged morphological measurements attained. This was repeated 6 times to equal 6 biological replicates (i.e., a total of 144 larvae measured in each treatment group). Larvae were imaged similar to past studies in our research group under a Nikon SMZ1 500 stereomicroscope with a Nikon Digital Sight DS-fil camera and NIS elements imaging software (Nikon Instruments Inc., Melville, NY) (Horzmann et al. 2017, 2018). Larvae were suspended in a low percent agar for measuring. The measurements assessed were total body length (measured snout to tail), head length (snout to operculum), head width (width of head at the level of the eyes), and brain length (rostral aspect of forebrain to caudal aspect of brainstem) with Nis-Elements D 3.2 software (Figure 2-2). In addition, ratios of head length to body length, head width to body length, and brain length to body length were determined. The order in which treatment groups were measured was randomized.

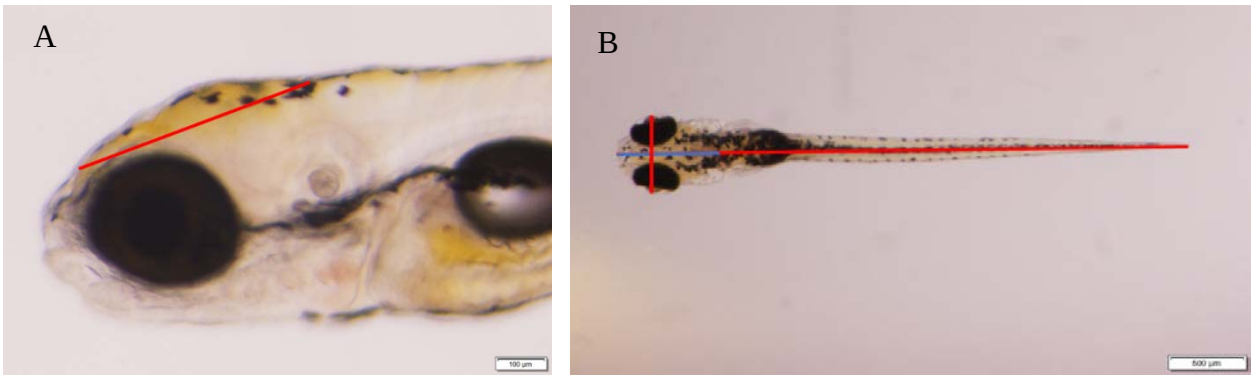


Figure 2-2. Image for general morphology measurements for larvae at 120 hpf. A shows brain length measurement (red line). Scale bar represents 100 µM. B shows an aerial view of the larvae at with the anterior to posterior red line indicating total length, the blue line indicating head length, and red line indicating head width. Scale bar represents 500 µM.

2.3.5 Statistical analysis

There was a total of 7-12 biological replicates (with 45-50 larvae pooled in each replicate) for targeted metabolomics. For behavior, there were a total of 8 replicates with 24 subsamples per treatment group per replicate. For morphology, there were a total of 6 replicates with 9-16 subsamples per treatment group per replicate. Each biological replicate represents a separate spawning event. A Grubb's outlier test was used on data organized by treatment group for each of the metabolomics, behavior, and morphology analyses separately to identify any outliers within treatment group for each outcome. All data was assessed and confirmed for normality before proceeding to further statistical analysis. For the targeted metabolomics, atrazine treatments below the quantitation limit were excluded in the statistical analysis. Metabolomic, cumulative behavior, and morphological endpoints were statistically analyzed with an analysis of variance (ANOVA) with a Fisher's least significant difference (LSD) post hoc test when a significant ANOVA was observed ($p<0.05$) using SAS Statistical Software (SAS Institute Inc., Cary, NC). Phasic behavior was analyzed using a repeated measures ANOVA for phase, treatment, and phase*treatment interaction using SAS. Data for behavior are represented as bar graphs (Bridi et al. 2017; Horzmann et al. 2018). Results are presented as mean ± standard deviation of the mean.

2.4 Results

2.4.1 Targeted Metabolomics

ATZ and the three major mammalian ATZ metabolites were identified in zebrafish tissue at 120 hpf following exposure from 1-120 hpf. Chromatographic retention times for DACT, DIA, DEA, and ATZ were 3.3, 5, 6, and 7.8 minutes, respectively (Figure 2-3). The quantitation limit was 2.5 ng per sample for ATZ, DEA, and DIA and 10 ng per sample for DACT. Samples were calculated in ng/mg (Table 2.1). ATZ and DACT were the most abundant with quantification in the 300, 1,000, 3,000, and 10,000 ppb treatment groups and with dose increasing as exposure concentration increased ($p<0.0001$ for ATZ and DACT). DEA and DIA were less abundant with quantification only in the 3,000 and 10,000 ppb treatment groups, but similarly a dose increase was observed as exposure concentration increased ($p=0.0055$ for DEA and $p=0.0028$ for DIA).

The percent of the major metabolites in the 3,000 and 10,000 ppb treatment groups was compared to a previous study in humans (Table 2.2). For the zebrafish, each metabolite is

represented as the percent of total for DACT, DEA, and DIA. In the human study, the metabolite ATZ mercapturate was also included and detected as 11.9% or less of total metabolite composition. Similar to humans, a higher percent of DACT was detected in comparison to DEA and DIA. The percent similarity for the highest zebrafish treatment (10,000 ppb) was most similar to the high-level acute exposure in humans, while the 3,000 ppb zebrafish treatment group was more similar to the low-level acute exposure in humans for DACT. The comparison for DEA and DIA was not as clear and could be limited with percent comparison assessment due to not assessing ATZ mercapturate in the zebrafish. Unfortunately, this assessment was not able to be completed for the lower zebrafish exposure concentrations due to limits of detection of the technique.

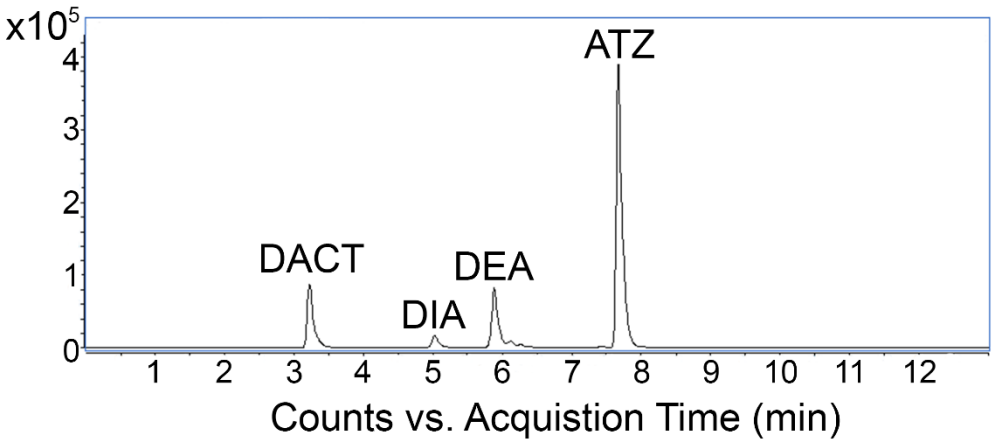


Figure 2-3. HPLC-MS-MS chromatogram of zebrafish tissue following developmental atrazine exposure. The same major atrazine metabolites were detected in zebrafish tissue following a developmental exposure as is detected in mammals. (Desethyl atrazine: DEA; Deisopropyl atrazine: DIA; Diaminochlorotriazine DACT).

Table 2-1. Average atrazine and atrazine metabolite distribution in zebrafish larvae at 120 hpf following atrazine exposure from 1-120 hpf.

Atrazine exposure concentration	Atrazine ng/mg	Desethyl atrazine (DEA) ng/mg	Deisopropyl atrazine (DIA) ng/mg	Diaminochloroatrazine (DACT) ng/mg
0 ppb	ND	ND	ND	ND
3 ppb	ND	ND	ND	ND
30 ppb	ND	ND	ND	ND
300 ppb	0.163^{C}	ND	ND	0.100^{B}
1000 ppb	0.526^{BC}	ND	ND	0.093^{B}
3000 ppb	0.997^{B}	0.080^{B}	0.073^{B}	0.152^{B}
10000 ppb	3.647^{A}	0.374^{A}	0.488^{A}	1.284^{A}

Different letters indicate statistically significant different groups at $p<0.05$.
ND: below quantification limit

Table 2-2. comparison of percent metabolite distribution among main ATZ metabolites in larval zebrafish versus human exposure

ATZ exposure concentration	Percent DEA	Percent DIA	Percent DACT	ATZ mercapturate*
10,000 ppb in zebrafish	17.4%	22.7%	59.8%	*
High-level acute exposure in humans	31.2%	5.3%	51.3%	11.9%
3,000 ppb in zebrafish	26.2%	23.9%	49.8%	*
Low-level acute exposure in humans	43%	2%	47.6%	7.5%
Environmental exposure in humans	15%	6%	77%	2%

* ATZ mercapturate is a metabolite analyzed in a human exposure study not analyzed in this study.

2.4.2 Larval visual motor assay

Larvae were exposed to ATZ, and at 120 hpf a visual motor response test was used to assess zebrafish behavior. Significant behavioral changes were identified when evaluated for cumulative testing time and then changes within specific phases were determined. The 0.3 ppb ATZ exposed larvae had hyperactivity in the cumulative total distance moved ($p=0.0105$), time spent moving ($p=0.0118$), and velocity ($p=0.0104$) (Figure 2-4).

Phasic behavioral data for total distance moved showed significance of phase ($f(5,3610)=1652$, $p<0.001$), treatment ($f(3,722)=2.59$, $p=0.0518$), and interaction of treatment and phase ($f(15,3610)=2.78$, $p=0.0013$) (Figure 2.5A). In the first light phase, larvae in the 0.3 ppb treatment group had increased movement and larvae in the 30 ppb treatment group had decreased movement in the second and third dark phases. For time spent moving, a significant effect of phase ($f(5,3610)=1696$, $p=0.0001$), treatment ($f(3,722)=3.13$, $p=0.0251$), and the interaction of treatment and phase ($f(15,3610)=1.66$, $p=0.0526$) was seen. The 0.3 ppb treatment group had more time spent moving in comparison to control larvae in the first dark phase and first light phase (Figure 2.5B). While a significant effect for phase was observed for velocity ($f(5,3610)=52.51$, $p=0.001$), no evidence of overall effect of treatment ($f(3,722)=1.05$, $p=0.3703$) or treatment and phase interaction ($f(15,3610)=0.99$, $p=0.4653$) was observed (Figure 2.5C). Hyperactivity in 0.3 ppb treatment group and hypoactivity in the 30 ppb treatment group indicates atrazine exposure alters swimming patterns of larvae at 120 hpf.

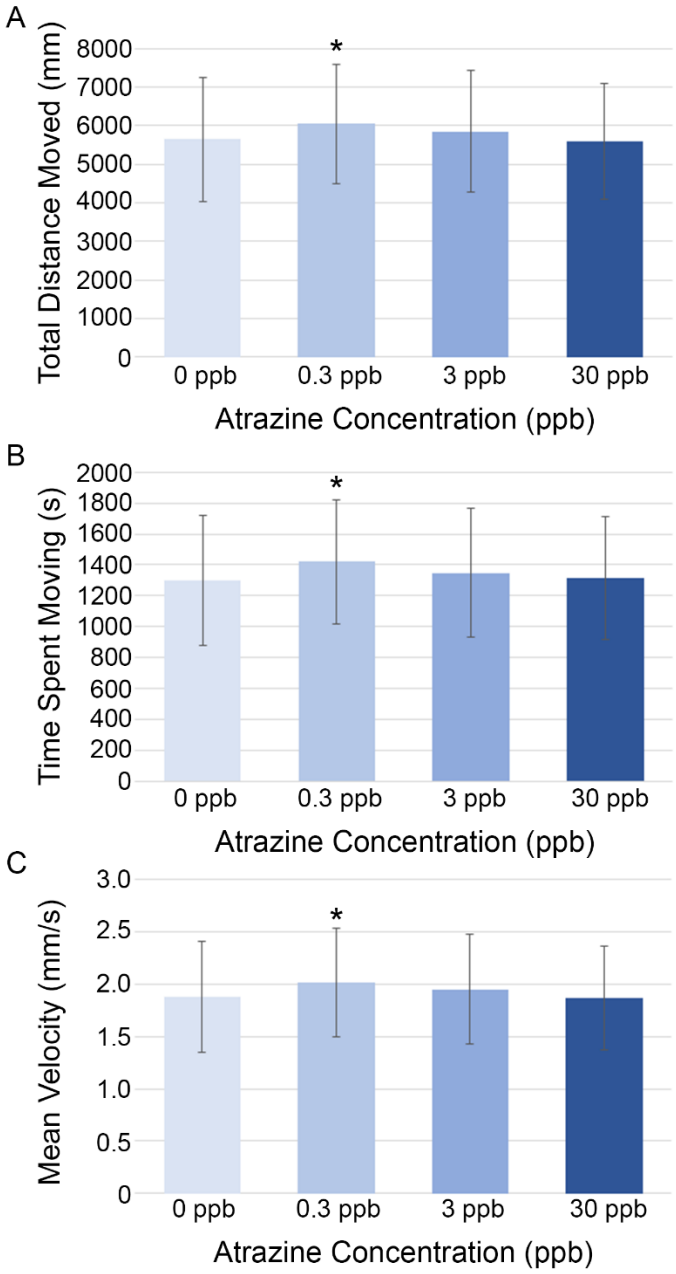


Figure 2-4. Cumulative behavior of zebrafish larvae at 120 hpf exposed to atrazine from 1-120 hpf. Zebrafish exposed to 0.3 ppb atrazine had an increase in (A) total distance moved, (B) center-point movement, and (C) velocity. Error bars represent standard deviation. *$p<0.05$, N=8 with 24 subsamples per treatment in each replicate to total 192 larvae per treatment.

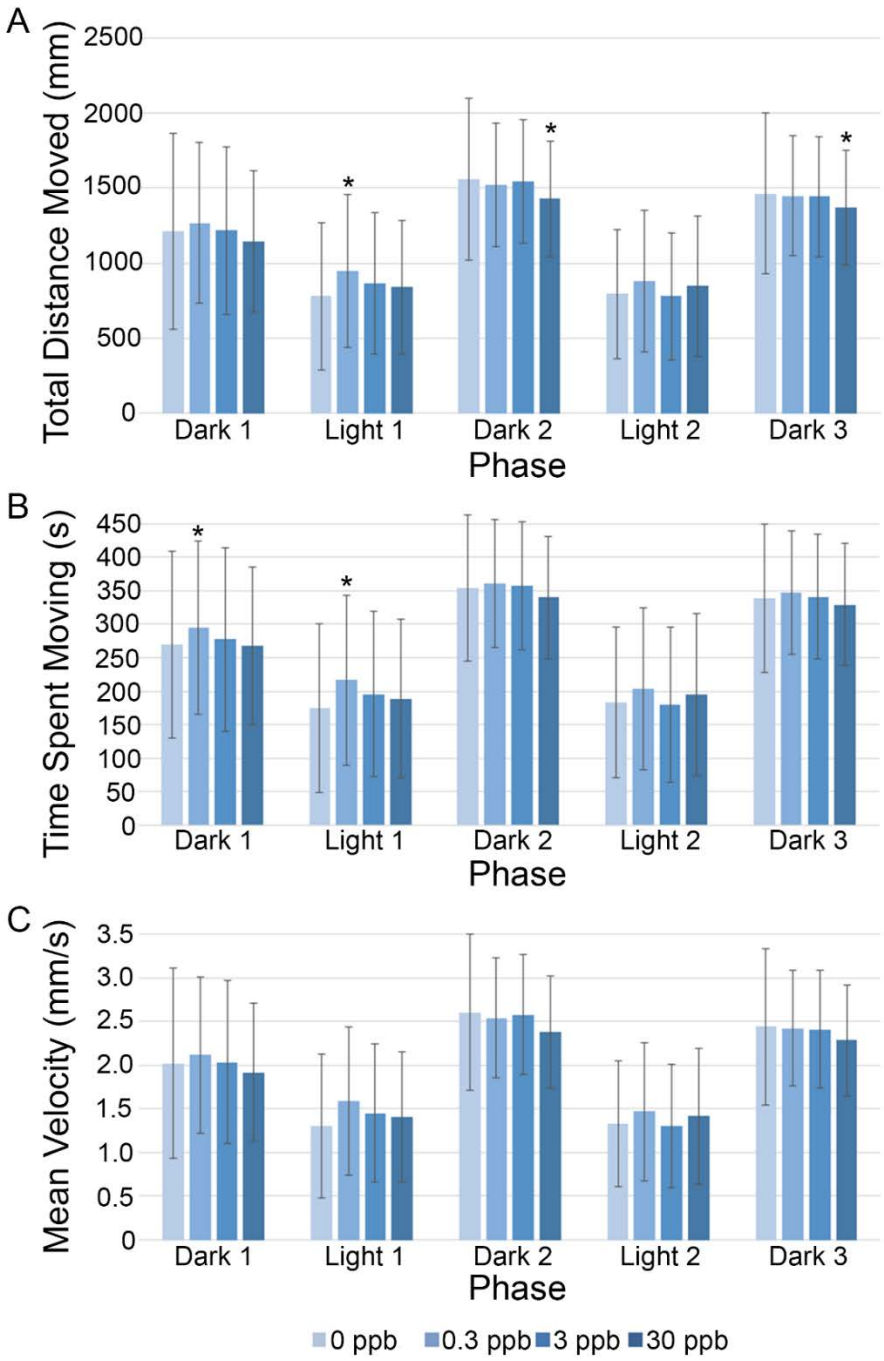


Figure 2-5. Light and dark phase behavior of zebrafish larvae at 120 hpf exposed to atrazine from 1-120 hpf. (A) Zebrafish exposed to 0.3 ppb atrazine had an increase in total distance moved in the first light phase and 30 ppb atrazine exposed fish had decreased movement in the second and third dark phases. (B) Larvae in the 0.3 ppb atrazine treatment group had increased time spent moving in the first dark and light phases. (C) No significant differences were observed for any atrazine treatment group for mean velocity. Error bars represent standard deviation. *$p<0.05$, N=8 with 24 subsamples per treatment in each replicate to total 192 larvae per treatment.

2.4.3 Morphology

Zebrafish larvae measured at 120 hpf, had no significant changes in mean total length (p=0.9121), mean head width (p=0.531), mean head length (p=0.551), or mean brain length (p=0.3997) (Table 2.2). Ratios for mean brain length over total length (p=0.6092), mean head length over total length (p=0.2503), and mean head width over total length (p=0.2644) also were not significantly different among treatment groups.

Table 2-3. No significant differences in morphological measurements were observed in zebrafish larvae at 120 hpf following atrazine exposure from 1-120 hpf (p>0.05).

Atrazine exposure concentration	Body length (µm)	Head length (µm)	Head width (µm)	Brain length (µm)	Head length/ body length ratio	Head width/ body length ratio	Brain length/ total length ratio
0 ppb	4235 ± 210[a]	805 ± 53	663 ± 29	956 ± 55	0.190 ± 0.011	0.157 ± 0.008	0.226 ± 0.012
0.3 ppb	4244 ± 193	799 ± 46	668 ± 28	967 ± 49	0.188 ± 0.009	0.158 ± 0.007	0.2280 ± 0.012
3 ppb	4232 ± 196	799 ± 47	669 ± 27	960 ± 46	0.227 ± 0.012	0.158 ± 0.007	0.227 ± 0.012
30 ppb	4223 ± 163	806 ± 43	658 ± 27	960 ± 37	0.228 ± 0.0126	0.156 ± 0.007	0.228 ± 0.013

[a]± Standard deviation

2.5 Discussion

Identifying adverse health outcomes associated with ATZ exposure is important due to its persistence in the environment and presence in potable water sources. ATZ and its metabolites are detected in the urine of mammalian models as well as humans with most mammalian studies indicating the parent compound as the major driver of toxic outcomes. A few studies have evaluated the toxicity of ATZ metabolites in zebrafish by direct exposure to these metabolites (Blahova et al. 2020; Liu et al. 2016, 2017), but there is limited information on if DEA, DIA, or DACT is present in zebrafish tissue after an ATZ exposure (i.e., in vivo ATZ metabolism). Embryotoxicity studies evaluating hatching rate, somite formation, eye and pigment development, as well as cardiotoxicity with ATZ, DIA, and DEA exposure found no potential risk at environmentally relevant concentrations (Blahova et al. 2020). In addition, pericardial edema was the only major malformation recorded and was only observed at the highest test concentration of 10,000 ppb ATZ (Blahova et al. 2020). Alternatively, immunotoxicity studies with developmental exposure to ATZ, DEA, DIA, or DACT at 30, 100, or 300 ppb through 120 hpf reported altered immune response through upregulated transcription for genes related to mast cell function and altered cytokine mRNA levels after an immune challenge (Liu et al. 2017). In addition, the same group reported cumulative hypoactivity following exposure to ATZ (100 and 300 ppb), DEA (30

and 300 ppb), DIA (100 and 300 ppb), or DACT (30, 100, and 300 ppb) through 120 hpf (Liu et al. 2016). These findings agree with the hypoactivity observed in this study following ATZ exposure, but does indicate the major metabolites may also be drivers of the behavioral alterations. The goal of the current study was to determine if zebrafish metabolized ATZ to produce DEA, DIA, and DACT in vivo and thus, further studies are needed to investigate if ATZ and/or metabolites are responsible for neurotoxicity outcomes.

So far, only one other study has evaluated ATZ metabolism is the zebrafish. In that study, the authors focused on microsomal and soluble metabolism of ATZ via glutathione-S-transferase with ATZ developmental exposure and determined that microsomal enzymes conjugated ATZ to glutathione effectively (Wiegand et al. 2001). Our current study is novel because it is the first to identify ATZ dealkylated metabolites that are found in mammals are also present in zebrafish tissue following a developmental ATZ exposure. Higher concentrations of DACT relative to the other metabolites were detected, which agrees with the human (Barr et al. 2007; Catenacci et al. 1993) and mouse (Ross et al. 2009) studies. Table 2.2 compares the percent distribution of ATZ metabolites in larval zebrafish in our study to the percent distribution of metabolites in a study with ATZ metabolite detection in human urine (Barr et al. 2007).

A limitation of the current study was that ATZ and DACT were not quantifiable in zebrafish in the 3 or 30 ppb treatment groups and that DEA and DIA were only quantifiable in the 3,000 and 10,000 ppb treatment groups. These findings signify a need for further methodological refinement if ATZ exposure occurs at lower concentrations. It should also be noted that ATZ solutions were not renewed during the exposure period. Taken together, our study confirms that similar ATZ dealkylated metabolites found in mammals are also observed in zebrafish tissue with ATZ and DACT being most abundant following the developmental ATZ exposure. There are also several studies suggesting that ATZ can bioaccumulate in different species. For example, a study of ATZ exposure in two bivalves species shows that ATZ can bioaccumulate in a tissue-specific manner (Jacomini et al. 2006). In addition, a study in *E. foetida* and *M. guillelmi*, two species of earth worms, found that E. *foetida* absorbed five times more ATZ than its counterpart when exposed to 4.25 mg ATZ/kg of soil due to this worm's dermal absorption (Wang et al. 2014). Alternatively, human studies indicate ATZ is eliminated from the body in urine over a few days (Barr et al. 2007). Thus, more studies are needed to further define ATZ bioaccumulation similarities and differences among species.

Behavioral data at 120 hpf in larval zebrafish showed hyperactivity for the 0.3 ppb treated larvae in cumulative total distance moved, time spent moving, and velocity. For phasic behavior, hyperactivity was seen in the first light phase of total distance moved for larvae in the 0.3 ppb treatment group. Behavioral hypoactivity occurred in the second and third dark phases for the 30 ppb treatment groups, which is in agreement with previous studies with ATZ exposure through 72 hpf evaluating cumulative behavior data (Horzmann et al. 2018). The previous study was limited in that only cumulative behavior data was assessed and thus, it is not known if hyperactivity at 0.3 ppb may have been also observed if phase analysis had been included. Overall, these two studies show agreement for hypoactivity at 30 ppb at 120 hpf for an ATZ exposure from 1-72 hpf or 1-120 hpf. These observations also agree with studies in other animals showing altered behavior. For example, hyperactivity was reported in mice of both sexes with a developmental ATZ exposure (Lin et al. 2014) and in tadpoles (Ehrsam et al. 2016) at environmentally relevant ATZ concentrations. Other studies have noted behavioral hypoactivity in adult rats (Rodríguez et al. 2013) and pacu fingerlings at higher exposure concentrations (Delcorso et al. 2020). In addition, adult male zebrafish aged 9 months exposed to ATZ only during embryogenesis (1-72 hpf) had decreased locomotor movement with brain transcriptome alterations associated with nervous and reproductive system function (Horzmann et al. 2020). Behavioral changes with ATZ are important to define as exposure has been connected to changes in neurochemistry related to anxiety-like behavior, (Chávez-Pichardo et al. 2020) as well as dopaminergic and serotonergic function (Lin et al. 2013). In addition, altered serotonergic neurotransmission and transcriptome changes in genes associated with behavior and the serotonergic pathway are reported in the brain of adult female zebrafish following an embryonic ATZ exposure (Wirbisky et al. 2015, 2016b). Overall, the findings of this study and others indicates treatment-dependent alterations in hyperactivity or hypoactivity. Further studies are needed to define the mechanisms that lead to these different behavioral alterations at the higher and lower ATZ exposures.

Morphological alterations are also important to study with ATZ exposure due to associations with babies being born small for gestational age (Chevrier et al. 2011; Migeot et al. 2013), as well as male genital birth defects (Agopian et al. 2013) in human epidemiology studies. No morphological changes were seen at 120 hpf in any parameter measured in the current study; however, our previous studies with ATZ exposure through 72 hpf in zebrafish showed increased head length in all treatments (Weber et al. 2013) and increased brain length in the 0.3 ppb treatment

groups (Horzmann et al. 2018). Discrepancies between the two studies could be due to differences in time of exposure (i.e., 1-72 hpf compared to 1-120 hpf), time of analysis (72 hpf compared to 120 hpf), or compensation events over the developmental period; however, more studies are needed to define. Other studies conducted with developmental ATZ exposure at environmentally relevant concentrations (0, 0.3, 3, and 30 ppb) in zebrafish found rare malformations, but focused on endpoints such as pericardial edema or eye development at 24, 48, 72 and 96 hpf (Blahova et al. 2020). In addition, our previous studies report later in life morphological alterations from an embryonic ATZ exposure with a decreased head length to body ratio in the 30 ppb treatment group and increased head width to body ratio in the 0.3 and 3 ppb treatment groups (Wirbisky et al. 2016c). Integration of these findings suggest that the body may compensate at certain developmental time points, but that the developmental exposure can lead to health consequences into adulthood, aligning with the developmental origins of health and disease paradigm (DOHaD) (Haugen et al. 2015; Heindel and Vandenberg 2015; Horzmann and Freeman 2018).

Overall, findings from this study support ATZ as a neurodevelopmental toxicant that can alter behavioral outcomes. Importantly, the same major metabolites as detected in humans and other mammals are present in the zebrafish following a developmental ATZ exposure, further validating zebrafish as a biomedical model with human relevance for ATZ toxicity studies.

2.6 References

1. 2016 Pesticide Use Maps -. Available: https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2016&map=ATRAZINE&hilo=L [accessed 4 November 2018].

2. Agopian AJ, Lupo PJ, Canfield MA, Langlois PH. 2013. Case-Control Study of Maternal Residential Atrazine Exposure and Male Genital Malformations. Am J Med Genet 161:977–982; doi:10.1002/ajmg.a.35815.

3. Almberg K, Turyk M, Jones R, Rankin K, Freels S, Stayner L. 2018. Atrazine Contamination of Drinking Water and Adverse Birth Outcomes in Community Water Systems with Elevated Atrazine in Ohio, 2006–2008. IJERPH 15:1889; doi:10.3390/ijerph15091889.

4. Atwood D and Paisley-Jones C.Pesticides industry Sales and Usage: 2008-2012 Market Estimates. Office of Chemical Safety and Pollution Prevention US Environmental Protection Agency, Washington.

5. Barr DB, Panuwet P, Nguyen JV, Udunka S, Needham LL. 2007. Assessing Exposure to Atrazine and Its Metabolites Using Biomonitoring. Environ Health Perspect 115:1474–1478; doi:10.1289/ehp.10141.

6. Bethsass J, Colangelo A. 2006. European Union Bans Atrazine, While the United States Negotiates Continued Use. International Journal of Occupational and Environmental Health 12:260–267; doi:10.1179/oeh.2006.12.3.260.

7. Blahova J, Cocilovo C, Plhalova L, Svobodova Z, Faggio C. 2020. Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (Danio rerio). Environmental Toxicology and Pharmacology 77:103370; doi:10.1016/j.etap.2020.103370.

8. Blanchard P.E., & Lerch R.N. (2000). Watershed Vulnerability to Losses of Agricultural Chemicals: Interactions of Chemistry, Hydrology and Land Use. *Environ. Sci. Technol. 34*, 3315-3322).

9. Brandhonneur N, Hutin V, Chevrier C, Cordier S, Le Corre P. 2019. Maternal and fetal blood pharmacokinetics and organ distribution of atrazine, propazine, simazine and their metabolites in pregnant rats after chronic oral administration. Toxicological Sciences kfz223; doi:10.1093/toxsci/kfz223.

10. Catenacci G, Barbieri F, Bersani M, Fereoli A, Cottica D, Maroni M. 1993. Biological monitoring of human exposure to atrazine. Toxicology Letters 69:217–222; doi:10.1016/0378-4274(93)90107-9.

11. Cheremisinoff NP and Rosenfeld PE. Handbook of Pollution Prevention and Cleaner Production: Best Practices in the Agrochemical Industry. 2011.Elsevier.

12. Chevrier C, Limon G, Monfort C, Rouget F, Garlantézec R, Petit C, et al. 2011. Urinary Biomarkers of Prenatal Atrazine Exposure and Adverse Birth Outcomes in the PELAGIE Birth Cohort. Environ Health Perspect 119:1034–1041; doi:10.1289/ehp.1002775.

13. Delcorso MC, de Paiva PP, Grigoleto MRP, Queiroz SCN, Collares-Buzato CB, Arana S. 2020. Effects of sublethal and realistic concentrations of the commercial herbicide atrazine in Pacu (Piaractus mesopotamicus): Long-term exposure and recovery assays. Vet World 13:147–159; doi:10.14202/vetworld.2020.147-159.

14. Ehrsam M, Knutie SA, Rohr JR. 2016. The herbicide atrazine induces hyperactivity and compromises tadpole detection of predator chemical cues: Atrazine reduces detection of predator chemical cues. Environ Toxicol Chem 35:2239–2244; doi:10.1002/etc.3377.

15. Garcia GR, Noyes PD, Tanguay RL. 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacology & Therapeutics 161:11–21; doi:10.1016/j.pharmthera.2016.03.009.

16. Haugen AC, Schug TT, Collman G, Heindel JJ. 2015. Evolution of DOHaD: the impact of environmental health sciences. J Dev Orig Health Dis 6:55–64; doi:10.1017/S2040174414000580.

17. Heindel JJ, Vandenberg LN. 2015. Developmental origins of health and disease: a paradigm for understanding disease cause and prevention. Current Opinion in Pediatrics 27:248–253; doi:10.1097/MOP.0000000000000191.

18. Horzmann KA, Freeman JL. 2018. Making Waves: New Developments in Toxicology With the Zebrafish. Toxicological Sciences 163:5–12; doi:10.1093/toxsci/kfy044.

19. Horzmann KA, Reidenbach LS, Thanki DH, Winchester AE, Qualizza BA, Ryan GA, et al. 2018. Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures. Journal of Proteomics 186:71–82; doi:10.1016/j.jprot.2018.07.006.

20. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496:498–503; doi:10.1038/nature12111.

21. LeBaron, H. M., McFarland, J.E., & Burnside, O.C. (2008). *The Triazine Herbicides*. Elseiver.

22. Lin Z, Dodd CA, Xiao S, Krishna S, Ye X, Filipov NM. 2014. Gestational and Lactational Exposure to Atrazine via the Drinking Water Causes Specific Behavioral Deficits and Selectively Alters Monoaminergic Systems in C57BL/6 Mouse Dams, Juvenile and Adult Offspring. Toxicol Sci 141:90–102; doi:10.1093/toxsci/kfu107.

23. Lin Z, Fisher JW, Ross MK, Filipov NM. 2011. A physiologically based pharmacokinetic model for atrazine and its main metabolites in the adult male C57BL/6 mouse. Toxicology and Applied Pharmacology 251:16–31; doi:10.1016/j.taap.2010.11.009.

24. McMullin TS, Hanneman WH, Cranmer BK, Tessari JD, Andersen ME. 2007. Oral absorption and oxidative metabolism of atrazine in rats evaluated by physiological modeling approaches. Toxicology 240:1–14; doi:10.1016/j.tox.2007.05.031.

25. Mendaš G, Vuletić M, Galić N, Drevenkar V. 2012. Urinary metabolites as biomarkers of human exposure to atrazine: Atrazine mercapturate in agricultural workers. Toxicology Letters 210:174–181; doi:10.1016/j.toxlet.2011.11.023.

26. Migeot V, Albouy-Llaty M, Carles C, Limousi F, Strezlec S, Dupuis A, et al. 2013. Drinking-water exposure to a mixture of nitrate and low-dose atrazine metabolites and small-for-gestational age (SGA) babies: A historic cohort study. Environmental Research 122:58–64; doi:10.1016/j.envres.2012.12.007.

27. Ochoa-Acuña H, Frankenberger J, Hahn L, Carbajo C. 2009. Drinking-Water Herbicide Exposure in Indiana and Prevalence of Small-for-Gestational-Age and Preterm Delivery. Environmental Health Perspectives; Research Triangle Park 117: 1619–24.

28. Rodríguez VM, Limón-Pacheco JH, Mendoza-Trejo MS, González-Gallardo A, Hernández-Plata I, Giordano M. 2013. Repeated exposure to the herbicide atrazine alters locomotor activity and the nigrostriatal dopaminergic system of the albino rat. NeuroToxicology 34:82–94; doi:10.1016/j.neuro.2012.10.012.

29. Ross MK, Jones TL, Filipov NM. 2009. Disposition of the Herbicide 2-Chloro-4-(ethylamino)-6-(isopropylamino)- *s* -triazine (Atrazine) and Its Major Metabolites in Mice: A Liquid Chromatography/Mass Spectrometry Analysis of Urine, Plasma, and Tissue Levels. Drug Metab Dispos 37:776–786; doi:10.1124/dmd.108.024927.

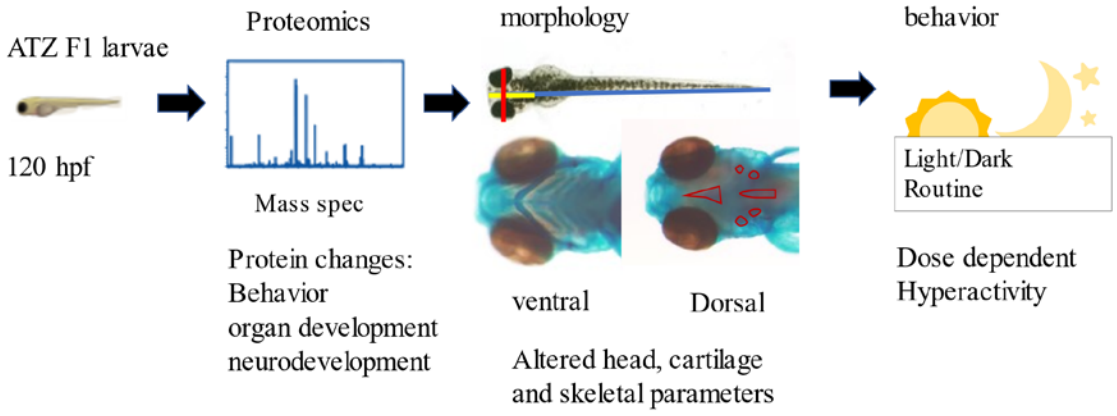
30. Weber GJ, Sepúlveda MS, Peterson SM, Lewis SS, Freeman JL. 2013. Transcriptome Alterations Following Developmental Atrazine Exposure in Zebrafish Are Associated with Disruption of Neuroendocrine and Reproductive System Function, Cell Cycle, and Carcinogenesis. Toxicological Sciences 132:458–466; doi:10.1093/toxsci/kft017.

31. World Health Organization. Atrazine and its metabolites in drinking water. Geneva. WHO 2010.

32. Wirbisky SE, Sepúlveda MS, Weber GJ, Jannasch AS, Horzmann KA, Freeman JL. 2016. Embryonic Atrazine Exposure Elicits Alterations in Genes Associated with Neuroendocrine Function in Adult Male Zebrafish. Toxicological Sciences 153:149–164; doi:10.1093/toxsci/kfw115.

CHAPTER 3. MULTIGENERATION ADVERSE DEVELOPMENTAL IMPACTS IN PROGENY OF ZEBRAFISH EXPOSED TO THE AGRICULTURAL HERBICIDE ATRAZINE DURING EMBRYOGENESIS

3.1 Abstract



Atrazine (ATZ) is an herbicide that is commonly used on crops in the Midwestern US and other select global regions. ATZ has a long half-life and is a common contaminant of drinking water sources. The US Environmental Protection Agency (EPA) ATZ regulatory limit is 3 parts per billion (ppb; µg/L), but this limit is often exceeded with ATZ indicated as an endocrine disrupting chemical in multiple species. The zebrafish model system was used to test the hypothesis that an embryonic parental ATZ exposure altered protein expression leading to modifications in morphology and behavior in developing progeny. Zebrafish embryos (F1) were collected from adults (F0) exposed to 0, 0.3, 3, or 30 ppb ATZ during embryogenesis. Proteomic, morphology, and behavior assays were completed with offspring aged 120 hours with no additional chemical treatment. Proteomic analysis identified differential expression associated with neurological development and disease; and organ and organismal morphology, development, and injury, specifically the skeletomuscular system. Head length and the ratio of head length to total length was significantly increased in the F1 of 0.3 and 30 ppb ATZ treated groups ($p<0.05$). Based on molecular pathway alterations, further craniofacial morphology was completed with decreased distance for cartilaginous structures, decreased surface area and distance between saccular otoliths, and a more posteriorly positioned notochord observed ($p<0.05$), indicating

delayed ossification and skeletal growth. The visual motor response assay showed hyperactivity in the F1 of the 30 ppb treatment group for total distance and time spent moving in the F1 of the 0.3 and 30 ppb treatment groups for all phases ($p<0.05$). These findings suggest that a single embryonic parental exposure alters proteomic pathways that lead to perturbations in craniofacial development and locomotor behavior.

3.2 Introduction

Atrazine (ATZ) is a triazine herbicide that is commonly applied to agricultural fields to control broad leafy weeds (LeBaron, McFarland, & Burnside, 2008). Although ATZ is the second most common agricultural herbicide in the US, the European Union banned its use in 2003 due to water contamination concerns (Bethsass and Colangelo 2006; European Commission 2003). Due to heavy application and low binding efficiency to soils, ATZ contaminates runoff and potable water sources (Guzzella et al. 2006; Meffe and de Bustamante 2014). The US Environmental Protection Agency (EPA) has set the maximum contaminant level of ATZ in potable water to 3 parts per billion (ppb; µg/L), but concentrations can exceed this limit (Blanchard and Lerch, 2000; WHO 2011).

In the general US population the most common ATZ exposure route is through drinking contaminated water and is associated with several health risks, which have been documented in epidemiological studies as well as various animal studies. Epidemiological studies suggest exposure during gestation increases the likelihood of birth defects and premature births defects (Agopian et al. 2013; Migeot et al. 2013; Stayner et al. 2017; Waller et al. 2010). For example, women with medium levels of residential ATZ exposure were found to have increased risk of offspring with male genital malformations in Texas, USA (Agopian et al. 2013). In addition, a study in France with women exposed in their second trimester with mixtures of ATZ metabolites and nitrates in their drinking water found that their offspring were more likely to be born small for gestational age (Migeot et al. 2013). An assessment of births in counties across the Midwestern US for water systems included in the US EPA's atrazine monitoring program found that there was increased rate of preterm birth (Stayner et al. 2017), while a study in Washington, USA found women who resided less than 25 km from a site with high ATZ concentrations had offspring with increased frequency of gastroschisis (Waller et al. 2010). Furthermore, studies in various model organisms support ATZ as an neuroendocrine disrupting compound, altering reproductive cycling

and development (Cooper 2000; Russart and Rhen 2016), stress response (Foradori et al. 2018; Fraites et al. 2009), and the dopaminergic system (Bardullas et al. 2011; Fraites et al. 2009; Li et al. 2019a; Ma et al. 2015).

Adverse health outcomes with direct ATZ exposure are well documented, but multigenerational and transgenerational studies are important to determine if an exposure affects subsequent generations, and whether the adverse health outcomes are comparable. Moreover, some studies have begun to assess ATZ toxicity in the context of the Developmental Origins of Health and Disease (DOHaD) paradigm, which states that exposures to environmental factors or chemical exposures during developmental periods can have long lasting impacts, into adulthood and even subsequent generations (Heindel and Vandenberg 2015). Transgenerational studies in Sprague Dawley rats at high concentrations support generational effects of ATZ exposure reporting reductions in body weight, increased testis disease, and early onset puberty in males and in their offspring, as well as hyperactivity and altered methylation patterns in sperm (DeSesso et al. 2014; McBirney et al. 2017). In mice, decreased sperm quality in the generation exposed to ATZ during development and in the following generation due to defects of proteins in meiosis and altered gene expression in several organs was observed (Hao et al. 2016). ATZ exposure during the first 12 days of life in medaka documented decreased sperm count and motility in offspring (Cleary et al. 2019). Previous studies in zebrafish with an embryonic ATZ exposure report alterations in proteins related to developmental and neurological disorders and cancer, as well as changes in morphology and genes associated with head development, neurogenesis, and behavior (Horzmann et al. 2018; Wirbisky et al. 2015b). Building upon these findings, we expect that an embryonic ATZ exposure in zebrafish will have generational consequences. Specifically, it was hypothesized that offspring of zebrafish exposed to ATZ only during embryogenesis will exhibit similar changes in protein expression, morphology, and behavior in comparison to the parental generation. Zebrafish are an established model in toxicology and environmental health research with several strengths for generational ATZ studies including high similarity of physiological structures and molecular pathways related to development and disease, ex vivo embryonic development, shorter developmental and generation times, and established behavioral assays (Bailey et al. 2013; Garcia et al. 2016; Horzmann and Freeman 2018). In addition, it was recently confirmed that zebrafish metabolize ATZ similar to mammals producing the same major metabolites (Ahkin Chin Tai et al. 2021).

3.3 Materials and methods

3.3.1 Zebrafish husbandry and ATZ exposure parameters

Adult zebrafish (Wild-type AB strain *Danio rerio*) were housed in standalone systems (Aquatic Habitats, Apopka, FL) on a 14:10 light-dark cycle and monitored twice daily for water quality. System water was maintained at 28°C with a pH range of 7.1-7.3 pH and conductivity of 470-550 µS. Adult fish were fed twice daily a combination of brine shrimp, Golden Pearls (500-800 µm) (Artemia international LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, PA). Zebrafish were bred to generate embryos for experiments in breeding tanks according to established protocols (Peterson et al. 2011). Embryos were collected at the 4-8 cell stage of development (1 hour post fertilization: hpf), rinsed, and randomly assigned to treatment groups of 0 (aquaria water), 0.3, 3, or 30 ppb (µg/L) ATZ. ATZ stock solutions were prepared from technical grade ATZ (98.1% purity) (CAS 1912-24-9; Chem Service, West Chester, PA) as previously described (Weber et al. 2013; Wirbisky et al. 2015b) and diluted to the respective ATZ treatment concentrations. Concentrations of stock solutions and treatment water were confirmed by an US EPA approved immunoassay kit (Abraxis Atrazine ELISA Kit, Warminster, PA) as previously described (Freeman et al. 2005; Wirbisky et al. 2016b) and were found to all be within expected concentration ranges ($\pm$0.1 ppb; data not shown).

Embryos were exposed via immersion in groups of 50 in petri dishes and were housed at 28.5°C through the end of embryogenesis (72 hpf). At 72 hpf, all eleutheroembryos were rinsed to terminate ATZ treatment and were raised to adulthood under control conditions. The exposure procedure was replicated several times to attain multiple adult groups. The adult zebrafish exposed to ATZ only during embryogenesis (ATZ F0) were then bred within treatment group to generate embryos. These progeny received no additional ATZ exposure and were maintained at 28.5°C in aquaria water in groups of 50 in a petri dish until 120 hpf for experimental procedures. All larvae within a single petri dish were considered as a subsample. For all experimental procedures larvae were collected from multiple petri dishes (biological replicates) from multiple breeding groups. Larvae with parental embryonic ATZ exposure are referred to as ATZ F1. The experimental design with chemical dosing times and analysis time points is summarized in Figure 3-1. All protocols were approved by the Purdue University Animal Care and Use Committee and all fish treated humanely with regards to prevention and alleviation of suffering.

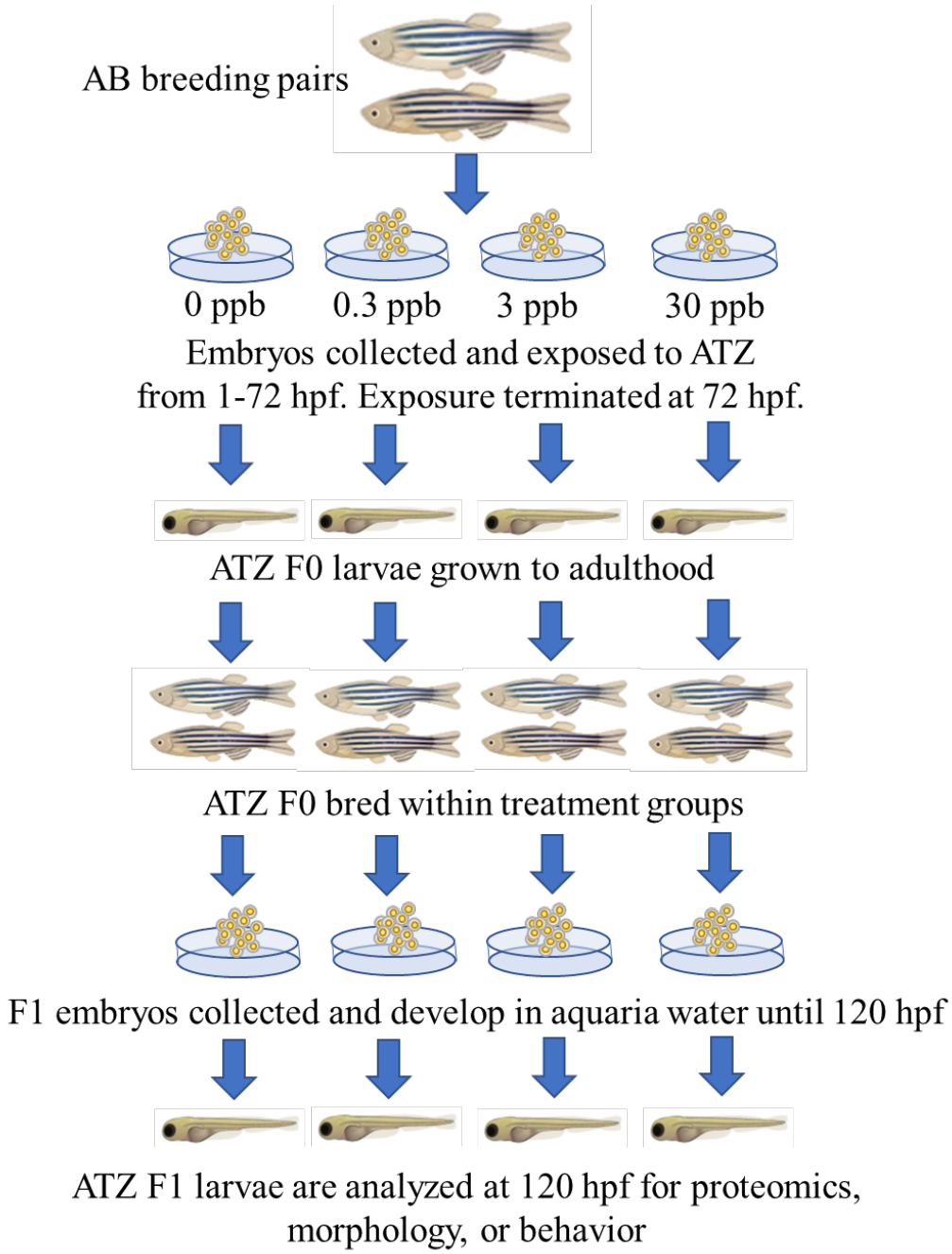


Figure 3-1. Parental ATZ exposure. AB adult zebrafish were bred and embryos were collected and exposed to ATZ at environmentally relevant concentrations for 1--72 hours post fertilization (hpf). ATZ chemical exposure was terminated at 72 hpf. Afterwards, these developmentally exposed larvae were grown to adult hood and then bred within treatment groups to get F1 embryos. These embryos were collected 1 hpf and placed in aquaria water only (no additional ATZ exposure) until 120 hpf when used in experiments.

3.3.2 Proteomics

Proteomic analysis was performed on larval F1 zebrafish at 120 hpf to determine whether parental exposure resulted in protein expression alterations using the same experimental parameters as in our previous study (Horzmann et al. 2018). For each biological replicate, 30 larvae per treatment group (parental exposure of 0, 0.3, 3, or 30 ppb ATZ) were pooled and euthanized via hypothermic shock. Six biological replicates were collected for each treatment group. Larval zebrafish were homogenized using a Preceyllys 24 homogenizer (bertin Instruments, Montignyle-Bretonneux, France) and a Bicinchoninic (BCA) assay used to determine protein concentration. Sample preparation and analysis was completed as described in Horzmann et al. (2018). Briefly, digestions were performed on a Barocycler NEP2320 at 50°C under 20,000 psi for 1 hour and then samples cleaned over C18 spin columns (Nest Group, Southborough, MA) and dried in a vacuum centrifuge. Pellets were solubilized in 97% purified water, 3% acetonitrile (ACN), 0.1% formic acid (FA). Samples were then analyzed through liquid chromatography/mass spectrometry (LC/MS) with 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. Samples were injected into the QE HF through the Nanospray Flex™ Ion Source fitted with an emission tip (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.

Data were processed using the MaxQuant computational proteomics platform (Max-Planck-Gesellschaft, München, Germany). The peak list was searched against *Danio rerio* sequences from UNIPROT and a common contaminants database (MaxQuant, Max-Planck-

Gesellschaft, München, Germany) with MaxQuant default Orbitrap parameters and minimum peptide length of seven amino acids. Data were analyzed with label-free quantification (LFQ) and the 'match between runs' interval set to 1 minute. Other analysis settings included protein FDR at 1%, enzyme trypsin and LysC allowing for two missed cleavages and three modifications per peptide. Fixed modifications were iodoethanol and variable modifications were set to acetyl (protein N-term) and oxidation. An in-house script was used within the MaxQuant results to remove all common contaminant proteins, to log transform [log2(x)] the LFQ intensity values, to input missing values using the average values of the other samples when one sample was missing, and to use half of the lowest intensity when all samples were missing in one group and present in all other samples in the other group. In total 1277 unique proteins were identified.

3.3.3 Morphology

F1 ATZ larvae from each parental treatment group were assessed to determine if morphology was altered at 120 hpf. Larvae were euthanized via anesthetic overdose with 0.4 mg/ml buffered tricaine-S (Western Chemical Inc.). For each F1 treatment group, 10-13 larvae were imaged as subsamples per biological replicate. A total of 8 biological replicates were assessed to total 80-100 larvae per treatment group. Measurements assessed were total body length, head length, head width, and brain length similar to our previous study (Horzmann et al. 2018). Total body length was measured dorsally from the top of the head to the caudal fin. Head width was measured dorsally as the distance between the widest part of the eyes. Head length was measured dorsally from the top of the head to the beginning of the yolk sac protrusion. Brain length was measured as the distance rostrally to the brainstem spinal cord junction (Peterson et al. 2013). Additionally, ratios of head length to body length, head width to body length, and brain length to body length were also assessed for relative comparison. Treatment groups were randomized and blinded during measurement. Images were collected 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).

3.3.4 Craniofacial morphology staining and measurements

To determine if craniofacial skeletal or cartilage morphology was altered, alcian blue and alizarin red costaining was completed. At 120 hpf, larvae were euthanized via anesthetic overdose as described above, and then stained simultaneously for alcian blue and alizarin red based on a previously established protocol (Walker and Kimmel 2007). Briefly, samples were fixed in paraformaldehyde, washed to cease fixation in a series of washes, stained with alcian blue overnight, bleached, and stained for alizarin red. After staining, fish were imaged on an Olympus SZX16 dissecting microscope. Images were taken using Cellsentry (Waltham, MA) and structures measured using ImageJ. Cartilaginous structures were assessed based on previously described protocols (Cohen et al. 2014; Staal et al. 2018; Walker et al. 2018) and included palatoquadrate cartilage (PQ) length, Meckel's length, ceratohyal cartilage (CH) angle, Meckel's angle, the distance between the CH and Meckel's structure, the angle between PQ and CH, the angle between PQ and Meckel's angle, and jaw distance (Figure 3-2). For skeletal structures, surface area of the utricular and saccular otoliths, length between the utricular otoliths and between the saccular otoliths, parasphenoid, notochord, and notochord to jaw length were measured as in previous studies (Aceto et al. 2015; Luo et al. 2016) (Figure 3-3). For each F1 treatment group 11-20 larvae were imaged as subsamples per biological replicate. A total of 5 biological replicates were assessed to achieve 55-75 larvae per treatment group.

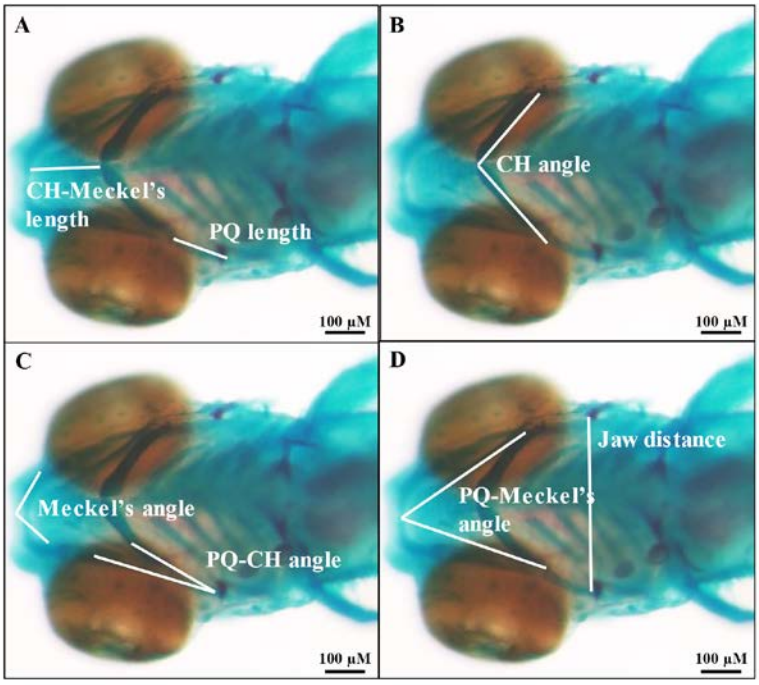


Figure 3-2. Morphology measurements of cartilaginous structures of ATZ F1 at 120 hpf.. At 120 hpf alcian blue staining was used to measure (A) CH-Meckel's length and PQ length. For angles, (B) CH angle, (C) Meckel's angle, (C) PQ-CH angle, and (D) PQ-Meckel's angle were measured. The length of the (D) jaw distance was also determined. All measurements were taken at 120 hpf and are indicated as white lines or angles. Scale bars represent 100 µM.

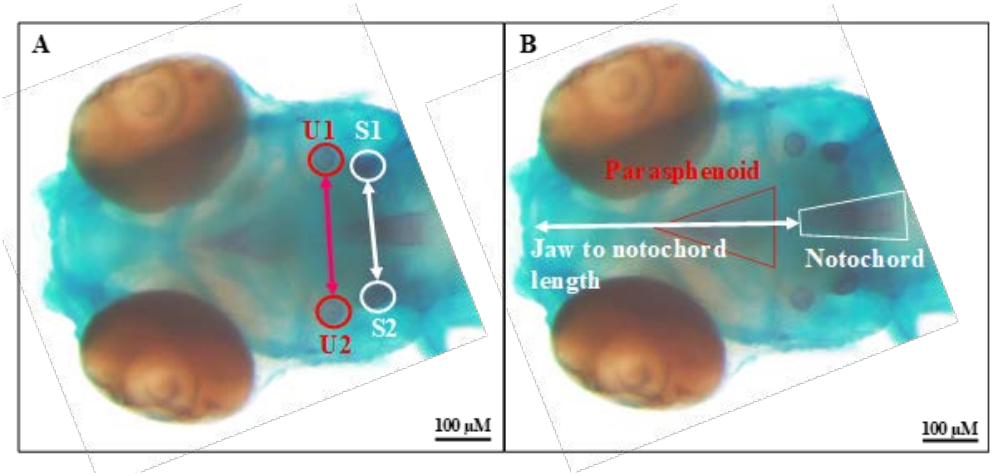


Figure 3-3. Morphology measurements of skeletal structures of ATZ F1 at 120 hpf. (A) Surface area of each otolith (U1 and U2: utriclar otoliths, red circles; S1 and S2 saccular otoliths, white circles. The length between the utricular otoliths (red line) and saccular otoliths (white line) was determined. (B) The surface area for the parasphenoid (red triangle) and notochord were measured (white quadrangle). In addition, the length from jaw to notochord was assessed (white line). Scale bar represents 100 µM.

3.3.5 Larval visual motor response assay

A larval visual motor response test was performed at 120 hpf to determine if F1 larvae exhibited altered behavior from parental ATZ exposure following an established protocol (Horzmann et al. 2018, Ahkin Chin Tai et al. 2021). Each larvae was individually placed into a 96 square well plate in 0.5 mL of aquaria water. Larvae were acclimated for 10 minutes at 28°C in the Noldus Daniovision Observation Chamber (Noldus Information Technology, Wagenningen, Netherlands). The Noldus white light routine consisting of alternating 10 minutes of dark and white light to test the visual motor response was performed over 50 minutes (3 dark phases and two light phases). Behavioral experiments were performed between 11 am to 1 pm to minimize circadian variability in movement. Infrared movement was recorded during behavioral experiments at a rate of 25 frames per second with a Basler GenICam acA 1300-60g camera and analyzed with Noldus EthoVision 12.0 software. Tracks were smoothed for total distance moved, velocity, and time spent moving. Each treatment group (0, 0.3, 3, or 30 ppb ATZ) had 8 biological replicates containing 24 subsample fish per biological replicate for a total of 192 fish per treatment group.

3.4 Statistics

For each experiment, one spawning event represents one biological replicate with fish within a petri dish considered as subsamples. Multiple spawning events from multiple groups of ATZ exposed adults were included among the biological replicates for all experimental procedures. For proteomics, statistical analysis was performed in R. An one-way analysis of variance (ANOVA) was performed on the LFQ intensities and proteins with a p-value <0.05 were analyzed with a Tukey's post hoc test to identify differences between treatment groups. The list of significantly altered 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.

For morphology and behavior, statistics were performed in SAS Statistical Software (SAS Institute Inc. Cary, NC). A Grubb's outlier test was used to detect outliers within treatment groups for morphology and behavior. All data was assessed and confirmed for normality before further statistical analysis. An ANOVA was used to analyze differences in morphological measurements among treatment groups and a Fisher's least significant difference (LSD) test at $\alpha = 0.05$ was

incorporated when a significant ANOVA was observed. For phasic behavioral analysis of the visual motor and acoustic assays, a repeated measures ANOVA was used to assess phase, treatment, and the interaction of phase*treatment. All behavioral data is represented in bar graphs (Bridi et al. 2017; Horzmann et al. 2018). All results are presented as mean ± standard deviation of the mean.

3.5 Results

3.5.1 Proteomic alterations in progeny

A total of 1,277 unique proteins were identified. From this list, 62 proteins had significant LFQ intensity values ($p<0.05$) in larvae of the 0.3 ppb treatment group, while 32 and 24 proteins were significantly altered in larvae of the 3 and 30 ppb treatment groups, respectively. In IPA, 54 of the 62 proteins in the 0.3 ppb treatment group, 26 of the 32 proteins in the 3 ppb treatment group, and 20 of the 24 proteins in the 30 ppb treatment group were recognized for mapping to their human ortholog (Figure 3-4). These 100 significant calls consisted of 70 unique mapped proteins including several ATP synthase proteins (ATP5F1A, ATP5F1C, ATP5MD, ATP5ME, ATP5MG, and ATP5PB), collagen proteins (COL10A1, COL18A1, and COL1A1), heterogeneous nuclear ribonucleoproteins (HNRNPM, HNRNPR, and HNRNPU), and ribosomal proteins (RPL10A, RPL11, RPL17-C18orf32, RPL3, RPL4, RPS11, RPS16, and Rps3a1) (Table 3.1). 24 proteins were altered in two treatment groups (23 proteins in the 0.3 and 3 ppb and 1 protein in the 0.3 and 30 ppb treatment groups) and 3 proteins were altered in all three treatment groups (HBE1, MATN3, and SF3B3) (Figure 3-4).

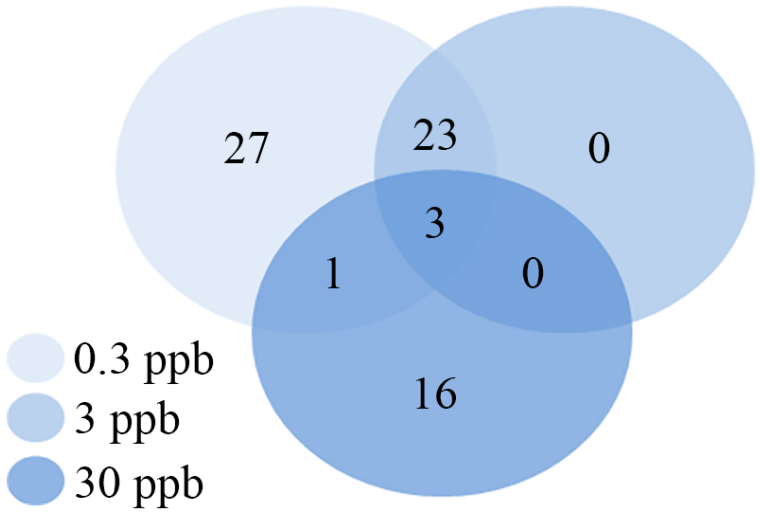


Figure 3-4. A venn diagram of proteins altered within treatment groups. ATZ F1 larvae in the 0.3 ppb treatment group had 54 proteins altered, in the 3 ppb treatment group had 26 proteins altered, and in the 30 ppb treatment group had 20 proteins altered. 24 proteins were altered in two treatment groups (23 proteins in the 0.3 and 3 ppb and 1 protein in the 0.3 and 30 ppb treatment groups) and 3 proteins were altered in all three treatment groups (HBE1, MATN3, and SF3B3).

Table 3-1. A list of the 70 uniquely mapped proteins found to be altered with parental ATZ exposure.

Symbol	Entrez Gene Name	Ensembl/GenPept/UniProt/ Swiss-Prot Accession	0.3 ppb/ 0 ppb Fold Change	3 ppb/ 0 ppb Fold Change	30 ppb/ 0 ppb Fold Change
2310057J18Rik	RIKEN cDNA 2310057J18 gene	A5PF61			0.577
Actn3	actinin alpha 3	Q6P0J5			0.793
ACTR2	actin related protein 2	F1QF15	0.344		
ADD3	adducin 3	F1R6L1	0.411		
ADSS1	adenylosuccinate synthase 1	ENSG00000185100			0.462
AGRN	agrin	F1R074	-0.837	-1.244	
ANXA11	annexin A11	Q804G3	0.366		
ATP1B2	ATPase Na+/K+ transporting subunit beta 2	Q9DGL2	-0.26	-0.474	
ATP5F1A	ATP synthase F1 subunit alpha	Q58P23	0.981		
ATP5F1C	ATP synthase F1 subunit gamma	Q6P959	1.192	1.34	
ATP5MD	ATP synthase membrane subunit DAPIT	ENSG00000173915	-1.109		
ATP5ME	ATP synthase membrane subunit e	ENSG00000169020	-7.41	-7.41	

Table 3-1 continued

ATP5MG	ATP synthase membrane subunit g	Q6P6E0	0.465		
ATP5PB	ATP synthase peripheral stalk-membrane subunit b	Q5XJJ3	0.563	0.682	
ATP6V1E1	ATPase H+ transporting V1 subunit E1	Q6NWK4	0.706	1.081	
CAPNS1	calpain small subunit 1	X1WFZ2			0.883
CES1	carboxylesterase 1	Q1LYL6			0.614
COL10A1	collagen type X alpha 1 chain	F1QXD5	-6.009	-0.658	
COL18A1	collagen type XVIII alpha 1 chain	ENSG00000182871	-0.448	-0.531	
COL1A1	collagen type I alpha 1 chain	ENSG00000108821			-0.852
CRYBA2	crystallin beta A2	Q6IQU2	-0.52	-0.541	
CRYBB1	crystallin beta B1	Q90WT1	-0.721		
EPRS1	glutamyl-prolyl-tRNA synthetase 1	A8WG07	0.418	0.511	
FAU	FAU ubiquitin like and ribosomal protein S30 fusion	Q6PC01	0.483		
GAPDHS	glyceraldehyde-3-phosphate dehydrogenase, spermatogenic	F1R3D3	0.62	0.515	
GATM	glycine amidinotransferase	ENSG00000171766	0.498		
GNB1	G protein subunit beta 1	Q803H5	0.312		
HBE1	hemoglobin subunit epsilon 1	Q5BLF6	0.485	0.573	0.344
HMGB1	high mobility group box 1	ENSG00000189403	-0.436		
HNRNPM	heterogeneous nuclear ribonucleoprotein M	ENSG00000099783			5.332
HNRNPR	heterogeneous nuclear ribonucleoprotein R	ENSG00000125944			0.339
HNRNPU	heterogeneous nuclear ribonucleoprotein U	Q6PYX3	-6.771		
HSPD1	heat shock protein family D (Hsp60) member 1	Q803B0	1.28	0.946	
IGFN1	immunoglobulin like and fibronectin type III domain containing 1	ENSG00000163395			0.282
ILF2	interleukin enhancer binding factor 2	Q6NZ06			0.956
KRT17	keratin 17	Q6DHU3	-0.387		
LUM	lumican	Q6IQQ7			-0.298
MARCKSL1	MARCKS like 1	ENSG00000175130	-1.522	-1.575	
MATN3	matrilin 3	ENSG00000132031	0.569	0.985	0.706
MDH1	malate dehydrogenase 1	Q7ZSY2	-0.725	-1.316	
MYL12A	myosin light chain 12A	Q6PHJ8	-7.32	-0.693	
NID1	nidogen 1	F1RAG3			-0.585
NOMO1 (includes others)	NODAL modulator 1	ENSG00000103512			-0.252

Table 3-1 continued

OPN1LW	opsin 1, long wave sensitive	A0A0N9P0E6	-1.298		
OPN1SW	opsin 1, short wave sensitive	Q6P981	-0.732		
PC	pyruvate carboxylase	Q7ZZ22			0.8
PFN1	profilin 1	ENSG00000108518	-0.409		
PHB2	prohibitin 2	Q6PC13	0.504		
PRPF8	pre-mRNA processing factor 8	A0A0R4J8E6	0.329	0.387	
PRSS2	serine protease 2	Q7SX90	0.767		
RAB10	RAB10, member RAS oncogene family	Q6DGV5	0.329		0.304
REELD1	reeler domain containing 1	ENSG00000250673	-0.907	-1.133	
RHO	rhodopsin	ENSG00000163914	-1.09		
RPL10A	ribosomal protein L10a	Q6PC69			-0.585
RPL11	ribosomal protein L11	Q6IQI6	0.483		
RPL17-C18orf32	RPL17-C18orf32	Q7T1K0	0.559	0.478	
RPL3	ribosomal protein L3	Q5BJJ2	1.001		
RPL4	ribosomal protein L4	Q1LUY6	0.721		
RPS11	ribosomal protein S11	Q7ZV05	0.543		
RPS16	ribosomal protein S16	Q1LWH1	1.337	1.195	
Rps3a1	ribosomal protein S3A1	Q6PBY1	0.883	0.773	
S100A10	S100 calcium binding protein A10	Q7ZVA4	-0.617		
S100A4	S100 calcium binding protein A4	Q6XG62	1.83	2.669	
SF3B3	splicing factor 3b subunit 3	Q1LVE8	-0.498	-0.475	-0.439
SLC25A12	solute carrier family 25 member 12	A0A0R4II73	0.421	0.412	
SNRPG	small nuclear ribonucleoprotein polypeptide G	Q66I63	0.628		
SSR3	signal sequence receptor subunit 3	B8JL82	0.263		
TMEM38A	transmembrane protein 38A	Q6P2T0	-0.635	-0.785	
TNNI2	troponin I2, fast skeletal type	Q71N42	-1.227		
VSNL1	visinin like 1	E7FCW3			0.498

Pathway analysis was completed on the mapped proteins within each treatment group to determine molecular pathways altered in the progeny. As expected with a high degree of similarity in proteins altered in the progeny of the 0.3 and 3 ppb treatment groups, similar pathways were altered among these two groups. The top physiological system development and function pathways in the 0.3 and 3 ppb treatment group offspring included embryonic and organismal development, organ development and morphology, and nervous system development and function (Table 3.2).

There were more differences in the top disease and disorder pathways that were altered in the 0.3 and 3 ppb treatment groups with neurological disease and organismal injury and abnormalities being similar (Table 3.3). In addition, psychological disorders, skeletal and muscular disorders, ophthalmic disease, and cancer were also top pathways. Progeny of the 30 ppb treatment group had some similarities with the other two treatment groups (e.g., organismal injury and abnormalities and cancer), but also hit on reproductive system development and disease (Tables 3.2 and 3.3).

Upstream regulator and causal pathway analysis identified similarities in the 0.3 and 3 ppb treatment group offspring in DDX5 (DEAD-box helicase 5; 0.3 ppb: p-value: 7.92E-06; 3 ppb: p-value: 8.25E-09) and MYCN (MYCN proto-oncogene; 0.3 ppb: p-value: 2.39E-05; 3 ppb: p-value: 2.70E-04). Top canonical pathways in the 0.3 and 3 ppb treatment group progeny included estrogen receptor signaling, oxidative phosphorylation, and mitochondrial dysfunction.

Table 3-2. Top physiological systems development and function pathways altered in F1 progeny.

Physiological System Development and Function Pathway	p-value[a]	Number of molecules[b]
Progeny of 0.3 ppb treatment group		
Embryonic development	4.73E-02 - 4.44E-04	7
Nervous system development and function	4.73E-02 - 4.44E-04	6
Organ development	4.96E-02 - 4.44E-04	7
Organ morphology	4.96E-02 - 4.44E-04	6
Organismal development	4.73E-02 - 4.44E-04	7
Progeny of 3 ppb treatment group		
Embryonic development	4.64E-02 - 1.53E-04	7
Nervous system development and function	4.95E-02 - 1.53E-04	8
Organ development	4.95E-02 - 1.53E-04	7
Organ morphology	4.95E-02 - 1.53E-04	9
Organismal development	4.95E-02 - 1.53E-04	8
Progeny of 30 ppb treatment group		
Reproductive system development and disease	3.19E-03 – 3.19E-03	1

[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; a protein may be present in more than one category.

Table 3-3. Top diseases and disorders pathway altered in F1 progeny at 120 hpf

Diseases and Disorders Pathway	p-value[a]	Number of molecules[b]
Progeny of 0.3 ppb treatment group		
Neurological disease	3.83E-02 - 1.98E-06	11
Organismal injury and abnormalities	4.90E-02 - 1.98E-06	14
Psychological disorders	3.74E-02 - 1.98E-06	9
Hereditary disorder	4.90E-02 - 3.80E-06	9
Skeletal and muscular disorders	4.67E-02 - 3.80E-06	12
Progeny of 3 ppb treatment group		
Neurological disease	5.00E-02 - 3.57E-06	18
Organismal injury and abnormalities	5.00E-02 - 3.57E-06	25
Cancer	4.55E-02 - 1.31E-05	24
Tumor morphology	2.16E-03 - 1.31E-05	5
Ophthalmic disease	4.95E-02 - 1.53E-04	7
Progeny of 30 ppb treatment group		
Cancer	1.45E-02 - 8.63E-05	1
Organismal injury and abnormalities	1.45E-02 - 8.63E-05	1
Reproductive system disease	8.63E-05 - 8.63E-05	1
Infectious disease	5.83E-03 - 2.59E-04	1
Hematological disease	1.45E-02 - 1.45E-02	1

[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; a protein may be present in more than one category.

3.5.2 Morphological changes in progeny

Length measurements were assessed at 120 hpf (Table 3.4). A significant increase in mean head length was seen in offspring of the 0.3 and 30 ppb treatment groups (p=0.0048). No other changes were seen in total length (p=0.5196), mean brain length (p=0.7411), or mean head width (p=0.1932) in the larvae. For morphological ratios to assess relative size compared to total body length, a significant increase was observed in head length to total body length ratio in the offspring of the 0.3 ppb and 30 ppb treatment groups (p=0.0082), indicating head length was greater than would be expected based on body length. No significant differences in brain length to total body length (p=0.4433) or head width to total body length (p=0.0690) was observed.

Table 3-4 Morphology measurements in larval offspring.

Parental ATZ treatment group	Body length (µm) ± SD[a]	Head length (µm) ± SD	Head width (µm) ± SD	Brain length (µm) ± SD	Head length/body length ratio ± SD	Head width/ body length ratio ± SD	Brain length/ total length ratio ± SD
0 ppb	4270 ± 218[a]	767 ± 61	692 ± 59	947 ± 59	0.181 ±0.013	0.163 ± 0.016	0.222 ± 0.013
0.3 ppb	4311 ± 170	792 ± 45*	683 ± 34	949 ± 52	0.184 ± 0.009*	0.159 ± 0.008	0.221 ± 0.015
3 ppb	4266 ± 208	780 ± 65	692 ± 51	954 ± 52	0.183 ± 0.013	0.162 ± 0.012	0.224 ± 0.015
30 ppb	4274 ± 185	795 ± 50*	680 ± 47	946 ± 46	0.186 ± 0.008*	0.159 ± 0.011	0.222 ± 0.012

*p<0.05 compared to progeny of the 0 ppb treatment group (no parental ATZ exposure)

[a]Standard deviation

3.5.3 Cartilage and skeletal modifications in progeny

Cartilaginous and skeletal structures were measured at 120 hpf. For cartilaginous structures, PQ length was decreased in all parentally exposed groups (p<0.0001, Figure 3-5A). In addition, the CH-meckel's length (p=0.003; Figure 3-5B) and jaw distance was decreased in the progeny of the 30 ppb treatment group (p=0.0036; Figure 3-5C) (p<0.05). No differences were detected in the Meckel's angle (p=0.065), PQ/Meckel's angle (p=0.5792), CH angle (p=0.9822), PQ/CH angle (p=0.0970), or Meckel's length (p=0.9822) (Figure 3-6A-E). For skeletal structures, decreased

surface area of the saccular otoliths (Figure 3-7A) was seen in the progeny of the 30 ppb treatment group ($p=0.0405$; Figure 3-7B and $p=0.0030$; Figure 3-7C), but no differences observed in utricular otolith areas in the progeny ($p=0.1711$; Figure 3-7D and $p=0.3443$; Figure 3-7E). Distance between the utricular and saccular otoliths was also measured, respectively (Figure 3-7A). The F1 larvae of the 30 ppb treatment group had decreased distance between the two saccular otoliths ($p<0.0001$; Figure 3-7F), but no changes in distance for utricular otoliths ($p=0.1393$; Figure 3-7G) was observed. In addition, larvae of the 30 ppb treatment group had a significant posteriorly positioned notochord as measured from the jaw (i.e., notochord to jaw length) ($p=0.0149$; Figure 3-7H), but no difference in the surface area of the notochord ($p=0.9773$) or parasphenoid ($p=0.0739$) was observed (Figure 3.7F-G).

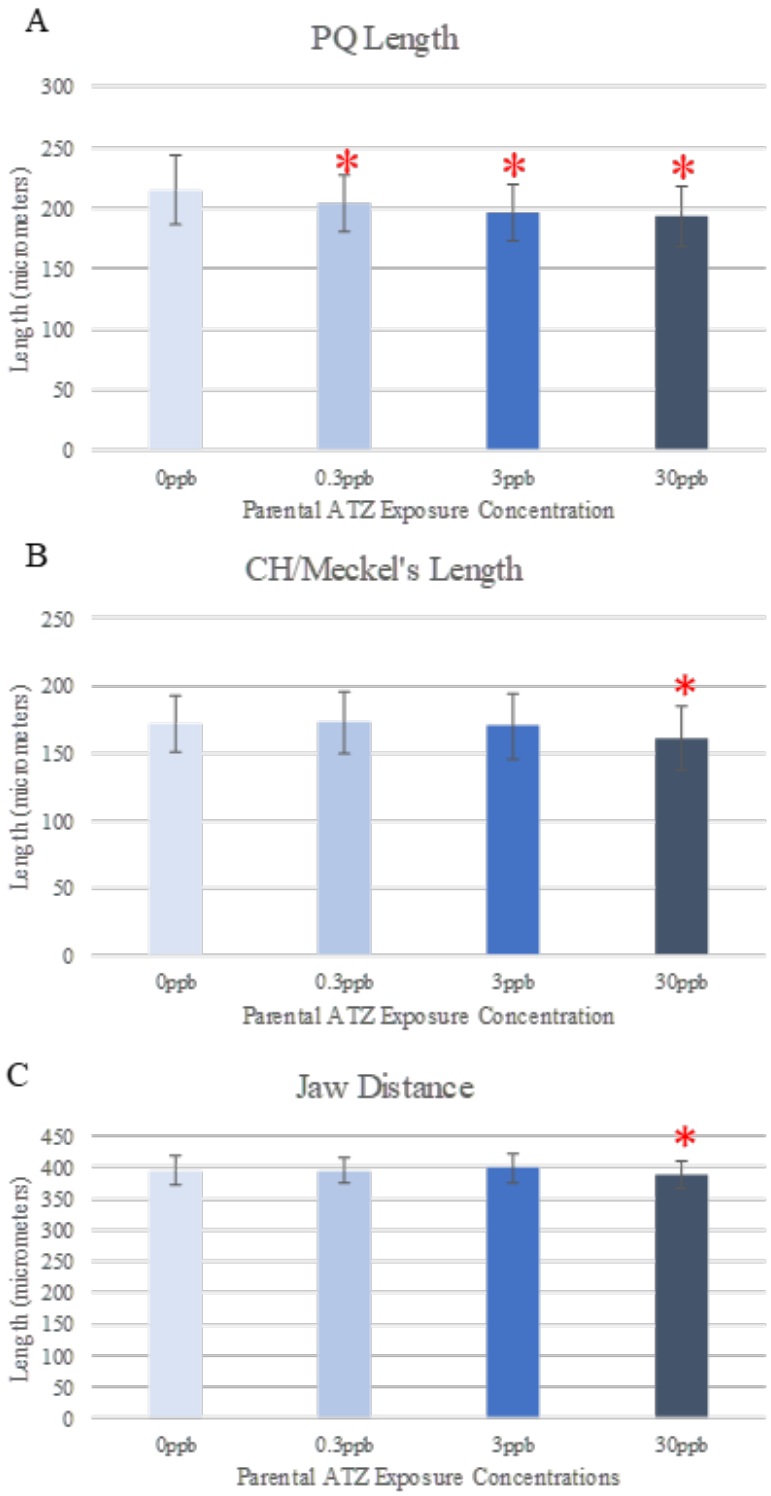


Figure 3-5. Significant cartilaginous structures. PQ length was found to be significantly smaller in all parentally exposed groups in comparison to controls (A). CH/Meckel's length was found to be significantly shorter in the F1 30 ppb group (B). Jaw distance was found to be significantly smaller in the F1 30 ppb group. *$p<0.05$. Error bars represent standard deviation (C).

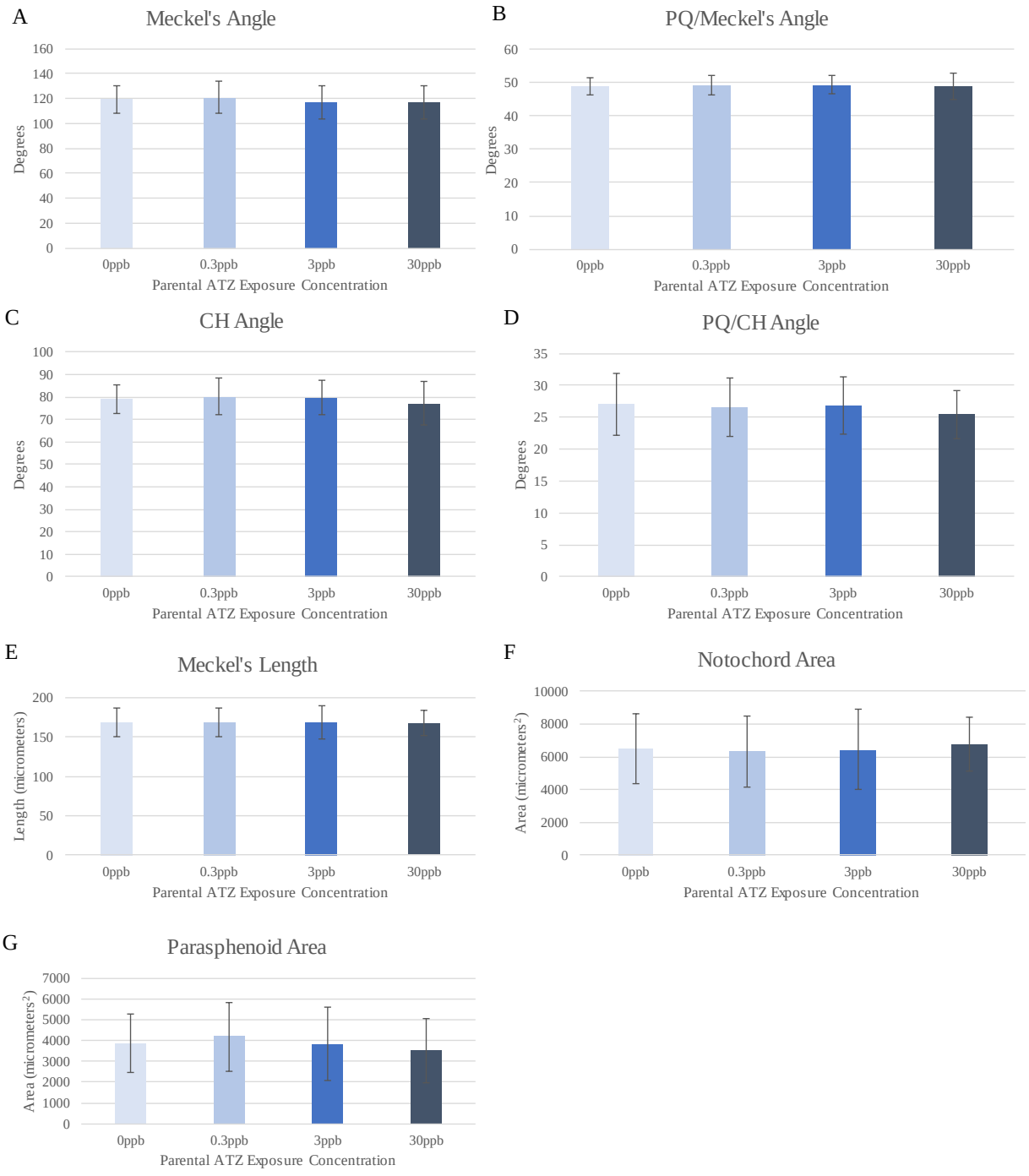


Figure 3-6. Cartilagenous and skeletal structure assessments. No significant difference was found between control animals and parentally exposed F1 larvae in Meckel's Angle (A), PQ/Meckel's Angle (B), CH Angle (C), PQ/CH Angle (D), Meckel's Length (E), Notochord Area (F), or Parasphenoid Area (G). $p>0.05$. Error bars represent standard deviation.

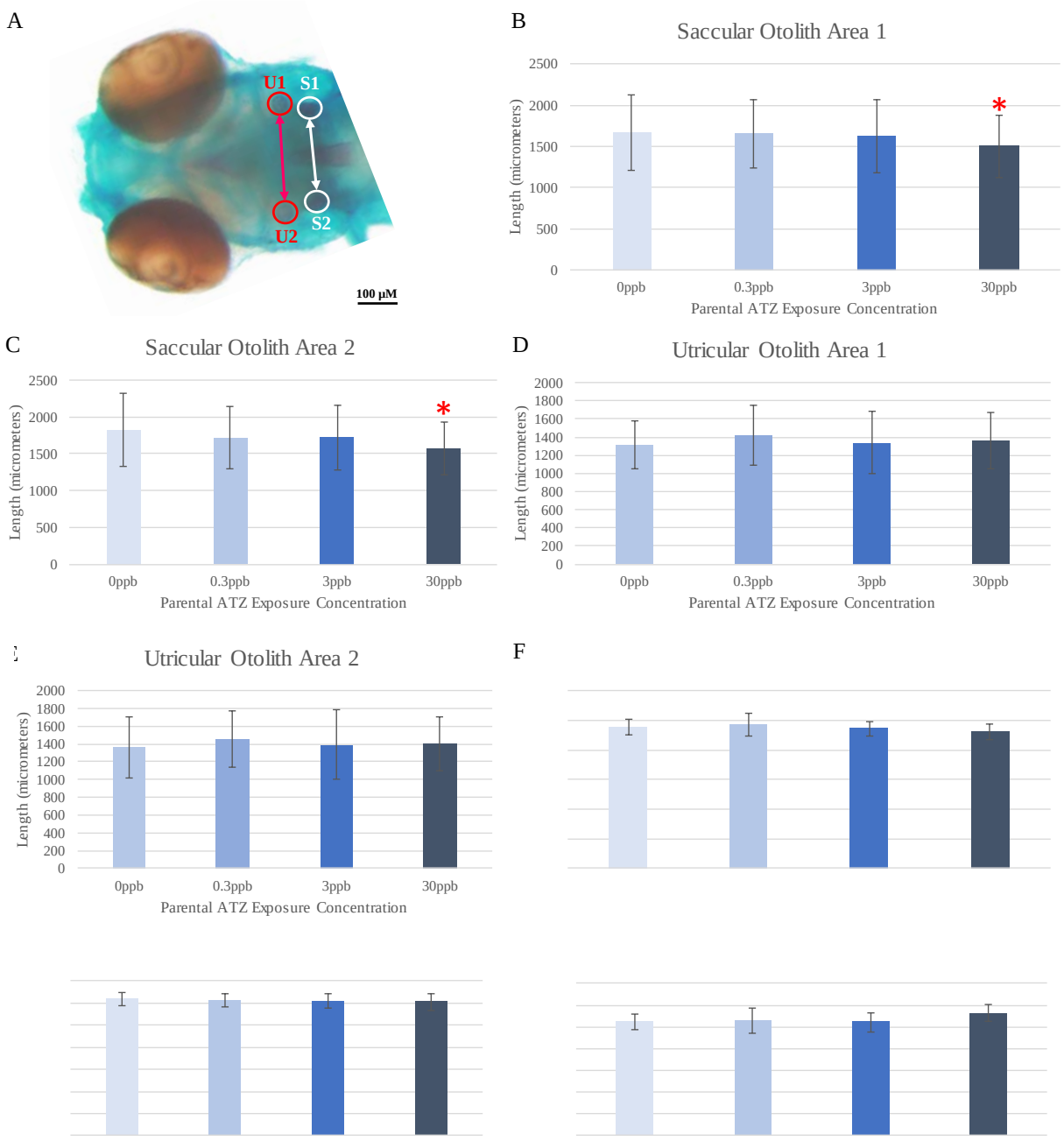


Figure 3-7. Otolith positioning and surface area. Utricular and saccular otoliths are labeled (A) for analysis. Saccular otolith Area 1 was significantly smaller in F1 30 ppb progeny (B), as well as for saccular otolith area 2 (C). No change in surface area was found for utricular otolith area 1 (D) or utricular otolith area 2 (E). The length between saccular otoliths was significantly smaller in F1 30 ppb animals (F), but no difference in length was found between utricular otoliths (G). Notochord to jaw length was found to be significantly increased in F1 30 ppb larvae (H). *$p<0.05$. Error bars represent standard deviation.

3.5.4 Visual motor response in progeny

Phasic behavioral data was assessed for each light and dark period. For phasic total distance, there was significance for phase ($F(5,2450)=1805.42$, $p<0.0001$), treatment ($F(3,490)=7.18$, $p=0.001$), and phase*treatment ($F(15,2450)=2.09$, $p=0.008$; Figure 3-8A). The 30 ppb F1 progeny moved more total distance in all light and dark phases. For time spent moving, phase ($F(5,2450)=2408$, $p<0.0001$), treatment ($F(3,490)=8.64$, $p<0.0001$), and phase*treatment ($F(15,2450)=3.59$, $p<0.0001$) were significant. Both the 0.3 ppb and 30 ppb F1 progeny had more time spent moving in all light and dark phases (Figure 3-8B). No significant differences were observed in velocity for treatment ($F(3, 490)=1.55$, $p=0.200$) or phase*treatment ($F(15, 2450)=0.79$, $p=0.6919$; Figure 3-8C).

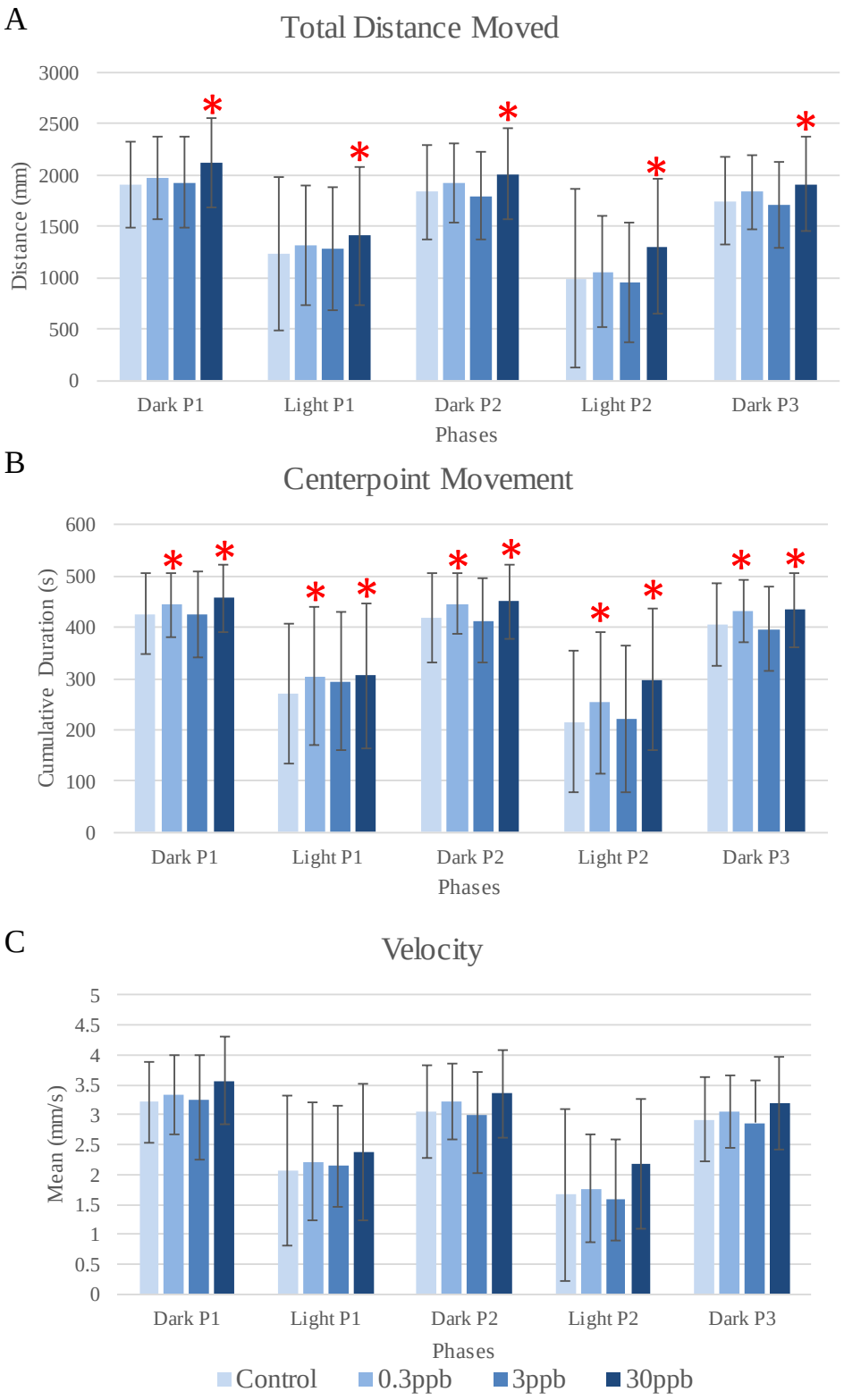


Figure 3-8. Phasic behavioral data. (A) F1 progeny had significance in 30 ppb hyperactivity in all light and dark phases for distance moved. (B) Center point movement had hyperactivity in 0.3 and 30 ppb in all phases. (C) No significance was found in velocity. *p<0.05. Error bars represent standard deviation.

3.6 Discussion

ATZ is an endocrine disrupting chemical that targets the neuroendocrine system resulting in developmental and reproductive consequences as reported in several epidemiological (Agopian et al. 2013; Migeot et al. 2013; Stayner et al. 2017; Waller et al. 2010) and animal model studies (DeSesso et al. 2014; Foradori et al. 2018; Hao et al. 2016; McBirney et al. 2017; Russart and Rhen 2016). Multi- and transgenerational studies have shown changes in sperm quality, hyperactivity, and change in weight in subsequent generations warranting further studies into generational adverse health impacts (Cleary et al. 2019; McBirney et al. 2017). Here, we first used a proteomics approach to identify altered proteins and molecular pathways in the ATZ F1 larvae. A total of 1,277 unique proteins were identified, which is similar to the total number of proteins detected in past studies (Horzmann et al. 2018), but does only represent a percent of the total proteins in the zebrafish. Regardless, imperative details on altered proteins and molecular pathways were ascertained in this study. Overall, more altered proteins were observed in progeny of the lowest ATZ treatment group (0.3 ppb). In addition, all proteins altered in offspring of the 3 ppb treatment group were also observed in the 0.3 ppb treatment group. Three proteins were common among all treatment groups: HBE1, MATN3, and SF3B3. HBE1 is an embryonic hemoglobin protein, which suggests changes in oxygen regulation with ATZ exposure (Aceto et al. 2015). MATN3 is an extracellular matrix protein expressed in the cartilage during development (Pullig et al. 2002). Pathogenic variants or alterations in expression of MATN3 is associated with bone disorders in humans such as multiple epiphyseal dysplasia (MED), which affects the epiphsyses in the long bone (Jackson et al. 2012; Pettersson et al. 2018) or osteoarthritis later on in life. SF3B3 is a component of U2 small nuclear ribonucleoprotein-associated protein complex SF3B (Das 1999). SF3B3 is present in the nucleus and partakes in DNA and RNA maintenance and function including chromatin modification, transcription, splicing, and DNA repair (Chen et al. 2017; Metselaar et al. 2021). Alterations in SF3B3 are linked with carcinogenesis (Chen et al. 2017). Furthermore, SF3B3 was identified along with HNRNPM (altered in offspring of the 30 ppb treatment group in the current study) to interact with RANBP9 (RAN-binding protein 9) in the testis to regulate alternative splicing in spermatogenic cells (Bao et al. 2014). This function is critical for normal spermatogenesis and male fertility, aligning with past studies on ATZ endocrine disrupting properties (Hao et al. 2016); McBirney et al. 2017; Cleary et al. 2019).

Several altered proteins in the ATZ F1 were within similar families including the ATP synthase proteins, collagen proteins, heterogeneous nuclear ribonucleoproteins, and ribosomal proteins. Past studies identified ATZ to alter ATP content, mitochondrial dysfunction, and collagen fibers and structures in multiple tissues in the initial exposed generation (Lim et al. 2009). The results of the current study indicate these targets are also altered in the subsequent generation. The heterogeneous nuclear ribonucleoproteins are complexes of RNA and protein that serve as a signal that the pre-mRNA is not yet processed; thus, being important for pre-mRNA processing and other aspects of mRNA metabolism and transport. The heterogeneous nuclear ribonucleoproteins localize to border regions of chromatin and are reported to function in multiple cellular processes (Fakan et al. 1984). Moreover, a number of ribosomal proteins were also altered in the ATZ F1 larvae. Ribosomal proteins are highly conserved regulators of translation. Ribosomal proteins altered in the ATZ F1 included both large and small subunits with a number of them known to be associated with Diamond-Blackfan Anemia and/or Shwachman-Diamond Syndrome (RPL11, RPL3, RPL4, RPS11). Although these disorders are inherited conditions, it may suggest similar adverse health targets (e.g., effects on growth, bone marrow, the skeletomuscular system, and/or osteosarcoma) for further study. Pathway analysis of the altered proteins identified associations with neurological development and disease and cancer as well as molecular pathways associated with organ and organismal morphology, development, and injury. These pathways align with many of the adverse health outcomes and molecular pathways indicated to play a role in atrazine toxicity in the exposed generation (F0) including our past proteomic study in the ATZ F0 larvae (Horzmann et al. 2018). These common molecular pathways included nervous system development, cancer, and organismal injury and abnormalities being altered in the exposed and subsequent generation.

When comparing the specific proteins altered in the ATZ F1 larvae to the ATZ F0 larvae in our previous study, a higher number of proteins were significantly changed in the larval progeny compared to the same aged larvae that had the embryonic ATZ exposure (ATZ F0) (Horzmann et al. 2018). In addition, there was only one common protein identified to be altered, ADD3 (adducin 3), in both generations. ADD3 is one of the family members of the adducins. The adducins are associated with membrane skeletal proteins and are involved in the spectrin-actin network in erythrocytes and in epithelial tissues at sites of cell-to-cell contact. In addition, members of the profilin family were also changed in both generations (F0: PFN2L; F1: PFN1). Profilins are also associated with the actin cytoskeleton and are essential for organ development. Mutations in PFN1

are linked to amyotrophic lateral sclerosis (ALS), while overexpression is associated with cancer (Alkam et al. 2017). One previous protein study in MCF-10A human breast epithelial cells also identified PFN1 to be differentially expressed with ATZ exposure (Huang et al. 2014).

Upstream regulator and causal pathway analysis of the altered proteins identified similarities in the 0.3 and 3 ppb treatment group offspring in DDX5. DDX5, a putative RNA helicase, is part of the estrogen receptor signaling canonical pathway (also one of the top canonical pathways in these treatment group offspring) involved in embryogenesis, spermatogenesis, and cellular growth and division (Legrand et al. 2019). Overall, this finding along with the other discussed proteome results greatly overlap with the wealth of literature on ATZ's association with the estrogen pathways and endocrine disrupting properties.

Morphology measurements were completed to assess impacts to larval growth parameters and brain development. An increase in head length, along with an increase in head length to total body length ratio were observed indicating head size was larger than would be expected based on total larval length. These findings agree with measurements completed at 72 hpf immediately following an embryonic ATZ exposure in zebrafish (F0) observing increased head length (Weber et al. 2013). Alternatively, when the F0 were exposed to ATZ from 1-120 hpf no significant differences in morphology were observed (Ahkin Chin Tai et al. 2021). Epidemiological studies have also reported alterations in head size and other birth defects related to in utero ATZ exposure (Chevrier et al. 2011; Stayner et al. 2017; Winchester et al. 2009). In addition, zebrafish studies using the DOHaD paradigm, report that an embryonic ATZ exposure leads to decreased body weight at 14 months of age in adult male zebrafish exposed to 3 ppb ATZ during embryogenesis (Horzmann et al. 2020) and altered brain to body weight ratio in adult female zebrafish aged 8 months with exposure to 0.3 or 30 ppb ATZ during embryogenesis (Wirbisky et al. 2016c).

Based on the molecular pathways identified in the proteomic analysis, further morphological analysis of craniofacial structures was completed. Cranioskeletal formation is conserved among vertebrates (Kuratani et al.) and bone remodeling and signaling in zebrafish and humans are also similar (Kwon et al. 2019; Mork and Crump 2015; Paul et al. 2016). Zebrafish are also an established modeled for bone remodeling and bone disease modeling (Carnovali et al. 2019; Kwon et al. 2019), making them an ideal model to understand how ATZ alters craniofacial structures. Furthermore, endocrine disrupting chemicals can cause changes in cartilage and bone development. A couple studies in different model organisms support developmental ATZ exposure

interfering with craniofacial bone and cartilage formation during development including reports in frogs (Lenkowski and McLaughlin 2010) and zebrafish (Walker et al. 2018) altering craniofacial cartilaginous structures. In addition, a study with developmental ATZ exposure in chick embryos also showed neural tube defects as well as craniofacial hypoplasia (Joshi et al. 2013). Conversely, a developmental exposure in South American river turtles found no visual changes present with ATZ exposure in whole body skeletal structures (dos Santos Mendonça et al. 2016).

In zebrafish, chondrogenesis is present 2 days after fertilization and cartilaginous structures begin to develop around 3 days after fertilization (Kimmel et al. 1998). Cranial bone development in the zebrafish starts from 3 days after fertilization (Cubbage and Mabee 1996). The jaw is derived from 7 pharyngeal arches by 5 days in the larval zebrafish and neural crest cells during development play a critical role in neurocranial cartilage and bone development. Malformations in the jaw is associated with bone formation or neural crest migrations issues (Kimmel et al. 1998; TeSlaa et al. 2013; Ton et al. 2006). Here, we observed a significant decrease in PQ length for progeny of all ATZ treatment groups and in CH-Meckel's length, jaw distance, and a more posteriorly positioned jaw as measured from the notochord in offspring of the 30 ppb ATZ treatment group. In addition, a decrease in surface area of the saccular otoliths and a decrease in distance between the saccular otoliths was measured in the offspring of the 30 ppb treatment group, suggesting a decrease in ossification. These observations are comparable to the craniofacial hypoplasia reported in developing chicks (Joshi et al. 2013). In addition, the current study agrees with two other zebrafish studies reporting decreased ossification of vertebrae in developmentally exposed zebrafish at 8 days post fertilization (Walker et al. 2018) and a visible phenotype of an underdeveloped jaw at 96 hpf (Ton et al. 2006). Alternatively, the decrease in size of various cartilaginous structures in the 30 ppb ATZ F1 treatment group at 120 hpf contradicts another study in zebrafish in which the authors found a trend of increasing size of cartilaginous structures at 120 hpf (Walker et al. 2018). However, this study was completed in the exposed generation (F0), in which zebrafish were exposed from 8-120 hpf to ATZ in a mixture. Overall, the findings in the current study suggest that ATZ exposure in the parental generation can alter craniofacial development in their progeny with alterations observed in cartilaginous and skeletal structures, along with supporting molecular pathway alterations identified in the proteomic analysis.

ATZ as an endocrine disruptor has also been shown to cause sex specific behavioral outcomes in various model organisms. A study in tadpoles observed hyperactivity in ATZ exposed

tadpoles, and that these tadpoles were also less likely to avoid predatory chemical cues (Ehrsam et al. 2016). A gestational ATZ exposure in C57BL/6 mice found that dams had decreased novel object recognition and displayed hyperactivity, while juvenile offspring were hyperactive with an increase in anxiety-like behavior (Lin et al. 2014). Furthermore, a transgenerational ATZ study in Sprague Dawley rats found that the F3 generation displayed hyperactivity (McBirney et al. 2017), showing that behavioral changes can be observed in subsequent generations.

In zebrafish, embryonic ATZ exposure resulted in altered transcriptome pathways associated with behavioral, cognitive, and locomotive changes at 72 hpf (Wirbisky et al. 2016c). Proteomic analysis at 120 hpf in zebrafish exposed to ATZ during embryogenesis (1-72 hpf) also supported molecular pathway alterations associated with behavior, similar to the current study (Horzmann et al. 2018). Furthermore, behavioral analysis with the visual motor response assay in the ATZ F0 zebrafish exposed during embryogenesis (1-72 hpf) showed cumulative hypoactivity in the 30 ppb treatment group at 120 hpf (Horzmann et al. 2018). When the ATZ exposure was extended through 120 hpf in the F0 and locomotion was evaluated by phase, hypoactivity was observed in the 30 ppb treatment group in the dark phases for total distance moved and hyperactivity was seen in the 0.3 ppb treatment group in the first light phase in total distance moved and time spent moving (Ahkin Chin Tai et al. 2021). No significant changes were seen for velocity. These findings are similar to what was observed in the current study in the ATZ F1 larvae at 120 hpf with behavioral alterations only observed in progeny of the 0.3 and 30 ppb treatment groups and no significant changes in velocity, but hyperactivity was observed in the 30 ppb treatment group progeny.

In the context of previous laboratory studies, as well as various developmental transgenerational studies of ATZ exposure, these studies fall in line with the Developmental Origin of Health and Disease. This paradigm suggests that exposures during development can have long lasting impacts seen later on in life and subsequent generations.

3.7 References

1. 2016 Pesticide Use Maps -. Available: https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2016&map=ATRAZINE&hilo=L [accessed 4 November 2018b].

2. Aceto J, Nourizadeh-Lillabadi R, Marée R, Dardenne N, Jeanray N, Wehenkel L, et al. 2015. Zebrafish Bone and General Physiology Are Differently Affected by Hormones or Changes in Gravity. P.E. Witten, ed PLoS ONE 10:e0126928; doi:10.1371/journal.pone.0126928.

3. Agopian AJ, Lupo PJ, Canfield MA, Langlois PH. 2013. Case-Control Study of Maternal Residential Atrazine Exposure and Male Genital Malformations. Am J Med Genet 161:977–982; doi:10.1002/ajmg.a.35815.

4. Ahkin Chin Tai JK, Horzmann KA, Franco J, Jannasch AS, Cooper BR, Freeman JL. 2021. Developmental atrazine exposure in zebrafish produces the same major metabolites as mammals along with altered behavioral outcomes. Neurotoxicology and Teratology 106971; doi:10.1016/j.ntt.2021.106971.

5. Alkam D, Feldman EZ, Singh A, Kiaei M. 2017. Profilin1 biology and its mutation, actin(g) in disease. Cell Mol Life Sci 74:967–981; doi:10.1007/s00018-016-2372-1.

6. Almberg K, Turyk M, Jones R, Rankin K, Freels S, Stayner L. 2018. Atrazine Contamination of Drinking Water and Adverse Birth Outcomes in Community Water Systems with Elevated Atrazine in Ohio, 2006–2008. IJERPH 15:1889; doi:10.3390/ijerph15091889.

7. Aluru N, Deak KL, Jenny MJ, Hahn ME. 2013. Developmental exposure to valproic acid alters the expression of microRNAs involved in neurodevelopment in zebrafish. Neurotoxicology and Teratology 40:46–58; doi:10.1016/j.ntt.2013.10.001.

8. Bailey J, Oliveri A, Levin ED. 2013. Zebrafish model systems for developmental neurobehavioral toxicology: Zebrafish Developmental Neurobehavioral Toxicology. Birth Defect Res C 99:14–23; doi:10.1002/bdrc.21027.

9. Bao J, Tang C, Li J, Zhang Y, Bhetwal BP, Zheng H, et al. 2014. RAN-Binding Protein 9 is Involved in Alternative Splicing and is Critical for Male Germ Cell Development and Male Fertility. G.S. Barsh, ed PLoS Genet 10:e1004825; doi:10.1371/journal.pgen.1004825.

10. Bardullas U, Giordano M, Rodríguez VM. 2011. Chronic atrazine exposure causes disruption of the spontaneous locomotor activity and alters the striatal dopaminergic system of the male Sprague–Dawley rat. Neurotoxicology and Teratology 33:263–272; doi:10.1016/j.ntt.2010.09.001.

11. Barr DB, Panuwet P, Nguyen JV, Udunka S, Needham LL. 2007. Assessing Exposure to Atrazine and Its Metabolites Using Biomonitoring. Environ Health Perspect 115:1474–1478; doi:10.1289/ehp.10141.

12. Bethsass J, Colangelo A. 2006. European Union Bans Atrazine, While the United States Negotiates Continued Use. International Journal of Occupational and Environmental Health 12:260–267; doi:10.1179/oeh.2006.12.3.260.

13. Blahova J, Cocilovo C, Plhalova L, Svobodova Z, Faggio C. 2020. Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (Danio rerio). Environmental Toxicology and Pharmacology 77:103370; doi:10.1016/j.etap.2020.103370.

14. Blanchard PE. 2000. Watershed Vulnerability to Losses of Agricultural Chemicals: Interactions of Chemistry, Hydrology, and Land-Use.

15. Brandhonneur N, Hutin V, Chevrier C, Cordier S, Le Corre P. 2019. Maternal and fetal blood pharmacokinetics and organ distribution of atrazine, propazine, simazine and their metabolites in pregnant rats after chronic oral administration. Toxicological Sciences kfz223; doi:10.1093/toxsci/kfz223.

16. Bridi D, Altenhofen S, Gonzalez JB, Reolon GK, Bonan CD. 2017. Glyphosate and Roundup® alter morphology and behavior in zebrafish. Toxicology 392:32–39; doi:10.1016/j.tox.2017.10.007.

17. British Crop Protection Council. 1977. *Pesticide manual: basic information on the chemicals used as active components of pesticides*. 5th ed. / edited by Hubert Martin, Charles R. Worthing.. The Council:Droitwich, Eng.

18. Carnovali M, Banfi G, Mariotti M. 2019. Zebrafish Models of Human Skeletal Disorders: Embryo and Adult Swimming Together. BioMed Research International 2019:1–13; doi:10.1155/2019/1253710.

19. Caron-Beaudoin É, Denison MS, Sanderson JT. 2016. Effects of Neonicotinoids on Promoter-Specific Expression and Activity of Aromatase (CYP19) in Human Adrenocortical Carcinoma (H295R) and Primary Umbilical Vein Endothelial (HUVEC) Cells. Toxicol Sci 149:134–144; doi:10.1093/toxsci/kfv220.

20. Cassar S, Adatto I, Freeman JL, Gamse JT, Iturria I, Lawrence C, et al. 2020. Use of Zebrafish in Drug Discovery Toxicology. Chem Res Toxicol 33:95–118; doi:10.1021/acs.chemrestox.9b00335.

21. CastaÃ±eda CortÃ©s DC, Langlois VS, Fernandino JI. 2014. Crossover of the Hypothalamic Pituitaryâ€“Adrenal/Interrenal, â€“Thyroid, and â€“Gonadal Axes in Testicular Development. Front Endocrinol 5; doi:10.3389/fendo.2014.00139.

22. Catenacci G, Barbieri F, Bersani M, Fereoli A, Cottica D, Maroni M. 1993. Biological monitoring of human exposure to atrazine. Toxicology Letters 69:217–222; doi:10.1016/0378-4274(93)90107-9.

23. Chávez-Pichardo ME, Reyes-Bravo DY, Mendoza-Trejo MS, Marín-López AG, Giordano M, Hernández-Chan N, et al. 2020. Brain alterations in GABA, glutamate and glutamine markers after chronic atrazine exposure in the male albino rat. Arch Toxicol 94:3217–3230; doi:10.1007/s00204-020-02806-2.

24. Chen K, Xiao H, Zeng J, Yu G, Zhou H, Huang C, et al. 2017. Alternative Splicing of EZH2 pre-mRNA by SF3B3 Contributes to the Tumorigenic Potential of Renal Cancer. Clin Cancer Res 23:3428–3441; doi:10.1158/1078-0432.CCR-16-2020.

25. Cheremisinoff N, Rosenfeld P. 2011. *Handbook of Pollution Prevention and Cleaner Production: Best Practices in the Agrochemical Industry*. Elsevier.

26. Chevrier C, Limon G, Monfort C, Rouget F, Garlantézec R, Petit C, et al. 2011. Urinary Biomarkers of Prenatal Atrazine Exposure and Adverse Birth Outcomes in the PELAGIE Birth Cohort. Environ Health Perspect 119:1034–1041; doi:10.1289/ehp.1002775.

27. Cleary JA, Tillitt DE, vom Saal FS, Nicks DK, Claunch RA, Bhandari RK. 2019. Atrazine induced transgenerational reproductive effects in medaka (Oryzias latipes). Environmental Pollution 251:639–650; doi:10.1016/j.envpol.2019.05.013.

28. Cohen SP, LaChappelle AR, Walker BS, Lassiter CS. 2014. Modulation of estrogen causes disruption of craniofacial chondrogenesis in Danio rerio. Aquatic Toxicology 152:113–120; doi:10.1016/j.aquatox.2014.03.028.

29. Cooper RL. 2000. Atrazine Disrupts the Hypothalamic Control of Pituitary-Ovarian Function. Toxicological Sciences 53:297–307; doi:10.1093/toxsci/53.2.297.

30. Cubbage CC, Mabee P. 1996. Development of the cranium and paired fins in the zebrafish Danio rerio (Ostariophysi, Cyprinidae). Journal of Morphology 229: 121–160.

31. Delcorso MC, de Paiva PP, Grigoleto MRP, Queiroz SCN, Collares-Buzato CB, Arana S. 2020. Effects of sublethal and realistic concentrations of the commercial herbicide atrazine in Pacu (Piaractus mesopotamicus): Long-term exposure and recovery assays. Vet World 13:147–159; doi:10.14202/vetworld.2020.147-159.

32. DeSesso JM, Scialli AR, White TEK, Breckenridge CB. 2014. Multigeneration Reproduction and Male Developmental Toxicity Studies on Atrazine in Rats: Reproductive Toxicity Studies of Atrazine. Birth Defects Res B 101:237–253; doi:10.1002/bdrb.21106.

33. Dong X, Zhu L, Wang J, Wang J, Xie H, Hou X, et al. 2009. Effects of atrazine on cytochrome P450 enzymes of zebrafish (Danio rerio). Chemosphere 77:404–412; doi:10.1016/j.chemosphere.2009.06.052.

34. dos Santos Mendonça J, Vieira LG, Valdes SAC, Vilca FZ, Tornisielo VL, Santos ALQ. 2016. Effects of the exposure to atrazine on bone development of Podocnemis expansa (Testudines, Podocnemididae). Ecotoxicology 25:594–600; doi:10.1007/s10646-016-1618-x.

35. Ehrsam M, Knutie SA, Rohr JR. 2016. The herbicide atrazine induces hyperactivity and compromises tadpole detection of predator chemical cues: Atrazine reduces detection of predator chemical cues. Environ Toxicol Chem 35:2239–2244; doi:10.1002/etc.3377.

36. Eldridgea JC, Wetzel LT, Tyrey L. 1999. Estrous cycle patterns of Sprague-Dawley rats during acute and chronic atrazine administration. Reproductive Toxicology 13:491–499; doi:10.1016/S0890-6238(99)00056-8.

37. Fakan S, Leser G, Martin TE. 1984. Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. Journal of Cell Biology 98:358–363; doi:10.1083/jcb.98.1.358.

38. Foradori CD, Healy JE, Zimmerman AD, Kemppainen RJ, Jones MA, Read CC, et al. 2018. Characterization of Activation of the Hypothalamic-Pituitary-Adrenal Axis by the Herbicide Atrazine in the Female Rat. Endocrinology 159:3378–3388; doi:10.1210/en.2018-00474.

39. Foradori CD, Hinds LR, Hanneman WH, Legare ME, Clay CM, Handa RJ. 2009. Atrazine Inhibits Pulsatile Luteinizing Hormone Release Without Altering Pituitary Sensitivity to a Gonadotropin-Releasing Hormone Receptor Agonist in Female Wistar Rats1. Biology of Reproduction 81:40–45; doi:10.1095/biolreprod.108.075713.

40. Fraites MJP, Cooper RL, Buckalew A, Jayaraman S, Mills L, Laws SC. 2009. Characterization of the Hypothalamic-Pituitary-Adrenal Axis Response to Atrazine and Metabolites in the Female Rat. Toxicological Sciences 112:88–99; doi:10.1093/toxsci/kfp194.

41. Freeman JL, Beccue N, Rayburn AL. 2005. Differential metamorphosis alters the endocrine response in anuran larvae exposed to T3 and atrazine. Aquatic Toxicology 75:263–276; doi:10.1016/j.aquatox.2005.08.012.

42. Freeman JL, Weber GJ, Sepúlveda MS. 2013. Fishing for microRNAs in Toxicology. In: *microRNAs in Toxicology and Medicine* (S.C. Sahu, ed). John Wiley & Sons, Ltd:Chichester, UK. 49–75.

43. Freeman LEB, Rusiecki JA, Hoppin JA, Lubin JH, Koutros S, Andreotti G, et al. Atrazine and Cancer Incidence Among Pesticide Applicators in the Agricultural Health Study (1994-2007). 8.

44. Gammon DW, Aldous CN, Carr WC, Sanborn JR, Pfeifer KF. 2005. A risk assessment of atrazine use in California: human health and ecological aspects. Pest Manag Sci 61:331–355; doi:10.1002/ps.1000.

45. Garcia GR, Noyes PD, Tanguay RL. 2016. Advancements in zebrafish applications for 21st century toxicology. Pharmacology & Therapeutics 161:11–21; doi:10.1016/j.pharmthera.2016.03.009.

46. Goldman JM, Davis LK, Murr AS, Cooper RL. 2013. Atrazine-induced elevation or attenuation of the LH surge in the ovariectomized, estrogen-primed female rat: role of adrenal progesterone. REPRODUCTION 146:305–314; doi:10.1530/REP-13-0011.

47. Goldstone JV, McArthur AG, Kubota A, Zanette J, Parente T, Jönsson ME, et al. 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC Genomics 11:643; doi:10.1186/1471-2164-11-643.

48. Guzzella L, Pozzoni F, Giuliano G. 2006. Herbicide contamination of surficial groundwater in Northern Italy. Environmental Pollution 142:344–353; doi:10.1016/j.envpol.2005.10.037.

49. Hallare A, Nagel K, Köhler H-R, Triebskorn R. 2006. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (Danio rerio) embryos. Ecotoxicology and Environmental Safety 63:378–388; doi:10.1016/j.ecoenv.2005.07.006.

50. Hao C, Gely-Pernot A, Kervarrec C, Boudjema M, Becker E, Khil P, et al. 2016. Exposure to the widely used herbicide atrazine results in deregulation of global tissue-specific RNA transcription in the third generation and is associated with a global decrease of histone trimethylation in mice. Nucleic Acids Res gkw840; doi:10.1093/nar/gkw840.

51. Haugen AC, Schug TT, Collman G, Heindel JJ. 2015. Evolution of DOHaD: the impact of environmental health sciences. J Dev Orig Health Dis 6:55–64; doi:10.1017/S2040174414000580.

52. He J-H, Gao J-M, Huang C-J, Li C-Q. 2014. Zebrafish models for assessing developmental and reproductive toxicity. Neurotoxicology and Teratology 42:35–42; doi:10.1016/j.ntt.2014.01.006.

53. Heindel JJ, Vandenberg LN. 2015. Developmental origins of health and disease: a paradigm for understanding disease cause and prevention. Current Opinion in Pediatrics 27:248–253; doi:10.1097/MOP.0000000000000191.

54. Horzmann et al. - 2020 - Embryonic atrazine exposure and later in life beha.pdf.

55. Horzmann KA, de Perre C, Lee LS, Whelton AJ, Freeman JL. 2017. Comparative analytical and toxicological assessment of methylcyclohexanemethanol (MCHM) mixtures associated with the Elk River chemical spill. Chemosphere 188:599–607; doi:10.1016/j.chemosphere.2017.09.026.

56. Horzmann KA, Freeman JL. 2018. Making Waves: New Developments in Toxicology With the Zebrafish. Toxicological Sciences 163:5–12; doi:10.1093/toxsci/kfy044.

57. Horzmann KA, Lin LF, Taslakjian B, Yuan C, Freeman JL. 2020. Embryonic atrazine exposure and later in life behavioral and brain transcriptomic, epigenetic, and pathological alterations in adult male zebrafish. Cell Biol Toxicol; doi:10.1007/s10565-020-09548-y.

58. Horzmann KA, Reidenbach LS, Thanki DH, Winchester AE, Qualizza BA, Ryan GA, et al. 2018. Embryonic atrazine exposure elicits proteomic, behavioral, and brain abnormalities with developmental time specific gene expression signatures. Journal of Proteomics 186:71–82; doi:10.1016/j.jprot.2018.07.006.

59. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496:498–503; doi:10.1038/nature12111.

60. Huang P, Xiao A, Zhou M, Zhu Z, Lin S, Zhang B. 2011. Heritable gene targeting in zebrafish using customized TALENs. Nat Biotechnol 29:699–700; doi:10.1038/nbt.1939.

61. Huang P, Yang J, Song Q. 2014. Atrazine Affects Phosphoprotein and Protein Expression in MCF-10A Human Breast Epithelial Cells. IJMS 15:17806–17826; doi:10.3390/ijms151017806.

62. International Programme on Chemical Safety, ed. 1990. *Atrazine: health and safety guide*. World Health Organization:Geneva.

63. Jackson GC, Mittaz-Crettol L, Taylor JA, Mortier GR, Spranger J, Zabel B, et al. 2012. Pseudoachondroplasia and multiple epiphyseal dysplasia: A 7-year comprehensive analysis of the known disease genes identify novel and recurrent mutations and provides an accurate assessment of their relative contribution. Hum Mutat 33:144–157; doi:10.1002/humu.21611.

64. Jacomini AE, Avelar WEP, Martinêz AS, Bonato PS. 2006. Bioaccumulation of Atrazine in Freshwater Bivalves Anodontites trapesialis (Lamarck, 1819) and Corbicula fluminea (Müller, 1774). Arch Environ Contam Toxicol 51:387–391; doi:10.1007/s00244-005-0238-x.

65. Joshi N, Rhoades MG, Bennett GD, Wells SM, Mirvish SS, Breitbach MJ, et al. 2013. Developmental Abnormalities in Chicken Embryos Exposed to *N* -Nitrosoatrazine. Journal of Toxicology and Environmental Health, Part A 76:1015–1022; doi:10.1080/15287394.2013.831721.

66. Karapanou O, Papadimitriou A. 2010. Determinants of menarche. Reprod Biol Endocrinol 8:115; doi:10.1186/1477-7827-8-115.

67. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203:253–310; doi:10.1002/aja.1002030302.

68. Kimmel CB, Miller CT, Kruze G, Ullmann B, BreMiller RA, Larison KD, et al. 1998. The Shaping of Pharyngeal Cartilages during Early Development of the Zebrafish. Developmental Biology 203:245–263; doi:10.1006/dbio.1998.9016.

69. Kimura M, Ishii MN, Seki N, Sakai Y, Yamashita T, Awatsuji H, et al. 2019. Reduction of *Kiss1* expression in the anteroventral periventricular nucleus is associated with atrazine-induced attenuation of the luteinizing hormone surge in female rats. Biology of Reproduction 100:41–48; doi:10.1093/biolre/ioy159.

70. Kokel D, Bryan J, Laggner C, White R, Cheung CYJ, Mateus R, et al. 2010. Rapid behavior-based identification of neuroactive small molecules in the zebrafish. Nat Chem Biol 6:231–237; doi:10.1038/nchembio.307.

71. Kuratani S, Matsuo I, Aizawa S. Developmental patterning and evolution of the mammalian viscerocranium: Genetic insights into comparative morphology. 17.

72. Kwon RY, Watson CJ, Karasik D. 2019. Using zebrafish to study skeletal genomics. Bone 126:37–50; doi:10.1016/j.bone.2019.02.009.

73. LeBaron HM, McFarland JE, Burnside OC. 2008. *The Triazine Herbicides*. Elsevier.

74. Legrand JMD, Chan A-L, La HM, Rossello FJ, Änkö M-L, Fuller-Pace FV, et al. 2019. DDX5 plays essential transcriptional and post-transcriptional roles in the maintenance and function of spermatogonia. Nat Commun 10:2278; doi:10.1038/s41467-019-09972-7.

75. Lenkowski JR, McLaughlin KA. 2010. Acute atrazine exposure disrupts matrix metalloproteinases and retinoid signaling during organ morphogenesis in Xenopus laevis. J Appl Toxicol 30:582–589; doi:10.1002/jat.1529.

76. Li B, Jiang Y, Xu Y, Li Y, Li B. 2019a. Identification of miRNA-7 as a regulator of brain-derived neurotrophic factor/α-synuclein axis in atrazine-induced Parkinson's disease by peripheral blood and brain microRNA profiling. Chemosphere 233:542–548; doi:10.1016/j.chemosphere.2019.05.064.

77. Li J, Li X, Bi H, Li B. 2019b. The MEK/ERK/CREB signaling pathway is involved in atrazine induced hippocampal neurotoxicity in Sprague Dawley rats. Ecotoxicology and Environmental Safety 170:673–681; doi:10.1016/j.ecoenv.2018.12.038.

78. Li Y, Sun Y, Yang J, Wu Y, Yu J, Li B. 2014. Age-dependent dopaminergic dysfunction following fetal exposure to atrazine in SD rats. Environmental Toxicology and Pharmacology 37:1275–1282; doi:10.1016/j.etap.2014.04.023.

79. Li Y-S, He X, Ma K, Wu Y-P, Li B-X. 2015. The Effect of Exposure to Atrazine on Dopaminergic Development in Pubertal Male SD Rats: THE EFFECT OF EXPOSURE TO ATRAZINE ON DOPAMINERGIC. Birth Defects Res B 104:184–189; doi:10.1002/bdrb.21151.

80. Lim S, Ahn SY, Song IC, Chung MH, Jang HC, Park KS, et al. 2009. Chronic Exposure to the Herbicide, Atrazine, Causes Mitochondrial Dysfunction and Insulin Resistance. G. Malaga, ed PLoS ONE 4:e5186; doi:10.1371/journal.pone.0005186.

81. Lin Z, Dodd CA, Filipov NM. 2013. Short-term atrazine exposure causes behavioral deficits and disrupts monoaminergic systems in male C57BL/6 mice. Neurotoxicology and Teratology 39:26–35; doi:10.1016/j.ntt.2013.06.002.

82. Lin Z, Dodd CA, Xiao S, Krishna S, Ye X, Filipov NM. 2014. Gestational and Lactational Exposure to Atrazine via the Drinking Water Causes Specific Behavioral Deficits and Selectively Alters Monoaminergic Systems in C57BL/6 Mouse Dams, Juvenile and Adult Offspring. Toxicol Sci 141:90–102; doi:10.1093/toxsci/kfu107.

83. Lin Z, Fisher JW, Ross MK, Filipov NM. 2011. A physiologically based pharmacokinetic model for atrazine and its main metabolites in the adult male C57BL/6 mouse. Toxicology and Applied Pharmacology 251:16–31; doi:10.1016/j.taap.2010.11.009.

84. Liu Z, Fu Z, Jin Y. 2017. Immunotoxic effects of atrazine and its main metabolites at environmental relevant concentrations on larval zebrafish (Danio rerio). Chemosphere 166:212–220; doi:10.1016/j.chemosphere.2016.09.100.

85. Luo S, Yang Y, Chen J, Zhong Z, Huang H, Zhang J, et al. 2016. Tanshinol stimulates bone formation and attenuates dexamethasone-induced inhibition of osteogenesis in larval zebrafish. Journal of Orthopaedic Translation 4:35–45; doi:10.1016/j.jot.2015.07.002.

86. Ma K, Wu H-Y, Zhang B, He X, Li B-X. 2015. Neurotoxicity effects of atrazine-induced SH-SY5Y human dopaminergic neuroblastoma cells via microglial activation. Mol BioSyst 11:2915–2924; doi:10.1039/C5MB00432B.

87. Magoffin DA. 2005. Ovarian theca cell. The International Journal of Biochemistry & Cell Biology 37:1344–1349; doi:10.1016/j.biocel.2005.01.016.

88. Marques P, Skorupskaite K, George JT, Anderson RA. 2000. Physiology of GNRH and Gonadotropin Secretion. In: *Endotext* (K.R. Feingold, B. Anawalt, A. Boyce, G. Chrousos, K. Dungan, A. Grossman, et al., eds). MDText.com, Inc.:South Dartmouth (MA).

89. McBirney M, King SE, Pappalardo M, Houser E, Unkefer M, Nilsson E, et al. 2017. Atrazine induced epigenetic transgenerational inheritance of disease, lean phenotype and sperm epimutation pathology biomarkers. C. Óvilo, ed PLoS ONE 12:e0184306; doi:10.1371/journal.pone.0184306.

90. McMullin TS, Hanneman WH, Cranmer BK, Tessari JD, Andersen ME. 2007. Oral absorption and oxidative metabolism of atrazine in rats evaluated by physiological modeling approaches. Toxicology 240:1–14; doi:10.1016/j.tox.2007.05.031.

91. Meffe R, de Bustamante I. 2014. Emerging organic contaminants in surface water and groundwater: A first overview of the situation in Italy. Science of The Total Environment 481:280–295; doi:10.1016/j.scitotenv.2014.02.053.

92. Meiser J, Weindl D, Hiller K. 2013. Complexity of dopamine metabolism. Cell Commun Signal 11:34; doi:10.1186/1478-811X-11-34.

93. Mendaš G, Vuletić M, Galić N, Drevenkar V. 2012. Urinary metabolites as biomarkers of human exposure to atrazine: Atrazine mercapturate in agricultural workers. Toxicology Letters 210:174–181; doi:10.1016/j.toxlet.2011.11.023.

94. Metselaar PI, Hos C, Welting O, Bosch JA, Kraneveld AD, de Jonge WJ, et al. 2021. Ambiguity about Splicing Factor 3b Subunit 3 (SF3B3) and Sin3A Associated Protein 130 (SAP130). Cells 10:590; doi:10.3390/cells10030590.

95. Migeot V, Albouy-Llaty M, Carles C, Limousi F, Strezlec S, Dupuis A, et al. 2013. Drinking-water exposure to a mixture of nitrate and low-dose atrazine metabolites and small-for-gestational age (SGA) babies: A historic cohort study. Environmental Research 122:58–64; doi:10.1016/j.envres.2012.12.007.

96. Mitchell PD. 2014. Market-level assessment of the economic benefits of atrazine in the United States: Economic benefits of atrazine. Pest Manag Sci 70:1684–1696; doi:10.1002/ps.3703.

97. Mork L, Crump G. 2015. Zebrafish Craniofacial Development. In: *Current Topics in Developmental Biology*. Vol. 115 of. Elsevier. 235–269.

98. [No title found].pdf.

99. Ochoa-Acuña H, Frankenberger J, Hahn L, Carbajo C. 2009. Drinking-Water Herbicide Exposure in Indiana and Prevalence of Small-for-Gestational-Age and Preterm Delivery. Environmental Health Perspectives; Research Triangle Park 117: 1619–24.

100. Paul S, Schindler S, Giovannone D, Terrazzani A de M, Mariani FV, Crump JG. 2016. Ihha induces hybrid cartilage-bone cells during zebrafish jawbone regeneration. 11.

101. Peng Y, Clark KJ, Campbell JM, Panetta MR, Guo Y, Ekker SC. 2014. Making designer mutants in model organisms. Development 141:4042–4054; doi:10.1242/dev.102186.

102. Peterson SM, Zhang J, Freeman JL. 2013. Developmental reelin expression and time point-specific alterations from lead exposure in zebrafish. Neurotoxicology and Teratology 38:53–60; doi:10.1016/j.ntt.2013.04.007.

103. Peterson SM, Zhang J, Weber G, Freeman JL. 2011. Global Gene Expression Analysis Reveals Dynamic and Developmental Stage–Dependent Enrichment of Lead-Induced Neurological Gene Alterations. Environmental Health Perspectives 119:615–621; doi:10.1289/ehp.1002590.

104. Pettersson M, Vaz R, Hammarsjö A, Eisfeldt J, Carvalho CMB, Hofmeister W, et al. 2018. *Alu-Alu* mediated intragenic duplications in *IFT81* and *MATN3* are associated with skeletal dysplasias. Human Mutation 39:1456–1467; doi:10.1002/humu.23605.

105. Priyadarshi A, Khuder SA, Schaub EA, Shrivastava S. 2000. A meta-analysis of Parkinson's disease and exposure to pesticides. Neurotoxicology 21: 435–40.

106. Pullig O, Weseloh G, Klatt AR, Wagener R, Swoboda B. 2002. Matrilin-3 in human articular cartilage: increased expression in osteoarthritis. Osteoarthritis and Cartilage 10:253–263; doi:10.1053/joca.2001.0508.

107. Qin L, Du Z-H, Zhu S-Y, Li X-N, Li N, Guo J-A, et al. 2015. Atrazine triggers developmental abnormality of ovary and oviduct in quails (Coturnix Coturnix coturnix) via disruption of hypothalamo-pituitary-ovarian axis. Environmental Pollution 207:299–307; doi:10.1016/j.envpol.2015.09.044.

108. Review report for the active substance atrazine Finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on 3 October 2003.

109. Reviews of environmental contamination and toxicology. 1987.Springer-Verlag:New York.

110. Riffle BW, Klinefelter GR, Cooper RL, Winnik WM, Swank A, Jayaraman S, et al. 2014. Novel molecular events associated with altered steroidogenesis induced by exposure to atrazine in the intact and castrate male rat. Reproductive Toxicology 47:59–69; doi:10.1016/j.reprotox.2014.05.008.

111. Rodríguez VM, Limón-Pacheco JH, Mendoza-Trejo MS, González-Gallardo A, Hernández-Plata I, Giordano M. 2013. Repeated exposure to the herbicide atrazine alters

locomotor activity and the nigrostriatal dopaminergic system of the albino rat. NeuroToxicology 34:82–94; doi:10.1016/j.neuro.2012.10.012.

112. Ross MK, Jones TL, Filipov NM. 2009. Disposition of the Herbicide 2-Chloro-4-(ethylamino)-6-(isopropylamino)- *s* -triazine (Atrazine) and Its Major Metabolites in Mice: A Liquid Chromatography/Mass Spectrometry Analysis of Urine, Plasma, and Tissue Levels. Drug Metab Dispos 37:776–786; doi:10.1124/dmd.108.024927.

113. Rusiecki JA, De Roos A, Lee WJ, Dosemeci M, Lubin JH, Hoppin JA, et al. 2004. Cancer Incidence Among Pesticide Applicators Exposed to Atrazine in the Agricultural Health Study. JNCI Journal of the National Cancer Institute 96:1375–1382; doi:10.1093/jnci/djh264.

114. Russart KLG, Rhen T. 2016. Atrazine alters expression of reproductive and stress genes in the developing hypothalamus of the snapping turtle, Chelydra serpentina. Toxicology 366–367:1–9; doi:10.1016/j.tox.2016.08.001.

115. Semchuk M, Love J, Lee G. 1992. Parkinson's disease and exposure to agricultural work and pesticide chemicals. Neurology 42: 1328–1335.

116. Staal YCM, Meijer J, van der Kris RJC, de Bruijn AC, Boersma AY, Gremmer ER, et al. 2018. Head skeleton malformations in zebrafish (Danio rerio) to assess adverse effects of mixtures of compounds. Arch Toxicol 92:3549–3564; doi:10.1007/s00204-018-2320-y.

117. Stayner LT, Almberg K, Jones R, Graber J, Pedersen M, Turyk M. 2017. Atrazine and nitrate in drinking water and the risk of preterm delivery and low birth weight in four Midwestern states. Environmental Research 152:294–303; doi:10.1016/j.envres.2016.10.022.

118. Stoker TE. 2002. The Effects of Atrazine Metabolites on Puberty and Thyroid Function in the Male Wistar Rat. Toxicological Sciences 67:198–206; doi:10.1093/toxsci/67.2.198.

119. Suhl J, Golla V, Rinsky JL, Hopenhayn C. 2016. Atrazine in Kentucky Drinking Water. Journal of Environmental Health 79: 7.

120. Tegelenbosch RAJ, Noldus LPJJ, Richardson MK, Ahmad F. 2012. Zebrafish embryos and larvae in behavioural assays. Behav 149:1241–1281; doi:10.1163/1568539X-00003020.

121. TeSlaa JJ, Keller AN, Nyholm MK, Grinblat Y. 2013. Zebrafish Zic2a and Zic2b regulate neural crest and craniofacial development. Developmental Biology 380:73–86; doi:10.1016/j.ydbio.2013.04.033.

122. tfacts153.pdf.

123. Ton C, Lin Y, Willett C. 2006. Zebrafish as a model for developmental neurotoxicity testing. Birth Defect Res A 76:553–567; doi:10.1002/bdra.20281.

124. Toufexis D, Rivarola MA, Lara H, Viau V. 2014. Stress and the Reproductive Axis. J Neuroendocrinol 26:573–586; doi:10.1111/jne.12179.

125. US EPA O. 2015. Atrazine Monitoring Program Data and Results. US EPA. Available: https://www.epa.gov/ingredients-used-pesticide-products/atrazine-monitoring-program-data-and-results [accessed 14 February 2021].

126. Walker BS, Kramer AG, Lassiter CS. 2018. Atrazine affects craniofacial chondrogenesis and axial skeleton mineralization in zebrafish (*Danio rerio*). Toxicol Ind Health 34:329–338; doi:10.1177/0748233718760419.

127. Walker M, Kimmel C. 2007. A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnic & Histochemistry 82:23–28; doi:10.1080/10520290701333558.

128. Waller SA, Paul K, Peterson SE, Hitti JE. 2010. Agricultural-related chemical exposures, season of conception, and risk of gastroschisis in Washington State. American Journal of Obstetrics and Gynecology 202:241.e1-241.e6; doi:10.1016/j.ajog.2010.01.023.

129. Wang F, Ji R, Jiang Z, Chen W. 2014. Species-dependent effects of biochar amendment on bioaccumulation of atrazine in earthworms. Environmental Pollution 186:241–247; doi:10.1016/j.envpol.2013.12.012.

130. Wang Q, Xie S. 2012. Isolation and characterization of a high-efficiency soil atrazine-degrading Arthrobacter sp. strain. International Biodeterioration & Biodegradation 71:61–66; doi:10.1016/j.ibiod.2012.04.005.

131. Weber GJ, Sepúlveda MS, Peterson SM, Lewis SS, Freeman JL. 2013. Transcriptome Alterations Following Developmental Atrazine Exposure in Zebrafish Are Associated with Disruption of Neuroendocrine and Reproductive System Function, Cell Cycle, and Carcinogenesis. Toxicological Sciences 132:458–466; doi:10.1093/toxsci/kft017.

132. Wetzel LT, Luempert LG, Breckenridge CB, Tisdel MO, Stevens JT, Thakur AK, et al. 1994. Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats. Journal of Toxicology and Environmental Health 43:169–182; doi:10.1080/15287399409531913.

133. World Health Organization. Atrazine and its metabolites in drinking water. Geneva. WHO 2010.

134. Wiegand C, Krause E, Steinberg C, Pflugmacher S. 2001. Toxicokinetics of Atrazine in Embryos of the Zebrafish (Danio rerio). Ecotoxicology and Environmental Safety 49:199–205; doi:10.1006/eesa.2001.2073.

135. Winchester PD, Huskins J, Ying J. 2009. Agrichemicals in surface water and birth defects in the United States. Acta Paediatrica 98:664–669; doi:10.1111/j.1651-2227.2008.01207.x.

136. Wirbisky SE, Sepúlveda MS, Weber GJ, Jannasch AS, Horzmann KA, Freeman JL. 2016a. Embryonic Atrazine Exposure Elicits Alterations in Genes Associated with Neuroendocrine Function in Adult Male Zebrafish. Toxicological Sciences 153:149–164; doi:10.1093/toxsci/kfw115.

137. Wirbisky SE, Weber GJ, Schlotman KE, Sepúlveda MS, Freeman JL. 2016b. Embryonic atrazine exposure alters zebrafish and human miRNAs associated with angiogenesis, cancer, and neurodevelopment. Food and Chemical Toxicology 98:25–33; doi:10.1016/j.fct.2016.03.027.

138. Wirbisky SE, Weber GJ, Sepúlveda MS, Lin T-L, Jannasch AS, Freeman JL. 2016c. An embryonic atrazine exposure results in reproductive dysfunction in adult zebrafish and morphological alterations in their offspring. Scientific Reports 6; doi:10.1038/srep21337.

139. Wirbisky SE, Weber GJ, Sepúlveda MS, Xiao C, Cannon JR, Freeman JL. 2015a. Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis. Toxicology 333:156–167; doi:10.1016/j.tox.2015.04.016.

140. Wirbisky SE, Weber GJ, Sepúlveda MS, Xiao C, Cannon JR, Freeman JL. 2015b. Developmental origins of neurotransmitter and transcriptome alterations in adult female zebrafish exposed to atrazine during embryogenesis. Toxicology 333:156–167; doi:10.1016/j.tox.2015.04.016.

141. Xia J, Lin J, Zhu S-Y, Du Z-H, Guo J-A, Han Z-X, et al. 2016. Lycopene protects against atrazine-induced hepatotoxicity through modifications of cytochrome P450 enzyme system in microsomes. Experimental and Toxicologic Pathology 68:223–231; doi:10.1016/j.etp.2015.12.004.

142. Xia J, Qin L, Du Z-H, Lin J, Li X-N, Li J-L. 2017. Performance of a novel atrazine-induced cerebellar toxicity in quail (Coturnix C. coturnix): Activating PXR/CAR pathway responses and disrupting cytochrome P450 homeostasis. Chemosphere 171:259–264; doi:10.1016/j.chemosphere.2016.12.075.

143. Zon LI, Peterson RT. 2005. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov 4:35–44; doi:10.1038/nrd1606.

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation studied the effects of developmental exposure to the herbicide atrazine (ATZ) in the immediate (F0) and the subsequent generation (F1) as part of the Developmental Origins of Health and Disease (DOHaD) paradigm. These studies took advantage of the strengths of the zebrafish as a model system for translation to human health. To characterize developmental consequences, -omics, morphological structures, and neurobehavioral alterations were assessed (Figure 4-1). Results from these studies characterized the immediate and long term impacts to a single developmental exposure. Results from these findings help define health risks to developmental ATZ exposure immediately, and in subsequent generations with contaminated potable water sources.

The first study focused on the effects of immediate developmental ATZ exposure. ATZ's metabolites were assessed to determine if the same major metabolites found in mammals are also found in humans. ATZ, desethyl atrazine (DEA), deisopropyl atrazine (DIA), and didealkyl atrazine (DACT) are the major metabolites found in mammals and through targeted metabolomics were also found present in larval zebrafish tissue, a novel finding in a non-mammalian model. ATZ and DACT were the most abundant with quantification in the 300, 1000, 3000, and 10000 ppb treatment groups and with dose increasing as exposure concentration increased. DEA and DIA were less abundant with quantification only in the 3000 and 10000 treatment groups, but still had a dose increase when exposure concentration increased. This finding shows that larval zebrafish metabolize ATZ into the same major metabolites as mammals, further validating zebrafish as a model organism for ATZ toxicity studies. No change in total length, head length, head width, brain length, or ratios were found in any ATZ exposed groups at 120 hpf, suggesting that morphological developmental alterations are not present at this time point. Behavioral findings show dose dependent behavioral alterations with the 0.3 ppb treatment group experiencing hyperactivity in total distance moved in the first light phase, and centerpoint movement for the first and second dark phases. Larvae in the 30 ppb treatment group were hypoactive for the second and third dark phases for total distance moved. These findings suggest that ATZ is being metabolized in the larval fish tissue, leading to altered neurobehavioral outcomes with implication for risks of neurotoxicity in humans.

The second study focused on health outcomes of progeny from parents that were exposed developmentally to ATZ to determine if the same health risks are present compared to the initially exposed generation. Proteomic analysis found 70 uniquely altered proteins among the parentally exposed larvae, which were involved in a variety of processes, including DNA repair, cartilage formation, and neural development and behavioral outcomes. To further assess these changes, morphological parameters were assessed with head length and head length to total length ratio being larger in the 0.3 and 30 ppb treatment groups. Since alterations were found in proteins related to cartilaginous structures, alcian blue and alizarin red costaining was used to assess cartilage and skeletal development. Alterations associated with a decrease in cartilaginous structures were found in the 30 ppb group and for PQ length in all treated groups. A decrease in otoliths and an increase in notochord to jaw length was also found when assessing skeletal structures. Phasic behavior was also altered in all phases for total distance and time spent moving for the 0.3 and 30 ppb F1 larvae. Taken together, these results suggest that parental exposure alters protein expression leading to delayed cartilage development and ossification of craniofacial structures, as well as behavioral hyperactivity. In addition, this study suggests that parental exposure not only results in health consequences in the subsequent generation, but that the health consequences at the same time point may differ from parental health outcomes. Further study is needed to determine if skeletal ossification deficits are seen later in life or potentially lead to bone metabolic disorders, and potentially sensory disorders related to hearing and balance for the otoliths.

Collectively, these results demonstrate zebrafish as a biomedically relevant model to investigate the legacy of ATZ exposure in a multigeneration study noting persistent impacts to developing morphological structures and developmental neurotoxicity. It will be important to further investigate mechanisms of multigeneration ATZ developmental neurotoxicity to better understand translation of the findings reported in this thesis to human health.

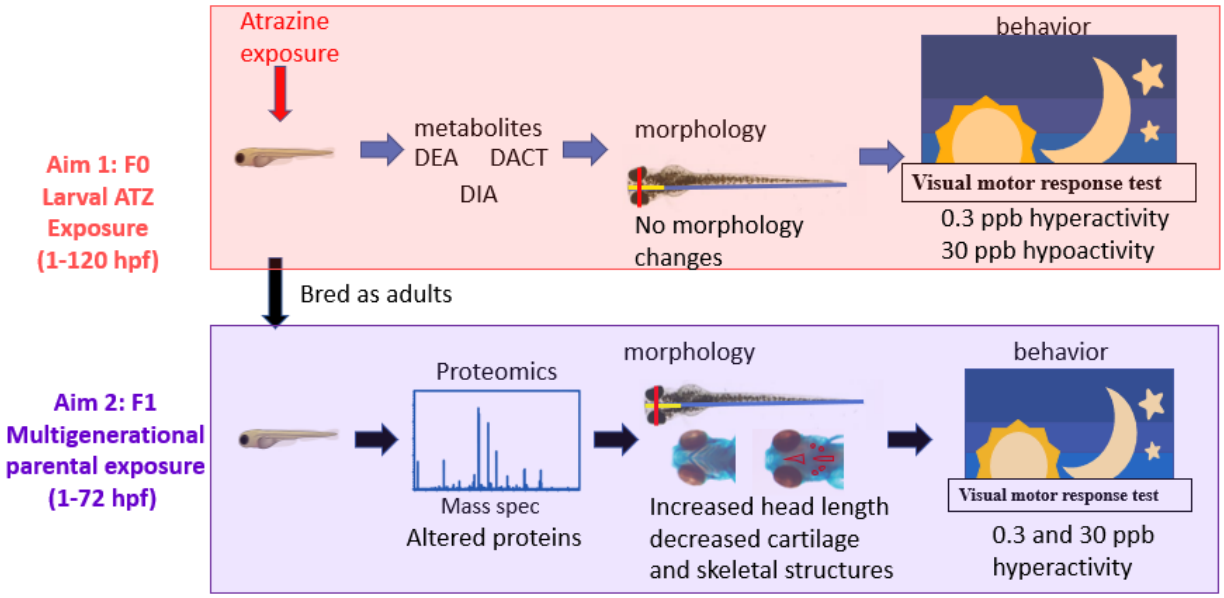


Figure 4-1. Overall results summary of thesis. Chapter 2 (aim 1) reports that targeted metabolomic analysis found that zebrafish produce the same major ATZ metabolites as mammals in the exposed generation. The visual motor response test at 120 hpf detected hyperactivity in larvae in the 0.3 ppb treatment group and hypoactivity in the 30 ppb treatment group, but no alterations in morphology. In Chapter 3 (aim 2), proteomic analysis in the subsequent generation (F1) identified differential expression associated with neurological development and disease and organ and organismal morphology, specifically the skeletomuscular system. Head length and the ratio of head length to total length was significantly increased in the F1 in the 0.3 and 30 ppb ATZ treated groups. Craniofacial morphology was assessed based on molecular pathway analysis and revealed decreased cartilaginous structure size, decreased surface area and distance between saccular otoliths, and a more posteriorly positioned notochord, indicating delayed ossification. The visual motor response assay showed hyperactivity in the F1 of the 30 ppb treatment group for total distance and time spent moving in the F1 of the 0.3 and 30 ppb treatment groups for all phases. Collectively, these results demonstrate persistent ATZ developmental toxicity in this multigeneration study.

VITA

JANIEL AHKIN CHIN TAI

www.linkedin.com/in/janielact/

EDUCATION

Purdue University, West Lafayette, IN (2016-2021)

- **PhD candidate**
  - Rotational life sciences PhD program http://www.purdue.edu/gradschool/pulse/
  - Admitted into **Health Sciences Program (HSCI)** with a focus on toxicology
- **Post bac Researcher**
  - **Purdue PREP**: Post-baccalaureate research education program in Biomedical Sciences
    - One-year research program that provides biomedical research training for entry into competitive PhD programs https://vet.purdue.edu/education/PREP-program.php

Florida International University (FIU), Miami, FL (2011- 2016)

- Bachelors of Science in Biology **(2016**)
- **QBIC**: Quantifying Biology in the Classroom **(2011- 2015)**
  - Hands on program that to approach concepts applied in research (http://qbic.fiu.edu/)

RESEARCH EXPERIENCE

Industry Experience: PepsiCo (June2020-August 2020): Scientific affairs intern

- Used Toxicology background and risk assessment to assess and recommend updates for an internal Generally Regarded as Safe (GRAS) document
- Cross functional team project across marketing, HR, and finance to create an ad campaign

Dr. Jennifer Freeman Lab (May 2018-current) PhD candidate

- Using morphology, behavior, metabolomics, proteomics and epigenetics to assess transgenerational atrazine exposure in *Danio rerio*
- Published a book chapter, a review and first author manuscript, with several to be published later

PULSe Rotations (September 2017-April 2018)

- Dr. Marisol Sepulveda lab: Determined whether organ weights in *Ambystoma tigrinum* were affected by exposure to perfluorinated chemicals
- Dr. Angeline Lyon Lab: Learned cell culture, protein preparation/purification, and chromatography techniques to generate, clean and quantify Phospholipase C Ɛ for structure characterization
- Dr. Robert Stahelin lab: learned cell culture and how to perform mini and maxi preps
- Dr. Jennifer Freeman lab: used qPCR to understand whether perfluorinated compounds alter gene expression in *Danio rerio*

Dr. Estuardo Robles Lab (June2017- July 2017) Purdue AGEP summer bridge

- Used confocal microscopy to identify retinal ganglion cells in *Danio rerio*
- Performed statistics, presented a poster and oral presentation

Dr. Russell P. Main Lab (June 2016-2017): Purdue PREP/Post baccalaureate

- Created a protocol for isolating cancellous bone from lumbar vertebrae
- Used μCT to study the role of estradiol and estrogen receptors in skeletal growth and remodeling
- Performed statistics to evaluate surgery and genotypic effects on bone

Dr. Christopher A. Blanar Lab (February 2015- May 2016): NSU Collaboration

- Established collaboration for training on necropsies and parasite identification in *Gambusia*

Dr. Philip K. Stoddard Lab (2012- May 2016): FIU MARC Fellow

- Capture and experimentation on sexual selection and parasitism in *Gambusia sp.*
- Managed budgets, experimental design, literature reviews, proposals, and IACUC permits
- Experiment on diet and survival rate in developing *brachyhypopomus gauderio*
- Successfully started a breeding program for albino *brachyhypopomus gauderio*

Dr. Tyrone B. Hayes Lab (June - August 2015): UC Berkeley NSF REU

- Experiment on estrogen metabolism and gene upregulation in male *xenopus laevis* including data collection and analysis, chemical stocking, experimental design and benchwork techniques

AWARDS/HONORS/FELLOWSHIPS

- **AGEP Scholar Award (2019-2021):** A semesterly program with professional development and mentorship for fall, spring and summer ($1000/semester)
- **Scientist Mentoring & Diversity Program (SMDP) (2019):** A national program seeking to diversify and prepare PhDs and postdocs for careers in industry through mentorship and networking. 1 of 36 of scholars chosen for the 2019 cohort.
- **1st place in HSCI Research Blitz (2019):** 3-minute oral research synopsis for the Health Sciences department. Won a $1000 travel grant.
- **AGEP Summer Bridge Program (2017):** A program that provides funding and mentoring for an eight-week summer research experience in a STEM related field

- **Purdue Doctoral fellowship (2017):** A 4-year award package that has 2 years of stipend support from the Graduate school and two additional years of support from the major professor ($24,000/year)
- **MARC U* STAR** (Maximizing Access to Research Careers- Undergraduate Student Training for Academic Research) **(2014- 2016)** **http://mbrs.fiu.edu/MARCUSTAR/**
  - **NIH** funded fellowship that provides opportunities to become competitive researchers through mentorship, funds, workshops, and conference opportunities
- Winner of the Guy Harvey's Save Our Gulf Essay and Art contest. Won an original signed print. **(2011)**

TRAVEL GRANTS

- **HSCI Travel Grant (2019):** A travel grant to attend a research conference($1000)
- **Ismail Travel Grant (2018):** A competitive travel grant for students to present interdisciplinary research at a conference ($700)
- **Annual Biomedical Research Conference for Minority Students (ABRCMS) Travel Grant (2016)**
  - A full travel grant to present a poster and attend the five-day conference in Tampa ($1500)
- **National Organization for the Professional Advancement of Black Chemists and Chemical Engineers** (**NOBBChE) Travel Grant (2016)**-received but declined

PUBLICATIONS

- Ahkin Chin Tai JK, Horzmann KA, Franco J, Jannasch AS, Cooper BR, Freeman JL. 2021. Developmental atrazine exposure in zebrafish produces the same major metabolites as mammals along with altered behavioral outcomes. Neurotoxicol Teratol 106971; doi:10.1016/j.ntt.2021.106971.

- Ahkin Chin Tai, JK, Freeman, JL. Developmental Neurotoxicity of the Herbicide Atrazine. 2021. In: Diagnosis, Management and Modelling of Neurodevelopmental Disorders: The Neuroscience Of Development. Academic Press. 219-228; https://doi.org/10.1016/B978-0-12-817988-8.00019-1.
- **Ahkin Chin Tai, JK,** Freeman JL. Zebrafish as an integrative vertebrate model to identify miRNA mechanisms regulating toxicity. 2020. Toxicology reports. 7: 559-570

ORAL/ POSTER PRESENTATIONS

- **Chin Tai, J.** Horzmann, K. Freeman, J. Metabolism, morphological effects and behavioral alterations following a developmental atrazine exposure in zebrafish. SOT. 2021. Virtual.

- **Chin Tai, J.** Horzmann, K. Freeman, J. Metabolism, morphological effects and behavioral alterations following a developmental atrazine exposure in zebrafish. OVSOT. Nov. 6. 2020. Virtual.
- **Chin Tai,J.,** Horzmann, K., Freeman, J. Adverse developmental effects in progeny of zebrafish that were exposed to atrazine during embryogenesis.
    - C4E mixer. Oct. 17. 2019. PMU East Faculty lounge. West Lafayette, IN.
    - PULSe Retreat. Aug. 1. 2019. PMU North Ballroom. West Lafayette, IN.
    - *HSCI research blitz*. Apr. 5.2019. Indianapolis Zoo HSCI retreat. Indianapolis, IN.
    - *Society of Toxicology*. Mar. 12 2019. Baltimore Convention Center. Baltimore, MD.
    - *Health and Disease Poster Session*. Feb. 28 2018. Purdue University. West Lafayette, IN.
    - *Ohio Valley SOT*. Nov. 2018. University of Louisville. Louisville, KY.
    - *PULSe Retreat*. Aug. 2019. Purdue University, West Lafayette, IN.
- **Chin Tai, J.,** Rugema, Y., Lyon, A. Protein purification of the Rap1A/PLC epsilon complex. *PULSe reception*. Mar. 2018. STEW center West Lafayette, IN.
- **Chin Tai, J.,** Demarco E, Robles E. A dedicated visual pathway connects photoreceptors directly to the brain*. SROP summer research*, Jul. 28, 2017. STEW center, West Lafayette, IN
- **Chin Tai, J.,** Main RP. Interactive role of sex hormones and osteocyte-specific ERβ in regulating bone mass.
    - *ABRCMS National conference,* Nov. 11. Tampa Convention Center, Tampa, FL;
    - *BMES Regional conference,* Nov. 4. I hotel conference center, Champaign, IL
    - *Purdue summer research poster session*, Jul. 2016. Purdue University, West Lafayette, IN
    - *College of Vet Med poster session*, Jul. 26 2016. Purdue University, West Lafayette, IN
- **Chin Tai, J.** Tyrone Hayes. Estradiol metabolites and gene expression in African clawed frogs.
    - *MARC U* STAR Mini symposium*, Oct. 2015. FIU, Miami, FL.
    - *NSF REU Berkeley Symposium*, Aug. 13 2015. UC Berkeley, Berkeley, CA.

  Chin Tai, J. Sexual selection in *Gambusia*. *MARC U* STAR Mini Symposium,* Oct. 2014.

  FIU, Miami, FL.

TEACHING

- HSCI 20200 TA:
    - I help proctor and grade quizzes and exams, I run exam review sessions, and help class run smoothly when Dr. Freeman is not able to make it to class.
- HSCI 5800 TA
    - I grade homework assignments and help proctor exams for this class

STUDENT ORGANIZATIONS/PROFESSIONAL DEVLOPMENT

- **Social Coordinator for PULSe GSO (Aug. 2018-2019)**
  - Manage events, transport, catering and budgets for social events
  - Submit Event planning forms (EPFs) and other administrative forms for events
- **Graduate Assistant (Dec. 2017-May 2018)**
  - Liaison between graduate students and OIGP administration
  - Escorted interviewing students
  - Ran the social media page for the OIGP office
  - Consolidated information for on campus facilities, local activities, and health
- **Graduate student Panel (Nov. 4 2017|Nov 9, 2017|Jun 30, 2021)**
  - Discussed opportunities for gap years before grad school and information on internships
  - Spoke on the diversity visitation program panel about grad school and opportunities
- **Science Fair (Mar 3, 2017|Mar 2, 2018)**
  - Volunteered as a judge to give feedback/encouragement to elementary and middle school students' science fair projects and for future STEM involvement
- **Adopt a Freshman (2015-2016)**
  - Coached and advised QBIC freshman in time management, effective learning and career building opportunities in the field
- **The Transition: High school to College (Mar 9, 2015)**
  - Spoke about research and STEM opportunities at MAST high school in Key Biscayne
- **President of Young Science (2014- 2015)**
  - Science communication club that involved the public in science via games and discussions
  - Founded club, organized events, designed displays, established networking for public outreach and events

OUTREACH ACTIVITIES

- **Letters to a Pre-Scientist (LPS) 2020-2021**
  - Pen pal program geared towards getting students at title one elementary schools paired with a science professional to learn science careers, opportunity and accessibility in higher education
- **Open Kitchen Night (Feb 12, 2017)**
  - Volunteered at the Lafayette Transitional Home Center (LTHC) preparing meals and interacting with community members that are homeless
- **Dr. Seuss Night (March 4, 2015)**
  - Decorated classroom and ran activities at Palm Cove Elementary to promote reading

- **Scientific Illustrator (2014- 2016)**
    - Designed logo for Anya Goldina's Research lab at Elizabethtown college hormones and crayfish behavior http://facultysites.etown.edu/goldinaa/research/
    - Designed lab logo for Philip Stoddard's electric fish research lab at FIU
- **Science interactive displays**
    - **Chin Tai J**., Ho B., How J., Mercado A. A sensory journey; an interactive display/talk. *Technology Fair,* Oct. 2014. South Regional Library, Pembroke Pines, FL.
    - **Chin Tai, J.,** Smatrakaleva, K., Martinez-Montalvo, A. A sensory journey; an interactive display/talk. *STEAM Event,* Jul. 2014. Southwest Regional Library, Pembroke Pines, FL.
    - **Chin Tai, J.** How do you perceive the world, a sensory journey, *21st Century CCLC,* Jul. 2014. Riverland Elementary, Ft. Lauderdale, FL.
    - **Chin Tai, J.,** Goldina A., Roach, A., Dos Santos, J. Electric signaling in *Brachyhypopomus pinnicaudatus*. *Brain Fair,* Mar. 2012. Miami Museum of Science, Miami, FL
- **Teacher's Aid at a title one school (2007-2016)**
    - Designed classroom themes to create a personalized atmosphere for students
    - Organized paperwork and classroom library
    - Helped guide students in science project concepts and procedures