PARENTALLY EXPOSED ZEBRAFISH LARVAE HAVE ALTERED CRANIOFACIAL MEASUREMENTS

Multigeneration Developmental Atrazine Toxicity

Abstract

Atrazine is a herbicide used throughout the midwestern United States to prevent broadleaf weeds in crops. The U.S. Environmental Protection Agency (EPA) has set the maximum contaminant level at 3 ppb (μg/L) in drinking water. Atrazine is an endocrine disrupter interfering with the function of hormones and disrupting normal physiology and homeostasis throughout development and the life course of an organism. The zebrafish model was used to test the hypothesis that an embryonic parental atrazine exposure will cause modifications in morphology in developing offspring. AB adult zebrafish were bred. Embryos were collected and exposed to atrazine concentrations of 0, 0.3, 3, or 30 ppb from 1 to 72 hours post fertilization (hpf; the end of embryogenesis). Atrazine exposure ceased at 72 hpf and larvae were grown into adulthood in aquaria water (F0). Atrazine F0 adult zebrafish were then bred within their treatment group. Their embryos were collected and placed in petri dishes in aquaria water until 120 hpf. At 120 hpf, larvae were collected for morphological analysis including general morphology measurements and co-staining with alcian blue and alizarin red for cartilage and skeletal assessments. Head length and ratio of head length to total length was significantly increased in the F1 0.3 and 30 ppb atrazine groups ($p < 0.05$). The posteriorly positioned notochord indicated delayed ossification and skeletal growth. These findings signify that a single embryonic parental exposure leads to changes in craniofacial development in their offspring.

Keywords

atrazine, cartilage, craniofacial, development, embryo, herbicide, morphology, multigeneration, skeletal, zebrafish

https://doi.org/10.7771/2158-4052.1642

Student Author

Isabelle Akoro graduated in May 2023. She majored in Biomedical Health Sciences with a minor in Psychology. She worked as an undergraduate researcher in Dr. Freeman's laboratory starting in the fall of 2019 and was involved in projects investigating the neurological and craniofacial effects of atrazine. After graduation, Isabelle plans on applying to physician assistant school.

Mentors

Jennifer L. Freeman is a Professor of Toxicology in the School of Health Sciences. Research efforts in the Freeman Environmental Molecular Toxicology Laboratory are defining the underlying genetic and epigenetic mechanisms of toxicity of environmental stressors in the developmental origins of health and disease (DOHaD) paradigm. Research projects are focused on pesticides, metals, radiation, PFAS, and other legacy and emerging contaminants. These projects are defining the immediate adverse impacts of a developmental exposure, the lasting adverse impacts of this developmental exposure throughout the lifespan, and/or the analysis of subsequent generations linking genetic, epigenetic, and phenotypic assessments using the zebrafish vertebrate model system. These studies are investigating a developmental origin of adult disease pathogenesis with a specific focus on neuroendocrine dysfunction, neurodegenerative diseases, and cancer with a goal of understanding the role of exposure to the environmental chemicals in these adverse health outcomes.

Sydney Stradtman is a third-year PhD candidate in Toxicology working in Dr. Jennifer Freeman's laboratory. She began her graduate studies in August 2020 following graduating with a bachelor's in Biomolecular Sciences and a minor in Environmental Sciences. Her project focuses on investigating a mechanism of atrazine toxicity using the zebrafish model. She utilizes environmentally relevant exposures to atrazine in developing zebrafish and examines the effects these exposures have on the neuroendocrine system both early in life as well as into adulthood. Aside from her research, she serves on her school's Graduate Student Organization Executive Committee and mentors multiple undergraduate students interested in taking on independent projects in toxicology research.

INTRODUCTION

Atrazine is the second most commonly used herbicide throughout the United States to prevent broadleaf weeds in crops (LeBaron et al., 2008). Heavy application and low binding efficiency to soils causes atrazine to run off and contaminate potable water sources. Atrazine also has a long half-life and persists in the environment (Guzzella et al., 2006; Meffe & de Bustamante, 2014). Based on these environmental and chemical characteristics, the most common human exposure route of atrazine is through drinking contaminated water. Because of this, the European Union banned the use of atrazine in 2003 (Bethsass & Colangelo, 2006). As of now, the current maximum contaminant level (MCL) of atrazine set by the U.S. Environmental Protection Agency (EPA) is 3 parts per billion (ppb; µg/L), but concentrations can exceed this limit in drinking water sources.

Atrazine is classified as an endocrine-disrupting chemical, meaning that it interferes with the function of hormones, disrupts homeostasis, and alters reproductive cycling and development (Cooper, 2000; Russart & Rhen, 2016). Epidemiological studies suggest exposure to atrazine during gestation increases the likelihood of birth defects and premature birth defects (Agopian et al., 2013; Migeot et al., 2013; Stayner et al., 2017; Waller et al., 2010). A study conducted in Texas found that women exposed to medium levels of residential atrazine had a higher risk of birthing offspring with male genital malformations (Agopian et al., 2013). Another study conducted in France found that women exposed to atrazine in their second trimester by drinking contaminated water were more likely to birth offspring that were small for their gestational age (Migeot et al., 2013). Furthermore, an assessment conducted across midwestern states in America found a higher risk of preterm birth (Stayner et al., 2017), while a study in Washington discovered that children of mothers who lived fewer than

25 kilometers from a location with high atrazine concentrations had a higher likelihood of having gastroschisis (Waller et al., 2010).

Although adverse health outcomes with direct atrazine exposure have been documented, few multigenerational and transgenerational studies have been completed to determine if exposure affects subsequent generations and whether the effects are comparable. Furthermore, some studies follow 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 both into adulthood and even in subsequent generations (Heindel & Vandenberg, 2015). A study of rats exposed to high concentrations supports the generational effects of atrazine exposure. Results found reductions in body weight, increased testis size, and early onset puberty in males and their offspring (DeSesso et al., 2014; McBirney et al., 2017). Due to protein abnormalities in meiosis and changed gene expression in various organs, it was shown that mice exposed to atrazine during development and the subsequent generation had lower-quality sperm (Hao et al., 2016). Reduced sperm motility and count were also seen in medaka (*Oryzias latipes*) exposed to atrazine during the first 12 days of life (Cleary et al., 2019).

There are few studies that have addressed the effects of atrazine on chondrogenesis or vertebral mineralization in vertebrates. Previous work performed in frogs investigated atrazine's effect on cranial cartilage formation (Lenkowski & McLaughlin, 2010) and found malformations. Another study exposing chicken embryos to atrazine found neural tube defects and craniofacial hypoplasia (Joshi et al., 2013). Previous studies using the zebrafish model with embryonic atrazine exposure found 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 & Freeman, 2018; Wirbisky et al., 2015). The current project was modeled after another research project that also focused on the effects of atrazine exposure on zebrafish craniofacial development but was completed in the exposed generation at concentrations ranging from 0.1 to 5 μM (equivalent to 21.5 to 1,078.4 ppb) (Walker et al., 2018). Cartilaginous structures measured were ceratohyal cartilage (CH) angle, palatoquadrate cartilage (PQ) length, Meckel's length, Meckel's angle, the angle between PQ and CH, the angle between PQ and Meckel's angle, and the distance between CH and Meckel's structure. Significant variations in craniofacial cartilage components were seen after treatment with atrazine at concentrations as low as 0.1 μM (21.5 ppb), and 2 μM (431 ppb) treatment concentrations and above were associated with lower survival and elevated heart rates. A delayed mineralization of the vertebrae was also observed in fish treated with 1 μM (215 ppb) atrazine. Greater atrazine concentrations resulted in severe craniofacial deformities and lowered hatching rates (Walker et al., 2018). The zebrafish exposed in this study were first generation and based on these findings, we expected an embryonic atrazine exposure in zebrafish would result in multigenerational effects. Our hypothesis was that an embryonic parental atrazine exposure caused modifications in morphology in developing zebrafish progeny similarly to the parental generation.

Zebrafish have become an established model for toxicology and environmental health research because they have many strengths. Zebrafish have a 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 & Freeman, 2018). In addition, zebrafish are an established model for bone and bone disease modeling, making them ideal for learning how atrazine alters craniofacial structures (Carnovali et al., 2019; Kwon et al., 2019). Furthermore, it was recently discovered that zebrafish metabolize atrazine similarly to mammals, producing the same major metabolites and increasing confidence in them as a model for studying atrazine toxicity (Ahkin Chin Tai et al., 2021).

MATERIALS AND METHODS

Adult zebrafish (wild-type AB strain, *Danio rerio*) were bred to create embryos in breeding tanks. Their embryos were collected at 1 hour post fertilization (hpf), rinsed, and randomly assigned to treatment groups of 0, 0.3, 3, or 30 ppb atrazine (F0 generation). Concentrations of atrazine treatment water were confirmed using an atrazine immunoassay approved by the U.S. EPA following the manufacturer's protocol (Abraxis). Embryos were

exposed via immersion in groups of 50 in petri dishes and held in the atrazine treatment until the end of embryogenesis (72 hpf). Next, all embryos were rinsed to rid them of the atrazine treatment and left to grow into adulthood in aquaria water. No changes in survival rates were observed at these exposure concentrations, which is in agreement with our past studies (Ahkin Chin Tai et al., 2021). In addition, no differences in sex ratios were observed in the F0 adults similar to our past studies within this same exposure regimen (Wirbisky et al., 2015). Once reaching adulthood, zebrafish (6 months of age) were bred within each treatment group to generate embryos (F1). Their embryos were collected and placed into petri dishes in aquaria water until 120 hpf at which time all major morphological structures are established. No changes in survival rates were observed among the treatment groups. At 120 hpf, the larvae were collected for morphological measurements.

The larvae from the F1 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 to 13 larvae were imaged as subsamples per biological replicate. A total of 8 biological replicates were assessed for a total of 80 to 100 larvae per treatment group. Measurements taken were total body length, head length, head width, and brain length. 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 near the brainstem spinal cord junction (Peterson et al., 2013). In addition, ratios of head length to body length, head width to body length, and brain length to body length were collected for comparison. A Nikon SMZ1500 dissecting microscope was used to collect images via light microscopy.

To determine if craniofacial skeletal or cartilage morphology was altered, zebrafish were stained with alcian blue stain and alizarin red stain. At 120 hpf, larvae were euthanized via anesthetic overdose as described above, then stained with alcian blue and alizarin red. Samples were placed in paraformaldehyde, washed, stained with alcian blue stain overnight, bleached, and then stained again with alizarin red. After staining, fish were imaged on an Olympus SZX16 dissecting microscope, and images were taken using Cellsentry. Both skeletal and cartilaginous structures were then measured using the program ImageJ. Cartilaginous structures measured were the distance between ceratohyal cartilage (CH) and Meckel's structure, palatoquadrate cartilage (PQ) length, Meckel's length, CH angle, Meckel's angle, the angle between PQ and CH, the angle between PQ and Meckel's angle, and jaw length (Figure 1). Skeletal structure measurements included the surface area of the utricular otoliths, the surface area of the saccular otoliths, length between the utricular otoliths, length between the saccular otoliths, parasphenoid, notochord, and jaw to notochord length (Figure 2). Eleven to twenty larvae were imaged as subsamples per biological replicate for each F1 treatment group. Five biological replicates were analyzed, creating 55–75 larvae per treatment group.

For each measurement, a Grubb's outlier test was used to identify outliers within treatment groups. Statistical analysis was then performed using SAS Statistical Software. All data was assessed and confirmed for normality before further statistical processing. Next, an analysis of variance (ANOVA) was completed and was followed by a post hoc least significant difference (LSD) test when a significant ANOVA was observed ($\alpha = 0.05$).

RESULTS

Length measurements were taken at 120 hpf (Table 1). A significant increase in mean head length was seen in the offspring of the 0.3 ppb and 30 ppb treatment groups ($p < 0.05$). However, no other changes were seen in total length, mean brain length, or mean head width in the larvae ($p > 0.05$). There was also an increase in the head length to total body length ratio in the offspring of the 0.3 ppb and 30 ppb treatment groups ($p < 0.05$), meaning head length was greater than what would be expected when compared to body length. No significant changes in brain length to total body length or head width to total body length were observed ($p > 0.05$).

Cartilaginous and skeletal structures were also measured at 120 hpf (as in Figures 1 and 2). For cartilaginous structures, PQ length was found to be shorter in all parentally exposed groups ($p < 0.05$) (Figure 3A).

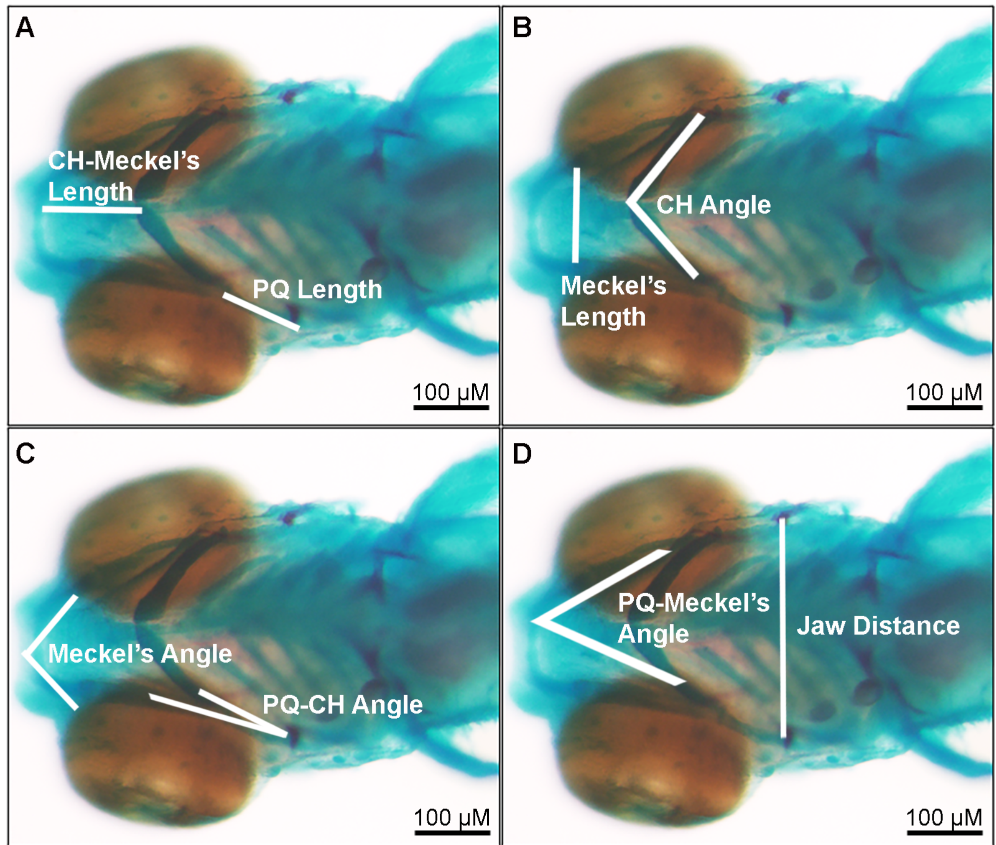

FIGURE 1. Morphology measurements of cartilaginous structures of F1 larvae at 120 hpf. Morphological parameters were measured in ImageJ for CH-Meckel's and PQ length (A), for CH angle and Meckel's length (B), for Meckel's angle and PQ-CH angle (C), and for PQ-Meckel's angle and jaw distance (D). All measurements are indicated as white lines or angles. Scale bar represents 100 µM.

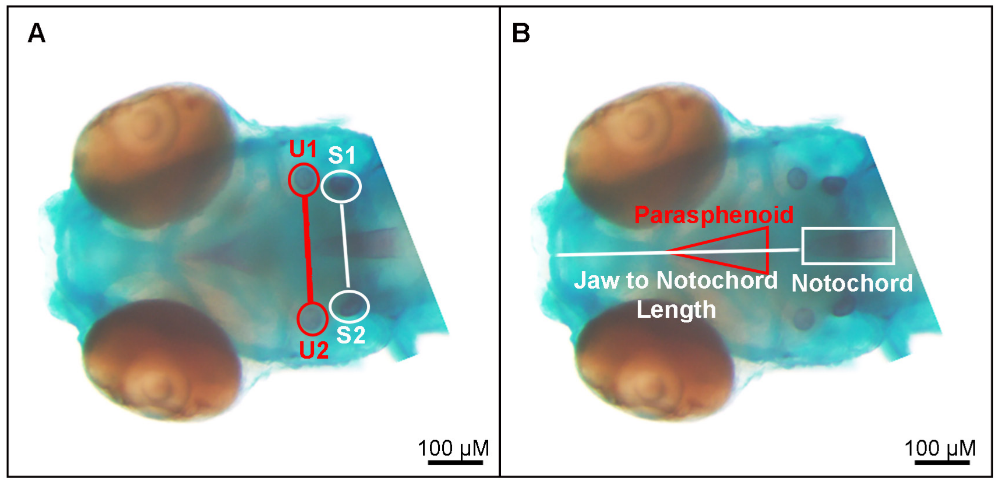

FIGURE 2. Morphology measurements of skeletal structures of F1 larvae at 120 hpf. Surface area of each otolith (U1 and U2: utricular otoliths, red circles; S1 and S2 saccular otoliths, white circles (A). The length between the utricular otoliths (red line) and saccular otoliths (white line) was determined (A). The area for the parasphenoid (red triangle) and notochord were measured (white quadrangle) (B). In addition, the length from jaw to notochord was assessed (white line) (B). Scale bar represents 100 µM.

TABLE 1. 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	4,270 ± 218	767 ± 61	692 ± 59	947 ± 59	0.181 ±0.013	0.163 ± 0.016	0.222 ± 0.013
0.3 ppb	4,311 ± 170	792 ± 45*	683 ± 34	949 ± 52	0.184 ± 0.009*	0.159 ± 0.008	0.221 ± 0.015
3 ppb	4,266 ± 208	780 ± 65	692 ± 51	954 ± 52	0.183 ± 0.013	0.162 ± 0.012	0.224 ± 0.015
30 ppb	4,274 ± 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

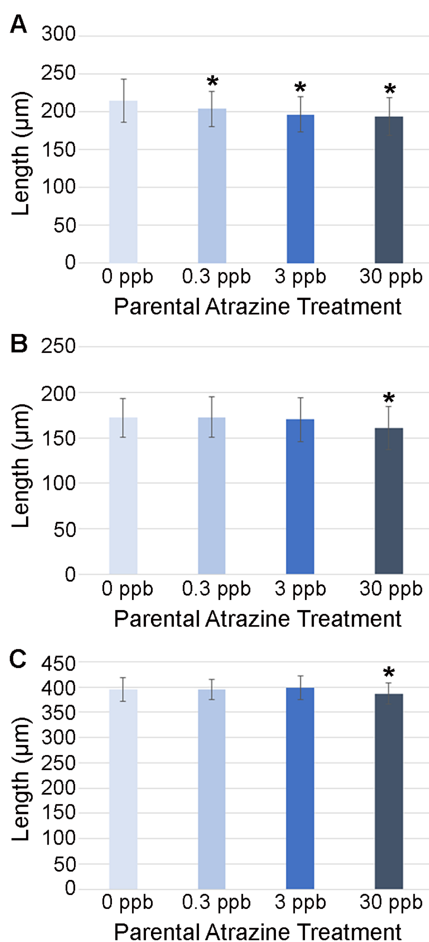

FIGURE 3. Significant alterations in cartilage measurements in F1 larvae at 120 hpf from adult zebrafish exposed to atrazine during embryogenesis. F1 larvae of all atrazine treatment groups had significantly shorter PQ length (A). CH-Meckel's length was shorter for 30 ppb F1 groups (B). Jaw distance was also shorter for 30 ppb F1 larvae (C). For each atrazine 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. *$p < 0.05$. Error bars represent standard deviation.

Furthermore, the CH-Meckel's length (Figure 3B) and jaw distance were smaller in the offspring of those parents exposed to 30 ppb atrazine during embryogenesis ($p < 0.05$) (Figure 3C). No differences were found in the Meckel's angle, PQ-Meckel's angle, CH angle, PQ-CH angle, or Meckel's length ($p > 0.05$). For skeletal structures, a decrease in surface area of the saccular otoliths of the 30 ppb treatment group were observed ($p < 0.05$) (Figures 4A and 4B), but no differences were found in the utricular otolith surface area ($p > 0.05$) (Figures 4C and 4D) (as measured in Figure 2A). The distance between the utricular and saccular otoliths were also measured (as in Figure 2A). The F1 larvae of the 30 ppb treatment group had a decreased distance between the two saccular otoliths ($p < 0.05$) (Figure 4E), but no changes were seen in the distance for utricular otoliths ($p > 0.05$) (Figure 4F). Furthermore, the larvae of the 30 ppb treatment group had a significant posteriorly positioned notochord as measured from the jaw ($p < 0.05$) (Figure 5A), but no difference in the surface area of the notochord (Figure 5B) or parasphenoid (Figure 5C) ($p > 0.05$), suggesting a decrease in ossification.

DISCUSSION

Atrazine is an endocrine-disrupting chemical targeting the neuroendocrine system, causing developmental and reproductive alterations. Multi- and transgenerational studies have shown changes in sperm quality, hyperactivity, and change in weight in subsequent generations (Cleary et al., 2019; McBirney et al., 2017). Morphology measurements were taken to assess impacts to larval growth parameters and brain development. An increase in head length as well as an increase in head length to

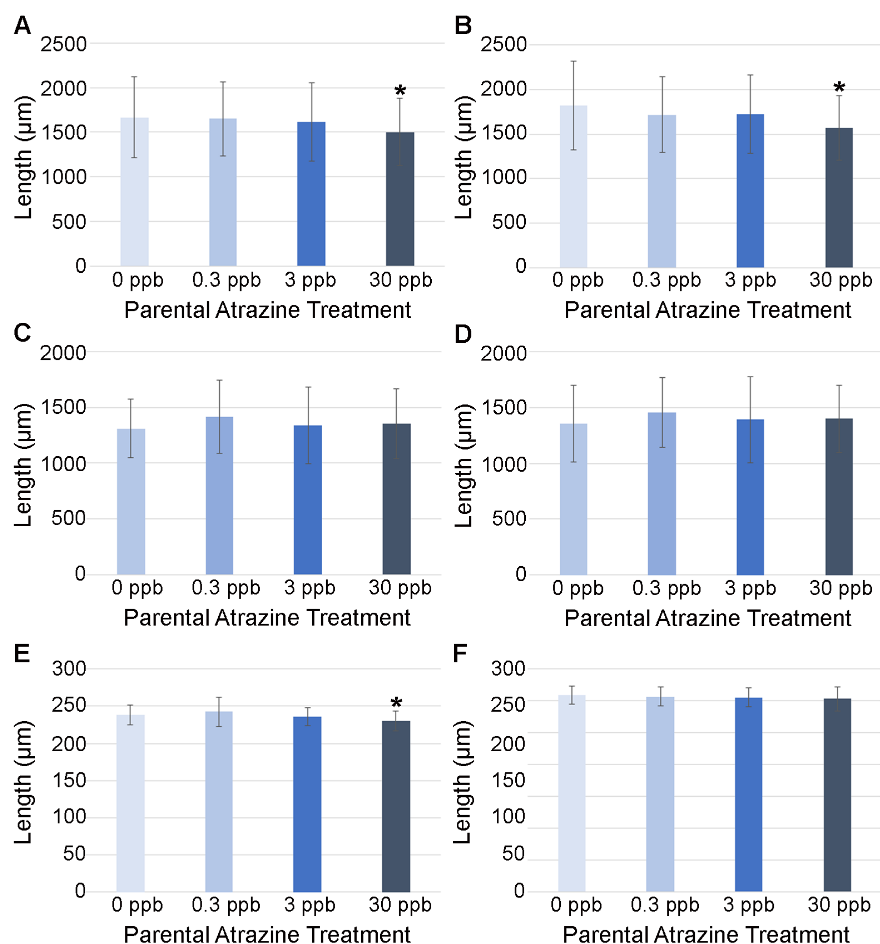

FIGURE 3. Significant alterations in cartilage measurements in F1 larvae at 120 hpf from adult zebrafish exposed to atrazine during embryogenesis. F1 larvae of all atrazine treatment groups had significantly shorter PQ length (A). CH-Meckel's length was shorter for 30 ppb F1 groups (B). Jaw distance was also shorter for 30 ppb F1 larvae (C). For each atrazine 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. *$p < 0.05$. Error bars represent standard deviation.

total body length ratio were observed, indicating head size was larger than would be expected based on total larval length. These findings align with measurements taken at 72 hpf immediately following embryonic atrazine exposure in zebrafish (F0), which showed an increase in head length (Weber et al., 2013). However, in a separate study when F0 zebrafish were exposed to atrazine from 1 to 120 hpf, no significant differences in morphology were found (Ahkin Chin Tai et al., 2021). Following the DOHaD paradigm, embryonic atrazine exposure caused a decrease in body weight at 14 months of age in adult male zebrafish exposed to 3 ppb of atrazine during embryogenesis (1–72 hpf) (Horzmann et al., 2021) and altered brain to body weight ratio in adult female zebrafish aged 8 months old with exposure to atrazine at 0.3 or 30 ppb during embryogenesis (1–72 hpf) (Wirbisky et al., 2015).

Cranioskeletal formation is conserved among vertebrates (Kuratani et al., 1997) and bone remodeling and signaling in zebrafish and humans are similar (Kwon et al., 2019; Mork & Crump, 2015; Paul et al., 2016). Zebrafish and mammals share similar cranial vaults. There is an overlapping of the calvarial bones in adult zebrafish, which form the same suture patterns found in mammals. The two frontal bones are separated from each other by

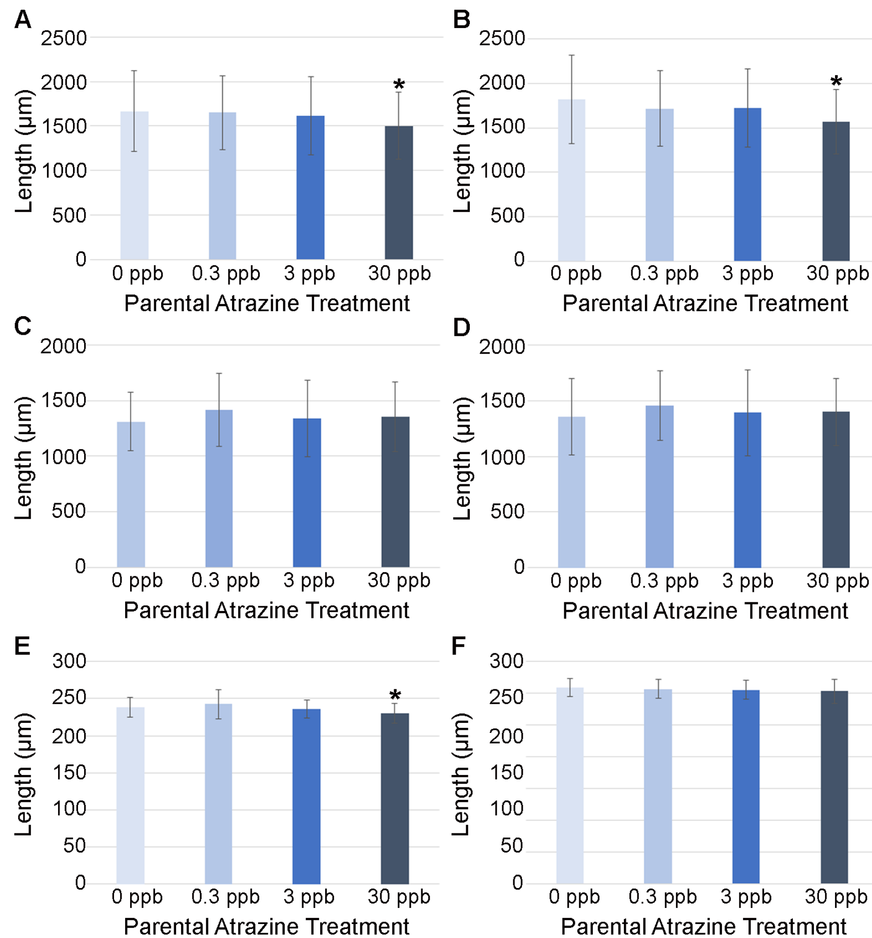

FIGURE 4. Otolith positioning and surface area in F1 larvae at 120 hpf from adult zebrafish exposed to atrazine during embryogenesis. Saccular otolith area 1 (A) and saccular otolith area 2 (B) were significantly smaller in the F1 30 ppb progeny. No change in surface area was found for utricular otolith area 1 (C) or utricular otolith area 2 (D). Length between saccular otoliths was significantly shorter in F1 30 ppb progeny (E), but no significant difference in length was observed between utricular otoliths (F). For each atrazine 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. *$p < 0.05$. Error bars represent standard deviation.

the interfrontal suture and from the parietal bones by the bilateral coronal sutures. The parietals are divided along the midline by the sagittal suture and posteriorly bound by the lambdoid suture (Kwon et al., 2019; Mork & Crump, 2015; Paul et al., 2016). In addition, zebrafish are an established model for bone remodeling and bone disease modeling, making them an ideal model to understand how atrazine alters craniofacial structures (Carnovali et al., 2019; Kwon et al., 2019). A previous study found that atrazine-exposed tadpoles had a lower percentage and severely distorted cartilage in Meckel's cartilage, CH cartilage, and branchial arches (Lenkowski & McLaughlin, 2010). In addition, another study exposing chicken embryos to atrazine found neural tube defects and craniofacial hypoplasia (Joshi et al., 2013). Conversely, a study conducted on river turtles in South America found no visual changes present with atrazine exposure in whole body skeletal structures (dos Santos Mendonça et al., 2016).

In zebrafish, cartilaginous structures and craniofacial bones begin to develop around 2 days post fertilization (Cubbage & Mabee, 1996; Kimmel et al., 1998). The jaw develops from 7 pharyngeal arches by 5 days in the larval

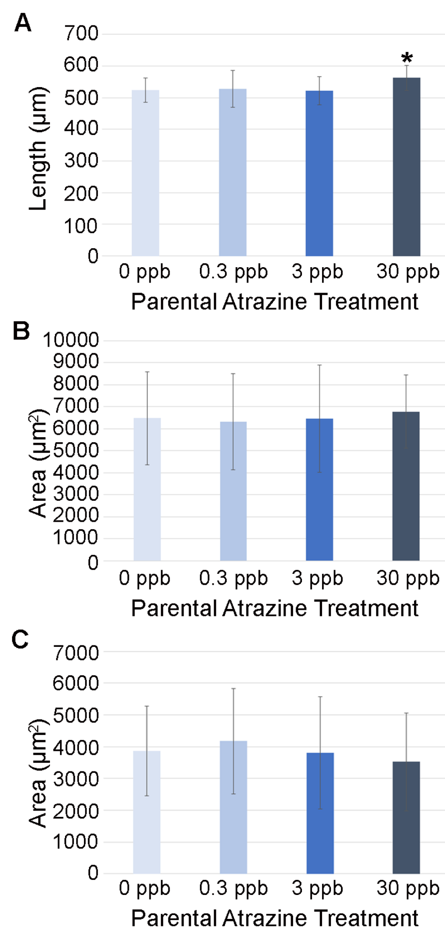

FIGURE 5. Jaw to notochord length and notochord and parasphenoid surface area in F1 larvae at 120 hpf from adult zebrafish exposed to atrazine during embryogenesis. Jaw to notochord length was significantly increased in F1 30 ppb larvae (A). There were no differences among the offspring of the different parental atrazine treatment groups for the notochord (B) or parasphenoid (C) surface area. For each ATZ 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. $*p < 0.05$. Error bars represent standard deviation.

zebrafish and neural crest cells during development, playing a critical role in neurocranial cartilage and bone development. Developmental issues in the jaw are associated with bone formation or neural crest migration issues (Kimmel et al., 1998; TeSlaa et al., 2013; Ton et al., 2006). For all atrazine treatment groups' offspring, we saw a substantial reduction in PQ length. For the 30 ppb atrazine treatment group's offspring, we saw significant increases in CH-length, Meckel's jaw distance, and posterior jaw position. Also, measurements of the saccular otoliths' surface area and distance from one another in the progeny of the 30 ppb treatment group indicated that ossification had decreased. Our findings are comparable to the craniofacial hypoplasia reported in developing chicks (Joshi et al., 2013) as well as two other zebrafish studies finding a decreased ossification in exposed zebrafish 8 days post fertilization (Walker et al., 2018) and an undeveloped jaw at 96 hpf (Ton et al., 2006). The decrease in size of various cartilaginous structures contradicts another study where researchers found an increasing size of cartilaginous structures at 120 hpf (Walker et al., 2018). However, this study was conducted with the parental generation instead of their offspring.

The current study findings aligned with some of the Walker et al. (2018) data in the fact of decreased ossification of vertebrae in developmentally exposed zebrafish at 8 days post fertilization. However, the decrease in size of various cartilaginous structures in the 30 ppb atrazine F1 treatment group at 120 hpf contradicts the Walker et al. (2018) study. The researchers found a trend of increasing size of cartilaginous structures at 120 hpf. Overall, the findings in the current study suggest an embryonic atrazine exposure in the parental generation can alter craniofacial development in their progeny with alterations observed in cartilaginous and skeletal structures. These findings also fall in line with the developmental origin of health and disease, suggesting that exposure during development can have long-lasting impacts seen later in life as well as in subsequent generations.

ACKNOWLEDGMENTS

Isabelle Akoro is supported through the School of Health Sciences Undergraduate Research Honors Program. The research is supported by the National Institutes of Health, National Institute of Environmental Health Sciences (R03 ES030545), and as part of AgSEED Crossroads funding to support Indiana's Agriculture and Rural Development.

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