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Effects of Waterborne Benzo[A]Pyrene Embryonic Exposure on Development, Behavior, Reproduction, and Mitochondrial Bioenergetics in Zebrafish

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EFFECTS OF WATERBORNE BENZO[A]PYRENE EMBRYONIC EXPOSURE ON DEVELOPMENT, BEHAVIOR, REPRODUCTION, AND MITOCHONDRIAL BIOENERGETICS IN ZEBRAFISH

By

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT

MEGHA PATEL: Effects of Waterborne Benzo[a]pyrene Embryonic Exposure on Development, Behavior, Reproduction, and Mitochondrial Bioenergetics in Zebrafish
(Under the direction of Dr. Kristine Willett)

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) that is a known carcinogen leading to adverse effects in the development of both humans and animals. BaP is also continuously present in the environment leading to regular exposure via inhalation or ingestion. Because organisms' early life stages can be more susceptible to contaminant exposure, our focus was on BaP's adverse impacts on survival, length, weight, behavior, bioenergetic state, and fecundity following developmental exposures. To study BaP's impacts, zebrafish (Danio rerio) were used as a model organism. BaP is a ligand for the aryl hydrocarbon receptor (*AHR* in humans; *Ahr* in fish). This receptor mediates some of BaP's adverse effects (i.e., metabolic activation of reactive oxygen species). To understand the role of *Ahr* in behavior and bioenergetics, wild-type (5D) and a presumptive *Ahr2*$^{-/-}$ line were used. Embryos were exposed to waterborne BaP exposure at confirmed concentrations of 0, 4.02, or 53.9 µg/L from 6 – 120 hours post fertilization (hpf). Fish were then raised in clean water until 4 months postfertilization (mpf). Subsequently, survival, hatch, size, and behavioral effects (locomotion and anxiety-like behaviors) were recorded at various life stages: larval photomotor response at 120 hpf (larval); and open field test at 1 mpf (juvenile); 3 mpf (adolescent); and 4 mpf (adult). In addition, at 4 mpf, fecundity was assessed by breeding the F0 adults who were developmentally exposed to BaP to produce the F1 generation. The Seahorse XFe96 Flux Analyzer was used to assess the relative mitochondrial bioenergetic state at 4 mpf in the F0 fish. Furthermore, F1 survival and hatch was recorded, and behavior was assessed using a larval photomotor response assay at 120 hpf. In the wildtype (5D) F0 generation, while there were no significant differences in survival between control and 53.9 µg/L BaP through adulthood, larvae from the 4.02 µg/L BaP treatment

group did not survive past 2 weeks post fertilization (wpf). The 53.9 µg/L BaP exposed males weighed significantly more than controls at 3 mpf and length was significantly increased at 4 mpf. With respect to behavior, BaP exposed larvae displayed increased activity in the dark phase compared to controls. No behavioral differences were observed in the open field assessment at 1 mpf. However, at 3 mpf, BaP exposed fish had a significant decrease in total distance traveled and a significant increase in freezing duration. At 4 mpf, adult females, regardless of treatment, spent significantly more time in the periphery than males indicating anxiety-related behavior, but there were no significant treatment effects. There were no significant differences in the bioenergetic state between treatments. No differences were observed in F0 fecundity or F1 survival and hatch. In the F1 generation, larvae whose parents were exposed to BaP were significantly less active in the dark phase compared to the F1 controls, which is the opposite effect of what was observed in the F0 larvae. The *Ahr2*$^{-/-}$ fish when genotyped represented a mix of *Ahr* genotypes so no conclusions were drawn from null animals. Overall, our wild-type results suggest that BaP-related behavioral impacts are present and can cause multigenerational effects and is potentially harmful in terms of cognition and development.

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LIST OF ABBREVIATIONS

AHR – aryl hydrocarbon receptor

ARNT – aryl hydrocarbon receptor nuclear translocator

BaP – Benzo[a]pyrene

CYP – cytochrome

DMEM – Eagle's minimal essential medium

DMSO – dimethyl sulfoxide

dpf – days post fertilization

FCCP - carbonyl cyanide-p-trifluoromethoxy phenylhydrazone

hpf – hours post fertilization

mpf – months post fertilization

OCR – oxygen consumption rate

PAH – polycyclic aromatic hydrocarbon

PPM – parts per million

s – seconds

1. INTRODUCTION

1.1 Benzo[a]pyrene

Benzo[a]pyrene (BaP), which is depicted in Figure 1, is a representative polycyclic aromatic hydrocarbon (PAH) that is found in the environment as a byproduct of incomplete combustion of organic matter in fossil fuels (Cousin & Cachot, 2014). BaP is also present in cigarette smoke and in many grilled and broiled foods such as fried chicken and smoke-dried beef (Lee & Shim, 2007). Hence, the primary routes of BaP exposure are via inhalation or ingestion. BaP is classified as a Group 1 carcinogen in humans, indicating that it is of the highest cancer-causing potential (No et al., 2016). BaP is also known to cause adverse reproductive and developmental effects in both humans and animals. In particular, BaP exposure has been linked to neurodegenerative syndromes, decreased dopamine levels, and decreased locomotor activity leading to lasting developmental effects (Gao et al., 2017). Additionally, BaP can cause adverse developmental effects in a transgenerational manner. Previous studies with a mice model have shown that exposure during sensitive windows such as pre- and post-natal development can pose long-term reproductive effects including depletion of ovarian follicles and increased ovarian and bone marrow mutations, which may be linked to tumorigenesis (Moura & Houten, 2010). Furthermore, prior longitudinal studies in humans have shown the adverse outcomes of prenatal PAH environmental exposure on the progeny's behavioral tendencies (i.e., anxiety, depression, and attention-deficit problems) (Perera et al., 2012).

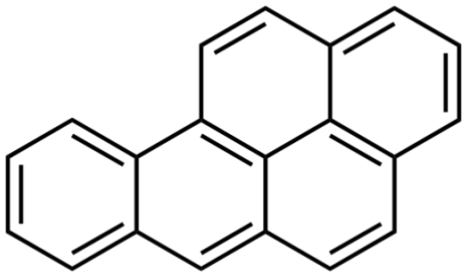


Figure 1:
Chemical Structure of Benzo[a]pyrene

1.2 Zebrafish Model

This study used zebrafish (*Danio rerio*) as a model organism to further elucidate the toxicities associated with BaP exposure. The use of zebrafish provides numerous scientific advantages such as their transparent chorion and a shared homology with other animals (Fitzgerald et al., 2021). While zebrafish have three Ahr receptors, the *Ahr2* receptor referred to in this study serves as on ortholog for the mammalian AHR receptor (Hahn et al., 2017). Furthermore, their high fecundity and rapid life cycle enable zebrafish to be useful model organisms. Their larvae are small, which allow them to be easily handled with a relatively low husbandry cost. They reproduce in large numbers and develop rapidly with a functioning brain, heart, pancreas, kidneys, bones, muscles, and sensory systems functioning within 5 dpf (days post fertilization) allowing for the study of their life cycle in a short amount of time (Kimmel et al., 1995). Additionally, this allows for a neurotoxic response to be observed at earlier time points rather than waiting to use adult fish, which requires more resources (Fitzgerald et al., 2021).

Zebrafish serve as useful models for behavioral observation because the behavioral traits of larvae and adults have been rigorously catalogued for neurotoxic research purposes (Kalueff et al., 2013). Behavioral tests such as open field tests can be done to show chemical and/or genotype specific responses compared to control conditions (Fitzgerald et al., 2021). One of the behavioral tendencies that is a focus of this study is thigmotaxis, which is the tendency of the fish to swim in the periphery of the arena. This measure is indicative of anxiety behavior, and using tracking software such as Viewpoint Zebrabox and Noldus Ethovision allows for the detection of thigmotactic behavior (Norton & Bally-Cuif, 2010).

1.3 Behavioral Effects of BaP Exposure

Behavior assessment can be used to determine neurotoxicity and neurocognitive decline for both humans and other animals (Vignet et al., 2014). Specifically, anxiety-related behavior, including as thigmotactic behavior, is a known indicator of psychiatric distress such as autism, anxiety, and depression (Luo et al., 2014; Settipani et al., 2012). Maternal BaP exposure in humans, due to environmental presence, has been linked to adverse behavioral effects indicative of anxiety/depressive and attention-deficit disorders based on the Child Behavioral Checklist (CBCL) standards (Perera et al., 2012). Additionally, prior studies have shown that postnatal BaP exposure in mice is linked to anxiety-like behavior and alterations of anxiety-related and neurodevelopmental genes (Yang et al., 2019).

PAH exposure also poses significant behavioral effects on zebrafish. Previous studies monitoring photomotor response have shown that dietary PAH exposure is linked to higher levels of anxiety-related behavior, and the measure of exploratory activity is significantly decreased as well (Vignet et al., 2014). BaP exposure in zebrafish also causes further adverse behavioral effects such as learning and memory deficits (Knecht et al., 2017). Furthermore, BaP exposure can cause multigenerational effects because F1 female offspring of exposed F0 fish showed significant increases in distance traveled and mobility, which are indicative of anxiety, compared to the controls (Gillespie, 2021).

1.4 Bioenergetic Effects

BaP exposure has long-term effects on bioenergetics and cellular respiration. With the buildup of toxic metabolites and reactive oxygen species, BaP ultimately leads to activation of multiple transcription factor networks related to metabolism, oxidative stress, and cell proliferation (Souza et al., 2016). In addition to affecting respiratory rates, airborne BaP exposure affects adiposity and weight-related metabolism (Ortiz et al., 2014). Prenatal exposure

in mice caused increased adipose tissue levels and weight gain (Ortiz et al., 2014). BaP exposure is linked to lasting cardiovascular effects. Oxygen consumption rate (OCR) increased in BaP-injected adult zebrafish (Gerger & Weber, 2015). Along with increased oxygen consumption, the overall ventricular heart rate decreased which is indicative of significant adverse cardiorespiratory effects (Gerger & Weber, 2015).

1.5 BaP Mechanism of Action

Despite studies indicating the presence of adverse effects of BaP exposure, the mechanism of action and full effects remain to be further elucidated. To cause mutagenic adverse outcomes, BaP is bioactivated into reactive species and metabolized by aryl hydrocarbon receptor mediated (*AHR* in humans; *Ahr* in fish) metabolic pathways. *AHR* is a ligand-activated transcription factor that is highly conserved across various animal phyla (Garcia et al., 2018). Upon ligand activation, the *AHR* undergoes a conformational change and relocates to the nucleus from the cytoplasm of the cell (Jayasundara et al., 2015). It then forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) causing xenobiotic responses (i.e. gene expression and transport of xenobiotic compounds) (Jayasundara et al., 2015). More specifically, *AHR* regulates the cytochrome P450 (CYP) genes (CYP1A1, CYP1A2, and CYP1B1), and the CYP1 enzymes are responsible for metabolically activating and detoxifying many of the PAHs present in the environment (Nebert et al., 2004). Thus, *AHR* activation causes the upregulation of CYP, which is responsible for metabolizing BaP into toxic metabolites (Genies et al., 2013; Souza et al., 2016). One of the primary goals of this study was to determine whether *AHR* is involved in BaP's behavioral and mitochondrial bioenergetic effects.

AHR has been identified by numerous studies as the primary mediator of early life toxicological effects in zebrafish (Goodale et al., 2012). *AHR* is involved in numerous endogenous functions such as immune, reproductive, and developmental processes (Hernández-Ochoa et al., 2009; Kerkvliet, 2009; Lahvis et al., 2005). To understand the role of specific receptors, animals can be genetically modified and selectively bred to compare the wild-type and knockout animals. There are significant physical differences between 5D wild-type and *Ahr2*$^{-/-}$ zebrafish as shown in Figure 2. *Ahr2*$^{-/-}$ fish have fin abnormalities, structural differences in the neurocrania, and bone structure differences such as extended ethmoid and mandibula (Goodale et al., 2012; Cubbage & Mabee, 1996). These studies suggest that AHR is involved in the proper physical and neuromuscular development and function of an organism.

Figure 2:
Representative Pictures of 3 mpf 5D and *Ahr2*-null Zebrafish

1.6 Study Goals

Due to known adverse behavioral and developmental effects of PAH exposure, we wanted to determine whether developmental waterborne BaP exposure has lasting developmental and behavioral effects and whether exposure adversely impacts the mitochondrial bioenergetics. The main goals of this study were the following:

1. Assess how a developmental waterborne embryo/larval BaP exposure results in acute and chronic changes in mitochondrial bioenergetics and behavior.
2. Further elucidate the role of *Ahr2* in mediating changes in bioenergetics, fecundity, and behavior following the waterborne exposure.

Our hypotheses were the following:

1. There will be no constitutive differences in behavior and mitochondrial bioenergetics between 5D and *Ahr2-null* controls.
2. BaP induces lasting impacts on behavior and mitochondrial bioenergetics that are *Ahr2*-dependent, sex-dependent, and treatment-dependent.
3. Larval BaP exposure has multigenerational effects on behavioral outcomes.
4. BaP exposure has negative impacts on fecundity.

2. MATERIALS AND METHODS

2.1 Zebrafish Husbandry

Wild-type (5D) and *Ahr2*^{OSU1} zebrafish were obtained from Oregon State University through Dr. Robyn Tanguay, and all the fish were raised according to the approved IACUC protocols. The fish were placed in Aquatic Habitats ZF0601 Stand-Alone System with zebrafish water (pH 7.0-7.5, 60 parts per million (ppm) Instant Ocean, Cincinnati, OH) at 25-28°C. Fish were fed twice daily with Gemma 300 micro food (Skretting USA, UT). If the fish were found to be sexually mature and free of sickness and visible deformities, they were selected to breed.

The fish selected to breed were placed in breeding tanks the evening prior to egg collection. The fish laid their eggs in response to the light, and within the next hour, eggs accumulated on the bottom of the breeding tank. Upon collection, water was poured through the sieve in the breeding tank to collect and transfer eggs to petri dishes. There, they were raised in embryo water (pH 7.5, 60 ppm Instant Ocean, 0.5% methylene blue) in an incubator (14:10 light dark cycle) at 28°C.

2.1.1 Genotyping of Ahr2^{OSU1}

To confirm the *Ahr2*-null fish, *Ahr2*^{OSU1} fish were genotyped. The breeding fish were anesthetized using buffered tricaine (MS-222; 150 mg/L), and a small portion of the zebrafish

tail fin was clipped. DNA was extracted from the fin upon lysing and protein degradation. The PCR primers used were: Forward 5'-TTC AAC AGT CCT CCT TAA GAA CG-3' and Reverse 5'- TGT AAA ATA ACA ACA TAA CTT GGC CC-3' (Garcia et al., 2018). Following PCR, the sample was digested with the Nde1 (New England Biolab) restriction enzyme and processed using gel electrophoresis to isolate the fish that are homozygous recessive. After observing for a singular band around 700 base pairs, the *Ahr2*-null fish were separated into their respective tanks.

2.2 Exposure

2.2.1 Embryo/Larval BaP Waterborne Exposure

BaP was prepared as a 1 µg/µL stock solution using 0.0015 grams of BaP from Sigma-Aldrich in 1.5 mL of dimethyl sulfoxide (DMSO). To make the 100 µg/L exposure solution, 1 µL of the BaP stock was added to 10 mL of zebrafish embryo water. To make the 10 µg/L exposure solution, a 1:10 sub-stock was prepared in DMSO. Then, 1 µL of the sub-stock was diluted with 10 mL of zebrafish embryo water. Each vial received 6 mL of its respective solution for the exposure. All treatment groups contained 0.01% DMSO.

Upon egg collection of *Ahr2*-null and 5D embryos, embryos were selected for the exposure at roughly 6 hours post fertilization (hpf). The treatment groups were the following: 0, 10, or 100 µg/L for both *Ahr2*-null and 5D wild-type fish. This exposure was conducted in triplicates – meaning that there were three vials of each experiment group per genotype. Each vial consisted of 10 embryos, and the embryos were screened daily to remove dead and/or unfertilized eggs and debris using transfer pipettes. The embryos were exposed to BaP in the vials for up to 96 hpf while undergoing a water change every two days. At 96 hpf, larvae were transferred to 96-well plates (1 larva/well; 300 µL exposure water/well) while continuing to be

exposed until 120 hpf. At 120 hpf, a majority of the 5D 10 µg/L larvae did not survive. However, we continued the experiment as outlined with the control and 100 µg/L groups in the 5D strain.

2.3.2 Extraction, Quantification, and Chemical Analysis

After exposure, an extraction and analysis on the dosing stock solutions were done to verify the concentrations used during the exposures. After preparing the respective solutions and exposing the fish, BaP-d12, the surrogate standard, was added to the remaining solution. Then, BaP and the standard were extracted from the solution using C18 cartridges and eluted using methylene chloride. The samples were then further processed in a Nitrogen-Evaporator to evaporate the methylene chloride off completely and dry the sample. Hexane was added to the remaining sample, transferred into a GC (gas chromatography) vial, and flourene-d10 was added as the internal standard. The calibration curve consisted of 0.1, 0.2, 0.5, 1, and 2 µg/mL BaP and 0.2 µg/mL BaP-d12 and Fl-d10. The samples were then transported to a collaborating lab to undergo gas chromatography time of flight mass spectrometry. Actual concentrations for the solutions were 4.02 µg/L and 53.9 µg/L for the nominally 10 and 100 µg/L concentrations, respectively.

2.2 Behavior Analysis

2.2.1 Larval Photomotor Response Assay

At 96 hpf, individual larvae (n=28-29 per treatment) were placed in a well on a 96-well plate to acclimate before undergoing behavioral analysis at 120 hpf. These fish continued to be exposed to BaP until 120 hpf, and each well contained 300 µL of the respective dosing solution. The fish were given 5 minutes to acclimate to the behavior room's darkened environment. Then, they underwent light-dark testing. This test monitors larval behavior over a 50-minute period, where the environment alternates between light and dark in 10-minute intervals starting with a

light phase. Zebrafish typically exhibit hyperactivity in darkened environments. The Viewpoint Zebrabox software tracked the fish's movement to analyze the total distance traveled by each treatment group in 2-minute intervals.

2.2.2 Open Field Test for 1 mpf, 3 mpf, and 4 mpf

After assessing the larval photomotor response, the exposed fish were further raised to adulthood in clean water. Behavioral assessment was conducted via open field testing at the following time points: 1 mpf (juvenile), 3 mpf (adolescent), and 4 mpf (adult). Prior to testing, the fish were acclimated to the darkened behavioral testing room (26° C) for 5 minutes. The testing consisted of each individual fish swimming for 5 minutes in a 0 Lux for 1 mpf and 9 Lux for 3 and 4 mpf open-field environment while Noldus Ethovision 14.0 software tracked movements to obtain data. The swim arena was divided into two areas for analysis: periphery (outer 50% of the area) and center (inner 50% of the area). A larger portion of time spent in the periphery is indicative of anxiety-related behaviors. Open field testing was routinely conducted within the 12 PM – 4 PM time-period to control for any time-related differences in energy or behavior.

For the 1 mpf tests, the fish (n = 21-22) were placed into 6-well plates (6 fish/plate) with a well diameter of 3.5 cm and 10 mL of system water per well. Individual plates were then placed into the Viewpoint Zebrabox to record the fish's movement for 5 minutes. Then, this footage was processed by the Noldus Ethovision 14.0 software to track the fish's movements to determine the distance traveled, velocity, freezing duration, and time spent in the periphery.

For the 3 mpf assessment, individual fish were placed in a 2-gallon bucket with a diameter of 23 cm filled with 2 L of system water. The arena size for the periphery is a 21 cm diameter, and the center zone diameter is 15 cm. For the 4 mpf testing, the fish were placed into

a 5-gallon bucket with a diameter of 30.5 cm and a volume of 10 L of system water to accommodate for their growth. The arena sizes also had to be modified to the following: 28 cm diameter for the outer zone and 20 cm diameter for the inner zone. Refer to Figure 3 for a depiction of arena sizes for the different time points of the behavior assessments. The behavioral data was obtained with cameras and lighting placed above the designated testing area. Total distance traveled (cm), average velocity (cm/s), the percentage of time spent in the periphery, and freezing duration (s) were calculated by the software. Additionally, the video tracks were manually cleaned to correct the tracking software's errors that were caused by glares or water ripples when needed.

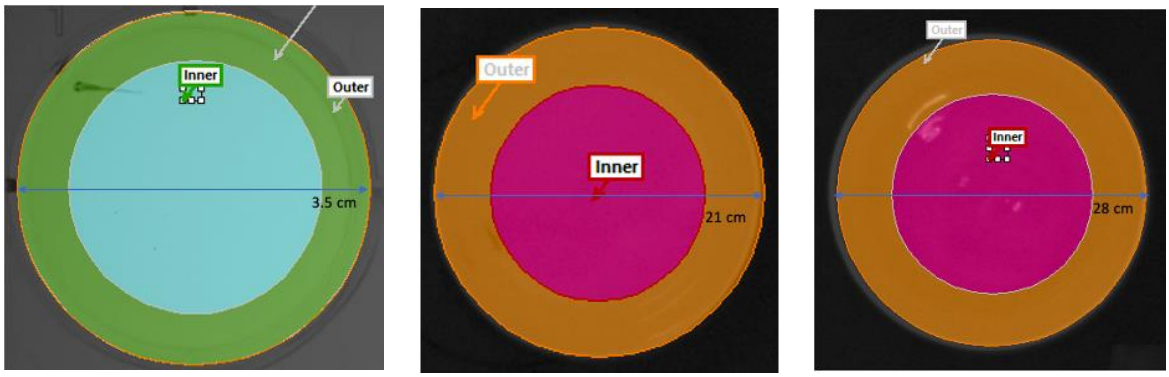


Figure 3: Arena Sizes for 1 mpf, 3 mpf, and 4 mpf, respectively
Outer represents the periphery region (outer 50% of the area), and inner represents the center. region (inner 50% of the area).

2.3 Fecundity and F1

The presumptive *Ahr2*-null fish did not have enough males to investigate reproductive success. However, the 5D fish had a balanced ratio of males and females to test for reproductive success. The 5D fish (3-5 breeding tanks of 2 male x 2 female per tank) were placed into breeding tanks following the 4 mpf open field testing to assess fecundity. Both control and 100 µg/L BaP were able to reproduce successfully. The resulting embryos were collected, and larvae are still being raised until 4 mpf. At 120 hpf, the larval photomotor response assay as described

in Section 2.3.1 was performed to determine if behavioral effects persisted into the F1 generation following a parental developmental BaP exposure.

2.4 Bioenergetic Analysis Using Seahorse Flux Analyzer

To assess to effects of acute BaP exposure, our lab attempted to optimize the Seahorse XFe96 Flux Analyzer for larval samples. We were able to make mesh screens with a diameter of 400 microns using a polymer O-ring and nylon mesh in the lab. These mesh screens were made with the intent of securing the larvae in the well by preventing them from being flushed out of the well or into the instrument. However, ultimately, it was determined that the size of the fish and thickness of the O-ring exceeded the threshold recommended by Agilent, and use of the O-rings could likely damage the larval sample and sensor cartridge. The recommended thickness is 200 microns from the bottom of the well; however, the O-ring alone was 700 microns thick. There was no smaller O-ring size available for purchase, and therefore, bioenergetic data was obtained only at 4 mpf using adult brain samples after mitochondria were isolated instead of on intact larvae.

On the day prior to bioenergetic analyses, the XFe96 sensor cartridge was hydrated and placed in a 28°C incubator, and each well of a 96-well plate was coated with 20 µL polyethylenimine (PEI) to ensure adherence of the isolated mitochondria. An hour prior to running the assay, the sensor cartridge solution was exchanged for the XF Calibrant solution. Then, the sensor cartridge was calibrated within the Seahorse XFe96 Analyzer for pH and O_2. Fish brains (3-4 biological replicates per treatment per sex; 2-4 pooled 4 mpf fish brains per biological replicate) were dissected and homogenized (20 up and down strokes) in mitochondrial extraction buffer (MEB- 125 mM sucrose, 250 mM mannitol, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM EGTA (Ethyleneglycol- *bis*(β-

aminoethyl)-N,N,N′,N′-tetraacetic Acid); pH 7.2) with 0.01% BSA (Bovine Serum Albumin) and 1x protease inhibitors. To isolate the mitochondria, homogenized brains were centrifuged twice at 700xg for 10 min at 4°C to remove any remaining tissues. The supernatant obtained was centrifuged at 10,000xg for 15 min at 4°C to pellet out the mitochondria. The pellet was rinsed with MEB and centrifuged again at 10,000xg for 15 min at 4°C. The pellet was resuspended in 50 µL of MEB. Mitochondrial protein was quantified using the Nanodrop 2000 (ThermoFisher). Mitochondria (30 µg) of each sample was added to the 96-well plate in 1 to 4 technical replicates of each biological sample. The plate was centrifuged at 2000xg for 20 minutes at 4°C to attach mitochondria to the bottom of the plate, buffer was removed from each well, and 180 µL Dulbecco's Modified Eagle's Medium (DMEM) complete (DMEM plus 1 mM pyruvate, 10 mM glucose, and 2 mM glutamine) was added to each well. After loading the samples, the respective metabolic activators and inhibitors were loaded into the cartridge through the injection ports (A to C). Port A was loaded with oligomycin which acts as a complex V (ATP synthase) inhibitor and shutting down respiration and lowering the oxygen consumption rate (OCR) (Technologies, 2019). Port B was loaded with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) which acts as a mitochondrial uncoupler and maximized the respiration rate and OCR by causing uninhibited electron flow and reaching maximal respiration (Technologies, 2019). Port C was loaded with rotenone and antimycin-A which serve as complex I/III inhibitors in the electron transport chain to calculate non-mitochondrial OCR and respiration rates (Technologies, 2019). Final metabolic activators/inhibitors were as follows: 1.5 µM oligomycin, 1 µM FCCP, and 0.5 µM rotenone/antimycin A.

Following the Cell Mito Stress Test manufacturer's protocol, for each condition (Baseline, oligomycin, FCCP, Rotenone/Antimycin A), OCR was measured 3 times over the course of 18

minutes (3-minute mixing, 0-minute waiting, and 3-minute measuring) in the Seahorse XFe96 Flux Analyzer.

2.6 Statistics

The data was analyzed using Sigma Plot 14.0 software and graphed using box and whisker plots or time-course plots. All the data preliminarily went through the Shapiro-Wilk Normality Test and the Brown-Forsythe Equal Variance Test to determine whether data was parametric or non-parametric. When two treatment groups were being compared (control vs. 100 μg/L in the 5D strain), an unpaired t-test or Mann-Whitney test was used to determine statistical significance for parametric and non-parametric data, respectively. In the case of multiple treatment groups (control, 10 μg/L, 100 μg/L in *Ahr2*^{mix} strain), a one-way ANOVA or ANOVA on ranks was used followed by Dunn's post hoc test when applicable. When two factors were compared (i.e., sex and treatment in 3 mpf and 4 mpf behavior, Seahorse XFe96), a two-way ANOVA was used followed by a Student-Newman-Keuls post hoc test to perform a pairwise multiple comparison. A $p \leq 0.05$ indicated a statistical difference.

3. RESULTS

Using both the *Ahr2*^{mix} and the 5D strains, various stages following a developmental BaP exposure were analyzed. Hatch and survival were assessed daily until 120 hpf, on a weekly basis from 120 hpf to 1 mpf, and monthly until 4 mpf. Zebrafish length and weight was recorded at the juvenile (1 mpf), adolescent (3 mpf) and adult (4 mpf) stages, and behavior was monitored at the larval (120 hpf), juvenile (1 mpf), adolescent (3 mpf) and adult (4 mpf) stages. At 4 mpf, reproductive success and F1 survival/hatch were assessed, and brain mitochondrial bioenergetics were analyzed. The *Ahr2*^{mix} F0 fish were genotyped at 4 mpf, and at this point, it was discovered that not all the fish were truly *Ahr2*$^{-/-}$. Therefore, the focus of this thesis will be on BaP effects in

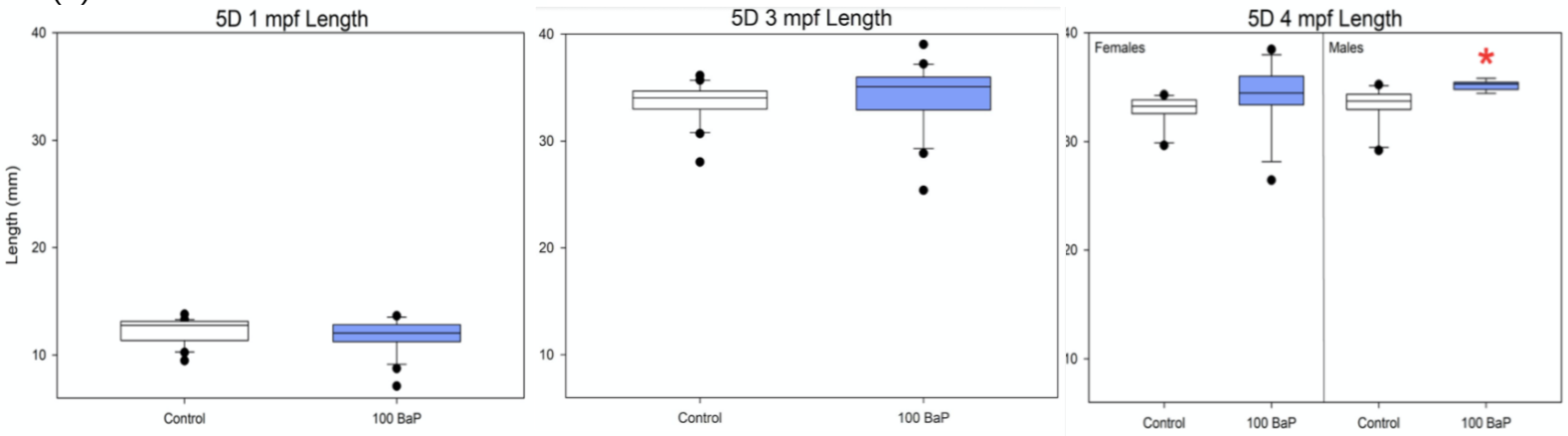
the wild-type 5D line with data on the initially presumed *Ahr2*$^{-/-}$ line included at the end of the results section.

In wild-type exposure at 120 hpf, there was 66% mortality in the 10 µg/L BaP treatment group and by 2 wpf, there was 100% mortality in this group (data not shown). The high mortality in the 10 µg/L BaP group was unexpected. In contrast, there was no significant difference in survival and hatch between the control and 100 µg/L BaP treatment groups in the 5D strain at any time point (Figure 4A). Lengths were measured at 1, 3 and 4 mpf while weights were only recorded at adolescence (3 mpf) and adulthood (4 mpf) (Figure 4B and C). For the 5D strain, there was a significant increase in length for the 100 µg/L BaP treatment group compared to the control for the 4 mpf males. There was also a significant increase in weight for the 100 µg/L BaP treatment group compared to the control for males at 3 mpf, and a similar trend continued at 4 mpf (p=0. 0562). There were no significant differences in terms of hatch/survival between the treatment groups or for length and weight within females.

(A)

F0	48 hpf % Hatch		96 hpf % Survival		4 wpf % Survival	
	Average	St. Error	Average	St. Error	Average	St. Error
5D Control	35.2%	10.6%	96.6%	3.33%	70%	3.33%
5D 100 BaP	16.7%	2.22%	100%	0%	73.3%	0%

(B)



(C)

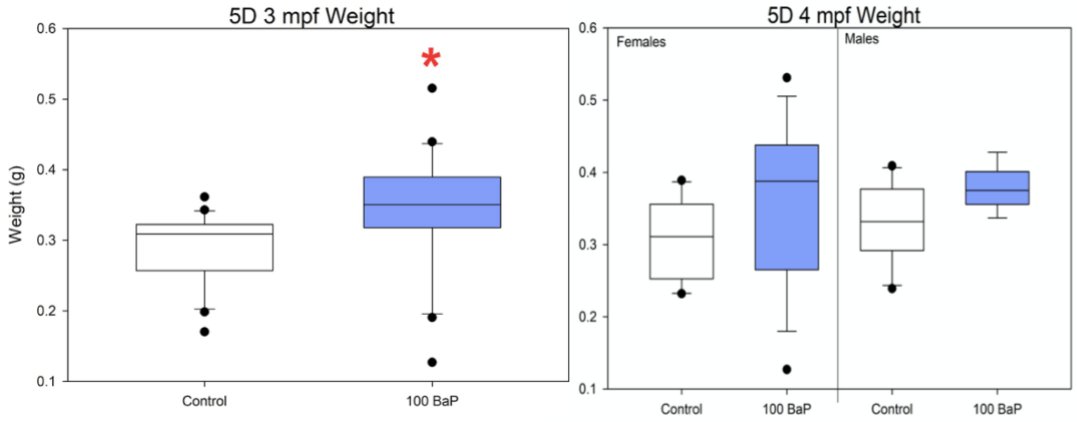


Figure 4: 5D Survival, Length, and Weight
Zebrafish hatch/survival (A) was recorded at various points throughout the study. A t-test was used to compare the 48 hpf % survival and the 96 hpf % survival with n=3 tanks. For the 4 wpf % survival, we consolidated the fish into two tanks resulting in an insufficient n to perform statistical analysis. Zebrafish length (B) at 1 mpf was compared across treatment group using a t-test (n=21-22). Zebrafish length (B) at 3 mpf (n=20-21) and 4 mpf (n=10-15 for females and n=6-10 for males) was compared using a t-test (n=20-21) to observe for any sex or treatment-related effects. Zebrafish weight (C) at 3 mpf and 4 mpf was recorded, and this data was also compared using a t-test to observe for significant differences between treatment for males and females. Statistically significant findings are denoted with the (*) symbol in red above ($p \leq 0.05$)

At 120 hpf, the larval photomotor response (light-dark) assay was performed, and this assay monitored the total distance traveled by each fish in a 96-well plate over a 50-minute time alternating between light and dark (Figure 5). In the dark phase (10-20 minutes and 30-40 minutes), 100 µg/L BaP larvae were significantly more active compared to control (Mann-Whitney), but not significantly different in the light phase. While this figure does include fish from the 10 µg/L BaP exposure group, these fish were very sickly and had many deaths (66% mortality); therefore, these fish were not included in the statistical analysis. Following the larval photomotor response assay, the zebrafish were further raised to 1 mpf, which is the juvenile stage of their life cycle. Open field testing was conducted over a 5-minute period to analyze the total distance traveled (cm), average velocity (cm/s), percent of time spent in the periphery, and freezing duration for each fish (Figure 6). For the 5D fish, there were no significant differences at 1 mpf.

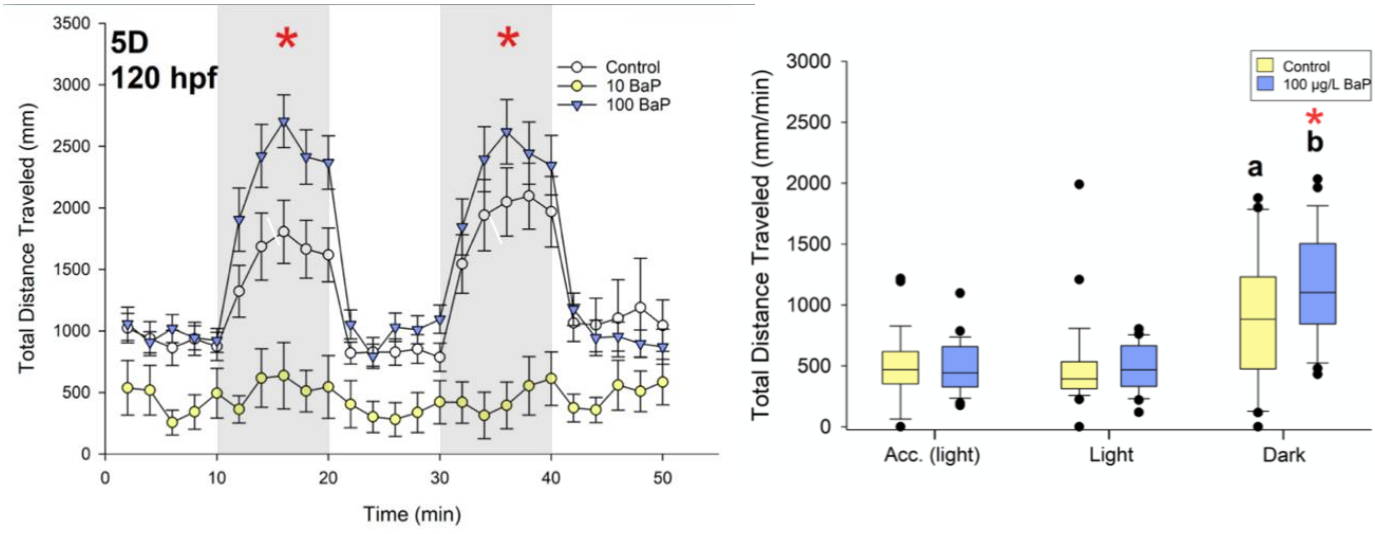


Figure 5: 5D Larval Photomotor Response Assay (120 hpf)
Distance traveled over a 50 min larval photomotor response assay (light: 0-10, 20-30, 40-50 min) (dark: 10-20, 30-40 min). There was significant hyperactivity in the 100 µg/L BaP group compared to the control during the dark phases. Statistical significance was determined using a t-test to compare the two groups (n = 28-29) and denoted with (*) in red. The 10 µg/L group was omitted from analysis due to a high mortality rate.

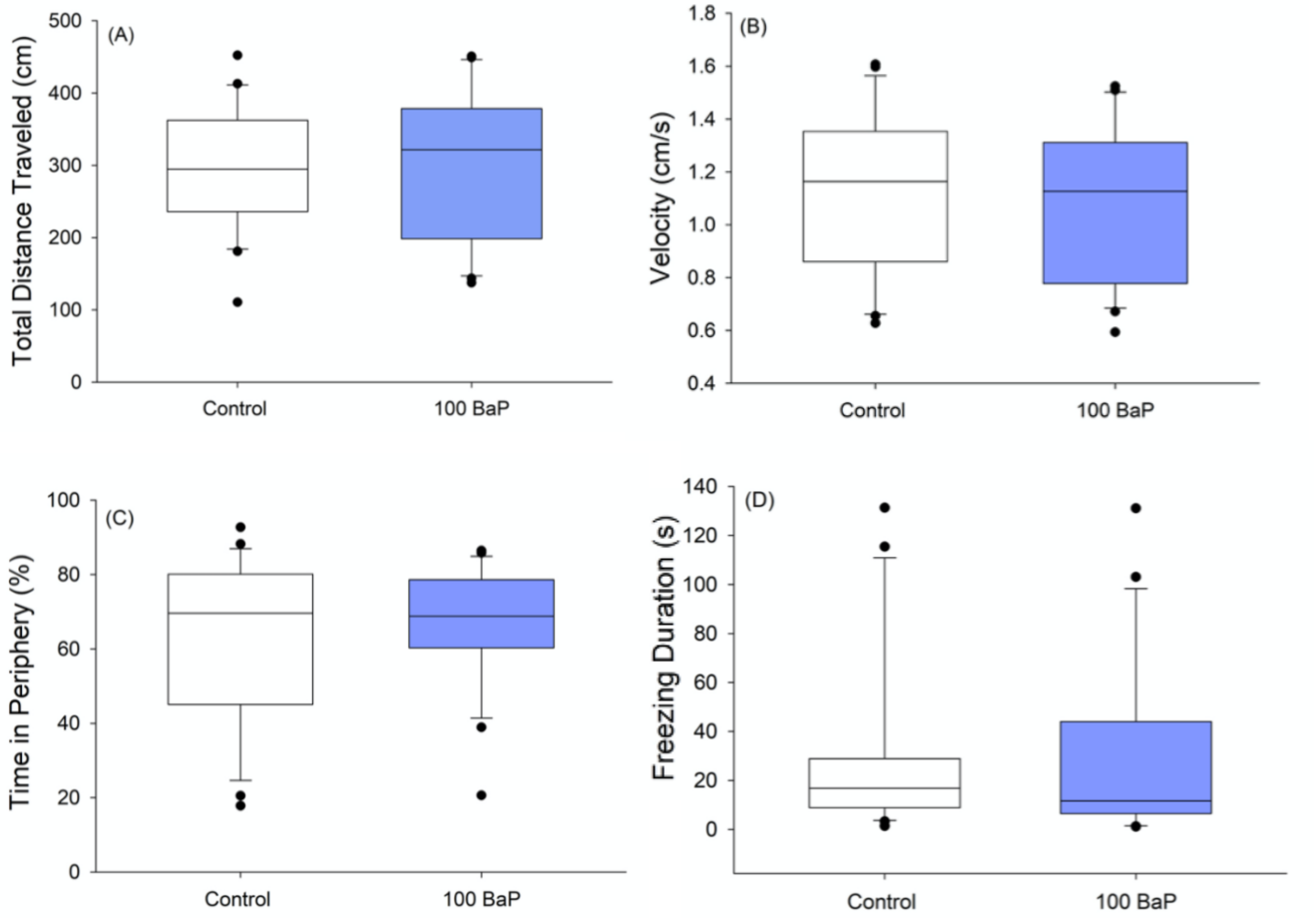


Figure 6: 5D Open Field Test Results for 1 mpf
Zebrafish behavior was assessed in the open field environment at the juvenile stage (1 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The total distance and velocity data was analyzed using a t-test to determine if behavior differs between treatment groups (n=21-22). The time in periphery and freezing duration data did not pass normality assumptions and was consequently analyzed using the Mann-Whitney test. Bars with the (*) symbol above them are significantly different ($p<0.05$).

Fish were further raised to adolescence (3 mpf) and behavior in an open-field test was assessed again for the total distance traveled (cm), average velocity (cm/s), percent of time spent in the periphery, and freezing duration for each fish (Figure 7). There was a significant decrease in the total distance traveled and a significant increase in freezing duration between the 100 µg/L group in comparison to the control.

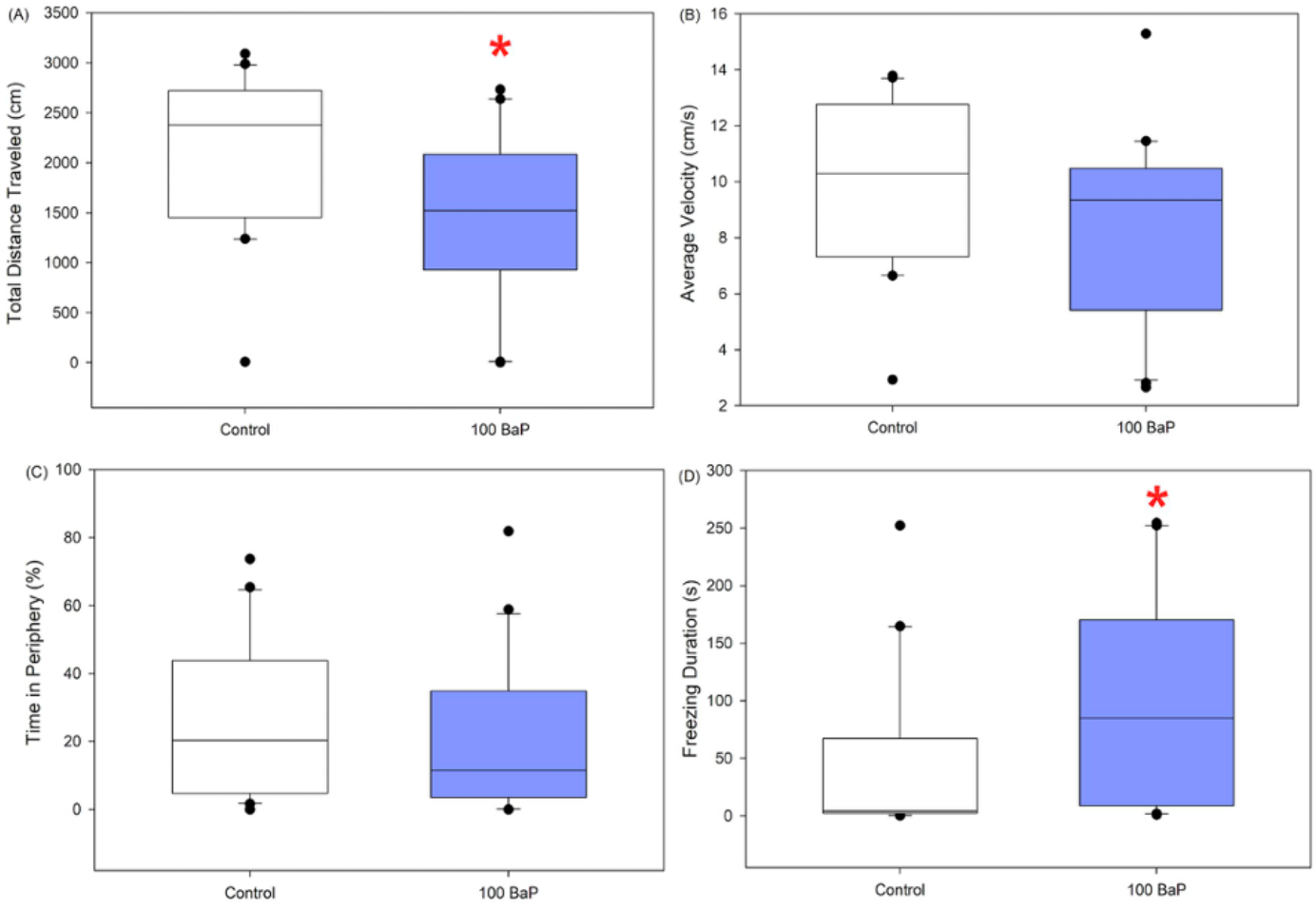


Figure 7: 5D Open Field Test Results for 3 mpf
Zebrafish behavior was assessed in the open field environment at the adolescent stage (3 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The data was analyzed using a t-test to determine if behavior differs between treatment groups (n=20-21). Bars with the (*) symbol above them are significantly different ($p<0.05$).

Open-field testing measuring the same parameters as mentioned previously was conducted again at 4 mpf, which is the adult stage in the zebrafish life cycle (Figure 8). There was a significant decrease in 5D males compared to females for the percent of time spent in the periphery, but there was no significant difference between the two treatment groups.

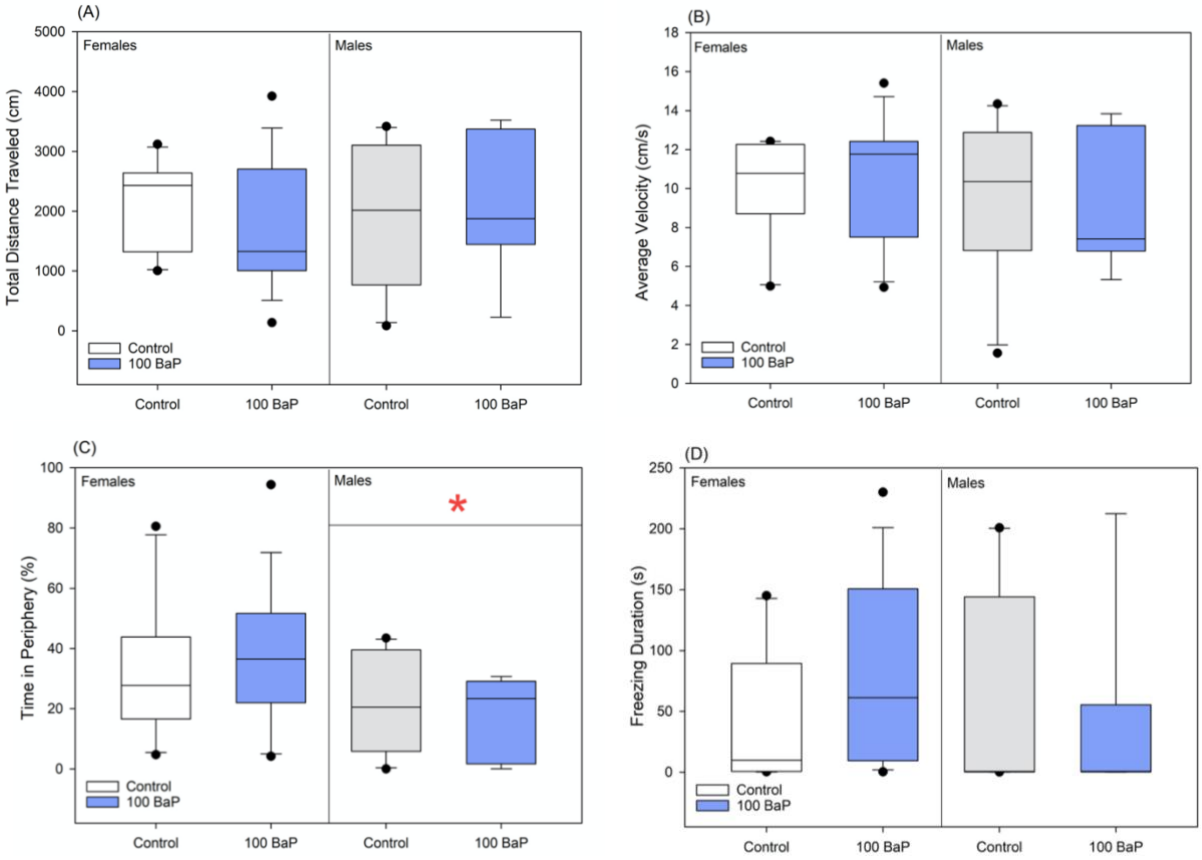


Figure 8: 5D Open Field Test Results for 4 mpf
Zebrafish behavior was assessed in the open field environment at the adult stage (4 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The data was analyzed using a two-way ANOVA to determine if behavior differs between treatment groups and between sex (n=10-15 for females and n=6-10 for males). Bars with the (*) symbol above them indicate that males significantly differed from females.

Following behavior analysis at 4 mpf, reproductive success of the 5D fish was assessed. Both the control and 100 µg/L BaP fish successfully reproduced and provided the F1 generation. F0 fecundity and F1 survival and hatch data are shown in Figure 9A, but this data was unable to be statistically analyzed. The 100 µg/L BaP fish only provided eggs in two of the three breeding tanks, and an n=2 is insufficient for statistical analysis. A larval photomotor response assay was conducted at 120 hpf for the F1 control and 100 µg/L BaP fish, and the results are shown in Figure 9B. The progeny of the exposed fish exhibited decreased activity in the dark phases of the assay as opposed to the hyperactivity in the F0 generation.

(A)

	Number of Eggs in F1		48 hpf % Hatch		96 hpf % Survival	
	Average	St. Error	Average	St. Error	Average	St. Error
5D Control	142.6	15.47	27.8%	5.79%	92.5%	3.87%
5D 100 BaP	94	77.63	3.75%	3.06%	98.75%	1.02%

(B)

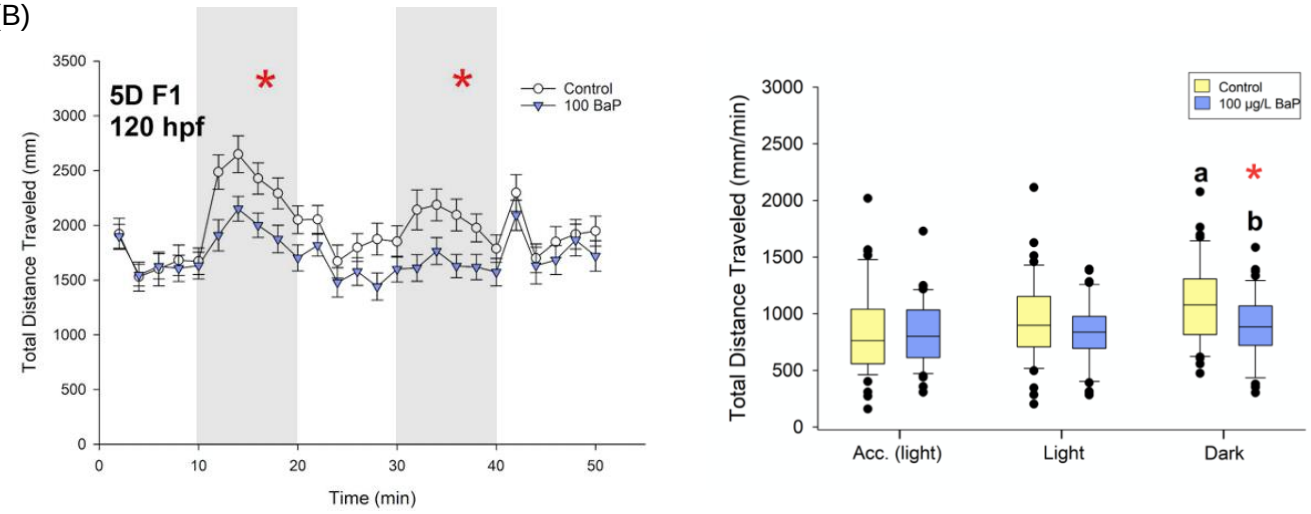


Figure 9: 5D F0 Fecundity/F1 Hatch & Survival (A) & F1 Behavior (B)
F1 generations were bred for both 5D control and 100 µg/L BaP. Due to a small sample size (n=2 for 100 µg/L BaP), statistics were not run for F0 fecundity/F1 survival/hatch. The control breeding tanks all provided eggs (n = 5). An n = 5 tanks is optimal to compare the fecundity, but only two of three tanks for the 100 µg/L BaP group provided eggs. F1 behavior (B) was assessed using a larval photomotor response assay (light: 0-10, 20-30, 40-50 min) (dark: 10-20, 30-40 min) with n= 49. Statistically significant findings are denoted with the (*) symbol in red above (t-test or Mann Whitney; p≤0.05).

Following the 4 mpf open field testing and reproductive success, the fish were euthanized and underwent brain mitochondrial bioenergetic analysis using the Seahorse XFe96 Flux Analyzer. The results are shown in Figure 10A, and a comparison between the measurements during the stages of this assay is depicted in Figure 10B. Bioenergetic results were not significantly different between treatment groups or sexes in the 5D fish based on a two-way ANOVA.

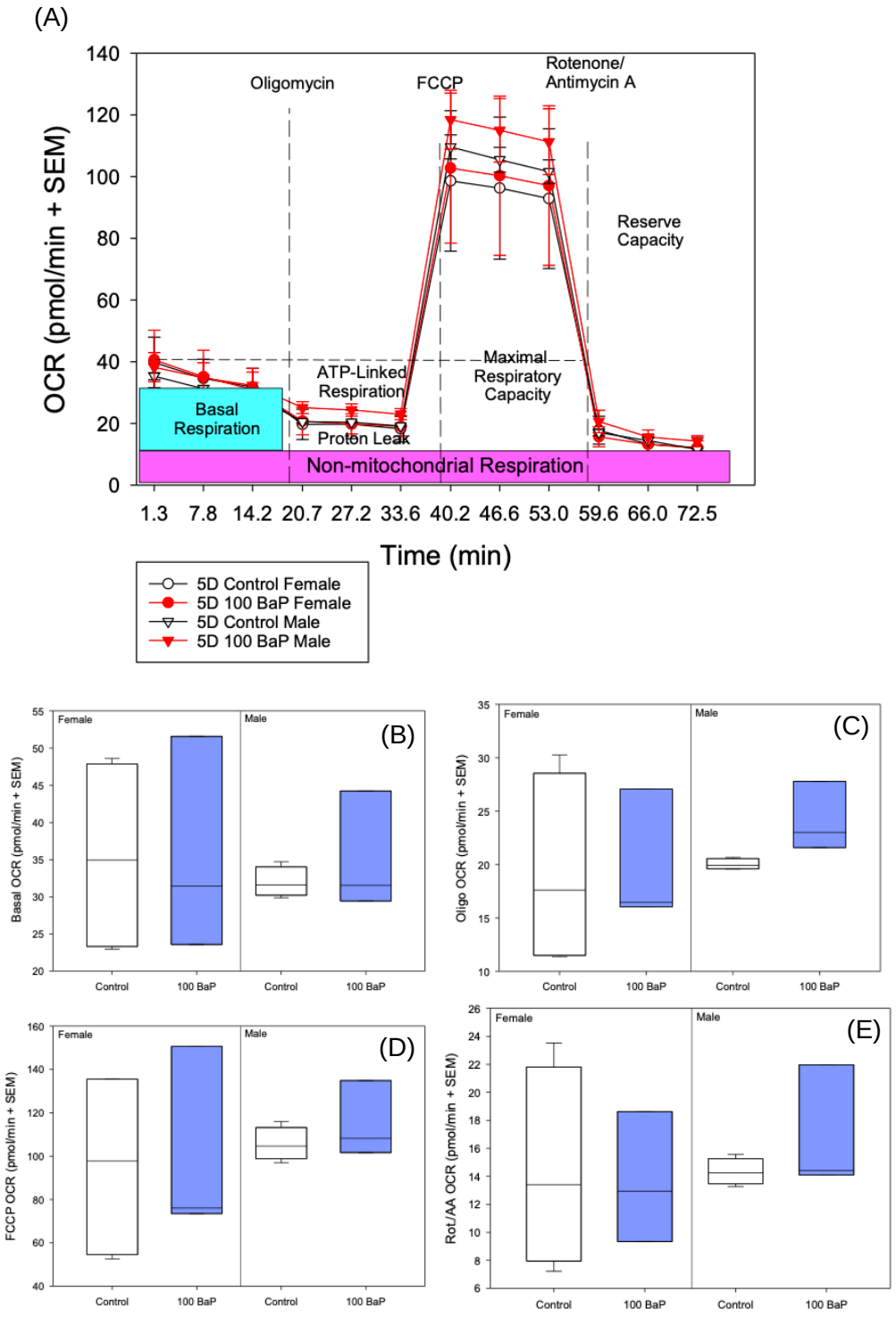


Figure 10: 5D Seahorse XFe96 Flux Analyzer Results
Based on the Seahorse XFe96 Flux Analyzer protocol, fish mitochondria was extracted for the Cell Mito Stress Test. The OCR relative to time is shown in (A), and the average OCR of the cycles is shown below: Basal OCR (B), Oligomyocin (C), FCCP (D), and Rotenone/Antimycin A (E). The data was compared between sex and treatment using a two-way ANOVA test using an n=3-4/sample.

Presumptive *Ahr2*$^{-/-}$ Results:

Half of the presumptive *Ahr2*$^{-/-}$ F0 fish were genotyped at 4 mpf, and at this point, it was discovered that not all the fish were *Ahr2*$^{-/-}$. Therefore, the figures are labeled *Ahr2*^{mix} to show that there was a mix of *Ahr2*$^{+/+}$(34%), *Ahr2*$^{+/-}$ (9%), and *Ahr2*$^{-/-}$ (38%), and 19% of the samples did not have a sufficient DNA sample for genotyping. Relative to length and weight (Figure 11), the differences in length between the various treatments of the *Ahr2*^{mix} fish were significant at 1 mpf showing a dose-dependent increase in length. However, there was no other significant differences for the *Ahr2*^{mix} fish when comparing length and weight at 3 and 4 mpf.

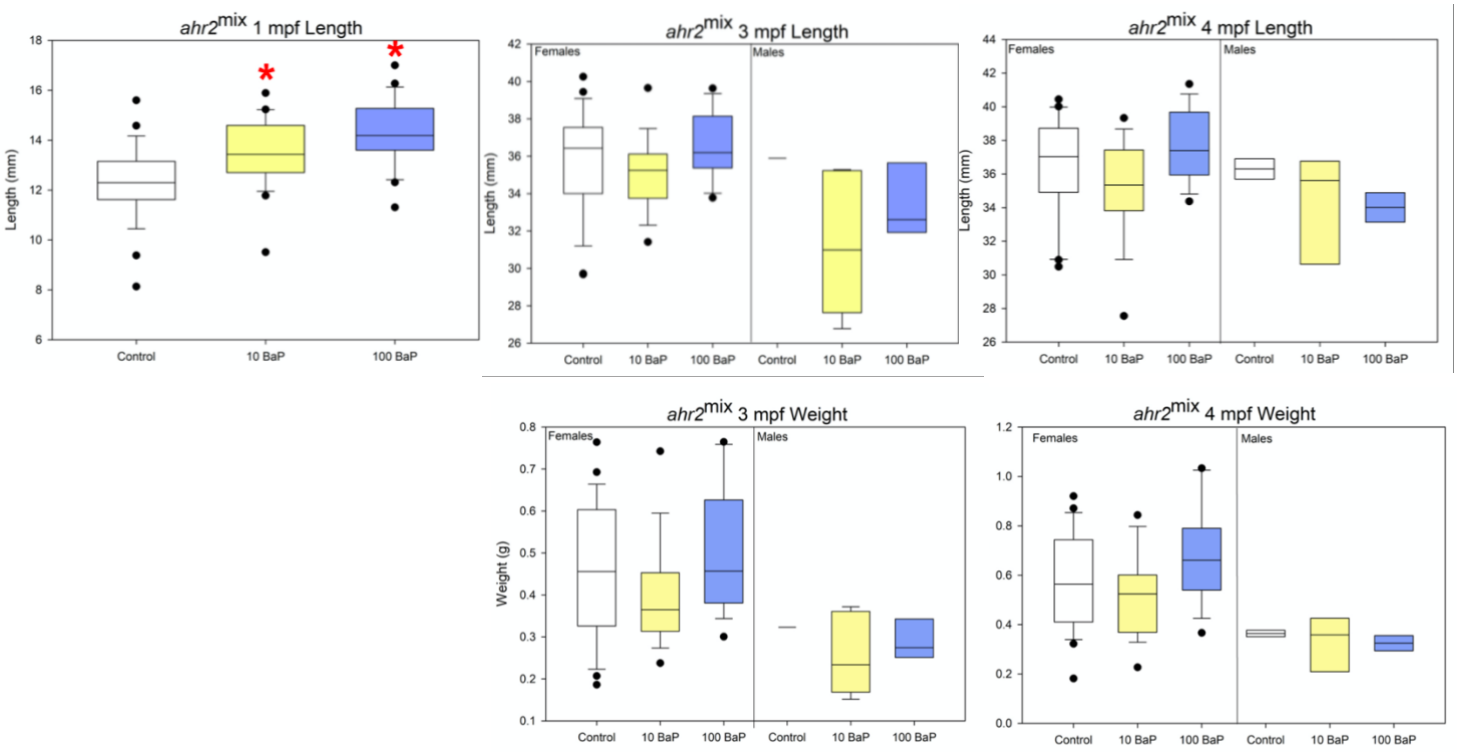


Figure 11: *Ahr2*^{mix} Average Lengths and Weights at 1, 3 and 4 mpf
Zebrafish length at 1 mpf was compared across treatment group using an ANOVA followed with a Dunnett's post-hoc test to compare each group to control (n=21-26). Zebrafish length at 3 mpf (n=21-26) and 4 mpf (n=20-25) was compared using a one-way ANOVA test to observe for any treatment-related effects. Zebrafish weight at 3 mpf and 4 mpf was recorded, and this data was also compared using a one-way ANOVA observe for significant differences between treatment. Comparisons between sex were unable to be determined due to an insufficient n for males. Statistically significant findings are denoted with the (*) symbol in red above ($p \leq 0.05$).

When analyzing behavior with the larval photomotor response assay at 120 hpf, the *Ahr2*^{mix} fish had no significant difference between treatment groups in either the light or dark phases (Figure 12). At 1 mpf, there were no significant differences between treatment groups for the *Ahr2*^{mix} fish in the open field tests (Figure 13). For the 3 mpf OFT (Figure 14), the fish were separated by sex, and there was only a total of 9 males (out of the 68 fish) in the *Ahr2*^{mix} strain across all three treatment groups. As a result of the small sample size, statistics could not be calculated for the *Ahr2*^{mix} male sample. In the *Ahr2*^{mix} females, there was a significant decrease in the percent of time spent in periphery for the 100 µg/L BaP exposure group in comparison to the control at 3 mpf. For the 4 mpf OFT (Figure 15), the statistics for the *Ahr2*^{mix} males were not calculated again due to the small sample size. For the female *Ahr2*^{mix} fish, there were no significant differences in behavior. Because there were not enough males in each treatment group, reproductive success was not assessed in the *Ahr2*^{mix} fish. Following behavior at 4 mpf, fish were euthanized, and brains were processed for the Cell Mito Stress Test with the Seahorse XFe96 Flux Analyzer. Due to too few male fish and issues with protein quantification, not enough protein was obtained to run mitochondria in this assay.

(A)

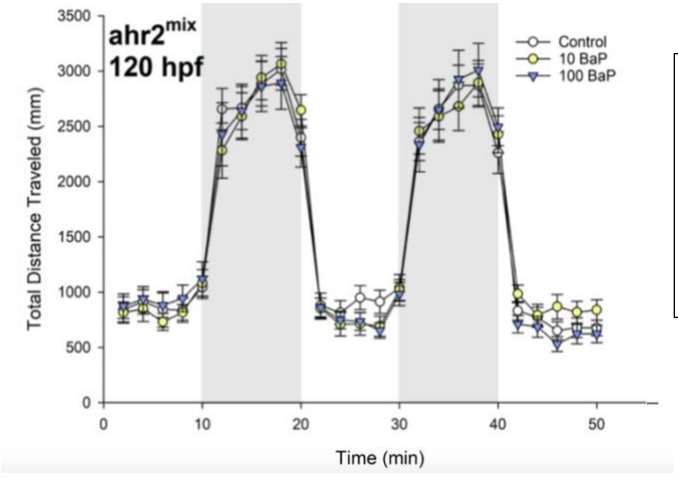


Figure 12: *Ahr2*^{mix} Larval Photomotor Response Assay (120 hpf)
Distance traveled over a 50 min larval photomotor response assay (light: 0-10, 20-30, 40-50 min) (dark: 10-20, 30-40 min). There was no significant difference between treatments and the control group. Statistical significance was determined using a one-way ANOVA to compare the three groups (n =28-29).

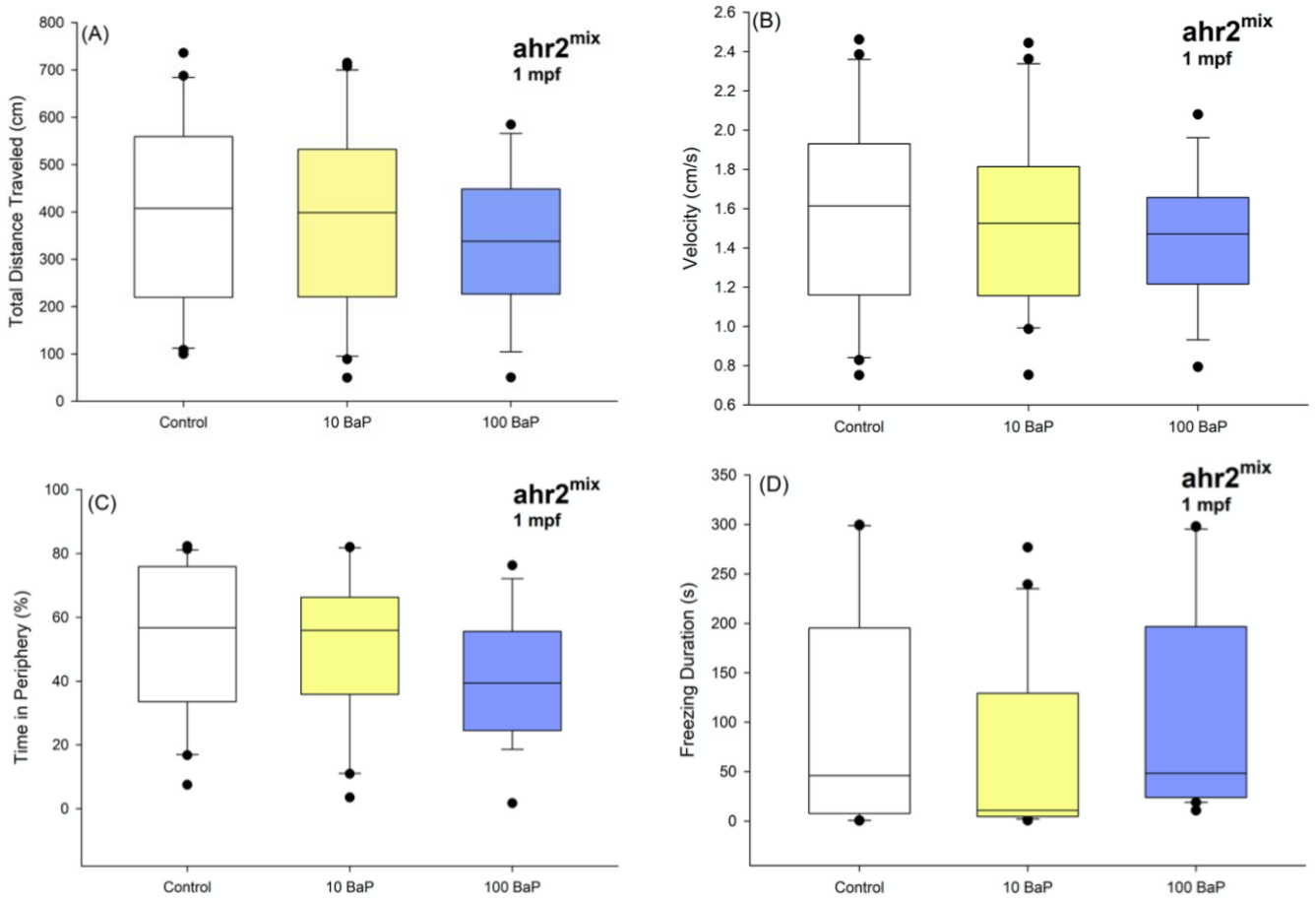


Figure 13: *Ahr2*^{mix} Open Field Test Results for 1 mpf
Zebrafish behavior was assessed in the open field environment at the juvenile stage (1 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The data was analyzed using a one-way ANOVA to determine if behavior differs between treatment groups (n=21-26). There were no significant differences.

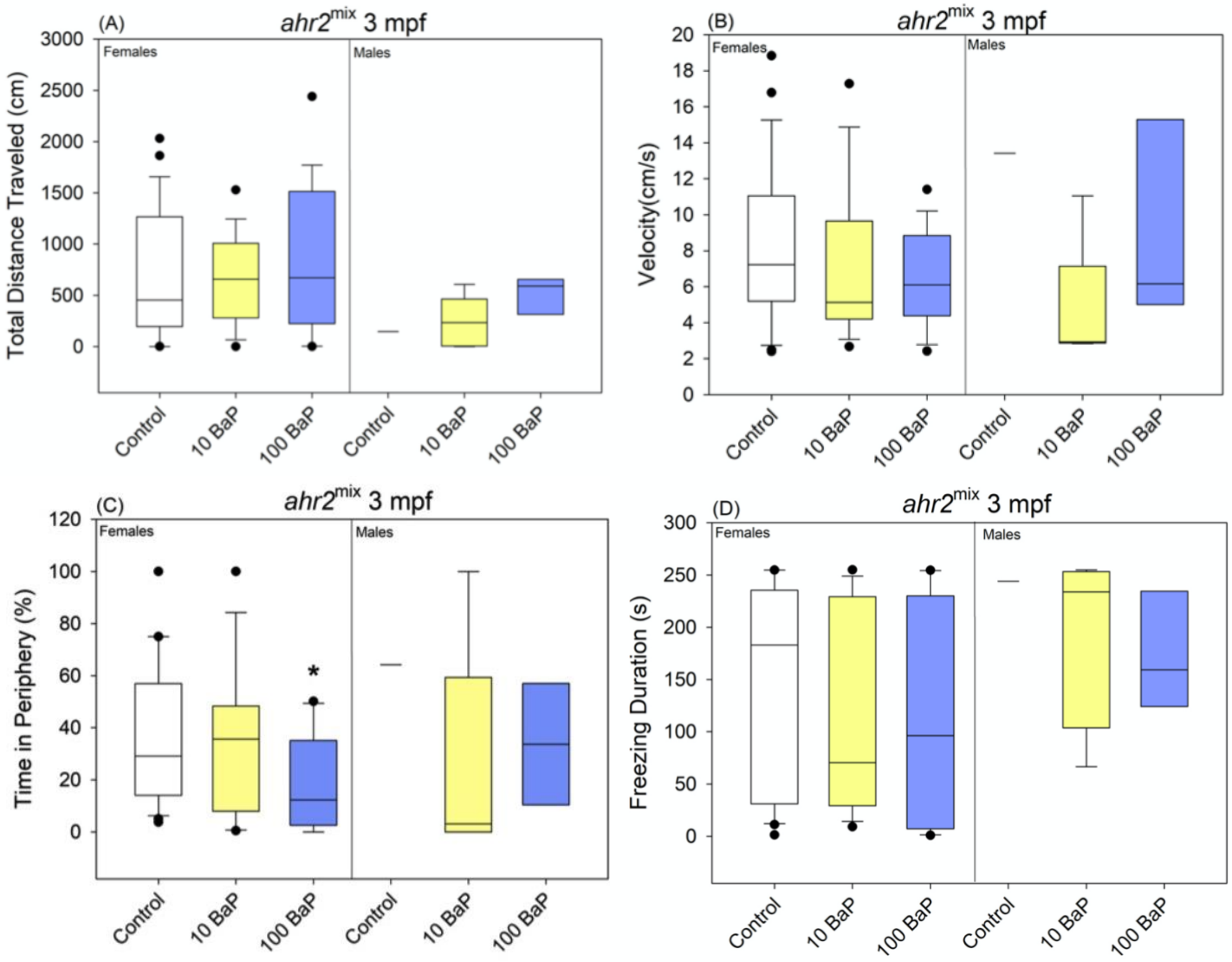


Figure 14: *Ahr2*^{mix} Open Field Test Results for 3 mpf
Zebrafish behavior was assessed in the open field environment at the adolescent stage (3 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The data was analyzed using a one-way ANOVA to determine if behavior differs between treatment groups (n=21-26/treatment) for females (n=59). Sex-dependent effects were indeterminable due to an insufficient n for males (n=9 males).

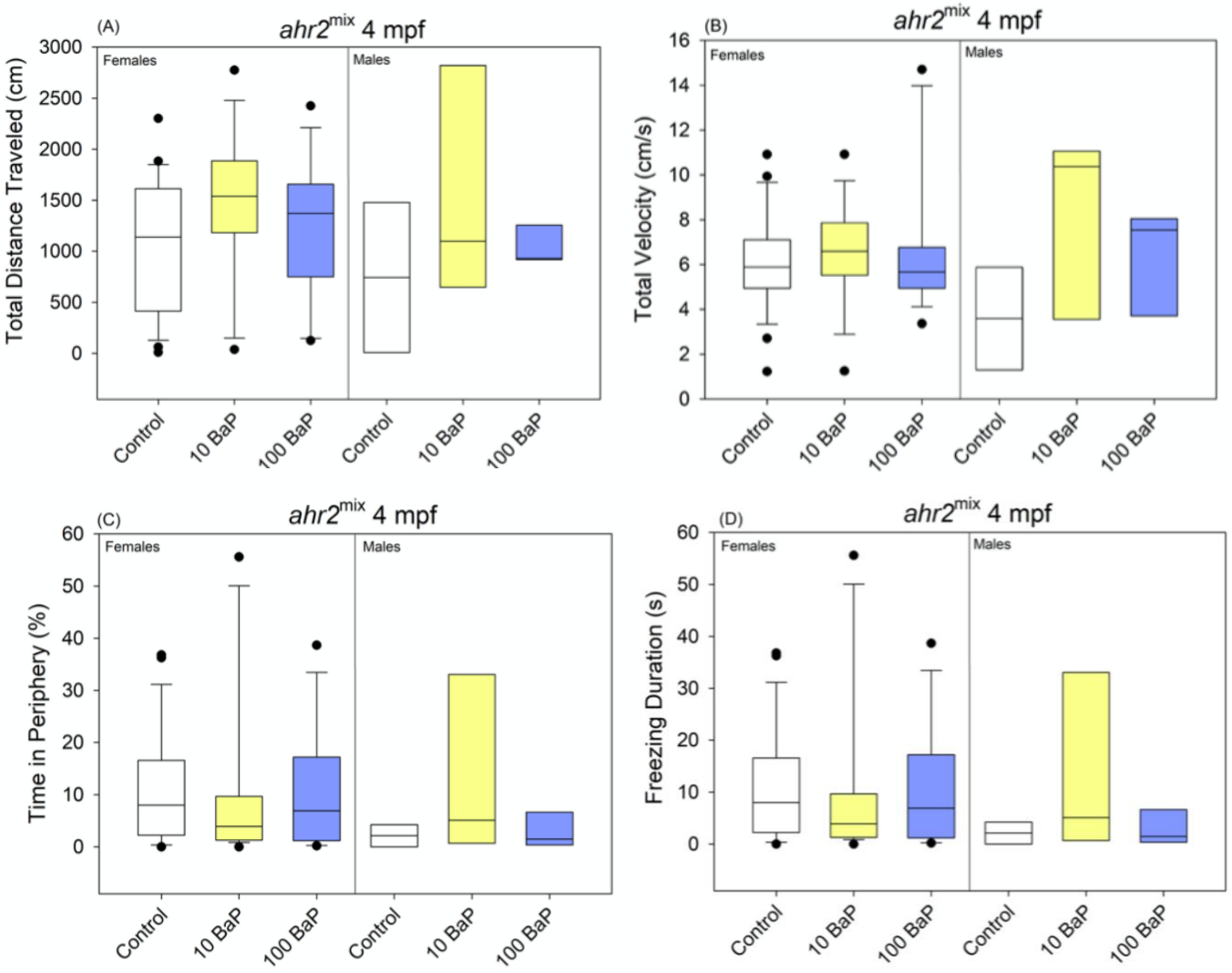


Figure 15: *Ahr2*^{mix} Open Field Test Results for 4 mpf
Zebrafish behavior was assessed in the open field environment at the adulthood stage (4 mpf) using the Noldus Ethovision 14.0 Software to record total distance (A), average velocity (B), time in periphery (C), and freezing duration (D) during a 5-minute period. The data was analyzed using a one-way ANOVA to determine if behavior differs between treatment groups (n=20-25/treatment) for females (n=59). Sex-dependent effects were indeterminable due to an insufficient n for males(n=7). There were no significant differences.

4. DISCUSSION:

The Department of Health and Human Services (DHHS) has determined that many polycyclic aromatic hydrocarbons (PAHs) including benzo[a]anthracene, benzo[b]fluoranthene, benzo[a]pyrene, etc. have cancer-causing potential in animals (Mumtaz, 1999). Additionally, the Agency for Toxic Substances and Disease Registry (ATSDR) currently ranks PAHs as the 9th most likely substance to cause harm to human health on their Substance Priority List (ATSDR, 2019). In addition to increased cancer potential, studies show that benzo[a]pyrene exposure via inhalation in factory workers is linked to extensive respiratory abnormalities such as bronchiovascular markings, pleural effusions, and chest and breathing problems (Mumtaz, 1999). Along with an increased predisposition to disease, PAH exposure poses long-term behavioral and reproductive effects. Prenatal PAH exposure has been positively associated with cognitive developmental effects and symptoms of anxiety/depression and attention problems in children (Perera et al., 2012). Furthermore, the aryl hydrocarbon receptor (*AHR*) is a highly conserved transcription factor required for proper developmental and reproductive functions, and it also mediates the toxic effects of PAH exposure. Because BaP is also an aryl hydrocarbon receptor (AHR) agonist and up-regulates CYP1, it increases the likelihood of CYP1-dependent bioactivation and ultimately leads to epoxide formation in the body (Vermillion Maier et al., 2022). Epoxides are chemically regarded as the ultimate carcinogenic metabolite from PAH exposure (Manson, 1980). Additionally, AHR activation via BaP activates the mitogen activated protein kinase (MAPK) signaling cascade, which alters cellular processes and leads to BaP-induced DNA damage (Vázquez-Gómez et al., 2018). Despite the receptor mediating toxic effects, AHR is required for proper development as *Ahr2*-null fish show a reduction of mature follicles, decreased survival, and impaired behavioral responses in zebrafish (Garcia et al., 2018).

Due to the known impacts of parental and prenatal PAH exposure, our interest increased in better understanding the toxicologic effects of varying levels BaP exposure between 5D and *Ahr2*-null zebrafish.

Zebrafish were used as a model organism to study the behavioral, developmental, reproductive, and bioenergetic effects of developmental BaP exposure due to their fully sequenced genome and shared homology with humans. In a prior study from our laboratory, it was established that parental exposure followed by embryonic exposure reduced egg production and offspring survival, and BaP exposure altered gene expression in cancer and development-related genes ultimately suggesting an increased risk of disease in adulthood (Corrales et al., 2014). Following the establishment of multigenerational developmental and toxicological effects from BaP exposure, the two goals of this study were to (1) assess how a developmental waterborne BaP exposure causes acute and chronic changes in behavior and mitochondrial bioenergetics and (2) elucidate the role of *Ahr2* in mediating changes in size, bioenergetics, fecundity, and behavior following the waterborne exposure. This study provides further insight on the effects of a developmental BaP exposure throughout key stages (larval, juvenile, adolescence, adulthood) in the zebrafish life cycle.

In previous studies, BaP exposure in parents led to larval deformities in the F1 generation, and the *Ahr2* receptor was implicated to have a key role in mediating those effects (Corrales et al., 2014). Our project goal was to further elucidate the role of *Ahr2* in mediating BaP toxicities, and therefore, the 5D strain was selected because the *Ahr2*-null ($Ahr^{-/-}$) fish were on the 5D background. Dose selection was based on prior studies conducted in the lab (Fang et al., 2015). However, the presumptive *Ahr2*-null group was later discovered to not be completely

Ahr2-null because of an error from the initial breeding. Therefore, the focus of the discussion is on the effects of the 5D BaP exposure rather than a comparison between strains.

The BaP exposure began at 6 hpf and continued to 120 hpf. Survival, hatch, length, and weight were recorded at various time points during and after the exposure. At 3 mpf, there was a significant increase in male weight for the 100 µg/L BaP group compared to controls, and while not statistically significant, a similar trend was present for the weight at 4 mpf ($p = 0.0562$). At 4 mpf, there was also a significant increase in length for the 5D males. The gain in size and weight coincides with a prior BaP exposure study in mice showing increased adipose tissue content with higher concentrations of BaP exposure indicative of weight gain and obesity (Ortiz et al., 2014), and ultimately suggests that BaP exposure can be linked to childhood obesity. BaP is also considered an environmental obesogen because a previous study conducting BaP exposure on mice led to the impairment of adipose tissue lipolysis and ultimately caused weight gain (Irigaray et al., 2006; Janesick, 2001). In turn, obesity can lead to an increased predisposition for health conditions such as high blood sugar and high blood pressure. These health conditions can also lead to more severe outcomes such as heart disease, kidney disease, strokes, etc.

In addition to recording size and survival, behavioral data was obtained. Locomotor activity endpoints are a widely-used and scientifically-accepted way to study the behavioral effects of various drugs and neurotoxic compounds (Bownik et al., 2020). Larval photomotor response assays and open field tests were performed to provide data to determine these endpoints. For the larval photomotor response assay, the difference in total distance traveled was significantly different during the dark phases with the 100 µg/L BaP being more hyperactive than the control. In previous studies, BaP exposure induced hyperactivity in male rats, and this hyperactivity also correlated with cognitive and motor impairments (Hawkey et al., 2019; Maciel

et al., 2014). This finding suggests that there is a potential link between BaP exposure and neurological effects, and these effects could present differently between sexes.

Behavioral testing continued with open field testing conducted at juvenile stage (1 mpf), but there were no significant differences based on treatment. The lack of findings is possibly due to the limited size of the arena at 1 mpf. The average length of all fish was 12 mm at 1 mpf, while the arena diameter was 3.5 cm (35 mm). At 3 mpf, the average fish length was 33.7 mm with a 21 cm (210 mm) arena diameter. The fish only increased in length by approximately a factor of 3 while their environment diameter was increased by a factor of 6. At 3 mpf and 4 mpf, the fish had a larger swimming area relative to their size compared to 1 mpf. Therefore, the fish were likely hindered in terms of locomotion and exploratory activity at 1 mpf due to the small-well environment. Open field testing was repeated at adolescence (3 mpf). There were no significant differences in average velocity and freezing duration between treatment groups. However, there was a significant decrease in total distance traveled along with a significant increase in the freezing duration for the exposed fish compared to the control. Previous studies suggest that BaP exposure can lead to a decrease in locomotion and exploratory activity in zebrafish (Gao et al., 2017; Vignet et al., 2014). These effects suggest the possibility of neurocognitive decline associated with BaP exposure (Vignet et al., 2014). These effects could also coincide with the weight gain seen in males at this time point because lack of movement can be relative to weight gain. Open field testing was again conducted in adults (4 mpf), and at this time-point, the sex of the fish was unable to be determined. There was no significant difference between treatments for any of the parameters measured. However, there was a significant difference in the percent of time spent in the periphery between males and females. Adult females were found to spend more time in the periphery compared to adult males potentially

indicating higher levels of anxiety-related behavior than adult males. Previous longitudinal studies have shown a link between maternal PAH exposure and their children having a higher chance of developing anxiety/depressive disorders (Perera et al., 2012). In past studies from our lab, the female F1 offspring of dietarily-exposed parents also showed significantly greater levels of larval hyperactivity indicating anxiety-related behaviors (Pandelides, 2021). Aside from behavior, there are also known differences in CYP1A1 gene expression between male and female smokers, and females showed higher levels of expression suggesting an increased likelihood of cancer (Mollerup et al., 1999). This led to our increased interest into the complexity of the multigenerational sex-dependent, BaP-induced effects. However, further research is needed to explore the possible implications of these sex-dependent effects.

After conducting behavioral analysis, the Seahorse XFe96 Flux Analyzer was used to compare bioenergetic state of the control fish to the BaP-exposed fish. Previous studies showed that BaP injections can induce increases in oxygen consumption rate (OCR) in zebrafish, and that OCR is directly proportional to weight/adipose-content (Gerger & Weber, 2015; Valverde et al., 2009). Despite significant differences in weight at 3 mpf between the treatment groups, there were no significant differences in weight at 4 mpf, and there was no significant difference in OCR at 4 mpf between treatment or sex for any of the measurement periods (baseline, oligo, FCCP, rotenone/antimycin A). The data contained a large variance, and this possibly suggests that due to the length of the exposure window being limited, initial effects did not persist into adulthood. If the Seahorse XFe96 Flux analyzer had been optimized for the larval stage, there would have been a greater likelihood for significant differences amongst the treatment groups.

Additionally, an F1 generation was bred to determine how the effects of a developmental BaP exposure persist and to see if BaP exposure affects the reproductive capabilities of

zebrafish. Contrary to the larval photomotor response assay for the F0 generation, the F1 larvae whose parents were exposed to BaP showed significant hypoactivity in the dark phase compared to controls. This suggests that there is a potential trend in the locomotive effects of developmentally exposing zebrafish to BaP causing initial hyperactivity and then leading to an increase in weight and hypoactivity.

Initially, it was hypothesized that BaP exposure causes lasting effects on the size, behavior, bioenergetics, and reproductive capabilities of organisms that are *Ahr2*-dependent, sex-dependent, and treatment-dependent. Overall, the developmental BaP exposure led to acute effects such as hyperactivity at 120 hpf, which is indicative of possible neurocognitive decline. Additionally, there were other significant outcomes such as the treatment-related weight gain at 3 mpf and an increase in length at 4 mpf for the 5D males. However, as the exposed fish aged, they exhibited less movement with a decrease in total distance traveled at 3 mpf and a subsequent increase in freezing duration at 3 mpf for the males. This shows that BaP exposure can affect locomotor and exploratory activity into adulthood suggesting adverse and persistent developmental effects. BaP toxicities can also present differently between sexes. Overall, this study does show that BaP exposure can potentially lead to adverse effects to human, and these effects can differ based on treatment or sex.

This study was initially designed to gain further insight into the role of the *Ahr2* receptor. Because all the presumed $Ahr2^{-/-}$ fish were not truly $Ahr2^{-/-}$, a comparative analysis on the effects of BaP exposure between 5D and $Ahr2^{-/-}$ strain fish was not performed. Appropriate data was acquired, but the $Ahr2^{-/-}$ component of this study was not able to provide reliable results as the fish were later discovered to be a mix of $Ahr2^{+/+}$, $Ahr2^{+/-}$, and $Ahr2^{-/-}$. Because it was also hypothesized that developmental BaP exposure would lead to multigenerational effects, a larval

photomotor response assay was performed on the F1 generation, and the progeny of exposed fish showed decreased levels of activity compared to the controls. Although the F0 exposed fish exhibited hyperactivity, the inverse effect in the F1 generation suggests that there is decreased locomotor activity in the long run which could ultimately affect cognition.

In terms of future BaP studies, discovering more about the *Ahr2* receptor is a primary goal, and further research is needed to understand the role of the receptor in mediating the toxic effects caused by BaP exposure. A better understanding of *Ahr* could ultimately help build the foundation to possibly prevent and/or treat the various toxicities associated with BaP exposure. With continued research, our lab strives to gain further insight into the molecular and phenotypic effects of BaP to gain a better understanding of the possible outcomes of PAH exposure.

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