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Developmental benzo[a]pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are produced from incomplete combustion of organic materials or fossil fuels, and are present in crude oil and coal; therefore, they are ubiquitous environmental contaminants present in urban air, dust, soil, and water. It is widely recognized that PAHs pose risks to human health, especially for the developing fetus and infant where PAH exposures have been linked to in-utero mortality, cardiovascular effects, and lower intelligence. Using the zebrafish model, we evaluated the developmental toxicity of benzo[a]pyrene (B[a]P). Zebrafish embryos were exposed from 6–120 hours post fertilization (hpf) to 0.4 and 4 μ M B[a]P. The Viewpoint Zebrabox systems were used to evaluate larval photomotor response (LPR) activity and we identified that exposure to 4 μ M B[a]P resulted in a hyperactive LPR phenotype. To evaluate the role of aryl hydrocarbon receptor (AHR) in this larval phenotype, we exposed *ahr2*^{hu2334} null larvae to 4 μ M B[a]P. Though *ahr2*^{hu2334} larvae did not display hyperactive swimming, these larvae had a decrease in LPR activity, suggesting AHR2 plays a role in B[a]P induced larval hyperactivity. To determine if developmental B[a]P exposures would produce adult behavioral deficits, a subset of exposed animals was raised to adulthood and tested in a conditioned stimulus test using shuttleboxes. Developmentally exposed B[a]P zebrafish exhibited decreased learning and memory. Together this data demonstrates that developmental B[a]P exposure adversely impacts larval behavior, and learning in adult zebrafish.

Keywords

neurotoxicity; zebrafish; benzo[a]pyrene

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Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

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1. Introduction

Benzo[a]pyrene (B[a]P) is a nearly ubiquitous polycyclic aromatic hydrocarbon (PAH) contaminant, formed by incomplete combustion of organic materials, especially petroleum-based fuels and coal (ATSDR 1995; Howsam and Jones 1998). As such it is often used as a surrogate for general PAH contamination. B[a]P is also found in cigarette smoke, grilled and broiled foods, and is a byproduct of some industrial processes such as coke ovens in metal processing plants (ATSDR 1995). Human exposure is primarily through ingestion and inhalation and B[a]P is categorized in the highest cancer-causing potential classification, group 1 carcinogenic in humans. As a pro-mutagen, B[a]P must be metabolized to reactive species before it can induce adverse effects, including carcinogenicity (Genies 2013; Souza 2015). One mechanism of toxicity for benzo[a]pyrene is through activation of the aryl hydrocarbon receptor, AHR, and subsequent metabolism by the CYP class of enzymes into more toxic intermediates known to cause oxidative stress, DNA adducts, DNA strand breaks, and gene mutations in cells and animal models (Genies 2013; Souza 2015). B[a]P also targets the cardiovascular system, and developmental exposures are associated with cardiac toxicity in zebrafish (Incardona, Collier et al. 2011; Huang, Wang et al. 2012; Huang, Zuo et al. 2015) and mice (Sagredo 2009; Kerley-Hamilton 2012). Developmental B[a]P cardiotoxicity is AHR dependent in mice (Kerley-Hamilton 2012) and AHR2 dependent in zebrafish (Incardona, Collier et al. 2011; Van Tiem and Di Giulio 2011). Additionally, both developmental and acute B[a]P exposure is associated with impaired motor activity, and lower cognitive performance in juvenile and adult rodents (Saunders, Das et al. 2006; McCallister, Maguire et al. 2008; Chen, Tang et al. 2012; Cheng, Xia et al. 2013; Brown 2016) and anxiety-like behavior in juvenile killifish and zebrafish developmentally exposed to B[a]P-containing PAH mixtures (Vignet, Menach et al. 2014; Brown 2016). The observed neurotoxicity is also hypothesized to result from toxic metabolites (Saunders, Das et al. 2006; Chepelev, Moffat et al. 2015). B[a]P is lipophilic, known to cross the blood-brain-barrier and has been detected, along with B[a]P metabolites, in brain tissue of rodents after exposure (Saunders, Das et al. 2006; McCallister, Maguire et al. 2008; Yan, Xiang et al. 2012; Chepelev, Moffat et al. 2015). Short term B[a]P exposure in killifish resulted in an increase in BPDE-dG adduct levels, and both larval and adult killifish exposed to B[a]P also showed a significant increase of nDNA and mtDNA damage, measured as DNA lesions in whole animal or in multiple tissues, including the brain (D Jung 2009). In addition, increased lipid peroxidation was detected in brains of rodents exhibiting decreased cognitive performance following B[a]P exposure (Saunders, Das et al. 2006; McCallister, Maguire et al. 2008; Chen, Tang et al. 2012; Cheng, Xia et al. 2013).

Studies have suggested that developmental stages are more sensitive than adults to the genotoxic effects of environmental PAHs (Tang, Li et al. 2006). PAHs, including B[a]P and Ba]P metabolites, are commonly detected worldwide in the human placenta, umbilical cord blood, maternal blood and breast milk samples (Perera, Whyatt et al. 1998) (Madhavan and Naidu 1995; Perera, Rauh et al. 2006; Tang, Li et al. 2006; Perera and Herbstman 2011). Exposure to PAH mixtures in humans have been linked to *in-utero* mortality, lower intelligence, and cardiovascular effects (Perera, Jedrychowski et al. 1999; Perera, Rauh et al. 2006; Tang, Li et al. 2006). Prenatal exposure to environmental PAHs, including B[a]P, is

associated with learning deficiencies and behavioral disorders. A cohort of newborns, with higher diverse PAH exposure measurements in umbilical cord blood and maternal blood at birth, had a significantly higher incidence of delayed mental development at age three (Perera, Rauh et al. 2006). Higher PAH-adduct levels in cord blood was significantly correlated to increased child behavior checklist (CBCL) scores at ages 5 and 7, reflective of increased anxiety, depression, and attention problems (Perera and Herbstman 2011). Though B[a]P is often detected in these human studies, it is important to note that environmental exposures occur in complex mixtures which can result in different exposure effects and mechanisms of action compared to individual PAH exposures. Various developmental or PAH mixtures can result in either synergetic or antagonistic effects compared to individual PAHs, depending on chemical composition. Though environmental PAH exposures occur in mixtures, B[a]P is highly toxic and one of the most commonly found PAHs in the environment (Gerger and Weber 2015), so it is important to identify B[a]P specific developmental and long-term effects of exposure.

In this study we used the zebrafish model to investigate the behavioral effects of developmental B[a]P exposure in larval animals and evaluated the long-term effects in adult animals. The larval photomotor response (LPR) assay was used to measure changes in swimming patterns and total movement. Unexposed zebrafish larvae normally exhibit low total movement in the light and increased movement in the dark following light-dark transitions (MacPhail, Brooks et al. 2009; Truong, Saili et al. 2012; Noyes, Haggard et al. 2015). Developmental exposure to multiple chemicals including ethanol, nicotine, nanomaterials, and halogenated organophosphate flame retardants can elicit a larval hypoactive or hyperactive phenotype in zebrafish (MacPhail, Brooks et al. 2009; Truong, Saili et al. 2012; Crosby, Bailey et al. 2015; Noyes, Haggard et al. 2015). Chemical exposures in adult zebrafish have produced differences in learning and memory retention during an active avoidance test. For example, nitro-L-arginine methyl-ester (L-NAME), (Xu, Scott-Scheiern et al. 2007) and methylmercury (Xu, Weber et al. 2012) resulted in significantly decreased avoidance and escape responses, reflective of impaired learning. Adult zebrafish developmentally exposed to polybrominated diphenyl ethers (PBDE-47) showed a significantly lower shock avoidance and performance deterioration between a train and test session, suggesting compromised memory in adult animals (Truong, Mandrell et al. 2014). These larval and adult assays demonstrate the zebrafish is a robust model to study the developmental and long term neurological effects of exposure.

We exposed embryonic zebrafish to 0.4 and 4 μ M B[a]P from 6–120 hours post fertilization (hpf) and evaluated behavioral changes at 120 hpf. Because B[a]P toxicity may not act exclusively through the AHR, we also evaluated the larval photomotor response behavior phenotypes in an AHR2 null background larvae (Goodale, La Du et al. 2012). Finally, to evaluate if developmental B[a]P exposure leads to life-long behavioral effects, a subset of exposed animals was raised to adulthood and tested for learning and memory deficits using custom built shuttle boxes. Together this data demonstrates developmental B[a]P exposure leads to effects in larval behavioral changes as well as impaired learning in adult zebrafish.

2. Materials and Methods

2.1. Fish Husbandry

Adult zebrafish were maintained with a water temperature of 28+/- 1°C on a recirculating system with a 14h light: 10h dark photoperiod at the Oregon State University Sinnhuber Aquatic Research Laboratory (SARL). All experiments were conducted with the wildtype 5D Tropical or AHR2-null (*ahr2*^{hu3335}) strains. The *ahr2*^{hu3335} line, with a point mutation in *ahr2* (*ahr2*^{hu3335} strain) is from the Hubrecht Institute. This line was identified from a library of N-ethyl-N-nitrosourea (ENU)-mutagenized zebrafish using the TILLING method and characterized by Goodale et al (Goodale, La Du et al. 2012). Fish husbandry, reproductive techniques, and adult learning tests were conducted according to Institutional Animal Care and Use Committee protocols at Oregon State University. All embryos used in exposure experiments were collected following group spawning of adult zebrafish as described previously (Kimmel, Ballard et al. 1995; Reimers, La Du et al. 2006).

2.2. Benzo[a]pyrene Exposure and Developmental Toxicity

A neat benzo[a]pyrene (B[a]P) powder standard was obtained from Sigma Aldrich and dissolved in dimethyl sulfoxide (DMSO) resulting in a maximum solubility master stock of 4.4mM. For exposure experiments, dilution stock solutions were made in 100% DMSO and during initial dose range experiments, solutions were made at a 1:100 dilution in E2 embryo medium, (Westerfield 2000) (1% DMSO final concentration). Zebrafish embryos were statically exposed to 1, 5, 10, 20, 25, 30, 40 µM B[a]P from 6–120 hpf in 96-well polystyrene plates, with one embryo per well in 100 µl of solution. At 24 hpf, zebrafish embryos were evaluated for 4 endpoints: mortality, developmental progress, somite, and notochord malformations; and at 120 hpf evaluated for total mortality and a suite of 17 morphological endpoints including yolk sac edema, pericardial edema, body axis, snout, jaw, eye, otic, brain, trunk, caudal and pectoral fin malformations, notochord, somites, circulation, pigment, swim bladder, and touch response (Truong, Harper et al. 2011). All subsequent behavior studies were conducted at 0 µM (0.1% DMSO), 0.4 and 4 µM B[a]P, concentrations that did not cause any significant increase in mortality or malformations at 120hpf.

2.3. Larval Photomotor Response (LPR)

The Viewpoint Zebrabox systems (Viewpoint Behavior Technology) were used to evaluate photo-induced larval locomotor activity in 120 hpf larvae. Zebrafish embryos were exposed to 0, 0.4 and 4 µM B[a]P in 96-well plates, with a minimum of 32 animals of each concentration tested and a final DMSO concentration of 0.1%. The plates were wrapped in foil from 6–120 hpf, and unwrapped for testing in the Zebrabox. The video tracking protocol on the Viewpoint Zebrabox software (version 3.2, Viewpoint Life Science, Lyon, France) was used to track total larval movement over light-dark cycles. The larval photo motor response (LPR) assay consisted of 3min light and dark alternating periods, with a total of four light-dark transitions, but only 3 light-dark transitions were analyzed. The level for the lighted period was set for 525 LUX. The integration time was set to 6 seconds and raw data files were processed using custom R scripts (Team 2013) to average the total distance traveled for each integration time point, and the area under the curve was computed.

Zebrafish were evaluated at 120 hpf for mortality and a suite of 17 physical malformations following the viewpoint assay (Truong, Harper et al. 2011), and any mortalities or animals with physical malformations (Reif, Truong et al. 2016) at 120 hpf were excluded from the data analysis. The overall area under the curve for the last 3 light-dark cycles was compared to the DMSO control using a Kolmogorov-Smirnov test ($p < 0.01$).

2.4. Adult Studies

For the adult studies, solution stocks were diluted 1:1000 in E2 embryo medium for a final DMSO concentration of 0.1% DMSO. Zebrafish embryos were exposed to 0, 0.4 and 4 μ M B[a]P from 6–120 hpf in 96 well plates. For the adult grow out studies, 288 embryos were exposed from 6–120 hpf and grown out to adulthood. At 15 months, adult survivorship in the vehicle controls for this cohort was 69% and similar among the treatment groups (68 and 72% respectively). A subset of ~60 animals from each treatment group were randomly chosen for learning assessments in the shuttlebox instruments.

2.5. Adult Learning in B[a]P exposed animals

An active avoidance conditioning test was used to assess learning and performance differences in B[a]P developmentally exposed zebrafish compared to controls. Custom built shuttleboxes, previously described (Truong, Mandrell et al. 2014), were used with a modified protocol (Xu, Scott-Scheiern et al. 2007; Truong, Mandrell et al. 2014) to test for learning deficiencies. Adult zebrafish developmentally exposed to either a vehicle control or B[a]P were evaluated at approximately 15–18 months of age. Briefly, zebrafish were placed into shuttleboxes that were designed to condition the fish to leave the blue lighted side to avoid a mild electrical shock (unconditioned stimulus), and swim to the dark side (conditioned stimulus). Thus, for purposes of data categorization, the lighted and dark sides were deemed “reject side” and “accept side”, respectively. Each shuttlebox trial was automatically initiated with the fish’s current location assigned as the reject side by the computer. At the initiation of each trial, a 4 sec “seek period” allowed the zebrafish time to choose to leave the reject side for the accept. Once the 4 seconds elapsed, or immediately upon the fish changing sides, whichever happened first, the conditioning mode began. Thus, a fish could choose to go to the accept side prior to the end of the 4 sec seek period and, if it remained there for the trial, would not receive a shock during that trial. If the fish did not swim to the accept side within the seek period, or crossed back to the reject side after occupying the accept side, the unconditioned stimulus (US), a mild shock (3V, 500ms duration, at a 1sec interval, for a maximum 20 sec duration) was administered until the fish returned to the accept side. After the 24 sec trial, there was a 60 sec inter-trial interval where both sides of the shuttlebox were dark (accept condition), before the next trial began. As a humane contingency, if the zebrafish did not leave the reject side for the entire 24 sec trial, for 8 trials in a row, the fish would “fault out” and would be removed from further testing. Prior to commencement of trials, a 10 minute acclimation period in the dark (accept condition) was allowed, and thereafter testing consisted of 50 consecutive trials. Two sets, a train session of 50 trials and a test session of 30 trials were conducted, separated by a 1 hour rest, with both compartments of the shuttlebox darkened.

Statistical analysis was done using code developed in R version 3.0.1 (R Development Core Team 2010) as previously described (Truong, Mandrell et al. 2014). Briefly, data for each exposure group was fit using linear regression models and intercept and slopes were calculated for each parameter. An analysis of variance (AOV) was used to identify differences in regression lines, followed by a Tukey's Honest Significant Differences (HSD) test for pairwise comparisons.

3. Results

3.1. Developmental Toxicity and Larval Photomotor Response (LPR)

Wild-type 5D Tropical zebrafish embryos (WT) were exposed to 0, 1, 5, 10, 20, 25, 30, 40 μM B[a]P in 1% DMSO to assess for developmental toxicity. The lowest effect level to cause an adverse effect was 25 μM (Supplemental Figure 1). To assess for potential neurotoxicity, we exposed embryos to two concentrations: a no observable adverse effect level (NOAEL; 4 μM), and concentration 10-fold lower than the NOAEL (0.4 μM) in 0.1% DMSO. Previous reports have demonstrated that higher concentrations of DMSO can alter activity in larval zebrafish (Chen, Wang et al. 2011). Embryos exposed to B[a]P from 6–120 hpf exhibited a statistically significant hyperactive swimming phenotype ($p < 0.01$) in the dark, following light-dark transitions. WT embryos exposed to 4 μM B[a]P exhibited a hyperactive larval photomotor response (LPR) as a 26% increase in movement in the dark, with higher activity sustained over the three dark phases, compared to control animals which show a decreasing maximum activity over each dark phase cycle. Embryos exposed to 0.4 μM B[a]P had a small decrease (-6.5% , $p = 0.014$) in the dark (Fig. 1A–C). To determine whether this hyperactivity was AHR2 dependent, *ahr2*^{hu2334} embryos were exposed to 0 or 4 μM B[a]P and evaluated for LPR behavior changes. *ahr2*^{hu2334} embryos exposed to the vehicle control had a similar LPR to WT larvae, with low activity in the light and increased movement in the dark periods. The overall activity was higher compared to WT controls (Fig. 2A–B). *ahr2*^{hu2334} embryos exposed to B[a]P did not show hyperactive swimming compared to unexposed *ahr2*^{hu2334} larvae in the dark. *ahr2*^{hu2334} larvae exposed to 4 μM B[a]P displayed a trend toward a smaller LPR response compared to *ahr2*^{hu2334} controls, but it was not statistically significant (Fig. 2C).

3.2. Learning and Memory in Adult Zebrafish

Developmental B[a]P exposure resulted in learning differences in adult zebrafish. Embryos were exposed to 0.4 and 4 μM B[a]P in 0.1% DMSO. Developmental exposure to 4 μM , but not 0.4 μM B[a]P, was associated with decreased shock avoidance in both the train phase and the test phase, with a higher intercept and smaller slope value for the total shocked time compared to controls (Fig. 3A, 3B). In addition, the 4 μM B[a]P exposed group spent less overall time on the non-shock side of the shuttlebox (Fig. 4A), where 4 μM B[a]P had a lower intercept and smaller slope value (Fig. 4B, 4C), with a significant difference ($p < 0.00046$, $p < 0.00016$) between treatment and control. Developmental exposure to 4 μM B[a]P was associated with detectable deficits in learning to associate the conditioned stimulus with the unconditioned stimulus.

4. Discussion

We used the zebrafish model to evaluate the developmental and long term behavior effects of benzo[a]pyrene exposure. Zebrafish embryos developmentally exposed to 0.4 and 4 μ M B[a]P were tested at 120 hpf using a larval photomotor response (LPR) assay. 4 μ M B[a]P exposure resulted in significant hyperactivity at 120 hpf. Developmentally exposed zebrafish were also tested as adults using an active avoidance shuttlebox test. Developmental B[a]P exposure decreased learning and memory in adult animals. Our results are consistent with PAH mixture behavioral effects in fish species. Naïve killifish larvae exposed to a PAH mixture from an Elizabeth river sediment extract (ERSE) showed hyperactivity in a repeated light-dark transition assay (Brown 2016). Juvenile zebrafish fed a pyrolytic PAH fraction mixture were also hyperactive following a light-dark challenge (Vignet, Menach et al. 2014). We demonstrate that developmental B[a]P exposure produce larvae that exhibit significant hyperactivity in the dark over 3 light-dark cycles. We evaluated AHR null fish and found unexposed larvae displayed greater movement in the dark compared to unexposed WT larvae (+13% increase). To our knowledge, this is the first report of differences in AHR2 null behavior. Mukai et al reported AHR knockout (KO) mice did not show behavioral abnormalities, where AHR KO animals re-entrained to a new light schedule similar to WT controls. However, in a 6 hour advance shift experiment, AHR KO mice had a tendency to take longer to adjust to the new light schedule, measured by higher wheel running activity (Mukai, Lin et al. 2008). Importantly, B[a]P AHR2 null larvae did not display a significant difference in LPR compared to unexposed AHR2 null fish, illustrating a role for AHR2 in this B[a]P induced hyperactivity. Previously, a PAH resistant killifish population from the Elizabeth River (ER) was also tested, and exposure to a PAH mixture from an Elizabeth river sediment extract (ERSE) did not lead to swimming differences in larval or juvenile killifish (Brown 2016). This ER population was shown to have resistance to induction of the phase I enzyme, CYP1A (Meyer 2002) and upregulation of phase II and phase III metabolizing enzymes (Gaworecki 2004) following PAH exposures. Mortality and developmental malformations associated with B[a]P exposure are dependent on AHR2 signaling in zebrafish (Incardona, Collier et al. 2011; Van Tiem and Di Giulio 2011). AHR dependent metabolism of B[a]P and its resulting metabolites have recently been hypothesized to play a role in B[a]P neurotoxicity (Chepelev, Moffat et al. 2015). Here, developmentally B[a]P exposed *ahr2*^{hu2334} larvae did not exhibit a hyperactive response, suggesting the AHR2-dependent metabolites of B[a]P are responsible for the behavioral as well as the previously reported morphometric phenotypes.

Our results are also consistent with the reported impacts of early life stage B[a]P exposures in rodent models and in human epidemiological studies of children exposed to PAHs *in utero*. Pregnant Long Evans Hooded rats developmentally exposed to 600 and 1200 μ g/kg bw B[a]P from embryonic day 14–17 exhibit a B[a]P dose dependent decrease in correct responses during spatial discrimination reversals in the adolescent pups (McCallister 2016). Postnatal B[a]P exposure in Sprague-dawley rats is associated with significant differences in the surface righting reflex test at postnatal day 12, 14, and 16; and the negative geotaxis test at postnatal day 12 and 14 (Chen, Tang et al. 2012). Developmental B[a]P exposure is associated with learning deficiencies and behavioral disorders in children. Perera et al found

a significant correlation between PAH-adduct levels in cord blood and an increased incidence of moderate mental developmental delay in three year olds (using the mental development index, MDI) (Perera, Rauh et al. 2006). A cohort study in Poland found associations between *in utero* PAH exposure and lower reasoning ability and intelligence (RCPM cognitive test) in five year old children (Edwards, Jedrychowski et al. 2010). In addition, high PAH-adduct levels in cord blood was positively correlated with increased anxiety, depression, and attention deficits in older children, ages five and seven (Perera and Herbstman 2011). We observed that developmental B[a]P exposures result in hyperactivity in the dark, with higher activity levels sustained over each dark period, which is unlike the control larvae's pattern of decreasing activity over each dark cycle, the B[a]P exposed larvae failed to habituate to the light-dark transitions.

Finally, we tested the hypothesis that B[a]P exposure would be associated with long term behavioral effects. Our finding that B[a]P exposure impaired learning in adults is consistent with several studies. B[a]P exposure produces neurotoxicity in adult rodents, where 6.25mg/kg benzo[a]pyrene exposure resulted in impaired learning of adult Sprague Dawley rats in the Morris water maze test (Cheng, Xia et al. 2013) and decreased motor activity was observed over 48 hours after B[a]P administration in F-344 rats (Saunders, Das et al. 2006). Fewer studies have shown long term effects resulting from developmental B[a]P exposures. Similar to our study design, impaired learning and brain function was found in developmentally exposed adult rodents (McCallister, Maguire et al. 2008); and adolescent Sprague Dawley rats (postnatal day 35) were significantly hyperactive in an open-field test, showed increased escape latencies in the Morris water maze (postnatal day 35 and 75), and had delayed changes in latency time in an elevated plus maze in adult rats (postnatal day 75) following postnatal B[a]P exposure (Chen, Tang et al. 2012). Using the zebrafish model we were able to investigate the behavioral effects resulting from developmental B[a]P exposure. We showed that developmental B[a]P exposure affected larval behavior, but also impaired adult learning and memory. The correlation between larval and adult behavior agrees with a B[a]P rodent study, where decreased sensory and mobility performance, using the righting reflex test and geotaxis test, in pre-weanling Sprague Dawley rats was associated with learning and memory deficiencies in the same animals as adults (Chen, Hamm et al. 2001).

We illustrated that the developmental zebrafish can be a useful tool to detect neurotoxicity at chemical exposure levels that do not otherwise produce visibly signs of toxicity such as mortality or malformation. Our results demonstrate a long term behavioral consequence from a transient developmental B[a]P exposure which contributes to a greater understanding of the developmental hazard of PAHs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Embryonic exposure of 0.4 and 4 μ M B[a]P induced behavior effects
- AHR2 plays a role in B[a]P induced larval hyperactivity
- Developmentally exposed B[a]P zebrafish exhibited decreased learning and memory

Fig. 1. Larval Photomotor Response (LPR) in B[a]P exposed wildtype (WT) zebrafish: Developmental exposure to 4 μ M B[a]P results in larval hyperactivity
 5D WT zebrafish embryos were exposed to 0, 0.4 and 4 μ M B[a]P from 6–120 hpf, and tested using the Viewpoint Zebrafish. (A) The larval photo motor response (LPR) assay consisted of three, 3minute periods of alternating light and dark periods. Embryos exposed to 4 μ M B[a]P exhibited hyperactivity in the dark period (A, C), resulting in significant hyperactivity over the entire assay (B). (C) shows total AUC for the dark cycle, * denotes statistical significance ($p < 0.01$, 25% fold change).

Fig. 2. Larval Photomotor Response (LPR) in B[a]P exposed *ahr2*^{hu2334} embryos: Developmental exposure to 4 μM B[a]P decreases larval photo-motor activity
ahr2^{hu2334} embryos were exposed to 0 or 4 μM B[a]P from 6–120 hpf and tested using the Viewpoint Zebrafish systems. (A) The larval photo motor response (LPR) assay consisted of four, 3minute periods of alternating light and dark periods. (B, C) Embryos treated with 4 μM B[a]P exhibited a trend toward a decreased response following light-dark transitions, but the difference was not significant, (C) shows total AUC for the dark

Fig. 3. Developmental B[a]P exposure impairs learning in adults

B[a]P developmentally exposed zebrafish were grown to adulthood and tested for learning difference in an active avoidance test using shuttleboxes. Zebrafish were conditioned to leave a lighted side to avoid a mild shock. Animals underwent a train (**A**), and test (**B**) session. Each point represents an individual animal for that one trial. A linear regression was fit for each treatment. The solid line represents the DMSO controls, while the dashed line represented 4 μ M B[a]P.

A Shocked

Treatment	Phase	Fault Outs	Learners	Intercept, Slope	Diff, p value
0 μ M	Train	8.7%	47.6%	9.25, -0.071	-
	Test	1.92%	35.3%	5.62, -0.086	-
0.4 μ M	Train	9.1%	46%	8.74, -0.062	0.27, p =0.55
	Test	13.24%	37.3%	7.19, -0.154	0.57, p =0.027*
4 μ M	Train	3.6%	42.9%	9.53, -0.058	0.61, p =0.089
	Test	1.92%	37.3%	6.54, -0.082	0.99, p =6.00E-05*

B Time to Accept

Treatment	Phase	Fault Outs	Learners	Intercept, Slope	Diff, p value
0 μ M	Train	8.7%	38.1%	9.268, -0.009	-
	Test	1.923%	31.3%	8.014, -0.067	-
4 μ M	Train	3.571%	22.22%	7.339, 0.022	1.15, 0.00046*
	Test	1.923%	23.53%	8.27, -0.051	0.501, 0.03*

C Accept

Treatment	Phase	Fault Outs	Learners	Intercept, Slope	Diff, p value
0 μ M	Train	8.7%	47.62%	10.96, 0.062	-
	Test	1.923%	31.37%	14.812, 0.087	-
4 μ M	Train	3.571%	48.15%	11.38, 0.052	0.19, 0.55
	Test	1.923%	31.37%	14.02, 0.079	0.91, 0.00016*

Fig. 4. Developmental exposure to B[a]P significantly affects learning and memory in adults Zebrafish were conditioned to leave a lighted side during the “seek period” to avoid a mild shock. Their performance in the active avoidance test for 3 parameters: **(A)** total shocked time, **(B)** time to accept and **(C)** total time on the accept time. * $p < 0.05$ ANOVA and Tukeys post hoc test.