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Embryonic exposure to benzo[*a*]pyrene causes age-dependent behavioral alterations and long-term metabolic dysfunction in zebrafish

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are products of incomplete combustion which are ubiquitous pollutants and constituents of harmful mixtures such as tobacco smoke, petroleum and creosote. Animal studies have shown that these compounds exert developmental toxicity in multiple organ systems, including the nervous system. The relative persistence of or recovery from these effects across the lifespan remain poorly characterized. These studies tested for persistence of neurobehavioral effects in AB* zebrafish exposed 5–120 h post-fertilization to a typical PAH, benzo[*a*]pyrene (BAP). Study 1 evaluated the neurobehavioral effects of a wide concentration range of BAP (0.02–10 μM) exposures from 5 to 120 hpf during larval (6 days) and adult (6 months) stages of development, while study 2 evaluated neurobehavioral effects of BAP (0.3–3 μM) from 5 to 120 hpf across four stages of development: larval (6 days), adolescence (2.5 months), adulthood (8 months) and late adulthood (14 months). Embryonic BAP exposure caused minimal effects on larval motility, but did cause neurobehavioral changes at later points in life. Embryonic BAP exposure led to nonmonotonic effects on adolescent activity (0.3 μM hyperactive, Study 2), which attenuated with age, as well as startle responses (0.2 μM enhanced, Study 1) at 6 months of age. Similar startle changes were also detected in Study 2 (1.0 μM), though it was observed that the phenotype shifted from reduced pretap activity to enhanced posttap activity from 8 to 14 months of age. Changes in the avoidance (0.02–10 μM, Study 1) and approach (reduced, 0.3 μM, Study 2) of aversive/social cues were also detected, with the latter attenuating from 8 to 14 months of age. Fish from study 2 were maintained into aging (18 months) and evaluated for overall and tissue-specific oxygen consumption to determine whether metabolic processes in the brain and other target organs show altered function in late life based on embryonic PAH toxicity. BAP reduced whole animal oxygen consumption, and overall reductions in total basal, mitochondrial basal, and mitochondrial maximum respiration in target organs, including the brain, liver and heart. The present data show that embryonic BAP exposure can lead to neurobehavioral impairment across the lifespan, but that these long-term risks differentially emerge or attenuate as development progresses.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous pollutants which are primarily found as products of incomplete combustion. Humans are exposed to PAH by diverse means, including smoke from tobacco or marijuana and through environmental contamination by coal ash, fossil fuels and creosote (Lee, 2010; Zhu et al., 2009). PAH are widely associated with risks to health, including cancer (Sarigiannis et al., 2015), cardiovascular function (Cunha et al., 2020), metabolic health (Yebra-Pimentel et al., 2015), and development (Billiard et al., 2008). With respect to development, the brain is particularly sensitive to PAH exposure (Schroeder, 2011). Epidemiological studies have associated prenatal exposures to these compounds, and the mixtures they typically appear in, to later changes in mood, behavior and cognition (Perera et al., 2012; Peterson et al., 2015), including an increased rate of attention deficit hyperactivity disorder (ADHD) (Perera et al., 2014). The connection between early PAH exposures and mental health later in life remains somewhat controversial, as exposures to prenatal smoking

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and pollution are conflated with a variety of other genetic and environmental risk factors for neurodevelopment (Perera et al., 2013; Salihu and Wilson, 2007; Vieira, 2015). To support this discussion, animal models have been developed to examine the causal relationships between developmental PAH exposure and neurobehavioral function in that absence of these confounders.

Based on available studies, PAH exposures during either prenatal (mammalian) or prehatching (nonmammalian) development cause changes in behavior at doses well below those that produce observable defects in sensitive systems like the heart and spinal cord (e.g. 10 μM in zebrafish). For example, exposure to individual PAH such as benzo[*a*]pyrene (BAP) or phenanthrene cause measurable behavioral changes in rodents (Chepelev et al., 2015; Hawkey et al., 2018) and zebrafish (Gao et al., 2017; Gao et al., 2015; Knecht et al., 2017b). These effects show some correspondence with the effects of complex PAH-containing mixtures, such as tobacco smoke extracts (Costello et al., 2014; Gellner et al., 2016; Hall et al., 2016), extracted particulates from air pollution (Patten et al., 2020; Woodward et al., 2018) and soils or extracts of soil contaminated by mixtures like creosote (Ataka et al., 2007). Observed deficits include changes in locomotor activity, affective behaviors, and cognition, showing face validity for neurobehavioral functions identified in epidemiological work. Furthermore, studies with zebrafish suggest that resulting neurobehavioral impairment may be observed very early in development, and persist into adulthood (Knecht et al., 2017a; Knecht et al., 2017b). For example, exposure to 4 μM BAP led to impaired heart rate and hyperactivity in the dark among 5–6 day-old zebrafish, as well as learning deficits in adulthood (15–18 months). Such studies indicate that early developmental exposures may contribute to behavioral effects that appear much later in life, raising additional concerns about future psychiatric needs.

The toxicity of PAH in developing organisms is often linked to its interactions with the aryl-hydrocarbon receptor (AHR), a transcription factor which mediates gene expression and metabolic processes (Vogel et al., 2020). A prominent link has been made between PAH exposure and glutamatergic signaling, where PAH-exposed neurons show changes in the number and sub-receptor make-up of glutamate-receptive ion channels (Brown et al., 2007). The net effect of these changes is a reduction in glutamatergic plasticity, as seen in long-term potentiation (Brown et al., 2007). Further, rodent studies have shown that prenatal PAH exposure can reduce the turnover of acetylcholine and enhance serotonergic receptor populations in brain tissue from rats (Slotkin et al., 2019). However, PAH may also alter cellular functions and development by inducing oxidative stress and mitochondrial dysfunction, which can damage DNA, modify enzymatic activity, and suppress ATP-dependent processes (Meyer et al., 2013). Relatedly, a broad pattern of genotoxicity has also been documented, affecting both nuclear and mitochondrial DNA (da Silva Junior et al., 2021; Jung et al., 2009; Pavanello et al., 2013; White, 2002). These effects are understood to be acutely neurotoxic (Saunders et al., 2006), although the long-term effects of such exposures on cellular metabolism remain unclear.

Within the literature on this topic, two prominent aspects remain unexamined. First, analyses with animal models have principally looked at behavioral function within quite restricted age ranges, usually conducting each test at only a single point in development. This approach supports the identification of phenotypic changes in behavioral function, but fails to place these effects within a developmental context, showing when in development a symptom may emerge, and the degree to which it persists or attenuates across development. One notable exception to this was in a rat study by Hawkey et al. (2018), where a locomotor test was performed in both adolescent and adult rats with prenatal exposure to BAP, nicotine, or both. This study found persistent BAP-induced hyperactivity across these ages among male offspring, although this was the only test out of six performed at multiple ages. Additional neurobehavioral research with comparable data from multiple ages is needed to place phenotypes within a developmental context, and to observe whether neurobehavioral symptoms are differentially or persistently expressed across early development and adulthood.

A second issue which remains to be explored concerns bioenergetic consequences of PAH exposure. A number of studies have shown that mitochondria are acutely sensitive to PAH exposures (Jung et al., 2009; Lindberg and Di Giulio, 2019), and that the pathways targeted by PAH can influence neuronal survival and function (Gao et al., 2017). While this work has typically focused on acute or short-term effects, the potential for long-term mitochondrial dysfunction should also be considered, as mitochondrial function has been identified as a limiting factor for neuronal function (Schon and Manfredi, 2003), and mitochondrial dysfunction has been associated with neurodegenerative disorders in aging, such as Alzheimer's disease and Parkinson's disease (Greenamyre et al., 1999; Wang et al., 2014).

To examine the neurobehavioral risk posed by PAH and its potential links to long-term metabolic changes, the current experiments were carried out with developing zebrafish exposed to a model PAH, BAP during embryonic development. For each exposure, fish embryos were housed in a BAP-dosed medium from 5 to 120 h post-fertilization. In Study 1, a wide range of BAP doses (0.02–10 μM) were tested for tolerability and neurobehavioral effects, testing locomotor motility under lit and dark conditions in larval development (144hpf) and performance in a behavioral battery in adulthood (6 months of age). The adult battery contained assays of locomotor and affective behaviors, reflexive startle, reactivity to social or predatory cues, and learning. Based on the findings of Study 1, a similar design was adopted for Study 2, with a more focused dose range (0.3–3 μM) at 4 developmental windows of testing representing juvenile (144hpf), adolescent (2.5 months), adult (8 months) and late adult (14 months) development. Fish from Study 2 were maintained to 18 months of age and tested for whole animal and tissue-specific respiration, to evaluate the potential for long-term changes in mitochondrial and non-mitochondrial metabolic function in the whole fish, brain, liver, heart and gonads. Exposure concentrations were selected to fall below the threshold for overt toxicity (i.e. increased mortality or dysmorphology rates), rather than any specific environmental level of contamination. As such, these model decending levels of exposure and/or toxicity which may be neurologically relevant, but would fail to be detected using gross developmental toxicity screens.

2. Materials and methods

2.1. Fish housing and husbandry

All subjects in the experiments consisted of AB* zebrafish bred in the Levin lab at Duke University. All housing and experimental procedures were approved by the Institutional Animal Care and Use Committee and Duke University (Protocol #A253-19-11). Zebrafish embryos were raised and exposed to BAP or the DMSO vehicle in glass petri dishes at a density of 1 embryo/ml of exposure medium. Embryos were incubated at 28 ± 1 °C. Following exposures, fish were transferred to 3 L housing tanks maintained on a recirculating water system (Aquatic Habitats, Inc., Apopka, FL, USA; Aquatic Enterprises, Inc., Bridgewater, MA, USA) at a density of ≤5 fish/L. System water consisted of sea salt (Instant Ocean, 0.5 parts per thousand) and buffer (Alkaline Regulator, 1 g/L, Neutral Regulator 0.03 g/L, Seachem Laboratories, Madison, GA, USA) in de-ionized water to maintain a consistent salinity and pH. This system water was used as both the base for embryonic exposure medium and in the recirculating housing systems. Water quality (chemistry, salinity and temperature) was monitored weekly, and adjusted to maintain the following parameters: 26 ± 1 °C, 6.8–7.0 pH, <0.25 ppm ammonia, <0.25 ppm nitrite, <20 ppm nitrate, and 0.5–1.0 parts per thousand salinity. Fish were maintained on a 14:10 h light:dark cycle throughout all testing procedures. Fish were fed with live brine shrimp (*Artemia Salina)* in the morning and afternoon (eggs from Brine Shrimp Direct, Ogden, UT, USA); and a dry food of GEMMA Micro 300 micro-pellets (Skretting USA, Tooele, UT, USA) at noon.

2.2. Chemicals

Dimethyl Sulfoxide ReagentPlus,® ≥99.5% (DMSO) and benzo[*a*]pyrene (BAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were made by dissolving BAP in 100% DMSO in glass vials. A base stock solution of 10 mM BAP was made and adjusted with serial dilutions to produce the necessary dosing solutions. Exposure medium was made by adding the stocks to freshly made system water at a ratio of 1 μL stock per 1 mL of system water, producing 0.1% DMSO solutions with varying concentrations of BAP (e.g. 10 mM stock produced 10 μM exposure medium).

2.3. Exposure of zebrafish embryos

Samples of zebrafish eggs (2–3 replicate breedings per study) were collected from groups of mixed-sex breeder tanks consisting of multiple male and female breeders (5–7 tanks represented per breeding) using removable egg traps and artificial vegetation. All eggs from a given replicate breeding were mixed together and rinsed once with a 0.01% bleach solution (system water + original Clorox bleach) and twice with clean system water. These mixed fertilized and unfertilized eggs were then placed in clean system water and moved to the incubator. At 3–4 h post-fertilization (hpf), eggs were viewed under a microscope and typically developing embryos were sorted into glass petri dishes at a density of 40 embryos per 40 mL of system water. Each dish was randomly assigned to a treatment group. At 5hpf, the clean system water was removed and replaced with exposure medium. The exposure medium was prepared daily using frozen stock solutions in 100% DMSO, diluted to exposure concentrations using fresh system water. The exposure medium was replaced every 24 h, at which time, researchers noted and removed any dead or deformed embryos. Spinal defects, as well as overall mortality, visible eye defects, and docked tail (failure of spine/tail to elongate) were noted. At 122hpf, the exposure medium was replaced with fresh system water and fish remained in incubated petri dishes until larval motility testing (24 h later). Preliminary testing observed that across two replicate plates, a concentration of 30 μM BAP caused spinal deformities in the majority (72.5%) of embryos, but lower concentration ranges produced defect rates indistinguishable from controls. The 24 h viability rate of selected embryos was 97.9% and further defect/mortality rates for subtoxic exposures were as follows: spinal deformity (0.4%), docked tail (4.4%), pericardial edema (2.1%) and unattributable death after the 48+ hr after fertilization (4.4%) (deformity not visible due to condition of dead embryo).

2.4. Experimental design

Two studies were conducted assessing the persisting behavioral effects of embryonic BAP exposure. Methods are discussed chronologically by developmental age and a full summary of the testing batteries, shown by age, developmental stage and study (1,2) is provided in Table 1. In Study 1, a preliminary study sought to assess the effects of embryonic BAP exposure on adult behavior using a wide dose range (0.02, 0.2, 2, 10 μM BAP). Each treatment group represented 2 separate exposure cohorts (derived from different egg collections), with target N's of 15–25 fish per group surviving to adulthood. At 6 months of age, these fish completed a battery of four behavioral tests, as described below. Based on those prior findings, Study 2 was designed to assess the incidence and persistence of behavioral effects over a much longer developmental time scale, beginning in larval development, and ending in late adulthood, as well as the presence of metabolic effects during aging. Each treatment group represented 3 separate exposure cohorts (derived from different egg collections), with target N's of 20–30 fish per group surviving into the aging period (18 months). A more restricted dose-range of 0.3–3 μM BAP was selected based on the prior study. An insufficient number of fish from the 3 μM group survived for testing at the 2.5 month time point, so this group is excluded from all analysis beyond larval motility.

At 6 days post fertilization (dpf), or 144 hpf, larval fish were tested in the larval motility assay. At 2.5 months old (±1 week), adolescent fish were then tested for locomotor and anxiety-like behaviors in the novel tank dive test. In full adulthood (8 months) and late adulthood (14 months), fish, completed a battery consisting of the novel tank dive test, tap startle test, shoaling social approach, and predator avoidance assay. Following the end of the 14-month battery, fish were maintained to 18-months of age and transferred to a second facility for subsequent testing in whole animal and tissue specific respiration.

2.5. Behavioral testing

Studies 1 and 2 used an established battery of tests for testing adult and adolescent zebrafish, although some refinements have been made to the procedures over time. Each fish was tested on each test only once at each age, and biological replicates consisted of multiple cohorts derived from independent egg collections and grouped exposures. Some notable changes were made to the standard testing procedure in the period between the completion of the first experiment and the beginning of the second experiment. These modifications are detailed below.

2.6. Larval behavior

Larval motility was conducted in study 1 and 2, as performed in previous studies (Glazer et al., 2018). At 144 hpf, larvae were loaded into 96-well plates, with one larval fish in each 0.5 ml glass well insert. Each 96-well plate contained fish from multiple treatment groups, and their positions on the plate were counterbalanced across the experiment. After a 1-h acclimation, the plate was placed into a DanioVision lightbox controlled by the EthoVision XT tracking software (v.14, Noldus, Wageningen, The Netherlands). Activity, measured as distance moved per minute, was then tracked across a 50-min testing session consisting of 5 phases. The first phase was in the dark, which allowed the fish to habituate to the lightbox. This was followed by two cycles of alternating light-dark conditions (light, dark, light, dark, switching every 10 min). Larval zebrafish show high levels of activity in the dark, and low levels of activity in the light.

Table 1
Sequence of Testing for Study 1 and Study 2, shown by age and developmental stage. Components of each test battery are reported chronologically from left to right.

	Age	Stage	Test battery			
Study 1	6 days	Larval	Larval motility			
	6 Months	Adult	Novel tank dive	Tap startle	Shoaling	Predator
Study 2	6 days	Larval	Larval motility			
	3 Months	Adolescent	Novel tank dive	Tap startle		
	8 Months	Adult	Novel tank dive	Tap startle	Shoaling	Predator
	14 Months	Older adult	Novel tank dive	Tap startle	Shoaling	Predator
	18 Months	Aging	Swim tunnel	Seahorse OCR		

2.7. Novel tank dive test

Novel tank dive test was conducted using identical procedures (Levin et al., 2007) across all ages tested, including adolescence (2.5 months, Study 2), adulthood (6 months Study 1, 8 months Study 2) and late adulthood (14 months, Study 2). Adolescent and adult zebrafish were placed into trapezoidal 1.5-L plastic tanks (Aquatic Habitats) filled with 10 cm of system water and their behavior was recorded for 5 min using an HD camcorder (VIXIA HFR700; Canon Inc., Tokyo, Japan). This camera was placed on a tripod 75 cm from the testing enclosure facing the long sides of two clear tanks, separated by an opaque barrier. Behavioral analysis consisted of tested for locomotor activity, measured as distance moved per min (cm) and attraction to the bottom of the tank, measured as average distance from the bottom (cm).

2.8. Startle tap test

Startle response and habituation were tested in adulthood (6 months Study 1, 8 months Study 2) and late adulthood (14 months, Study 2) using an identical protocol developed in our laboratory (Eddins et al., 2010). Each fish was placed into one of eight clear cylindrical arenas arranged in a 2 × 4 arrangement, with the HD camera mounted 50 cm above. Each arena was positioned over a solenoid which could then deliver a startling tap to the arena floor. In zebrafish, the acoustic startle response consists of a brief period of rapid locomotion. Startle responses are assessed by measuring the amount of locomotor activity in the 5-s immediately before the tap (pre-tap) and the amount of locomotor activity for 5-s immediately after the tap (post-tap), and taking a difference score between them (startle magnitude). Generally, the size of the startle response will diminish over successive taps in a short period of time. The testing session consisted of a 30-s acclimation period followed by 10 taps delivered at 1 min intervals.

2.9. Shoaling social approach

Social approach was tested in adulthood (6 months Study 1, 8 months Study 2) and late adulthood (14 months, Study 2), with the two experiments using slightly different methods. Study 1 was performed in a trapezoidal tank identical to a housing tank (Crosby et al., 2015), while study 2 used a custom-made rectangular partitioned tank (Hawkey et al., 2020). In each testing sequence, the fish began with a 30 min isolation period in 1.5 L tanks, after which each fish was individually placed into a testing enclosure. The testing enclosure in Study 1 consisted of a trapezoidal 3 L plastic tank which was identical to the tanks used for housing (dimensions, filled to 10 cm deep). The testing enclosure in Study 2 was a rectangular partitioned experimental tank (30 cm long, 6 cm wide, filled to 10 cm deep) made with clear Plexiglas on both ends and black Plexiglas on the two long side walls. In each case, the enclosure was positioned over a light box (Huion Technology, Shenzhen, China) with each end facing a flat computer screen, and the HD camera mounted 55 cm above. The trial then began with a 2-min habituation phase, during which the two screens showed control shapes (striped ovals which did not move). After the habituation phase was complete was collected, a close-up video of a shoal of swimming zebrafish was projected onto one of the two screens for 4 min (Study 1) or 5 min (Study 2). Approach was measured as the average distance of the fish from the relevant screen (in cm) before vs. during the video.

2.10. Predator avoidance test

Predator cue avoidance and fleeing was tested in adulthood (6 months Study 1, 8 months Study 2) and late adulthood (14 months, Study 2), were tested using slightly different methods. Study 1 used a previous multi-stage iteration of the procedure, while study 2 used a single-session version of the procedure (Crosby et al., 2015; Hawkey et al., 2020). In each experiment, the same testing apparatus' and set-ups were adopted for predator avoidance as in the shoaling test. For the predator avoidance test, fish were exposed to alternating 1-min periods with either a blank screen (control) or a video with an aversive dot (predator cue), which grows from very small to very large. This is a 2-dimensional representation of a large object moving towards the viewer. In zebrafish, this stimulus can trigger fleeing behavior and avoidance in that size of the enclosure.

In Study 1, fish completed two sessions of predator avoidance, each of which presented either a slow-growing blue dot (duration of 1–23 cm increase 4-s), or a fast-growing red dot (duration of size increase 1-s). The fast-growing dot generally produces a greater fleeing and avoidance response than the slow-growing dot. Predator cues were presented twice in a given session, during the second and fourth minutes of the 5 min session respectively. Study 2 used these same stimuli in a different presentation format. Here, fish completed a single 9-min session which presented slow-dots in minutes 2 and 4, and fast dots in minutes 6 and 8, with blank screens at all other times. Avoidance was measured as the average distance from the relevant screen during the cue presentations vs during the blank periods.

2.11. Three-chamber learning test

The final test conducted on fish in Study 1 was the three-chamber learning task, which uses operant punishment to train the fish to avoid one chamber and to prefer another instead. This test was performed using a previously established protocol (Sledge et al., 2011). The apparatus consisted of a PVC cylinder with closed ends, split lengthwise to create a long channel (14.5 cm wide x 28 cm long). This channel was then subdivided into three chambers using two thin plastic walls which could be repositioned and rotated using two exterior handles. Each wall was equipped with a single 6 cm hole, or doorway, which could be presented or removed by rotating that wall. When both doorways were presented, the fish had access to each of the side chambers, one of which was designed as "correct" and one as "incorrect". If a fish entered the "incorrect chamber", the doorway closed behind them and the wall was moved to confine the animal in a 1 cm space between the wall and the end of the channel. Zebrafish are claustrophobic and will tend to avoid the chamber which is tied to confinement. Testing consisted of a preference assessment, after which the preferred side was designated as "incorrect", and seven test trials separated by 30 s intertrial intervals. Entries into the incorrect chamber resulted in confinement, while entries into the "correct" chamber did not. Choice accuracy and latency was scored on each trial, and percent-accuracy score was calculated for each animal across the session.

2.12. Whole animal oxygen consumption rate

Fish from Study 2 were maintained to an age of 18 months and underwent respiration testing. The first component of respiration testing consisted of whole animal oxygen consumption, conducted in vivo. A Beamish-style swim tunnel (4000 mL vol; Loligo Systems, Viborg, Denmark) with 170-ml swim tunnel respirometers was used to measure oxygen consumption rate (OCR). Each fish was isolated and fasted for approximately 24 h prior to OCR measurements In this way, energy expenditure associated with digestion and storage of food (i.e., specific dynamic action (SDS) or thermic effect of food (TEF)) would not affect basal metabolic rate during measurements. Following placement into the swim tunnel, a fish was allowed 1 h to acclimate under a black tarp to prevent disturbances. Then OCRs were measured for 1 h.

Measurements consisted of recording oxygen concentrations (μmol L^{-1}) using OxyView software (PreSens, Regensburg, Germany). Each measurement contained two values. First, the oxygen value of the tunnel was recorded as the lowest value within a 5 s period. Immediately after the pump was shut off for 5 min during which fish behavior was noted since the level of physical activity could affect the oxygen content of the tunnel. After 5 min, the second oxygen value was recorded and the pump turned on. Intervals of 10 min were between each set of measurements so the oxygen level could return to normal. A total of 4 sets of measurements were taken for each individual after which fish were returned to their tanks. For each set of measurements, end values were subtracted from start values to yield OCR (μmol L^{-1}). OCR values were averaged and then divided by the fish's body weight to get the resting metabolic rate (RMR) and then divided by 5 to determine RMR per min.

2.13. *Tissue-specific oxygen consumption rate*

The second component of respiration testing consisted of tissue-specific oxygen consumption, conducted ex vivo, as in Boyda et al. (2021). A Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA; hereafter referred to as "Seahorse") was used to analyze organ-specific OCR. Dissections were performed on each fish one day after its swim tunnel measurements. The fish continued to be isolated and fasted between swim tunnel measurements and dissection. The fish were immobilized in an ice water bath for approximately 15 s, until loss of equilibrium and movement. Then fish were removed from the bath, weighed (mg wet weight; Mettler Toledo, Columbus, OH, d = 0.01 mg/0.1 mg) and total length (mm) was recorded. Then, cervical dislocation was performed immediately prior to dissection.

On the day of dissection, fresh Ringer's solution was prepared using 8.4 mmol L^{-1} sodium pyruvate, 5.6 mmol L^{-1} $NaHCO_3$, 0.97 mmol L^{-1} HEPES, and 3.2 mmol L^{-1} HEPES sodium salt that was added to a stock of 115 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 1.2 mmol L^{-1} $MgCl_2$, 0.64 mmol L^{-1} NaH_2PO_4, and 2.1 mmol L^{-1} $CaCl_2$. The pH was adjusted to 7.0. 525 μl of Ringer's solution was placed into each well of a Seahorse islet capture microplate. For each fish, the brain, liver, heart, and gonads were removed and individually placed into wells and capture screens placed on top to secure the organs in place. After all of the organs were plated, 250 ul of Ringer's solution was removed and replaced with 250 ul of fresh Ringer's. Then plated organs were placed into the Seahorse. The first measurement taken is the total basal respiration, or total basal oxygen consumption rate (basal respiration plus non-mitochondrial respiration in Fig. 1). Then, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) is injected via the drug ports. FCCP is an uncoupler of oxidative phosphorylation in mitochondria. It simulates a cellular demand for extra energy and causes the mitochondria to act at maximum capacity, giving the value for maximal mitochondrial respiration (Demine et al., 2019). The difference between the maximal mitochondrial respiration and the total basal respiration is known as the spare capacity. The spare capacity of the mitochondria is the potential of the mitochondria to produce extra energy when the cell has to meet a higher energy demand (Desler et al., 2012).

The second injection consists of a mixture of antimycin A and rotenone. Rotenone is a complex I inhibitor and antimycin A is a complex III inhibitor, so the mixture of the two causes mitochondrial respiration to cease (Wang et al., 2015). With mitochondrial respiration no longer in effect, the remaining oxygen consumption can be attributed to non-mitochondrial respiration. This consists of oxygen consuming processes outside of the mitochondria, including the activity of certain enzymes in the cell (Wang et al., 2018). The non-mitochondrial respiration is subtracted from the total basal respiration in order to calculate the mitochondrial basal respiration.

For this study, eight measurements were taken first to measure basal OCR. Next, 2 μM FCCP in 1% DMSO was injected and then eight measurements of maximal respiration were taken. Next, a 44.4 μM mixture of antimycin A and rotenone was injected to measure non-mitochondrial respiration for 25 cycles. These measurements were used calculate total basal OCR, total maximal respiration, non-mitochondrial respiration, basal mitochondrial respiration, maximal mitochondrial respiration, and spare capacity. Calculations were normalized by organ weight to get respiration values per mg wet weight.

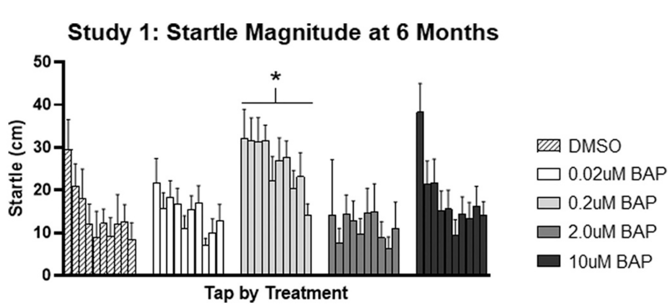

Fig. 1. Study 1: Tap Startle Test. Startle responses were measured as a change in activity, measured as distance moved, from the 5-s before each tap to the 5-s after the tap (cm). Main effect of treatment is shown. Fish with 0.2 μM BAP exposure showed elevated startle responses relative to controls ($p < 0.05$). Data are shown as mean (cm) +/− SEM. Asterisks (*) indicate significance relative to controls.

2.14. *Statistical analysis*

Statistical analyses were performed using SPSS v.27 (IBM Corp.). Each analysis consisted of either a mixed-factorial or univariate analysis of variance (ANOVA), based on the number of dependent variables under analysis. For all behavioral data, mixed factorial ANOVAs were performed to assess within-session effects including time (e.g. 1 min-block), stimulus presentation (e.g. tap in sequence) or stimulus type (e.g. fast or slow predator cue), with the primary between-subjects variable set as treatment group. Sex and exposure cohort were treated as covariates, with sex only included in adult ages where sex can be reliably determined visually (6+ months). Violations of homogeneity of variance are common in behavioral data, and these violations were addressed using the Greenhouse-Geisser correction (Greenhouse and Geisser, 1959). This method led to degrees of freedom that were not whole numbers. Main effects and/or interactions were further investigated using Tukey's correction for multiple testing to ensure that non-monotonic effects could be adequately assessed. Significance was set at $p < 0.05$ for all analysis of variance (ANOVA) and follow-up testing. As the identity of fish could not be maintained across different ages and behavioral tests, each stage in development was treated as an independent test, rather than a repeated-measures factor. Main effects of age and interactions with treatment were assessed as a part of the overall analysis and are provided separately within the results section.

Analysis of whole animal and organ-specific tissue respiration proceeded as follows. Raw respiration scores, which vary considerably across different organs, were standardized by conversion to a percent control score ((value/mean of control group)*100). Whole animal respiration was assessed using a univariate ANOVA, with treatment and sex as between-subjects variables. Tissue-specific respiration values (total basal, mitochondrial basal, mitochondrial maximum and spare capacity) were assessed using organ (brain, liver, heart) as a repeated measure and treatment/sex as between-subjects variables. Gonads were assessed with separate one-way ANOVAs of treatment effects for males and females, due to the lack of equivalent organs between males and females.

3. Results

The results of the statistical analysis are summarized in Table 2. (See Tables 3 and 4.)

3.1. *Study 1*

3.1.1. *Larval motility*

Analyses of larval motility performance revealed a main effect of light phase, $F(3.05, 799.05) = 101.18$, $p < 0.05$. No effects of treatment or relevant interactions were observed. Performance is summarized in supplemental table S1.

Table 2
Summary of results across studies, ages, and tests.

Study	Age	Test	Statistic	Difference from control
1	6 days	Larval motility	–	–
	6 Months	Novel tank dive	–	–
		Tap startle	–	0.2 enhanced startle
		Shoaling	–	–
		Predator	Main effect, Treatment in Fast Session	All BAP groups closer to screen during no-cue phases
2	6 days	Larval motility	Treat*Lighting phase	3.0 μM hypoactive on Dark 1
	3 Months	Novel tank dive	Main effect, Treatment	0.3 μM hyperactive
	8 Months	Novel tank dive	–	–
		Tap startle	Main effect, Pretap	1.0 μM reduced
		Shoaling	Treat*Cue Phase	0.3 μM further from screen when video present
		Predator	–	–
	14 Months	Novel tank dive	–	–
		Tap startle	Main effect, Posttap	1.0 μM enhanced
		Shoaling	–	–
		Predator	–	–
	18 Months	Swim tunnel	Main effect, Treatment	0.3 μM Significantly reduced, 1.0 - > p = 0.051
		Seahorse OCR		Total basal and Mit. Basal OCr reduced in both BAP groups, Mit. Max = 0.3 μM sign.*, 1.0 - > p = 0.055. Spare Cap. = No effect

Table 3
Tissue-Specific Oxygen Consumption: Raw Data. Four means are provided for individual target organs (Brain, Liver, Heart): (a) Total Basal OCR (Tot. Basal), (b) Mitochondrial Basal OCR (Mit. Basal), (c) Mitochondrial Maximal OCR (Mit. Max) and (d) Spare Capacity (Spare Cap.). Rows designate sex and treatment groups. Data are presented as mean (μmol/L/mg/5 min) +/− SEM.

Organ	Sex	Control	± *SEM*	0.3 μM	± *SEM*	1.0 μM	± *SEM*
A. Tot. Basal							
Brain	Male	214.1	43.8	174.8	27.4	189.5	27.1
	Female	281.4	46.8	192.4	17.8	189.4	23.2
Liver	Male	310.7	54.0	187.1	12.0	181.1	24.7
	Female	157.7	16.8	133.4	15.1	117.2	18.9
Heart	Male	396.3	49.3	406.5	46.4	333.2	34.3
	Female	332.2	25.2	285.6	27.5	352.0	22.1
B. Mit. Basal							
Brain	Male	121.4	42.2	80.0	22.0	91.6	22.6
	Female	185.4	42.1	98.2	15.2	97.3	19.6
Liver	Male	81.2	16.8	42.2	5.2	46.2	12.9
	Female	34.7	10.1	29.1	10.0	23.2	6.0
Heart	Male	300.7	37.8	273.5	37.6	225.8	31.3
	Female	240.9	28.6	169.1	27.6	239.5	21.6
C. Mit. Max.							
Brain	Male	129.3	42.4	87.5	23.6	105.2	26.8
	Female	195.1	41.3	115.3	18.6	114.4	22.1
Liver	Male	94.9	26.0	60.3	4.4	52.3	19.5
	Female	54.6	12.2	39.6	9.1	29.2	7.1
Heart	Male	371.1	52.9	348.3	44.6	293.4	37.7
	Female	338.4	55.2	253.5	47.6	312.1	38.4
D. Spare Cap.							
Brain	Male	7.9	1.3	7.5	2.0	12.1	2.6
	Female	9.6	3.4	21.0	6.6	17.1	4.7
Liver	Male	16.8	6.0	14.5	6.8	11.1	4.8
	Female	13.3	4.8	5.9	1.5	5.9	1.9
Heart	Male	75.8	14.7	74.8	12.4	67.6	13.9
	Female	104.1	26.6	84.4	31.3	72.6	20.1

3.1.2. Novel tank

Analyses of locomotor activity observed a main effect of time, $F(2.70, 231.78) = 4.98$, $p < 0.05$, whereby activity increased across the five-min testing session. Main effects were also observed for sex, $F(1, 86) = 11.26$, $p < 0.05$, and treatment, $F(4, 86) = 3.13$, $p < 0.05$. However, no pairwise comparisons of treatment groups reached significance in post-hoc testing. For distance from bottom of the tank, a main effect of time, $F(1.62, 139.34) = 3.49$, $p < 0.05$, was observed whereby distance from the bottom increased across the five-min testing session. A time by treatment interaction was also detected, $F(6.48, 139.34) = 11.26$, $p < 0.05$, however, no pairwise comparisons reached significance in post-hoc testing. Performance is summarized in supplemental table S2.

3.1.3. Tap test

No main effects of time, treatment or relevant interactions were observed for activity prior to each tap. For post-tap activity, a main effect of treatment was observed, $F(4, 59) = 2.84$, $p < 0.05$. Post hoc Tukey tests showed that the moderate dose of 0.2 μM BAP had significantly elevated post-tap activity relative to controls and to fish in the 2 μM BAP group ($p < 0.05$). A similar effect relative to the 0.02 μg/L group was nearly significant ($p = 0.06$). No other main effects or interactions were observed. For the startle response magnitude (Fig. 1), a main effect of treatment was observed, $F(4, 59) = 4.80$, $p < 0.05$. Startle magnitude was elevated in the 0.2 μM BAP group relative to controls and fish exposed to 0.02 or 2uM BAP ($p < 0.05$).

3.1.4. Shoaling

No main effects of time, treatment or relevant interactions were observed for activity during the shoaling session. For distance from the screen, no main effects of treatment or relevant interactions were observed for activity during the 2-min habituation phase, or during the video presentation. No main effects of treatment or relevant interactions were observed on the attraction score (average distance from the screen at baseline – distance during the video). A general, nonsignificant trend was noted whereby controls showed the highest attraction score and zero was within 1 SEM of the mean (no shoaling observed) for the 0.2–10 μM BAP groups. Performance is summarized in supplemental table S3.

3.1.5. Predator avoidance

During the slow-predator cue session, a main effect of time was detected, $F(3.29, 282.61) = 3.39$, $p < 0.05$, but no main effects of treatment or relevant interactions. For distance from the screen, no main effects of time, treatment or relevant interactions were observed (Fig. 2a). No effects were observed on the tendency to approach the screen after the cue was removed.

During the fast-predator cue session, a main effect of treatment on locomotor activity was nearly significant, $F(4, 89) = 2.34$, $p = 0.061$, but no other main effects or relevant interactions were detected. For distance from the screen, main effects were detected for time, $F(3.16, 281.39) = 10.17$, $p < 0.05$, and treatment, $F(4, 89) = 2.90$, $p < 0.05$, as was a time by treatment interaction, $F(12.65, 281.39) = 2.25$, $p < 0.05$, (Fig. 2b). Post hoc testing showed treatment effects during the two cue presentations and during the blank screen period between the two presentations. Specifically, the 0.02 μM group remained closer to the screen than either the 0.2 or the 2.0 μM groups during the first stimulus presentation ($p < 0.05$), while a similar trend relative to controls failed to reach significance ($p = 0.10$). During the inter-stimulus period, all treatment groups were closer to the screen than controls ($p < 0.05$). During the second stimulus presentation the 0.02 μM group remained

Table 4
Tissue-Specific Oxygen Consumption: Gonadal Raw Data. Four means are provided for individual target organs (Brain, Liver, Heart): (a) Total Basal OCR (Tot. Basal), (b) Mitochondrial Basal OCR (Mit. Basal), (c) Mitochondrial Maximal OCR (Mit. Max) and (d) Spare Capacity (Spare Cap.). Rows designate sex and treatment groups. Data are presented as mean (μmol/L/mg/5 min) +/− SEM.

	Gonads	Measure	Control	± *SEM*	0.3 μM BAP	± *SEM*	1.0 μM BAP	± *SEM*
A	Testes	TB	264.0	27.3	225.6	19.3	245.5	18.4
		MB	202.3	27.9	164.3	18.6	185.8	15.3
		MM	257.5	42.3	202.6	21.3	253.3	24.7
		SC	55.2	19.3	38.3	4.8	67.5	14.5
B	Ovaries	TB	97.4	11.9	82.8	7.8	102.6	9.6
		MB	62.3	10.5	42.4	8.5	57.9	9.2
		MM	81.0	14.5	51.5	9.2	68.5	11.8
		SC	18.8	5.0	9.1	2.4	10.7	2.8

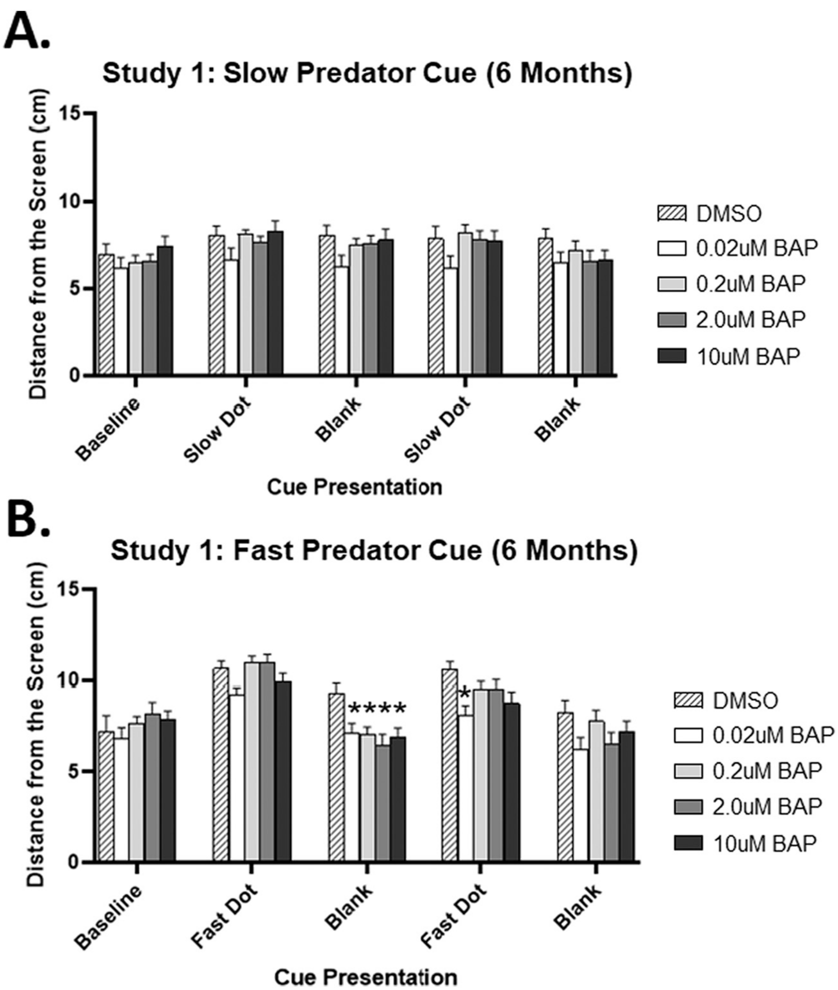

Fig. 2. Study 1: Predator avoidance. Avoidance was measured as a change in exploration, measured as distance from the predator cue screen (cm) during cue present (slow or fast dot) and absent (baseline, blank) conditions. (A) No group differences were observed during the slow-predator cue session. (B) Main effect of treatment is shown. All BAP-exposed conditions explored closer to the screen relative to controls after the first fast predator cue was removed ($p < 0.05$). Fish exposed to 0.02 μM BAP also remained closer to the screen when the fast cue was presented a second time ($p < 0.05$). Data are shown as mean (cm) +/− SEM. Asterisks (*) indicate significance relative to controls.

closer to the screen than controls ($p < 0.05$), while a similar trend was not significant in the 10.0 μM group ($p = 0.09$).

3.1.6. 3-chamber tank

Analyses of performance in the 3-chamber task revealed no significant main effects or interactions. Performance is summarized in supplemental table S4.

3.2. Study 2

3.2.1. Larval motility

Analyses of larval locomotor behavior observed a main effect of lighting phase, $F(3.14, 561.58) = 20.67$, $p < 0.05$, whereby activity was reduced under lit conditions and elevated under dark conditions. A lighting phase by treatment interaction was also detected, $F(9.41, 561.58) = 2.53$, $p < 0.05$ (Fig. 3). In post hoc testing, the 3.0 μM BAP group was significantly hypoactive during the first lit period ($p < 0.05$), while a similar effect relative to the lowest dose group 0.3 μM) was nearly significant ($p = 0.06$). During the second lit phase, 0.3uM BAP group was significantly hyperactive relative to the 1.0 μM group ($p < 0.05$), and while a similar effect relative to controls was nearly significant ($p = 0.053$).

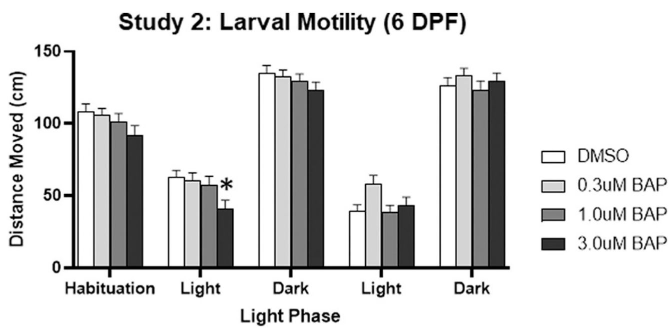

Fig. 3. Study 2: Larval Motility. Locomotor activity was measured as distance moved (cm) across 5 alternating dark and lit conditions. Treatment by lighting phase (light/dark, repetition 1–2) interaction is shown. Fish with 3 μM BAP exposure showed reduced locomotor activity relative to controls ($p < 0.05$) during the first presentation of the light. Data are shown as mean (cm/10 min) +/− SEM. Asterisks (*) indicate significance relative to controls.

3.2.2. *Novel tank*

Novel tank testing was conducted at three separate ages: adolescence (2.5 months), adulthood (8 months), and late adulthood (14 months). The data from each of these tests are presented in Fig. 5 for ease of age comparison.

In adolescent fish, analyses of locomotor activity (Fig. 4a) observed a main effect of treatment, $F(2, 75) = 6.22$, $p < 0.05$. Follow-up tests showed that fish exposed to 0.3 μM BAP were hyperactive relative to both controls and those exposed to the higher 1.0 μM dose. For distance from bottom of the tank (Fig. 4b), a main effect of time, $F(2.10, 157.35) = 3.20$, $p < 0.05$, was observed, as was a main effect of treatment, $F(2, 75) = 4.48$, $p < 0.05$. Post hoc testing showed that fish exposed to 0.3 μM BAP swam significantly further from the tank floor than fish exposed to 1.0 μM ($p < 0.05$), although a similar trend relative to controls failed to reach significance ($p = 0.11$).

In adult fish, analyses of locomotor activity (Fig. 4a) found no main effects of time or treatment, and no significant interactions. Similarly for distance from bottom of the tank (Fig. 4b), no main effects of time or treatment were observed, and no significant interactions.

Among fish in later adulthood, analyses of locomotor activity (Fig. 4a) observed a main effect of time, $F(2.16, 200.55) = 4.90$, $p < 0.05$, but no effects of treatment and no interactions. Similarly for distance from bottom of the tank (Fig. 4b), observed a main effect of time, $F(2.60, 166.38) = 4.10$, $p < 0.05$, but no effects of treatment and no interactions.

3.2.3. *Tap test*

Tap startle testing was conducted at two ages: adulthood (8 months), and late adulthood (14 months). Pre-tap and post-tap activity scores from each of these tests are presented in Fig. 5. for ease of age comparison.

Among adult fish, a main effect of tap on pre-tap activity (Fig. 5a) was observed, $F(7.16, 487.06) = 2.69$, $p < 0.05$, whereby pre-tap activity generally increased across the session, as was a main effect of treatment, $F(2, 68) = 4.55$, $p < 0.05$. Post hoc testing showed that fish exposed to the higher dose of 1.0uM BAP were significantly less active than controls prior to the tap ($p < 0.05$). A similar trend relative to the lower 0.3 μM dose was not significant ($p = 0.09$). For post-tap activity (Fig. 5b) and startle magnitude (*not shown*), no main effects or interactions were detected.

Among fish in late adulthood, a main effect of time was detected on pre-tap activity (Fig. 5a), $F(7.27, 712.64) = 4.24$, $p < 0.05$, but no other main effects or interactions were found. For post-tap activity (Fig. 5b), a

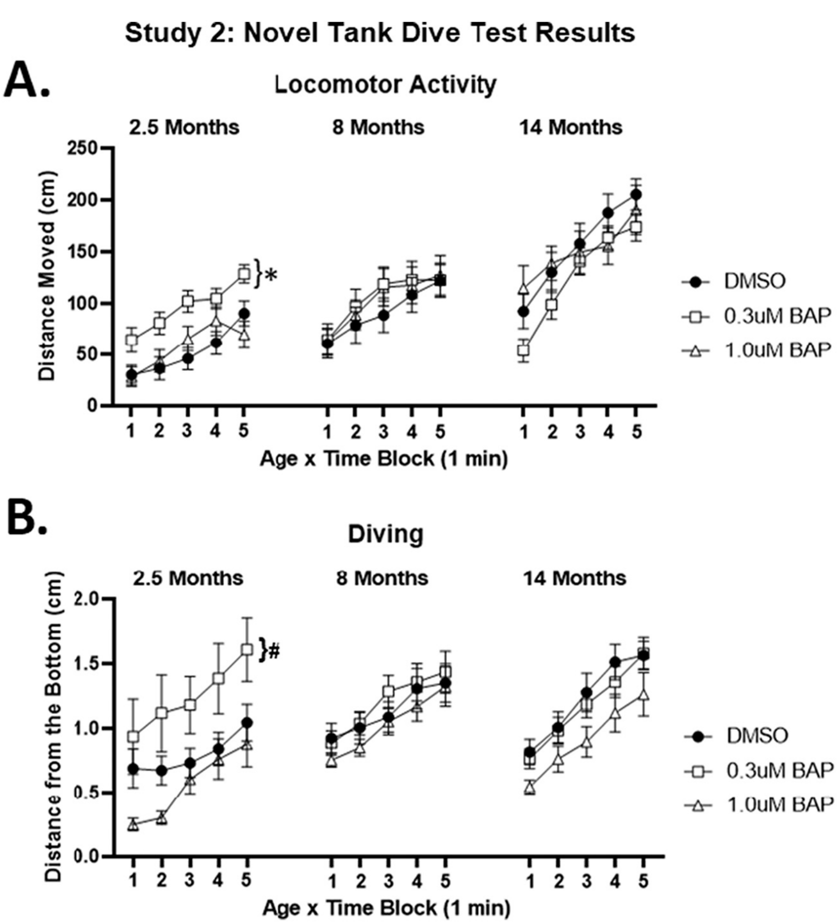

Fig. 4. Study 2: Novel Tank Diving Test. Locomotor activity was measured as distance moved (cm) during each 1-min bin within the session. (A) Main effect of treatment in adolescence, and non-effect at other ages, are shown. Fish exposed to 0.3 μM BAP were hyperactive compared to controls at 2.5 months ($p < 0.05$) (left) but not at 8- (center) or 14-months of age (right). (B) Main effect of treatment in adolescence, and non-effect at other ages, are shown. At 2.5 months of age (left), fish exposed to 0.3 μM BAP showed elevated exploration away from the floor relative to those exposed to 1.0 μM BAP ($p < 0.05$), but not controls. Data are shown as mean (cm) +/− SEM. Asterisks (*) indicate significance relative to controls. Pound (#) indicates significance relative to the 1.0uM group.

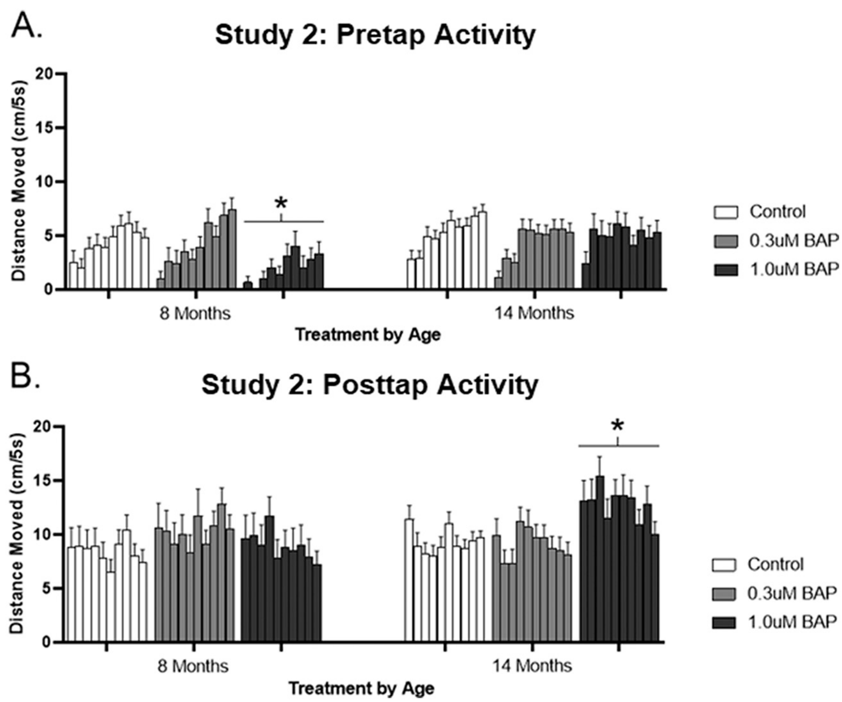

Fig. 5. Study 2: Tap Startle Test. Startle responses were measured as a change in activity, measured as distance moved, from the 5-s before each tap to the 5-s after the tap (cm). (A) Main effect of treatment is shown. Fish exposed to 1.0 μM BAP showed reduced pre-tap activity compared to controls at 8 months of age ($p < 0.05$) (left) but not at 14-months of age (right). (B) Main effect of treatment is shown. Fish exposed to 1.0 μM BAP showed increased post-tap activity compared to controls at 14 months of age ($p < 0.05$) (left) but not at 8-months of age (right). Data are shown as mean (cm) +/− SEM. Asterisks (*) indicate significance relative to controls.

main effect of treatment was observed, $F(2, 98) = 8.20$, $p < 0.05$. Post hoc testing showed that fish exposed to the higher dose of 1.0 uM BAP had significantly elevated post-tap activity relative to controls and to fish in the lower 0.3 uM BAP group ($p < 0.05$). No other main effects or interactions were observed. For the startle response magnitude (data not shown), a main effect of tap was observed, treatment was observed, $F(6.91, 676.75) = 3.42$, $p < 0.05$, whereby startle magnitudes decreased across the session, as was a main effect of treatment, $F(2, 98) = 6.54$, $p < 0.05$. Startle magnitude was elevated in fish exposed to the higher dose of 1.0 μM BAP relative to both controls and to fish in the lower 0.3 μM BAP group ($p < 0.05$).

3.2.4. *Shoaling*

Shoaling social approach testing was conducted at two ages: adulthood (8 months), and late adulthood (14 months). Locomotor activity and average distance from the screen data from each of these tests are presented in Fig. 6 for ease of age comparison.

Among adult fish, no main effects of time or treatment, or relevant interactions were detected for locomotor activity. For distance from the video screen (Fig. 6), univariate analyses detected a main effect of treatment during the video presentation, $F(2, 61) = 3.74$, $p < 0.05$, but not during the 2-min habituation period. Post hoc testing showed that fish exposed to 0.3 μM BAP remained further from the video screen during the video presentation relative to controls ($p < 0.05$). A similar trend among the 1.0 μM BAP group relative to controls failed to reach significance ($p = 0.07$). No treatment effects reached significance for the approach score (distance from the screen prior to the video – during the video).

Among fish in late adulthood, main effects on locomotor activity were detected for time, $F(3.40, 253.11) = 265.24$, $p < 0.05$, but not treatment. For distance from the video screen (Fig. 6), no treatment effects were evident during either phase of testing session (habituation or video). Additionally, no treatment effects were observed for the approach score (distance from the screen prior to the video – during the video).

3.2.5. *Predator avoidance*

Predator avoidance testing was conducted at two ages: adulthood (8 months), and late adulthood (14 months). Performance is summarized in supplemental table S5. Among adult fish, no main effects of time or treatment, or relevant interactions were detected for locomotor activity. For distance from the video screen (*not shown*), a main effect of predator cue (present/absent) was observed, $F(1, 73) = 26.10$, $p < 0.05$, as was a predator cue by cue speed (fast/slow) interaction, $F(1, 73) = 7.23$, $p < 0.05$. No main effects of treatment or relevant interactions were detected.

Among fish in late adulthood, a main effect of time was detected for locomotor activity, $F(4.58, 416.40) = 2.37$, $p < 0.05$, but no effects of treatment or relevant interactions were detected. For distance from the video screen (*not shown*), a main effect of predator cue (present/absent) was observed, $F(1, 91) = 27.78$, $p < 0.05$. No main effects of treatment or relevant interactions were detected.

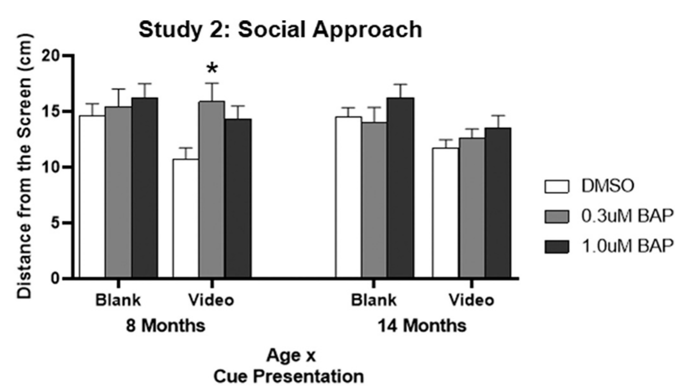

Fig. 6. Study 2: Shoaling Social Approach Test. Social approach was measured as a change in exploration, measured as distance from the video cue screen (cm) during cue present (video) and absent (blank) conditions. (cm). Treatment by cue condition interaction is shown at 8 months, and no effect is shown at 14 months. Fish exposed to 0.3 μM BAP showed remained further from the screen during the video presentation compared to controls at 8 months of age ($p < 0.05$) (left) but not at 14-months of age (right). A similar trend among 1.0 μM-treated fish did not reach significance. Data are shown as mean (cm) +/− SEM. Asterisks (*) indicate significance relative to controls.

3.2.6. Behavioral performance by age

Further analyses were performed to determine which behaviors show an age-effect function and whether this significantly interacted with treatment. As the individual identity of zebrafish could not be specified between the different testing phases, the age of testing was treated as a between-subjects variable.

In the novel tank dive test, analyses of locomotor activity (Fig. S1a) showed a main effect of age, $F(2, 255) = 22.31$, $p < 0.05$, and a treatment x age interaction $F(4, 255) = 2.21$, $p = 0.07$ triggered tests of the simple main effects. Post hoc tests showed that 14-month old fish were significantly more active relative to both 2.5 month old and 8 month old fish ($p < 0.05$) and 8-month old fish were more active relative to 2.5 months ($p < 0.05$). Analyses of the diving response (Fig. S2) showed a main effect of age, $F(2, 255) = 3.99$, $p < 0.05$, but no treatment x age interaction. Post hoc tests showed that both 8 and 14 month old fish swam significantly further from the bottom relative to 2.5 month old fish ($p < 0.05$) and these adult ages did not differ from one another ($p > 0.05$).

In the tap startle test (Fig. S3), main effects of age were observed for pre-tap activity, $F(1, 125) = 12.56$, $p < 0.05$, and post-tap activity, $F(1, 126) = 7.57$, $p < 0.05$, but not startle magnitude. Post hocs observed that older adults showed higher pre- and post-tap activity relative to adults ($p < 0.05$). A marginal age x treatment interaction was uniquely observed for post-tap activity, $F(2, 126) = 2.95$, $p = 0.056$. In the shoaling test (Fig. S1b), a main effect of age was observed for locomotor activity, $F(1, 165) = 4.11$, $p < 0.05$, and no relevant interactions were observed. Older adults were more active relative to adults ($p < 0.05$). No effects of age or interactions were detected on distance from the screen (raw values during baseline or video blocks) or on the approach score. In the predator avoidance test (Fig. S1c), no main effect of age was detected for locomotor activity, and no relevant interactions were observed. For distance from the screen (*not shown*), there was a main effect of age on distance from the screen regardless of cue condition, $F(1, 168) = 6.97$, $p < 0.05$, although the difference across ages was quite small (Difference = 1.32 cm) (Means: 8 m = 22.39 +/− 0.33, 14 m = 21.07 +/− 0.37). No other effects or interactions were detected, and no effects were detected for the magnitude of the fleeing response.

3.2.7. Whole organismal basal respiration

For swim tunnel whole animal respiration, there were no significant interactions between treatment and sex, however there were main effects of sex, $F(1, 45) = 4.25$, $p < 0.05$, and treatment, $F(2, 45) = 4.51$, $p < 0.05$. Post hoc testing indicated a significant difference between the 0.1 μM group and controls ($p = 0.016$, Fig. 2), whereby these fish showed reduced respiration. A similar difference for the 0.3 μM group was nearly significant ($p = 0.051$, Fig. 7).

3.2.8. Tissue-specific respiration

Data analysis was performed using standardized respiration scores, expressed as percent control (below). Raw data is summarized in supplemental tables 3 and 4 to allow the trends across target organs to be qualitatively compared.

<u>Total basal respiration</u>. Repeated measures ANOVA of total basal respiration (Fig. 8a) observed a main effect of organ, $F(2, 86) = 5.26$, $p < 0.05$, an interaction of organ by sex, $F(2, 86) = 7.16$, $p < 0.05$, and main effects of sex, $F(1, 43) = 5.59$, $p < 0.05$, and treatment, $F(2, 43) = 6.18$, $p < 0.05$, but no interactions of treatment with sex or organ. Both 0.3 and 1.0 μM BAP resulted in reduced total basal respiration ($p < 0.05$).

<u>Mitochondrial basal respiration</u>. Analyses of mitochondrial basal respiration (Fig. 8b) observed an interaction of organ by sex, $F(2, 86) = 6.77$, $p < 0.05$, and a main effect of treatment, $F(2, 43) = 5.68$, $p < 0.05$, but no interactions of treatment with sex or organ. Both 0.3 and 1.0 μM BAP reduced mitochondrial basal respiration ($p < 0.05$).

<u>Mitochondrial maximum respiration</u>. Analyses of mitochondrial maximum respiration (Fig. 8c) observed a main effect of treatment, $F(2, 29) = 8.20$, $p < 0.05$, but no interactions of treatment with sex or organ. Exposure to 0.3 μM BAP significantly reduced mitochondrial maximum respiration ($p < 0.05$), while 1.0 μM BAP was nearly significant ($p = 0.055$).

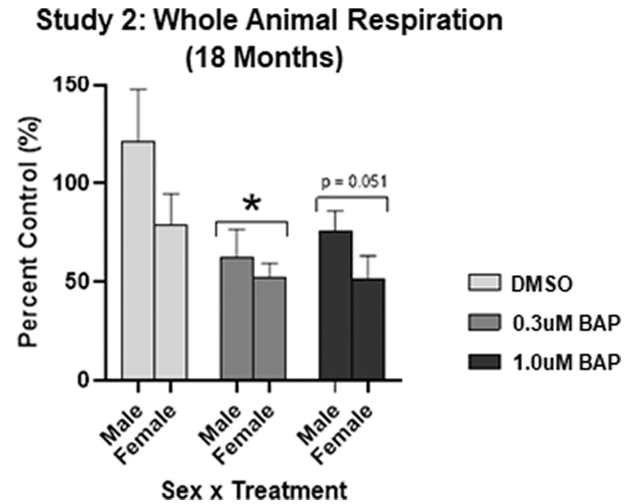

Fig. 7. Study 2: In Vivo Oxygen Consumption. Standardized OCR data from aged fish were generated recorded in the swim tunnel task. Main effect of treatment is shown. Fish with 0.3uM BAP exposure showed lower oxygen consumption relative to controls ($p < 0.05$). A similar pattern among 1.0 μM-treated fish failed to reach significance. Data are shown as mean (percent control (%)) +/− SEM. Asterisks (*) indicate significance relative to controls.

<u>Spare capacity</u>. Analyses of total spare capacity (Fig. 8d) observed a main effect of organ, $F(2, 58) = 8.20$, $p < 0.05$, an interaction of organ by sex, $F(2, 58) = 4.77$, $p < 0.05$, but no main effects of treatment or interactions of treatment with sex or organ.

<u>Sex-specific gonadal measures</u>. One way ANOVAs of gonadal respiration and spare capacity did not reveal any main effects of treatment on any measures taken.

4. Discussion

The present studies were conducted to assess the effects of early life exposures to BAP on neurobehavioral functions across the lifespan, with particular attention to the emergence and persistence of these effects across ages and the potential role of long-term metabolic dysfunction in their persistence. These studies investigated effects of developmental BAP on behavioral function from the larval stage to late adulthood. The exposure concentrations of BAP included in this study focused on those below the threshold for causing gross anatomical effects. As the concentrations were not selected to represent any single environmental condition, but show descending levels of exposure and toxicity which would go without detection using gross anatomical screening. This concentration selection process accommodates the unique toxicokinetic and toxicodynamic characteristics of the zebrafish and provides a point of comparison with other species commonly used to predict human health risk (invertebrates, rodents, etc). The exact nature of the resulting behavioral effects were largely mediated by the age of testing and were generally specific to certain tests, with other being spared from impairment, at any given age. It is likely that developmental processes play a role in the expression or attenuation of these problems, although further research is needed to understand what these relationships may be. These effects showed a non-monotic dose-effect function in multiple cases, indicating that it should not be assumed that higher levels of exposure lead to more severe outcomes in all cases. In parallel with these behavioral effects are long-term reductions in metabolic function, and while they were not specific to brain function, they appear to be associated with prolonged risks to health, including neurological health. We

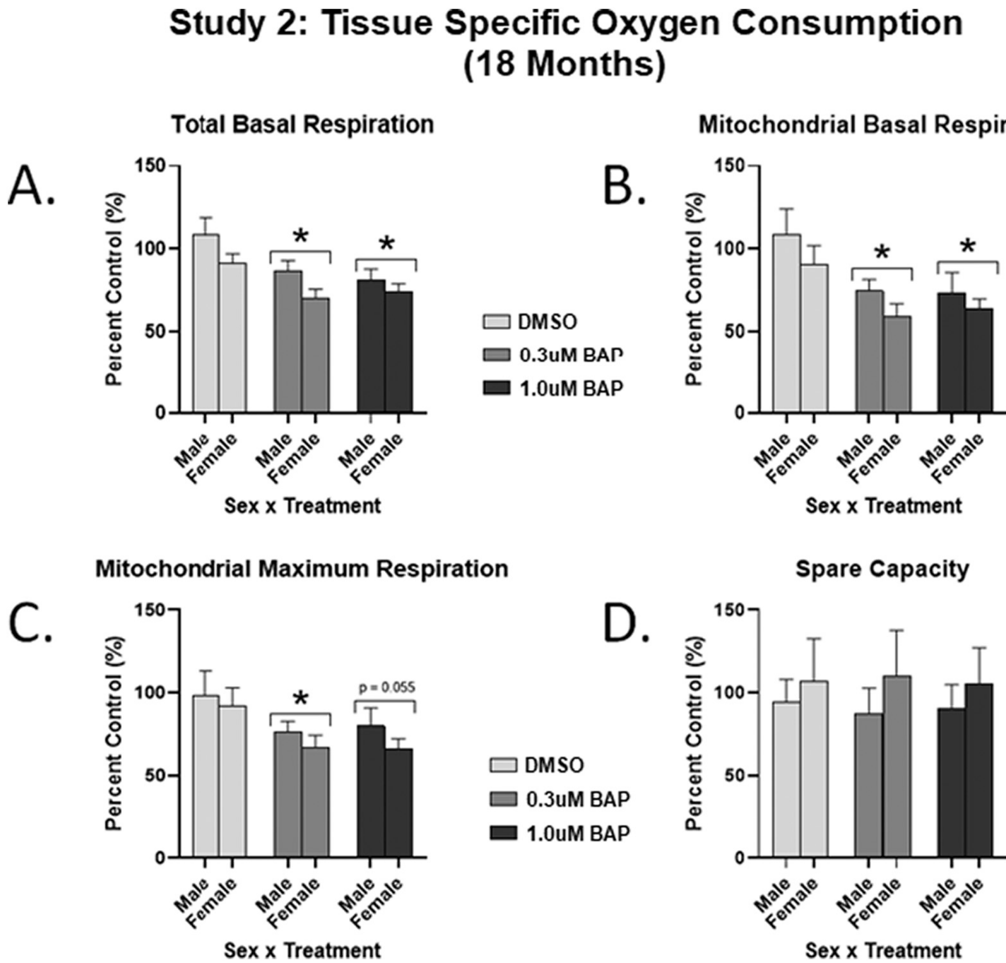

Fig. 8. Study 2: Tissue-Specific Oxygen Consumption. Standardized OCR data from the brain, liver and heart tissue of aged fish were generated recorded in the Seahorse Assay. Main effects of treatment are plotted for each of the four outcomes. (A) Main effect of treatment is shown. Fish exposed to either 0.3 or 1.0 μM BAP showed reduced total basal respiration compared to controls ($p < 0.05$). (B) Main effect of treatment is shown. Fish exposed to either 0.3 or 1.0 μM BAP showed reduced mitochondrial basal respiration compared to controls ($p < 0.05$). (C) Main effect of treatment is shown. Fish exposed to 0.3 μM BAP showed reduced mitochondrial maximum respiration compared to controls ($p < 0.05$). A similar pattern among 1.0 μM-treated fish failed to reach significance. (D) No effect of treatment is shown. No differences were found for spare capacity. Data are shown as mean (percent control (%)) +/− SEM. Asterisks (*) indicate significance relative to controls.

have previously shown that PAHs including BaP produce similar respiratory dysfunction immediately after exposure (Raftery et al., 2017). Such effects may be due to PAH/PAH-containing mixture's ability to potently induce CYP1A, the key enzyme in fish that activates PAHs to genotoxic metabolites in zebrafish (Brown et al., 2015; Timme-Laragy et al., 2007) and killifish (Wills et al., 2010), and to cause mitochondrial DNA damage in exposed embryos and larvae (Jung et al., 2009; Lindberg and Di Giulio, 2019; Wills et al., 2010). These effects are expected to be biologically significant for the zebrafish, as the affected neurobehavioral outcomes are associated with species-critical functions: sensory processing and responsiveness, threat detection and minimization of predation risk, and social affliliation; and as mitochondrial respiration is a limiting factor in many cellular functions. These disruptions would likely lead to impaired survival in the wild, and cellular functions that mediate these effects are expected to control similarly critical functions in other affected species, including humans.

The behavioral findings in this experiment indicate that while some behavioral alterations following early life BAP exposure last across much of the lifespan, the symptom profile may not be persistent, at least in the traditional sense. Single-age analyses, such as those in Study 1, could be easily interpreted as representing a stable and consistent phenotype, a hypothesis which is challenged by the findings of Study 2. For example, Studies 1 and 2 both show that BAP-exposed fish have minimal impairment in the larval stage and later, in adulthood, fail to show changes in novel tank exploration and instead show enhanced tap startle. Study 2 elaborates on this, showing that phenotypes displayed at one age may systematically differ from those displayed, or in multiple cases, spared, at another age. Likewise, Study 2 showed an impairment of social approach at 8 months of age which was attenuated by 14 months of age, and the effect at 8 months matched a nonsignificant trend in the same direction seen at 6 months in Study 1 (0.2–10 uM, 0 < 1 SEM unit from approach score mean). What this study emphasizes is the need for clarity on the developmental regulation of neurobehavioral dysfunction, both in preclinical models and our broader understanding of developmental neurotoxicity. A number of human epidemiological studies have associated early life exposure to PAH with attention deficit hyperactivity disorder (ADHD) (Kalantary et al., 2020), as have a range of studies examining PAH-containing mixtures, such as air pollution (Aghaei et al., 2019; Zhang et al., 2020) and cigarette smoke (Huang et al., 2018; Knopik et al., 2016). Related to the current data, human studies do suggest that a portion of those with ADHD, even a majority in some studies, experience a full or partial "remission" of their symptoms as they mature (Sudre et al., 2018), with observable differences on both behavioral and neurological features of the disorder based on age. Such remission is interpreted as evidence of developmental processes which can correct or compensate for alterations in neural function in the developing brain, although the identity and nature of those compensatory processes remain unclear. Based on the current findings, zebrafish may be an ideal model for mechanistic investigations of compensatory developmental processes, as well as additional mechanisms which may allow a phenotype to emerge or shift as aging continues.

The general conclusion that BAP is neurotoxic, despite its developmental nuances, fits well with prior work in this area. Prior neurobehavioral studies have established that developmental PAH exposure causes neurotoxicity in multiple fish species, including zebrafish (Holloway et al., 2021; Knecht et al., 2017b; Massarsky et al., 2015; Perrichon et al., 2014, medaka *(Oryzias Latipes)* (Le Bihanic et al., 2014; Perrichon et al., 2014) and killifish *(Fundulus Heteroclitus)* (Brown et al., 2016). The specific effects in larval zebrafish are mixed however, as studies with 5D or TU-strain zebrafish have reported that BAP enhances swimming behavior in the dark (Knecht et al., 2017a; Knecht et al., 2017b; Perrichon et al., 2014), while a prior study with AB* strain fish showed that BAP (5 μM) leads to locomotor impairment (Holloway et al., 2021). While the concentrations used in those studies were comparable to those used in Study 1, more specific factors, including zebrafish strain, which determine those short term effects may require further research. With respect to long-term effects, Knecht et al. (2017b) reported that cognitive-like deficits were present in prescenescent adulthood (15–17 months), supporting the current conclusion that the potential for neurobehavioral effects is maintained well into adulthood. The extensive battery used in the present study expands this conclusion, by noting that the neurobehavioral effects are highly test-specific, and so may target only specific neurobehavioral processes within any given stage of development. This compliments rodent studies, which commonly use batteries covering a variety of endpoints, and typically report that certain tests are impacted while others are spared (Chen et al., 2012; Hawkey et al., 2018; Honaker et al., 2022) Likewise, there is longitudinal evidence of behavioral deficits may not be stable across maturation (Chen et al., 2012), as in the current study.

One additional finding which was observed on multiple tests was the non-monotonic relationship between BAP concentration and behavioral function. In study 1, increases in tap startle were associated with a moderate concentration of BAP (0.2 μM), rather than a high concentration, and in study 2, locomotor and diving effects were observed in adolescents with moderate (0.3 μM) BAP exposure, but not with the higher concentration. These patterns are distinct from discrepancies in statistical significance, such as in the Study 2 shoaling results and certain outcomes in the metabolic results, where the lower concentration group uniquely reached significance, but was statistically equivalent to the higher concentration group. Non-monotonic effects are common in environmental toxicology studies, particularly among endocrine disrupting chemicals (Beausoleil et al., 2013). Although it is often assumed that higher levels of exposure should produce more severe phenotypes, it is acknowledged that toxins can induce compensatory responses in exposed cells and may produce multiple forms of toxicity which could interact with one another. In the present case, moderate concentrations enhanced the response, rather than inducing a deficit, per se. The ability of a toxin to do so may be limited to concentrations that are too low to induce a compensatory response (e.g. upregulation or downregulation of an impacted neurotransmitter receptor), or too low to produce a parallel form of toxicity which impairs this function through another mechanism. The basis of non-monotonic dose effects on behavior is unclear for BAP, and future studies using similar models will be needed.

With respect to the behavioral findings, certain limitations are present which should be considered. Although the observed relationships across development are generally attributed to differences in age, it should be noted that longitudinal designs do have one consistent limitation; that age and experience are conflated. While this observation is clear, long-term memory capacities of zebrafish are poorly understood to date, and further research will be necessary to determine whether prior experiences, particularly over this time span, are relevant to the interpretation of these and similar findings. Another limitation surrounds the procedural updates between Studies 1 and 2. Variants of the shoaling and predator tests are conceptually equivalent, but the influences of apparatus size and session design cannot be ruled out. Study 1 found significant effects of developmental BaP exposure on performance in the predator test, while Study 1 did not. For shoaling, the trends across treated and control groups are similar, with one simply reaching significance while the other did not, perhaps suggesting a difference in sensitivity rather than a difference in the effect of BAP. Predator results appear more clearly different from Study 1 to Study 2, with the second study notably showing stronger control fleeing responses throughout the testing. Larger differences between cued and non-cued phases of the session are hypothesized to reduce the sensitivity of the test to changes in the return to the screen after the cue is removed, although specific parameters of the test should also be considered. Parameters such as apparatus size and session design have not been well studied in the zebrafish to this point, but future research in this area may clarify such differences and support the current comparison.

As with neurobehavioral function, metabolic effects were observed to be present well into adulthood, despite that fact that 18 months had passed before metabolic testing. In particular, aged fish with embryonic BAP-exposure showed a pattern of respiratory depression, with whole animal and tissue-specific scores largely showing an overall reduction in oxygen consumption. Within the primary target organs (brain, liver, heart), total basal OCR, mitochondrial basal OCR and mitochondrial maximal OCR were all suppressed. However, the respiratory range between basal and maximal respiration remained consistent, as evidenced by unaltered spare capacity. This suggests two important conclusions. First, metabolic changes following an embryonic exposure can be quite persistent, in this case lasting well into aging. We and others have previously documented changes in metabolic and mitochondrial function immediately after PAH exposures (Elie et al., 2015; Lindberg and Di Giulio, 2019; Raftery et al., 2017), and the present results indicate that such effects are not limited to that immediate time-span. Changes experienced during aging may be developmentally significant, as aging is a period commonly associated with a slowing of metabolic functions, overall declines in neurological health and onset of neurodegenerative diseases (Błaszczyk, 2020; Chistiakov et al., 2014; Ivanisevic et al., 2016). Second, the overall change in oxygen consumption in live animals was not differentially driven by specific target organs. Although many studies have directly examined the ramifications of metabolic effects in the aging brain (Moeller et al., 1996; Yang et al., 2019; Yin et al., 2016), such effects are unlikely to be brain-specific in the case of PAH exposures. Therefore, these consequences may be relevant to a wider range of adverse outcomes than just altered neurobehavioral function. One caveat to such a conclusion in the present study is the lack of significant effects found in the parallel gonadal analysis. Trends observed in the gonads (Table S2), particularly the 0.3 μM group, were generally consistent with respiratory depression but failed to reach statistical significance. This lack of significance may be due to reduced sample sizes within sex-specific analyses, and a general loss of statistical power, rather than a resistance of the gonads to PAH-induced mitochondrial dysfunction. The overall sex differences in whole animal respiration rates are notable and we are unaware of studies that have addressed this. One possibility is that the larger mass of female versus male gonads, which have low respiration rates, may contribute to this difference, although followup analyses will be necessary to fully address this factor, and its potential contributions to long term metabolic dysfunction.

As the final behavioral testing occurred prior to senescence (14 months), it would not be appropriate to consider those effects direct consequences of mitochondrial aging perturbations. However, it is clear that mitochondrial dysfunction is a highly persistent form of toxicity across the lifespan. Such persistence is necessary to maintain the persistent potential of phenotypic changes across the lifespan. Prior studies have noted that impairments in mitochondrial function also disrupt core functions of neurons, likely through restriction of ATP-dependent processes. For example, the motichondrial toxin rotenone disrupts neuronal electrophysiological outcomes and alters the release of excitatory neurotransmitters and dopamine in striatal neurons (Chan et al., 2016; Costa et al., 2008). A secondary effect of this dysfunction may be the promotion of apoptosis (Joza et al., 2001; Rego and Oliveira, 2003; Xiong et al., 2014), which could influence neurological function and development more broadly. Mitochondrial dysfunction has also been implicated more broadly in psychiatric disorders and cognitive dysfunction (Netto et al., 2018; Sharma et al., 2021; Verma et al., 2016), within and beyond the processes of aging or neurodegeneration. At present, it remains unclear what degree of mitochondrial dysfunction is required to cause such neurological effects, but the present findings suggest that future studies should address the interrelatedness of these outcomes across multiple stages of development. Additionally, the present study did not produce similar metabolic data across a range of ages (e.g. adolescent, adult, aged), so it cannot be clearly stated whether the present effects represent an age-related decline that BAP enhances, or a stable pattern of metabolic depression which begins earlier in life.

In conclusion, the current studies provide further evidence for the neurotoxicity and mitochondrial toxicity of the prototypic PAH, BaP. Developmental BaP exposure of zebrafish to low doses of BaP which are below the threshold for overt dysmorphogenesis or increased leathality cause persisting neuroberhavioral impairments. The expression of these effects are dependent on the perameters of behavioral assessment and effects are often non-monotonic in character. Previous studies generally focused on more immediate impacts BaP exposure; the present study shows that neurobehavioral and bioenergetic consequences of embryonic PAH exposure can cause deficits across the lifespan. However, these data also demonstrate that neurobehavioral consequences vary in their expression over the lifespan. They may be differentially expressed in immature, maturing, mature and aged animals. In some cases, this can result in deficits that emerge early in the lifespan but are transient, while others may persist or even emerge later in the lifespan. Future studies will be required to clarify the role of development in the expression of toxin-induced functional deficits, and in particular how these processes interact with more stable effects, like mitochondrial dysfunction.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Edward D. Levin reports financial support was provided by National Institute of Environmental Health Sciences. Edward D. levin reports a relationship with National Institute of Environmental Health Sciences that includes: funding grants. None

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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