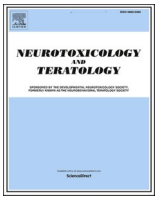
Neurotoxicology and Teratology 102 (2024) 107339

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera



Embryonic exposures to cadmium and PAHs cause long-term and interacting neurobehavioral effects in zebrafish

Alexandra Stickler [a], Andrew B. Hawkey [a,b], Anas Gondal [a], Sarabesh Natarajan [a], Mikayla Mead [a], Edward D. Levin [a,*]

[a] Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710, USA
[b] Department of Biomedical Sciences, Midwestern University, Downers Grove, IL 60515, USA

ARTICLE INFO

Editor: Dr. D Volz

Keywords:
Cadmium
Benzo[*a*]pyrene
Fluoranthene
Zebrafish
Neurobehavioral
Developmental
Behavior

ABSTRACT

Developmental exposure to either polycyclic aromatic hydrocarbons (PAHs) or heavy metals has been shown to cause persisting and overlapping neurobehavioral effects in animal models. However, interactions between these compounds have not been well characterized, despite their co-occurrence in a variety of environmental media. In two companion studies, we examined the effects of developmental exposure to cadmium (Cd) with or without co-exposure to prototypic PAHs benzo[*a*]pyrene (BaP, Exp. 1) or fluoranthene (FA, Exp. 2) using a developing zebrafish model. Zebrafish embryos were exposed to Cd (0–0.3 μM), BaP (0–3 μM), FA (0–1.0 μM), or binary Cd-PAH mixtures from 5 to 122 h post fertilization (hpf). In Exp. 1, Cd and BaP produced independent effects on an array of outcomes and interacting effects on specific outcomes. Notably, Cd-induced deficits in dark-induced locomotor stimulation were attenuated by BaP co-exposure in the larval motility test and BaP-induced hyperactivity was attenuated by Cd co-exposure in the adolescent novel tank test. Likewise, in Exp. 2, Cd and FA produced both independent and interacting effects. FA-induced increases on adult post-tap activity in the tap startle test were attenuated by co-exposure with Cd. On the predator avoidance test, FA- and 0.3 μM Cd-induced hyperactivity effects were attenuated by their co-exposure. Taken together, these data indicate that while the effects of Cd and these representative PAHs on zebrafish behavior were largely independent of one another, binary mixtures can produce sub-additive effects for some neurobehavioral outcomes and at certain ages. This research emphasizes the need for detailed risk assessments of mixtures containing contaminants of differing classes, and for clarity on the mechanisms which allow cross-class toxicant interactions to occur.

1. Introduction

Environmental pollution presents exposures that are complex mixtures. The traditional approach to this problem has been to identify specific candidate compounds and evaluate their individual toxicities, and to estimate their cumulative toxicities based on those data (Sarigiannis and Hansen, 2012). Based on this, it has become common to test mixtures against the "additivity hypothesis" (Rider et al., 2018), which states that co-exposures produce effects which are mathematically equivalent to the collective effects of the constituent exposures. Interactions would then be defined as sub-additive or supra-additive effects, corresponding with effects that are over- or under-estimated using the additivity assumption. Studies utilizing this approach show considerable promise, particularly those focusing on within-class mixtures (Cao et al., 2011; Jarvis et al., 2014; Kamo and Yokomizo, 2015; Meyer et al., 2015). However, the neurobehavioral impacts of mixtures containing multiple classes of chemicals remain poorly understood.

Among the most concerning environmental toxicants are polycyclic aromatic hydrocarbons (PAHs) and heavy metals. These classes of compounds are frequently co-localized, as noted in diverse media, such as tobacco smoke (Richter et al., 2016), wildfire smoke (Boaggio et al., 2022), coal and coal-byproducts (Sarver et al., 2019; Verma et al., 2015), air pollution from combustion products (Kamila et al., 2018; Singh et al., 2011), urban soils and runoff (Azzolina et al., 2016; Lau et al., 2009; Morillo et al., 2008; Wang et al., 2004), and creosote-contaminated soils (Volkoff et al., 2019). Metal and PAH co-exposures are common, and evaluation of their interactive toxicities is of substantial importance for risk assessment and management.

Metals and PAHs provide a complex context for mixtures toxicology, as they act through quite different mechanisms of action. Metals, such as

* Corresponding author at: Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Box 104790, Durham, NC 27710, USA.
E-mail address: edlevin@duke.edu (E.D. Levin).

https://doi.org/10.1016/j.ntt.2024.107339
Received 27 November 2023; Received in revised form 15 February 2024; Accepted 3 March 2024
Available online 6 March 2024

cadmium (Cd), have been found to exert toxicity through direct binding with cellular proteins and disruption of membrane permeability, leading to genotoxicity and interference with critical cellular processes, including Ca-homeostasis, mitochondrial function, DNA repair, cell growth, proliferation and differentiation (Balali-Mood et al., 2021; Nagaraju et al., 2022). PAHs, on the other hand, have been found to act through direct activation or inhibition of select proteins. Classical PAHs, like benzo-a-pyrene (BAP), act as agonists of aryl-hydrocarbon receptors (AHRs), which are ligand-activated transcription factors that mediate the expression of a variety of key proteins. These include CYP450 enzymes, cell adhesion molecules, and proteins involved in mitochondrial, immune and inflammatory functions (Das et al., 2017; Fu et al., 2022; Malott and Luderer, 2023; Vondracek et al., 2011). Other PAHs, like fluoranthene (FA), have AHR-independent mechanisms of toxicity, acting as inhibitors of the CYP1a1 enzyme (Brown et al., 2015), which take part in the metabolism of exogenous compounds and endogenous signals including retinol, melatonin, sex hormones, and linoleic acid (Lu et al., 2020). While these compounds act with different primary mechanisms and levels of specificity, it is notable that they have considerable overlap in their broader effects in the developing nervous system.

Both PAHs and metals are associated with spinal cord defects (Le Bihanic et al., 2014; Sfakianakis et al., 2015) and developmental alterations of neurotransmitter systems including glutamate, GABA, acetylcholine, and monoamines (Abd El Naby et al., 2023; Bensoussan et al., 2009; Karri et al., 2016; Leret et al., 2002; Marchetti and Gavazzo, 2005; McCallister et al., 2008; Slotkin et al., 2017). Additionally, these compounds influence key processes in nervous system development, including neurogenesis, synaptic plasticity, and long-term potentiation (Chow et al., 2008; Dou and Zhang, 2011; Sadiq et al., 2012; Salehi et al., 2015; Zhang et al., 2022; Zhao et al., 2018). These toxicants and their neurochemical effects are further connected to overlapping sets of neurobehavioral outcomes in rodent models, including changes in activity levels, affective responses, and cognitive functions (Chen et al., 2012; Hawkey et al., 2019; Kamel and Abd El-Razek, 2011; Lamtai et al., 2021; Sprowles et al., 2018; Zhang et al., 2016). These effects are believed to support links between developmental exposures to these compounds and issues such as attention deficit hyperactivity disorder, mood disorders, and impaired cognitive performance (Bauer et al., 2020; Chandravanshi et al., 2021; Jedrychowski et al., 2015; Rezaei Kalantary et al., 2020; Zhen et al., 2023). As these compounds appear to influence the same neurodevelopmental processes through differing mechanisms, it remains unclear whether those effects would be additive within a mixture.

Research in neurotoxicology has relied on model organisms for decades as a means of predicting how developmental and neurological health can be impacted by toxicant exposures. The zebrafish has become a prominent model organism for this work due to several advantages that zebrafish hold. Zebrafish share 71.4% of their genes with humans, and of those genes, 84% of those genes are associated with a human disease (Howe et al., 2013). With respect to neuroscience, their brains are anatomically simple compared to humans, but align notably in organizational structure, neurochemistry and synaptic organization (Shams et al., 2018). Further, zebrafish are small, inexpensive and densely housed, allowing for evaluation of a greater number of chemicals, concentrations, mixtures, and ages than is typically possible for rodent studies. Like rodents, a variety of behavioral techniques are available which can allow the effects of a toxicant to be longitudinally evaluated and characterized at the *in vivo* level. Given these advantages, zebrafish were selected for the current study to allow mixture analyses using multiple concentrations of Cd, multiple representative PAHs, and behavioral analyses spanning development from the larval stage to maturity.

To date, zebrafish have contributed to our understanding of the neurobehavioral toxicology of PAHs and metals, although significant gaps in this literature still remain. A number of studies have examined the behavioral effects of BAP, including embryonic/larval studies and those assessing behavioral function into adulthood (Gao et al., 2017; Hawkey et al., 2022; Holloway et al., 2021; Knecht et al., 2017a; Knecht et al., 2017b). BaP shows mixed effects at the larval stage, although additional deficits such as altered locomotor activity, startle responses and memory performance have been noted in later stages of development. Comparatively, there is little data on the neurobehavioral toxicity of FA, with existing studies limited to embryo/larval testing (Geier et al., 2018; Shankar et al., 2019). A number of studies have tested the neurobehavioral toxicity of CD in zebrafish, although this too is generally limited to embryo/larval testing (Han et al., 2019; LeFauve and Connaughton, 2017; Liao et al., 2021; Shankar et al., 2021; Xu et al., 2022). No known studies have examined binary mixture effects of these PAHs and CD. Future studies examining such mixture effects would expand the literature for both the mixture and those constituents alike.

The purpose of the current study was to use the developing zebrafish model to assess the consistency with which PAHs and a representative heavy metal (Cd) produce longitudinal neurobehavioral effects when presented alone or in a mixture with one another. In the current study, BaP and FA were selected as the representative PAHs, due to their differing mechanisms of action, and Cd as the neurotoxic metal of interest. Exposures were conducted by housing zebrafish embryos in contaminated housing water from 5 to 120 h post fertilization (hpf). In experiment 1, a six-group design was used including a 0.1% DMSO control, two Cd-only groups (0.1 and 0.3 μM), a BaP-only group (3 μM) and two binary mixtures (0.1 μM Cd + 3 μM BAP, 0.3 μM Cd +3 μM BAP). Experiment 2 used the same design as Experiment 1, with 1 μM FA used in place of 3 μM BaP in the single and mixture exposure groups. Exposure concentrations were selected to be in a neurotoxic and behaviorally toxic range without increases in dysmorphology and/or mortality, so that consistent effects and/or interactions could be observed. This places them above typical regulatory and environmental levels. For example, US EPA minimum contaminant level (MCL) values for PAHs and Cd are 0.2 μg/l and 5 μg/l respectively, compared to current study levels of 756.9 μg/l BAP, 202.2 μg/l FA, and 18.3–55.0 μg/l Cd (https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations#two). Behavioral function was assessed using a battery of tests covering locomotor activity, reflexive behavior, affective-like behavior, and approach or avoidance responses. These batteries sampled behavioral performance at four points in development: larval (144 hpf), juvenile (30 days of age), adolescence (2.5 months of age), and adulthood (7–8 months of age).

2. Methods

2.1. Animals, exposures and husbandry conditions

The subjects for this study were the offspring of untreated adult AB* zebrafish, housed and bred in the Levin lab at Duke University. All husbandry and testing procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at Duke University (Protocol #A169–22-10), and in accordance with federal standards for the use of vertebrate animals in research.

Housing water was made using deionized water, sea salt (Instant Ocean, 0.5 parts per thousand), and buffering powders (Alkaline Regulator, 1 g/l, Neutral Regulator 0.3 g/l, Seachem Laboratories, Madison, GA, USA). This was used to make exposure media and for long-term housing. Embryos/larvae were housed in glass petri dishes in an incubator set to 28 ± 1 °C and maintained on a 14:10 light/dark cycle. After larval motility testing, larvae were moved to 3 l acrylic tanks maintained on a recirculating water system (Tecniplast, West Chester, PA, USA). These larvae/juveniles were fed 3× per day with larval diet powder (Golden Pearl Active Spheres, Brine Shrimp Direct, Ogden, UT, USA) until 30 days of age, and then transitioned to the adult food regime of live *artemia salina* (Brine Shrimp Direct, Ogden, UT, USA) twice per day and dry adult diet (GEMMA Micro 300 micro-pellets, Skretting USA, Tooele, UT, USA) once per day. Water quality in the recirculating system

was monitored weekly and managed to remain within 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 ppt salinity. As in the incubator, developing fish on the recirculating system were maintained on a 14:10 h light/dark cycle.

The offspring collection and exposure procedure proceeded as follows. Zebrafish eggs were collected from multiple mixed-sex tanks of untreated AB* zebrafish (> 10 fish per tank) to provide 2–3 replicate cohorts of mixed parentage (3 cohorts in Exp. 1, 2 cohorts in Exp. 2). At 3-4hpf, successfully fertilized and normally developing embryos were sorted out and randomized into 6 glass petri dishes to a density of 1 embryo/ml (40 embryos per petri dish). At 5hpf, the housing water in each plate was removed and replaced with freshly made exposure medium. The exposure for both experiments was renewed every day, ending at 120hpf. At this point, larvae were transferred to clean housing water.

Due to the low solubility of the selected PAHs, a 0.1% dimethyl sulfoxide (DMSO) solution was used as a vehicle for exposures. Exposure media was made by dissolving powdered cadmium chloride (Cd), benzo-a-pyrene (BAP), or fluoranthene (FA) into 100% DMSO to make stocks, then diluting these stocks with newly made housing water at a ratio of 1 µl stock per 1 ml of housing water. Stocks used in mixtures were made separately for Cd and the PAHs, and these were combined/diluted to make the mixture solution immediately before each exposure began or was renewed.

These doses of BaP and FA were selected as ones producing neurobehavioral effects without significant impairments in survival or increases in dysmorphology during the embryo/larval stage. Pilot tolerability studies (*not shown*) indicated increases in embryonic lethality and/or dysmorphology at and above the following thresholds: Cd 1 µM, BaP 10 µM, FA 3 µM. Concentrations used for neurotoxicity assessment were selected to fall below this threshold, with concentrations of 0.1–0.3 µM Cd, 3 µM BaP and 1 µM FA. The suitability of these concentrations was verified within each study through tracking of survival and dysmorphologies (including pericardial edema, spinal curvature, eye defects, yolk sac defects and docked tail). Concentrations for CD, BAP and FA were selected to meet a lethality and defect criterion rate of <10% in viable embryos during the pilot tolerability studies. Each exposure cohort contained six treatment groups: DMSO control, 0.1 µM Cd, 0.3 µM, the PAH reference group, and two mixture groups (0.1 µM Cd + PAH; 0.3 µM Cd + PAH). In Exp. 1, the PAH treatment was 3 µM BaP. In Exp. 2, the PAH treatment was 0.3 µM FA. Observed lethality/defect rates within each study are shown in supplemental tables (Tables S1 and S2) for reference.

2.2. Light/dark larval motility test

The Light/Dark Motility Test was used to test motor function during early stages of zebrafish development (Hawkey et al., 2022). The test was first conducted at 144 h post-fertilization, 24 h post the final exposure. Prior to testing, larvae were transferred to a 96 well plate (one larva per 7 mm diameter well) and acclimated for 1 h in the dark. This plate was then loaded into the Daniovision Observation Chamber (Noldus, Wageningen, The Netherlands) for testing. The testing session lasted 50 min, consisting of alternating 10-min periods of light and dark (dark – light – dark – light – dark). The first 10 min in the dark were treated as a habituation period. Zebrafish larvae show increased locomotor activity in the dark, and reduced movement in the light periods. The primary measure of locomotor activity in this test was distance moved in cm. A secondary endpoint, stimulation index, was calculated (dark phase activity – light phase activity). This represented the level of reactive stimulation that occurred when switching from lit to dark conditions. This metric was only used for follow-up analyses of lighting x treatment interactions, to allow a more direct interpretation of the lighting response. For each replicate, 24 larvae were tested.

At 30 days post-fertilization, the juvenile fish completed a second session of light/dark motility testing. The testing protocol and analysis was identical to that used at 6 days of age, except that 24-well plates (16 mm diameter wells) were used to accommodate the increased size of animals at this age.

2.3. Novel tank dive test

The novel tank dive test was used to test locomotor activity and anxiety-like behavior (Hawkey et al., 2022). This test was performed identically in adolescence (2.5 months) and adulthood (6–8 months). Briefly, fish were individually netted and placed into 1.5 l acrylic tanks (Aquatic Habitats housing tanks) filled to a depth of 10 cm with clean system water (approximately 1250 ml of water). These tanks were trapezoidal in shape [22.9 cm long on the bottom, 27.9 cm long at the top, 15.2 cm tall, and 5.1 cm wide]. Each fish was then allowed to explore the tank for 5 min, while a digital webcam (Logitech HD C310), placed to face the side of each tank, recorded the session. Videos of this session were collected and analyzed with Ethovision software (v.14, Noldus, Wageningen, The Netherlands) to measure locomotor activity and anxiety-like diving responses, represented as distance moved and distance from the bottom.

2.4. Tap startle test

The tap startle test was used to test sensorimotor reflex in adolescence (2.5 months) and adulthood (6–8 months) (Hawkey et al., 2022). Briefly, fish were individually placed into one of 8 clear plexiglass wells (5.7 cm diameter) arranged in a 2 × 4 setup. White plastic dividers were in place to prevent the fish from seeing each other. The platform under each well was fitted with a 24-V DC push solenoid to strike the well bottom with a metal pin when triggered at set intervals. After a 30 s delay, 10 such tapping stimuli were delivered at 1 min intervals. This session was recorded using a digital webcam mounted 50 cm above the wells. Movement data was collected during the 5 s prior to the tap (pre-tap) and the 5 s after each tap (post-tap). The magnitude of each startle response was then calculated as a change in movement due to the delivery of the tap (post-tap - pretap).

2.5. Shoaling test

The shoaling test was used to test the social behavior of adult fish (6–8 months of age) (Hawkey et al., 2022). This test was performed in a rectangular partitioned tank placed on top of an LED light pad (Huion Technology, Shenzen China). Each lane of the partitioned tank (30 × 6 cm, filled to a depth of 10 cm) was bordered by black plexiglass on the long sides and clear plexiglass on the short ends. The lane ends faced LCD monitors where stimuli could be presented. Testing began with a 30 min isolation period in visually separated 1.5 l tanks. This was done to promote social attraction during the test. After the isolation period, the fish were individually placed into the lanes and testing began immediately. The first two minutes of the session served as a baseline phase, where each monitor showed only control shapes, which were statis ovals with zebrafish stripes. After two minutes, a 5-min video of a shoal of adult zebrafish swimming was presented on one of the two screens. The webcam mounted 55 cm above the tank recorded this session. Videos were analyzed for locomotor activity (distance moved in cm) and distance from the video-adjacent end of the lane. Social attraction, or the shoaling response, was calculated as the change in position due to the video (distance to wall at baseline – video phase).

2.6. Predator avoidance test

The predator avoidance test was used to test threat recognition in adult zebrafish (6–8 months) (Hawkey et al., 2022). Predator avoidance testing was conducted using the same apparatus as used in the shoaling test. Fish were individually removed from their home tanks and placed

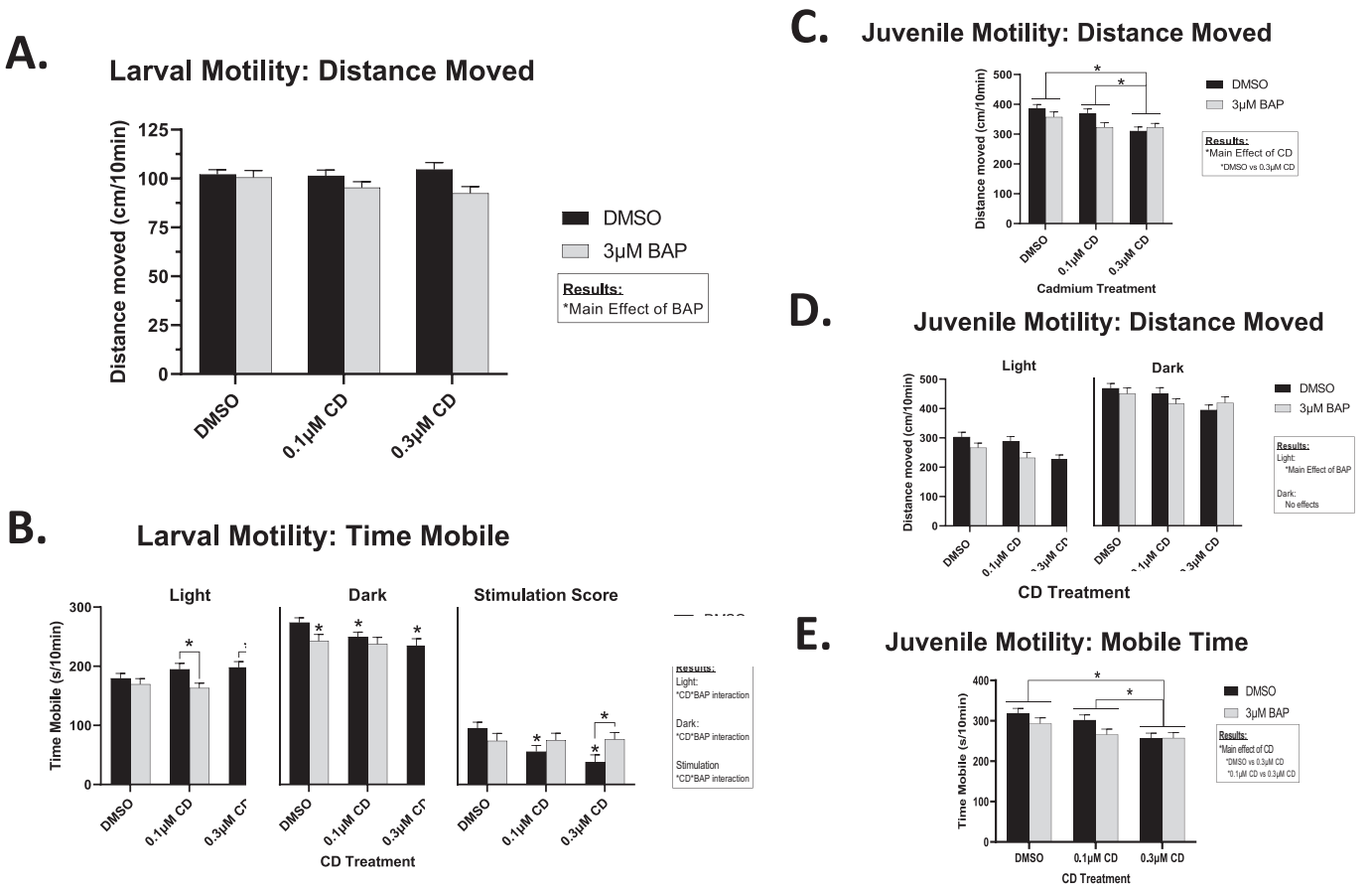


Fig. 1. Experiment 1: Larval and Juvenile Effects of Cd and BaP. Motility testing measured locomotor activity as distance moved (cm/10 min), and time mobile (*sec*/10 min). (A) BaP treatment reduced distance moved in larvae regardless of Cd exposure. (B) In the light (left), BaP reduced time mobile in larvae, but only within 0.1 and 0.3 μM Cd-exposed groups. In the dark (right), BaP, 0.1 μM CD and 0.3 μM Cd reduced time mobile in larvae compared to controls. Co-exposure had no additional effect. Dark-induced stimulation (right) was reduced in larvae by 0.1 and 0.3 μM CD. BaP co-exposure attenuated the effect of 0.3 μM Cd. (C) 0.3 μM Cd treatment reduced juvenile distance moved relative to non-Cd and 0.1 μM Cd treated groups, regardless of BAP exposure. (D) BaP treatment reduced juvenile distance moved under lit (left) but not dark (right) conditions. (E) 0.3 μM Cd treatment reduced juvenile time mobile relative to non-CD and 0.1 μM Cd treated groups, regardless of BaP exposure. Data expressed as mean ± SEM. Asterisk (*) indicates significance at $p < 0.05$. Ns = Larval 67–72/group; Juvenile 25–48/group.

into the lanes of the partitioned tank. The first minute of the session served as a baseline phase, where each monitor was blank (white). After one minute, a sequence of four threatening cues (presented for 1 min each), separated by blank screen phases (1 min each), was presented on one of the two screens. The threatening cues consisted of a colored dot which grew from 1 to 23 cm in size at a fixed speed, to provide a two-dimensional representation of a large object approaching. On minutes 2 and 4, the predator cue grew from 1 to 23 cm in size over 4 s (slow cue). On minutes 6 and 8, the predator cue grew from 1 to 23 cm in size over 1 s (fast cue). Videos were analyzed for locomotor activity (distance moved in cm) and distance from the cue-adjacent end of the lane. Predator avoidance, or the fleeing response, was calculated as the change in position due to the video (distance to wall when cue present – pre-cue blank phase).

2.7. Data Analysis

Statistical analyses were performed with IBM SPSS Statistics v. 24. Data were analyzed with mixed factorial analysis of variance (ANOVA). Each toxicant comprising the mixture (Cd, BaP, FA) was treated as a between-group factor (coded by concentration), to allow identification of main effects of each toxicant and interactions between the toxicants. Sex was also treated as a between-subjects factor at ages when sex could be reliably identified (adulthood). Repeated-measures analyses included within-session parameters (*e.g.* time block, stimulus type or repetition, phase of the trial, etc). Cohort was treated as a covariate. Main effects and interactions were then investigated using Fisher's LSD *post hoc* tests. For interactions, these tests were examined based on an *a priori* design, which compared each single-treatment group to the DMSO control, and each mixture to the single Cd and single PAH groups. For initial identification of interactions, a significance cutoff of $p < 0.10$ was used (Snedecor and Cochran, 1967). However, for all main effects and *post hoc* tests, $p < 0.05$ was set as threshold for statistical significance. For ease of interpretation, the results report all methodological and treatment main effects, and the highest order interaction regarding either the combined treatment (if present) or a given single-treatment, as relevant. Where applicable, within-session alterations in treatment effects, indicated by interactions of treatments and sequential within-session factors, such as time block or repetition of a given stimulus, are provided as supplemental data. The analysis of variance presentation includes the F-ratio, degrees of freedom and the *p*-value. The control group refers to the group that was administered only vehicle with no Cd or PAH (BaP, Exp. 1 or PA, Exp. 2) when individual treatment groups re compared. Non-BaP, Non-FA or Non-Cd groups are used when discussing the main effects of these treatments when the groups with and without these treatments are compared regardless of the exposure to the other treatment.

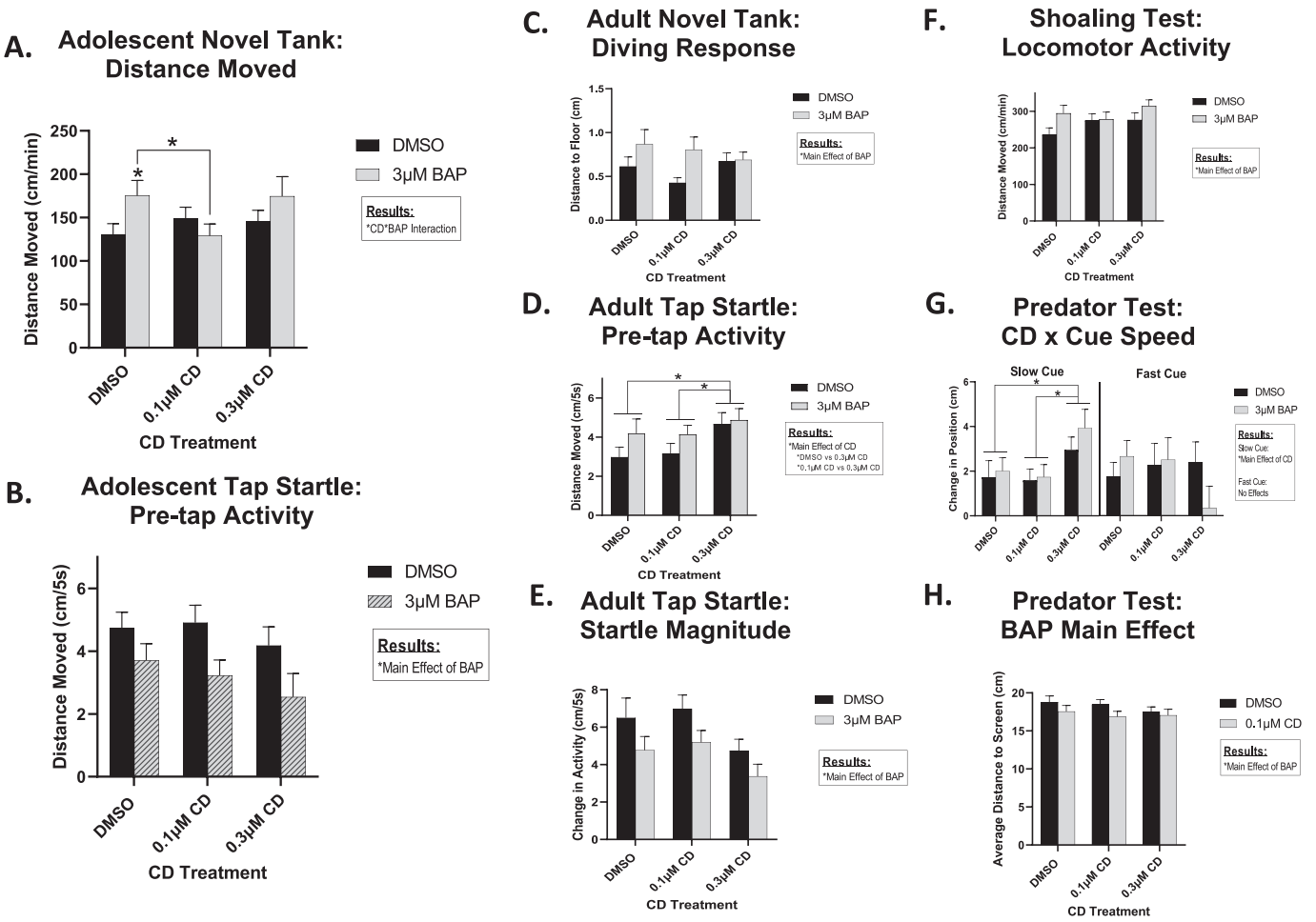


Fig. 2. Experiment 1: Adolescent and Adult Effects of Cd and BaP. Figures represent results of the adolescent and adult test batteries. (A) BaP treatment increased locomotor activity (cm/min) in the adolescent novel tank, but co-exposure to 0.1 μM Cd attenuated this effect. (B) BaP reduced baseline (pre-tap) activity (cm/5 s) in the adolescent tap startle test, regardless of CD exposure. (C) BaP impaired the diving response (distance to bottom in cm) in the adult novel tank, regardless of Cd exposure. (D) 0.3 μM Cd treatment increased baseline (pre-tap) activity (cm/5 s) in the adult tap startle test relative to non-Cd and 0.1 μM Cd treated groups, regardless of BaP exposure. (E) BaP impaired the startle response (change in activity, cm/5 s) in the adult tap startle test, regardless of Cd exposure. (F) BAP increased locomotor activity (cm/min) in the adult shoaling test, regardless of Cd exposure. (G) 0.3 μM Cd enhanced fleeing responses (change in position, in cm) to the slow predator cue (left) in the adult predator avoidance test regardless of BaP exposure. (H) BaP reduced the average distance from the screen (in cm) in the adult predator avoidance test, regardless of Cd exposure. Data expressed as mean ± SEM. Asterisk (*) indicates significance at $p < 0.05$. Ns = Adolescent 19–31/group; Adult 22–36/group.

3. Results

3.1. Experiment 1 - Cadmium and BaP mixtures

The primary results of Exp. 1 are shown in Figs. 1 and 2 and are summarized in Table 1.

3.1.1. Cadmium - BaP larval testing

With the distance moved measure (Fig. 1a), significant main effects of lighting, $F(1, 408) = 45.92$, $p < 0.05$, and BaP treatment, $F(1, 408) = 5.56$, $p < 0.05$, were detected. As typical in this test the fish moved less during the lit condition than in the dark. BAP-treated groups moved less than non-BAP treated groups, regardless of Cd treatment or lighting condition. Time spent moving was also recorded (Fig. 1b). In this analysis similar to the distance moved measure, main effects of lighting, $F(1, 408) = 38.56$, $p < 0.05$, and BaP, $F(1, 408) = 10.40$, $p < 0.05$, were observed. There was also a lighting x Cd x BaP interaction, $F(2, 408) = 3.53$, $p < 0.05$. Under lit conditions (Fig. 1a, left), 0.1 μM and 0.3 μM Cd each differed from their respective mixture (Cd + BaP) ($p < 0.05$), with the mixture group showing reduced activity. No single treatments differed from controls. Under dark conditions (Fig. 1a, center), 0.1 μM Cd, 0.3 μM Cd and 3 μM BaP groups were each hypoactive relative to controls ($p < 0.05$). Based on this interaction, a stimulation index score was evaluated (change in activity due to lighting) (Fig. 1a, right). Both Cd-alone treatment groups showed reduced dark-induced stimulation relative to controls ($p < 0.05$). Additionally, the 0.3 μM CD group showed reduced dark-induced stimulation relative to its respective mixture with BaP ($p < 0.05$).

3.1.2. Cadmium - BAP juvenile testing

For distance moved (Fig. 1c/d), main effects of lighting, $F(1,233) = 162.31$, $p < 0.05$, and Cd treatment, $F(2,233) = 5.56$, $p < 0.05$, were detected, as was an interaction of lighting x BaP, $F(2, 233) = 3.29$, $p < 0.10$. For the Cd effect (Fig. 1c), *post hoc* tests indicated that groups treated with 0.3 μM Cd were hypoactive relative to non-Cd and 0.1 μM Cd treated groups, regardless of BaP treatment ($p < 0.05$). For the lighting x BaP treatment, 3 μM BaP treatment led to hypoactivity under lit (Fig. 1d, left), but not dark (Fig. 1d, right), conditions, regardless of Cd treatment ($p < 0.05$).

For the time spent moving measure (Fig. 1), a main effect of Cd was observed, $F(2, 233) = 5.76$, $p < 0.05$. No interactions reached significance. For the Cd main effects, *post hoc* testing determined that groups

Table 1
Experiment 1: Summary of CD, BAP and Mixture Effects.

Test	Age	Measure	Nature of Cadmium Effect	Nature of Benzo [a] Pyrene Effect	Nature of Interaction
Motility	Larval	Distance Moved		Hypoactive, regardless of lighting	**Light;** BaP reduces mobility, but only when in a mixture with 0.1–0.3 μM Cd. **Dark;** All single treatments reduce mobility. No effect of BaP on Cd treated groups. **Stimulation;** 0.1–0.3 μM Cd impairs dark-induced stimulation. 3 μM BaP attenuates effect of 0.3 μM Cd.
		Time Mobile			
	Juvenile	Distance Moved	Hypoactive (0.3 μM)	Hypoactive in light, but not dark	
		Time Mobile	Reduced mobility (0.3 μM)		
Novel Tank	Adolescent	Distance Moved			BAP single treatment is hyperactive. 1 μM Cd attenuates this effect.
		Distance to Bottom			
	Adult	Distance Moved			
		Distance to Bottom		Impaired diving response	
Tap Startle	Adolescent	Distance Moved		Reduced baseline activity (pre-tap)	
	Adult	Distance Moved	Increased baseline activity (pre-tap, 0.3 μM)	Impaired startle response	
Shoaling	Adult	Distance Moved		Hyperactive	
		Change in position			
Predator	Adult	Distance Moved			
		Change in Position	Hypersensitive to slow cue (0.3 μM)		

For each age, test, and endpoint (rows), the presence of each main effect (CD and/or BaP, in the absence of an interaction) and interactions (Cd x BaP) are noted. A brief summary of those effects is provided for comparison. Summary covers all panels contained in Fig. 1–2.

treated with 0.3 μM Cd were hypoactive relative to both the non-Cd treated and 0.1 μM Cd treated groups, regardless of BaP treatment ($p < 0.05$).

3.1.3. Cadmium - BAP adolescent testing

Within the adolescent novel tank analysis, a Cd x BaP treatment interaction was observed for distance moved, $F(2, 156) = 2.67$, $p < 0.10$ (Fig. 2a). Post-hoc analysis observed that the 3.0uM BaP group was hyperactive relative to Controls, and to its relevant mixture with 0.1 μM Cd ($p < 0.05$). No other pairwise comparisons reached significance. For the diving response (*not shown*), measured as distance from the bottom, no significant main effects or relevant interactions were observed.

Within the adolescent tap startle analysis, a main effect of BaP treatment, $F(1, 138) = 8.81$, $p < 0.05$, was observed on activity prior to the tap stimulus (Fig. 2b), whereby BaP-treated groups generally showed lower baseline activity than non-BaP treated groups, regardless of Cd treatment ($p < 0.05$). For activity after the tap stimulus, no effects of treatment were observed. For startle magnitude, no effects of treatment were observed.

3.1.4. Cadmium - BAP adult testing

Within the adult novel tank analysis, no main effects or interactions were observed for distance moved (*not shown*). For the diving response (Fig. 2c), measured as distance to the bottom, a main effect of BaP treatment was observed, $F(1, 169) = 5.33$, $p < 0.05$, whereby BaP-treated groups generally swam further from the bottom than non-BaP treated groups, regardless of Cd treatment ($p < 0.05$).

Within the adult tap startle analysis, main effects of sex (M > F), $F(1, 180) = 7.17$, $p < 0.05$, and Cd treatment, $F(1, 180) = 4.04$, $p < 0.05$, were observed on activity prior to the tap stimulus (Fig. 2d), whereby 0.3 μM Cd-treated groups showed higher baseline activity than Controls or 0.1 μM Cd-treated groups, regardless of BaP treatment ($p < 0.05$). For activity after the tap stimulus (*not shown*), no effects of treatment were observed. For startle magnitude (Fig. 2e), a main effect of BaP, $F(1, 180) = 6.25$, $p < 0.05$, was observed, whereby BaP-treated groups generally had a lower startle magnitude than non-BAP-treated groups, regardless of Cd treatment ($p < 0.05$).

Within the shoaling test, main effects of time block (x 7), $F(6, 1062) = 4.07$, $p < 0.05$, sex (M > F), $F(1, 177) = 4.22$, $p < 0.05$, and BaP, $F(1, 177) = 4.81$, $p < 0.05$, were detected for locomotor activity (Fig. 2f). *Post hoc* analysis of the BaP effect indicated that groups treated with BaP were hyperactive relative to non-BaP exposed groups ($p < 0.05$), regardless of Cd treatment. No effects were detected for the magnitude of the shoaling response (*not shown*).

Within the predator avoidance test, a main effect of sex (M > F), $F(1, 162) = 12.69$, $p < 0.05$, was detected for locomotor activity, but no effects of treatment reached significance (*not shown*). For the distance from the screen, main effects of cue presence, $F(1, 162) = 21.19$, $p < 0.05$, and BaP, $F(1, 162) = 4.17$, $p < 0.05$, were detected. Additionally, an interaction of cue speed (slow, fast) x cue presence (present, absent) x Cd treatment, $F(2, 162) = 4.18$, $p < 0.05$, was detected. The Cd interaction (Fig. 2g) was then investigated as the fleeing score (cue presence effect, present – absent) by cue speed and treatment. *Post hoc* testing indicated that during the slow cue presentations (Fig. 2g, left), treatment with 0.3 μM Cd led to increased fleeing responses relative to the DMSO control and 0.1 μM Cd groups, regardless of BaP treatment. No differences were detected during the fast cue presentation (Fig. 2g, right). With respect to BaP (Fig. 2h), BaP-treated groups remained closer to the screen on average compared to non-BaP-treated groups ($p < 0.05$), regardless of predator cue presence or absence.

3.2. Experiment 2 - Cadmium and Fluoranthene mixtures

The primary results of Exp. 2 are shown in Figs. 3 and 4 and are summarized in Table 2.

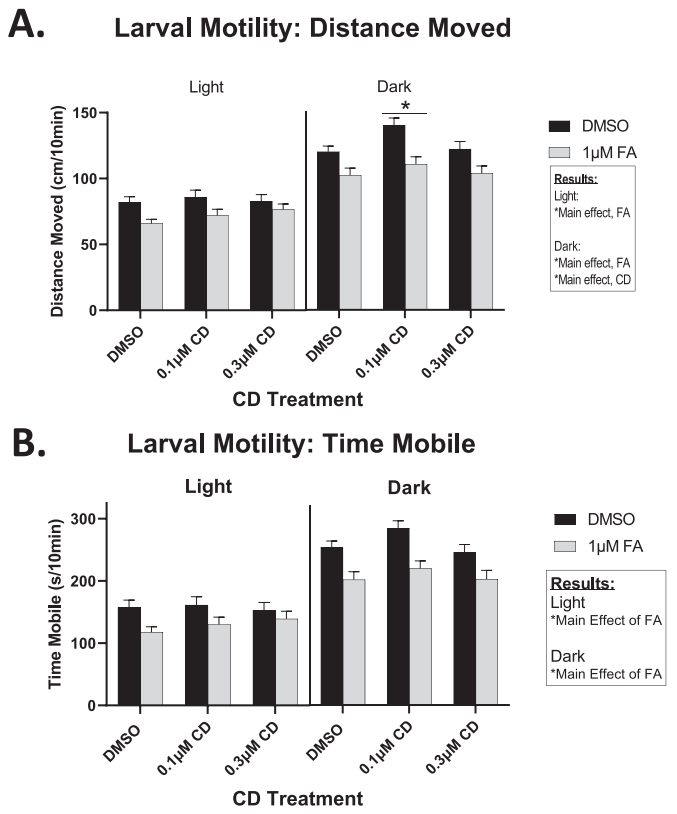


Fig. 3. Experiment 2: Larval Effects of Cd and FA. Locomotor activity was measured as distance moved (cm/10 min), and time mobile (sec/10 min). (A) FA treatment reduced distance moved under both lit (left) and dark (right) conditions, regardless of Cd exposure. 0.1 μM Cd treatment increased distance moved in larvae in the dark (right), but not the light (left), regardless of FA exposure. (B) In the light (left), FA reduced time mobile in larvae under both lit (left) and dark (right) conditions, regardless of Cd exposure. Data expressed as mean ± SEM. Asterisk (*) indicates significance at $p < 0.05$. Ns = Larval 38–44/group.

3.2.1. Cadmium - FA larval testing

Larval motility was measured using two metrics; distance moved per 10 min lighting phase, and mobile time per 10 min lighting phase. For distance moved (Fig. 3a), main effects of lighting, $F(1, 249) = 77.29$, $p < 0.05$, lighting repetition, $F(1, 249) = 6.13$, $p < 0.05$, and FA treatment, $F(1, 249) = 26.63$, $p < 0.05$, were detected, as were interactions of a lighting x CD, $F(2, 249) = 2.66$, $p < 0.10$, and lighting x FA, $F(1, 249) = 4.49$, $p < 0.05$. *Post hoc* investigation of this interaction was conducted separately for the lit and dark phases of the session. Under lit conditions (Fig. 3a, left), FA-treated groups were hypoactive relative to non-FA-treated groups ($p < 0.05$), regardless of Cd treatment. Under dark conditions (Fig. 3a, right), groups treated with 0.1 μM Cd were hyperactive relative to non-Cd-treated groups ($p < 0.05$), regardless of FA treatment. Also, in the dark, FA-treated groups were hypoactive relative to non-FA-treated groups ($p < 0.05$), regardless of Cd treatment.

Parallel to these effects were effects on mobile time (Fig. 3b). In this analysis, main effects of lighting, $F(1, 249) = 64.11$, $p < 0.05$, lighting repetition, $F(1, 249) = 7.67$, $p < 0.05$, and FA, $F(1, 249) = 26.98$, $p < 0.05$, were observed, as was a lighting x FA interaction, $F(1, 249) = 4.76$, $p < 0.05$. *Post hoc* analyses were performed separately for dark and light conditions. FA-treated groups were hypoactive relative to non-FA treated groups, regardless of Cd treatment, under both lit (Fig. 3b, left) and dark conditions (Fig. 3b, right).

3.2.2. Cadmium - FA juvenile testing

For distance moved (*not shown*), a main effect of lighting, $F(1, 208) = 5.58$, $p < 0.05$, was detected, as was a lighting repetition x FA interaction, $F(1, 208) = 5.34$, $p < 0.05$. However, pairwise comparisons between FA and non-FA-treated groups did not reach significance during either the first or second presentations of the lighting cues. For mobile time (*not shown*), no significant effects were detected.

3.2.3. Cadmium - FA adolescent testing

Within the adolescent novel tank analysis, no effects of treatment were detected for distance moved (*not shown*). For the diving response (Fig. 4a), a main effect of FA treatment, $F(1, 186) = 4.04$, $p < 0.05$, was observed. FA-treated groups swam a further distance from the bottom than non-FA treated groups, regardless of Cd treatment ($p < 0.05$).

Within the adolescent tap startle analysis, a main effect of FA treatment, $F(1, 215) = 6.78$, $p < 0.05$, was observed on activity prior to the tap stimulus (Fig. 4b, left), whereby FA-treated groups generally showed lower baseline activity than non-FA treated groups, regardless of Cd treatment ($p < 0.05$). For activity after the tap stimulus (Fig. 4b, right), a main effect of FA treatment, $F(1, 215) = 6.78$, $p < 0.05$, was observed, whereby FA-treated groups generally showed activity than non-FA treated groups, regardless of Cd treatment ($p < 0.05$). For startle magnitude, no effects of treatment were observed.

3.2.4. Cadmium - FA adult testing

Within the adult novel tank analysis, no effects were observed (*not shown*). For the diving response (*not shown*), no effects were observed.

Within the adult tap startle analysis, an interaction of Cd and FA treatment, $F(1, 139) = 4.29$, $p < 0.05$, was observed on activity prior to the tap stimulus (Fig. 4c, left). *Post hoc* analyses observed that the 0.1 μM Cd alone group showed higher baseline activity compared to controls ($p < 0.05$). No other pairwise comparisons reached significance. For activity after the tap stimulus (Fig. 4c, right), a main effect of sex (M > F), $F(1, 139) = 5.64$, $p < 0.05$, and an interaction of Cd and FA treatment were observed, $F(1, 139) = 4.67$, $p < 0.05$. *Post hoc* analyses observed that the 1 μM FA-alone group showed higher post-tap activity compared to controls ($p < 0.05$) and the 0.1 μM Cd + FA mixture group ($p < 0.05$). A similar trend between the 1 μM FA group and the 0.3 μM Cd mixture failed to reach significance ($p = 0.08$). For startle magnitude (Fig. 4d), a main effect of FA was observed, $F(1, 139) = 4.66$, $p < 0.05$, whereby FA-treated groups generally had a higher startle magnitude than non-FA-treated groups, regardless of Cd treatment ($p < 0.05$).

Within the shoaling test, main effects of sex (M > F), $F(1, 137) = 5.36$, $p < 0.05$, and FA, $F(1, 137) = 4.98$, $p < 0.05$, were detected for locomotor activity (Fig. 4e). *Post hoc* analysis of the FA effect indicated that groups treated with FA were hypoactive relative to non-FA exposed groups ($p < 0.05$), regardless of Cd treatment. No effects were detected for the magnitude of the shoaling response (*not shown*).

Within the predator avoidance test, main effects of sex (M > F), $F(1, 148) = 8.60$, $p < 0.05$, and CD, $F(2, 148) = 3.89$, $p < 0.05$, were detected for locomotor activity, as was a Cd x FA interaction, $F(1, 148) = 3.03$, $p < 0.10$ (Fig. 4f). *Post hoc* analyses indicated that the 0.3 μM Cd group was hyperactive relative to controls and the 0.3 μM Cd + FA mixture group ($p < 0.05$). A trend towards hyperactivity in the 1 μM FA group approached but did not reach significance ($p = 0.056$). Additionally, the 1 μM FA group differed from both Cd + FA mixture groups ($p < 0.05$). For the distance from the screen, no effects were detected.

4. Discussion

The present study was performed to assess the additive and/or non-additive effects of developmental exposures to Cd and PAHs on behavioral function in a zebrafish model. This study focused on two concentrations of Cd, 0.1 and 0.3 μM, and two representative PAHs associated with differing mechanisms of action, (3uM) BaP and (1.0) FA. The present data indicated that these compounds partially overlap in their profile of neurobehavioral effects, with some tests impacted by both Cd and PAH treatment and others impacted by only one treatment. When the two treatments impacted the same test, the resulting patterns were

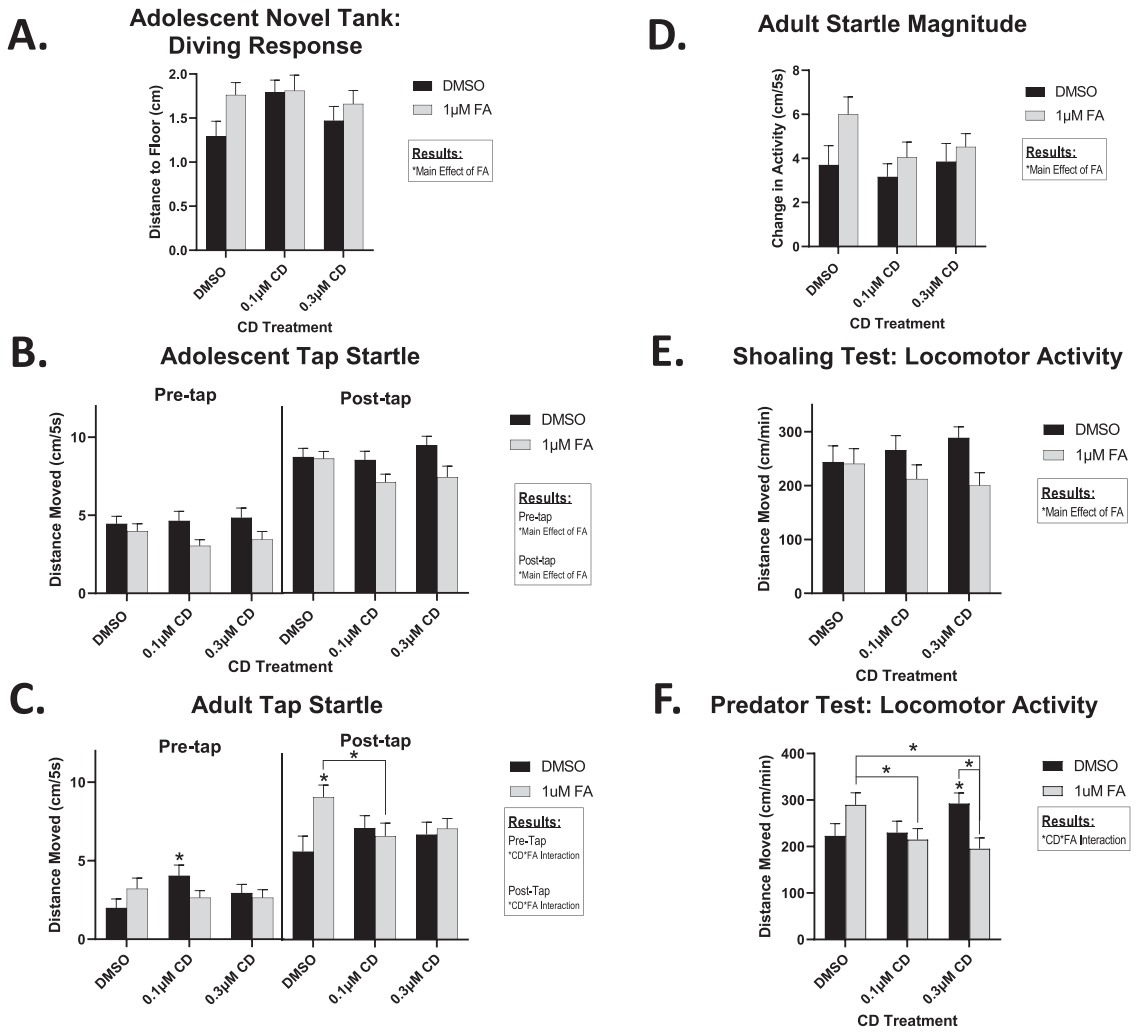


Fig. 4. Experiment 2: Adolescent and Adult Effects of Cd and FA. Figures represent results of the adolescent and adult test batteries. (A) FA treatment impaired the adolescent diving response (distance to bottom in cm) in the novel tank diving test, regardless of Cd exposure. (B) FA exposure reduced baseline (pre-tap) activity (cm/5 s) (left) and post-tap activity (right) in the adolescent tap startle test, regardless of Cd exposure. (C) 0.1 μM Cd enhanced baseline (pre-tap) activity (cm/5 s) (left) in the adult tap startle test when administered alone, but not in a Cd mixture. FA treatment enhanced post-tap activity (right) in the adult tap startle test, but co-exposure to 0.1 μM Cd attenuated this effect. (D) FA exposure increased startle magnitude (change in activity, cm/5/s) in the adult tap startle test, regardless of Cd exposure. (E) FA exposure reduced locomotor activity (cm) in the adult shoaling test, regardless of Cd exposure. (F) FA and 0.3 μM Cd each increased locomotor activity (cm) in the adult predator avoidance test relative to controls. The FA effect was attenuated by co-exposure to either 0.1 μM or 0.3 μM Cd. The 0.3 μM Cd effect was attenuated by co-exposure with FA. Data expressed as mean ± SEM. Asterisk (*) indicates significance at $p < 0.05$. Ns = Adolescent 28–35; Adult 25–28/group.

complex and poorly predicted by the additivity hypothesis. In both experiments, instances were noted where the effect of one toxicant systematically differed based on the presence or absence of the second toxicant. In each of these cases, the mixture effects were sub-additive. In general, these data indicate that CD and PAH interactions can occur, but are impactful on only select endpoints, and at select stages of development. These concentrations were selected to be sub-dysmorphogenic and matched with one another according to tolerability within this specific species. Based on this, the selected concentrations are higher than typical environmental and regulatory levels, but were necessary in order to observe consistent responses across animals and their potential interactions. For example, the US EPA minimum contaminant level (MCL) values for PAHs and Cd are 0.2 μg/L and 5 μg/L respectively, compared to current study levels of 756.9 μg/L BAP, 202.2 μg/L FA, and 18.3–55.0 μg/L Cd. As these concentrations were not selected based on environmental levels and known metal:PAH ratios, this data does not necessarily represent effects and interactions resulting from a specific source and level of human exposure (including from coal waste and wildfire smoke). Rather, its intended purpose is to test the general hypothesis that Cd and PAH co-exposures interactively modulate neurobehavioral outcomes.

Across the present study, it was observed that on a majority of affected outcomes, behavioral deficits could be attributed to a single exposure, regardless of the presence or absence of the second exposure. This was observed even in cases where the two constituent treatments impacted the same test. In Exp. 1, BaP- and 0.3 μM Cd treatment each reduced distance moved metrics in the juvenile motility test, but the lack of a BaP x CD interaction suggests that these effects were additive. Indeed, these effects were also qualitatively different, with BaP effects being lighting-dependent, and Cd effects being lighting independent. Likewise, in the adult tap startle test, BaP and Cd both altered performance but independently and in qualitatively different ways. Cd reduced baseline activity without significantly altering startle magnitude and BaP impaired startle magnitude without significantly altering the pre- and post-tap scores. In Exp. 2, larval locomotor activity was altered in qualitatively different ways by Cd and FA, with Cd increasing distance moved in the dark, and FA suppressing mobile time regardless of lighting condition. Taken together, these data indicate that when CD

Table 2
Experiment 1: Summary of CD, BAP and Mixture Effects.

Test	Age	Measure	Nature of Cadmium Effect	Nature of Fluoranthene Effect	Nature of Interaction
Motility	**Larval**	**Distance Moved**	Hyperactive in the dark (0.1 μM)	Hypoactive under both lit and dark conditions	
		Time Mobile		Reduced mobility under both lit and dark conditions	
	Juvenile	**Distance Moved**			
		Time Mobile			
Novel Tank	**Adolescent**	**Distance Moved**			
		Distance to Bottom		Impaired diving response	
	Adult	**Distance Moved**			
		Distance to Bottom			
Tap Startle	**Adolescent**	**Distance Moved**		Reduced pre- and post-tap activity.	Pre-tap; Increased for 0.1 μM Cd alone.
	Adult	**Distance Moved**		Increased startle magnitude	Post-tap; Increased for FA alone.0.1 μM Cd attenuates this effect.
Shoaling	**Adult**	**Distance Moved**		Reduced locomotor activity	
		Change in position			Increased for 0.3 μM Cd alone. FA attenuates this effect.
Predator	**Adult**	**Distance Moved**			Increased for FA. Both 0.1 μM and 0.3 μM Cd attenuate this effect.
		Change in Position			

For each age, test, and endpoint (rows), the presence of each main effect (Cd and/or FA, in the absence of an interaction) and interactions (Cd x FA) are noted. A brief summary of those effects is provided for comparison. Summary covers all panels contained in Fig. 3–4.

and PAHs alter the same test, they can do so independently, additively, and in qualitatively different ways. While this suggests that co-exposure effects can be statistically and qualitatively parsed out in some cases, this did not apply to all instances where both constituents impacted the data.

A subset of combined Cd and PAH effects resulted in Cd x PAH interactions, indicating non-additive relationships. In Exp. 1, two tests showed such effects. In the larval motility test, multiple non-additive relationships were observed. In the dark, BaP, 0.1 μM Cd and 0.3 μM Cd each significantly reduced mobile time. However, these effects were not seen to interact with one another, meaning that the binary mixtures were equivalent to each of their constituent mixtures. By contrast, under lit conditions, the effect of BaP was facilitated by co-exposure with Cd, meaning that BaP-induced suppression of mobile time was only observed among Cd-treated fish. When taking these patterns together, the stimulation score revealed that Cd impaired dark-induced stimulation, an effect which was attenuated by co-exposure with BAP. Even by itself, this data indicates that mixture effects can be quite complex. The reversal effect of combined exposure was observed in multiple other endpoints across the two experiments. Later in Exp. 1, BaP-induced hyperactivity in the adolescent novel tank test was attenuated by co-exposure to 0.1 μM Cd. In Exp. 2., FA-induced increases in adult post-tap activity were attenuated by co-exposure to 0.1 μM Cd. A similar pattern emerges in the pre-tap component of this test, although this should be interpreted cautiously, as the 0.1 μM Cd group was significantly hyperactive compared to controls, but not its respective mixture group. In the predator avoidance test, FA and 0.3 μM Cd individual exposures both caused hyperactivity, but their co-exposure attenuated both individual effects. Notably, the FA effect was also attenuated by co-exposure with the lower concentration (0.1 μM) of Cd. Taken together, these data indicate that Cd and PAH co-exposures can result in non-additive effects, and the predominant interaction appears to be the reversal of individual toxicant effects. Further, the presence, developmental timing and endpoint-relevance of these interactions may differ significantly between Ahr-agonist and CYP1a1-antagonist PAHs.

The present analysis complements a range of prior studies with toxicant mixtures, particularly those containing PAHs. BaP has been found to reduce accumulation of Cd *in vitro* (Zhang et al., 2019). In zebrafish there is subadditivity of toxicity of the mixture of BaP and Cd (Kodzhahinchev et al., 2021). Within our own work, we have analyzed tobacco smoke constituents and found that in a rat model, a binary nicotine and BaP mixture (Hawkey et al., 2019) generated a range of individual, redundant and reversal effects across a behavioral battery. Other labs have also found similar non-additive relationships relevant to PAHs. In a range of models, particularly fish models, combinations of Ahr- and CYP1a-acting compounds have been found to produce synergistic effects during early development, with further indications that these mechanisms directly interact biologically (Billiard et al., 2008). Notably, the synergism between Ahr and CYP1a1 activity extends beyond relevant classes of PAHs, as noted in fish-based studies of PAHs with polychlorinated biphenyls (Wassenberg and Di Giulio, 2004) and the pesticide chlorpyrifos (Endirlik et al., 2023). However, as the present results did not generally find synergistic relationships between PAH and Cd, the nature of the interactions may be distinct from these other environmental compounds, and more similar to that of nicotine (Hawkey et al., 2019).

In light of growing interest in mixtures and mixture effects, it should be noted that the present data do suggest developmental timing of testing as a key parameter. Our recent work in the zebrafish has increasingly added longitudinal testing and observed that when the same test is performed at multiple ages, the effect of a given toxicant can radically change (Boyda et al., 2021; Hawkey et al., 2019; Hawkey et al., 2022; Hawkey et al., 2023a, 2023b). The present study added an additional testing point to this array, an adapted version of the classic larval motility test. When comparing Cd, PAH, or co-exposures, effects generally differed from one time point to the next, in subtle or striking ways depending on the test. This should not, perhaps, be surprising given that neural circuit development goes through a series of stages

between birth and adulthood (Edde et al., 2021), but this does provide necessary context for current and future zebrafish screens which are heavily reliant on very early time points. Within the present study, larval data suggest that non-additive interactions are a significant feature of Cd-BaP mixtures, but not Cd-FA mixtures. When viewing that data with additional time points and tests, a more accurate conclusion would be that Cd-BaP interactions are more relevant within early and mid-maturation stages of development, and that Cd-FA interactions are more relevant in adulthood. Future studies elaborating on the potential for developmental processes to diminish or facilitate neurobehavioral effects will be needed so that early, late and longitudinal data can be integrated and used to guide risk management.

The present study presents a unique analysis of mixture effects generated by Cd, two representative PAHs, and mixtures thereof, with relevance to neurobehavioral function. The effects and interactions of these compounds were found to be highly specific, with certain tests and endpoints detecting non-additive interactions, and others detecting only additive or single-compound effects. The interactions observed were generally in agreement that Cd and PAHs have can produce sub-additive effects to the point of reversing a single-compound effect outright. Future studies will be needed to determine how to best understand and apply these findings to mixture modelling and risk management for mixtures. However, this work indicates that laboratory studies with zebrafish can be used to advance this work, and to further observe core characteristics of toxicant interactions, including their neurobehavioral specificity and developmental impact.

Author contributions

AS contributed to data collection and management, data analysis, and preparation of the manuscript. ABH participated in protocol design, data collection and management, data analysis and preparation/editing of the manuscript. AG contributed to data collection and management and to editing of the manuscript. SN contributed to data collection and management and to editing of the manuscript. MM contributed to data collection and management and to editing of the manuscript. EDL contributed to project and protocol design, data analysis and preparation/editing of the manuscript.

CRediT authorship contribution statement

Alexandra Stickler: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Andrew B. Hawkey:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Anas Gondal:** Writing – review & editing, Methodology, Investigation. **Sarabesh Natarajan:** Writing – review & editing, Methodology, Investigation. **Mikayla Mead:** Writing – review & editing, Methodology, Investigation. **Edward D. Levin:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interests for this article.

Data availability

Data will be made available on request.

Acknowledgement

This research was supported by the Duke University Superfund Research Center ES010356.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ntt.2024.107339.

References

Abd El Naby, W.S.H., Zong, C., Fergany, A., Ekuban, F.A., Ahmed, S., Reda, Y., Sato, H., Ichihara, S., Kubota, N., Yanagita, S., Ichihara, G., 2023. Exposure to benzo [a] pyrene decreases noradrenergic and serotonergic axons in Hippocampus of mouse brain. Int. J. Mol. Sci. 24 (12).

Azzolina, N.A., Kreitinger, J.P., Skorobogatov, Y., Shaw, R.K., 2016. Background concentrations of PAHs and metals in surface and subsurface soils collected throughout Manhattan, New York. Environ. Forensic 17 (4), 294–310.

Balali-Mood, M., Naseri, K., Tahergorabi, Z., Khazdair, M.R., Sadeghi, M., 2021. Toxic mechanisms of five heavy metals: mercury, lead, chromium, cadmium, and arsenic. Front. Pharmacol. 12, 227.

Bauer, J.A., Fruh, V., Howe, C.G., White, R.F., Claus Henn, B., 2020. Associations of metals and neurodevelopment: a review of recent evidence on susceptibility factors. Curr. Epidemiol. Rep. 7, 237–262.

Bensoussan, H., Grancolas, L., Dhieux-Lestaevel, B., Delissen, O., Vacher, C.M., Dublineau, I., Voisin, P., Gourmelon, P., Taouis, M., Lestaevel, P., 2009. Heavy metal uranium affects the brain cholinergic system in rat following sub-chronic and chronic exposure. Toxicology 261 (1–2), 59–67.

Billiard, S.M., Meyer, J.N., Wassenberg, D.M., Hodson, P.V., Di Giulio, R.T., 2008. Nonadditive effects of PAHs on early vertebrate development: mechanisms and implications for risk assessment. Toxicol. Sci. 105 (1), 5–23.

Boaggio, K., LeDuc, S.D., Rice, R.B., Duffney, P.F., Foley, K.M., Holder, A.L., McDow, S., Weaver, C.P., 2022. Beyond particulate matter mass: heightened levels of lead and other pollutants associated with destructive fire events in California. Environ. Sci. Technol. 56 (20), 14272–14283.

Boyda, J., Hawkey, A.B., Holloway, Z.R., Trevisan, R., Di Giulio, R.T., Levin, E.D., 2021. The organophosphate insecticide diazinon and aging: neurobehavioral and mitochondrial effects in zebrafish exposed as embryos or during aging. Neurotoxicol. Teratol. 87.

Brown, D.R., Clark, B.W., Garner, L.V., Di Giulio, R.T., 2015. Zebrafish cardiotoxicity: the effects of CYP1A inhibition and AHR2 knockdown following exposure to weak aryl hydrocarbon receptor agonists. Environ. Sci. Pollut. Res. 22, 8329–8338.

Cao, Z., Shafer, T.J., Crofton, K.M., Gennings, C., Murray, T.F., 2011. Additivity of pyrethroid actions on sodium influx in cerebrocortical neurons in primary culture. Environ. Health Perspect. 119 (9), 1239–1246.

Chandravanshi, L., Shiv, K., Kumar, S., 2021. Developmental toxicity of cadmium in infants and children: a review. Environ. Anal. Health Toxicol. 36 (1).

Chen, C., Tang, Y., Jiang, X., Qi, Y., Cheng, S., Qiu, C., Peng, B., Tu, B., 2012. Early postnatal benzo (a) pyrene exposure in Sprague-Dawley rats causes persistent neurobehavioral impairments that emerge postnatally and continue into adolescence and adulthood. Toxicol. Sci. 125 (3), 248–261.

Chow, E.S.H., Hui, M.N.Y., Lin, C.C., Cheng, S.H., 2008. Cadmium inhibits neurogenesis in zebrafish embryonic brain development. Aquat. Toxicol. 87 (3), 157–169.

Das, D.N., Panda, P.K., Naik, P.P., Mukhopadhyay, S., Sinha, N., Bhutia, S.K., 2017. Phytotherapeutic approach: a new hope for polycyclic aromatic hydrocarbons induced cellular disorders, autophagic and apoptotic cell death. Toxicol. Mech. Methods 27 (1), 1–17.

Dou, C., Zhang, J., 2011. Effects of lead on neurogenesis during zebrafish embryonic brain development. J. Hazard. Mater. 194, 277–282.

Edde, M., Leroux, G., Altena, E., Chanraud, S., 2021. Functional brain connectivity changes across the human life span: from fetal development to old age. J. Neurosci. Res. 99 (1), 236–262.

Endirlik, B.Ü., Wincent, E., Dreij, K., 2023. Non-additive mixture effects of benzo [a] pyrene and pesticides in vitro and in vivo: role of AhR signaling. Environ. Pollut. 316, 120510.

Fu, C., Li, Y., Xi, H., Niu, Z., Chen, N., Wang, R., Yan, Y., Gan, X., Wang, M., Zhang, W., Zhang, Y., 2022. Benzo (a) pyrene and cardiovascular diseases: an overview of pre-clinical studies focused on the underlying molecular mechanism. Frontiers in Nutrition 9.

Gao, D., Wang, C., Xi, Z., Zhou, Y., Wang, Y., Zuo, Z., 2017. Early-life benzo[a]pyrene exposure causes neurodegenerative syndromes in adult zebrafish (Danio rerio) and the mechanism involved. Toxicol. Sci. 157 (1), 74–84.

Geier, M.C., Chlebowski, A.C., Truong, L., Massey Simonich, S.L., Anderson, K.A., Tanguay, R.L., 2018. Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. Arch. Toxicol. 92, 571–586.

Han, J., Liu, K., Wang, R., Zhang, Y., Zhou, B., 2019. Exposure to cadmium causes inhibition of otolith development and behavioral impairment in zebrafish larvae. Aquat. Toxicol. 214, 105236.

Hawkey, A., Junaid, S., Yao, L., Spiera, Z., White, H., Cauley, M., Levin, E.D., 2019. Gestational exposure to nicotine and/or benzo [a] pyrene causes long-lasting neurobehavioral consequences. Birth Defects Res. 111 (17), 1248–1258.

Hawkey, A.B., Piatos, P., Holloway, Z., Boyda, J., Koburov, R., Fleming, E., Di Giulio, R. T., Levin, E.D., 2022. Embryonic exposure to benzo[a]pyrene causes age-dependent behavioral alterations and long-term metabolic dysfunction in zebrafish. Neurotoxicol. Teratol. 93, 107121.

Hawkey, A.B., Mead, M., Natarajan, S., Gondal, A., Jarrett, O., Levin, E.D., 2023a. Embryonic exposure to PFAS causes long-term, compound-specific behavioral alterations in zebrafish. Neurotoxicol. Teratol. 97, 107165.

Hawkey, A.B., Unal, D., Holloway, Z.R., Levin, E.D., 2023b. Developmental exposure of zebrafish to neonicotinoid pesticides: long-term effects on neurobehavioral function. Neurotoxicology 96, 240–253.
Holloway, Z., Hawkey, A., Asrat, H., Boinapally, N., Levin, E.D., 2021. The use of tocofersolan as a rescue agent in larval zebrafish exposed to benzo[a]pyrene in early development. Neurotoxicology 86, 78–84.
Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496 (7446), 498–503.
Jarvis, I.W., Dreij, K., Mattsson, Å., Jernström, B., Stenius, U., 2014. Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment. Toxicology 321, 27–39.
Jedrychowski, W.A., Perera, F.P., Camann, D., Spengler, J., Butscher, M., Mroz, E., Majewska, R., Flak, E., Jacek, R., Sowa, A., 2015. Prenatal exposure to polycyclic aromatic hydrocarbons and cognitive dysfunction in children. Environ. Sci. Pollut. Res. 22, 3631–3639.
Kamel, M.M., Abd El-Razek, A.H., 2011. Perinatal exposure to cadmium affects neurobehavioural development and anxiety-like behaviour in rat offspring. Life Sciences Journal 8, 529–536.
Kamila, W., Wioletta, R.K., Krzysztof, L., Karolina, K., Grzegorz, M., 2018. Health risk impacts of exposure to airborne metals and benzo (a) pyrene during episodes of high PM10 concentrations in Poland. Biomed. Environ. Sci. 31 (1), 23–36.
Kamo, M., Yokomizo, H., 2015. Explanation of non-additive effects in mixtures of similar mode of action chemicals, 335, pp. 20–26.
Karri, V., Schuhmacher, M., Kumar, V., 2016. Heavy metals (Pb, cd, as and MeHg) as risk factors for cognitive dysfunction: a general review of metal mixture mechanism in brain. Environ. Toxicol. Pharmacol. 48, 203–213.
Knecht, A.L., Truong, L., Marvel, S.W., Reif, D.M., Garcia, A., Lu, C., Simonich, M.T., Teeguarden, J.G., Tanguay, R.L., 2017a. Transgenerational inheritance of neurobehavioral and physiological deficits from developmental exposure to benzo [a]pyrene in zebrafish. Toxicol. Appl. Pharmacol. 329, 148–157.
Knecht, A.L., Truong, L., Simonich, M.T., Tanguay, R.L., 2017b. Developmental benzo[a] pyrene (B[a]P) exposure impacts larval behavior and impairs adult learning in zebrafish. Neurotoxicol. Teratol. 59, 27–34.
Kodzhahinchev, V., Shekh, K., Weber, L.P., Niyogi, S., 2021. Interactive effects of cadmium and Benzo[a]pyrene in adult zebrafish (Danio rerio) during short-term aqueous co-exposure. Environ. Poll.(Barking, Essex : 1987) 272, 116027.
Lamtai, M., Azirar, S., Zghari, O., Ouakki, S., El Hessni, A., Mesfioui, A., Ouichou, A., 2021. Melatonin ameliorates cadmium-induced affective and cognitive impairments and hippocampal oxidative stress in rat. Biol. Trace Elem. Res. 199, 1445–1455.
Lau, S.L., Han, Y., Kang, J.H., Kayhanian, M., Stenstrom, M.K., 2009. Characteristics of highway stormwater runoff in Los Angeles: metals and polycyclic aromatic hydrocarbons. Water Environ. Res. 81 (3), 308–318.
Le Bihanic, F., Clérandeau, C., Le Menach, K., Morin, B., Budzinski, H., Cousin, X., Cachot, J., 2014. Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in Japanese medaka. Environ. Sci. Pollut. Res. 21, 13732–13743.
LeFauve, M.K., Connaughton, V.P., 2017. Developmental exposure to heavy metals alters visually-guided behaviors in zebrafish. Curr. Zool. 63 (2), 221–227.
Leret, M.L., Garcia-Uceda, F., Antonio, M.T., 2002. Effects of maternal lead administration on monoaminergic, GABAergic and glutamatergic systems. Brain Res. Bull. 58 (5), 469–473.
Liao, G., Wang, P., Zhu, J., Weng, X., Lin, S., Huang, J., Xu, Y., Zhou, F., Zhang, H., Tse, L. A., Zou, F., 2021. Joint toxicity of lead and cadmium on the behavior of zebrafish larvae: an antagonism. Aquat. Toxicol. 238, 105912.
Lu, J., Shang, X., Zhong, W., Xu, Y., Shi, R., Wang, X., 2020. New insights of CYP1A in endogenous metabolism: a focus on single nucleotide polymorphisms and diseases. Acta Pharm. Sin. B 10 (1), 91–104.
Malott, K.F., Luderer, U., 2023. 30 - the effects of polycyclic aromatic hydrocarbons on mitochondria. Mitochondrial Intoxication 663–681.
Marchetti, C., Gavazzo, P., 2005. NMDA receptors as targets of heavy metal interaction and toxicity. Neurotox. Res. 8, 245–258.
McCallister, M.M., Maguire, M., Ramesh, A., Aimin, Q., Liu, S., Khoshbouei, H., Aschner, M., Ebner, F.F., Hood, D.B., 2008. Prenatal exposure to benzo (a) pyrene impairs later-life cortical neuronal function. Neurotoxicology 29 (5), 846–854.
Meyer, J.S., Ranville, J.F., Pontasch, M., Gorsuch, J.W., Adams, W.J., 2015. Acute toxicity of binary and ternary mixtures of cd, cu, and Zn to Daphnia magna. Environ. Toxicol. Chem. 34 (4), 799–808.
Morillo, E., Romero, A.S., Madrid, L., Villaverde, J., Maqueda, C., 2008. Characterization and sources of PAHs and potentially toxic metals in urban environments of Sevilla (southern Spain). Water Air Soil Pollut. 187, 41–51.
Nagaraju, R., Kalahasthi, R., Balachandar, R., Bagepally, B.S., 2022. Cadmium exposure and DNA damage (genotoxicity): a systematic review and meta-analysis. Crit. Rev. Toxicol. 52 (10), 786–798.
Rezaei Kalantary, R., Jaffarzadeh, N., Rezapour, M., Hesami Arani, M., 2020. Association between exposure to polycyclic aromatic hydrocarbons and attention deficit hyperactivity disorder in children: a systematic review and meta-analysis. Environ. Sci. Pollut. Res. 27, 11531–11540.
Richter, P., Steven, P.R., Bravo, R., Lisko, J.G., Damian, M., Gonzalez-Jimenez, N., Gray, N., Keong, L.M., Kimbrell, J.B., Kuklenyik, P., Lawler, T.S., 2016. Characterization of SPECTRUM variable nicotine research cigarettes. Tob. Regul. Sci. 2 (2).
Rider, C.V., Dinse, G.E., Umbach, D.M., Simmons, J.E., Hertzberg, R.C., 2018. Predicting mixture toxicity with models of additivity. Chem. Mixt. Comb. Chem. Nonchem. Stress. 235–270.
Sadiq, S., Ghazala, Z., Chowdhury, A., Büsselberg, D., 2012. Metal toxicity at the synapse: presynaptic, postsynaptic, and long-term effects. J.Toxicol. 2012, 1–42.
Salehi, I., Karamian, R., Komaki, A., Tahmasebi, L., Taheri, M., Nazari, M., Shahidi, S., Sarihi, A., 2015. Effects of vitamin E on lead-induced impairments in hippocampal synaptic plasticity. Brain Res. 1629, 270–281.
Sarigiannis, D.A., Hansen, U., 2012. Considering the cumulative risk of mixtures of chemicals – a challenge for policy makers. Environ. Health 11.
Sarver, E., Keles, C., Rezaee, M., 2019. Beyond conventional metrics: comprehensive characterization of respirable coal mine dust. Int. J. Coal Geol. 207, 84–95.
Sfakianakis, D.G., Renieri, E., Kentouri, M., Tsatsakis, A.M., 2015. Effect of heavy metals on fish larvae deformities: a review. Environ. Res. 137, 246–255.
Shams, S., Rihel, J., Ortiz, J.G., Gerlai, R., 2018. The zebrafish as a promising tool for modeling human brain disorders: a review based upon an IBNS symposium. Neurosci. Biobehav. Rev. 85, 176–190.
Shankar, P., Geier, M.C., Truong, L., McClure, R.S., Pande, P., Waters, K.M., Tanguay, R. L., 2019. Coupling genome-wide transcriptomics and developmental toxicity profiles in zebrafish to characterize polycyclic aromatic hydrocarbon (PAH) hazard. Int. J. Mol. Sci. 20 (10), 2570.
Shankar, P., Dashner-Titus, E.J., Truong, L., Hayward, K., Hudson, L.G., Tanguay, R.L., 2021. Developmental toxicity in zebrafish (Danio rerio) exposed to uranium: a comparison with lead, cadmium, and iron. Environ. Pollut. 269, 116097.
Singh, D.P., Gadi, R., Mandal, T.K., 2011. Characterization of particulate-bound polycyclic aromatic hydrocarbons and trace metals composition of urban air in Delhi, India. Atmos. Environ. 45 (40), 7653–7663.
Slotkin, T.A., Skavicus, S., Card, J., Di Giulio, R.T., Seidler, F.J., 2017. In vitro models reveal differences in the developmental neurotoxicity of an environmental polycylic aromatic hydrocarbon mixture compared to benzo [a] pyrene: Neuronotypic PC12 cells and embryonic neural stem cells. Toxicology 377, 49–56.
Snedecor, G.W., Cochran, W.G., 1967. Statistical Methods. Iowa State University Press, Ames, IA, USA.
Sprowles, J.L., Amos-Kroohs, R.M., Braun, A.A., Sugimoto, C., Vorhees, C.V., Williams, M.T., 2018. Developmental manganese, lead, and barren cage exposure have adverse long-term neurocognitive, behavioral and monoamine effects in Sprague-Dawley rats. Neurotoxicol. Teratol. 67, 50–64.
Verma, S.K., Masto, R.E., Gautam, S., Choudhury, D.P., Ram, L.C., Maiti, S.K., Maity, S., 2015. Investigations on PAHs and trace elements in coal and its combustion residues from a power plant. Fuel 162, 138–147.
Volkoff, S.J., Osterberg, J.S., Jayasundara, N., Cooper, E., Hsu-Kim, H., Rogers, L., Gehrke, G.E., Jayaraman, S., Di Giulio, R.T., 2019. Embryonic Fundulus heteroclitus responses to sediment extracts from differentially contaminated sites in the Elizabeth River, VA. Ecotoxicology 28, 1126–1135.
Vondracek, J., Umannova, L., Machala, M., 2011. Interactions of the aryl hydrocarbon receptor with inflammatory mediators: beyond CYP1A regulation. Curr. Drug Metab. 12 (2), 89–103.
Wang, G., Mielke, H.W., Quach, V.A.N., Gonzales, C., Zhang, Q., 2004. Determination of polycyclic aromatic hydrocarbons and trace metals in New Orleans soils and sediments. Soil Sediment Contam. 13 (3), 313–327.
Wassenberg, D.M., Di Giulio, R.T., 2004. Synergistic embryotoxicity of polycyclic aromatic hydrocarbon aryl hydrocarbon receptor agonists with cytochrome P4501A inhibitors in Fundulus heteroclitus. Environ. Health Perspect. 112 (17), 1658–1664.
Xu, Y., Zhao, H., Wang, Z., Gao, H., Liu, J., Li, K., Song, Z., Yuan, C., Lan, X., Pan, C., Zhang, S., 2022. Developmental exposure to environmental levels of cadmium induces neurotoxicity and activates microglia in zebrafish larvae: from the perspectives of neurobehavior and neuroimaging. Chemosphere 291, 132802.
Zhang, W., Tian, F., Zheng, J., Li, S., Qiang, M., 2016. Chronic administration of benzo (a) pyrene induces memory impairment and anxiety-like behavior and increases of NR2B DNA methylation. PLoS One 11 (2), e0149574.
Zhang, L., Zhou, L., Han, L., Zhao, C., Norton, J.M., Li, H., Hu, F., Xu, L., 2019. Benzo(a) pyrene inhibits the accumulation and toxicity of cadmium in subcellular fractions of Eisenia fetida. Chemosphere 219, 740–747.
Zhang, Y., Du, L., Yan, J., Bai, Q., Niu, Q., Mo, Y., Zhang, Q., Nie, J., 2022. Prenatal benzo [a] pyrene exposure impairs hippocampal synaptic plasticity and cognitive function in SD rat offspring during adolescence and adulthood via HDAC2-mediated histone deacetylation. Ecotoxicol. Environ. Saf. 246, 114180.
Zhao, Z.H., Zheng, G., Wang, T., Du, K.J., Han, X., Luo, W.J., Shen, X.F. Chen, J.Y., 2018. Low-level gestational lead exposure alters dendritic spine plasticity in the hippocampus and reduces learning and memory in rats. Sci. Rep., 8(1), 3533, 2018.
Zhen, H., Zhang, F., Cheng, H., Hu, F., Jia, Y., Hou, Y., Shang, M., Yu, H., Jiang, M., 2023. Association of polycyclic aromatic hydrocarbons exposure with child neurodevelopment and adult emotional disorders: a meta-analysis study. Ecotoxicol. Environ. Saf. 255, 114770.