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Assessment of unique behavioral, morphological, and molecular alterations in the comparative developmental toxicity profiles of PFOA, PFHxA, and PFBA using the zebrafish model system

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Abstract

Perfluoroalkyl substances (PFAS) are a class of synthetic chemicals that are persistent in the environment. Due to adverse health outcomes associated with longer chain PFAS, shorter chain chemicals were used as replacements, but developmental toxicity assessments of the shorter chain chemicals are limited. Toxicity of three perfluoroalkyl acids (PFAAs) [perfluorooctanoic acid (PFOA), composed of 8 carbon (C8), perfluorohexanoic acid (PFHxA, C6), and perfluorobutanoic acid (PFBA, C4)] was compared in developing zebrafish (*Danio* rerio). LC_{50}s at 120 h post fertilization (hpf) assessed potency of each PFAA by exposing developing zebrafish (1–120 hpf) to range of concentrations. Zebrafish were then exposed to sublethal concentrations (0.4–4000 ppb, μg/L) throughout embryogenesis (1–72 hpf). Effects of the embryonic exposure on locomotor activities was completed with the visual motor response test at 120 hpf. At 72 hpf, morphological changes (total body length, head length, head width) and transcriptome profiles to compare altered molecular and disease pathways were determined. The LC_{50} ranking followed trend as expected based on chain length. PFOA caused hyperactivity and PFBA hypoactivity, while PFHxA did not change behavior. PFOA, PFHxA, and PFBA caused morphological and transcriptomic alterations that were unique for each chemical and were concentration-dependent indicating different toxicity mechanisms. Cancer was a top disease for PFOA and FXR/RXR activation was a top canonical pathway for PFBA. Furthermore, comparison of altered biological and molecular pathways in zebrafish exposed to PFOA matched findings reported in prior epidemiological studies and other animal models, supporting the predictive value of the transcriptome approach and for predicting adverse health outcomes associated with PFHxA or PFBA exposure.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107642.

Keywords

Behavior; Development; Perfluoroalkyl; PFAS; Transcriptomic; Zebrafish

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of man-made chemicals that have been used since the 1940s in a wide range of consumer and industrial products (OECD, 2018). These substances are composed of alkyl chains of partially or fully fluorinated carbons. Although this chemical structure provides these chemicals some unique characteristics (e.g., low surface tension, hydrophobicity, and lipophobicity) the strength of the carbon-fluoride bond leads to their persistence in the environment. PFAS are resistant to degradation by oxidizing agents, strong acids, alkalis, and photolysis (Wang et al., 2015). Some PFAS are biodegradable, but their biodegradation leads to the formation of other PFAS that are stable in the environment or accumulate in biological organisms (Giesy and Kannan, 2002; Nilsson et al., 2013).

The most studied PFAS are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS). Epidemiological studies suggest association between PFOA and PFOS exposure and many adverse health effects such as pregnancy-induced hypertension, increased serum hepatic enzymes, increased lipids, decreased immune response, and decreased birth weight (Fenton et al., 2021; ATSDR, 2021). The serum concentrations of PFOA and PFOS in PFAS workers ranged between 423 and 1000 ng/mL and 960–1400 ng/mL, respectively (ATSDR, 2021). In 2002, 3 M, the main manufacturer of PFOA and PFOS announced voluntary phase out in the United States, but based on the persistence of PFOA, PFOS, and other longer chain PFAS these substances remain in the environment presenting continual risk of exposure. Moreover, these chemicals are still used in other countries (EPA, 2020). Recently, the United States Environmental Protection Agency (US EPA) proposed regulations to control the entry of imported products containing long chain PFAS [≥ 6 perfluorocarbons which translates to perfluoroalkyl carboxylic acids (PFCAs) with alkyl chains of ≥ 7 carbons and perfluoroalkyl sulfonic acids (PFSAs) with alkyl chains of ≥ 6 carbons] (EPA, 2017).

Alternatives were introduced into industry to replace the use of these longer chain PFAS. Those alternatives include PFAS with chemical modifications to their alkyl chain where one or more oxygen atoms have been inserted leading to perfluoroalkyl ether carboxylic acids (PFECAs). These ether alternatives have lower bioaccumulation potential compared to legacy PFAS (ATSDR, 2021). Unfortunately, the next generation are also stable and persist in the environment similar to the legacy PFAS. Thus, there is a concern that these PFAS alternatives can bioaccumulate leading to toxicity; however, current toxicity data are too limited to know if they indeed represent safer alternatives in regards to both human and ecological health (Wang et al., 2015).

Additional alternatives include shorter chain PFAS that are more water soluble and partition less to solids; thus, these shorter chain PFAS have higher mobility in the environment compared to PFOA and PFOS (Wang et al., 2015). Additionally, shorter chain PFAS may

bioaccumulate but in different tissues than PFOA or PFOS (Ghisi et al., 2019). For example, in plants the shorter chain compounds (C4-C6) tend to accumulate in leaves and fruits, while PFOA and PFOS tend to accumulate in plant roots. As such, there is an increased risk of exposure through diet for these shorter chain PFAS. Furthermore, a study showed that perfluorobutanoic acid (PFBA, C4) was detected in higher levels in water, soil, and produce in several cities in Minnesota, USA compared to the longer chain compounds (e.g., PFOA and PFOS) (Scher et al., 2018). In this study, the maximum PFBA concentrations detected were 2.5 μg/L in water, 33,000 μg/kg in produce, and 13 μg/kg in soil; while the maximum PFOA concentrations were 0.12 μg/L in water, 0.26 μg/kg in produce, and 3 μg/kg in soil. Interestingly, this study was completed six years after installation of carbon filter systems in the municipal water treatment plant or in water treatment units in homes that used private wells to remove these substances (Scher et al., 2018). Altogether, the alterative shorter chain PFAS have shorter half lives in organisms [e.g., the half-life of PFBA is estimated to be 0.14 years in human serum compared to 1.7 years for PFOA (Xu et al., 2020)] but are still being detected in the environment presenting risk of continual exposure through contaminated water or food sources. Therefore, thorough toxicity investigations into the safety of the shorter chain alterative PFAS are needed especially during development, a susceptible life stage.

Developmental toxicity is one of the main concerns regarding PFAS exposure. PFOA and PFOS have been detected in the serum of pregnant women, in cord blood, and in breast milk, indicating risk for in utero and early developmental PFAS exposure (Kim et al., 2011; Barbarossa et al., 2013). Specifically, the median level of PFOA was 0.46 ng/mL in serum of pregnant women, 1.15 ng/mL in cord blood of infants, and 0.05 ng/mL in breast milk. While, the median level of PFOS was 2.93 ng/mL in serum of pregnant women, 1.26 ng/mL in cord blood of infants, and 0.06 ng/mL in breast milk (Kim et al., 2011; Barbarossa et al., 2013). Animal studies showed that developmental PFAS exposure results in many adverse health effects including a reduction in neonate weight, delays in eye opening [low observed adverse effect level (LOAEL) in rodents for oral PFOA exposure is 3 mg/kg/day], altered bone development (LOAEL for oral PFOA exposure is 0.3 mg/kg/day in rodents), delays in mammary gland differentiation (LOAEL for oral PFOA exposure is 0.0024–0.3 mg/kg/day in rodents), and neurodevelopmental alterations (LOAEL for oral PFOA exposure is 0.1 mg/kg/day in rodents) (ATSDR, 2021; Vuong et al., 2021). Prenatal exposure to PFOS or perfluorononanoic acid (PFNA) was associated with measures related to hyperactive-impulsive type attention deficient/hyperactivity disorder (ADHD); however, PFOA prenatal exposure wasn't associated with childhood neurobehavioral alterations (Barbarossa et al., 2013). Overall, there is a need to increase our knowledge on the developmental toxicity of the shorter chain PFAS replacements compared to what is known for PFOA and PFOS. To this end, the zebrafish presents as a complementary vertebrate model to investigate developmental toxicity outcomes. Strengths of the zebrafish model include ease in husbandry, high fecundity, and ex vivo development. In addition, the great similarity in gene function through vertebrate evolution (i.e., 82% similarity in disease-related genes) has secured their widespread use in developmental biology and toxicology studies allowing for translation of results observed in zebrafish to human health (Howe et al., 2013). Additionally in recent years, there has been an expansion of behavioral assays

in the zebrafish resulting in application of this animal model in neuropharmacology and neurotoxicity research (Basnet et al., 2019; Irons et al., 2010; de Esch et al., 2012).

In this study, we aimed to compare the developmental toxicity of legacy PFAS exposure, represented by PFOA, to the shorter chain PFAS, represented by perfluorohexanoic acid (PFHxA) and perfluorobutanoic acid (PFBA), using the zebrafish. The US EPA is in the process of conducting an IRIS toxicological review for PFHxA (U.S. EPA, 2022a) and PFBA (U.S. EPA, 2021) but currently there are no federal regulatory levels for PFAS in drinking water (i.e., no Maximum Contaminant Level, MCL). In June 2022, the US EPA released an interim health advisory level for PFOA exposure at 0.004 parts per trillion (ppt, ng/L) in drinking water (U.S. EPA, 2022b) However, some states adopted more strict policies due to high concerns of PFAS. For example, Michigan Department of Environment, Great Lakes, and Energy (EGLE) has set regulatory limits for seven PFAS, including PFOA at 8 ppt and PFHxA at 400,000 ppt (or 400 parts per billion, ppb, μg/L) (EGLE, 2020). Globally, the European Chemicals Agency (ECHA) proposed restricted use of PFHxA in textile, food-contact product, and firefighting foam due to its mobility and persistence in the environment (ECHA, 2019). Given limited developmental toxicity information, a comparative toxicity study was completed to first compare lethality. Next, an assessment of additional outcomes at a wide range of sublethal concentrations including behavioral, morphological and growth, and transcriptome alterations were completed to address observed similarities and differences for the different chain lengths of PFOA, PFHxA, and PFBA. The study hypothesized that greater lethality, along with more significant impacts to behavior, morphology, and molecular alterations would be observed with increased chain length. In addition, it was hypothesized that perturbed biological and disease pathways for PFOA would align with adverse health outcomes reported in epidemiological and animal studies such as cancer, thyroid alterations, changes in liver function, altered cholesterol pathways, and immunotoxicity.

2. Materials and methods

2.1. Zebrafish husbandry

Zebrafish (*Danio rerio*) of the wild-type AB strain were housed in a Z-Mod system (Aquatic Habitats/Pentair, Apopka, FL) on a 14:10 light–dark cycle. Water was maintained at 28 ± 1 °C, pH at 7.0–7.2, and conductivity at 550 μS/cm. Water quality was assessed twice a day. Fish were fed a mixture of brine shrimp (Artemia International LLC., Fairview, Texas), Golden Pearls 500–800 μm (Artemia International LLC., Fairview, Texas), and Zeigler adult zebrafish food (Zeigler Bros Inc., Gardners, PA). Adult fish were bred in spawning tanks according to established protocols (Peterson et al., 2011; Peterson et al., 2013). Embryos were collected within 1 h post fertilization (hpf). The embryos were rinsed with embryo water (filtered reverse osmosis water of pH 7.2 and conductivity at 550 μS/cm) and randomly distributed in groups of 50 embryos into 100 mm × 20 mm polystyrene Petri dishes for each treatment group within each biological replicate. All fish used in experiments were incubated at 28 °C through 72 or 120 hpf. Protocols were approved by the Purdue University Animal Care and Use Committee (PACUC protocol # 1110000088) and all fish treated humanely with regard to prevention and alleviation of suffering.

2.2. Chemical treatments for zebrafish assays

PFOA (CAS# 335–67–1, 95% purity, Sigma, St. Louis, MO), PFHxA (CAS# 307–24–4, ≥ 97.0% purity, Sigma, St. Louis, MO), and PFBA (CAS# 375–22–4, 98% purity, Sigma, St. Louis, MO) were used in the study (Table S1). The chemicals were solubilized in reverse osmosis water and all were within solubility limits (Table S1). Stock solutions of PFOA [900 parts per million (ppm), mg/L], PFHxA (10,000 ppm), and PFBA (10,000 ppm) were neutralized to pH 7 with 5 M sodium hydroxide (Wasel et al., 2021).

2.3. Acute toxicity assay

To assess the lethal concentration at which 50% mortality was observed (LC50) of the test chemicals, three biological replicates (embryos from 3 different clutches) were used. Each replicate composed of 50 embryos per treatment (with total 150 fish per treatment). Each 50 embryos were placed into a Petri dish and dosed with 20 mL of a range of concentrations of each PFAS within one hour after spawning (Peterson et al., 2011; Weber et al., 2013). Selected concentrations were 0, 300, 500, 600, 700, and 900 ppm (724.5–2173.5 μM) of PFOA; 0, 5000, 7000, 8000, 9000, or 10,000 ppm (15,921.03–31,842.06 μM) of PFHxA; and 0, 5000, 7000, 8000, 9000, and 10,000 ppm (23,360–46,720 μM) of PFBA. All solutions were adjusted to a neutral pH (Wasel et al., 2021). The developing zebrafish were exposed to the test chemicals until 120 hpf. The negative control was filtered water only. Mortality rates were monitored every 24 h. Mortality rates of treatment groups were normalized to the control treatment group.

2.4. Visual motor response behavior assay

Larval locomotion activities were assessed to evaluate if the developmental exposure to sublethal concentrations of test chemicals caused behavioral alterations. A visual motor response test was performed using Noldus DanioVision Observation Chamber (Noldus Information Technology, Wageningen, Netherlands). In each biological replicate, 50 zebrafish embryos were exposed to 0, 0.4, 4, 40, 400, or 4000 parts per billion (ppb, μg/L) of PFOA (0.0009–9.66 μM), PFHxA (0.00127–12.737 μM), or PFBA (0.0018–18.688 μM) within 1 hpf. Concentrations were chosen to encompass a log scale of sublethal concentrations with inclusion of and concentrations above an earlier US EPA provisional health advisory limit for PFOA at 0.4 ppb (EPA, 2021). At the time, there were no federal health advisory levels for PFHxA or PFBA but the maximum contaminant level for PFHxA in drinking water set by the state of Michigan for PFHxA is 400 ppb (EGLE, 2020). 0 ppb represents the negative control treatment consisting of filtered water.

At 72 hpf, exposure to the chemicals was terminated by rinsing the fish with filtered water. Fish were then incubated in water only at 28 °C until 120 hpf when behavioral analysis was completed similar to our past studies (Horzmann et al., 2018). Subsamples from each replicate were placed in separate wells in a 96-well plate (i.e., 16 subsamples of each treatment). The wells were filled with 500 μl filtered water, the plate placed in the DanioVision observation chamber, and temperature maintained at 28 °C throughout the experiment using the Noldus temperature control unit. After a 10-minute dark acclimation period, the test was started by exposing larvae to 10 min of alternating periods of dark and light for a total of 50 min (dark-light–dark-light–dark) (Horzmann et al., 2018). Infrared

light that is not visible to zebrafish larvae was used for tracking the movement. During the light phase, a 5000-lux light was activated from underneath the DanioVision observation chamber. The infrared movement traces were recorded at a rate of 25 frames per second with a Basler GenICam acA 1300–60 gm camera. Tracks were smoothed via a minimum distanced moved profile set to >0.2 mm. The exposure to dark or light was controlled by EthoVision 12 software. All behavioral experiments were performed at 11 am-2 pm to minimize circadian variability in movement. Total distance moved, mean velocity, and cumulative time spent moving were calculated using EthoVision 12 software. A total of 8 biological replicates were performed for each chemical with 16 subsample fish per treatment in each replicate to total up to 128 fish per treatment group.

2.5. Morphological growth assessment

Morphological growth evaluation of zebrafish at 72 hpf was performed to assess abnormalities in PFAS treatment groups compared to the control group. Zebrafish embryos were collected within 1 hpf. For each replicate, 50 embryos were dosed with 0, 4, 40, or 400 ppb PFOA, PFHxA, or PFBA through the end of embryogenesis (72 hpf). Twenty eleuthero-embryos were randomly selected from each replicate for the morphological analysis, euthanized using 0.4 mg/mL buffered tricaine-S (ethyl m-amino benzoate methane sulfonate; Western Chemical Inc., Ferndale, WA), and analyzed using a Nikon SMZ1500 dissecting microscope with NIS Elements imaging software (Melville, NY) to obtain measurements. Endpoints included body length, head width, and head length. The body length was measured as the distance from snout to tail, head length was measured as the distance from snout to operculum, and head width (intraocular space) was measured as the length between the midpoint of each eye (Horzmann et al., 2017; Horzmann et al., 2020). Ratios of head length to body length and head width to body length were calculated to assess changes in overall eleuthero-embryo size. Three biological replicates were included with 20 subsample fish per treatment per replicate for a total 60 fish assessed per treatment group.

2.6. Analysis of eleuthero-embryo transcriptome

Zebrafish embryos were exposed to 0, 4, 40, or 400 ppb PFOA, PFHxA or PFBA within 1 hpf through 72 hpf (end of embryogenesis) in groups of 50 in a Petri dish. At 72 hpf, fish were rinsed, pooled, homogenized in TRIzol (Life Technologies), and flash-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and quality confirmed by an Agilent Bioanalyzer (RIN > 9.0) and spectrometry. Transcriptome changes were assessed using the one-color hybridization strategy on a microarray containing 180,000 probes interrogating 36,000 gene targets with 3–5 probes per target based on Ensembl and UCSC genome databases (Agilent Technologies, Santa Clara, CA) (Wirbisky et al., 2016). Each microarray slide contained 4 arrays allowing comparison of gene expression profiles between the control treatment (0 ppb) and the three PFAS treatments (4, 40, 400 ppb) on the same slide to block and account for any potential slide to slide variation. Four biological replicates were included for each PFAS treatment. Microarray procedures and analyses were performed as previously described (Horzmann et al., 2018; Wirbisky et al., 2016). Briefly, RNA was hybridized to the array, arrays were washed in buffer solutions, and scanned using an Agilent Technologies SureScan Microarray Scanner (Agilent Technologies, Santa Clara, CA). Array image data were extracted and normalized using Agilent Feature Extraction

software 12.0. Microarray analysis was performed according to MIAME guidelines (Brazma et al., 2001) using GeneSpring 14.9 (Agilent Technologies) for statistical analysis. Gene lists were imported into Ingenuity Pathway Analysis (IPA) and core analysis was performed for gene ontology and molecular pathway analysis. IPA identifies human orthologs of zebrafish genes, which are subsequently used for pathway analysis. Thus, genes mentioned are the human nomenclature. All transcriptomic data are available in the gene expression omnibus (GEO) as GSE211169 (PFOA), GSE211171 (PFHxA), and GSE11172 (PFBA).

2.7. Statistical analyses

For the acute toxicity assessment, the LC50 and associated 95% confidence limits at 120 hpf in zebrafish larvae was determined using non-linear regression with a hill slope curve fitting using GraphPad Prism 8.4.1. A Grubb's outlier test was used to detect outliers within a treatment group for each outcome of the behavioral and morphological analyses. In the visual motor response assay, total distance moved, mean velocity, and time spent moving were analyzed using a two-way repeated measures analysis of variance (ANOVA) using SAS 9.4 software ($\alpha = 0.05$). The phase (dark or light) and chemical treatments were the independent variables and locomotor activity was the dependent variable. A one way ANOVA with SAS 9.4 software was used to analyze survival rates and morphological outcomes with a least significant difference post hoc test when a significant ANOVA was observed ($\alpha = 0.05$). Significance of transcriptome results was determined using GeneSpring 14.9 (Agilent Technologies) with an ANOVA ($\alpha = 0.05$) and a Tukey's post hoc test. A 1.5-fold change in gene expression criterion was also included for biological significance. All data are presented as mean ± standard deviation.

3. Results

3.1. Acute toxicity comparison of PFOA, PFHxA, and PFBA

Survival rates were monitored every 24 h after exposure to a range of PFOA, PFHxA, or PFBA concentrations (Figure S1) to serve as anchors for sublethal experiments and for comparison of findings with other studies that used slightly different exposure conditions. By 24 hpf, significant mortality was observed in the 600, 700, and 900 ppm PFOA treatment groups ($p < 0.0001$) (Fig. S1A). In addition, at 48 hpf a significant decrease in mortality was also seen in the 500 ppm PFOA treatment group ($p = 0.0002$), which continued for these four treatment groups through the end of the experiment (72 hpf: $p = 0.0002$; 96 hpf: $p = 0.0004$; 120 hpf: $p = 0.0004$) (Fig. S1A). No significant decrease in mortality was observed in the lowest treatment of 300 ppm (Fig. S1A) ($p > 0.05$). As a result, the PFOA 120 hpf-LC50 was 561.05 ppm (95% CI: 465.58–659.17) (Fig. 1).

In the PFHxA treatment groups, no increase in mortality occurred in the lowest treatment group of 5000 ppm (Fig. S1B) ($p < 0.0001$), while decreased survival occurred in all other treatment groups (7000, 8000, 9000, and 10,000 ppm) beginning at 24 hpf though the end of the exposure period (48 hpf: $p < 0.0001$; 72 hpf: $p = 0.0001$; 96 hpf: $p < 0.0001$; 120 hpf: $p < 0.0001$). The PFHxA 120 hpf-LC50 was calculated at 8394.5 ppm (95% CI: 7925.01–871.56) (Fig. 1).

No change in lethality occurred for the PFBA treatment groups up to 10,000 ppm throughout the exposure period ($p > 0.05$ at all developmental time points) (Fig. S1C) and thus, the 120 hpf-LC50 is >10,000 ppm (Fig. 1).

3.2. Locomotor behavior activity of zebrafish larvae exposed to PFOA, PFHxA, or PFBA during embryogenesis

As expected, zebrafish larvae were more active in dark phases and less active in light phases as measured in all parameters. Two-way repeated measure ANOVA was used to analyze the data, because the locomotor activities of the same sample was measured multiple times (during different lighting phases). PFOA exposure resulted in a significant overall effect of treatment [$(F5,743) = 2.54$, $p = 0.02$)] on the total distance moved (Fig. 2A) and mean velocity (Fig. 2D). In the first and second dark phase, exposure to 400 or 4000 ppb PFOA caused an increase in total distance moved and velocity, while hyperactivity was also observed in the first light phase at 400 ppb only (Fig. 2A, 2D). Although trends of hyperactivity were observed for time spent moving there was no significant difference in treatment [$(F5,743) = 1$, $p = 0.4$)] or in the interaction of treatment and time [$(F5, 743) = 1.14$, $p = 0.29$)] (Fig. 2G).

No significant effects were seen in the PFHxA exposure experiment for treatment [$(F5, 754) = 0.58$, $p = 0.7$)] or for interaction of treatment and time [$(F20, 3016) = 1.11$, $p = 0.3$)] for total distance moved or mean velocity (Fig. 2B, 2E). Similarly, no significant effect of treatment [$(F5,854) = 0.18$, $p = 0.18$)] or interaction between treatment and time [$(F20, 3016) = 1.26$, $p = 0.19$)] was observed for time spent moving (Fig. 2H).

There was a significant effect for PFBA exposure for treatment [$(F5, 754) = 2.18$, $p = 0.05$)] and also a significant interaction between treatment and time [$(F20, 3016) = 2.67$, $p < 0.0001$)] for total distance moved and mean velocity (Fig. 2C, 2F). Specifically, hyperactivity was observed in the first dark phase at 4 ppb and hypoactivity at 4000 ppb. Additionally, hypoactivity was seen in the first light phase at the lowest treatment concentration included in this study (0.4 ppb), along with hypoactivity at 400 and 4000 ppb in the second dark phase, which persisted at 4000 ppb in the third dark phase. In addition, there was a significant effect for treatment [$(F5, 754) = 2.56$, $p = 0.02$)] for time spent moving (Fig. 2I). A similar pattern was observed; however, hypoactivity was observed in the three dark phases in the 4000 ppb treatment group only and in the 0.4 ppb treatment group in the first light phase. Overall, these results indicate a more complex behavioral effect including a biphasic response among the treatment group concentrations.

3.3. Morphological assessment of eleuthero-embryos after PFOA, PFHxA, or PFBS embryonic exposure

Impacts to developmental growth were non-monotonic as measured at 72 hpf immediately following chemical exposure (Table 1; Figures S2–S4). A significant effect for treatment on body length (ANOVA: $p = 0.02$), head width (ANOVA: $p < 0.0001$), and head length (ANOVA: $p < 0.0001$) were observed. In addition, a significant effect of PFOA treatment on head length to body length ratio compared to unexposed fish was seen (ANOVA: $p < 0.0001$). Specifically, a decrease in body length and head width occurred within the 4 ppb

PFOA treatment group ($p < 0.05$) with no significant differences observed in measurement ratios ($p > 0.05$). Exposure to 400 ppb PFOA decreased head width and head length significantly and resulted in a significant decrease in head length to body length ratio.

PFHxA exposure also had a significant treatment effect for body length (ANOVA: $p < 0.0001$), head length (ANOVA: $p < 0.01$), head width (ANOVA: $p = 0.04$), and head length to body length ratio (ANOVA: $p = 0.02$). In the PFHxA 40 ppb treatment group, a decrease in body length, head length, and head width were observed compared to the 0 ppb treatment group ($p < 0.05$), but no differences occurred in the ratio of the measurements ($p > 0.05$). In addition, fish in the 400 ppb PFHxA treatment group had a significant decrease in head length, along with a lower head length to body length ratio compared to the 0 ppb treatment group ($p < 0.05$).

PFBA treatment had a significant effect on body length (ANOVA: $p = 0.0004$), head width (ANOVA: $p = 0.02$), and head width to body length ratio (ANOVA: $p < 0.0001$). The effects of PFBA included a significant decrease in body length in the 4 and 400 ppb treatment groups and a significant increase in head width in the 400 ppb exposure group. A significant increase in the head width to body length ratio was detected in all three treatment groups.

3.4. Transcriptome alterations of eleuthero-embryos exposed to PFOA, PFHxA, or PFBA during embryogenesis

Transcriptomic analysis of developing zebrafish exposed to PFOA identified 36 mapped genes with altered expression in the 4 ppb PFOA exposure group, 77 genes with altered expression in the 40 ppb PFOA exposure group, and 66 genes with altered expression in the 400 ppb PFOA exposure group. There were 9 common genes (*BATF, CIT, DOCK7, JDP2, NEDD4, Nedd4-2, PLCB2, PLCG1,* and *TASL)* altered in all three treatment groups (Figure S5).

Gene ontology and molecular pathway analyses with IPA revealed the most enriched pathways associated with diseases and disorders, physiological system development and function, and molecular and cellular functions based on gene expression changes for each treatment of each chemical (Tables 2, 3, and 4). Cancer, organismal injury and abnormalities, and reproductive system disease were among the top five enriched diseases and biological functions for the embryonic exposure to 4, 40, and 400 ppb PFOA (Table 2). In addition, immune cell trafficking, cell-mediated immune response, and hematological system development and function were among the top enriched physiological system development and function pathways in all PFOA treatment groups (Table 3). Concentration-dependent pathways included endocrine diseases for the embryonic exposure to 40 ppb and 400 ppb PFOA, while neurological diseases were one of the top enriched pathways in the 4 ppb PFOA exposure group (Table 2). Glioma was the most enriched disease for 4 ppb PFOA exposure group under neurological disease category. Molecular and cellular function groups were more variable among the PFOA treatment groups with alterations associated with cell cycle and cellular development, growth, proliferation, movement, signaling, function, maintenance, death, and survival (Table 4), with the ERBB pathway specifically identified (Figure S6).

There were 32 mapped genes with altered expression in the 4 ppb PFHxA exposure, 113 genes with altered expression in the 40 ppb PFHxA exposure, and 50 genes with altered expression in the 400 ppb PFHxA exposure (Figure S5). In addition, there were 9 common genes altered in all three PFHxA treatment groups including *DUSP29, EXOSC6, KLF9, LRRC3, PFKB4, PHLPP2, RHCG, TLN2*, and *UCP3*. The embryonic exposure to 4 and 400 ppb PFHxA resulted in the same disease and disorder pathways including endocrine system disorders, gastrointestinal disease, hereditary disorder, metabolic disease, and organismal injury and abnormalities, while exposure to 40 ppb PFHxA resulted in disease and disorder pathways related to organismal injury and abnormalities and hematological, immunological, dermatological, and infectious diseases (Table 2). For the physiological system development and function pathways, hematological system development and function was identified to be altered in all three PFHxA treatment groups similar to PFOA (Table 3). In addition, the embryonic development pathway was enriched in all three PFHxA treatment groups (Table 3). Other similarities included connective tissue development and function and hematopoiesis pathways in the 4 and 400 ppb PFHxA treatment groups and the humoral immune response pathway in the 40 and 400 ppb PFHxA treatment groups (Table 3). Similar to PFOA, there was more variability in the top enriched pathways associated with molecular and cellular functions with some similarities in cell cycle and cell development, growth, proliferation, morphology, function, maintenance, death, and survival (Table 4). The top canonical pathways in the three PFHxA treatment groups indicated immune system-related pathways such as the cross talk between dendritic cells and natural killer cells at 4 ppb PFHxA; altered T cell and B cell signaling in Rheumatoid Arthritis, Toll-like Receptor signaling, and communication between innate and adaptive immune cells at 40 ppb PFHxA; and primary immunodeficiency signaling at 400 ppb PFHxA. Significant expression changes of B-cell linker protein (*BLNK*) and *CD40* was observed in 40 and 400 ppb PFHxA treatment groups. The *BLNK* gene encodes a protein that plays a role in B-cell development, while *CD40* is a receptor protein on antigen-presenting cells of the immune system.

Transcriptomic analysis of 72 hpf zebrafish with embryonic exposure to PFBA identified substantially more genes compared to PFOA and PFHxA with 171 mapped genes with altered expression in the 4 ppb PFBA exposure, 165 genes with altered expression in the 40 ppb PFBA exposure, and 469 genes with altered expression in the 400 ppb PFBA exposure (Figure S5). There were 5 common genes altered in all three treatment groups, which were *ACE2, BC01, C3, CEL,* and *ENPP7*. There were no similar disease and disorder pathways for all three PFBA treatments group, but the nutritional disease pathway was identified for the 4 and 400 ppb PFBA treatment groups and the organismal injury and abnormalities pathway for the 40 and 400 ppb PFBA treatment groups (Table 2). All other pathways were only present in a single PFBA treatment group including neurological diseases in the 40 ppb PFBA exposure groups with genes specifically associated with neurodegenerative diseases such as Alzheimer's disease, dementia, onset of amyotrophic lateral sclerosis, and schizophrenia. Cancer was the top enriched disease for the 400 ppb PFBA exposure group (Table 2) with results showing genes associated with skin cancer, specifically cutaneous tumor which was among the top significantly altered cancer-related genes in the 400 ppb PFBA and PFOA treatment groups (Table 2). For the pathways related to physiological system development and function, again there were no pathways common to all three PFBA

treatment groups with only organ development in the 4 and 40 ppb PFBA treatment groups and hematological system development and function in the 4 and 400 ppb PFBA treatment groups (Table 3). All other pathways were concentration-dependent with endocrine system development and function and behavior in the 4 ppb PFBA treatment group; nervous system development and function along with organ morphology and organismal development in the 40 ppb PFBA treatment group; and tissue morphology and development along with connective tissue and skeletal and muscular system development and function in the 400 ppb PFBA treatment group (Table 3). There were more similarities in the pathways related to molecular and cellular function in the PFBA treatment groups compared to PFOA and PFHxA (Table 4). Cell-to-cell signaling and interaction pathway was identified in the 4 and 40 ppb PFBA exposure groups; while multiple pathways including lipid metabolism, molecular transport, and small molecule biochemistry were in both the 40 and 400 ppb PFBA treatment groups (Table 4) with several regulators and functions associated with lipid oxidation, absorption, uptake, and transport (Fig. 3). In addition, FXR/RXR activation was among the most enriched canonical pathway in all three PFBA treatment groups with several pathways showing activation and/or inhibition including the role of FXR in lipoprotein and lipid metabolism, *trans*-repression of FXR during acute phase response, role of FXR in bile acid homeostasis, and role of FXR in glucose homeostasis (Figure S7).

3.5. Comparison of transcriptome alterations with an embryonic exposure to PFOA, PFHxA, or PFBA

Comparative enrichment pathway analysis among the three PFAS overall identified unique signatures for each chemical and were also sometimes limited to a single treatment group for that chemical. For example, altered genes associated with neurotransmitter and nervous system signaling were primarily limited to the 4 ppb PFOA exposure group showing changes in multiple related canonical pathways (Figure S8). On the other hand, the 4 ppb PFHxA treatment group had association with netrin signaling and the 4 ppb PFBA treatment group affected dopamine-DARPP32 feedback in cAMP signaling (Figure S8). Interesting all of these neurological associated signaling pathways were only observed in the lowest exposure group (4 ppb) of each chemical tested in this study.

The comparison analysis also showed that the FXR/RXR and LXR/RXR activation pathways were mainly limited to the PFBA treatment groups (Fig. 4). In addition, TR/RXR and PXR/RXR activation and LPS/IL-1 mediated inhibition of RXR function was limited to only the 400 ppb PFBA exposure group (Fig. 4). Alternatively, alterations in aldosterone signaling in epithelial cells pathway was only observed in the PFOA treatment groups (Fig. 4).

Furthermore, exposure to PFOA or PFBA affected many cellular immune response canonical pathways, but there was no overlap between the affected pathways for the two chemicals (Fig. 5). PFOA treatment groups showed a non-monotonic concentration response in the number of canonical pathways that were associated with cellular immune response between the 4, 40, and 400 ppb treatment groups. In addition, additional unique pathways were only identified in the 40 ppb PFBA treatment group and only 1 pathway in the 400 ppb PFHxA treatment group (Fig. 5).

4. Discussion

PFAS are a class of chemicals that are widely used in consumer products and industrial products. Legacy PFAS (e.g., PFOA) were replaced by other shorter chain PFAS (e.g., PFHxA and PFBA) to increase their degradability in the environment and decrease their bioaccumulation in humans and other biological organisms, but limited toxicological data are available for those emerging PFAS. The goal of our study was to compare the developmental toxicity and developmental neurotoxicity of shorter chain PFAS to legacy compounds. We utilized the zebrafish model that allowed PFAS direct exposure to embryos and avoided any confounding effects of maternal toxicity that may be observed. For example, Iwai et al. assessed developmental toxicity of PFHxA in mice and concluded that no effect level for developmental toxicity will occur at levels higher than the concentrations that cause maternal toxicity (>175 mg/kg/day) (Iwai et al., 2019). Our results first revealed that the 120 hpf-LC50 of PFOA, PFHxA, and PFBA were 561.05, 8394.5, and > 10,000 ppm, respectively. These results are in agreement with previously published data for these chemicals and also where the LC50 decreases with increasing chain length (Wasel et al., 2021; Hagenaars et al., 2011; Ulhaq et al., 2013; Godfrey et al., 2017; Ding et al., 2013). On the other hand, other studies reported the 120 hpf-LC50 of PFOA to be around 72 ppm (continuous exposure started at < 4 hpf) (Gebreab et al., 2020) and the 120 hpf-LC50 of PFHxA to be 290 μM (91 ppm) (AB wild type zebrafish embryos exposed at 3 hpf for 120 hpf) (Annunziato et al., 2019). These values are much lower than our results. PFOA, PFHxA, and PFBA are acids, and highly concentrated solutions will overcome the buffering capacity of most solutions that zebrafish researchers use in their toxicity studies (Wasel et al., 2021). As such, if the solutions are not neutralized, the low pH of the dosing solution will greatly affect the survival of zebrafish embryos and larvae, which is hypothesized to be responsible for the difference observed in these two studies compared to the current findings. These findings also align with rodent studies where exposure to PFBA during pregnancy in mice didn't cause mortality or gross developmental abnormalities in pups at the same concentrations at which developmental toxicity was observed for PFOA (Das et al., 2008).

LC50 is a quick measure for acute toxicity and it is used for environmental risk assessment, but it is less relevant to the environmental exposures and general human population exposure. It is very important to assess the effect of sublethal exposure. Our research is showing that although the acute toxicity of PFOA is much higher than shorter chain PFAS, the short chain PFAS can cause adverse effects at sublethal exposures.

Behavioral changes in developing zebrafish with PFAS exposure has been investigated in multiple studies, but the majority of these studies used relatively high concentrations (summarized in Table S2). In our study, sublethal concentrations (0.4, 4, 40, 400, and 4000 ppb) were used to assess effects of exposure on neurobehavior by assessing locomotor activity alterations. Evaluation of locomotor activity of zebrafish has been used to identify neuroactive drugs and assessment of neurobehavior of environmental toxicants (Basnet et al., 2019). In the visual motor response assay, zebrafish larvae are exposed to alternating periods of dark and light with locomotor activity increased in dark periods and decreased in light periods. Our goal was to assess the effect of an embryonic exposure to PFOA, PFHxA, or PFBA. Thus, the chemical treatments were ceased at the end of embryogenesis

(at 72 hpf) and behavioral endpoints measured at 120 hpf, when the larvae have an inflated swim bladder and reliable movement patterns (Padilla et al., 2011). The results showed that exposure to 400 or 4000 ppb PFOA increased the total distance moved and mean velocity, which agrees with Rericha et al. where hyperactivity in 120 hpf larvae was observed with exposure to 414 ppb PFOA at 120 hpf (Rericha et al., 2021). Two additional studies also observed that PFOA exposure, albeit at higher concentrations, caused hyperactivity in 144 hpf larvae (Menger et al., 2020; Ulhaq et al., 2013). Ulhaq et al. showed an increase in activity in dark phases in PFOA exposed larvae (concentrations weren't specified) (Ulhaq et al., 2013). Menger et al. found exposure to 4,968 ppb PFOA increased swimming distance in 144 hpf-larvae (Menger et al., 2020). Alternatively, a study using similar concentrations showed that a 10, 100, or 1000 ppb PFOA exposure decreased locomotor activity in zebrafish larvae aged 168 hpf and linked behavior changes to alterations in development and function of dopaminergic neurons (Yu et al., 2021). On the other hand, (Khezri et al., 2017) at 96 hpf and (Gaballah et al., 2020) at 144 hpf did not find that a developmental PFOA exposure altered behavior.

In our study, the embryonic PFHxA exposure did not induce any behavioral changes, which agrees with two other studies (Annunziato et al., 2019; Menger et al., 2020). (Menger et al., 2020) did not observe significant changes in locomotor activities in 144 hpf zebrafish larvae exposed to 0.1–100,000 ppb PFHxA from 1 to 144 hpf. In addition, no significant alterations in total distance moved or mean velocity in 120 hpf zebrafish larvae exposed to 0.2–20 μM PFHxA from 3 to 120 hpf (62.8–6280 ppb) was observed in another study (Annunziato et al., 2019). Alternatively, Gaballah et al. observed hyperactivity at 144 hpf in the first dark, second dark, and second light phases but was at concentrations greater than were tested in the current study [i.e., 14 μM (4397 ppb) and 25 μM (7851 ppb)] (Gaballah et al., 2020). Furthermore, Guo et al. found that exposure to 480 ppb PFHxA caused an increase in swimming activity in 120 hpf zebrafish larvae but the effect wasn't observed in the higher exposure groups at 2,400 or 12,000 ppb (Guo et al., 2021).

PFBA exposure caused hypoactivity in all dark phases with effects in the highest treatment group (4000 ppb) being the most persistent in all outcomes measured. In addition, in the first light phase, hypoactivity was observed only in the 0.4 ppb exposure group in all outcomes. Furthermore, hyperactivity was also observed in the 4 ppb PFBA treatment group in only the first dark phase. As a result, PFBA caused behavioral changes at lower concentrations compared to PFOA. These biphasic effects on behavior have also been observed for other toxicants including ethanol where 1% and 2% ethanol increased activity of 144 hpf larvae and 4% ethanol caused a severe decrease in activity (MacPhail et al., 2009). Our findings are in contrast to two other studies reporting hyperactivity at 120 or 144 hpf in dark and light or only dark phases (Rericha et al., 2021; Ulhaq et al., 2013).

Overall, the behavioral results showed that PFOA (mainly hyperactivity) and PFBA (mainly hypoactivity) induced behavioral alterations but were absent for PFHxA. According to Gaballah et al. if a statistically significant difference from the negative control treatment is observed in multiple phases for the same concentration of a chemical or two concentrations of a chemical within a certain phase, the chemical should be considered developmentally neurotoxic (Gaballah et al., 2020). Those two criteria were met in our study for PFOA

and PFBA. In addition, these behavioral alterations were more prominent at lower exposure concentrations for PFBA compared to PFOA leading to the conclusion that the behavioral perturbations do not increase with chain length as is observed in acute toxicity studies. Furthermore, the overall trend of hyperactivity for PFOA and hypoactivity for PFBA suggest different toxicity mechanisms. The concentration-dependent and biphasic effects may also help to explain contradicting epidemiological results regarding developmental neurotoxicity. For example, some epidemiological studies observed an increase in risk of ADHD and reduced executive functioning (Oulhote et al., 2016) with PFAS exposure, while other epidemiological studies showed no association between PFAS exposure and behavioral outcomes and motor coordination (Liew et al., 2018).

As noted above (and as summarized in Table S2), our behavioral results agree with some studies but that there are differences in outcomes reported in the literature among the zebrafish larvae PFAS studies. These discrepancies may be attributed to differences in length of exposure (e.g., through 72 hpf compared to through 120 hpf) and/or the use of DMSO as a solvent. Although, DMSO at concentrations of 0.5% or less is reported not to alter behavior of zebrafish larvae (Padilla et al., 2011), DMSO can affect the uptake of the chemicals (Kais et al., 2013). It is important to note that multiple factors can affect results of behavioral analysis including concentration of chemicals, age of larvae, lighting conditions (length of phases and pattern), dosing regimen (static or daily renewal of dosing solutions), and rearing temperature (Padilla et al., 2011; Fraser et al., 2017; Yu et al., 2022) (Table S2). Another important factor that can affect results is the number of biological replicates and subsamples used in the experiments to influence statistical power. In our study, we used more than 100 subsamples per treatment from multiple biological replicates to account for intra-larvae variability to increase reliability of results (Ingebretson and Masino, 2013).

Morphological measurements are reliable indicators of proper development of zebrafish (Kimmel et al., 1995). Most studies evaluate gross malformation but few studies have measured fine morphological changes after PFAA exposure. Our study showed that exposure to 4 ppb PFOA decreased the total body length and head width; however, there was no change in the ratio of head width to total body indicating that 4 ppb PFOA exposure resulted in an overall smaller fish. Exposure to 400 ppb PFOA resulted in a decreased head length, head width, and ratio of the head length to body length suggesting that the 400 ppb PFOA exposure caused an unproportionate decrease in the head length. These findings align with Jantzen et al. in which an exposure to 838 ppb PFOA from 3 to 120 hpf but not 8.3 or 83 ppb increased total body length and yolk sac area and decreased interocular distance (similar to the head width) (Jantzen et al., 2016). For PFHxA, exposure to 40 ppb decreased total body length, head width, and head length with no changes in the morphological ratios. In addition, 400 ppb PFHxA exposure decreased head length and the head length to body length ratio. This finding is similar to Annunziato et al. that observed a decrease in body length and a decrease in yolk sac size of 120 hpf zebrafish following 83 ppb PFHxA exposure (Annunziato et al., 2019). Additionally, in rats an oral maternal exposure to PFHxA decreased body weight in offspring (Loveless et al., 2009). PFBA exposure also caused concentration-dependent morphological alterations with a decrease in body length in the 4 and 400 ppb PFBA exposure groups. There was also an increase in head width in the

400 ppb PFBA-treated fish and an increase in the head width to body length ratio suggesting that the decrease in head width wasn't proportional to the decrease in body length.

Lastly, transcriptome analysis was completed in whole eleuthero-embryos following the embryonic PFAA exposure to compare genes and molecular and disease pathway alterations among the different chain lengths. In addition, PFOA molecular pathways were compared to the literature to determine if perturbations in the developing zebrafish were concordant to adverse health outcomes observed in human populations. Overall, chemical- and concentration-dependent genes and molecular pathway alterations were detected with only a small number of genes being the same among the treatment groups of each PFAA (i.e., 5 for PFBA and 9 for PFHxA and PFOA).

For PFOA, the higher treatment groups (40 and 400 ppb) had a larger number of genes altered that were associated with cancer including breast, liver, prostate, ovarian, pancreatic, and testicular cancer. The C8 Health Project, which included thousands of participants from the Mid-Ohio Valley during the period between 2005 and 2013 studied the link between PFAS exposure, specifically PFOA (C8), and many diseases including cancer. The C8 science panel concluded a probable link between PFOA exposure and testicular and kidney cancer, along with high cholesterol, ulcerative colitis, thyroid disease, and pregnancy-induced hypertension. The C8 Health Project is revered as the most extensive environmental epidemiology study conducted to date. The ERRB pathway, which is associated with many human cancers, was specifically identified in the transcriptome analysis for PFOA. PFOA is classified as possibly carcinogenic to humans by IARC due to a positive association between PFOA exposure and testicular and kidney cancers (IARC, 2007). A National Toxicological Program (NTP) study also indicated strong evidence of association between PFOA exposure and increased incidence of hepatocellular neoplasms and increased incidence of acinar cell neoplasms of the pancreas, based on a 2-year study in male rats (NTP, 2020). While, both 40 and 400 ppb PFOA exposure groups had enrichment in endocrine system disease disorders, each treatment group had unique pathways with exposure to 400 ppb PFOA alterations associated with thyroid cancer and the 40 ppb PFOA exposure associated with multiple pathways related to the female reproductive system. A 2017 review assessed the effects of PFOA on the thyroid gland using in vitro studies, animal studies, and human data and concluded that PFOA exposure reduces the circulating levels of thyroid hormones in animals (Coperchini et al., 2017). This conclusion supports findings from the C8 Science Panel mentioned above that reported a probable link with thyroid disease. This review also concluded though that the risk of thyroid cancer from PFOA exposure was low (Coperchini et al., 2017). Furthermore, among the 9 common altered genes in the three PFOA treatment groups and in the multiple molecular pathways, genes associated with the immune system were identified. For example, *BATF* and *PLCG1* are associated with cell movement of $CD4^+$ *T*-lymphocytes, homing of $CD4^+$ *T*-lymphocytes, homing of TREG cells, and migration of $CD4^+$ *T*-lymphocytes. These results align with epidemiological studies that link PFOA to immunological diseases and the consideration that PFOA is an immune hazard due to its roles in suppression of the antibody response and increased hypersensitivity (NTP, 2016). Interestingly, an additional study also found that exposure of zebrafish embryos to PFOA resulted in multigenerational effects on immune system development (Haimbaugh et al., 2022). Haimbaugh et al. showed that parental exposure to 7 ng/L PFOA lead to down

regulation of the innate immunity-related c3a.2 in 7 day old F1 offspring. In addition, zebrafish embryos exposed to 7 or 70 ng/L PFOA through 5 days had changes in genes involved in immune cell function and trafficking in the 7 day-old F2 generation offspring (Haimbaugh et al., 2022).

There are limited available studies on the toxicological effects of PFHxA and even less studies focused on its developmental toxicity. It was reported in a toxicological review for PFHxA released by the US EPA in 2022 that PFHxA exposure is likely to cause developmental, hematopoietic, and hepatic effects in humans (U.S. EPA, 2022a), which aligns well with the observed perturbed molecular pathways in our study. For example, our results showed that exposure to 4, 40, or 400 ppb PFHxA caused alterations in genes associated with embryonic development. Other studies in rats have shown that 200 mg/kg/day of PFHxA decreased hemoglobin and red blood cells (RBCs) and in chronic and subchronic studies decreased reticulocytes (Loveless et al., 2009; Chengelis et al., 2009; NTP, 2018; Klaunig et al., 2015). Hematological disease was the top enriched disease in the 40 ppb PFHxA treatment group and hematological system development and function was a common physiological system development and function pathway in all PFHxA treatment groups. We also observed alterations in genes involved in the immune system in all the PFHxA exposure groups. Animal studies have shown an unclear pattern of the potential effects of PFHxA on the immune system including decreased spleen weights in male rats receiving 500 mg/kg/day PFHxA sodium salt for 90 days and alterations in immune cell counts in PFHxA exposures ranging between 20 and 500 mg/kg/day (Loveless et al., 2009). On the hand, no changes in immune cell counts or weight of spleen or thymus was observed after exposure to 200 mg/kg/day for 90 days in rats (Chengelis et al., 2009). In addition, a case-control study in children in Taiwan showed no association between PFHxA exposure and immunological markers (Dong et al., 2013). Metabolic disease was also among the top enriched disease pathways in the 4 and 400 ppb PFHxA exposure groups with expression alterations in uncoupled protein 3 (*UCP3*). *UCP3* has a role in fatty acid metabolism, energy homeostasis, and modulates insulin sensitivity (Liu et al., 2013). *UCP3* alterations are associated with familial non-insulin-dependent diabetes mellitus, severe obesity, and type 2 diabetes pathways, which is concordant with a study in pregnant women reporting links with PFHxA exposure and gestational diabetes mellitus and impaired glucose homeostasis (Liu et al., 2019).

The PFBA exposures resulted in the highest number of gene alterations compared to PFOA and PFHxA. With PFBA as one the top replacements for PFOA, there is currently a lot of focus on furthering the understanding of health risks associated with PFBA. In the most recent toxicological review of PFBA by the US EPA, it was concluded that PFBA exposure may cause developmental, thyroid, and liver effects in humans in utero or during adulthood (U.S. EPA, 2021). PFBA treatment groups in our study resulted in distinct molecular pathways including neurodegenerative diseases in the 40 ppb PFBA exposure group and cancer in the 400 ppb PFBA exposure group. Additionally, the endocrine system development and function pathway was enriched in the 4 ppb PFBA exposure group and was associated with proliferation of pancreatic cells, proliferation of endocrine cells, proliferation of islet cells, and proliferation of beta islets cells. Endocrine disease was also one of the top disease pathways for the 40 ppb PFBA treatment group, which was associated

with benign thyroid nodule, diabetes mellitus, hyperparathyroidism, and carcinoma of the pancreas. Moreover, canonical pathways were similar among the three PFBA treatment groups for their association with lipid metabolism, molecular transport, and small molecular biochemistry. Activation of the FXR/RXR pathway was one of the most significant findings present within all three PFBA treatment groups in a concentration–response manner (i.e., 22% overlap at 400 ppb PFBA, 9.3% overlap at 40 ppb PFBA, and 5.6% overlap at 4 ppb PFBA for this pathway). In addition, comparative analysis revealed that the 4 and 40 ppb PFBA treatment groups also had LXR/RXR activation among the most enriched canonical pathways. The farnesoid X receptor (FXR), liver X receptor (LXR), and retinoid X receptor (RXR) are nuclear receptors that control metabolic pathways such as bile acid regulation, lipid and glucose homeostasis, and metabolism (Zheng et al., 2018; Ding et al., 2014). This finding is in agreement with others that report activation of FXR and LXR by PFAS [reviewed in (Andersen et al., 2021)] but are discordant with findings in human HepG2 hepatocarcinoma cells that identified PFOA, PFBA, and PFOS exposure activated the peroxisome proliferator-activated receptor alpha (PPARα) but did not affect FXR, LXR, or RXR (Behr et al., 2020). In addition, another study showed that zebrafish exposed to PFOA caused alterations in genes involved in LXR, RXR, AhR, and FXR (IARC, 2007), although in our study this finding was more apparent in the PFBA exposed groups.

5. Conclusions

The results of this study were in agreement with past studies showing that acute developmental toxicity of PFAAs is driven by chain length. Alternatively, embryonic exposure to PFOA and PFBA caused behavioral changes in zebrafish larvae, while PFHxA did not. These results support that the neurobehavioral changes are not dependent on the chain length and that the observed behavioral alterations are likely to function through different mechanisms given differences among PFOA and PFBA responses. Morphological alterations were observed among treatment groups of all three PFAAs in distinct patterns. The transcriptome analysis validated concordance of several of the reported human adverse health outcomes associated with PFOA exposure based on similarity of molecular pathways and networks and canonical pathways observed following the embryonic exposure in the developing zebrafish. In addition, although current information is limited, molecular pathways also aligned with literature reports for PFHxA and PFBA [as summarized by the US EPA in the 2021–2022 IRIS toxicological reports (U.S. EPA., 2021; U.S. EPA, 2022a)]. These findings support the predictive value of this approach as we have observed in similar studies with other environmental toxicants (e.g., Weber et al., 2013; Peterson et al., 2013) and can be used to build hypotheses for future studies addressing mechanisms of specific adverse health outcomes based on these identified molecular pathways. Overall, unique alterations were seen for each of the three PFAAs demonstrating the need to assess the toxicity risks of the replacement PFAS. Furthermore, future studies are needed to compare toxicokinetics of these three PFAS for additional comparison in this exposure paradigm but a past study reported that the bioconcentration factor (BCF) of PFOA is 50 and PFBA is 0.8 in zebrafish (Vogs et al., 2019). Considering this BCF in zebrafish within the findings of our study (e.g., PFBA behavioral changes at concentrations lower than PFOA) further highlights the need to consider the toxicity of the replacement PFAS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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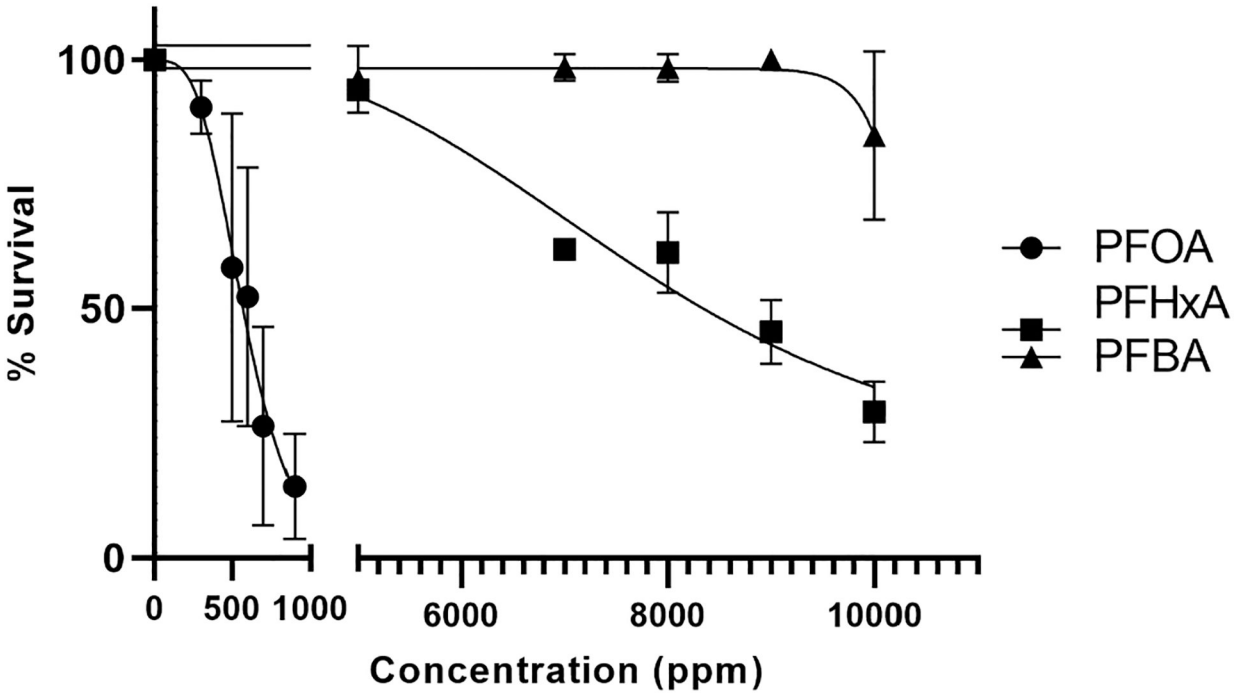

Fig. 1.

120 hpf-LC50 curves of developing zebrafish exposed to PFOA, PFHxA or PFBA from 1 to 120 hpf. The overall toxicity ranking is PFOA > PFHxA > PFBA. The LC50 for PFOA = 561.01 ppm, for PFHxA = 8394.5 ppm, and for PFBA > 10,000 ppm. N = 3 with 50 fish per treatment per replicate. Error bars are standard deviation.

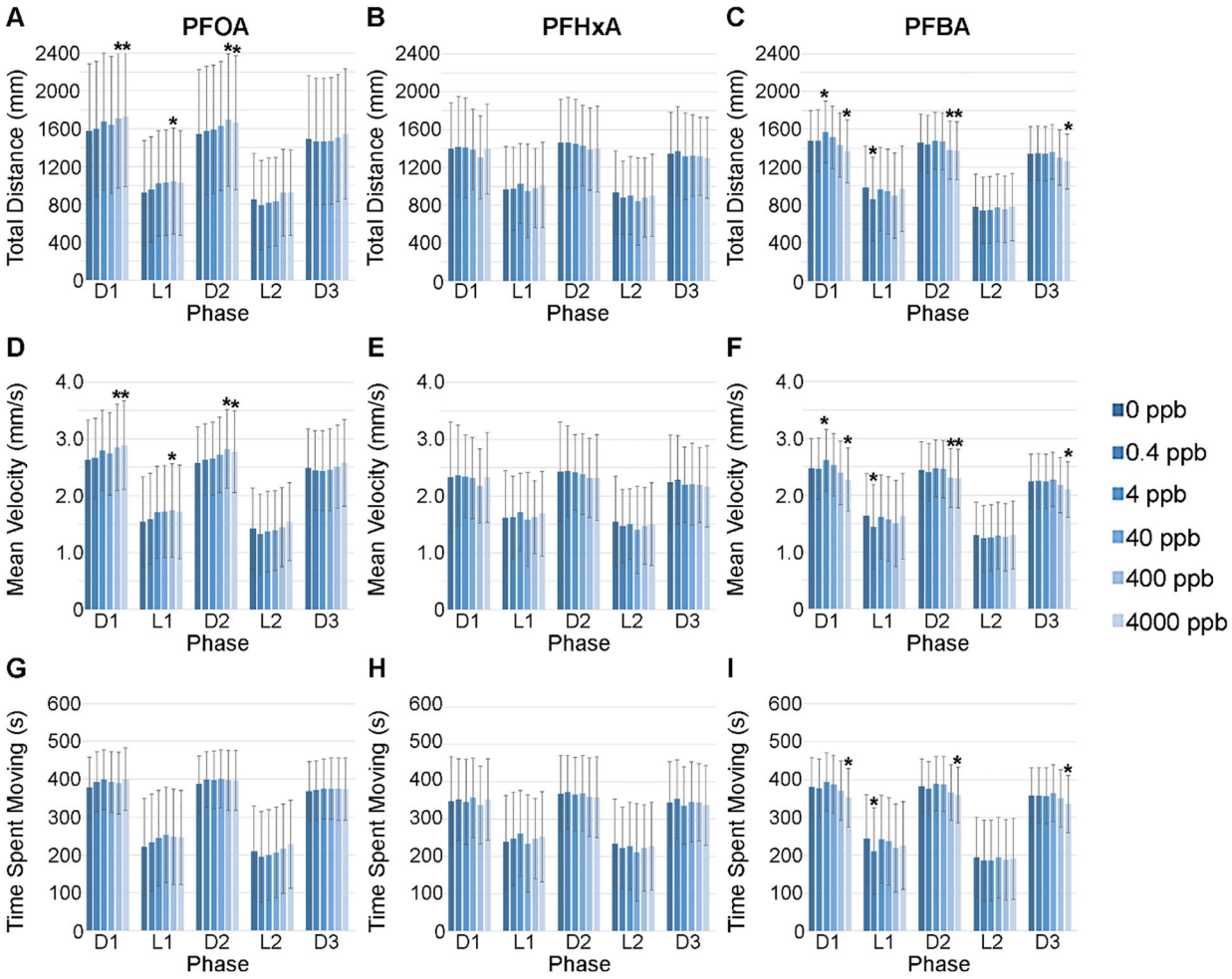

Fig. 2.

Visual motor response behavior assay in zebrafish larvae at 120 hpf following an embryonic exposure (1–72 hpf) to PFOA (A, D, G), PFHxA (B, E, H), or PFBA (C, F, I). N = 8 biological replicates with 13–16 subsamples per treatment per replicate to total 122–128 fish per treatment group. Error bars represent standard deviation. *$p < 0.05$ compared to the control treatment group within each phase based on lsmeans table when a significant ANOVA for treatment or interaction occurred. (D1: first dark phase; L1: first light phase; D2: second dark phase; L2: second light phase; D3: third dark phase).

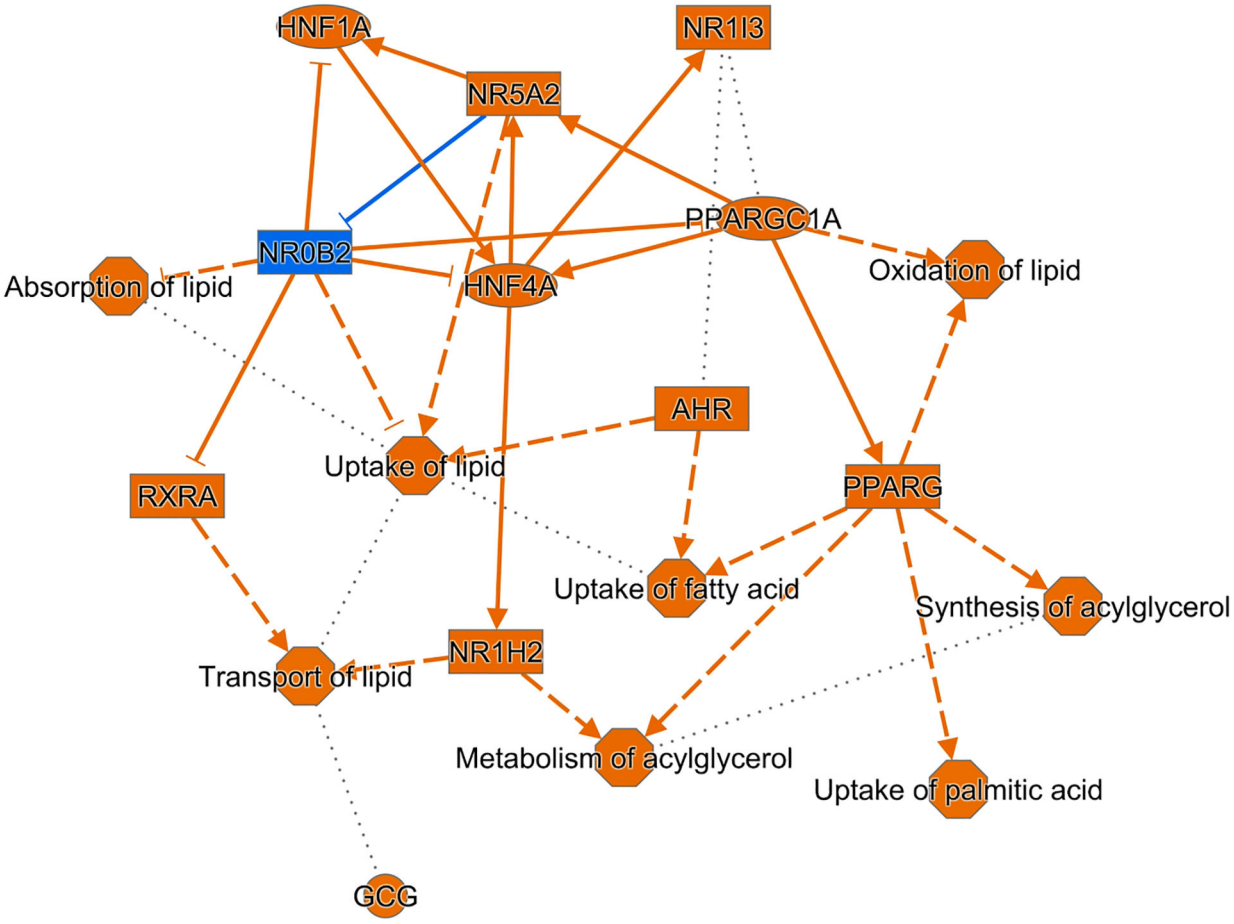

Fig. 3.

Top altered regulators and functions in zebrafish exposed to 400 ppb PFBA during embryogenesis. The top regulators and functions are primarily associated with lipid pathways. Rectangles represent ligand-dependent nuclear receptor, hexagons represent function, ovals represent transcription regulator, and circles represent other molecules. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates direct interaction and a dashed line indicates indirect interaction. A dotted line indicates that the association is predicated but not confirmed in literature.

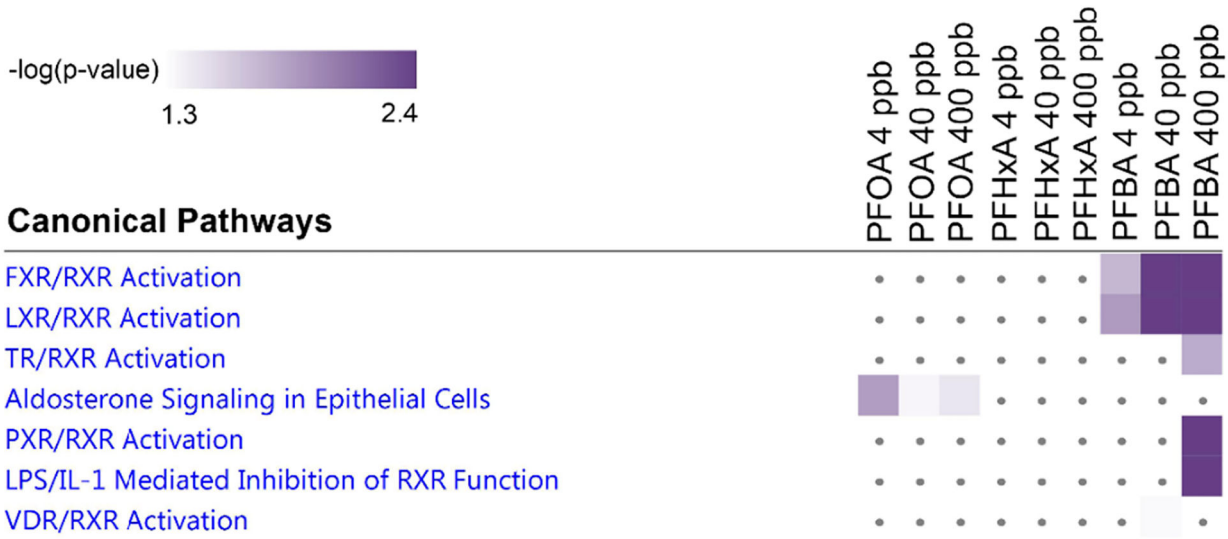

Fig. 4.

Comparison analysis showing canonical pathways related to nuclear receptor signaling following embryonic exposure to PFOA, PFHxA, or PFBA. Purple color indicates statistical significance (−log (p-value) = 1.3). Intensity of color correspond to – log (p-value).

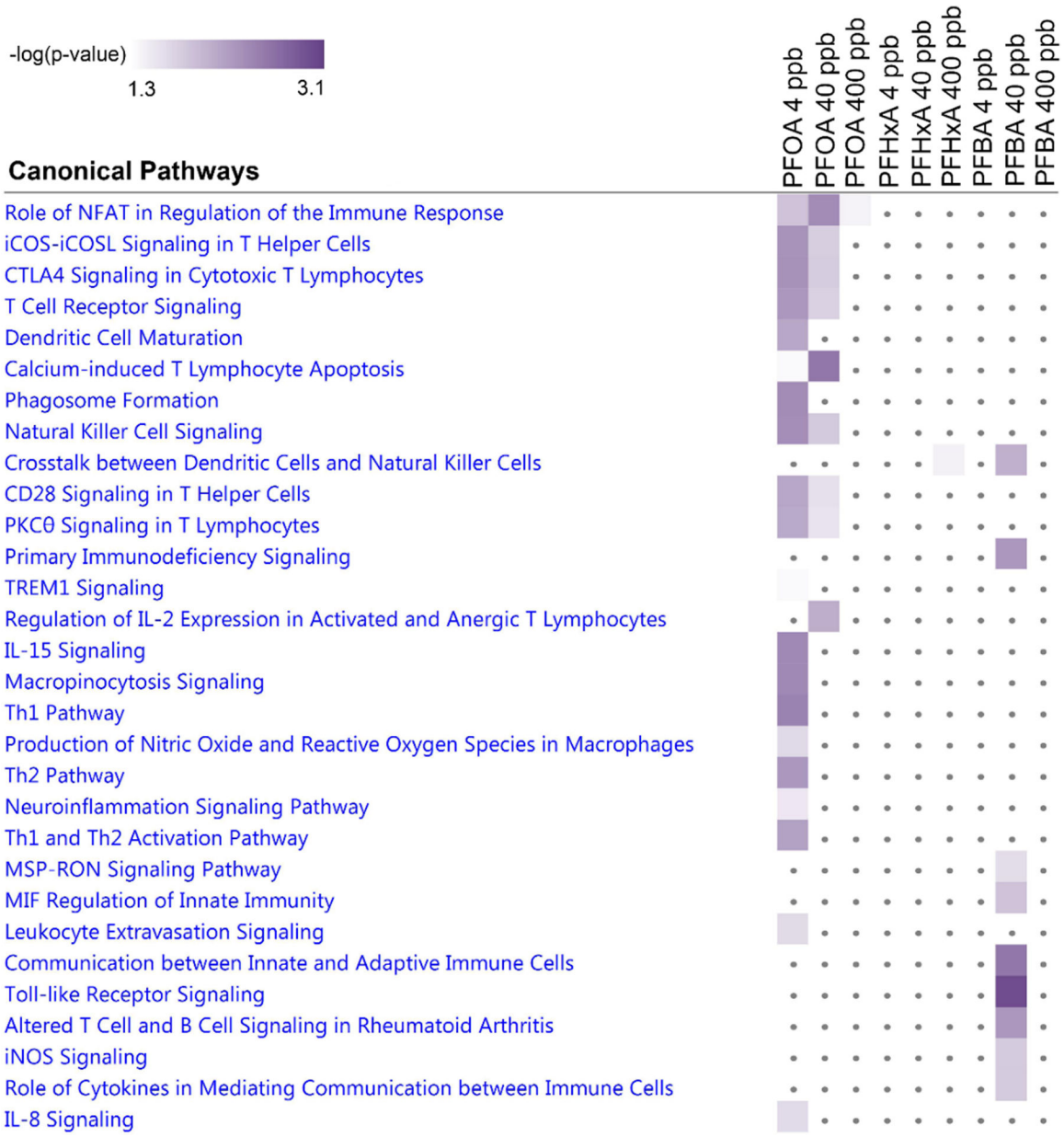

Fig. 5.

Comparison analysis showing canonical pathways related to cellular immune response following embryonic exposure to PFOA, PFHxA, or PFBA. Purple color indicates statistical significance (−log (p-value) = 1.3). Intensity of color correspond to – log (p-value).

Table 1

Morphological alterations at 72 hpf after embryonic exposure to PFOA, PFHxA, or PFBA.[a]

Concentration	Whole Body Length (μM)	Head Length (μM)	Head Width (μM)	Head Length/Body Length	Head Width/Body Length
PFOA					
0 ppb	3878.3 ± 133.0	650.3 ± 31.1	630.9 ± 34.1	0.168 ± 0.008	0.163 ± 0.006
4 ppb	3813.5 ± 152.4*	639.4 ± 33.3	616.4 ± 37.6*	0.168 ± 0.006	0.162 ± 0.006
40 ppb	3860.2 ± 176.4	642.0 ± 32.8	636.8 ± 32.9	0.166 ± 0.007	0.165 ± 0.006
400 ppb	3857.7 ± 197.5	623.0 ± 48.2*	619.4 ± 45.3*	0.162 ± 0.009*	0.161 ± 0.008
PFHxA					
0 ppb	3877.5 ± 105.9	663.9 ± 27.6	650.3 ± 24.8	0.173 ± 0.007	0.168 ± 0.006
4 ppb	3753.4 ± 104.4	656.7 ± 34.2	642.3 ± 28.9	0.171 ± 0.007	0.167 ± 0.006
40 ppb	3753.4 ± 101.9*	644.1 ± 36.2*	634.8 ± 30.7*	0.172 ± 0.001	0.169 ± 0.007
400 ppb	3891.4 ± 209.8	651.4 ± 47.9*	643.1 ± 39.9	0.168 ± 0.001*	0.165 ± 0.007
PFBA					
0 ppb	3782.5 ± 126.9	620.9 ± 38.9	624.9 ± 20.9	0.164 ± 0.008	0.165 ± 0.005
4 ppb	3706.4 ± 106.6*	607.3 ± 34.6	623.7 ± 26.3	0.163 ± 0.008	0.169 ± 0.007*
40 ppb	3773.5 ± 98.9	617.3 ± 32.4	632.7 ± 25.2	0.164 ± 0.008	0.168 ± 0.006*
400 ppb	3731.9 ± 115.1*	615.0 ± 36.1	634.7 ± 23.4*	0.165 ± 0.007	0.170 ± 0.005*

[a]Data are presented as mean ± standard deviation,

*$p < 0.05$ compared to the control group (0 ppb).

N = 3 with 20 subsamples per replicate to total 60 fish per treatment group.

Table 2

Disease and disorder enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA.[a,b]

PFOA			PFHxA			PFBA		
4 ppb								
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Cancer	4.40E-02–6.33E-04	29	Endocrine System Disorders	3.85E-02–1.58E-04	3	Nutritional Disease	1.05E-02–6.67E-05	16
Neurological Disease	1.37E-02–6.33E-04	10	Gastrointestinal Disease	4.35E-02–1.58E-04	22	Cardiovascular Disease	1.67E-02–1.06E-04	21
Organismal Injury and Abnormalities	4.40E-02–6.33E-04	31	Hereditary Disorder	4.60E-02–1.58E-04	7	Developmental Disorder	1.31E-02–1.06E-04	17
Reproductive System Disease	4.19E-02–7.88E-04	17	Metabolic Disease	4.35E-02–1.58E-04	5	Hereditary Disorder	1.31E-02–1.06E-04	24
Hematological Disease	4.34E-02–1.21E-03	12	Organismal Injury and Abnormalities	4.97E-02–1.58E-04	27	Metabolic Disease	1.57E-02–1.06E-04	23
40 ppb								
Cancer	4.92E-02–4.52E-04	68	Hematological Disease	2.42E-02–1.73E-04	13	Gastrointestinal Disease	2.07E-02–1.34E-04	107
Organismal Injury and Abnormalities	4.92E-02–4.52E-04	68	Immunological Disease	3.21E-02–1.73E-04	12	Organismal Injury and Abnormalities	2.07E-02–1.34E-04	136
Reproductive System Disease	4.65E-02–4.52E-04	48	Dermatological Diseases and Conditions	3.21E-02–1.93E-04	72	Neurological Disease	2.07E-02–2.20E-04	21
Infectious Disease	8.37E-03–5.99E-04	5	Organismal Injury and Abnormalities	3.21E-02–1.93E-04	90	Endocrine System Disorders	2.07E-02–2.38E-04	57
Endocrine System Disorders	4.12E-02–9.31E-04	34	Infectious Diseases	2.44E-02–3.85E-04	8	Renal and Urological Disease	2.07E-02–3.59E-04	20
400 ppb								
Cancer	4.76E-02–8.99E-05	57	Endocrine System Disorders	4.60E-02–6.49E-04	12	Cancer	1.66E-02–3.38E-06	422
Endocrine System Disorders	3.82E-02–8.99E-05	32	Gastrointestinal Disease	4.60E-02–6.49E-04	33	Dermatological Diseases and Conditions	1.25E-02–3.38E-06	281
Gastrointestinal Disease	4.19E-02–8.99E-05	50	Hereditary Disorder	3.47E-02–6.49E-04	9	Organismal Injury and Abnormalities	1.69E-02–3.38E-06	430
Organismal Injury and Abnormalities	4.97E-02–8.99E-05	57	Metabolic Disease	4.97E-02–6.49E-04	13	Nutritional Disease	1.90E-03–1.46E-05	52
Reproductive System Disease	4.76E-02–3.31E-04	33	Organismal Injury and Abnormalities	4.97E-02–6.49E-04	39	Hematological Disease	1.69E-02–2.38E-05	135

[a] p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b] Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 3

Physiological system development and function enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA.[a,b]

PFOA			PFHxA			PFBA		
4 ppb								
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Renal and Urological System Development and Function	3.97E-02–1.41E-05	3	Organismal Functions	2.71E-02–1.49E-03	3	Endocrine System Development and Function	1.73E-02–9.22E-05	10
Cell-mediated Immune Response	3.32E-02–9.65E-04	6	Connective Tissue Development and Function	4.97E-02–1.96E-03	5	Organ Development	1.11E-02–9.22E-05	19
Hematological System Development and Function	4.40E-02–9.65E-04	9	Embryonic Development	3.28E-02–1.96E-03	3	Behavior	1.62E-02–4.22E-04	29
Immune Cell Trafficking	3.75E-02–9.65E-04	8	Hematological System Development and Function	4.03E-02–1.96E-03	5	Hematological System Development and Function	1.48E-02–6.29E-04	15
Connective Tissue Development and Function	2.66E-02–2.25E-03	3	Hematopoiesis	3.66E-02–1.96E-03	1	Immune Cell Trafficking	1.04E-02–6.29E-04	3
			40 ppb					
Cell-mediated Immune Response	3.71E-02–3.39E-03	5	Embryonic Development	3.21E-02–1.93E-04	19	Nervous System Development and Function	2.07E-02–2.09E-05	14
Hematological System Development and Function	4.92E-02–3.39E-03	17	Organismal Development	3.21E-02–1.93E-04	24	Digestive System Development and Function	2.07E-02–1.34E-04	8
Immune Cell Trafficking	3.71E-02–3.39E-03	11	Tissue Morphology	3.21E-02–1.93E-04	20	Organ Morphology	2.07E-02–1.34E-04	25
Connective Tissue Development and Function	4.92E-02–4.19E-03	7	Hematological System Development and Function	3.21E-02–2.22E-04	26	Organismal Development	2.07E-02–1.34E-04	33
Digestive System Development and Function	2.08E-02–4.19E-03	1	Humoral Immune Response	3.21E-02–3.18E-04	14	Organ Development	2.07E-02–3.59E-04	18
			400 ppb					
Hematological System Development and Function	4.76E-02–5.78E-04	19	Connective Tissue Development and Function	4.97E-02–8.79E-05	10	Tissue Morphology	1.51E-02–1.28E-05	104
Immune Cell Trafficking	4.09E-02–5.78E-04	10	Embryonic Development	4.97E-02–2.42E-03	18	Hematological System Development and Function	1.63E-02–2.16E-04	86

Lymphoid Tissue Structure and Development	4.76E-02–1.81E-03	17	Hematological System Development and Function	4.97E-02–2.42E-03	11	Connective Tissue Development and Function	4.85E-03–2.61E-04	10
Hematopoiesis	3.68E-02–2.45E-03	8	Hematopoiesis	4.97E-02–2.42E-03	8	Skeletal and Muscular System Development and Function	1.07E-02–2.61E-04	12
Cell-mediated Immune Response	3.64E-02–2.70E-03	5	Humoral Immune Response	4.97E-02–2.42E-03	4	Tissue Development	7.66E-03–2.61E-04	31

[a] p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b] Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.

Table 4

Molecular and cellular function enriched pathways associated with genes altered following embryonic exposure to PFOA, PFHxA, or PFBA.[a,b]

PFOA			PFHxA			PFBA		
			4 ppb					
Pathways	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***	***Pathways***	***p-value range***	***No. of genes***
Cell Cycle	4.14E-02–1.41E-05	12	Molecular Transport	4.97E-02–2.11E-05	4	Cell Cycle	1.91E-02–4.95E-06	14
Cellular Development	4.19E-02–2.63E-04	16	Nucleic Acid Metabolism	1.55E-02–2.11E-05	2	Cellular Development	1.79E-02–9.22E-05	50
Cellular Growth and Proliferation	3.63E-02–2.63E-04	13	Small Molecule Biochemistry	4.97E-02–2.11E-05	5	Cellular Growth and Proliferation	1.67E-02–9.22E-05	47
Cellular Movement	4.34E-02–2.75E-04	18	Cell Cycle	2.13E-02–1.96E-03	2	Cell Morphology	1.31E-02–6.29E-04	18
Cell Death and Survival	4.40E-02–3.07E-04	17	Cell Morphology	3.85E-02–1.96E-03	5	Cell-to- Cell Signaling and Interaction	1.46E-02–6.29E-04	13
			40 ppb					
Cell Cycle	4.52E-02–2.52E-04	8	Cell Morphology	3.21E-02–1.93E-04	18	Cell-to- Cell Signaling and Interaction	2.07E-02–2.09E-05	13
Cell Death and Survival	4.76E-02–7.47E-04	10	Cellular Function and Maintenance	2.77E-02–2.22E-04	29	Cellular Assembly and Organization	2.07E-02–2.09E-05	16
Cellular Movement	3.85E-02–3.39E-03	11	Protein Synthesis	2.91E-02–3.18E-04	12	Lipid Metabolism	2.07E-02–2.09E-05	37
Amino Acid Metabolism	4.19E-03–4.19E-03	1	Cell Death and Survival	3.21E-02–3.85E-04	15	Molecular Transport	2.07E-02–2.09E-05	34
Carbohydrate Metabolism	4.92E-02–4.19E-03	5	Cellular Development	3.21E-02–3.85E-04	27	Small Molecule Biochemistry	2.07E-02–2.09E-05	46
			400 ppb					
Cellular Development	4.59E-02–5.78E-04	24	Cell Morphology	4.97E-02–8.79E-05	12	Drug Metabolism	1.04E-02–3.56E-07	18
Cell Signaling	4.59E-02–7.17E-04	10	Cellular Development	4.97E-02–8.79E-05	15	Lipid Metabolism	1.56E-02–4.82E-07	99
Molecular Transport	4.59E-02–7.17E-04	11	Cellular Growth and Proliferation	4.97E-02–2.42E-03	11	Molecular Transport	1.35E-02–4.82E-07	94
Vitamin and Mineral Metabolism	4.59E-02–7.17E-04	10	Carbohydrate Metabolism	4.22E-02–3.91E-03	9	Small Molecule Biochemistry	1.56E-02–4.82E-07	126
Cellular Function and Maintenance	4.59E-02–1.19E-03	16	Cell Cycle	4.60E-02–3.91E-03	6	Vitamin and Mineral Metabolism	1.41E-02–1.97E-06	50

[a] p-value range derived from the likelihood of observing the degree of enrichment in a gene set of a given size by chance alone.

[b] Number of genes are classified as being differentially expressed that relate to the specified function category; a gene may be present in more than one category.