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PFOS, PFNA, and PFOA Sub-Lethal Exposure to Embryonic Zebrafish Have Different Toxicity Profiles in Terms of Morphometrics, Behavior and Gene Expression

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Abstract

Polyfluorinated compounds (PFC) are a class of anthropogenic, persistent and toxic chemicals. PFCs are detected worldwide and consist of fluorinated carbon chains of varying length, terminal groups, and industrial uses. Previous zebrafish studies in the literature as well as our own studies have shown that exposure to these chemicals at a low range of concentrations (0.02 μ M – 2.0 μ M; 20–2000 ppb) resulted in chemical specific developmental defects and reduced post hatch survival. It was hypothesized that sub-lethal embryonic exposure to perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), or perfluorooctanoic acid (PFOA) would result in different responses with regard to morphometric, behavior, and gene expression in both yolk sac fry and larval zebrafish. Zebrafish were exposed to PFOS, PFOA, and PFNA (0.02, 0.2, 2.0 μ M) for the first five days post fertilization (dpf) and analyzed for morphometrics (5 dpf, 14 dpf), targeted gene expression (5 dpf, 14 dpf), and locomotive behavior (14 dpf). All three PFCs commonly resulted in a decrease in total body length, increased *tfc3a* (muscle development) expression and decreased *ap1s* (protein transport) expression at 5dpf, and hyperactive locomotor activity 14 dpf. All other endpoints measured at both life-stage time points varied between each of the PFCs. PFOS, PFNA, and PFOA exposure resulted in significantly altered responses in terms of morphometric, locomotion, and gene expression endpoints, which could be manifested in field exposed teleosts.

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Keywords

PFOS; PFNA; PFOA; morphometrics; zebrafish locomotion; *Danio rerio*

1. Introduction

Polyfluorinated compounds (PFCs) are anthropogenic, emerging contaminants of environmental concern. PFCs are composed of a long carbon backbone that is fully fluorinated with either a carboxyl, alcohol, or sulfonate terminal group (Conder et al. 2008). Long chain PFCs (greater than 8 carbons) were produced from the 1950s until 2000 (Lehmle 2005), when the manufacturers began a voluntary phase-out of the long chain PFC in favor of shorter chain compounds (6 carbons). Both the long chain and the replacement PFCs pose serious environmental concerns because of their persistence due to the carbon fluoride bonds and limited toxicity data for a number of the PFCs. The three main long chain PFCs that are most commonly found at elevated levels in the environment are perfluorooctanoic acid (PFOA; C8), perfluorooctane sulfonate (PFOS; C8) and perfluorononanoic acid (PFNA; C9) (Figure 1). At the time the phase-out began, it was estimated that in total 3500 metric tons of PFOS and 500 metric tons of PFOA were produced (Lau et al. 2007).

Large quantities of PFCs were produced since the 1950s because they have a wide range of applications (Lindstrom et al. 2011; Renner 2001). They are stable at very high temperatures, nonflammable and non-volatile (Lau et al. 2007). Additionally, they are not degraded by strong acids, bases, or oxidizing agents (Lau et al. 2007). In particular, PFOS has been used for industrial purposes such as fire-fighting foams and aviation hydraulic fluid as well as in consumer products such as water resistant coatings on clothing, furniture, and carpets (Renner 2001; Seacat et al. 2002). Both PFNA and PFOA were mainly used as an emulsifier for producing fluoropolymers (Lau et al. 2007). While these properties and uses made PFCs very appealing to manufacturers, they also caused PFCs to be non-degradable and in turn, persistent in the environment (Houde et al. 2006).

Although all three compounds are PFCs, the subtle structural differences shown in Figure 1 affect the toxicity, toxicokinetics, and biodynamics of each compound. The bioaccumulation and biomagnification of each PFC modeled by Houde et al. (2011) is based on the octanol-water partition coefficient (log K_{ow}). Additionally, the rate of bioaccumulation has been shown to increase as the carbon chain length (C8 to C13) of the PFC increases (Houde et al. 2011). PFCs containing a sulfonate end group are reported to bioaccumulate more than those of the same carbon chain length with a carboxylate end group. One explanation might be due to the sulfonate end group binding tighter to tissue proteins (Conder et al. 2008). PFNA and PFOA are also present in the biota to a lesser extent.

Even though PFCs have been mainly produced in North America and Asia, many have been detected in both the environment and animal tissue around the world, particularly PFOS and PFOA. These C8 compounds have been found in surface water in the Atlantic, central Pacific and eastern Pacific Ocean samples in the part per trillion range (Yamashita et al. 2005). PFOA, PFOS, and PFNA have been detected in the parts per trillion range in surface

water grab samples from the Delaware Rivers and the Delaware Bay estuaries (DRBC 2016). Fish fillets from these same waters contain a number of long chain and shorter chain PFCs. The concentrations used in this study span the range of surface water PFC concentrations (0.3 – 8.9 ppm) that have been reported from marsh habitats adjacent to the Wurtsmith Airforce base where a fire fighting school had been heavily contaminated with PFCs (Services 2013). The specific surface water concentrations for Clarks Marsh were PFOA 14–2200 ppt, PFOS 65–7400 ppt, PFNA <2.0– 85 ppt (Cooper 2015).

PFCs have been detected in animal tissue and plasma samples on nearly every continent and in a wide variety of animal species (Lindstrom et al. 2011). Pumpkinseed (*Lepomis gibbosus*) collected from the Clark's Marsh at Wurtsmith Airforce base had the following tissue concentrations in filet and liver: PFOA filet 1.86–1.25, liver 5.19–7.0; PFOS filet 3050–4210, liver 11300– 13900; PFNA filet 0.651–4.28, liver 2.03–12.0 (Cooper 2015). PFCs can bioaccumulate in the food chain (Lindstrom et al. 2011), and as a result species higher in the food chain often have increased levels of PFCs in their tissue (Kannan et al. 2002). Globally, PFOS is the most prevalent PFC found. Studies have shown PFOS in tissues of polar bears (180–680 ppb), river otters (340–990 ppb), albatrosses (<35 ppb), bald eagles (1–2570 ppb), fish (21–87 ppb), and many bird species in North America. In Europe, PFOS was detected in arctic seals (100 ppt) and Mediterranean fish, mammals, and birds (100– 270 ppt) and in Asia it was seen in dolphins, birds, and tuna (10–170 ppt). PFOS has even been detected in Antarctic wildlife in penguins and seals (Giesy and Kannan 2001). In that same study, PFOA was also analyzed, but very few animal samples were above the detection limit. PFNA was the highest PFC contaminant detected in Baikal seals in Russia (1000 ppt) (Houde et al. 2011).

In mammalian studies, decreased body weight and lipid metabolism defects in rodents were observed after exposure to PFOS (Seacat et al. 2003; Wang et al. 2014) and PFOA, (Biegel et al. 2001). These effects were also seen in PFOS exposure to monkeys (Seacat et al. 2002). An increase in liver size was reported for exposure to all three PFCs in mice (Das et al. 2015), as well as for PFOA exposure in birds (Mattsson et al. 2015). PFOS and PFOA exposure to juvenile mice resulted in hyperactivity and reduced habituation behaviors when in adulthood (Johansson et al. 2009). PFNA is less widespread, and fewer developmental and exposure studies are available.

In the present studies, we use a zebrafish embryo-larval toxicity paradigm to evaluate the developmental effects of PFOA, PFOS, and PFNA. The zebrafish has emerged as a powerful vertebrate model used to link the adverse developmental effects from environmental exposures with molecular endpoints to elucidate mechanisms of actions for toxicants *in vivo* (Bugel et al. 2014). Previous studies with zebrafish have demonstrated PFAs to be developmentally bioactive and teratogenic at high micromolar concentrations (Zheng et al. 2011).

Our studies report on the comparative toxicity profiles following sub-lethal PFOA, PFOS, and PFNA exposure (0, 0.02, 0.2, 2.0 μ M) to embryonic zebrafish. Control and continuously exposed 5 dpf zebrafish were evaluated for morphometric endpoints including total body length, area of yolk sac, and interocular distance. Gene expression was also analyzed in

both 5 days post fertilization (dpf) and 14 dpf juveniles. Swimming activity analysis included the following: total distance traveled, thigmotaxis, and swimming velocity. The data collected show that even minor structural differences between the three tested PFCs resulted in different toxicity profiles and effects on gene expression.

2. Methods

2.1 Zebrafish Husbandry and Exposure Protocols

The AB strain zebrafish (Zebrafish International Resource Center, Eugene, OR) were used for all experiments. Breeding stocks were bred and housed in Aquatic Habitats (Apopka, FL) recirculating systems under a 14:10 hour light:dark cycle. System water was obtained by carbon/sand filtration of municipal tap water and water quality was maintained at <0.05 ppm nitrite, <0.2 ppm ammonia, pH between 7.2 and 7.7, and water temperature between 26 and 28°C. All experiments were conducted in accordance with the zebrafish husbandry protocol and embryonic exposure protocol (#08-025) approved by the Rutgers University Animal Care and Facilities Committee. Zebrafish embryos were exposed at 3 hours post fertilization (hpf) to PFOS, PFOA, or PFNA at concentrations of 0, 0.02 µM, 0.2 µM, or 2.0 µM (0, 20, 200, 2000 ppb) for 120 hours in a static non-renewal protocol. All compounds were dissolved in water. After this time, fish were transferred to non-treated system water and fed 2 times daily with Zeigler Larval AP50 (Aquatic Habitats, Apopka, Florida). Therefore, the only exposure was through the water from 3hpf to 120hpf (5 days), which corresponds to embryonic to yolk sac larval exposure.

Shown in Figure 2 is the exposure and data collection timeline. Zebrafish embryos were exposed to PFOS, PFOA or PFNA (Sigma-Aldrich, St. Louis, MO) from 3 hpf until 120 hpf. The exposure followed modified OECD 212 protocol (OECD, 2011), where in addition to the endpoints of lesion presence, length, weight, and mortality as stated in the protocol, cranial facial development and gene expression were also analyzed. At 120 hpf, morphometric measurements were recorded and gene expression analyzed. Further modification to the OECD protocol was to extend the study beyond the exposure timepoints which allowed for removing any chemical exposure from 120hpf to 14 dpf. Morphometric measurements were also taken at 7 days post fertilization (dpf) and 14 dpf. At 14 dpf, gene expression data and swim activity endpoints were collected. Each treatment compound and corresponding control group was set up as individual experiments, and the sample size was dependent on number of embryos produced from the stock breeding sets. No experiment had mortality greater than 20% of the starting sample size.

2.2 Morphometric Analysis

Approximately thirty individual animals from each treatment and control group were fixed in formalin and then stained for bone and cartilage following a two-color acid free Alcian Blue/ Alizarin red stain (Walker and Kimmel 2007). Photographs were taken using a Scion digital camera model CFW-1310C mounted on an Olympus SZ-PT dissecting microscope and cartilage/bone were measured using Adobe Photoshop. Endpoints examined included total body length, interocular distance, and yolk sac size to assess larval growth, cranial

facial development, and nutrient storage and usage, respectively. Measurements could be made at the nanometer level. Each experiment was independently replicated three times.

2.3 Swim Activity Assay

Four replicates of each treatment and control each consisting of 25 animals were exposed for 120 hours and then transferred to clean water until they were two weeks old. The swim activity was performed in 24 well plates with a single animal in each well. After 1 hour incubation under fluorescent light, the light was turned off and zebrafish recorded with an infrared filter for 30 minutes. The recordings were analyzed with Noldus Ethovision Software (Leesburg, VA) for endpoints of total distance traveled, average swim velocity, and time and frequency of swimming in the middle of the well (Figure 3). The total distance traveled and swimming velocity measurements are indicators of general locomotion and activity. The time and frequency in the middle of the well is a measurement of stress and anxiety (Schnorr et al. 2012). These innate behaviors have been assumed to play important roles in predator-prey interactions (Kalueff et al. 2013). Control and each treatment group had approximately N=50 fish/replicate. Each experiment was independently replicated twice.

2.4 Gene Expression Analysis

Four replicates (N=25 fish/replicated) from each treatment and control were snap frozen in liquid nitrogen and RNA extracted using RNeasy reagent (Sigma-Aldrich, St. Louis, MO). DNA contamination was removed with the DNA-free™ kit (Life Technologies). Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) and real-time qPCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules CA). The qPCR protocol was used: 35 cycles of: 95°C for 15 seconds and 60°C for 1 minute. The housekeeping gene used was *b-actin*, which has been determined to be unaffected by any treatments in this study. Analysis was performed using a standard curve method. The four genes examined and primer sequences are listed in Table 1. Each independent experiment was replicated 3 times.

To analyze a broader selection of transcripts, messenger RNA expression was analyzed for a suite of 100 developmentally relevant transcripts using qRT-PCR methods previously described (Bugel et al. 2014). Transcripts selected for this analysis were broadly part of pathways involved in tissue remodeling, calcium signaling, cell cycle and cell death, growth factors, angiogenesis and hypoxia (Suppl. Table 1). For this gene expression analysis, embryonic zebrafish were exposed to 2.0 uM of PFOA, PFOS or PFNA until 120 hpf. Animals were observed daily and no lesion occurrence was recorded. Four replicates (N=25 animals/replicate) were snap frozen as whole animal pool replicates at 120 hpf and analyzed. Briefly, total RNA was isolated using RNeasy® RT (Molecular Research Center, Inc., Cincinnati, OH) and complementary DNA was synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA). qRT-PCR was performed using a StepOnePlus™ Real-Time PCR System with Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). All primers used are listed in (Bugel et al. 2014). β -*actin* was used as a housekeeping transcript for normalization, and relative expression was quantified using the $\Delta\Delta$ Ct method (Pfaffl 2001).

2.6 Statistical Analysis

All statistical analysis were performed using SigmaPlot™ (v. 11.0) and R (v. 3.2.2). Morphometric measurements were analyzed using a one-way analysis of variance (ANOVA). Swim activity was analyzed using a two way ANOVA based on treatment and 5-minute time intervals. Gene expression data was evaluated using either ANOVA, or Student's t-test when the data passed normality and variance tests. If the data was not normal, a log transformation was used, and t-test performed. After log transformation, if data cannot be normalized, a Wilcoxon test was used. Statistical significance was at a p-value 0.05

3. Results and Discussion

3.1 Morphometric Data

Morphometric endpoints of interocular distance, total body length, and yolk sac area were assessed to determine if exposure to PFOS, PFOA, or PFNA affected embryonic development. For all treatment groups, all concentrations (0.02, 0.2, 2.0 μM) were sub-lethal, and there was no significant difference for the prevalence of death, embryonic abnormalities, or delayed development. Summary of all measurements for each compound can be seen in Table 2.

The 5 dpf total body length measurement was used to determine if exposure to PFOS, PFOA or PFNA during the embryonic life stages had an effect on larval growth. PFOA, PFOS, and PFNA all resulted in significantly reduced body length at the 2.0 μM treatment. Additionally, PFOS exposure at 0.2 μM also significantly reduced the total body length. No significant differences were observed at lower concentrations of PFOA, PFOS, or PFNA. The data presented in Figure 4 plot the total body length for each compound at 5, 7 and 14 dpf.

Interocular distance was used to indicate changes in craniofacial development following embryonic PFC exposure (Table 2). PFOA treatment at 2.0 μM resulted in a significant increase of interocular distance, while PFOS at all concentrations (0.02 μM , 0.2 μM , 2.0 μM) significantly decreased interocular distance. PFNA exposure at all doses, and PFOA exposure at the lower concentrations had no effect on this measurement.

The yolk sac is comprised of vitellogenin derived yolk-proteins, maternally supplied by the oocyte to fully support nutritional needs of the embryo/larvae prior to beginning feeding after 120 hpf. Measuring the yolk sac size is an important endpoint to determine if PFOS, PFOA, or PFNA affected the volume of the available nutrients and utilization in embryonic zebrafish. PFOS (2.0 μM) treated zebrafish had a significantly decreased yolk sac size, while PFOA (2.0 μM) and PFNA (2.0 μM) treated animals both had a significantly increased yolk sac size (Table 2).

3.2 Swim Activity

Swim activity data were collected in five minute time bins for a total of 25 minutes. Cumulative data for each measurement are listed in Table 3. PFOS (0.02 μM , 2.0 μM) PFOA

(0.2 μ M and 2.0 μ M) and PFNA (0.2 μ M) exposure caused a significant increase in the distance traveled.

Swimming velocity is a measurement used to assess the average swimming speed of zebrafish for the duration of the activity assay (Table 3). Data points were obtained in five minute intervals. PFOS (0.02 μ M) and PFNA (0.02, 0.2, 2.0 μ M) exposure both resulted in a significant decrease in swimming velocity. In PFOA exposure (2.0 μ M) swimming velocity was significantly increased.

Zebrafish can exhibit thigmotaxis (movements towards or away from a stimulus) as a stress response to new environments. The measurement of time spent swimming in the middle of the assay well and the number of times the animal swam across the well is used as an indicator of stress or anxiety (Blaser et al. 2010). PFNA exposure (0.02, 0.2, μ M) significantly increased the time spent in middle of the well. PFOS and PFOA treatment had no significant effects on this endpoint. PFOS (0.02, 0.2, 2.0 μ M) showed a significant increase in crossings. PFNA and PFOA did not show any significant effects on this endpoint.

3.3 Two Week Growth Curve

The total body length of PFOS, PFNA, PFOA treated zebrafish were measured at three time points during development; 5 days, 7 days, and 14 days post fertilization (Figure 4). At 5 dpf there was a significant decrease in total body length for PFOS (0.2 μ M, 2.0 μ M), PFNA (2.0 μ M) and PFOA (0.2, 2.0 μ M) (Table 1). PFOS exposed animals were not significantly different from the controls at the 7 and 14 dpf timepoints (Figure 4A). PFNA exposure resulted in decrease body length at the 7 dpf timepoint (2.0 μ M), but at 14 dpf the larvae were significantly larger (Figure 4B). PFOA exposed animals (Figure 4C) remained significantly smaller than the controls at all time points and exhibited very little growth between 7 and 14 dpf.

3.4 Gene Expression Data 120 hpf

Targeted gene expression was analyzed for organic anion transporter 2b1 (*slco2b1*) and striated muscle development (*tfc3a*) transcripts in 120 hpf zebrafish across all concentrations (Figure 5). *Slco2b1* was significantly upregulated in PFOS (2.0 μ M) and PFOA (all treatments), and significantly down-regulated in PFNA (all concentrations). *Tfc3a* expression was significantly higher in PFOS (2.0 μ M) and PFOA (0.2, 2.0 μ M). *Ihha* (hedgehog gene) (data not shown) showed no significant difference in expression at any PFC concentration. *Wnt5b* (calcium modulation pathway) (data not shown), had a significant increase in expression after PFOS exposure (2.0 μ M, fold change +2.18).

Gene expression was performed on a battery of genes involved in tissue remodeling, cell cycle, cell death, angiogenesis, hypoxia, calcium signaling, and growth factors (Supplemental Figure 1). Genes that were significantly different in expression after exposure to PFOS, PFOA, or PFNA are listed in Table 4 below. Of the 106 genes analyzed, *ap1s1* was the only gene that was significantly different (decreased) across all three PFCs. Of the three compounds, PFOS significantly affected the greatest number of genes (*calm3a*, *cdkn1a*, *cyp1a*, *flk1*, *tgfb1a*). PFOA and PFNA each only significantly altered one gene (*c-fos* and *tgfb1a*, respectively).

3.5 Gene Expression Data 14 dpf

Organic anion transporter 2b1 (*slco2b1*) and striated muscle development (*tfc3a*) transcripts were analyzed in 14 dpf zebrafish (Figure 6). *Slco2b1* was significantly upregulated in PFOS (0.2 μ M, 2.0 μ M), PFOA (2.0 μ M), and PFNA (0.02 μ M, 0.2 μ M). *Tfc3a* expression was significantly higher in PFOS (all treatments) and PFOA (2.0 μ M)

4. Discussion

The toxic effects following PFOA, PFNA, and PFOS exposure to embryonic and larval zebrafish have different biomarker profiles. It is likely that both the carbon chain length and the terminal group play a role in the observed effects on morphometrics, gene expression, and behavior. A number of these changes are reported in this paper at PFC concentrations ranging from 5.0–25.0 fold below our previously calculated LC50 values (PFOS 25 μ M, PFNA 10 μ M, PFOA 35 μ M), and, to our knowledge, at lower concentrations than previously reported in the teleost literature. The exposure to the developing embryos and yolk sac fry (exposure 3–120 hpf, Figure 1) resulted in significant changes that were also observed at 120 hpf and persisted for up to 7 and 14 days in larva no longer being exposed through water. This would suggest that some biochemical and physiological pathways were sufficiently altered to cause more permanent effects without the direct, continuous, waterborne exposure to the compounds. However, considering the tissue half-life for these compounds in zebrafish are not known there could be residual PFCs contributing to these effects.

4.1 Compound Specific Toxicity or Behavioral Modifications

Figure 6A and 6B (below) is a Venn diagram representing each compound studied and the endpoints that were significantly changed at 5 dpf and 14 dpf, respectively. PFOS exposure resulted in the greatest number of significantly altered endpoints. Behavior analysis at 14 dpf showed an increase in the middle crossing frequency. In terms of morphometrics, PFOS was unique in decreasing the yolk sac size and interocular distance (Table 3). Gene expression changes after PFOS exposure were an increase in *calm3a* (calcium ion binding), and a decrease in *cdkn1a* (cell cycle regulations), *cyp1a* (aromatic compound metabolism), and *flk1* (angiogenesis). The changes in these genes and their downstream pathways are critical for normal development. The relationship between gene expression and the possible effects on morphometric and behavior outcomes is summarized in Table 5.

PFOA exposure resulted in an increase in expression of the *c-fos* (transcription factor complex) transcript, an increase in interocular distance, and a decrease in total body length at 14 dpf. *C-fos* is a transcription factor complex that is involved in stress response and regulation of neuronal excitability in the central nervous system (Buhrke et al. 2015). This gene is often induced as a result of seizures or other stress response situations. PFOA exposures in vitro (human hepatocytes) (Buhrke et al. 2015) and in mice have also resulted in an increase in *c-fos* expression (Cheng et al. 2013). After activation, *c-fos* forms into a heterodimer with Jun family proteins that then activate the AP-1 protein pathway (Jin et al. 2002), which plays an important role in larval growth and protein transport in zebrafish. This gene pathway could be one possible way to explain the increase in swimming activity

observed due to disruptions in the central nervous system, as well as play a role in the decreased body length due to impacts of protein transport during development.

PFNA exposure resulted in the same number of significantly altered endpoints as PFOA. PFNA exposed zebrafish showed a decrease in *slco2b1* (organic anion transporter), decrease in velocity and an increase in the time spent in middle of the well as well as the total body length at 14 dpf.

4.2 Overlapping Endpoints Between Two Compounds

PFOS and PFOA shared two transcripts, *tfc3a* (striated muscle, 14 dpf), and *slco2b1* (organic anion transporter, 5 dpf) that were both significantly elevated. No other significantly endpoints were shared between only these two compounds.

PFOS and PFNA both resulted in a decrease of *tgfb1a* (growth activity factor). *Tgfb1a* is responsible for growth factor activity and knocking down this gene results in disrupted lateral line formation (Xing et al. 2015). The lateral line of the zebrafish is important in sensing water flow and obstacles while swimming, and a defect in development effects their swim ability and energy efficiency (Yanase et al. 2012). This correlates with the observed decrease in total body size, as the animal would need to expend more energy to swim rather than for growth. This could also explain the reduced swimming velocity in exposed animals. However, this change in gene expression was not seen in the PFOA exposed fish, but their body size was significantly decreased at all of the time points in this study. This indicates that either PFOA is interacting with a different pathway than PFOS or PFNA to affect total body size, or that there are multiple pathways being affected that result in this endpoint.

PFNA and PFOA both significantly increased the yolk sac size, but did not share any other endpoints between only these two compounds. An increase in yolk sac size could indicate a disruption of the transport of essential proteins from the yolk sac for growth. However, this could also indicate edema, where the yolk sac is larger due to fluid accumulation rather than stored proteins.

4.3 Significantly altered endpoints in PFOS, PFOA and PFNA

In the morphometric endpoints examined, the total body length was the single measurement in which all three PFCs resulted in a similar outcome. PFOS, PFOA, and PFNA all resulted in a decrease in the total body length. In similar mammalian studies, PFOS and PFOA exposure were reported to decrease the body weight of treated mice (Berthiaume and Wallace 2002).

Only one transcript (*ap1s1*) of the 106 measured was significantly decreased for all three compounds. *Ap1s1* is involved in extracellular matrix organization and acts as a protein transporter during development (Montpetit et al. 2008). This gene is also responsible for protein cargo sorting and vesicular trafficking between organelles within the cell. When *ap1s1* was knocked down in zebrafish, larvae were significantly smaller in size and had many other developmental defects including disorganized fin structure and severe motor deficits (Montpetit et al. 2008). In this study, zebrafish exposed to PFOA, PFOS, or PFNA were smaller in total length. There were also changes in locomotion and swimming activity,

which could be a result of fin structure. The *ap1s1* transcript could be a critical gene in the alterations on growth to partially explain the decrease in body size observed for all three PFCs. Genes commonly associated with *ap1s1* such as *c-jun* and many matrix metalloproteinase (*mmps*) were not significantly altered. This would suggest there are some non-traditional targets being affected and a more global method of expression analysis would be needed to detect these targets.

Exposure to all three PFCs significantly altered the yolk sac size; PFOA and PFNA caused a significant increase, while PFOS resulted in a significant decrease. At this stage of development (larval, 5 dpf), the zebrafish are not feeding, have no external food source, and are reliant on only their yolk sac for nutrients. A change in yolk sac size would indicate a disruption with nutrient storage, transport, and/or utilization, which could be a result of the down-regulation of *ap1s1*. However, additional pathways (i.e. *c-fos* in PFOA, *tgfb3a* in PFNA) and genes not included in the transcripts analyzed can also play a role in determining if the yolk sac is larger (PFOA, PFNA) or smaller (PFOS) than controls. Further quantification of the lipid components, which comprises the yolk sac, may indicate other affected pathways.

Tcf3a expression was significantly increased in all PFCs at 5 dpf. *Tcf3a* is involved in striated muscle development, and can allow expression of genes that are responsible for eye and brain formation in embryonic zebrafish. A knockdown of this gene causes a “headless” phenotype, in which the eyes and brain of the embryos do not develop (Kim et al. 2000). The increased expression of this gene could also be affecting these downstream developmental pathways, and it could correlate to the increase in interocular distance in PFOA exposed zebrafish and decrease in interocular distance in PFOS animals depending on which pathways and how they were altered. The interocular distance may be affected by several independent alterations, including brain size, cranial formation, and edema.

All three PFC exposures resulted in an increase in swimming activity at 14 dpf, which correlates to previous studies indicating hyperactivity in zebrafish larvae exposed to PFOS (Spulber et al. 2014). However, this does not correlate with the difference in total body size (PFOA significantly smaller, PFNA significantly larger, PFOS no change). PFOS fish appear to be able to recover and obtain the nutrients needed for normal growth in spite of the fact that they are possibly expending more energy for increased swimming activity. PFOA and PFNA both appear to exhibit a disruption with nutrient storage, transport, or utilization. PFOA exposed animals are expending energy on swimming increased distances but the slope of the growth was reduced compared to the controls (Figure 4C). PFNA exposed animals had decreased in total initially, but were then significantly larger than the 14 day control fish.

Organic anion transporters are responsible for the transport of many substance into and out of cells, including bile acids, steroid hormones, thyroid hormones, taurocholate, statins, and xenobiotics. In mammals, PFCs of various chain length and end groups have been shown to be both an inhibitor and a substrate for these transporters (Yang et al. 2010). In zebrafish, PFOS has been shown to be a substrate of the *Slco1d1* transporter, while PFOA has been shown to be an inhibitor of this transporter (Popovic et al. 2014). Both of these can interfere

with the normal transport functioning by either competing or inhibiting transport of the natural substrates. The natural substrates of *Slco1d1* include conjugated steroid hormones such as dehydroepiandrosterone and estrone sulfate. These substrates have been shown to be important in bone formation, maturation, and homeostasis (Muir et al. 2004). At 5 dpf, the organic anion transporter *slco2b1* was significantly increased in PFOS and PFOA exposed animals, and significantly decreased in PFNA exposed animals. Therefore, disruption in uptake of the preferred substrate due to PFC hindrance or inhibition could lead to deficiencies in the bone development, and in turn impact endpoints such as total body length and craniofacial formation (interocular distance) observed in this study. At 14 dpf, *slco2b1* was significantly up-regulated by all PFCs tested, however each PFC resulted in a different outcome regarding total size (PFOA decrease, PFNA increase, PFOS no change). This suggests that while *slco2b1* could be playing an important role in decrease in total body size at 5dpf, there could be other transporters or pathways that are contributing to either the recovery or ongoing effect at 14dpf.

4.4 Conclusions

The data presented in this study support the hypothesis that sub-lethal embryonic exposure to PFOS, PFNA, or PFOA will result in different responses in regards to morphometric, behavior, and gene expression in both yolk sac fry and larval zebrafish. All three PFCs commonly resulted in a decrease in total body length, increased *tfc3a* (muscle development) expression and decreased *ap1s* (protein transport) expression at 5dpf, and hyperactive locomotor activity 14 dpf. All other endpoints measured at both life-stage time points varied between each of the PFCs.

At 5 dpf, PFCs are having subcellular effects, which are being translated into morphological and behavioral effects at concentrations well below the lowest observed sub-lethal concentrations (PFOS 20 uM, PFOA 30 uM, PFNA 5 uM). PFOS was more potent than PFOA and PFNA in altering gene expression, growth, behavior and yolk sac utilization. This correlates to studies in many other organisms including daphnia, medaka, rats, and aquatic invertebrates (Cui et al. 2009; Ji et al. 2008; Li 2009). PFOS had the greatest number of significant detrimental outcomes in the endpoints studied. While PFOA exposure at 5dpf had a smaller number of significant endpoint effects, at 14dpf it had the most persistent effect on growth in the juvenile zebrafish.

Our studies have focused on the embryo to juvenile life stages, but additional studies are needed to determine what the effects of altered nutrient transport, production, and storage will be later in life in adult animals as well as in the subsequent unexposed generation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- PFOS, PFOA, and PFNA exposure in zebrafish embryos caused changes in gene expression, and morphological and behavioral effects
- All three compounds significantly decreased total body length and expression of *tcf3a* and *ap1s1* at 5dpf and hyperactivity at 14dpf.
- PFOA, PFOS, and PFNA caused different effects on other endpoints examined.
- PFCs appear to affect transport pathways including *ap1s1* and organic anion transporters (OATps) that may explain the morphological effects

Figure 2.

Exposure timeline of embryonic zebrafish to PFOS, PFOA or PFNA. All exposures occurred between 3 and 120 hpf. Exposure time period is indicated by dashed line.

Figure 3.

An image of a 24-well plate used to analyze swim activity. Each well contained one animal. Time spent in the middle of the well was calculated by recording the time in seconds that the animal was detected in the central area labeled “middle”. The number of crossing was how many times the fish crossed into or out of the middle circle.

Figure 4.

Growth curve of [A] PFOS, [B] PFNA, [C] PFOA exposed embryos from 5–14 days post fertilization (dpf). Measurements of total body length were taken at 5, 7, and 15 dpf. Statistical significance was determined using a one-way ANOVA at each time point for each compound ($p < 0.05$). PFOS 0.2 and 0.02 μM treated fish were significantly smaller in size at the 5dpf timepoint. PFNA fish at the 2.0 μM concentration were significantly smaller at 5dpf but significantly larger at 14 dpf. PFOA fish at 2.0 μM were significantly smaller at 5dpf and

7dpf, and all treatments were significantly smaller at 14dpf. No other significant differences were observed.

Figure 5. Embryonic zebrafish gene expression (5dpf) after exposure to [A] PFOS, [B] PFNA, and [C] PFOA. Bars represent mean fold change and standard deviation. N= 4 replicates of 25 pooled animals for each exposure group. An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA.

Figure 6.

Embryonic zebrafish gene expression (14dpf) after exposure to [A] PFOS, [B] PFNA, and [C] PFOA for 120 hours and remove to control water from 5 to 14 dpf. Bars represent mean fold change and standard deviation. N= 4 replicates of 25 pooled fish for each exposure group. An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA.

14 dpf

Figure 7.

Venn diagram of morphometric, gene expression, and swimming activity endpoints for PFOS, PFOA and PFNA exposure at all concentrations examined for [A] 5 days post fertilization (dpf) endpoints and [B] 14 dpf endpoints. Up arrows (↑) indicate significant increase compared to control ($p < 0.05$). Down arrows (↓) indicate a significant decrease compared to control ($p < 0.05$).

Table 1

List of transcripts and primer sequences for gene expression analysis.

Gene Symbol	Gene Name	Primer Sequences
<i>slco2b1</i>	Solute carrier organic anion transporter 2b1	F: 5'- TTG CCC TGC CTC ACT TCA TT-3' R: 5'-AGG CTG GAG TTG AGT CTG GT-3'
<i>tfc3a</i>	Transcription factor 3a	F: 5'-TGA GAA ACC GCA GAC CA ACT -3' R: 5'-CTT GCT GCT CCA GGT TGA GA-3'
<i>lhha</i>	Indian hedgehog homolog a	F: 5'-TGA GTC CAA AGC TCA CAT CCA-3' R: 5'-AGG CTG GAA AAC AAC CAC CG-3'
<i>Wnt5b</i>	Wingless-type MMTV integration site family 5b	F: 5'-GCA AAG CCA TCT TTC CCT GAA-3' R: 5'-TGT ATC CCG AGC AAA AAC CTG-3'

Table 2

Summary of morphometric endpoints measured in 5 days post fertilization (dpf) zebrafish after exposure to PFOS, PFOA, or PFNA.

Total Body Length (mm)				
	Control	0.02	0.2	2.0
PFOS (N=26–29)	4.76±0.23	4.75±0.16	4.63±0.22 *	4.67±0.23 *
PFNA (N= 20–23)	4.72±0.24	4.74±0.167	4.64±0.19	4.63±0.11 *
PFOA (N= 30–38)	4.79±0.12	4.83±0.11	4.83±0.22	4.68±0.13 *
Interocular (mm)				
	Control	0.02	0.2	2.0
PFOS (N=26–29)	0.23±0.02	0.21±0.02 *	0.22±0.01 *	0.22±0.02 *
PFNA (N= 20–23)	0.24±0.03	0.24±0.02	0.23±0.02	0.23±0.03
PFOA (N= 30–38)	0.18± 0.05	0.19±0.04	0.19±0.03	0.20±0.04 *
Yolk Sac Area (mm ²)				
	Control	0.02	0.2	2.0
PFOS (N=26–29)	0.45±0.06	0.48±0.08	0.47±0.04	0.43±0.06 *
PFNA (N= 20–23)	0.43±0.04	0.43±0.04	0.43±0.03	0.45±0.05 *
PFOA (N= 30–38)	0.48±0.06	0.50±0.08	0.49±0.04	0.55±0.08 *

Values are the average ± standard deviation from the mean.

An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA compared to corresponding control.

Table 3

Summary of swim activity endpoints.

Distance Traveled (mm)				
	Control	0.02	0.2	2.0
PFOS (N=24–35)	88.75±9.31	98.15±12.95 *	95.63±10.41	108.15±11.22 *
PFNA (N= 14–27)	78.40±6.87	73.78±7.89 *	83.01±9.06	79.55±10.36
PFOA (N= 30–38)	93.39±9.95	104.50±12.41	97.24±10.72 *	106.95±11.23 *
Time in Middle of Well (seconds)				
	Control	0.02	0.2	2.0
PFOS (N=24–35)	166.02±22.84	229.60±41.69	209.43±32.68	169.70±31.35
PFNA (N= 14–27)	211.39±33.34	200.44±27.67	243.53±30.83 *	241.92±29.68 *
PFOA (N= 30–38)	208.74±37.57	195.78±29.88	195.84±30.43	193.91±37.43
Crossing Frequency (crosses/ 25 minutes)				
	Control	0.02	0.2	2.0
PFOS (N=24–35)	71±10	76±9 *	83±13 *	78±10 *
PFNA (N= 14–27)	84±10	73±8	67±7	79±9
PFOA (N= 30–38)	74±9	88±13	85±12	90±12
Velocity (mm/s)				
	Control	0.02	0.2	2.0
PFOS (N=24–35)	0.39±0.08	0.37±0.10	0.42±0.15	0.43±0.14 *
PFNA (N= 14–27)	0.42±0.14	0.36±0.08 *	0.35±0.09 *	0.37±0.07 *
PFOA (N= 30–38)	0.42±0.10	0.43±0.10	0.41±0.09	0.45±0.14

Values are the average ± standard deviation from the mean.

An asterisk (*) indicates a statistical significant value, p< 0.05, one-way ANOVA compared to corresponding control.

Table 4

List of genes that were significantly increased or decreased in transcript analysis of 120 hpf zebrafish exposed to PFOS, PFOA, and PFNA (2.0 μ M).

Gene symbol	Gene Name	Function	Compound	Fold Change $\pm$ SD
<i>ap1s1</i>	Adaptor related protein complex 1, sigma subunit 1	Protein transport	PFOS	0.58 $\pm$ 0.14 **
			PFOA	0.59 $\pm$ 0.21 **
			PFNA	0.57 $\pm$ 0.09 **
<i>calm3a</i>	Calmodulin 3a	Calcium ion binding	PFOS	1.17 $\pm$ 0.06 *
<i>cdkn1a</i>	Cyclin-dependent kinase inhibitor 1A	Apoptosis, mitotic cell cycle regulation	PFOS	0.72 $\pm$ 0.13 *
<i>cyp1a</i>	Cytochrome P450 1A	Aromatic compound metabolism	PFOS	0.60 $\pm$ 0.20 *
<i>flk1</i>	Kinase insert domain receptor like	angiogenesis	PFOS	0.66 $\pm$ 0.17 *
<i>tgfb1a</i>	Transforming growth factor beta 1a	Growth factor activity	PFOS	0.82 $\pm$ 0.07 *
			PFNA	0.82 $\pm$ 0.09 *
<i>c-fos</i>	v-fox FBJ murine osteosarcoma viral oncogene homolog Ab	Transcription factor complex	PFOA	1.63 $\pm$ 0.19 *

A list of all genes analyzed can be viewed in supplemental figure 1.

An asterisk (*) indicates a statistical significant value $p < 0.05$.

A double asterisk (**) indicates a statistical significant value $p < 0.01$.

Table 5

Critical genes and the relationship to morphological and behavior endpoints observed.

Gene symbol	Gene Name	Gene Function*	Possible Morphological / Behavioral Endpoint
<i>ap1s1</i>	Adaptor related protein complex 1, sigma subunit 1	Protein transport, transcription factor complex	Body size, protein transport; disruption can cause nutrient deficiency during development
<i>tgfb1a</i>	Transforming growth factor beta 1a	Growth factor activity, lateral line formation	Body size, Swimming activity; disruption in lateral line can result in swimming impairment, sensing prey and swimming energy usage
<i>c-fos</i>	v-fox FBJ murine osteosarcoma viral oncogene homolog Ab	Transcription factor complex	Multiple endpoints; effects on many downstream pathways that may affect normal development
<i>slco2b1</i>	Solute carrier organic anion transporter 2b1	Organic anion transporter	Body size; disruption of normal transport of substrates and hormones; altering endogenous substrate pharmacokinetics
<i>tfc3a</i>	Transcription factor 3a	Striated muscle development	Interocular distance; defects in head and brain formation can result in cranial facial deformities

* This is discussed in further detail in the text