# Toxicological Profiles of Perfluoroctanoic Acid (PFOA), Perfluorooctane sulfonate (PFOS) and Perfluornonanoic acid (PFNA) in zebrafish (*Danio rerio)*

By

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# ABSTRACT OF THE DISSERTATION

Toxicological Profiles of Perfluoroctanoic Acid (PFOA), Perfluorooctane sulfonate (PFOS) and Perfluornonanoic acid (PFNA) in zebrafish (*Danio rerio)*

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Perfluorooctane sulfonate (PFOS), perfluorononanoic acid (PFNA), and perfluorooctanoic acid (PFOA) are all members of the anthropogenic and persistent perfluoroalklyated class of compounds (PFASs). These compounds have similar structures and have been commonly grouped together in toxicity, treatment, and disposal analyses. It was hypothesized that PFOS, PFNA, and PFOA would result in similar toxicity profiles throughout different life stages of zebrafish (*Danio rerio*). Zebrafish were exposed from 3-120 hours post fertilization (hpf) and endpoints of morphometrics, behavior, and gene expression were analyzed at 5 dpf, 14 dpf, and 6 months (adults). At 5dpf, all PFASs resulted in gene expression changes of transforming growth factor *tcf3a*

and adaptor protein *ap1s1* and all fish were smaller size. PFOS exposed fish had the greatest number of endpoint and gene expression changes. At 14 dpf, all PFAS exposed fish showed hyperactivity and increased organic ion transporter *slco2b1* expression. All other endpoints analyzed at these time points varied between PFASs. In adult fish, PFNA males were the most affected in behavior but all three PFASs resulted in gene expression changes in *slco* transcripts. Adult fish chronically exposed to PFOA had reproductive and fecundity affects, including reduced egg production, morphometric effects, and delayed development of the offspring. Chronic PFNA exposure had similar but less severe effects, and PFOS exposure resulted in $P_0$ affects but no immediate reproductive changes. The null hypothesis of this dissertation was rejected at each time point and exposure; PFOS, PFNA, and PFOA exposure resulted in dissimilar toxicity profiles between compounds. The development age of the fish and the endpoints assessed determine which compound was having the greatest effect. In acute embryonic studies PFOS appeared to have the greatest effect. PFNA, in particular the males, are the most affected at the adult stage in terms of behavior. PFOA shows the greatest negative effects on reproduction after a chronic exposure. Additionally, multiple pathways such as *ap1s1*, *slco* and *tgfb1a* were identified as affected by PFASs and further studies are needed to determine if these altered genes during development and maturation may underlie the mechanism(s) of action for these compounds.

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## Abbreviations

| | |
|---|---|
| AB | |
| dpf | days post fertilization |
| FTOH | fluorotelomer alcohol |
| hpf | hours post fertilization |
| mg | milligrams |
| mL | milliliters |
| MS222 | tricaine methane sulphonate |
| nM | nanomolar |
| OATP | organic anion transporting polypeptide (human nomenclature, general use) |
| *oatp* | organic anion transporting polypeptide (zebrafish nomenclature, protein ) |
| PFASs | perfluoroalkylated substances |
| PFNA | perfluorononanoic acid |
| PFOA | perfluorooctanoic acid |
| PFOS | perfluorooctane sulfonate |
| pg | picagrams |
| pM | picamolar |
| ppb | parts per billion |
| ppm | parts per million |
| ppt | parts per trillion |

| | |
|---|---|
| qPCR | quantitative polymerase chain reaction |
| *slco* | organic anion transporting polypeptide (zebrafish, gene nomenclature) |
| *tcf3a* | transcription factor 3a (zebrafish, gene nomenclature) |
| *tgfb1a* | transcription growth factor beta-1-alpha (zebrafish, gene nomenclature) |
| µg | microgram |
| µM | micromolar |

# CHAPTER 1: INTRODUCTION

## 1.1 General Introduction

This dissertation aimed to examine the toxicological profiles of perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorooctane sulfonate (PFOS) exposure to embryonic zebrafish through multiple life stage time points. Each compound's toxicity was compared and contrasted for a number of different end points, and possible pathways responsible for these toxicities were identified. Chapter 2 describes the general methods employed in these studies. In Chapter 3, the effects of PFOS, PFNA, and PFOA exposure to embryonic zebrafish were examined at 5 and 14 dpf. Endpoints included morphometric measurements, behavioral and locomotion changes, and gene expression analysis of a suite of 100 developmentally relevant transcripts. While some of these endpoints overlapped between two or three of the compounds, each perfluoroalkylated substance (PFAS) had a unique toxicological profile, with PFOS eliciting the greatest number of significantly different end points. In Chapter 4, the effects of PFOS, PFNA, and PFOA on adult zebrafish after an embryonic exposure were examined for the same endpoints as the previous chapter. This study demonstrated that PFNA exposed male zebrafish were the most affected in altered behavioral endpoints. Chapter 5 discusses the effects of chronic, low dose PFOS, PFOA, and PFNA exposure from embryo though adult life stages induced toxicological effects in both the P0 and F1 generations. Based on these studies, it can be concluded that PFOS, PFOA, and PFNA have different toxicity profiles in zebrafish at multiple life stages. Each PFAS had specific endpoints and time points in which they were the most potent. For PFOS, the

critical exposure period was 5 dpf at endpoints of morphometrics and gene expression changes. PFNA exposure was especially important for adult male zebrafish in terms of behavior. PFOA exposure was most detrimental in reproductive studies in the morphometrics of the P0 generation and fecundity and survival of the F1 offspring.

**1.2 Perfluoroalkylated substances (PFASs)**

Perfluoroalkylated substances (PFASs) are a class of anthropogenic compounds that pose world-wide environmental concerns. The structure of these compounds consists of a fully fluorinated carbon chain 4-12 carbons long with either a carboxylic acid, sulfonate, or alcohol end chain group. PFAS manufacturing began in the 1950s for use in a number of applications. Their stable structure due to the carbon-fluorine bonds allows them to be non-flammable, non-volatile, non-oxidized even with strong acids or bases, stable in extremely high temperatures, and highly resistant to biodegradation (Lau 2012). These properties make PFASs useful for a number of industrial applications, including an emulsifier for producing fluoropolymers, hydraulic fire-fighting foams, as well as consumer products such as water and stain resistant coatings on clothing, furniture and carpets and cookware (Renner 2001).

The same properties that make PFASs useful for this wide variety of applications also make them persistent in the environment. It is estimated that since production began, up to 45,300 metric tons of PFASs have been released into the environment, and more than 95% have been released directly into aquatic environments (Ahrens et al. 2011).

Environmental contamination occurs in all aspects of the chemical's life cycle including production, supply chain, product use, and disposal. PFASs generally have a relatively low octanol-water coefficient, which makes them water-soluble and as a result are often detected in drinking water. PFASs have also been detected in measureable levels in animal tissue and serum, water, sediment, and human tissue samples on nearly every continent including some remote locations (Houde et al. 2011).

In the year 2000, a voluntary phase-out of long chain (> 8 carbon chain length) PFASs, in particular PFOS, was introduced. This agreement was to reduce emissions by 95% by 2010 and by 2015 eliminating emissions completely (Society et al. 2010). However, due to the persistence of these compounds, overseas production, and ability for long term transport, PFAS levels are still present in the environment world-wide (Houde et al. 2006). Additionally, there is no such agreement or ban on the shorter chain PFASs, and they continue to be produced and persist in the environment.

The three long chain PFASs most commonly found in environmental and human samples are perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA)(Figure 3.1) (Giesy and Kannan 2001; Kannan et al. 2005; Yamashita et al. 2005). These compounds are typically detected as a mixture of two or three PFASs. PFOS is an eight chain PFAS with a sulfonate end group. PFOA is also an eight carbon chain PFAS but with a carboxylic acid end group. PFNA is nine carbons long with a carboxylic acid end group. The structures of each compound can be seen in Figure 3.1. These compounds are often grouped together and analyzed as one class of

compounds even though they are structurally distinct. The subtle structural changes between each PFAS could affect its sorption into water and sediment as well as its uptake and binding into human and animal tissue, and in turn result in different toxicity profiles.

PFASs have been identified in many organisms as endocrine disrupting compounds. In an *in vivo* study of tilapia hepatocytes, PFASs induced estrogen dependent vitellogenin production (Liu et al. 2007). In rainbow trout cells, PFASs were identified as weak environmental xeno-estrogens (Benninghoff et al. 2011). In fat head minnows, plasma androgens and estrogen levels were affected by PFOS exposure (Ankley et al. 2005). In rodents, PFAS exposure affected serum thyroid hormone levels (Butenhoff et al. 2004). It is unclear how PFASs elicit their effects, however previous literature reported that PFASs, particularly PFOS and PFOA, have been shown to be peroxisome proliferators in rats through expression of PPARα (Berthiaume and Wallace 2002; White et al. 2011). However, when rats with PPARα knocked out were exposed to PFOS, neonatal lethality and delayed eye opening defects were observed in both the wild type and the knockout mode, indicating that PPARα is not the sole mediator of PFOS toxicity (Filgo et al. 2015; Rosen et al. 2010). Additionally, PFOA induced toxicities in fish species that do not have an active PPARα ortholog transcript such as zebrafish (Den Broeder et al. 2015). This is evidence that PFASs mode of action is not through PPARα and other pathways need to be investigated.

To investigate other candidate pathways involved in PFAS toxicity, an array of 100 genes involved in critical developmental pathways were analyzed in 5 day post fertilization

zebrafish for expression change after PFAS exposure. Based on the results of this analysis, genes that were significantly altered and likely play a role in PFAS toxicity were analyzed at additional timepoints as well as with different exposure routes. Genes found to be altered in by multiple PFASs in multiple life stages are discussed in section 1.9.

**1.2 Perfluorooctanoic acid (PFOA)**

Perfluorooctanoic acid (PFOA) is an 8-carbon chain PFAS that is fully fluorinated and has a carboxylic acid end group (Figure 3.1). This compound exists as both linear and branched isomers (Naile et al. 2016).Under normal environmental conditions, PFOA is an anion and is highly water soluble (Davis et al. 2007). Sources of PFOA in the environment can be from direct sources, or from the breakdown of its parent compound fluorotelomer alcohol (FTOH) (Post et al. 2012) Long term transport could either be due to transport of FTOH which breaks down into PFOA after deposition, or PFOA in its anion form is carried by ocean surface currents (Houde et al. 2011).

PFOA had been produced for use an emulsifier for the production of fluoropolymers (Butt et al. 2010). Fluoropolymers are used in a number of consumer applications such as non-stick, water-resistant, or stain-resistant surfaces. Some of these include cookware, electrical wire casings, and carpet coatings (Renner 2001). Sources of environmental PFOA contamination include waste water treatment plants (Sinclair et al. 2006), street runoff (Murakami et al. 2009), storm water runoff (Kim and Kannan 2007), and fire - fighting foams (Moody et al. 2003)

PFOA had been voluntarily phased out of use in 2000. However, it has still been detected in drinking water, human and animal tissue and serum, and environmental samples on every continent (Arvaniti and Stasinakis 2015). PFOA concentrations in human blood and serum samples have remained the same since that time (Calafat et al. 2007). Diet and drinking water were identified as the two predominant exposure sources for humans. In the United States, PFOA was detected in a number of drinking water samples ranging from 2 – 395 ng/L median levels (Post et al. 2012).

In the NHANES study, PFOA was detected (>0.1 ng/mL) in 99.9% of the participants sampled(Kato et al. 2011). Human exposure to PFOA was also seen in Greece (2 ng/mL) (Vassiliadou et al. 2010) Belgium (3.6 ng/mL)(Cornelis et al. 2012), and Australia (6.4 ng/mL)(Toms et al. 2014). The highest maximum exposure concentrations seen were above 100,000 ng/mL in workers making and using PFOA. A variety of health effects have been correlated with PFOA exposure. Some of these include elevated cholesterol and serum lipids (Steenland et al. 2015), thyroid disease, and osteoarthritis (Innes et al. 2014). Reproductive effects such as decreased sperm count and reduced birth weight and size have also been observed (Liu et al. 2015). The half-life of this compounds in humans ranges from 2-4 years after removal from exposure (Zhang et al. 2013)

The structure of PFOA resembles a fatty acid, but since it is non-reactive it cannot be a substrate for fatty acid biochemical reactions (Post et al. 2012). PFOA accumulates predominantly in the liver, followed by plasma and kidney (Houde et al. 2011). Once in

the body, similar to the environment, it does not undergo any processing or further metabolism and is often eliminated as the parent compound in the urine (Consoer et al. 2014).

PFOA exposure in mammalian models resulted in a number of adverse outcomes, including liver enlargement (Butenhoff et al. 2004), suppressed immune system (DeWitt et al. 2016) and hyperactivity(Johansson et al. 2009). Reproductive studies found a delay in parturition, decreased postnatal survival and growth, and development defects such as delayed eye opening (Lau et al. 2006).

PFOA exposure in aquatic species has also been studied. In rare minnows, PFOA led to changes in gene expression in transcripts involved in lipid metabolism and transport, hormone function, and mitochondrial function (Wei et al. 2008). In adult medaka (*Oryzias latipes*), PFOA exposure resulted in negative impacts on offspring development and survival. Some of these impacts included increased mortality, hyperplasia, hypertrophy, and reduced thyroid hormone homeostasis (Ji et al. 2008). PFOA has been shown to affect thyroid function in zebrafish by interfering with the thyroid hormone receptor (Du et al. 2013). Additionally, other endocrine disrupting effects such as interference with the estrogen receptor, increases in estrogen and decreases in testosterone were reported in zebrafish after PFOA exposure (Du et al. 2013). In teleost species, thyroid hormone levels have been shown to play a critical role in growth, development, reproduction, and behavior (Power et al. 2001). Zebrafish exposed to PFOA exhibited other adverse outcomes such as developmental abnormalities, yolk sac

edema, and at high concentrations delay embryo hatching (Hagenaars et al. 2011; Zheng et al. 2011). This dissertation examines possible phenotypic outcomes that could be due to endocrine disruption, such as changes in body size, behavior affects (anxiety, aggression), and reproductive success and possible genetic pathways associated with them.

**1.4 Perfluorooctane sulfonate (PFOS)**

Perfluorooctane sulfonate (PFOS) is an 8-carbon PFAS with a sulfonate end chain group. This compound is an anion and can be in the form of either branched or linear isomers. Generally the linear isomer is more commonly identified in samples (70%) compared to the branched isomer (30%) (De Silva and Mabury 2004). The linear form is typically detected in 77-93% of wildlife samples contaminated with PFOS (Houde et al. 2011). PFOS is an environmental contaminant that has multiple routes of release. Direct release from manufacturing and the breakdown of parent compounds such a perfluoroalkyl sulfonates or perfluorooctanesulfonamides both lead to increased PFOS concentration in the environment (Houde et al. 2006; Tomy et al. 2004)

PFOS was manufactured for a use in a wide number of applications, including fire-fighting foams and aviation hydraulic fluids. Additionally, it was a component in many consumer products for its water and stain resistant properties, such as coatings on carpets, furniture, cookware, and clothing(Renner 2001). The uptake kinetics of PFOS are shown to be somewhat similar to hydrophobic compounds, and their elimination rate is similar to that of metals (de Vos et al. 2008). Similar to most PFASs, PFOS tends to accumulate

in the liver and has been shown to bioaccumulate more than other PFASs (Ahrens et al. 2011).In contrast to other PFASs which see large sex specific differences in elimination rates, PFOS excretion is relatively similar between sexes.

In the year 2000, manufacturers in the United States agreed to voluntarily ban production of PFOS. Since this time, the concentration of PFOS in human and wildlife serum have decreased. However, there are still detectable amounts of PFOS in animal tissue and environmental samples around the world. In Tokyo Bay, Japan, PFOS was detected at concentrations of 145±3.5 pg/g in the sediment (Miyake et al. 2014).The Brisbane river in Australia had water PFOS concentration of 0.18-15 ng/L (Gallen et al. 2014). Gull eggs from the great lakes region contained 14.0 ±2.8 ng/g PFOS (Gewurtz et al. 2016)and Belgian wood mouse kidneys had >13.7 ng/g (D'Hollander et al. 2014). In China, PFOS was the predominant PFAS in fish muscle tissue in high elevation lakes and rivers (Shi et al. 2010). Bluegill fillet from the Mississippi River have median PFOS levels of 50-100 ng/g (Delinsky et al. 2009). Open ocean and off shore tuna samples near the Pacific Rim had elevated PFOS in liver tissue (Hart et al. 2008). While PFOS is identified in many drinking water sources, it is believed that food intake in the general public is responsible for 90% of PFOS exposure (Lindstrom et al. 2011).

In humans, increased serum PFOS concentration was associated with increased cholesterol and triglycerides (Olsen et al. 2003). Effects on fatty acid levels were also seen in rodent studies in which PFOS exposure resulted in increases in liver weight, liver hypertrophy, but had a decrease in serum cholesterol and triglyceride levels (Elcombe et

al. 2012; Seacat et al. 2003). Rodents exposed to PFOS had decreased body weight and lipid metabolism defects (Wang et al. 2014). Mice were hyperactive and had reduced habituation behaviors after juvenile PFOS exposure (Johansson et al. 2009). In rats there was a negative correlation between PFOS levels and glomerular filtration rates, as well as reduced serum thyroid hormone levels (Chang et al. 2008). Reproductive effects after PFOS exposure include decreased sperm count and reduced birth weight and size (Lau et al. 2006).

Endpoints analyzed in this study are similar to those assessed in the rodent literature. Measurements in zebrafish such as body weight and length, behavior, and reproductive effects after PFOS exposure can be compared to those seen in the mammalian literature.

**1.5 Perfluorononanoic acid (PFNA)**

Perfluorononanoic acid (PFNA) is a nine-carbon PFAS with a carboxylate end chain group. This compound can be directly released into the environment as PFNA, or it can be formed as a result of the breakdown of the parent compound FTOH. PFNA has multiple isomers: linear, iso-branched and multiple branched. It was observed that branched isomers were more common near industrial sites and the linear isomer more common in remote areas (De Silva and Mabury 2004).

Since the voluntary ban of production in 2000, the concentration of PFNA has not decreased but actually increased (NHANES). PFNA has been found at detectable levels in a number of different species found over a wide range of geographical locations and

environments. This includes Baikal seals in Russia (Houde et al. 2011), tilapia in Bejing China (0.40 pg/mL)(Bao et al. 2014), black sea bass from Georgia, United States (1.2 pg/mL) (Kumar et al. 2008)little ringed plover in Lake Shihwa Korea (8.4 pg/mL)(Yoo et al. 2009), atlantic fur seal (3.3 pg/mL) (Schiavone et al. 2009)

Currently, the toxicity PFNA has been less widely studied compared to PFOS and PFOA. However, a number of rodent studies report PFNA that resulted in a number of detrimental outcomes. Mice exposed to PFNA had increased neonatal death, delayed eye opening and delayed puberty (Das et al. 2015). In rats, PFNA exposure led to oxidative stress and caspase-independent apoptotic signaling in spleen cells (Fang et al. 2012)and increased apoptosis in the testes (Feng et al. 2009)

PFNA exposure has also resulted in many effects in zebrafish. In a proteomic analysis, 57 proteins involved in metabolism, structure, motility, stress and defense, signal transduction, and cell communication were significantly altered after PFNA exposure (Zhang et al. 2012). At higher doses of PFNA exposure, zebrafish embryos were delayed in development, had reduced hatching rates, and exhibited edema and spine malformation (Liu et al. 2015). Similar to PFOS and PFOA, PFNA has been shown to have endocrine relate effects, such as elevated thyroid hormone levels and histological changes in thyroid follicles of male zebrafish (Liu et al. 2011). Further analysis into endpoints affected by endocrine and hormone changes would bring further knowledge and PFNA toxicity and allow for comparisons both between PFNA exposure in higher vertebrate as well as comparisons between PFAS compounds.

### 1.6 The Zebrafish Model

Zebrafish have been developed as a vertebrate model for developmental toxicity analysis for a variety of reasons. They are easily housed in a laboratory setting, have a high fecundity rate, short generation times (reproductive maturation at approximately 3 months) and are cost effective compared to rodent models (Hill et al. 2005). The large clutch size allows for high throughput toxicity screenings with a large sample size. The short generation times allows for trans-generational endpoint and reproductive analysis (Hill et al. 2005). Zebrafish embryos have a translucent chorion, which allows visual monitoring of organogenesis and lesion presence during early, critical life stages as described(Kimmel et al. 1995). Each of the developmental stages in zebrafish and indicated specific markers for each time point is described based off of fish raised at 26°C. Zebrafish embryos can be monitored and staged *in vivo* developmental progression or defects can be analyzed.

Another benefit of the zebrafish model is that the genome has been mapped, and many genes and organ systems are highly similar to those in human and higher mammals (Lieschke and Currie 2007; Seth et al. 2013). This allows for gene expression analysis of pathways associated with specific phenotypes to narrow down possible pathways involved in phenotypic effects. Additionally, a number of genes in the zebrafish genome have functional orthologs in higher vertebrates. Zebrafish have a number of transgenic and mutant lines that allow further study to understand the phenotypes produced that resemble human malformations (Lieschke and Currie 2007).

### 1.7 Zebrafish Behavior Assays

Zebrafish have also been used as a model for behavior and locomotion analysis. Fish can be video recorded during a number of assays and then analyzed using tracking software Noldus Ethovision. Assays can be performed for basic locomotive movement, habituation, anxiety, aggression, prey capture, and predator avoidance behaviors. Locomotion, feeding, anxiety, and aggression are important endpoints to assess with regards to population fitness. Defects or changes in any of these endpoints could result in maladaptive behaviors, such as reduced ability to find prey or inability to mate, which would in turn drive population dynamics (Norton et al. 2011).

Locomotive endpoints can be measured in free-swimming larvae through adult life stages. Endpoints include the total distance traveled, the swimming speed, the amount of "meander" or changes in direction of swimming, and spatial body movements independent of swimming. At 14 dpf, zebrafish larvae are large enough to begin free feeding on live brine shrimp and a feeding assay can be conducted. The number of shrimp eaten per fish over a defined period of time can be measured and used to determine ingestion rate.

There a number zebrafish behaviors that would indicate an anxiety-like response, including thigmotaxis (tendency to swim near walls of arena), light/dark preference, freezing behavior (immobility independent of swimming), and erratic movements (Kalueff et al. 2013). One of these tests is the open field test, as described by (Champagne et al. 2010). This is a way to measure exploratory and thigmotaxis behavior.

In this test, the zebrafish is placed into a novel environment, and time spent in inner and outer parts of the tank are recorded. Fish that display wall following, or thigmotaxis, are thought to have increased anxiety. Thigmotaxis has been classified in a number of organisms as a way of seeking shelter, protection, or an escape from an environment or situation that they fear (Simon et al. 1994; Walz et al. 2015).

Scototaxis, or light-dark preference, is another assay to measure anxiety and has also been described by (Champagne et al. 2010). A zebrafish is placed into a novel tank that is half translucent and half opaque, and the percentage of time spent in each compartment is recorded. This assay was developed from rodent behavior models in which light-aversion has been found to be an anxiety driven behavior (Hascoet et al. 2001) .However, there have been discrepancies in zebrafish studies on whether they naturally prefer the light or dark areas (Gerlai 2010) (Serra et al. 1999). In the light, zebrafish are able to find food and mates, and the advantage of being in the dark is predatory avoidance. It was found that light-dark preference was determined by the ambient light levels during the assay (Stephenson et al. 2011).

Aggressive behavior in zebrafish can manifest as mouth opening, biting, charging, and chasing (Kalueff et al. 2013). The zebrafish aggression assay described by (Norton et al. 2011) and (Gerlai 2010) consists of fish placed into a tank with a mirror, and the number of hits against the mirror was recorded. These hits are considered attacks against what is perceived to be another fish, and this measurement determines the level of aggression.

### 1.8 Zebrafish Reproduction

One aspect that makes zebrafish a good model for toxicology studies is the high fecundity rate and short generation time. Each breeding set (~8 fish of each sex) can produce approximately 400 embryos per breeding event, and can be bred every 1-2 weeks. While the zebrafish reproductive system varies from humans in a number of aspects, there are many similarities in hormones and hormone receptors, which make it a viable model for vertebrate reproductive studies (Laan et al. 2002). However, there are a number of physiological differences between human and zebrafish reproduction.

In zebrafish, sex differentiation is determined mainly by genetic factors with some environmental aspects involved. These genetics factors are not based predominately on the sex chromosomes, but rather multiple autosomal gene pathways that affect the gonadal differentiation (Liew and Orban 2014). The gonad differentiation process is somewhat complex in that all larval zebrafish form a juvenile ovary made up of oogonia and primary oocytes between 2.5 – 4 weeks post fertilization (Maack 2003) .After this organ develops, the fish either continues develop into a female with a functional ovary, or the juvenile ovary goes through a transformation to a testis in the male zebrafish (Liew and Orban 2014). This transformation is likely signaled through a change in hormones which induces apoptosis of the oocytes in the juvenile kidney (Uchida et al. 2002).

After development, adult zebrafish reach sexual maturity and can be bred at 3-4 months post fertilization. Both environmental and social factors can reproductive success and quality of offspring produced.  The addition of males into a tank with only females

introduces pheromones that induce ovulation in the females. After ovulation, females release a pheromone to attract males to fertilize the eggs (Nasiadka and Clark 2012). The fitness of the males is important, as it has been seen that females prefer to mate with larger males (Skinner and Watt 2007) and more dominant males (Paull et al. 2010). Other environmental factors such as light cycles, fat content of diet, and overall fish health also contribute to egg production and viability.

Zebrafish reproduction assays have been used to analyze toxicity of a variety of compounds, including nanoparticles (Wang et al. 2011), phthalates (Carnevali et al. 2010), phenols (Deng et al. 2010), PCBs (Orn et al. 1998), and many others. Endpoints assessed consisted of total zebrafish eggs produced, embryo mortality, gene expression of hormone-related pathways, and hatching rate of the offspring. This dissertation analyzes reproduction effects after exposure to PFOS, PFOA, and PFNA in Chapter 5. The endpoints examined are similar to those studies with other compounds, such as total egg production, egg viability, offspring developmental progression, and gene expression of hormone transporter pathways (*slco;* oatp) and transforming growth factor involved in oocyte maturation (*tgfb1a*).

**1.9 Gene Expression**

Since it has been proven that pparα is not the only mechanism of action for PFAS toxicity, a number of other pathways were analyzed as possible candidates. Gene expression analysis on embryo-larval zebrafish after PFAS exposure was performed at Oregon State University. This analysis evaluated 100 genes that are involved a number of critical developmental pathways in zebrafish, including tissue remodeling, calcium signaling, cell

death and cell cycle regulation, growth factors, and angiogenesis and hypoxia (Bugel et al. 2016). Out of these 100 genes, the following were significantly altered by multiple PFASs: *slco2b1;* organic anion transporting polypeptide 2b1, *ap1s1*; adaptor protein 1s1, and *tgfb1a*; transforming growth factor b1a. Based on the data from this gene array, these genes were selected for analysis at additional time points (14 dpf, 180 dpf) as well as in a different exposure route (long term exposure, Chapter 5).

### 1.9.1 Organic Anion Transporting Polypeptides (OATps)

A group of pathways that have been shown to interact with PFASs and have the potential to be critical in PFAS toxicity are the organic anion transporting polypeptides (OATps). OATps are responsible for the transport of both exogenous and endogenous compounds into and out of cells (Klaassen and Aleksunes 2010). These transporters are highly conserved between species and found in multiple organs. Many transporters have functional orthologs that are comparable between species. There are a number of variations of OATps, and many have overlapping substrates and inhibitors, and are found in multiple organ systems with varying degrees of specificity. Additionally, other transporters can also overlap and play a role in the uptake or elimination of similar substrates. An example of this is seen in 1.1, which demonstrates the complexity of the interaction between various transporters in hepatocytes. Due to their presence in various tissues and cell types, regulation of each transporter is specific to its organ or cell type. However, expression of many OATps is regulated by cytokines, which cause activation of nuclear receptors (Svoboda et al. 2011).

Generally, endogenous substrates of OATps include bile acids, steroid hormones, thyroxine, bile acids, estron-3-sulfonate, bilirubin and prostaglandins, and exogenous substrates can include taurocholate, statins, and other xenobiotics (Klaassen and Aleksunes 2010). Therefore, disruption of these transporters can alter both normal endogenous compound toxicokinetics as well as medications.

In this thesis, four transporters in particular were evaluated: *slco2b1, slco3a1, slco4a1, slco1d1*. *Slco2b1* has a functional ortholog Oatp2b1 and OATP2b1 in rodents and humans, respectively. This transporter is expressed throughout the zebrafish and specifically transports estron-3-sulfonate, fexofenadine, statins, and glibenclamide(Popovic et al. 2014). The human ortholog has been proven to transport sulfated steroids from the fetus to the mother during pregnancy (Klaassen and Aleksunes 2010). *Slco3a1* and *slco4a1* both transport thyroid hormones, taurocholate and prostaglandins (Klaassen and Aleksunes 2010).These transporters can play a role in regulating hormone levels and in turn have a number of endpoints affected such as growth, reproduction, and anxiety type behaviors.

*Slco1d1* is a transporter that uptakes steroid hormone conjugates into hepatocytes which then allows elimination through the bile (Popovic et al. 2014). This transporter has a high affinity for conjugated steroid hormones, and can be inhibited by non-conjugated steroid hormones (Popovic et al. 2014). The kinetics of this transporter after PFOA exposure has been studied in many models. In vitro studies of zebrafish *slco1d1* (HEK293 cell line) as well as the functional orthologs in humans and rodents (OATP1a2, Oatp1a2) found

PFOA to be an inhibitor of this transporter (Popovic et al. 2014). Since it is involved in steroid and hormonal signaling and concentration, inhibition could lead to a number of toxic outcomes.

Expression of organic anion transporting polypeptides is likely to play in role in the half lives of PFASs. The half- lives of PFASs vary between sexes and between compounds. In aquatic species, PFOA half-life can range from 6.3 hours in female fat head minnows to 68.5 hours in male fat head minnows (Lee and Schultz 2010) and from 1.4 hours in male tilapia to 0.35 hours in female tilapia (Han et al. 2012). In rodent models this trend was also present in that males had a higher lower clearance rate of PFNA (2%) and PFOA (55%) than females (51% and 81%, respectively).

### 1.9.2 Tgfb1a

The transforming growth factor beta 1 (*tgfb1*) is a gene that has functional orthologs in both zebrafish and mammals. This growth factor is involved in a number of critical biochemical functions in early development such as cell proliferation, differentiation, apoptosis and cell migration (Verrecchia and Mauviel 2002). Other pathways affected by *tgfb1a* include wound healing and immune response regulation (Beanes et al. 2003; Li and Flavell 2008).

The general pathway for *tgfb* activation is shown in figure 1.2. Smad proteins mediate tgf-B pathway signaling(Kohli et al. 2003). First, a *tgfb* ligand binds to a tgfb type II receptor, which is a serine/threonine kinase. This causes the transphosphorylation and

subsequent activation of the type I receptors (Derynck and Zhang 2003). There are three types of Smads involved in this pathway: R-smads (receptor regulated Smads), common mediator smad, and inhibitor smads. The activation of tgfb type I phosphorylates R-Smad, which then forms a complex with Smad4 (mediator) and then translocates into the nucleus. Here, transcription is controlled by interacting with co-activators or repressors or through DNA binding cofactors (Derynck and Zhang 2003; Kohli et al. 2003; Xing et al. 2015). The *tgfb* ligands go through a complex synthesis and disposition process to be able to bind to the receptor. This involves proteases, scaffolding protein, chaperone proteins, and matrix metalloproteases. Changes in *tgfb1a* expression could be due to direct alterations in the promoter region, or more subtle changes to any of these proteins that would effect the mobility or binding of the ligand to the receptor.

In zebrafish, *tgfb1a* is critical for normal lateral line development. This pathway regulates the primordium migration and neuromast deposition during development, and when knocked down the formation of the lateral line was disrupted (Xing et al. 2015). The lateral line in teleost fish is extremely important for sensing water flow and detecting obstacles (Yanase et al. 2012). A defect in development of this structure could cause disruptions in normal swimming behavior and swimming efficiency, which in turn could affect energy efficiency and usage. This makes this pathway a target for further analysis to explain effects of PFOS, PFNA, or PFOA on morphometric or locomotive behavior endpoints.

*Tgbf1a* can also be an important gene to analyze to understand anxiety type behaviors as well as reproductive and fecundity deficits in zebrafish after PFAS exposure. In both mammals and fish, *tgfb1a* has been found to play a role in hormone production and cycling (Ingman and Robertson 2008; Kohli et al. 2003) In rodents, *tgfb1a* deficiency causes functional defects of the hypothalamic-pituitary-gonadal axis, which results in defects of ‘ production in males and estrous cycle in females (Ingman and Robertson 2008). Changes in hormone production and concentration can result in anxiety driven behavior as well as reproductive effects. This manifests in rodents as oocytes not developing, early embryo toxicity, and inability to mate (Ingman and Robertson 2008) and in zebrafish as inhibited oocyte maturation.

### 1.9.2 Ap1s1

Adaptor protein (AP) complexes are responsible for mediating protein trafficking between organelles in the cell as well as protein transport between the trans-Golgi network, endosomes, lysosomes, and plasma membrane (Montpetit et al. 2008). Each AP complex is comprised of four subunits; the large subunits mediate membrane binding and clathrin uptake, the small subunit is thought to stabilize the complex, and medium subunit’s main function is cargo sorting.

This protein has been studied in many animal models including mice and *C. elegans*, and in both species a knockdown of AP-1A resulted in embryonic lethality (Boehm and Bonifacino 2001; Ohno 2006). In humans, a mutated AP complex can result in a variety of genetic disorders. Mutations in AP-3 lead to albinism in the eyes, skin, and hair, and

increased susceptibility to hemorrhage ((Montpetit et al. 2008). AP-1 mutations have been associated with neurological issues, specifically abnormal synaptic development which can result in mental retardation (Tarpey et al. 2006) . Mice models also show defects in AP-3 knockouts such as increased epileptic seizures (Nakatsu et al. 2004)and neurological disorders (Kantheti et al. 1998)

In zebrafish, a knockdown of *ap1s1* resulted in embryo lethality after 48 hpf. Before that time point, the fish had a reduced pigmentation, were smaller in total size, and had disorganized fin structure (Montpetit et al. 2008). Additionally, these fish also showed behavioral deficits in their reaction to touch stimuli. The normal response to touch stimuli is swimming away, but the *ap1s1* knockdown fish coiled their body in response to the stimulus. It was shown that these fish also had neurological deficiencies in the form of reduced interneuron numbers (Montpetit et al. 2008)

Ap1s1 pathway may play a role in mediating many of the phenotypic responses see after PFAS exposure, listed in sections 1.3-1.5. A disruption in this gene could play a role in both morphometrics, as seen in changes in total body size in many models analyzed. Additionally, this gene has been seen to play a critical role in locomotive behavior and responses, and could be one possible mode of action for PFAS toxicity.

### 1.12 Research Objectives and Hypothesis

The overall objective of this dissertation was to determine the toxicity profiles of PFOS, PFNA, and PFOA using the zebrafish developmental model for assessing vertebrate

toxicity. Furthermore, this dissertation aimed to identify possible pathways that could be studied to detect mechanism(s) of action for these compounds. The null hypothesis is that the similar structure of each of these three PFASs will cause them to have similar toxicity profiles.

The specific aims of this thesis were to:

(1) Identify the toxicity profiles in an embryo-larval zebrafish model after embryonic exposure to PFOS, PFNA, and PFOA in terms of morphometrics, behavior and gene expression

(2) Study the continued effects on adult zebrafish after an embryo-only exposure to PFOS, PFNA, and PFOA in terms of morphometrics, gene expression, and behavior

(3) Examine the chronic and reproductive effects of a low dose long term PFAS exposure in zebrafish.

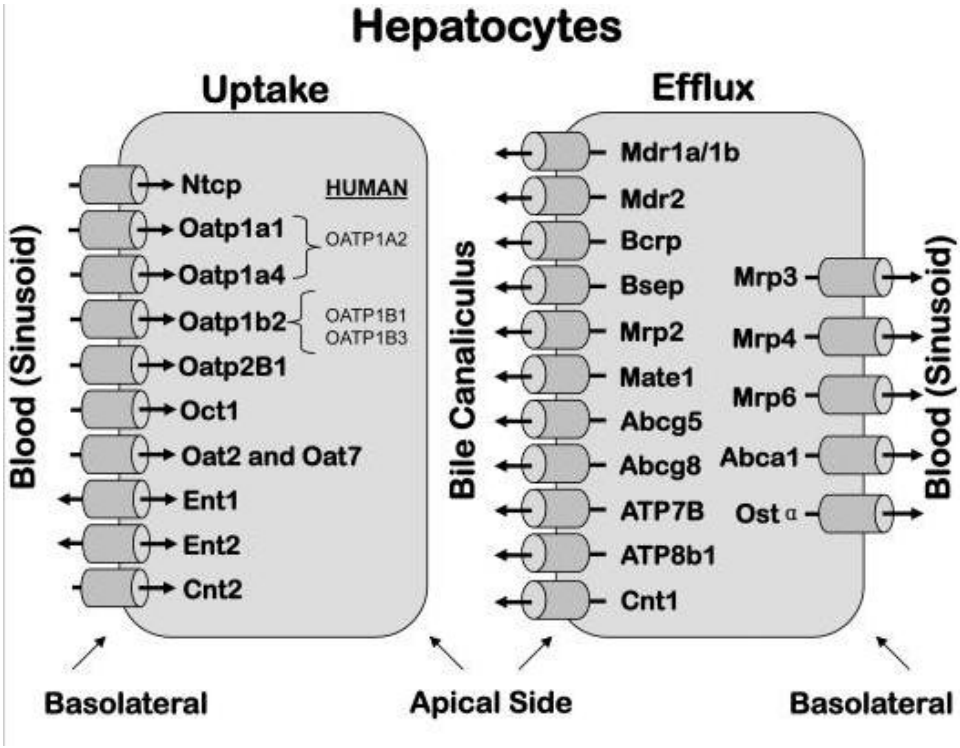


Figure 1.1 Modified from (Klaassen and Aleksunes 2010). This figure identifies the uptake and efflux transporters in rodent hepatocyte. Interaction and overlap between various transporters can be seen.

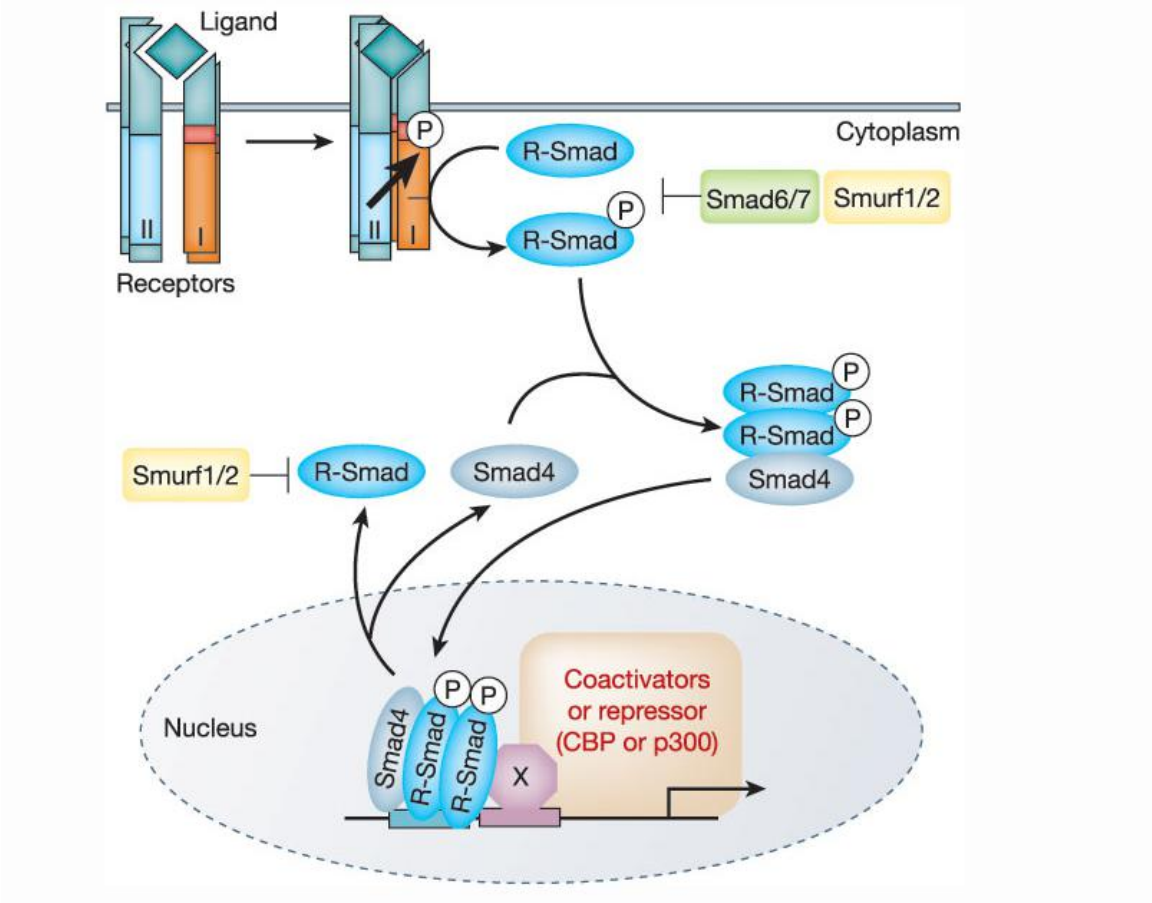


Figure 1.2. Modified from (Derynck and Zhang 2003)Pathway of *tgfb* receptor activated through ligand binding. This initiates a kinase pathway that translocates to the nucleus and affects transcription.

# CHAPTER 2: GENERAL METHODS

## 2.1 Zebrafish Husbandry

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.

## 2.2 Chemical Stocks

Perfluorooctane sulfonate (PFOS, 96%), perfluorooctanoic acid (PFOA, 95%), and perfluorononanonaoic acid (PFNA, >98%) were purchased from sigma Aldrich in a powder form. PFAS was weighed and added to a sterile conical containing 50 mL of Millipore filtered water for a stock concentration of 2000 μM. All compounds were dissolved by shaking. For exposures, stock concentrations were diluted into new conical tubes and diluted for system water described in section 2.1.

### 2.3 Embryonic Exposure Protocol

Zebrafish embryos were exposed at 3 hours post fertilization (hpf) to PFOS, PFOA, or PFNA for 120 hours in a static non-renewal protocol.. 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 3 hpf to 120 hpf (5 days), which corresponds to embryonic to yolk sac larval exposure. This protocol was used in chapters 3-4.

### 2.4 Chronic Exposure Protocol

Zebrafish embryos were exposed in glass vials to either control (non treated) or 2 nM PFOA, PFOS, or PFNA (> 95%, Sigma Aldrich, St. Louis, MO) through a waterborne exposure from 3 hpf – 120 hpf. Each trial had four replicates per treatment, and 20 fish per replicate. Three independent experiments were carried out using this protocol. At 120 hpf, fish were transferred to non-exposed water until 30 dpf, in which they were separated by treatment and placed in 5 liter fish tanks (~20 fish per treatment, per trial). At 1 month post fertilization, the fish were separated by sex and began a feeding regimen consisting of non-treated brine shrimp in the morning feeding and 0.04 g of either control or PFOA, PFOS, or PFNA treated flake food in the afternoon feeding.

When the fish were 90 dpf, they were bred once a week for 10 weeks for 9 successful breeding weeks. The number of eggs produced, the viability of the embryos, and the developmental staging progression (Kimmel et al. 1995) of the embryos were recorded.

After 10 breeding events, the morphometric measurements of body length and weight were recorded for the adult fish. Livers from adult fish were isolated for gene expression analysis. Detailed exposure water and food preparation is described in Chapter 5.

**2.5 Gene Expression Analysis**

In chapter 3, 100 genes involved in critical pathways of zebrafish development were analyzed by our collaborated at Oregon State University. This was to narrow down potential target genes to continue to analyze in later chapters. Specific protocol for this gene analysis is described in section 3.2.5.

For chapters 3, 4, and 5, gene expression analysis was performed using RT-qPCR with the following protocol. The genes examined were selected based on the results from the 100 gene screening. A list of genes and primer sequences are listed in Table 2.. Four replicates (N=25 fish/replicate for larval, N=5-8 fish livers for adults) from each treatment and control were snap frozen in liquid nitrogen and RNA was extracted using RNAzol 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, Carslbad, 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 to normalize our samples for mass was *b-actin*, which has been determined to be unaffected by any treatments in this study. Analysis was

performed using a standard curve method. Gene expression data is represented as fold change in expression compared to the control group.

### 2.5 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 $\leq 0.05$.

Table 2.1. List of gene symbol, gene name, and primer sequences of transcripts analyzed.

| Gene Symbol | Gene Name | Primer Sequences |
|---|---|---|
| *b-actin* | Beta-actin; Housekeeping gene | F: 5′-CGAGCAGGAGATGGGAACC-3′<br>R: 5′-CAACGGAAACGCTCATTGC-3′ |
| *slco2b1* | Solute carrier organic anion transporter 2b1 | F: 5’- TTG CCC TGC CTC ACT TCA TT-3’<br>R: 5’-AGG CTG GAG TTG AGT CTG GT-3’ |
| *tfc3a* | Transcription factor 3a | F: 5’-TGA GAA ACC GCA GAC CA ACT -3’<br>R: 5’-CTT GCT GCT CCA GGT TGA GA-3’ |
| *Ihha* | Indian hedgehog homolog a | F: 5’-TGA GTC CAA AGC TCA CAT CCA-3’<br>R: 5’-AGG CTG GAA AAC AAC CAC CG-3’ |
| *Wnt5b* | Wingless-type MMTV integration site family 5b | F: 5’-GCA AAG CCA TCT TTC CCT GAA-3’<br>R: 5’-TGT ATC CCG AGC AAA AAC CTG-3’ |
| *slco3a1* | Organic anion transporter | F: 5′-CTCATCTGCGGTGCCTTACT-3′<br>R: 5′-CAGGCACTCCTTCCATCTCC-3′ |
| *tgfb1a* | Transcription growth factor; lateral line development | F: 5’-CCGCATCCAAAGCCAACTTC-3’<br>R: 5’-CGCCCGAAAACATTCCCAAG-3’ |
| *slco4a1* | Organic anion transporter | F: 5’-GATCTTCTACACAGCCGCCA-3’<br>R: 5’-AATCCACCAAGCTCCAACCC-3’ |
| *slco1d1* | Organic anion transporter | F: 5’-GCCGCATTTCTTCCAAGGAC-3’<br>R: 5’-TGTAAGGCACGGCAGAACAT-3’ |
| *ap1s1* | Protein transporter | F: 5’-CCGTCGAAATGATGCGCTTT-3’<br>R: 5’-GTACTTATCCAGCACCACCTG-3’ |
| *Bdnf* | Brain-derived neurotrophic factor | F: 5'-AGGTCCCCGTGACTAATGGT-3'<br>R: 5'-CGCTTGTCTATTCCTCGGCA-3' |

# Chapter 3: 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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## 3.1. Introduction

Polyfluorinated compounds (PFASs) are anthropogenic, emerging contaminants of environmental concern. PFASs 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 PFASs (greater than 8 carbons) were produced from the 1950s until 2000 (Lehmler 2005), when the manufacturers began a voluntary phase-out of the long chain PFAS in favor of shorter chain compounds (6 carbons). Both the long chain and the replacement PFASs pose serious environmental concerns because of their persistence due to the carbon fluoride bonds and limited toxicity data for a number of the PFASs. The three main long chain PFASs that are most commonly found at elevated levels in the environment are perfluorooctanoic acid (PFOA; C8), perfluorooctane sulfonate (PFOS;

C8) and perfluoronanonanoic acid (PFNA; C9)(Figure 3.1). 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).

Although all three compounds are PFASs, the subtle structural differences shown in Figure 3.1 affect the toxicity, toxicokinetics, and biodynamics of each compound. The bioaccumulation and biomagnification of each PFAS modeled by Houde et al. (2011) is based on the octanol-water partition coefficient (log Kow). Additionally, the rate of bioaccumulation has been shown to increase as the carbon chain length (C8 to C13) of the PFAS increases (Houde et al. 2011). PFASs 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 PFASs 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 River and the Delaware Bay estuaries (DRBC 2016). Fish fillets from these same waters contain a number of long chain and shorter chain PFASs. The concentrations used in this study span the range of surface

water PFAS concentrations (0.3 – 8.9 ppm; 0.6 – 23.6 μM) that have been reported from marsh habitats adjacent to the Wurtsmith Airforce base where a fire fighting school had been heavily contaminated with PFASs (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).

PFASs 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 Wurthsmith 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). PFASs 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 PFASs in their tissue(Kannan et al. 2002). Globally, PFOS is the most prevalent PFAS 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 artic 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 PFAS 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 monkeys exposed to PFOS (Seacat et al. 2002). An increase in liver size was reported for exposure to all three PFASs 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 PFASs to be developmentally bioactive and teretogenic at high micromolar concentrations (Zheng et al. 2011).

Our study reports on the comparative toxicity profiles following sub-lethal PFOA, PFOS, and PFNA exposure (0, 0.02, 0.2, 2.0 μM; 8 ng/L to 1 μg/L) to embryonic zebrafish. Control and continuously exposed 5 dpf zebrafish were evaluated for morphometric endpoints including total body length, area of yolk sac, and interoccular 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 PFASs resulted in different toxicity profiles and effects on gene expression.

## 3.2. Methods

### 3.2.1 Zebrafish Husbandry and Exposure Protocols

Zebrafish husbandry and rearing conditions were as described in chapter 2.1. Chemical solution preparation were as described in section 2.2.

Shown in Figure 3.2 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 to concentrations of control, 0.02, 0.2, or 2.0 μM (8 ng/L – 1 μg/L). 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 120 hpf 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.

### 3.2.2 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, interoccular distance, and yolk sac size to assess larval growth, cranial facial development, and nutrient storage and usage, respectively. Measurements could be made at the micrometer level. Each experiment was independently replicated three times.

### 3.2.3 Swim Activity Assay

Four replicates of each treatment and control each consisting of 25 animals were exposed for 120 hours and then transferred a raised in non-treatment system water in 600 mL beakers until they were two weeks old. The swim activity was performed in 24 well plates with a single animal in each well. After one 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. 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.

### 3.2.4 Shrimp Feeding Assay

Four replicates of each treatment and control consisting of 20 animals were exposed for 120 hours and then transferred to non-treated system water in 600 mL beakers until they were two weeks old. The feeding assay was performed in white plastic weight boats with 20 animals in each boat and four replicates per treatment. Fish were habituated for one hour, after which time 20 brine shrimp were added into the container. After ten minutes, the fish were anesthetized with MS-222 (Sigma Aldrich, St. Louis MO) and the number of shrimp remaining was counted. The number of shrimp eaten per number of fish was calculated. After the assay was stopped, the fish were also weighed.

### 3.2.5 Gene Expression Analysis

Gene expression analysis was performed as describe in general methods chapter 2.3. Each independent experiment was replicated 3 times. The primer sequences are listed in Table 2.1

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 RNAzol® 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 usedare listed in (Bugel et al. 2014). *β-actin* was used as a housekeeping transcript for normalization, and relative expression was quantified using the ΔΔCt method (Pfaffl 2001).

### 3.2.6 Statistical Analysis

All statistical analyses 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 could not be normalized, a Wilcoxon test was used. Statistical significance was at a p-value $\leq 0.05$

## 3. Results and Discussion

### 3.1 Morphometric Data

Morphometric endpoints of interoccular 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 3.1.

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 3.4 plot the total body length for each compound at 5, 7 and 14 dpf.

Interoccular distance was used to indicate changes in craniofacial development following embryonic PFAS exposure (Table 3.1). PFOA treatment at 2.0 μM resulted in a significant increase of interoccular distance, while PFOS at all concentrations (0.02 μM, 0.2 μM, 2.0 μM) significantly decreased interoccular 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 3.1).

### 3.3.2 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 3.2. PFOS (0.02 μM, 2.0 μM) PFOA (0.2 μM and 2.0 μM) and PFNA (0.2 μM) 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 3.2). Data points were obtained in five minute intervals. PFOS (0.02 μM) and PFNA (0.02,0.2, 2.0 μM) exposure both resulted in a significant decrease in swimming velocity. In PFOA exposure (2.0 μM) 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, uM) 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 μM) showed a significant increase in crossings. PFNA and PFOA did not show any significant effects on this endpoint.

### 3.3.3 Shrimp Feeding Assay

The amount of shrimp eaten per fish during the ten minute assay was measured. PFOA and PFNA both resulted in significant decrease of feeding activity. This was correlated with a decrease in size for PFOA, indicating that the decreased weight and length may be a function of eating less. This was not the case for PFNA in that although they were eating less, their weight was increased, however not significantly. PFOS had a reduction of food intake at one dose, which correlated to a decreased body weight at the same dose.

### 3.3.4 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 3.4). At 5 dpf there was a significant decrease in total body length for PFOS (0.2 μM, 2.0 μM), PFNA (2.0 μM) and PFOA (0.2, 2.0 μM) (Table 3.1). PFOS exposed animals were not significantly different from the controls at the 7 and 14 dpf timepoints (Figure 3.4A). PFNA exposure resulted in decrease body length at the 7 dpf timepoint (2.0 μM), but at 14 dpf the larvae were significantly larger (Figure 3.4B). PFOA exposed animals (Figure

3.4C) remained significantly smaller than the controls at all time points and exhibited very little growth between 7 and 14 dpf.

### 3.3.5 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 3.5). *Slco2b1* was significantly upregulated in PFOS (2.0 μM) and PFOA (all treatments), and significantly down-regulated in PFNA (all concentrations). *Tfc3a* expression was significantly higher in PFOS (2.0 μM) and PFOA (0.2, 2.0 μM). *Ihha* (hedgehog gene) (data not shown) showed no significant difference in expression at any PFAS concentration. *Wnt5b* (calcium modulation pathway) (data not shown), had a significant increase in expression after PFOS exposure (2.0 μM, fold change +2.18).

Gene expression analysis was performed on a battery of genes involved in tissue remodeling, cell cycle, cell death, angiogenesis, hypoxia, calcium signaling, and growth factors (Supplemental Figure 3.1). Genes that were significantly different in expression after exposure to PFOS, PFOA, or PFNA are listed in Table 3.4. Of the 106 genes analyzed, *ap1s1* was the only gene that was significantly differently expressed (decreased) across all three PFASs. Of the three compounds, PFOS significantly affected expression of the greatest number of genes (*calm3a, cdkn1a, cyp1a, flk1, tgfb1a*). PFOA and PFNA each only significantly altered one gene (*c-fos* and *tgfb1a*, respectively).

### 3.3.6 Gene Expression Data 14 dpf

Organic anion transporter 2b1 (*slco2b1*) and striated muscle development (*tfc3a*) transcripts were analyzed in 14 dpf zebrafish (Figure 3.6). *Slco2b1* was significantly upregulated in PFOS (0.2 μM, 2.0 μM), PFOA (2.0 μM), and PFNA (0.02 μM, 0.2 μM). *Tfc3a* expression was significantly higher in PFOS (all treatments) and PFOA (2.0 μM)

**3.4. Discussion**

The toxic effects following PFOA, PFNA, and PFOS exposure to embryonic and larval zebrafish have different biomarker profiles. There was no clear correlation between either the two C8 compounds (PFOS, PFOA) or the two carboxylic end change compounds (PFOA, PFNA). Therefore, 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 PFAS concentrations ranging from 5.0 -25.0 fold below our previously calculated LC50 values (PFOS 25 μM, PFNA 10 μM, PFOA 35 μM), and, to our knowledge, at lower concentrations than previously reported in the teleost literature. The exposure of the developing embryos and yolk sac fry (exposure 3-120 hpf) 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 PFASs contributing to these effects.

### 3.4.1 Compound Specific Toxicity or Behavioral Modifications

Figure 3.7A and 3.7B (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 interoccular distance (Table 3.1). 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 3.4.

PFOA exposure resulted in an increase in expression of the *c-fos* (transcription factor complex) transcript, an increase in interoccular 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 and decreased prey capture 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.

### 3.4.2 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 significant endpoints were shared between these two compounds.

PFOS and PFNA both resulted in a decrease of *tgfb1a* (transforming growth 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.

Exposure to 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.

### 3.4.3 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 PFASs 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 change in expression of the *ap1s1* transcript could indicate that it is a critical gene contributing to the alterations on growth and could partially explain the decrease in body size observed for all three PFASs. Genes commonly associated with *ap1s1* such as *c-jun* and many matrix metallopeptidase (*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 PFASs 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, *tgfb1a* 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 PFASs 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 (Kim et al. 2000). 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 interoccular distance in PFOA exposed zebrafish and decrease in interoccular distance in PFOS animals depending on which pathways and how they were altered. The interoccular distance may be affected by several independent alterations, including brain size, cranial formation, and edema.

Exposure to all three PFAS 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 3.4C). PFNA exposed animals had decreased in total size 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, PFASs 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 PFAS hindrance or inhibition could lead to deficiencies in the bone development, and in turn impact endpoints such as total body length and craniofacial formation (interoccular distance) observed in this study. At 14 dpf, *slco2b1* was significantly up-regulated by all PFASs tested, however each PFAS 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.

### 3.4.4 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. Exposure toe each of the three PFASs commonly resulted in a decrease in total body length, increased *tfc3a* (muscle development) expression and decreased *ap1s* (protein transport) expression at 5 dpf, and hyperactive locomotor activity 14 dpf. All other endpoints measured at both life-stage time points varied between each of the PFAS.

At 5 dpf, PFASs 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.

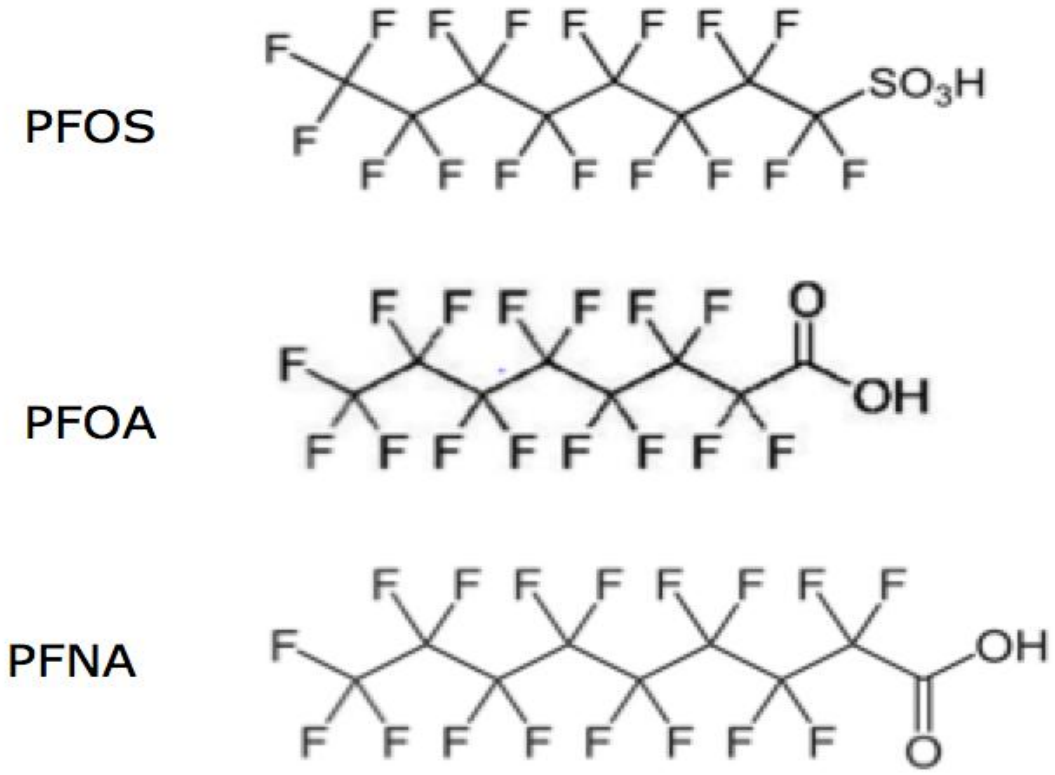


Figure 3.1. Molecular structures of PFOS, PFOA, and PFNA. PFOS has an eight-carbon chain backbone with a sulfonate end group, PFOA has an eight-carbon chain backbone with a carboxyl end group, and PFNA has a nine-carbon chain backbone with a carboxyl end group.

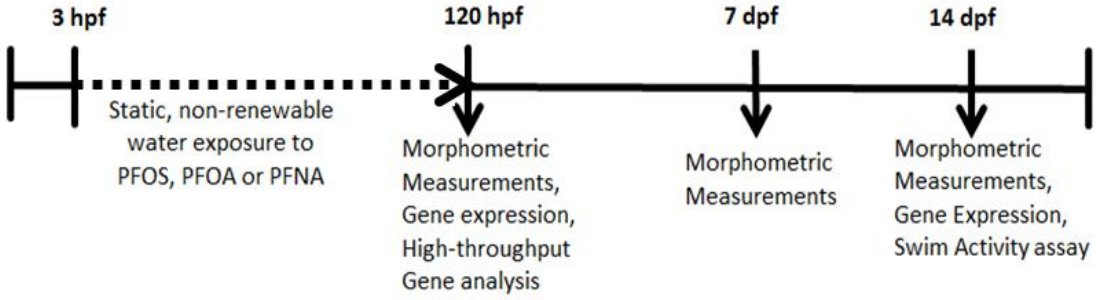


Figure 3.2: Exposure timeline of embryonic zebrafish to PFOS, PFOA or PFNA. All exposures occurred between 3 and 120 hpf. Exposure time period is indicated by dashed line.

| 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* |
| **Interoccular (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* |

Table 3.1. Summary of morphometric endpoints measured in 5 days post fertilization (dpf) zebrafish after exposure to PFOS, PFOA, or PFNA. 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.

| 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 |

Table 3.2. Summary of swim activity endpoints. 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.

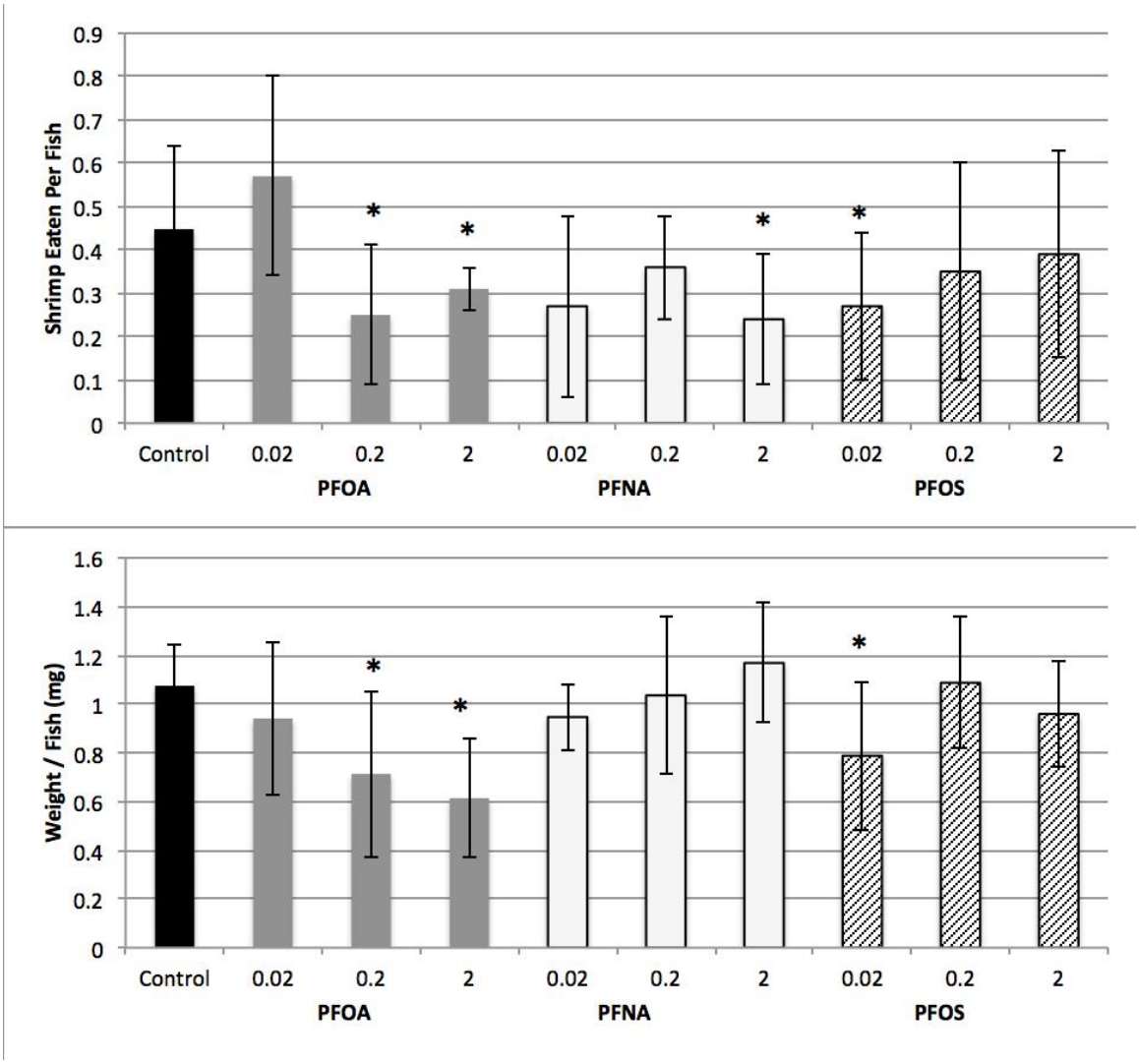


Figure 3.3. Shrimp Feeding Assay and total weight of fish at 14 dpf after PFOA, PFNA, and PFOS exposure. Bars represent average ± standard deviation. An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA compared to corresponding control.

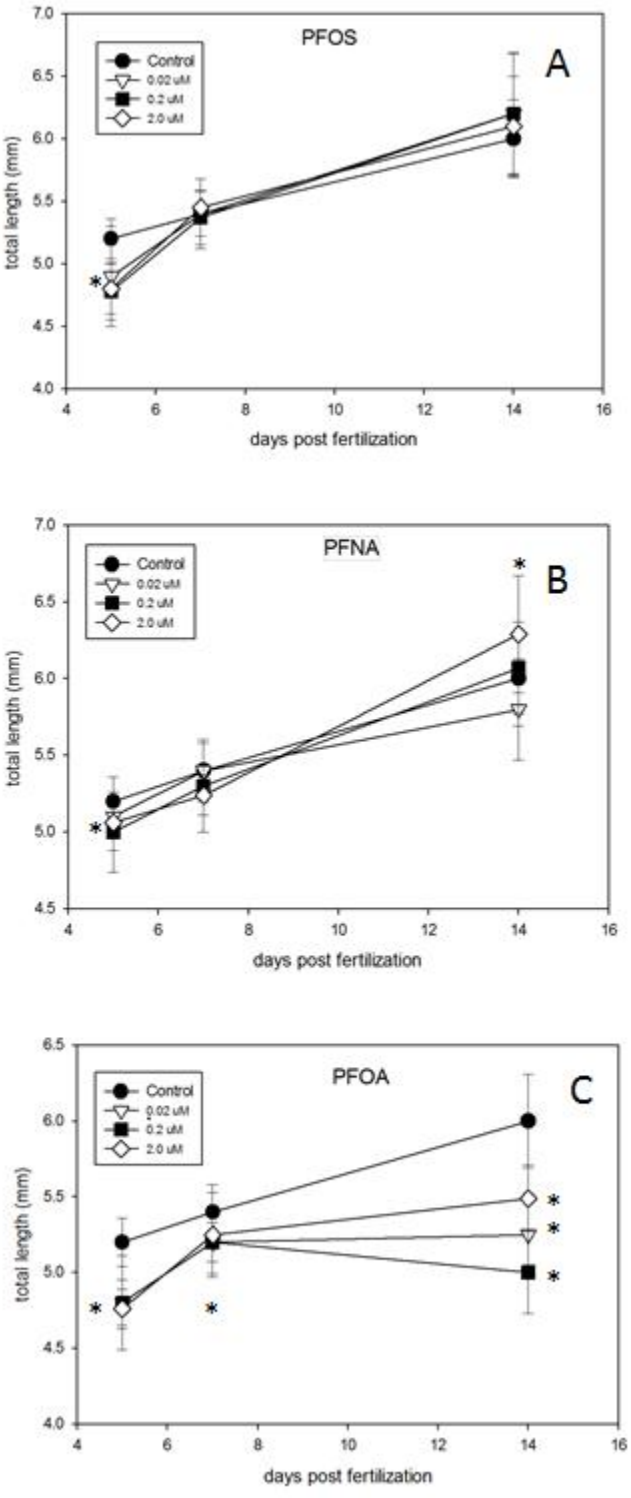


Figure 3.4. Growth curve of [A] PFOS, [B] PFNA, [C] PFOA exposed embryos from 5-14 days post fertilization (dpf). Measurements of total body length were taken at 5, 7, and 15 dpf. Statistical significance was determined using a one-way ANOVA at each time point for each compound ($p < 0.05$). PFOS 0.2 and 2.0 µM treated fish were significantly smaller in size at the 5 dpf timepoint. PFNA fish at the 2.0 µM concentration were significantly smaller at 5 dpf but significantly larger at 14 dpf. PFOA fish at 2.0 µM were significantly smaller at 5 dpf and 7 dpf, and all treatments were significantly smaller at 14 dpf. No other significant differences were observed.

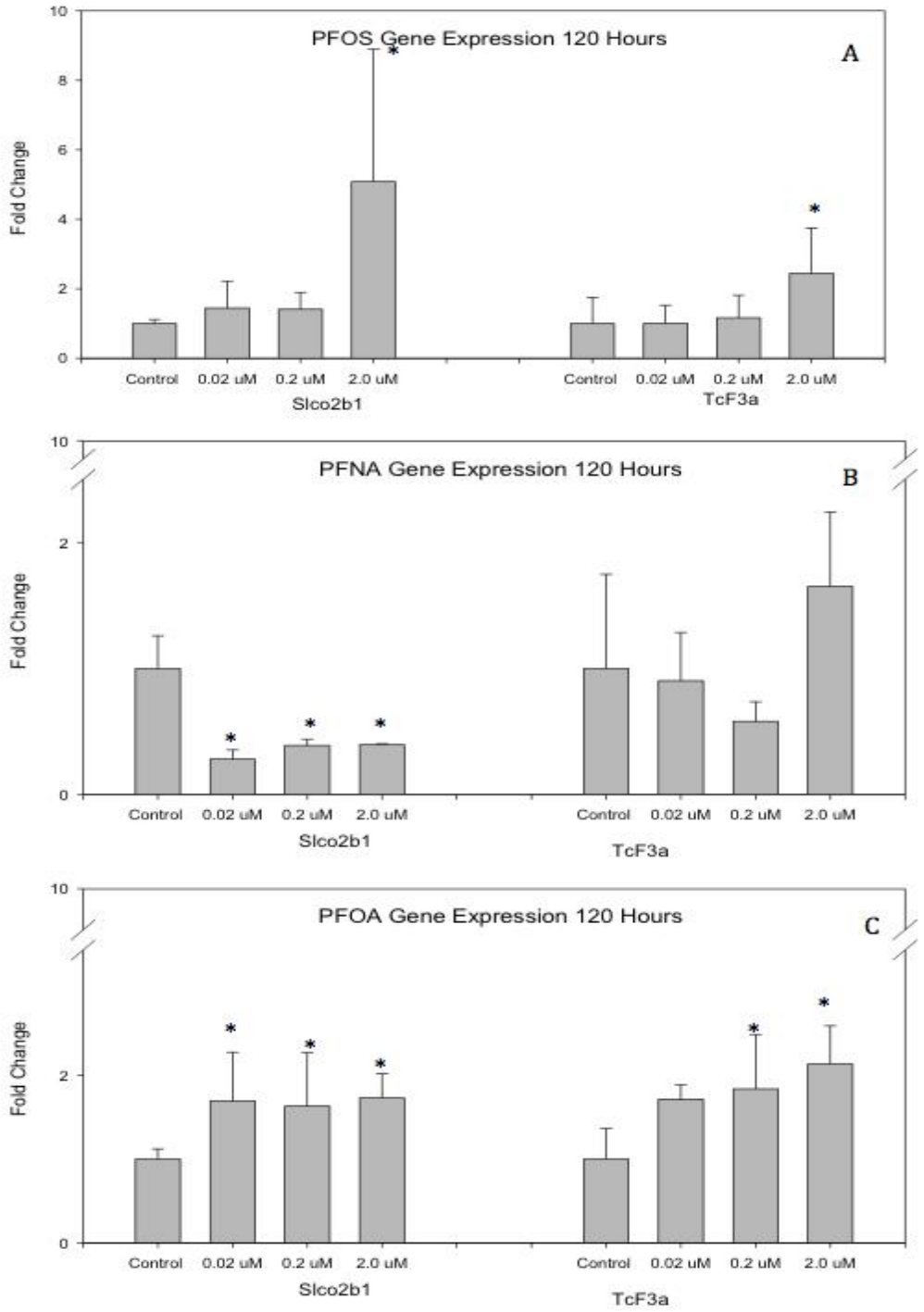


Figure 3.5. Embryonic zebrafish gene expression (5 dpf) after exposure to [A] PFOS, [B] PFNA, and [C] PFOA. Bars represent mean fold change and standard deviation. N= 4 replicates of 25 pooled animals for each exposure group. An asterisk (*) indicates a statistical significant value, $p< 0.05$, one-way ANOVA.

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

Table 3.3. List of genes that were significantly increased or decreased in transcipt analysis of 120 hpf zebrafish exposed to PFOS, PFOA, and PFNA (2.0 μM). 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 $p < 0.01$.

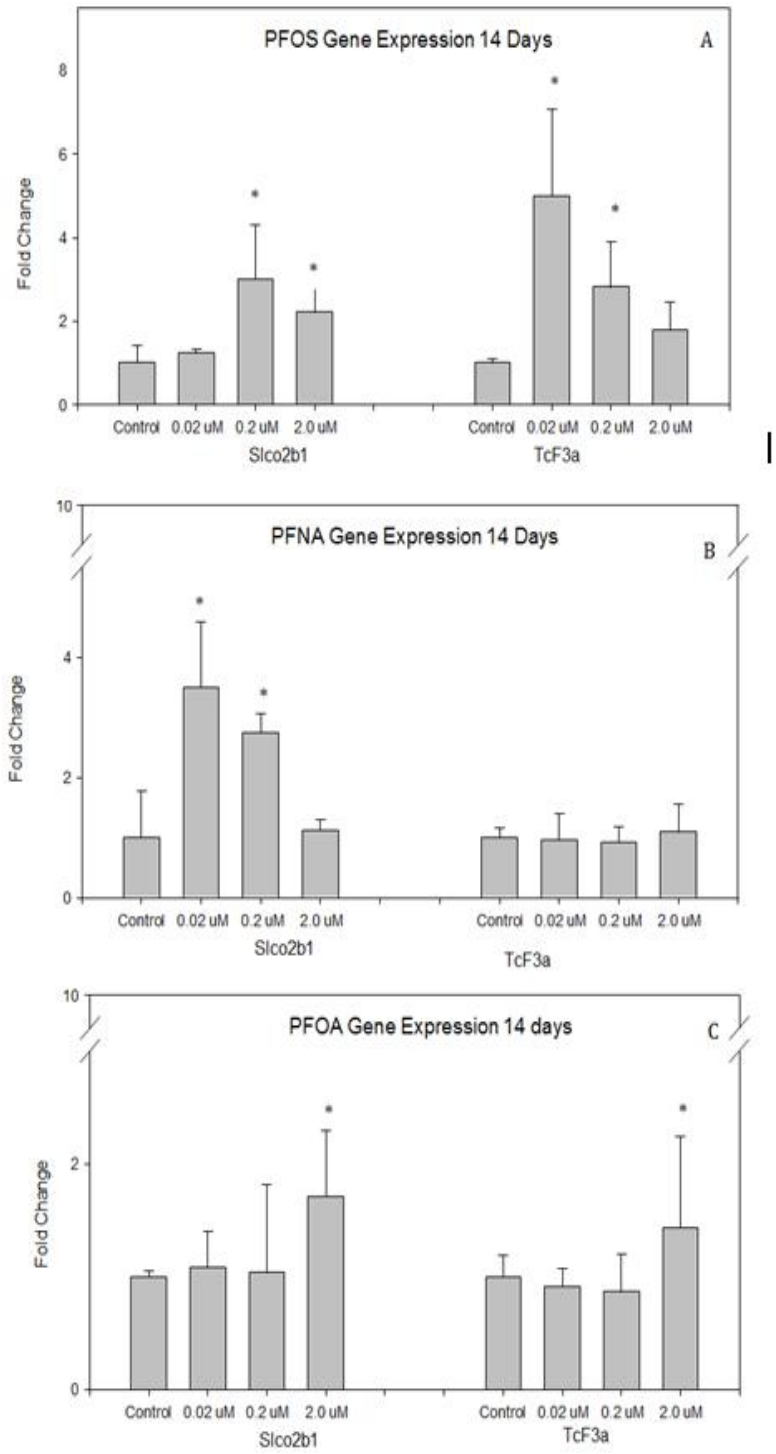


Figure 3.6. Embryonic zebrafish gene expression (14 dpf) after exposure to [A] PFOS, [B] PFNA, and [C] PFOA for 120 hours and remove to control water from 5 to 14 dpf. Bars represent mean fold change and standard deviation. N= 4 replicates of 25 pooled fish for each exposure group. An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA.

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

Table 3.4. Critical genes and the relationship to morphological and behavior endpoints observed.

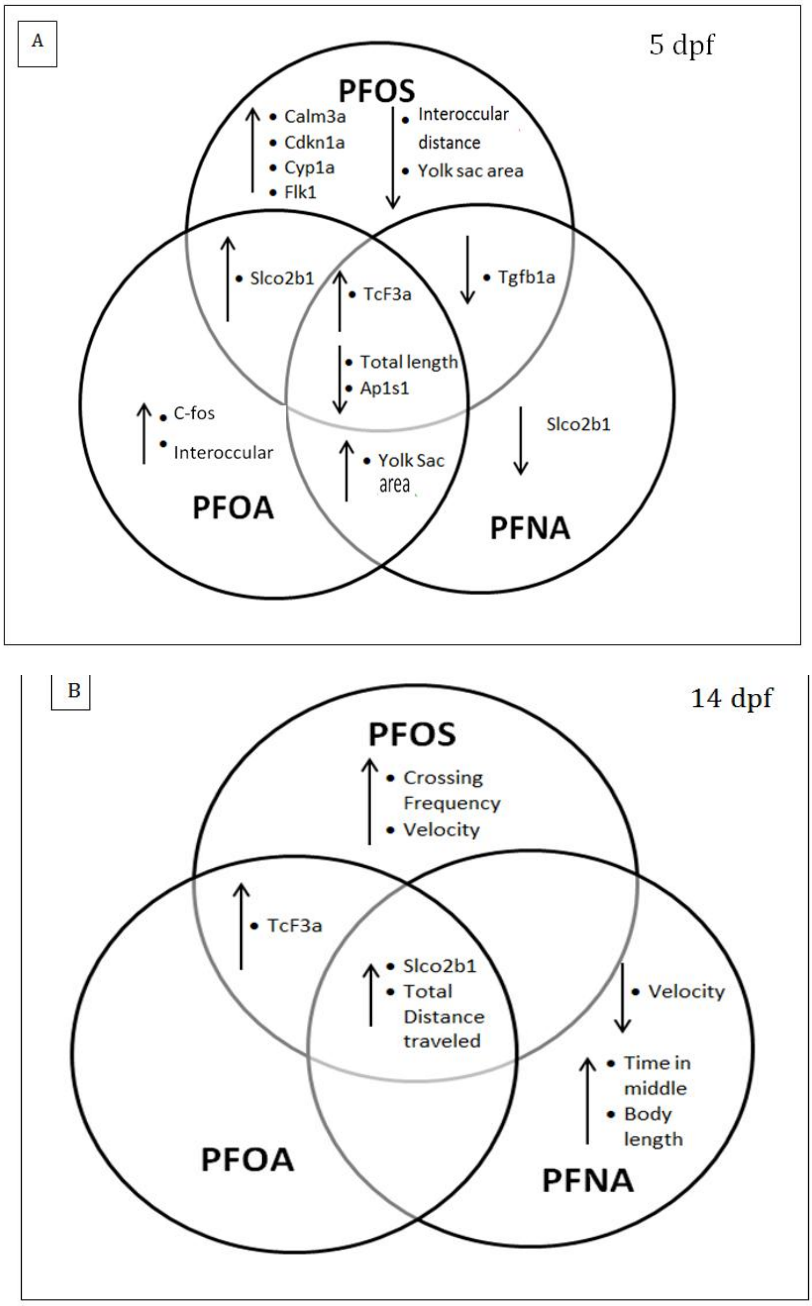


Figure 3.7. Venn diagram of morphometric, gene expression, and swimming activity endpoints for PFOS, PFOA and PFNA exposure at all concentrations examined for [A] 5 days post fertilization (dpf) endpoints and [B] 14 dpf endpoints. Up arrows (↑) indicate significant increase compared to control ($p < 0.05$). Down arrows (↓) indicate a significant decrease compared to control ($p<0.05$).

Supplemental Figure 1. List of genes of all genes analyzed in 100 transcript suite.

Listed are the average fold change ± one standard deviation and p-value obtained using a student's t-test or Wilcoxon

| Gene Name | Gene Symbol | Controls | PFNA | PFNA | PFOA | PFOA | PFOS | PFOS |
|---|---|---|---|---|---|---|---|---|
| | | avg ± stdev | avg ± stdev | p-value | avg ± stdev | p-value | avg ± stdev | p-value |
| **Tissue Remodelling** | | | | | | | | |
| basigin | *bsg* | 1.00 ± 0.12 | 1.01 ± 0.20 | 9.18E-01 | 1.17 ± 0.24 | 3.11E-01 | 1.21 ± 0.22 | 1.88E-01 |
| matrix metalloproteinase 11a | *mmp11a* | 1.00 ± 0.21 | 0.98 ± 0.12 | 8.70E-01 | 1.09 ± 0.21 | 5.81E-01 | 1.00 ± 0.15 | 9.74E-01 |
| matrix metalloproteinase 11b | *mmp11b* | 1.00 ± 0.12 | 1.11 ± 0.29 | 5.89E-01 | 0.96 ± 0.11 | 6.83E-01 | 0.95 ± 0.08 | 5.02E-01 |
| matrix metalloproteinase 13a | *mmp13a* | 1.00 ± 0.38 | 0.81 ± 0.16 | 3.95E-01 | 0.83 ± 0.30 | 5.26E-01 | 0.77 ± 0.12 | 3.01E-01 |
| matrix metalloproteinase 13b | *mmp13b* | 1.00 ± 0.29 | 1.05 ± 0.08 | 7.34E-01 | 1.40 ± 0.49 | 2.73E-01 | 1.05 ± 0.47 | 8.87E-01 |
| matrix metalloproteinase 14a | *mmp14a (mt1a)* | 1.00 ± 0.11 | 0.88 ± 0.10 | 2.11E-01 | 0.92 ± 0.10 | 2.29E-01 | 0.90 ± 0.11 | 2.69E-01 |
| matrix metalloproteinase 14b | *mmp14b (mt1b)* | 1.00 ± 0.04 | 0.96 ± 0.08 | 5.14E-01 | 1.00 ± 0.10 | 9.83E-01 | 0.92 ± 0.18 | 4.00E-01 |
| matrix metalloproteinase 15a | *mmp15a* | 1.00 ± 0.06 | 1.03 ± 0.05 | 4.75E-01 | 1.07 ± 0.06 | 1.58E-01 | 0.97 ± 0.16 | 8.04E-01 |
| matrix metalloproteinase 16a | *mmp16a* | 1.00 ± 0.14 | 1.00 ± 0.12 | 9.86E-01 | 0.84 ± 0.08 | 1.24E-01 | 0.93 ± 0.18 | 5.75E-01 |
| matrix metalloproteinase 16b | *mmp16b* | 1.00 ± 0.03 | 0.91 ± 0.06 | 5.48E-02 | 1.08 ± 0.24 | 4.00E-01 | 0.96 ± 0.10 | 5.20E-01 |
| matrix metalloproteinase 17a | *mmp17a (mt4a)* | 1.00 ± 0.27 | 1.04 ± 0.35 | 8.61E-01 | 0.89 ± 0.23 | 5.92E-01 | 0.91 ± 0.18 | 6.06E-01 |
| matrix metalloproteinase 17b | *mmp17b (mt4b)* | 1.00 ± 0.22 | 0.91 ± 0.17 | 5.75E-01 | 0.90 ± 0.22 | 5.66E-01 | 0.90 ± 0.07 | 8.57E-01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| matrix metalloproteinase 2 | *mmp2* | 1.00 ± 0.03 | 1.05 ± 0.09 | 3.74E-01 | 1.11 ± 0.17 | 3.37E-01 | 1.10 ± 0.27 | 6.29E-01 |
| matrix metalloproteinase 9 | *mmp9* | 1.00 ± 0.36 | 0.85 ± 0.37 | 6.13E-01 | 0.83 ± 0.30 | 5.30E-01 | 0.64 ± 0.17 | 1.29E-01 |
| brachyury homolog a (no tail a) | *ntla* | 1.00 ± 0.50 | 1.13 ± 0.81 | 9.40E-01 | 0.83 ± 0.40 | 6.13E-01 | 0.65 ± 0.14 | 2.03E-01 |
| brachyury homolog b (no tail b) | *ntlb* | 1.00 ± 0.21 | 1.03 ± 0.49 | 9.37E-01 | 0.92 ± 0.44 | 4.00E-01 | 0.92 ± 0.34 | 7.48E-01 |
| transcription factor 20 | *tcf20* | 1.00 ± 0.14 | 0.90 ± 0.21 | 5.28E-01 | 1.02 ± 0.24 | 9.07E-01 | 0.94 ± 0.03 | 6.29E-01 |
| transcription factor 3a | *tcf3a* | 1.00 ± 0.23 | 0.98 ± 0.14 | 8.87E-01 | 1.05 ± 0.08 | 8.57E-01 | 0.97 ± 0.25 | 8.86E-01 |
| tissue inhibitor of metalloproteinases 2a | *timp2a* | NA | NA | NA | NA | NA | NA | NA |
| tissue inhibitor of metalloproteinases 2b | *timp2b* | 1.00 ± 0.19 | 0.83 ± 0.05 | 1.18E-01 | 0.88 ± 0.11 | 3.19E-01 | 0.84 ± 0.15 | 2.60E-01 |
| tissue inhibitor of metalloproteinases 4 | *timp4* | 1.00 ± 0.28 | 1.28 ± 0.34 | 2.97E-01 | 1.02 ± 0.17 | 9.25E-01 | 0.99 ± 0.09 | 9.29E-01 |
| wingless-type MMTV integration site family, member 3A | *wnt3a* | 1.00 ± 0.23 | 1.09 ± 0.14 | 5.60E-01 | 1.17 ± 0.17 | 3.10E-01 | 1.15 ± 0.18 | 3.79E-01 |
| wingless-type MMTV integration site family, member 5b | *wnt5b* | 1.00 ± 0.37 | 1.00 ± 0.16 | 9.89E-01 | 1.07 ± 0.12 | 7.21E-01 | 1.00 ± 0.28 | 9.97E-01 |
| wingless-type MMTV integration site family, member 8A | *wnt8a* | 1.00 ± 0.33 | 1.07 ± 0.46 | 8.41E-01 | 0.63 ± 0.23 | 1.32E-01 | 0.68 ± 0.26 | 2.10E-01 |
| **Growth Factors** | | | | | | | | |
| epidermal growth factor | *egf* | 1.00 ± 0.20 | 1.00 ± 0.40 | 8.57E-01 | 1.04 ± 0.32 | 8.57E-01 | 0.86 ± 0.06 | 6.29E-01 |
| epithelial cell adhesion molecule | *epcam* | 1.00 ± 0.31 | 1.05 ± 0.11 | 7.79E-01 | 1.29 ± 0.08 | 1.31E-01 | 0.98 ± 0.14 | 9.28E-01 |
| keratinocyte growth factor (fibroblast growth factor 7) | *fgf7* | 1.00 ± 0.15 | 0.87 ± 0.10 | 2.41E-01 | 0.91 ± 0.21 | 5.50E-01 | 0.94 ± 0.14 | 1.00E+00 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| insulin-like growth factor 1a | *igf1a* | 1.00 ± 0.06 | 0.86 ± 0.06 | 7.45E-02 | 0.97 ± 0.09 | 1.00E+00 | 0.83 ± 0.13 | 1.14E-01 |
| insulin-like growth factor 1b | *igf1b* | 1.00 ± 0.25 | 1.04 ± 0.42 | 8.76E-01 | 1.24 ± 0.51 | 4.91E-01 | 1.36 ± 0.68 | 4.27E-01 |
| insulin-like growth factor 2a | *igf2a* | 1.00 ± 0.07 | 0.98 ± 0.13 | 8.57E-01 | 1.16 ± 0.09 | 5.21E-02 | 1.00 ± 0.13 | 1.00E+00 |
| insulin-like growth factor 2b | *igf2b* | 1.00 ± 0.19 | 0.85 ± 0.19 | 3.54E-01 | 0.94 ± 0.12 | 6.06E-01 | 0.70 ± 0.15 | 6.32E-02 |
| insulin-like growth factor binding protein 1a | *igfbp1a* | 1.00 ± 0.58 | 0.75 ± 0.14 | 5.05E-01 | 0.97 ± 0.34 | 6.29E-01 | 0.74 ± 0.10 | 1.00E+00 |
| insulin-like growth factor binding protein 1b | *igfbp1b* | 1.00 ± 0.59 | 0.66 ± 0.29 | 3.54E-01 | 0.63 ± 0.23 | 2.92E-01 | 0.77 ± 0.21 | 4.91E-01 |
| transforming growth factor alpha | *tgfa* | 1.00 ± 0.08 | 0.85 ± 0.12 | 1.10E-01 | 0.89 ± 0.17 | 3.58E-01 | 0.85 ± 0.23 | 3.24E-01 |
| transforming growth factor beta 1a | *tgfb1a* | 1.00 ± 0.09 | 0.82 ± 0.09 | 4.88E-02 | 0.85 ± 0.12 | 1.22E-01 | 0.82 ± 0.07 | 2.91E-02 |
| transforming growth factor beta 3 | *tgfb3* | 1.00 ± 0.16 | 0.89 ± 0.05 | 2.36E-01 | 1.03 ± 0.07 | 7.73E-01 | 0.94 ± 0.07 | 5.50E-01 |
| **Calcium Signaling** | | | | | | | | |
| calmodulin 1a | *calm1a* | 1.00 ± 0.32 | 1.09 ± 0.28 | 6.98E-01 | 1.13 ± 0.10 | 4.57E-01 | 1.00 ± 0.37 | 9.90E-01 |
| calmodulin 1b | *calm1b* | 1.00 ± 0.10 | 0.88 ± 0.13 | 2.43E-01 | 0.93 ± 0.16 | 5.56E-01 | 0.88 ± 0.05 | 1.12E-01 |
| calmodulin 2a | *calm2a* | 1.00 ± 0.06 | 0.94 ± 0.14 | 5.11E-01 | 1.00 ± 0.27 | 9.98E-01 | 0.97 ± 0.09 | 6.18E-01 |
| calmodulin 3a | *calm3a* | 1.00 ± 0.07 | 1.16 ± 0.19 | 2.46E-01 | 1.13 ± 0.31 | 5.15E-01 | 1.17 ± 0.06 | 2.20E-02 |
| calmodulin 3b | *calm3b* | 1.00 ± 0.18 | 0.98 ± 0.23 | 8.57E-01 | 0.98 ± 0.16 | 1.00E+00 | 0.97 ± 0.04 | 6.29E-01 |
| calpain 1a | *capn1a* | 1.00 ± 0.09 | 0.91 ± 0.09 | 2.77E-01 | 0.89 ± 0.12 | 2.44E-01 | 0.88 ± 0.07 | 1.05E-01 |
| calpain 1b | *capn1b* | 1.00 ± 0.27 | 1.04 ± 0.11 | 8.11E-01 | 1.12 ± 0.12 | 4.51E-01 | 1.04 ± 0.07 | 7.95E-01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| calpain 2a | *capn2a* | 1.00 ± 0.07 | 0.90 ± 0.10 | 2.13E-01 | 0.91 ± 0.21 | 5.42E-01 | 0.88 ± 0.13 | 2.22E-01 |
| calpain 2b | *capn2b* | 1.00 ± 0.08 | 0.96 ± 0.14 | 6.96E-01 | 0.92 ± 0.19 | 5.05E-01 | 0.96 ± 0.08 | 5.10E-01 |
| calpain 3a | *capn3a* | 1.00 ± 0.11 | 1.04 ± 0.28 | 8.25E-01 | 1.01 ± 0.07 | 8.57E-01 | 0.91 ± 0.15 | 4.15E-01 |
| calpain 3b | *capn3b* | 1.00 ± 0.16 | 1.03 ± 0.37 | 8.88E-01 | 0.82 ± 0.21 | 2.84E-01 | 0.82 ± 0.14 | 1.69E-01 |
| calpain 4a | *capn4a (capns 1a)* | 1.00 ± 0.10 | 0.92 ± 0.16 | 4.83E-01 | 0.95 ± 0.21 | 7.19E-01 | 0.93 ± 0.10 | 3.95E-01 |
| calpain 4b | *capn4b (capns 1b)* | 1.00 ± 0.16 | 0.96 ± 0.22 | 8.14E-01 | 0.96 ± 0.14 | 7.51E-01 | 0.92 ± 0.17 | 5.45E-01 |
| calpain 5a | *capn5a* | 1.00 ± 0.12 | 0.82 ± 0.15 | 1.48E-01 | 1.01 ± 0.22 | 9.37E-01 | 0.97 ± 0.15 | 8.10E-01 |
| calpain 5b | *capn5b* | 1.00 ± 0.07 | 0.88 ± 0.11 | 2.29E-01 | 0.99 ± 0.30 | 6.29E-01 | 0.96 ± 0.09 | 6.29E-01 |
| calpain 7 | *capn7* | 1.00 ± 0.19 | 1.04 ± 0.12 | 6.29E-01 | 1.06 ± 0.22 | 8.57E-01 | 1.03 ± 0.21 | 8.57E-01 |
| calpastatin | *cast* | 1.00 ± 0.07 | 0.97 ± 0.12 | 7.14E-01 | 1.00 ± 0.05 | 9.20E-01 | 1.03 ± 0.13 | 7.56E-01 |
| cadherin 1 | *cdh1* | 1.00 ± 0.16 | 0.90 ± 0.12 | 4.05E-01 | 0.91 ± 0.17 | 5.32E-01 | 0.85 ± 0.06 | 1.53E-01 |
| cadherin 11 | *cdh11* | 1.00 ± 0.10 | 1.05 ± 0.10 | 6.29E-01 | 1.09 ± 0.14 | 6.29E-01 | 0.97 ± 0.10 | 1.00E+00 |
| cadherin associated protein beta 1 | *ctnnb1* | 1.00 ± 0.19 | 0.83 ± 0.11 | 1.99E-01 | 0.95 ± 0.14 | 7.02E-01 | 0.81 ± 0.14 | 1.82E-01 |
| cadherin associated protein beta 2 | *ctnnb2* | 1.00 ± 0.03 | 0.94 ± 0.18 | 5.98E-01 | 1.05 ± 0.17 | 6.22E-01 | 1.02 ± 0.12 | 7.95E-01 |
| protein kinase C alpha | *prkca* | 1.00 ± 0.03 | 0.97 ± 0.02 | 2.10E-01 | 0.93 ± 0.11 | 3.51E-01 | 0.86 ± 0.11 | 8.88E-02 |
| protein kinase C beta a | *prkcba* | 1.00 ± 0.12 | 0.91 ± 0.12 | 3.60E-01 | 1.04 ± 0.11 | 6.50E-01 | 1.00 ± 0.06 | 9.82E-01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| protein kinase C beta b | *prkcbb* | 1.00 ± 0.11 | 1.09 ± 0.22 | 5.65E-01 | 0.94 ± 0.22 | 6.98E-01 | 0.94 ± 0.09 | 4.59E-01 |
| protein kinase C gamma | *prkcg* | 1.00 ± 0.12 | 0.83 ± 0.12 | 1.25E-01 | 0.92 ± 0.14 | 4.31E-01 | 0.89 ± 0.22 | 4.74E-01 |
| **Angiogenesis and Hypoxia** | | | | | | | | |
| erythropoietin | *epo* | 1.00 ± 0.23 | 0.94 ± 0.34 | 8.11E-01 | 0.92 ± 0.34 | 7.44E-01 | 1.04 ± 0.39 | 8.76E-01 |
| kinase insert domain receptor (vegf receptor) | *flk1* | 1.00 ± 0.16 | 0.89 ± 0.16 | 4.24E-01 | 0.83 ± 0.07 | 1.13E-01 | 0.66 ± 0.17 | 4.61E-02 |
| hypoxia inducible factor 1, alpha subunit a | *hif1aa* | 1.00 ± 0.15 | 0.87 ± 0.07 | 1.80E-01 | 1.00 ± 0.16 | 1.00E+00 | 1.08 ± 0.12 | 4.84E-01 |
| hypoxia inducible factor 1, alpha subunit b | *hif1ab* | 1.00 ± 0.34 | 0.95 ± 0.18 | 8.05E-01 | 1.06 ± 0.11 | 7.66E-01 | 0.95 ± 0.33 | 8.44E-01 |
| hypxia inducible factor 1, alpha subunit like 1 | *hif1al1* | 1.00 ± 0.35 | 0.96 ± 0.05 | 5.93E-01 | 1.03 ± 0.08 | 6.29E-01 | 0.89 ± 0.32 | 6.90E-01 |
| hypxia inducible factor 1, alpha subunit like 2 | *hif1al2* | 1.00 ± 0.51 | 1.02 ± 0.39 | 9.47E-01 | 0.89 ± 0.11 | 8.71E-01 | 0.70 ± 0.23 | 3.27E-01 |
| vascular endothelial growth factor Aa | *vegfaa* | 1.00 ± 0.39 | 0.92 ± 0.20 | 7.22E-01 | 1.05 ± 0.11 | 8.08E-01 | 0.78 ± 0.29 | 4.39E-01 |
| vascular endothelial growth factor Ab | *vegfab* | 1.00 ± 0.33 | 0.91 ± 0.05 | 8.57E-01 | 0.88 ± 0.11 | 5.21E-01 | 0.77 ± 0.24 | 3.26E-01 |
| **Cell Cycle and Cell Death** | | | | | | | | |
| apoptosis-inducing factor 1, mitochondrion-associated | *aifm1* | 1.00 ± 0.02 | 0.80 ± 0.15 | 5.71E-02 | 0.86 ± 0.26 | 4.00E-01 | 0.72 ± 0.14 | 5.71E-02 |
| bcl2-associated X protein | *baxa* | 1.00 ± 0.07 | 1.03 ± 0.08 | 6.56E-01 | 1.03 ± 0.13 | 7.30E-01 | 0.95 ± 0.09 | 4.55E-01 |
| bcl2-associated death promoter | *bcl2 (bad)* | 1.00 ± 0.02 | 0.89 ± 0.19 | 4.00E-01 | 1.00 ± 0.18 | 4.00E-01 | 0.95 ± 0.24 | 4.00E-01 |
| caspase 2 | *casp2* | 1.00 ± 0.16 | 0.90 ± 0.09 | 3.31E-01 | 0.96 ± 0.22 | 8.13E-01 | 0.87 ± 0.09 | 2.19E-01 |
| caspase 3a | *casp3a* | 1.00 ± 0.26 | 0.88 ± 0.52 | 7.23E-01 | 0.91 ± 0.45 | 7.81E-01 | 0.89 ± 0.12 | 4.58E-01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| caspase 6 | *casp6* | 1.00 ± 0.31 | 1.02 ± 0.14 | 9.20E-01 | 0.85 ± 0.16 | 4.35E-01 | 0.87 ± 0.15 | 4.81E-01 |
| caspase 7 | *casp7* | 1.00 ± 0.25 | 1.28 ± 0.27 | 2.29E-01 | 1.14 ± 0.12 | 6.29E-01 | 0.82 ± 0.14 | 6.29E-01 |
| caspase 8 | *casp8* | 1.00 ± 0.32 | 1.04 ± 0.09 | 8.13E-01 | 0.99 ± 0.12 | 9.41E-01 | 0.85 ± 0.07 | 4.26E-01 |
| caspase 9 | *casp9* | 1.00 ± 0.26 | 0.98 ± 0.23 | 8.99E-01 | 0.97 ± 0.13 | 8.45E-01 | 0.86 ± 0.20 | 4.41E-01 |
| cyclin-dependent kinase 2 | *cdk2 (p33)* | 1.00 ± 0.03 | 0.93 ± 0.08 | 2.04E-01 | 1.06 ± 0.11 | 3.92E-01 | 0.90 ± 0.12 | 2.11E-01 |
| cyclin-dependent kinase inhibitor 1a | *cdkn1a (p21)* | 1.00 ± 0.07 | 0.89 ± 0.32 | 5.84E-01 | 1.19 ± 0.35 | 4.15E-01 | 0.72 ± 0.13 | 2.32E-02 |
| FBJ murine osteosarcoma viral oncogene homolog | *c-fos* | 1.00 ± 0.33 | 1.48 ± 0.36 | 1.34E-01 | 1.63 ± 0.19 | 2.36E-02 | 1.16 ± 0.11 | 3.89E-01 |
| jun proto-oncogene | *c-jun* | 1.00 ± 0.15 | 0.86 ± 0.10 | 1.87E-01 | 0.97 ± 0.16 | 8.41E-01 | 1.03 ± 0.19 | 8.12E-01 |
| glycogen synthase kinase 3 beta | *gsk3b* | 1.00 ± 0.01 | 1.00 ± 0.22 | 1.00E+00 | 1.07 ± 0.16 | 4.00E-01 | 0.91 ± 0.12 | 1.14E-01 |
| mitogen-activated protein kinase 3 | *mapk3 (erk1)* | 1.00 ± 0.07 | 1.04 ± 0.12 | 5.92E-01 | 1.10 ± 0.13 | 3.01E-01 | 1.08 ± 0.09 | 2.69E-01 |
| microphthalmia-associated transcription factor a | *mitfa* | 1.00 ± 0.06 | 1.13 ± 0.28 | 4.74E-01 | 1.04 ± 0.18 | 7.06E-01 | 0.96 ± 0.17 | 6.85E-01 |
| myelocytomatosis oncogene | *myca* | 1.00 ± 0.10 | 1.10 ± 0.20 | 4.00E-01 | 1.15 ± 0.17 | 2.33E-01 | 1.12 ± 0.03 | 7.30E-02 |
| nuclear factor NF-kappa-B p100 subunit | *nfkb2* | 1.00 ± 0.21 | 0.73 ± 0.10 | 7.08E-02 | 0.80 ± 0.19 | 2.43E-01 | 0.77 ± 0.22 | 2.24E-01 |
| tumor necrosis factor alpha | *tnfa* | 1.00 ± 0.29 | 0.97 ± 0.64 | 9.46E-01 | 0.61 ± 0.36 | 1.87E-01 | 0.62 ± 0.20 | 8.86E-02 |
| tumor necrosis factor beta | *tnfb* | 1.00 ± 0.62 | 0.81 ± 0.48 | 6.62E-01 | 0.76 ± 0.19 | 4.89E-01 | 0.66 ± 0.23 | 3.45E-01 |
| tumor protein P53 | *tp53* | 1.00 ± 0.18 | 0.88 ± 0.14 | 3.80E-01 | 1.10 ± 0.19 | 4.98E-01 | 0.88 ± 0.16 | 3.81E-01 |
| tumor protein 63 | *tp63* | 1.00 ± 0.17 | 1.18 ± 0.31 | 4.05E-01 | 1.25 ± 0.25 | 1.96E-01 | 1.10 ± 0.10 | 3.82E-01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **AHR and ER** | | | | | | | | |
| cytochrome P450, family 19, subfamily A, polypeptide 1b | *cyp19a 1b* | 1.00 ± 0.11 | 1.03 ± 0.35 | 8.87E-01 | 0.98 ± 0.40 | 9.39E-01 | 0.86 ± 0.11 | 1.51E-01 |
| cytochrome P450, family 1, subfamily A | *cyp1a* | 1.00 ± 0.21 | 0.80 ± 0.17 | 2.22E-01 | 0.75 ± 0.24 | 1.99E-01 | 0.60 ± 0.20 | 4.79E-02 |
| estrogen receptor α | *esr1* | 1.00 ± 0.38 | 1.07 ± 0.30 | 7.97E-01 | 1.03 ± 0.54 | 9.36E-01 | 0.85 ± 0.24 | 5.52E-01 |
| estrogen receptor βa | *esr2a* | 1.00 ± 0.21 | 1.07 ± 0.60 | 8.63E-01 | 0.88 ± 0.33 | 6.02E-01 | 0.83 ± 0.26 | 3.87E-01 |
| estrogen receptor βb | *esr2b* | 1.00 ± 0.25 | 0.90 ± 0.21 | 6.08E-01 | 0.93 ± 0.19 | 7.11E-01 | 0.95 ± 0.18 | 7.51E-01 |
| glutathione S-transferase pi 2 | *gstp2* | 1.00 ± 0.17 | 0.74 ± 0.12 | 5.78E-02 | 0.96 ± 0.09 | 6.70E-01 | 0.97 ± 0.33 | 8.95E-01 |
| luteinizing hormone beta | *lhb* | 1.00 ± 0.83 | 0.89 ± 0.37 | 6.29E-01 | 0.85 ± 0.25 | 6.29E-01 | 0.93 ± 0.77 | 8.57E-01 |
| vitellogenin 1 | *vtg1* | 1.00 ± 0.38 | 0.53 ± 0.12 | 6.27E-02 | 0.76 ± 0.44 | 4.85E-01 | 0.93 ± 0.12 | 7.48E-01 |
| **Others** | | | | | | | | |
| AP-1 complex subunit sigma-1A | *ap1s1* | 1.00 ± 0.10 | 0.57 ± 0.09 | 1.76E-03 | 0.59 ± 0.21 | 2.92E-02 | 0.58 ± 0.14 | 7.19E-03 |
| cAMP response element binding protein binding protein a | *crebbp a* | 1.00 ± 0.12 | 0.99 ± 0.13 | 9.07E-01 | 1.20 ± 0.24 | 2.42E-01 | 1.02 ± 0.21 | 9.06E-01 |
| cAMP response element binding protein binding protein b | *crebbp b* | 1.00 ± 0.32 | 0.94 ± 0.34 | 8.23E-01 | 0.99 ± 0.29 | 9.64E-01 | 0.92 ± 0.21 | 6.92E-01 |
| indian hedgehog homolog a | *ihha* | 1.00 ± 0.28 | 0.93 ± 0.19 | 7.23E-01 | 0.98 ± 0.29 | 9.38E-01 | 0.79 ± 0.25 | 3.57E-01 |
| solute carrier organic anion transporter family member 2B1 | *slco2b 1* | 1.00 ± 0.42 | 1.08 ± 0.12 | 7.13E-01 | 1.23 ± 0.12 | 3.24E-01 | 1.08 ± 0.43 | 8.06E-01 |

# Chapter 4: Behavioral, morphometric, and gene expression effects in adult zebrafish (Danio rerio) embryonically exposed to PFOA, PFOS, and PFNA

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**This manuscript has been prepared for submission**

## 4.1 Introduction

Perfluoroalkylated substances (PFASs) are anthropogenic compounds 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 PFASs (greater than 8 carbons) were produced from the 1950s until 2000 (Lehmler 2005) because they are extremely stable which allows them to be used in a number of manufacturing applications (Renner 2001). The three most prevalent long chain PFASs 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).

PFASs are of particular concern because they are persistent in the environment (Houde et al. 2011), have been detected in animal tissue samples world-wide (Lindstrom et al. 2011), and have been detected in both ground and surface waters (Hu et al. 2016; Post et al. 2013). PFNA in particular, since a voluntary ban of long chain PFASs in 2000, has seen increased concentrations in human serum in U.S populations (Kato et al. 2011).

Although all three compounds belong to the same chemical class, the subtle structural differences affect the toxicity, toxicokinetics, and toxicodynamics within organisms. The occurrence in both environmental and animal samples as well as the fate and transport of these compounds has been well reviewed (Houde et al. 2011; Kannan et al. 2005; Lau 2012; Lindstrom et al. 2011).

Our previous study results are summarized in Table 4 and formed the basis for the endpoints examined in this paper. The effects on morphometrics, behavior analysis, and gene expression in zebrafish after the acute, embryonic exposure (3- 120 hours post fertilization, 0.02 – 2.0 μM) of yolk sac larvae (5 dpf) and free swimming larvae (14 dpf) time points were reported. The current paper analyzes the long term effects of an acute embryonic exposure (3- 120 hours post fertilization, 2.0 μM) in adult zebrafish, 6 months after exposure has stopped.

At 5 dpf, exposure to all three PFASs (2.0 μM) resulted in a significantly decreased total body size (Jantzen et al 2016). However, when the fish reached 14 dpf, only PFOA exposed fish continued to be significantly smaller. PFOS exposed fish had recovered and were not significantly different from controls, while PFNA exposed fish were significantly larger. Behavior analysis at 14 dpf (2.0 μM) resulted in an increased swimming distance for all PFAS treatments. Additional behavior effects included changes in swimming velocity (PFNA, PFOS) and thigmotaxis behaviors (PFNA, PFOS). Similar observations have been made in mammalian studies, in which exposure to PFOS in mice (0.3 mg/kg/day) induced spontaneous hyperactivity (Spulber et al. 2014) and

mice exposed to 0.3 mg/kg PFOA showed changes in exploratory behavior (Onishchenko et al. 2011). Analyzing similar behavior endpoints in adult zebrafish will determine if the growth and behavior affects seen in larval zebrafish persist through adulthood as well as understand the long term effects of PFAS exposure in our teleost model system.

In the Jantzen et al 2016 study, a battery of 100 genes relevant to critical development pathways were analyzed in 5 dpf zebrafish exposed to 2.0 µM PFASs. From this analysis, 4 specific pathways were identified to be affected by two or more PFASs. All three PFASs had a significantly decreased expression of adaptor related protein complex 1, sigma subunit 1 (*ap1s1)* and a significant increase transcription factor 3a (*tcf3a*). Transforming growth factor beta 1a (*tgfb1a)* was significantly decreased after PFOS and PFNA exposure, and solute carrier organic anion transporting polypeptide 2b1 (*slco2b1*) was significantly increased in PFOA and PFOS and decreased in PFNA. The current study aims to determine if these gene expression changes observed in 5 dpf larvae zebrafish after PFAS exposure persists to the adult life stage. Both *ap1s1* and *tcf3a* are transcripts that are only expressed in the developing zebrafish, and therefore could not be analyzed in adult zebrafish. *Tgfb1a* and *slco2b1* are both expressed throughout the lifetime of the zebrafish and were assessed in this study.

*Slco1d1* is a solute carrier organic anion transporting polypeptide that is expressed in all zebrafish life stages. This transcript was chosen for this study based on results shown in (Popovic et al. 2014) which determined *in vivo* that PFASs could be both a substrate and an inhibitor to this transporter. Due to the number of behavior endpoints analyzed in this

study, brain derived neurotrophic factor (*bdnf)* was also assessed. This is a transcription growth factor involved in a number of neurological processes including regulation of neuron differentiation (Reference Genome Group of the Gene Ontology 2009), serotonin transporter function (Mossner et al. 2000) and has a possible relation to stress responses (Pavlidis et al. 2015)

It is hypothesized that the toxicities induced by these compounds at 5 and 14 dpf in terms of morphometric measurements, behavior, and gene expression will be persistent to adult life stage (6 months). Exposure to PFASs during embryonic development appears to result in altered gene expression of transporters and behavior into adulthood, particularly in PFNA exposed male fish. In the case of finfish, these biochemical alterations could have detrimental effects on endogenous substrate pharmacokinetics thereby altering normal homeostatic pathways. The behavioral alterations could have detrimental effects on prey survival from predators or other behavioral related cues. Concordance between lower and higher vertebrate studies indicate that embryonic developmental stages are the most sensitive to PFASs and that those alterations can be manifested later in life (Lau et al. 2006; Yang et al. 2002) .

## 4.2 Methods

### 4.2.1 Animal Handling

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.

### 4.2.2 Exposure

Shown in Figure 1 is the exposure and data collection timeline. Zebrafish embryos were exposed to PFOS, PFOA or PFNA (Sigma-Aldrich, St. Louis, MO) from 3 hpf to 120 hpf hours in a static non-renewal protocol. All compounds were dissolved in water. The exposure followed a modified OECD 212 protocol (OECD. 2011), where the endpoints of lesion presence, length, weight, and mortality were recorded. Modification to the OECD protocol was to extend the study beyond the exposure time-point which allowed for the analysis of adult zebrafish. After the exposure was terminated (120 hpf), fish were transferred to non-treated system water and fed 2 times daily with Zeigler Larval AP50 (Aquatic Habitats, Apopka, Florida) and brine shrimp. Therefore, the only exposure was through the water from 3 hpf to 120 hpf (5 days), which corresponds to embryonic to yolk sac larval exposure. Morphometric measurements, gene expression, and swim activity endpoints were collected at 6 months post fertilization, which is during the adult life stage. One biological replicate from each control and treatment group consisted of 10 males and 10 females, for a total of 20 fish per treatment group. Two biological replicates

were performed. No experiment had mortality greater than 20% of the starting sample size. All treatment water was collected and disposed of through the Rutgers Environmental Health and Safety for proper disposal.

### 4.2.3 Animal Rearing After Exposure

After 120 hpf, fish larvae from both control and treated groups were transferred into system water as described above in 600 mL beakers and fed Zeigler Larval AP50 (Aquatic Habitats, Apopka, Florida). until 30 dpf. This food consists of marine, animal and vegetable proteins, test, vegetable starches, fish and vegetable oils, and vitamin and mineral premixes with a minimum protein content of 50%. When fish reached 30 dpf they were transferred onto the aquatic habitats system (described above) and fed a regimen of brine shrimp (1 mL/ tank) in the morning feeding and a Tetramin/Aquatox flake food combination (0.04g/tank) in the evening. Tetramin/Aquatox combination consists 43% protein, 13% crude fat, 1.5% crude fiber, 10% moisture and 10.5% ash. Each treatment group was housed together (N = 10-12) in a 3L tank. Two weeks before the study was performed, the sex of each fish was recorded and the fish were individually housed in a divided 1.5 L tank. The sex ratio of control and all treatment groups was approximately 50%. Feeding amounts for individual fish were adjusted to brine shrimp 0.25 mL/tank and Tetramin/Aquatox 0.01 g/tank.

### 4.2.4 Light/Dark Assay

One behavior that is classified as anxiety type behavior is light-avoidance; in which zebrafish prefer the dark, opaque compartment of a tank rather than the light, clear compartment (Champagne et al. 2010). The proportion of time the zebrafish prefer light versus dark is dependent upon the ambient light level (Stephenson et al. 2011).

A modified version of the light/dark box test assay described by (Champagne et al. 2010) was performed. Fish were habituated in tanks in our behavior room for 30 minutes. After this habituation period, each fish was placed in the assay tank and video recorded for a total of ten minutes. Four Ikegami ICD-49 CCD cameras (Noldus Information Technology, Leesburg, VA) were mounted to the ceiling above each tank. The videos were analyzed with Noldus Ethovision Software (Leesburg, VA) for endpoints of time spent in light or dark part of tank and number of crossings between compartments.

### 4.2.5 Open field test and Aggression Assay

The open field apparatus was modified from (Champagne et al. 2010), and consisted of 3L aquatic habitats tank filed with 1.5L clean system water to minimize vertical swimming. Each camera was able to capture two tanks in one frame. Illumination via fluorescent lights was consistent with housing conditions (300-400 lux).

Adult zebrafish were individually placed into a novel empty tank, and video recording using Noldus MPEG recorder 2.1 (Noldus Information Technology, Leesburg, VA) began immediately after transfer. The total trial length was 30 minutes, after which the

fish were removed and individually housed for the remainder of the experiment. The tanks were rinsed and water renewed between trials to remove waterborne pheromones. Testing occurred between 12:00 and 16:00 hours each day to limit circadian rhythm effects. Each fish was subject to this procedure once a day for four days. On the fourth day, the video was analyzed using Noldus Ethovision Software (Leesburg, VA) for endpoints of total distance traveled, mobility (a spatially independent measure of body movement) and thigmotaxis behaviors.

Thigmotaxis is the tendency of an animal to avoid the center of their tank (Sharma et al. 2009; Treit and Fundytus 1988). This behavior has been identified teleosts(Ahmad and Richardson 2013), rodents(Simon et al. 1994), and in humans (Walz et al. 2015). It is thought that thigmotaxis is a way of finding shelter, protection or a way of escape from a predator or stressor (Sharma et al. 2009)

After four consecutive days in the open field assay, on the fifth day the zebrafish were subject to a mirror-induced stimulation assay for aggression adapted from (Norton et al. 2011). A mirror (7.5 X 7.5 cm) was slotted into the trapezoidal end of the tank, and video was recorded for 10 minutes. Videos were viewed by two independent blind reviewers and the number of attacks against the mirror was counted.

### 4.2.6 Gene Expression Analysis

Prior to dissection for gene expression analysis, morphometric measurements of total body length and weight were recorded for each fish. Fish were anesthetized with tricane MS222 (Sigma Aldrich, St Louis MO) and measurements were taken. Each fish was placed into a weigh boat, and measured on an analytical balance. Then, fish were placed into the dissection tray, and total body length was measured from the tip of the mouth to the end of the spinal cord. Immediately after measurements, fish were dissected for liver isolation used in gene expression analysis.

### 4.2.7

Livers isolated from adult fish (N= 5-8 per treatment group per sex) were snap frozen in liquid nitrogen and RNA extracted using RNAzol reagent (Sigma-Aldrich, St. Louis, MO). 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 following 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*. It was ascertained that *b-actin* expression was not effected by any PFAS treatment. Analysis was performed using a standard curve method for all of the $P_0$ transcripts examined. The genes examined and primer sequences are listed in Table 1. Each independent experiment was replicated 2 times.

### 4.2.8 Statistical Analysis

Using SigmaPlot® 11, one-way ANOVA was used for analysis of all endpoints (Gene expression, morphometric measurements, and behavioral assays) to determine significance. Statistical significance was at a p-value $\leq 0.05$. The SigmaPlot software tests for normality and power of the test prior to statistical analysis.

## 4.3 Results

### 4.3.1 Morphometric Measurements

The total body weight and length of the adult zebrafish was measured at 6 months post fertilization. There were no significant differences between males and females in any control or treatment group. Additionally, between treatment groups of combined sexes, there was no significant difference in either weight (Figure 4.2A) or length (Figure 4.2B). However, the PFNA treated group was trending towards a decrease in body weight ($p = 0.061$).

### 4.3.2 Locomotion Activity

The locomotion activity was assessed in six month zebrafish. The fish were separated by sex and males (Figure 4.3) and females (Table 4.1) were analyzed separately. The total distance traveled is a measure of distance swum throughout the duration of the assay.

Males exposed to PFNA (Figure 4.3A) were the only group affected, in which a significant decrease in total distance was observed.

Swimming velocity is a measurement of the average speed traveled per 1 minute time bins for the duration of assay. No significant effects were observed in any treatment except for PFNA-exposed males, which exhibited significantly faster swimming velocity (Figure 4.3D).

The duration in the middle of the tank is a measure of thigmotaxis, an anxiety type behavior in zebrafish in which they will tend to stay along the walls of the assay arena. PFNA treated males (Figure 4.3B) spent a significantly less amount of time in the middle, meaning that there were exhibiting an increase in thigmotaxis.

Immobility duration is an endpoint to assess the amount of movement of a fish independent of swimming. This can include tail and fin movements and body angle changes. Male fish treated with PFNA showed a decrease in the amount of time immobile (Figure 4.3C), indicating more body movement independent of swim activity.

### 4.3.3 Light/Dark Assay

In the light/dark assay for activity, the amount of time spent in each area of the assay arena as well as the number of times crossed between the areas was recorded. This assay

was performed with a light intensity of 375 lux. Based on (Stephenson et al. 2011), we would expect control fish at this light intensity to spend approximately 49±15 percent of the time in the dark, which our results corroborate. PFOA exposed females spent a significantly less amount of time in the light compared to controls, while PFNA males (Table 4.2) spent a significantly larger amount of time in light. There were no significant differences for any treatment in either sex in terms of crossings between the two areas (Table 4.2).

### 4.3.4 Aggression Assay

The level of aggression is based on the number of times the fish attacked their reflection in the mirror. Fish were analyzed by sex and treatment group. PFNA exposed males had an increased level of aggression, and PFOS males showed a decrease in aggressive behavior (Figure 4.4B). Females exposed to any PFAS showed no significant difference in aggression compared to controls (Figure 4.4A).

### 4.3.5 Gene Expression

Gene expression of two organic anion transporting polypeptides *slco1d1* and *slco2b1* were analyzed as well as growth factor *tgfb1a*. In male zebrafish (Figure 5A), PFNA and PFOS treatment resulted in a significant decrease in *slco2b1* and significant increase *tgfb1a* transcript expression. Male zebrafish from all PFASs had an increased expression of *bdnf*. *Slco1d1* was increased in males exposed to PFOA and PFOS. In female zebrafish

(Figure 5B), there was no change in *tgfb1a* expression in any treatment group. All three PFASs examined significantly decreased *slco1d1* expression, and PFNA and PFOS exposed fish had a significantly reduced *slco2b1* expression (Figure 5).

**4.4 Discussion**

A summary of the morphometric, behavior, and gene expression endpoints after PFAS exposure at 5, 14, and 180 dpf is listed in table 4. In adult zebrafish, no PFAS treatment at any dose resulted in significant changes in total body weight or length compared to the control group (Figure 2). This is in contrast to the results found at 5 dpf (all PFAS decreased total body length) and at 14 dpf (PFOA significantly decreased, PFNA significantly increased total body length) at the same exposure concentration and similar study design previously published. Based on these results 6 months after exposure, all PFAS exposed fish were able to recover from changes in size during development and that the behavioral changes reported in the current study are likely not due to physical changes.

A summary of adult behavior and gene expression endpoints is presented in Table 4. Of the three PFASs tested, PFNA exposure appears to have the greatest persistent effects on behavior but only in male zebrafish (Figure 4.3, Table 4.1). PFNA exposed males exhibited a significant decrease in total distance traveled, an increased speed while swimming, a smaller time of immobility, and a higher tendency for thigmotaxis. Additionally, these fish also had a preference for light rather than dark (Table 4.2), and

had a higher level of aggression (Figure 4B). Males exposed to PFOA showed no significant behavioral deficits in any assay, and PFOS exposed males only showed one significantly altered endpoint which was a decrease of aggression (Figure 4B). Both PFNA and PFOS exposed females exhibited no behavioral changes, while PFOA exposed females had a preference for the dark compartment of the light/dark assay.

*Tgfb1a* is one possible pathway that can play a role in locomotive effects observed (Table 4). This gene is a transcription growth factor that can be involved in cell migration, proliferation, apoptosis, and tissue homeostasis (Xing et al. 2015). It was found that when this gene is knocked down, the lateral line of the zebrafish does not form correctly (Xing et al. 2015). The lateral line development is important because it allows the zebrafish to sense water movement, find prey, and avoid predators (Coombs 2005). Therefore, the *tgfb1a* transcript was examined to determine if altered gene expression could be correlated to changes in swimming behavior endpoints observed in Figure 3.

In the present study, the adult fish embryonically exposed to PFASs show a significant increase in *tgfb1a* expression in males exposed to PFNA and PFOS (Figure 6). This correlates to a previous acute embryonic exposure to PFOS and PFNA that also resulted in increased *tgfb1a* expression, indicating that this change is persistent from juvenile until adult zebrafish (Jantzen et al. 2016b). However, while both PFOS and PFNA had an increased expression of this transcript, the majority of significantly altered behavioral changes were exhibited by PFNA exposed males(Figure 3). No female groups from any treatment showed a significant change in *tgfb1a* expression (Table 4). While the change in *tgfb1a* expression could be affecting the development of lateral line formation and in

turn swimming behavior, this pathway alone is unlikely to account for all of the behavioral changes observed.

Brain derived neurotrophic factor (*bdnf*) is a transcription growth factor that is expressed throughout all life stages of the zebrafish, and has also been found to affect lateral line formation (Gasanov et al. 2015). *Bdnf* regulates the migration of the lateral line primordium, and enhances the differentiation of sensory and sympatic neurons (Diekmann et al. 2009). Therefore, an increase in this gene would appear to be beneficial for neurological and central nervous system development. In this study, males from each PFAS had a significantly increased level of *bdnf*, but PFNA exposed males still expressed many altered behavior endpoints. There are a number of possible scenarios relating *bdnf* to the endpoints observed in this study. One scenario is that due to the decreased expression of *tgfb1a* as embryos, *bdnf* is increased in order enhance neuron development, repair any possible damage of the lateral line, and compensate for this developmental loss. Other possibilities relating *bdnf* to behavior effect could be the large number of downstream pathways that interact with this transcript. For example, b*dnf* has been shown to affect the glucocorticoid receptor, which is directly involved in stress response (Lambert et al. 2013). Many of the behavior endpoints tested, such as thigmotaxis, the light/dark box or the aggression assay are indicators of stress (Champagne et al. 2010). However, this increase in expression due to a stress stimulus was transient and was reduced after the stimulus was removed so it would seem unlikely that *bdnf* expression alone accounts for the anxiety type behaviors observed. *Bdnf* has also shown to be involved in synaptic plasticity, longer term potentiation, and memory (Yamada and

Nabeshima 2003). It appears likely that *bdnf* and its downstream pathways may be directly and indirectly affected by PFAS exposure, the exact role and mechanism of each one are currently not known.

In behavioral endpoints as well as gene expression, there is a sex-specific difference between the compounds. A factor that could be contributing to the different outcomes observed between sexes and compounds is differences in body clearance rates of either the PFASs or endogenous bioactive compounds through competing with transporters. Difference in body clearance rates were observed in PFOA exposure in fat head minnows (Lee and Schultz 2010) and tilapia(Han et al. 2011), as well as in PFOA and PFNA exposure to rats (Kudo et al. 2001). The elimination of PFASs from tissues in various organisms has been associated with the organic anion transporting polypeptides, oatps (*slco*). The expression of these transporters is likely one of the reasons for the variable half-lives observed (Klaassen and Aleksunes 2010). Gene expression analysis of *sclo2b1* and *slco1d1* gives insight to both differences between compounds and between sexes due to elimination rates.

Previous studies have found that PFASs can be either a substrate or inhibitor of oatps, in particular s*lco1d1* (Popovic et al. 2014). In our previous manuscript, we reported expression of *slco2b1* was significantly changed at both 5 dpf (PFOS significantly decreased; PFNA and PFOA significantly increased) and at 14 dpf (PFOS, PFOA, PFNA significantly increased) (Table 4). In the current study, expression of organic anion transporting polypeptide *slco2b1* was significantly decreased in both sexes treated with

PFNA and PFOS. Changes in expression of *slco2b1* have persisted from larval through adult life stages for PFNA and PFOS treated fish, however there was a change from increased expression at 14 dpf to decreased expression in adults. The mechanism for the altered expression at these different time points will need to be further studied to understand these observations. What is striking is the inhibition at 180 days from exposure during the first five days following fertilization.

*Slco1d1 and slco2b1* are only two of the numerous organic ion transporting polypeptides, many of which have overlapping functions and substrates (Klaassen and Aleksunes 2010). *Slco1d1* was significantly decreased in expression in all sex and treatment groups except for males exposed to PFNA. Another role of oatps is to transport steroid conjugate and hormone precursor compounds (Klaassen). Changes in these transporters expressions could result in variations of normal hormone production and cycling (Popovic et al. 2014) which in turn could lead to many of the behavior and anxiety-type changes observed in the zebrafish. Our results show that these transporters are dramatically altered both during exposure and long after termination of exposure (180 days). However, at this time the direct relationship between transporter expression and specific hormone functioning resulting in behavioral changes cannot be made. Therefore, while it appears that after PFAS exposure both behavior and transporter expression are affected, more in depth studies will need to be performed to determine the exact role these transporters play in PFAS toxicity.

This study was designed to examine the long term effect on adult zebrafish from a sub-lethal exposure to PFOS, PFNA, or PFOA in embryonic zebrafish (5 days) will result in

ongoing morphometric, gene expression, and behavioral defects in adult zebrafish similar to those observed at 5 and 14 dpf. At six months post exposure the morphologic changes observed at 14 dpf did not persist in either sex. Therefore, a short-term exposure resulted in initial growth affects that are mitigated by six months. In terms of locomotive behavior, light/dark anxiety, and aggression, PFNA exposed fish exhibited the greatest number of significantly altered endpoints. These endpoints were sex specific in that only the male zebrafish were affected. Gene expression of *slco2b1* and *tgfb1a* remained altered. Both *tgb1a* and *bdnf* were altered in a sex dependent manner in that males exposed to all three PFASs had higher expression of these transcripts. Behavioral measurements are the result of a complex set of pathways that can be manifested in teleosts and further studies are needed to determine the mechanism by which these compounds are modifying these behaviors. This suggests that PFAS exposure, particularly to PFNA, at the embryonic level is sensitive to persistent effects into adulthood. These behavior changes could have impacts at the population level, which can be extrapolated to other teleost species in the ability to find mates, food, and avoid predators.

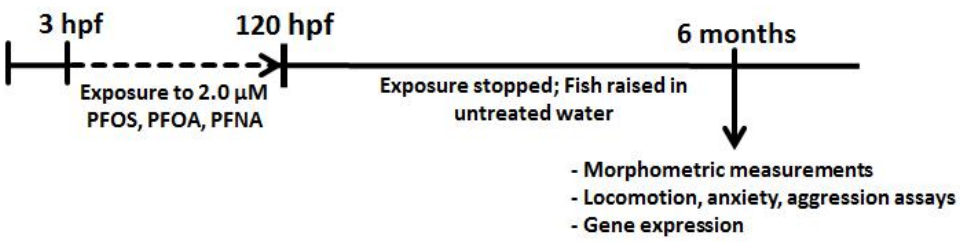


Figure 4.1. Timeline of zebrafish exposure to PFOA, PFOS and PFNA. Water-borne exposure occurred between 3 and 120 hpf. Dashed lines represent exposure periods.

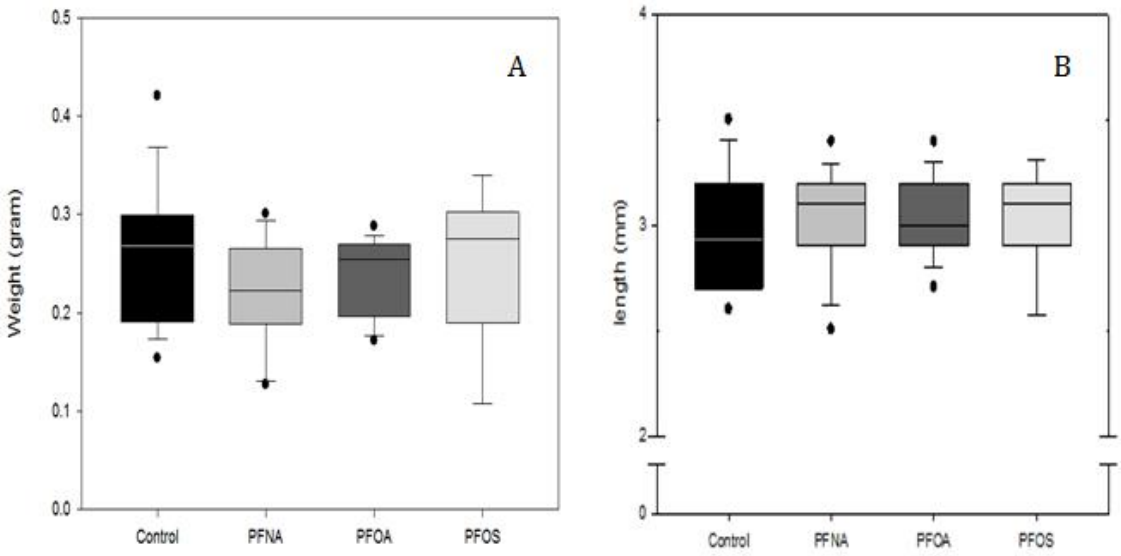


Figure 4.2. Morphometric measurements of adult zebrafish following acute, embryonic exposure (2.0 μM). No significant difference in weight (A) or length (B) were observed for any treatment group. N= 10 fish per treatment group. Statistical significance was tested using a one-way ANOVA, $p<0.05$.

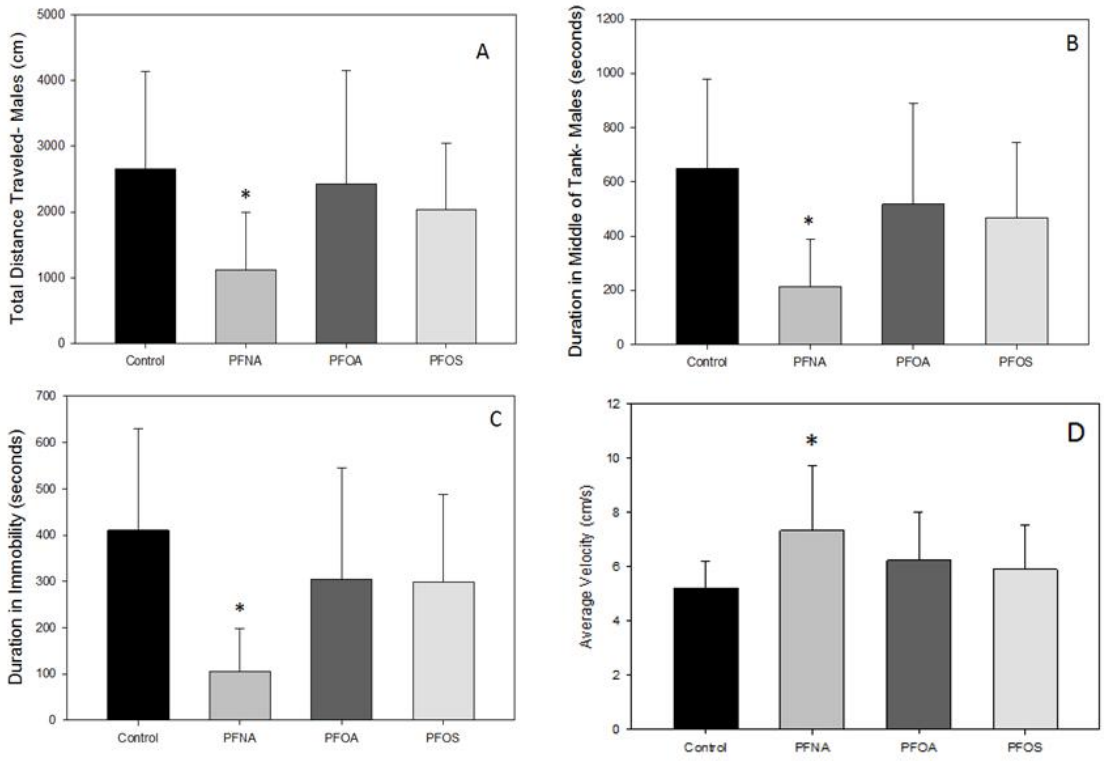


Figure 4.3. Locomotive behavioral endpoints of total distance traveled (A), middle duration (B), immobile duration (C) and velocity (D) in adult male zebrafish embryonically exposed to PFNA, PFOA, or PFOS (2.0 μM). Bars represent mean value and standard deviation. N=10 animals. An asterisk (*) indicates a statistical significant value, $p < 0.05$, one-way ANOVA compared to control.

| | Total Distance Traveled (cm; average ±standard deviation) | Duration in middle of tank (seconds; average ±standard deviation) | Time of immobility (seconds; average ±standard deviation) | Average velocity (cm/s; average ±standard deviation) |
|---|---|---|---|---|
| **Control** | 1997.7 ± 1672.5 | 438.8 ± 382.2 | 263.8 ± 343.9 | 6.3 ± 1.7 |
| **PFNA** | 2399.2 ± 1058.7 | 544.2 ± 272.4 | 294.7 ± 161.8 | 6.1 ± 1.0 |
| **PFOA** | 1994.3 ± 882.8 | 445.1 ± 232.4 | 268.9 ± 162.6 | 5.7 ± 1.2 |
| **PFOS** | 2087.7 ± 1320.6 | 440.9 ± 263.5 | 270.1 ± 153.5 | 6.1 ± 1.43 |

Table 4.1**.** Locomotive behavioral endpoints of total distance traveled, middle duration, immobile duration, and velocity in adult female zebrafish embryonically exposed to PFNA, PFOA, or PFOS (2.0 μM). N=10 animals. No statistically significant differences were observed, $p < 0.05$, one-way ANOVA compared to control.

| | Number of Crossings between Light and Dark Areas | | Time Spent in Light Compartment (seconds) | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| Control | 26± 20 | 25±14 | 266±75.12 | 365±112 |
| PFNA | 25± 14 | 22±16 | 430±130* | 275±137 |
| PFOA | 29±19 | 23±10 | 361±134 | 153±56* |
| PFOS | 37±26 | 27±12 | 198±1390 | 334±118 |

Table 4.2. Average number of crossings between light and dark compartments, and duration of time in the light compartment of the tank. Values represent the average ± standard deviation. Asterisk (*) represents statistical significance, one-way ANOVA between treatments for each sex ($p<0.05$).

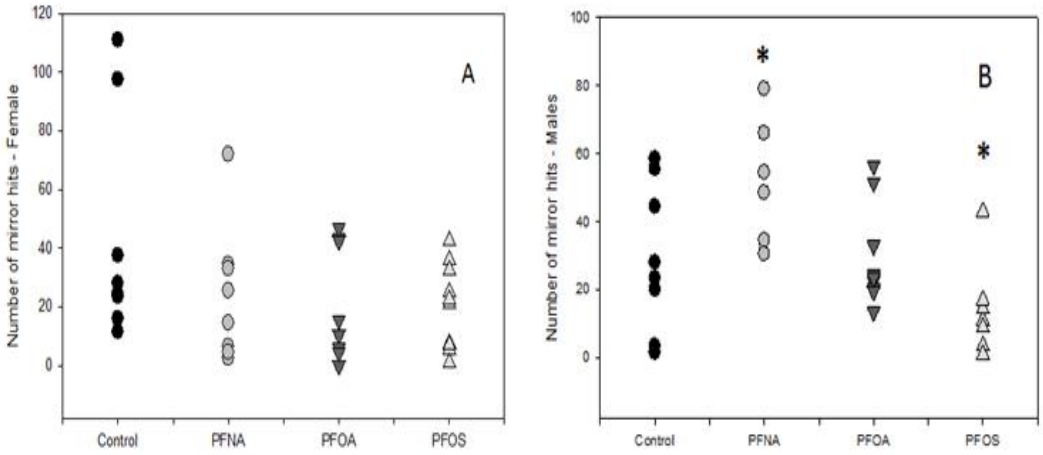


Figure 4.4 The number of mirror attacks in adult zebrafish in (A) females and (B) males. Each data point represents an individual fish (N=8-10 fish per treatment per sex). Asterisks represent statistical significance was determined using a one-way ANOVA compared to control ($p<0.05$).

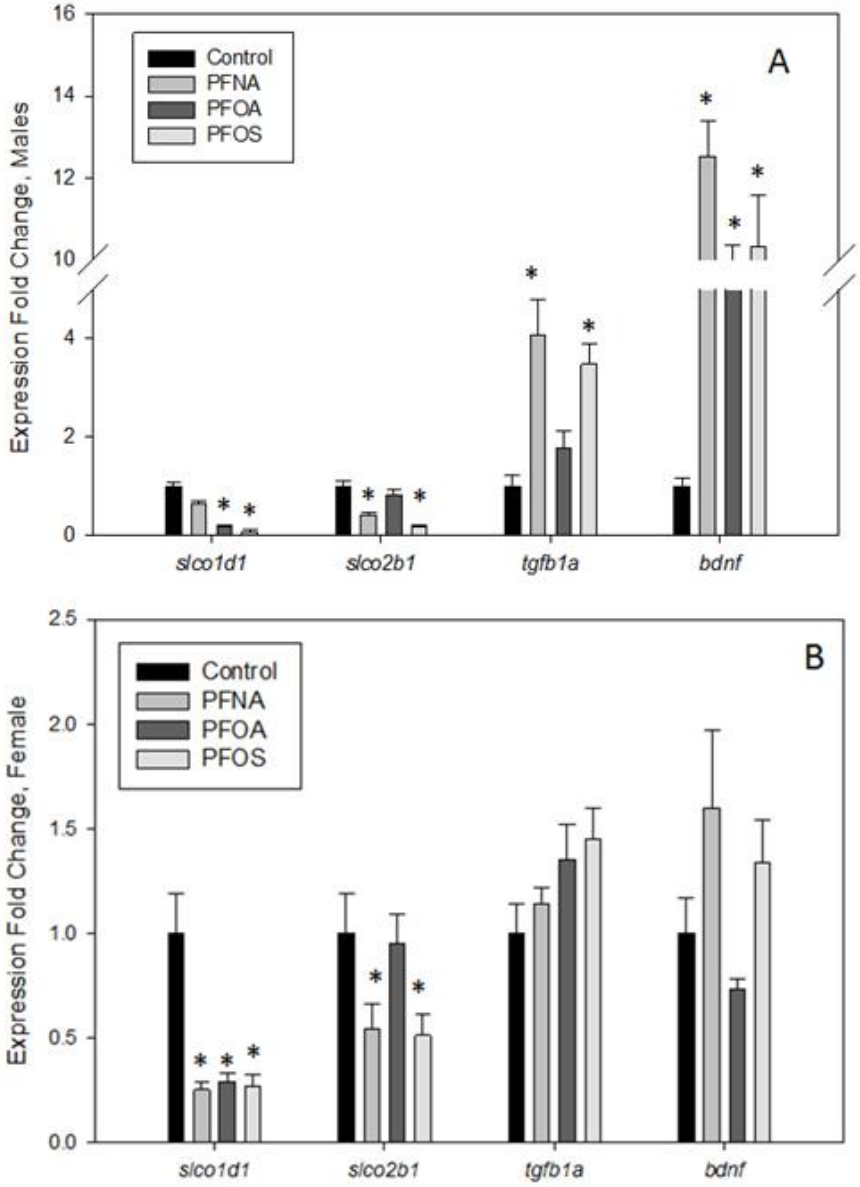


Figure 4.5. Gene expression of adult (A) male and (B) female zebrafish embryonically exposed to PFOS, PFOA, or PFNA. Bars represent mean fold change and standard deviation. N= 8-10 fish per treatment per sex. An asterisk (*) indicates a statistical significant value, $p<0.05$, one-way ANOVA.

| | PFNA | | | | PFOS | | | | PFOA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| dpf: | 5* | 14* | 180 | 180 | 5 | 14* | 180 | 180 | 5 | 14* | 180 | 180 |
| | | | Males | Females | | | Males | Females | | | Males | Females |
| **Morphometric** | | | | | | | | | | | | |
| Total Body Length | ↓ | ↑ | NS | NS | ↓ | NS | NS | NS | ↓ | ↓ | NS | NS |
| **Locomotion** | | | | | | | | | | | | |
| Distance | --- | ↑ | ↓ | NS | --- | ↑ | NS | NS | --- | ↑ | NS | NS |
| Middle | --- | ↑ | ↓ | NS | --- | NS | NS | NS | --- | NS | NS | NS |
| Immobile | --- | NS | ↓ | NS | --- | NS | NS | NS | --- | NS | NS | NS |
| Velocity | --- | ↓ | ↑ | NS | --- | ↑ | NS | NS | --- | NS | NS | NS |
| **Light/Dark Assay** | | | | | | | | | | | | |
| Time in Light | --- | --- | ↑ | NS | --- | --- | NS | NS | --- | --- | NS | ↓ |
| **Aggression Assay** | | | | | | | | | | | | |
| Number of hits | --- | --- | ↑ | NS | --- | --- | ↓ | NS | --- | --- | NS | NS |
| **Gene Expression** | | | | | | | | | | | | |
| *slco2b1* | ↑ | ↑ | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↑ | ↑ | ↓ | NS |
| *slco1d1* | --- | --- | NS | ↓ | --- | --- | ↓ | ↓ | --- | --- | ↓ | ↓ |
| *tgfb1a* | ↓ | --- | ↑ | NS | ↓ | --- | ↑ | NS | NS | --- | ↓ | NS |
| *bdnf* | --- | --- | ↑ | NS | --- | --- | ↑ | NS | --- | --- | ↑ | NS |

Table 4.3. Summary of total body length, locomotion, light/dark sensitivity, aggression, and gene expression after exposure to PFNA, PFOS, and PFOA at 5, 14, and 180 days post fertilization. (*) indicate data from (Jantzen et al. 2016b). "NS" indicates no significant. "---" indicates endpoint not assessed for this time/compound. Arrows represent a significantly increased (↑) or significantly decreased (↓) endpoint ($p < 0.05$).

# Chapter 5: Effects of Chronic Perfluorooctanoic Acid (PFOA), Perfluorononanoic acid (PFNA) and Perfluorooctane sulfonate (PFOS) at Low Concentration on Morphometrics, Gene Expression, and Fecundity in Zebrafish (*Danio rerio)*

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## 5.1 Introduction

PFOS, PFNA, and PFOA are persistent, anthropogenic compounds detected in environmental and animal tissue samples worldwide. In contrast to other organic pollutants, which tend to collect in fat, research has shown PFASs to accumulate predominantly in the liver, plasma, and kidney (Houde et al. 2011; Kudo and Kawashima 2003; Post et al. 2012), Falk 2015). They generally do not undergo any metabolism within the body, and in many of the organisms, they are commonly eliminated as the parent compound in the urine (Consoer et al. 2014; Han et al. 2012; Han et al. 2003;

Steenland et al. 2010). In the mammalian literature, PFOA has been shown to be eliminated from the kidney utilizing the OAT1 and OAT3 transporters (Yang et al. 2010).

PFOA exposure has led to gene expression changes (both up and down-regulated) in pathways involved in lipid metabolism, lipid transport, hormone action, and mitochondrial function in rare minnows (Wei et al. 2008). PFOS exposure in mice and PFNA exposure in rats also resulted in altered lipid metabolism pathways (Wang 2014, Fang 2015). A reproductive study of PFOA (0.01 mg/L) in adult medaka (Oryzias latipes) indicates negative impacts on offspring development and survivability, manifesting as an increased mortality rate and hyperplasia, hypertrophy, and colloidal depletion in the thyroid gland (Ji et al. 2008). PFNA exposed mice had decreased offspring viability, developmental delays, and increased liver weight (Das 2015). There appears to be multiple biochemical pathways that are disrupted following exposure to PFASs and the early assumption that this class of compounds exerts their toxicity solely through the peroxisome proliferator activated receptor-alpha (PPARα) is not substantiated by literature reports.

Studies in rodents have established that PFASs can act as a peroxisome proliferator (Berthiaume and Wallace 2002; Guruge et al. 2006), Das 2015) specifically through expression of PPARα (White et al. 2011). PPARα is a nuclear receptor involved in the regulation of fatty acids and lipid metabolism and activated though ligand binding (Escher and Wahli 2000). When pregnant mice were exposed to PFOA, it was found that

PPARα activation was associated with specific postnatal morphological delays, such as in eye opening, and the reduced survivability of offspring (Abbott et al. 2007).

However, additional studies in rats found PFASs to be a hormone disruptor and it's mode of action is independent of PPARα (Wang 2014, Yang 2002). One possible alternative pathway to examine the hormone disruption effect is the organic anion transporting polypeptides (oatps; *slco*). These transporters can have a significant impact on the uptake and clearance of both toxic and endogenous compounds (Klaassen and Aleksunes 2010). Some of the endogenous compounds transported include thyroxin, steroid conjugates, bile acids, bilirubin, and prostaglandins (Klaassen and Aleksunes 2010). These substrates are important for both hormone regulation, as well as for growth and development. PFASs has been classified as both an inhibitor and substrate of various OATps (Popovic et al. 2014). A disruption in the normal function, either through inhibition or competition, of these transporters would impact the pharmacokinetics of the natural substrates. This in turn could lead to disruptions in hormonal pathways as well as energy uptake, utilization, and storage. Disruption of these critical biochemical pathways would impact reproductive success and $F_1$ development across species.

The disruption of temporal and spatial expression during early embryonic development and altered receptor expression during critical organ development by PFASs can result in permanent structural alterations. An acute embryo-larval PFOA, PFNA, or PFOS exposure resulted in sac-fry larvae that were smaller in total length and had a changes in yolk sac size at 5 dpf (Jantzen et al. 2016a). Additionally, a decrease in gene expression

of organic anion transporting polypeptide *slco2b1* and protein transporter *ap1s1* pathway was observed. In the present study, we examined the effects of a chronic, environmentally relevant exposure concentration of PFASs in zebrafish from the embryo through adult life stages. $P_0$ adult fish were measured for body weight and length, number of eggs produced during breeding and gene expression of relevant *slco* transporter pathways. The $F_1$ generation was analyzed for percentage of viable embryos, developmental staging, and gene expression of *ap1s1,* a critical developmental pathway. The results presented in this paper demonstrate that chronic PFAS exposure to critical life-stages in zebrafish caused detrimental effects in both the parental ($P_0$) and offspring ($F_1$) generations.

## 5.2 Methods

### 5.2.1 Animal Handling and Exposure

Animal handling and rearing was performed according to the protocol described in Chapter 2.1. The chronic PFAS exposure protocol is described in Chapter 2.3. Figure 5.1 shows the exposure timeline.

### 2.2 Exposure

Shown in Figure 2 is the exposure and data collection timeline. Zebrafish embryos were exposed in glass vials to either 2nM or 0 nM PFOA (95%, Sigma Aldrich, St. Louis, MO) through a waterborne exposure from 3 hpf – 120 hpf. At 120 hpf, fish were transferred to non-exposed water until 30 dpf, in which they were separated by treatment and placed in 5 liter fish .In our laboratory, the greatest period of mortality has been observed to be between 7 dpf when feeding begins through 30 dpf after fish have been acclimated to the

feeding and water change schedule. In order to not further stress the fish and increase mortality during this critical time, exposure was terminated at 120 hpf and resumed at 30 dpf. At 1 month post fertilization, the fish were separated by sex and began a feeding regimen consisting of non-treated brine shrimp in the morning feeding and 0.04g of either control or PFOA treated flake food in the afternoon feeding. Feeding aliquots of 0.04 g of food were based on food quantities determined for our stock breeding sets (N=~8 fish per tank). No food was remaining in tank after feeding.

Figure 3 represents the experimental design for one independent biological replicate. Three biological replicates were performed, each from a different initial stock breeding set. Initially, embryos were collected and exposed to 2nM or 0 nM PFOS in 20 mL vials with 20 fish per vial. At 120 hpf, each group of fish from a vial were transferred to 600 mL beakers with no-treatment system water. These fish were raised in non-treated water until 30 dpf, in which 20 fish from each treatment group were placed into one 5-L tank and exposure continued via a food exposure (0.04g of PFOA or control food). After 90 days, the fish were separated by sex into two tanks per treatment, one for males (N=10) and one for females (N=8) to have what is considered one "breeding set". Each week, 5 males were moved into the female tank for a "breeding event". Generally, the first breeding event does not produce an adequate amount of embryos for accurate analysis. Therefore, they were bred together 10 times for 9 "successful" breeding events. The number of eggs produced, the viability of the embryos, and the developmental staging progression (Kimmel et al. 1995) of the embryos were recorded. After 10 breeding events,

the morphometric measurements of body length and weight were recorded for the adult fish, as well as livers isolated for gene expression analysis.

All tanks were constantly aerated and temperature was measured daily to ensure accurate range (25-27C) was maintained throughout the entire experiment. Twice weekly (day of breeding event, 3 days before next breeding event) a 50% water change was performed.

### 5.2.3 Exposure Water and Food Preparation

The PFOA study was done independently of the PFOS and PFNA study. Both experiments followed the same protocols as described for animal handling, exposure, exposure water and food preparation, staging, fecundity measures and gene expression analysis.

PFOA, PFNA, and PFOS solution for the water exposure was prepared by dissolving powdered perfluorooctanoic acid (Sigma Aldrich, St Louis MO) into Millipore filtered water for a stock solution (200 μM). The working solution of 2.0 nM was made by diluting the stock solution into filtered fish system water (described above). Control groups were raised in system water with 0 nM PFAS. The food was prepared by mixing either stock PFOA, PFNA, and PFOS or water with 95% ethanol for a final concentration of 2 nM (treated) or 0 nM (control). This solution was added to 10g fish flake food to make a slurry. The slurry was stirred overnight. The following day, the food-ethanol slurry was placed into a shallow Pyrex tray in the fume hood, and the ethanol was evaporated off. The remaining dried food was crushed into a powder and separated into

0.04g aliquots. The treated food had a final calculated concentration of 8.0 pM PFOA, PFNA, and PFOS per aliquot.

### 5.2.4 Gene expression analysis

Gene expression analysis was performed according to Chapter 2.3.

### 5.2.5 Reproductive Success and $F_1$ Embryo Staging

Each week, control and PFOA, PFNA, and PFOS -treated sets were bred at the same time. The total number of eggs produced and the percentage of eggs that were viable were recorded. Viability was determined by the appearance of dividing cells on the yolk mass. Ten embryos from each breeding set were randomly selected and raised individually in 1-mL glass vials. Staging of each embryo was performed daily from 24- 96 hpf using the parameters described by Kimmel (1995). Table 5.1 lists zebrafish embryo-larval developmental periods and an example of a specific stage in each period.

### 5.2.6 Statistics

Using SigmaPlot® 11, student t-test or paired student t-test, (gene expression, morphometric measurements) and chi-squared tests (developmental stages, viable offspring) were performed to determine significance. Statistical significance was at a p-

value ≤ 0.05. The SigmaPlot software tests for normality and power of the test prior to statistical analysis.

## 5.3 Results

### 5.3.1 Parental ($P_0$) Morphometrics

At 6 months post fertilization, the total body length and weight of the adult animals were measured. PFOA exposed fish had significantly shorter total body length (Figure 5.2A) as well as a significantly reduced body weight (Figure 5.2B) compared to control fish. Both PFNA and PFOS exposure increased the total body weight of the fish, but only PFNA exposure increased the total length (Figure 5.3). No measurement in any exposure or control grouped resulted in a significant difference between sexes.

### 5.3.2 Gene Expression

Gene expression of 4 organic anion transporting polypeptides (*slco1d1, slco2b1, slco3a1,* and *slco4a1*) and growth factor *tgfb1a* was performed on livers of the parent generation in the PFOA study(Figure 5.4). *Slco2b1, slco3a1*, and *slco4a1* all resulted in significantly decreased expression (0.5, 0.03, and 0.17 fold, respectively). *Tgfb1a* was also significantly decreased in expression in the treated group (0.30 fold). Organic anion

transporter *slco1d1* was the only gene examined that resulted in a significant increase in expression after PFOA exposure (9.12 fold). Analysis of protein transporter *ap1s1* was performed on the F1 embryos at 48 hpf (Figure 5.5). There was a significant increase in *ap1s1* expression (1.71 fold) in F1 embryos from PFOA exposed parents.

Expression of *slco2b1, slco1d1, slco3a1* and *tgfb1a* genes was determined on reverse transcribed mRNA recovered from livers extracted from adult fish in the PFOS and PFNA study. Each sex was analyzed separately. Females from both PFOS and PFNA treatments showed a significantly decreased expression of every gene analyzed (Figure 5.6A). PFOS males had a significant increase in *slco1d1*, but no other genes were altered. PFNA males exhibited significantly increased *slco1d1 and tgfb1a*, and significantly decreased *slco2b1* and *slco3a1* expression (Figure 5.6B). In 48 hpf embryos, there was no significant difference between control and either PFAS treatment in *ap1s1* expression (Figure 5.7)

### 5.3.3 Offspring production and viability

After nine breeding events, the cumulative number of eggs produced by the control-fed fish was significantly larger (2184 eggs) compared to number produced from the PFOA exposed fish (1754 eggs) (Figure 5.8). No difference in total number of eggs was observed in the PFOS and PFNA exposures (Figure 5.9). The average percent of viable embryos produced over the nine breeding events was significantly decreased in the PFOA and PFNA exposure groups (Figure 5.10 and Figure 5.11).

### 5.3.4 $F_1$ Generation Effects

The $F_1$ embryos were photographed and their developmental stage recoded at 24, 48, 72, and 96 hpf (Figure 5.12 and 5.13) for PFOA exposure and 3, 24, and 48 hpf for PFOS and PFNA exposures. The general staging criteria (based on (Kimmel et al. 1995)) are listed in Table 1. At each time point examined, there is a significant stage delay in the development of embryos from the PFOA exposed adults. At 24 hpf, 40% of the PFOA $F_1$ embryos are in gastrula period, which is when the germ ring and embryonic shield become visible, and the brain and notochord begin to form. At this time point, all of the control embryos are in the segmentation stage, which is when primary organogenesis occurs, the body and tail structures become distinct, and structures such as the somite, pharyngeal and neuromeres develop. After 48 hours, all of the PFOA embryos have reached the segmentation stage, whereas 75% of the control embryos have moved into the pharyngula period, in which the body axis straightens, circulation and pigmentation are visible, and fins begin to form. At 72 hpf, the majority of the embryos from the control group are in the long-pec or pec fin stages, which are part of the hatching period, and involve completion of morphogenesis of primary organs, and cartilage formation in the head and fins. PFOA embryos at this time point are in the pharyngula period. After 96 hours, 95% of the control embryos are at the pec fin or protruding mouth stage, which is classified as early free swimming larval stage. PFOA embryos at this time point are mainly in the segmentation stage (70%), and many have not hatched from the chorion.

A similar result is observed in embryos from PFNA exposed adults. Initially at 3 hpf, there is a significant stage delay compared to the controls (Figure 5.13). This persists

through 24 hpf, in which 60% of the control fish are in the late segmentation stages but 85% of the PFNA fish are just entering this development time-point. At 48 hpf, the delay is no longer present and control and PFNA fish are approximately the same stage.

PFOS embryos showed the opposite outcome in that at 3 hpf they had significantly accelerated development compared to the controls (Figure 5.13). However, this effect did not persist at all other timepoints measured there was no significant difference between PFOS and control embryo staging.

### *5.*4. Discussion

Chronic, low dose, PFAS exposure to zebrafish has detrimental effects both in the $P_0$ and $F_1$ generations. These effects are seen in this study at a concentration (3.4 ng/L) that is similar to those detected in drinking water sources in a number of localities throughout the country, such as the North Carolina river basin (median 12.6 ng/L)(Nakayama et al. 2010) and New Jersey drinking water sources (4-5 ng/L)(Post et al. 2013). In our study, fish chronically exposed to PFOA were significantly smaller in size, produced fewer eggs, had smaller percentage of embryo viability, and decreased expression of growth factor *tgfb1a* as well as three organic anion transporting polypeptides (*slco2b1, slco4a1, slco3a1),* and an increase in expression of transporter *slco1d1*. Chronic PFOS exposure resulted in fish that were larger in body weight, had initially accelerated development and changes in transporter and growth factor gene expression. Fish chronically exposed to PFNA were larger in size, had a smaller percentage of embryo viability, and also saw many gene expression changes in transporters and growth factors assessed. These

changes in gene expression during critical windows of development could explain the morphologic and toxic effects observed.

In the $P_0$ generation, a significant decrease of body size in terms of both length and weight was observed after PFOA exposure (Figure 5.2). This correlates with previous studies in our laboratory which found that zebrafish exposed to PFOA during development decreased in total body size both immediately following the exposure period (120 hpf) as well as after a recovery period (14 dpf) (Jantzen et al. 2016a). PFNA fish were also significantly smaller at 120 hpf, but at 14 dpf were significantly larger than the control (Jantzen 2016), and this correlates to the measurements observed in this study in that P0 PFNA exposed fish were larger in size and weight.

Zebrafish acutely exposed to PFASs (2.0 μM) also had a significantly changed yolk sac size; PFNA and PFOA increased, while PFOS decreased (Jantzen 2016). It had been shown that the yolk sac size can have a direct effect on the total size of the zebrafish; when the yolk sac was manipulated to be smaller, it resulted in a decrease in body size (Jardine 2003). The yolk sac is where the energy and nutrients supplied from the mother are stored for the developing embryo, and it is the embryo's only source of nutrition until feeding begins. In the current study, during the initial embryonic exposure of 2nM PFAS between 3 hpf and 120 hpf no significant changes in yolk sac size were observed. A decrease in body size could be due to a dysfunction of energy uptake, storage, or subtle changes in yolk sac function in which yolk sac size is not affected.

One pathway that could possibly be involved in the observed alterations in energy usage and possibly other reproductive effects observed is the *tgfb1a* gene, which was significantly decreased in expression in the PFOA and PFOS exposed animals and increased in PFNA exposed males. Previously, this gene was analyzed to assess embryo development because it had been shown to be critical for lateral line development. However, there are additional functions and pathways affected by this gene.

In mammals, the *tgfb1* family of genes has been shown to regulate follicle development, steroidogenesis, oocyte maturation, ovulation and follicular atresia (Kohli et al. 2003). In zebrafish, it was discovered that TGF-B1 is likely involved in regulating ovarian function through hormone signaling and has been shown to inhibit gonadotropin and maturation inducing hormone (MIH) oocyte maturation (Kohli et al. 2003). A decrease in expression of this gene could have effects on zebrafish reproductive hormone cycling as well as oocyte maturation, which could account for the reduced number of embryos and viability observed in the PFOA and PFNA exposures (Figures 5.10 and 5.11). Since PFOS exposure also down-regulated this gene in both sexes but fecundity effects were not observed, there appears to be other pathways involved.

Changes in developmental progression seen in the F1 treated embryos (Figures 5.12 and 5.13 ) may be related to the tgfb1a pathway or other transporters, such as the organic ion transporters. It is currently unknown whether these effects are due to PFASs affecting the embryo's nutrient and hormone availability, or potentially affecting the maternal circulating hormone levels during spawning.

As discussion in chapter 4, OATps are responsible for the transport of a number of hormone and endocrine related compounds into and out of the cell. PFASs, in particular PFOA, have been found to interact with OATps in a number of different species. In rats, PFOA had been determined to be both a substrate and inhibitor for Oatp1a1 and a strong inhibitor for human OATP1A2 (Yang et al. 2010). Acute exposure to PFOA resulted in a significant increase of *slco2b1* expression in zebrafish at both 5 dpf and 14dpf (Jantzen et al. 2016a) and has been shown to be a strong inhibitor of *slco1d1* (Popovic et al. 2014). Rodent Oatp1a1, human OATP1a2, and zebrafish Oatp1d1 are all considered functional orthologs of each other in their respective species.

The disruption of hormone concentrations could have a number of adverse impacts in zebrafish. In zebrafish, thyroid hormones are involved in the differentiation of pectoral fins, growth of pelvic fins, and necessary for the progression from larval to juvenile stages (Brown 1997). Changes in transport and serum concentration of these hormones through changes in organic anion transporter function could have resulted in the reduced growth observed in the PFOA treated adults (Figure 5.2) and increase in PFOS and PFNA treated adults (Figure 5.3). Prostaglandins are lipid compounds that can act in a similar manner to hormones, and have been shown to play a role in the maturation and ovulation of the zebrafish oocyte (Kohli et al. 2003). Alteration in the transport of these compounds could result in reproductive defects, such as a reduction of egg production observed in the current study.

*Slco1d1* is the only transporter transcript examined in this study to be significantly increased in expression and this affect was seen in all three PFASs tested. Previous studies *in vitro* (HEK293 cell line) found that PFOA was an inhibitor of *slco1d1* in zebrafish as well as its orthologs in humans and rodents (Popovic et al. 2014). The normal function of *slco1d1* in zebrafish is to uptake steroid hormone conjugates into hepatocytes, which allows elimination through the bile (Popovic et al. 2014). While the regulation mechanisms of this pathway are currently not known, a change in expression could affect the amount of circulating hormone conjugates. This could have effects on egg production in adult zebrafish and is another plausible pathway to account for the decrease in egg production (Figure 5.8) and viability observed (Figure 5.10 and 5.11).

In the $F_1$ generation, PFOA and PFNA exposed embryos showed a significant development delay compared to the control embryos (Figures 5.12 and 5.13). This could be an indirect effect from alterations to the $P_0$ females, in that they did not produce enough vitellogenin for the embryos to use for nutrients to grow until they are large enough to ingest their own food. This seems more likely for PFOS exposure because the developmental delay was seen immediately after fertilization. Another possible explanation would be that there is a defect in the embryos that affects their ability to transport nutrients from their yolk sac.

In an acute PFASs exposure, all three compound significantly decreased *ap1s1* expression at 5 (Jantzen et al. 2016a). The *ap1s1* pathway is involved in protein cargo sorting and vesicular trafficking in the cell in early zebrafish development (Montpetit et

al. 2008). Knockdown of this pathway results in zebrafish larvae that are smaller in size, disorganized fin structure, and severe motor deficits (Montpetit et al. 2008). Gene expression of the $F_1$ generation in the current study at 48 hpf showed a significant increase of the *ap1s1* for PFOA, but no difference for PFOS and PFNA

One explanation for this increase in *ap1s1* expression in PFOA could be that expression is dependent upon the development stage. At 48 hpf, the $F_1$ control fish are primarily in the prim-6 stage, in which the brain and notochord are fully formed (Table 5.1; (Kimmel et al. 1995)). At this time, development is less rapid and each stage begins to last for a longer duration. In contrast, the majority of the $F_1$ treated fish are in the 14-18-somite stage. During these stages organogenesis is rapidly occurring. Some characteristics of this stage include the subdivision of the brain becomes visible, the yolk sac constricts which allows the animal to begin to straighten, and muscular contractions begin. The $F_1$ from treated fish are at a stage where protein transport and utilization is needed more than those of the $F_1$ control fish, which could account for the increase in *ap1s1* expression. In contrast, at 48 hpf both PFNA and PFOS fish are at the same developmental stage as the control group, and no difference in *ap1s1* expression was observed.

Chapters 4 and 5 both analyzed adult zebrafish exposed to PFASs, but through different exposure protocols. Chapter 4 studied fish exposed only embryonically (3- 120 hpf), and Chapter 5 studied fish that were exposed long term through the majority of their life stages. A comparison between these two exposures is presented in table 5.3. Acutely exposed fish from all PFASs had no significant changes in total body weight or total

body length, while chronically exposed adult zebrafish exhibited effects at these endpoints. Gene expression of *slco2b1* and *slco1d1* were altered in most exposure groups, except for acutely exposed PFOA fish and acute exposed PFNA male fish, respectively. In both exposure types, *tgfb1a* expression was significantly in male groups of each PFAS. While gene expression alterations appeared to be somewhat similar in all groups, morphometric measurements were different.

**5.5 Conclusions:**

A chronic, environmentally relevant PFOA and PFNA exposure through water and food had detrimental effects on both the $P_0$ and $F_1$ generations with similar outcomes to those observed in other model organisms (Table 5.2). In the $P_0$ generation, this manifested as a decreases in body weight and body size. The reproductive effects observed were a significant decrease in total eggs production and viability. In the $F_1$ generation, there was a significant developmental delay in PFOA and PFNA exposed offspring. P0 PFOS exposed fish had morphometric and gene expression changes, but no immediate effect on fecundity was observed. Possible pathways that could account for these effects could be the decrease of growth factor *tgfb1a* expression, or a combination of organic anion transporters (*slco2b1, slco1d1, slco3a1, slco4a1)*. Currently, very little is known about the role of maternal versus embryonic production and utilization of these transporters after PFAS exposure. The reproductive defects observed in this study after chronic PFOA exposure in zebrafish could also manifest in field-exposed teleost species, which would in turn have severe population level effects.

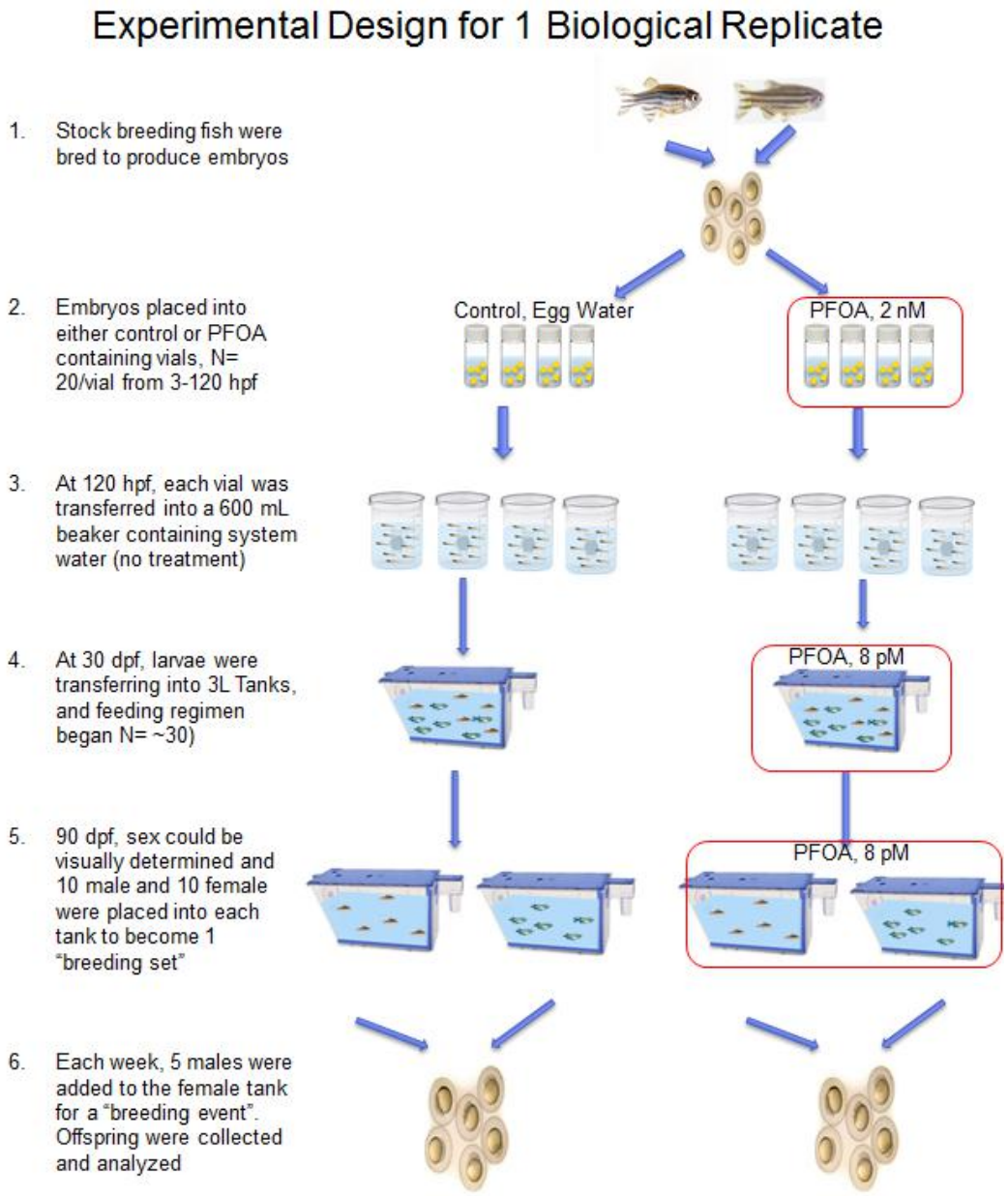


Figure 5.1**.** Timeline of zebrafish exposure to PFOA. Water-borne exposure occurred between 3 and 120 hpf. Food exposure occurred from 30 dpf – 6 months post fertilization.

| Developmental Period | Specific Developmental Stage | Hours Post Fertilization (26°C) | Description |
|---|---|---|---|
| Gastrula | Bud | 17 | Tail begins to be visible, head and notochord formation begins |
| Segmentation | 18-somite | 24 | Extension of tail, brain formation |
| Pharyngula | Prim-6 | 48 | Retina pigmented, melanophores present, heart beat prominent, |
| Hatching | Long Pec | 72 | Pectoral fin buds elongated, chondrocyte formation, olfactory development, lateral line formation |
| Larval | Protruding Mouth | 96 | Gill formations visible, distinct cartilage cells, hatching occurs, mouth is open |

Table **5.1.** Zebrafish developmental periods and examples of specific developmental stage. The timing of each stage is based on zebrafish being raised at 26C (Modified from (Kimmel et al. 1995)).

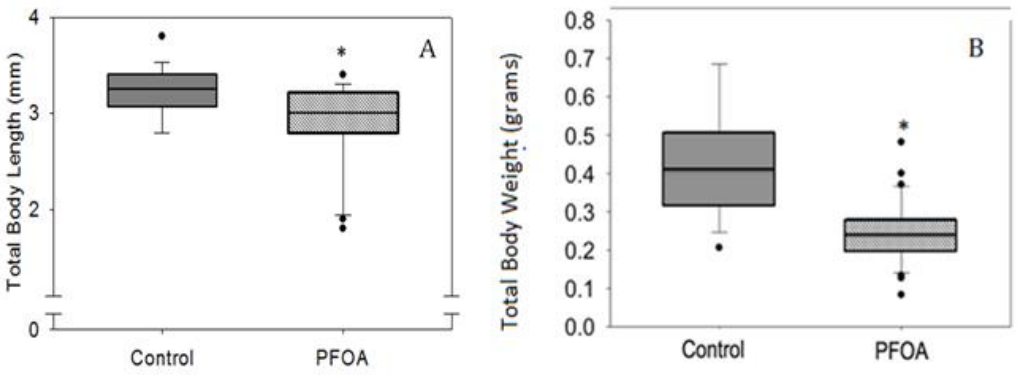


Figure 5.2**.** Morphometric measurements of PFOA $P_0$ fish at 6 months following embryonic exposure and 30 dpf – 6 months feeding exposure. The total body length (A) is reduced in the treatment group (average ±SD 2.9±0.5) compared to control (average ±SD 3.2± 0.3). The total body weight (B) is reduced in the treatment group (average ±SD .29±0.1) compared to control (average ±SD 4.0± 0.1). N = 8-12 fish per treatment group. An asterisk (*) indicates a statistical significant value, $p< 0.05$, student t-test.

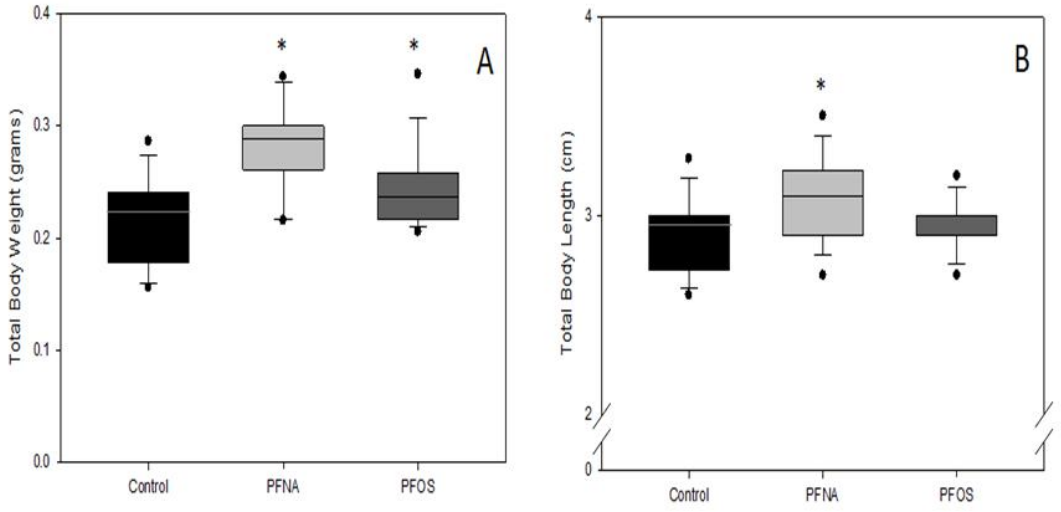


Figure 5.3**.** Morphometric measurements of PFNA and PFOS $P_0$ fish at 6 months following embryonic exposure and 30 dpf – 6 months feeding exposure. The total body weight (A) is increased in the both treatment groups (average ±SD 0.29±0.05, 0.26±0.03) compared to control (average ±SD 0.22± 0.01). The total body length (B) is increased in the PFNA treatment group (average ±SD 3.2±0.1) compared to control (average ±SD 2.9± 0.09). N = 8-12 fish per treatment group. An asterisk (*) indicates a statistical significant value, $p< 0.05$, student t-test.

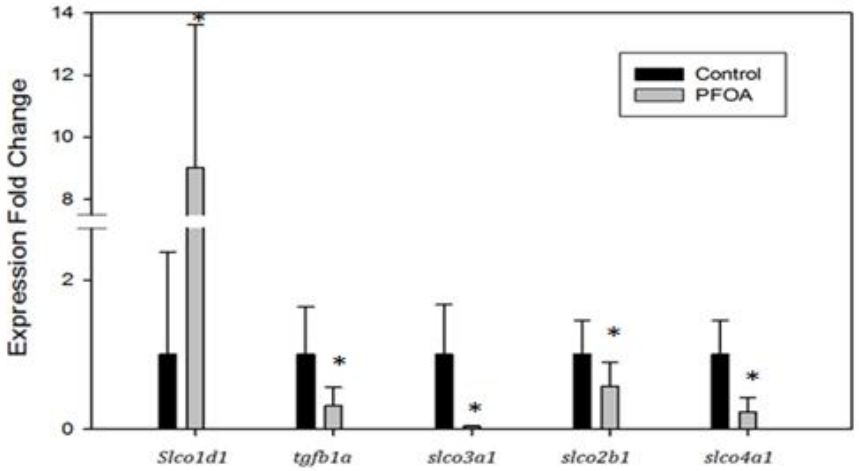


Figure 5.4**.** Gene expression of control and PFOA-fed (8.28 pM) fish of *slco1d1, tgfb1a, slco3a1, slco2b1, and slco4a1* transcripts. N = 5-8 for each exposure group. An asterisk (*) indicates a statistical significant value from control, $p < 0.05$, student t-test.

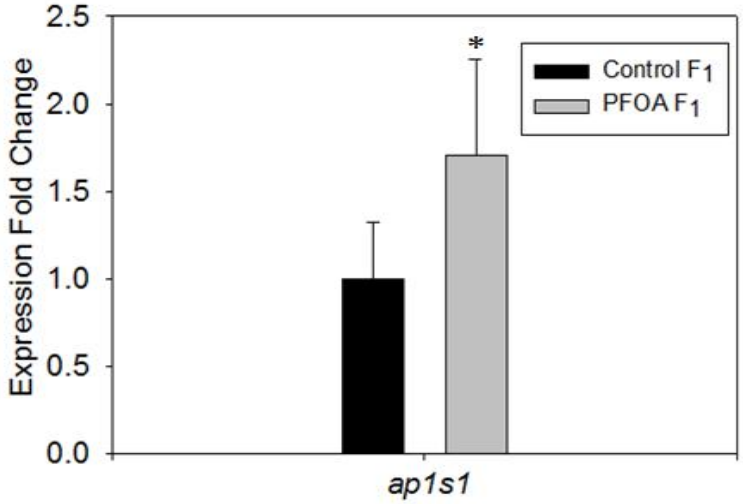


Figure 5.5. Gene expression of $F_1$ offspring from control and PFOA-fed (8.28 pM) fish of *ap1s1* transcript. N = 4 pooled sampled of 25 fish each per exposure group. An asterisk (*) indicates a statistical significant value from control, $p < 0.05$, student t-test.

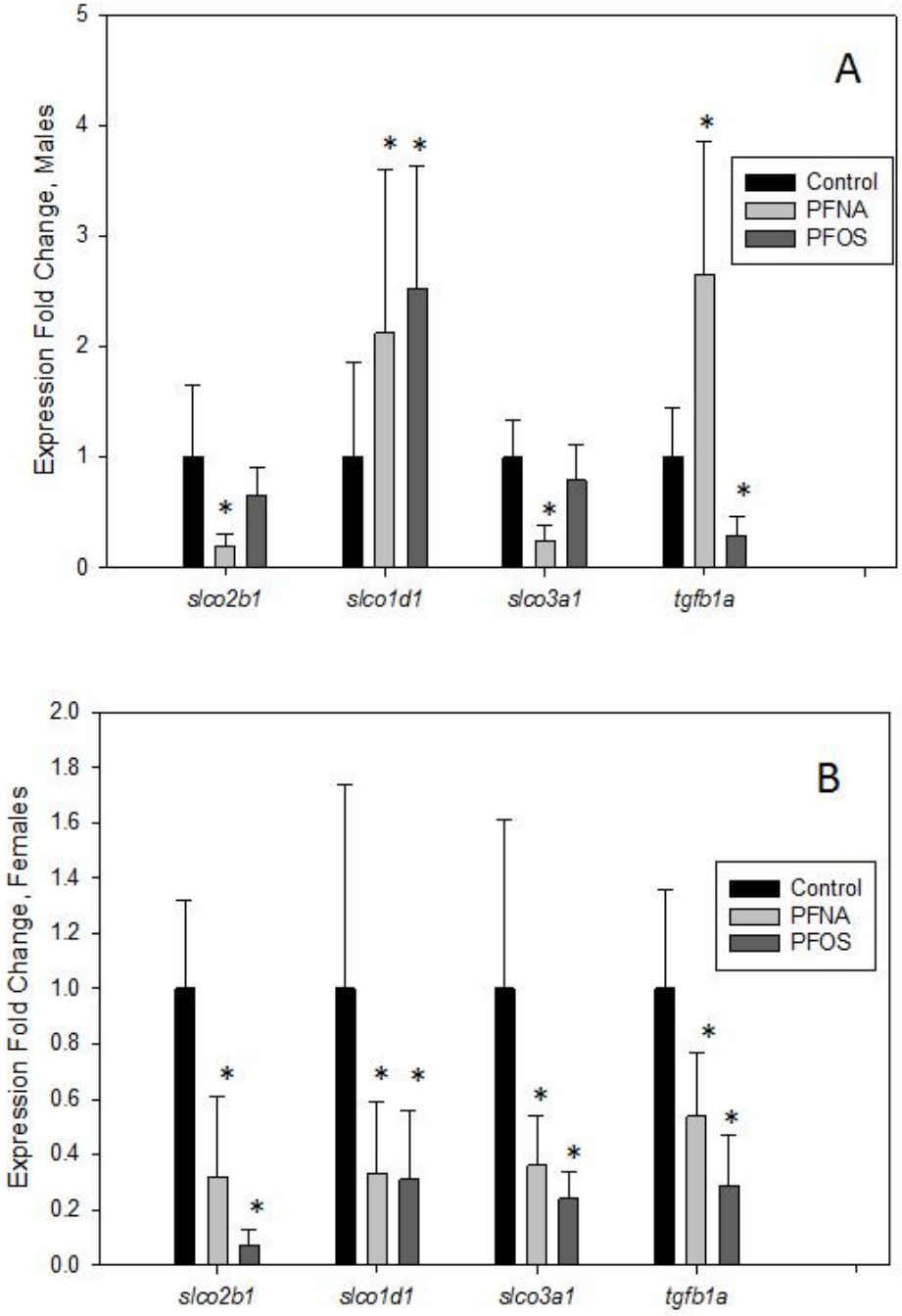


Figure 5.6**.** Gene expression of control and PFOS and PFNA-fed fish of *slco1d1, tgfb1a, slco3a1, slco2b1, and slco4a1* transcripts in males (A) and females (B). N = 5-8 for each exposure group. An asterisk (*) indicates a statistical significant value from control, p< 0.05, student t-test.

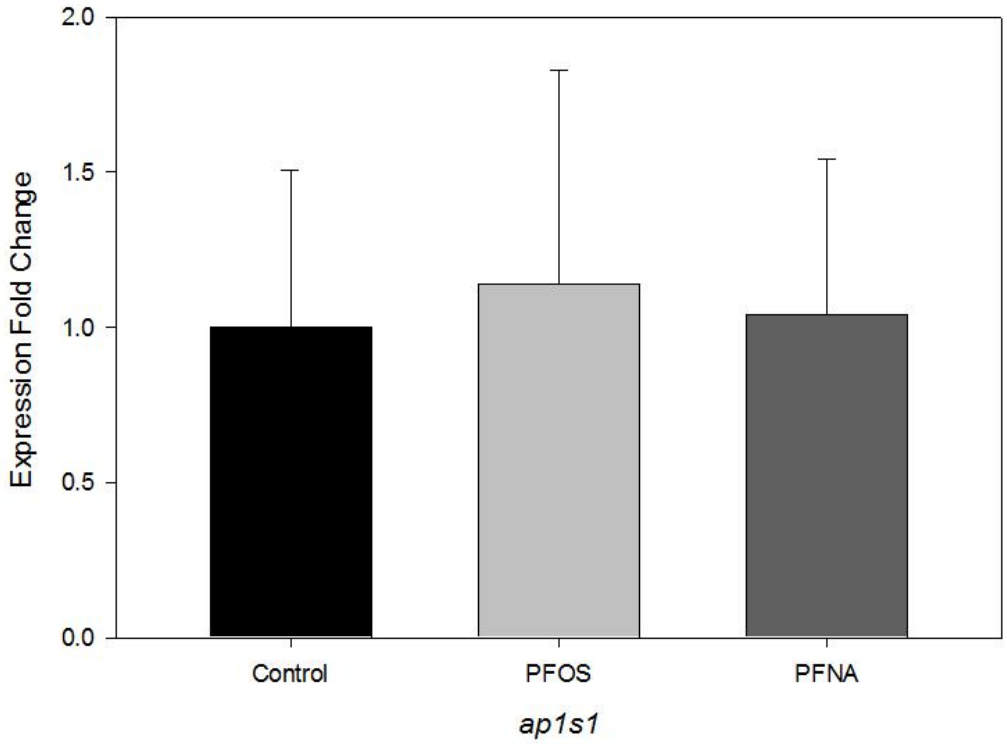


Figure 5.7 Gene expression of $F_1$ offspring from control and PFNA and PFOS-fed (8.28 pM) fish of *ap1s1* transcript. N = 4 pooled sampled of 25 fish each per exposure group.

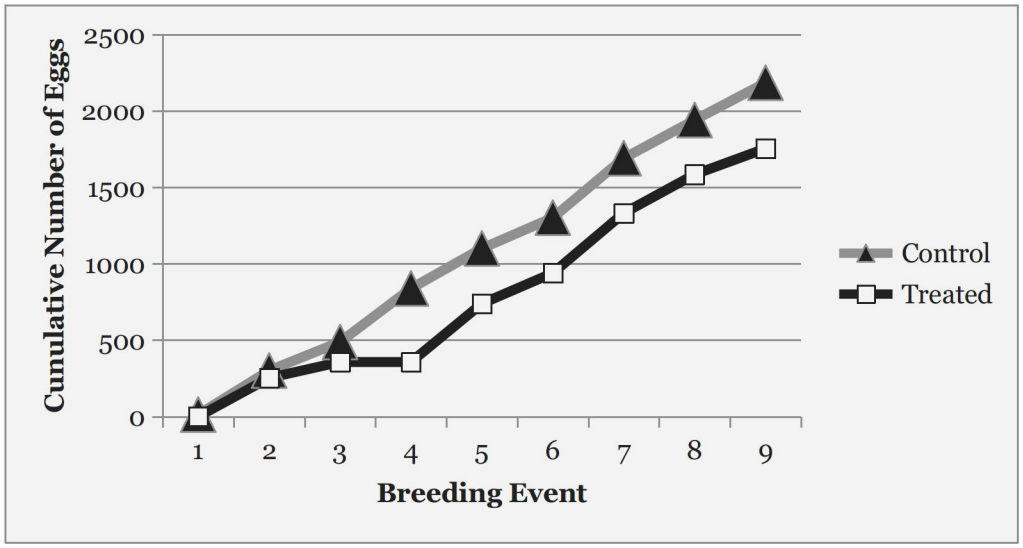


Figure 5.8. Cumulative number of eggs produced over the course of 9 breeding events for PFOA and control groups. Each breeding set had six females and five males. Statistical analysis using chi-squared test of the cumulative data indicated the control groups produced significantly more eggs.

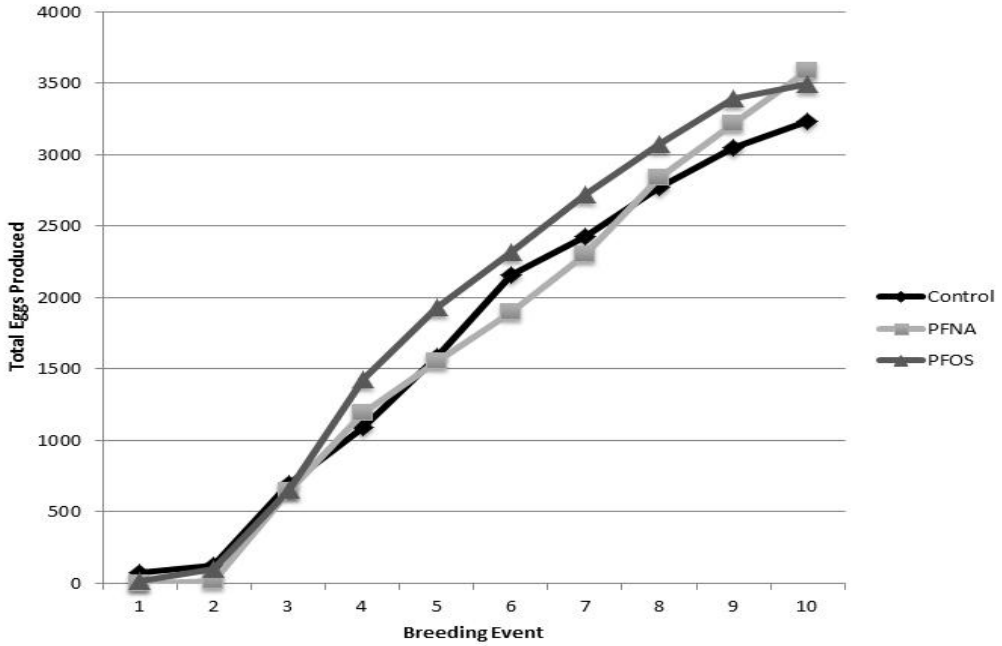


Figure 5.9. Cumulative number of eggs produced over the course of 9 breeding events for PFNA, PFOS and control groups. Each breeding set had six females and five males. Statistical analysis using chi-squared test of the cumulative data

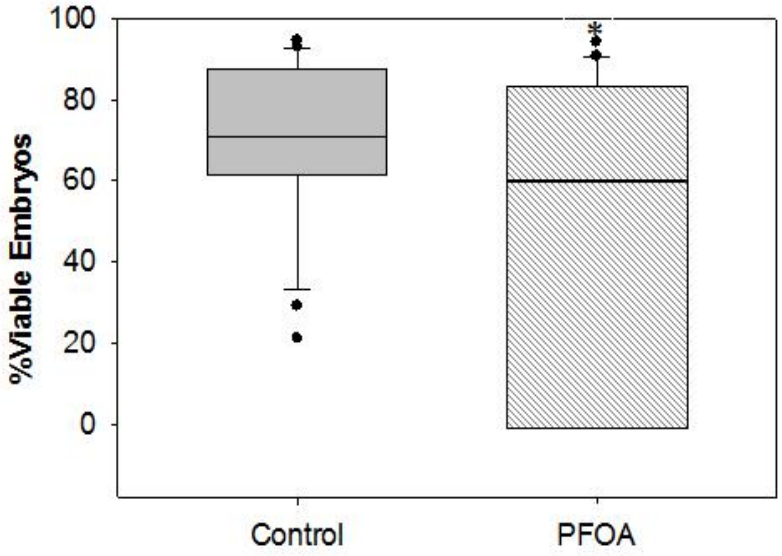


Figure 5.10. Average percentage of viable embryos/ eggs produced at collection 3 hpf. Middle bars represent median, error bars represent 95$^{th}$ and 5$^{th}$ percentiles. An asterisk (*) indicates a statistical significant value, $p< 0.05$, chi-squared test

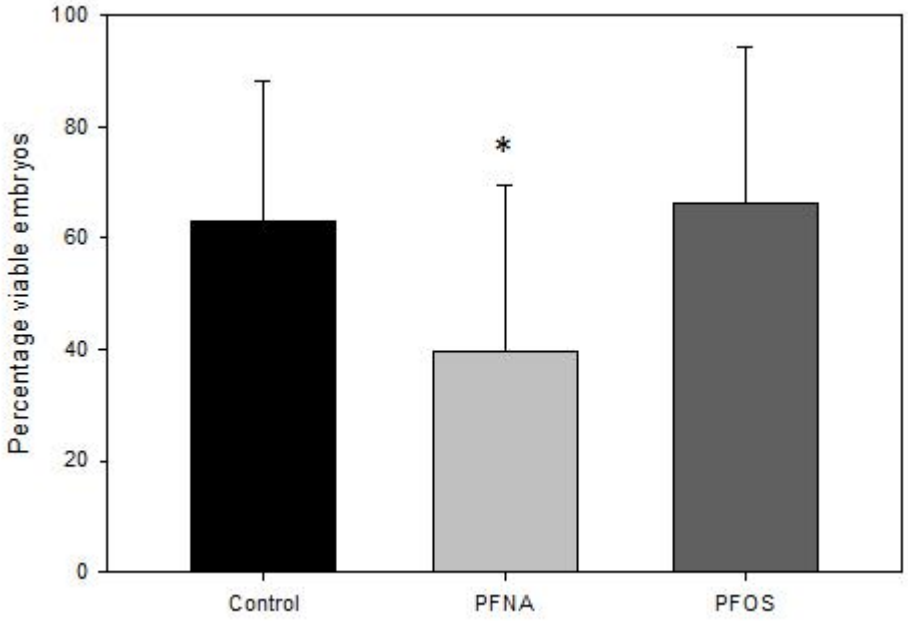


Figure 5.11. Average percentage of viable embryos/ eggs produced at collection 3hpf. Bars represent average and standard deviation. An asterisk (*) indicates a statistical significant value, $p < 0.05$, chi-squared test

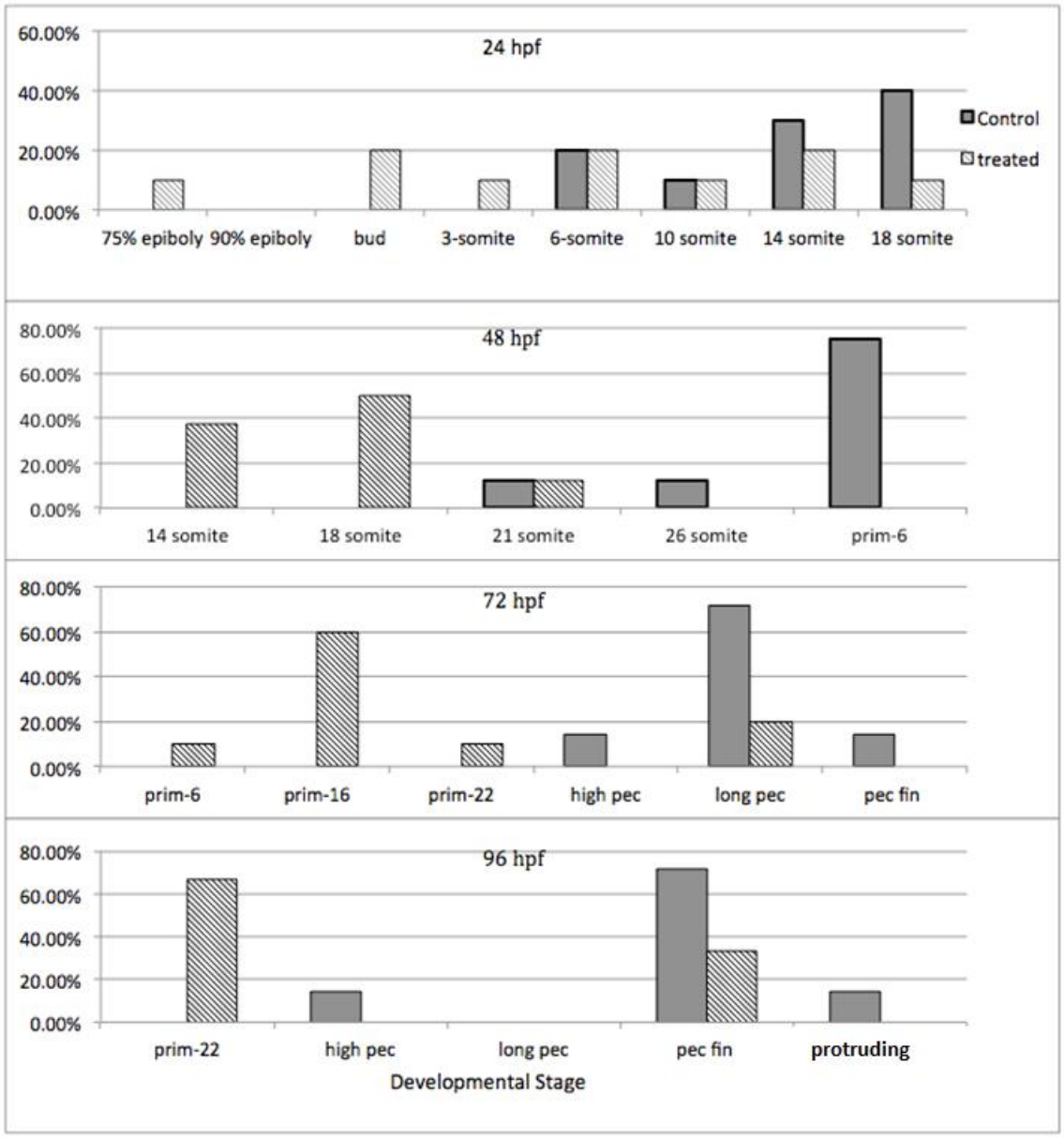


Figure 5.12. Embryo developmental staging based on (Kimmel et al. 1995) at 24, 48, 72, and 96 hpf for embryos collected from chronically exposed parental stock and raised in rearing solution free of treatment. Developmental stages along the x-axis increase from left to right. Statistical analysis using chi-squared test at each stage found the PFOA $F_1$ embryos were significantly developmentally delayed ($p < 0.05$).

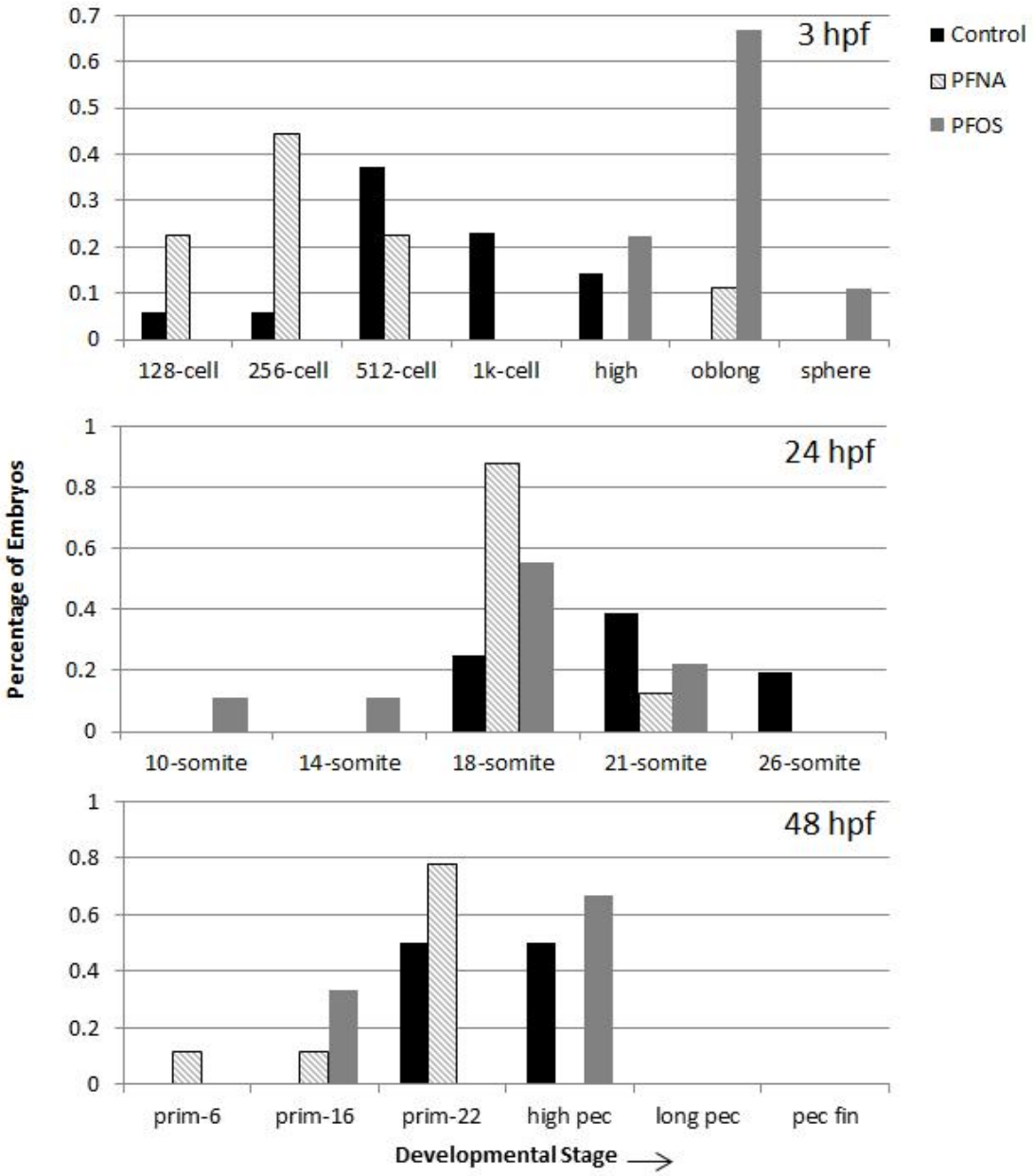


Figure 5.13. Embryo developmental staging based on (Kimmel et al. 1995) at 3, 24, and 48 hpf for embryos collected from chronically exposed PFOS and PFNA parental stock and raised in rearing solution free of treatment. Developmental stages along the x-axis increase from left to right. Statistical analysis using chi-squared test at each stage found the PFNA $F_1$ embryos were significantly developmentally delayed and PFOS embryos significantly accelerated ($p < 0.05$).

| **Organism** | **PFOA Concentration** | **Reproductive Effects** | **Citation** |
|---|---|---|---|
| Zebrafish (*Danio rerio)* | 0.002 μM | Reduced egg production, reduced viability, delayed $F_1$ development | Current study |
| Medaka (Oryzias latipes) | 4.14 μM | Increased $F_1$ mortality, thyroid development defects | (Ji et al. 2008) |
| Mice | 5 kg/mg | Reduced fetal weight, Increased post-partum mortality | (Yahia et al. 2010) |

Table 5.2. Summary of reproductive effects after PFOA exposure in various model species

| | PFNA | | | | PFOS | | | | PFOA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **dpf:** | Acute (3-120 hpf exposure) | | Chronic (3-120 hpf; 30-180 dpf exposure) | | Acute (3-120 hpf exposure) | | Chronic (3-120 hpf; 30-180 dpf exposure) | | Acute (3-120 hpf exposure) | | Chronic (3-120 hpf; 30-180 dpf exposure) | |
| | M | F | M | F | M | F | M | F | M | F | M | F |
| **Morphometric** | | | | | | | | | | | | |
| Total Body Length | NS | NS | ↑ | ↑ | NS | NS | ↑ | ↑ | NS | NS | ↓ | ↓ |
| Total Body Weight | NS | NS | ↑ | ↑ | NS | NS | NS | NS | NS | NS | ↓ | ↓ |
| **Gene Expression** | | | | | | | | | | | | |
| *slco2b1* | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | NS | NS | ↓ | ↓ |
| *slco1d1* | NS | ↓ | ↑ | ↓ | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ | ↑ | ↓ |
| *tgfb1a* | ↑ | NS | ↓ | NS | ↑ | NS | ↓ | NS | ↑ | NS | ↑ | NS |

Table 5.3. Summary of chapters 4 (acute) and 5 (chronic) exposures in adult zebrafish.

# Chapter 6: General Discussion and Conclusions

## 6.1 General Discussion

The data presented in this thesis reject the null hypothesis that perfluorooctane sulfonate (PFOS), perfluorononanoic acid (PFNA), and perfluorooctanoic acid (PFOA) would elicit the same toxicity profiles in zebrafish. While there was no clear correlation between end chain group (carboxylic acid vs sulfonate group), or between chain length (C8 vs C9) throughout these studies, there were similarities between the compound groups at certain endpoints. At the 5 dpf time point, end-chain group may play a larger role in the toxicity, as PFOA and PFNA appeared to have a more similar toxicity profile with each other when comparing yolk sac size and gene expression as compared to PFOS exposure, which showed alteration in yolk sac size in the opposite direction (PFOS smaller, PFNA and PFOA larger) as well as induced a larger number of gene expression changes (Figure 3.7). This appears to also hold true at the 14 dpf, in which PFOS was the only PFASs to elicite a hyperactive phenotype (swimming further distance, swimming faster, and crossing more frequencty), while both PFNA and PFOA exposure resulted in a decrease prey capture ability. In the adult studies, it appears that carbon chain length could be a determining factor particularly in terms of behavior. PFNA exposed males had a number of behavioral defects, while only few altered endpoints were observed in either of the C8 compound exposures. In terms of reproduction, while the effects seen after PFOA exposure appeared to be more severe, they were similar to that of PFNA, while PFOS elicited very few effects.

### 6.1.1 Similarities between three PFASs

The similarities between the three compounds observed at 5 dpf were: decrease in total body size, and *ap1s1* and *tcf3a* expression. Changes in yolk sac size were also observed, although PFNA and PFOA exposure increased this measurement while PFOS exposure resulted in a decrease. Hyperactivity and altered *slco2b1* expression at 14 dpf were observed after exposure to all PFASs. In adults, increased expression in *bdnf* and *tgfb1a* was seen in all exposed adult males, and down-regulation of *slco* genes was observed in all exposure groups. In the chronic reproductive studies, the $P_0$ fish from each compound were significantly different in size, but not in the same direction (PFNA and PFOS larger, PFOA smaller). Overall, it appears that each compound has a different critical time point where exposure is most severe relative to the other compounds (Figure 6.1)

## 6.2 PFOS

A summary of effects of PFOS exposure at various life stages is seen in Table 6.1. While all three compounds affected morphometric measurements (total length, interoccular, and yolk sac size), PFOS exposure was the only one that caused all to decrease. This would indicate that the exposed fish as a whole is not as large compared to the controls, rather than there being malformation of specific structures. This correlates with previous studies in other animals that found a significant decrease in body weight associated with PFOS exposure (Fang et al. 2012; Houde et al. 2013; Spulber et al. 2014).

In comparison to PFNA and PFOA, PFOS elicited the greatest number of gene expression changes at 5 dpf. These genes (*calm3a, cdkn1a, cyp1a, flk, tgfb1a, tcf3a, slco2b1*) are involved in a number of critical developmental pathways, including protein transport, calcium ion binding, apoptosis, mitotic cell cycle regulation, angiogenesis and growth activity factors (Bugel et al. 2014). At 5 dpf, PFOS exposed fish were significantly shorter in length, and had a smaller interoccular distance and yolk sac size (Table 3.1). While it is difficult at this time to pinpoint an exact gene pathway that could account for the morphometric affects observed, it is likely that any combination of alterations in these transcripts could be affecting the growth of the fish.

After 14 dpf, in which the fish were taken out of treatment and allowed to recover for 9 days, there was no longer any significant difference in morphometric measurements in either length or weight. This indicates that the morphological effects seen at 5 dpf are not persistent after exposure was terminated. However, these fish did have more altered swimming behaviors (increased in total distance traveled, traveling speed, and crossing frequency) compared to PFNA and PFOA. Gene expression of *slco2b1* and *tcf3a* were also significantly increased. This indicates that even though these fish have recovered from an initial decrease in size, there are still morphological or behavioral alterations that affect locomotive ability.

As expected based on data collected at 14 dpf, adult fish embryonically exposed to PFOS showed no changes in total weight or length. These fish had decreased aggression levels but no other significantly altered behavior endpoints. Gene expression of *slco2b1,*

*slco1d1, bdnf* and *tgfb1a* was significantly altered. In Chapter 4, we discussed the possible correlation between these transcripts affecting hormone levels, which could account for increased anxiety levels (Blaser et al. 2010; Champagne et al. 2010). This demonstrates that there may be more subtle effects occurring with PFOS exposure and further analysis into hormone and endocrine related pathways would be beneficial.

In terms of adult behavior studies PFOS exposed males had a reduced aggression level, and were trending towards spending less time in the light area of the light/dark test. *Bdnf* is a neurotrophic factor that has altered expression in the PFOS exposed male group, and could be contributing to some of these altered endpoints. Further analysis of *bdnf* activity or receptor activity would provide a better insight to how alterations in this pathway affect behavior.

Of the three PFASs tested, PFOS elicited the fewest reproductive effects after a chronic exposure. The P0 generation had an increase in total body size, and sex specific changes in *slco2b1*, *slco3a1, slco1d1 and tgfb1a* were observed. There was no effect on fecundity or offspring viability. F1 embryos initial exhibited an accelerated development, but this affect was corrected by 48 hpf. F1 embryos also showed no difference in ap1s1 expression at 48 hpf. Therefore, it appears that PFOS exposure is most detrimental during the embryonic exposure period (3-120 hpf) and as the fish age, the effects become less pronounced.

### 6.3 PFNA

Table 6.2 summarizes the effects of PFNA elicited at various life stages in the zebrafish. In terms of morphometrics at 5 dpf, these fish were significantly smaller in length but had a larger yolk sac. Previous studies had shown that the yolk sac size had a direct positive correlation to the size of the fish (Jardine and Litvak 2003). However, this was not observed in this exposure, so it is likely that nutrient uptake and transport from the yolk sac were being affected.

Exposure only resulted in one gene expression change at 5 dpf, *tgfb1a*, which was not observed in both PFOS and PFOA. This, along with other significantly altered genes (slco2b1 ap1s1, tcf3a) play a critical role in embryonic development (Kim et al. 2000; Montpetit et al. 2008; Popovic et al. 2014) and it is possible that changes in any of these could contribute to the morphological changes observed.

While PFOS recovered from the initial 5 dpf decrease in body length by 14 dpf, PFNA fish actually overshot the controls and were significantly larger. Even though there was an increase in body size, the number of shrimp eaten/fish was decreased, and the fish had increased hyperactivity. This further shows that there appears to be a defect in nutrient uptake or storage after PFNA exposure.

Behavior assays in PFNA exposed adult showed by far a greater number of effects compared to PFOS and PFOA. These fish had decreased activity (distance traveled) but increased body movement independent of swimming activity. These fish also swam faster

and had a number of anxiety-like behaviors such as thigmotaxis, preference for light rather than dark, and increase aggression (Blaser et al. 2010; Champagne et al. 2010; Schnorr et al. 2012; Simon et al. 1994). *Slco2b1, slco1d1, bdnf* and *tgfb1a* were all significantly changed in expression. Many of these transcripts (*slco2b1, slco1d1, tgfb1a)* are involved in some aspect of hormone transport or signaling (Klaassen and Aleksunes 2010; Popovic et al. 2014), and changes in expression could manifest as some of the anxiety behaviors observed. However, further analysis of specific transporters and hormones would have to be performed to fully identify the role they play in zebrafish behavior. Alteration in *bdnf* expression could also be contributing to the changes observed in these behavior endpoints. However, *bdnf* was increased in expression for three PFAS male exposure groups, but only PFNA exposed males exhibited many of these changes. Therefore, further analysis into the regulation of this gene and consequent pathways is necessary.

While embryonically exposed adults had no morphometric differences, chronically exposed adults had an increased length and weight. Changes in gene expression of transcripts *slco1d1, slco2b1, slco3a1 and tgfb1a* were also observed. PFNA elicited more reproductive effects than PFOS, in that there was a decrease in offspring viability as well as delayed development through 24 hpf.

Through these analyses, it is clear that many detrimental effects of PFNA exposure manifest at the adult life stage, particularly in behavior modifications.

### 6.4 PFOA

At 5 dpf, the morphometrics of PFOA exposure appeared to be similar to PFNA in terms of decreased size and increased yolk sac size. PFOA fish also had an increased interoccular size. The absence of a positive correlation between yolk sac and body size leads to the conclusion that there is a problem with yolk sac production or usage. The increased interoccular distance also implicates that cranial-facial morphological defects are occurring after PFOA exposure. A summary of effects are listed in Table 6.3.

PFOA had one uniquely significantly altered transcript, *c-fos*. Downstream targets of their transcript have been previously shown to affect larval growth and development in zebrafish. However, additional studies of this transcript as well as other possible transcript pathways identified (*slco, ap1s1, tcf3a*) should be performed to determine a mode of action for this compound.

Unlike PFOS and PFNA, the decreased body size phenotype persisted in PFOA treated fish through 14 dpf. These fish were hyperactive, and increased swimming could be one possibility for a decreased body size. However, both PFNA and PFOS fish displayed hyperactivity as well, so other mechanisms must also be at play, such as nutrient uptake or transport.

When in adulthood, there was no different in total body size between PFOA exposed fish and the control group. In contrast to PFOS and PFNA, which saw changes in a number of organic anion transporters, PFOA exposed adults exhibited changes in one, *slco1d1*.

Additionally, gene expression changes of *bdnf* and *tgfb1a* were observed in the PFOA exposed male group. The only behavior modification present was a preference for the dark in the light/dark anxiety assay. However, this could be a beneficial effect in that in the dark it is more difficult for predators to find them.

The most effects seen with PFOA were after a chronic exposure. The P0 adults were significantly smaller in size and had a number of negative reproductive effects, including decreased egg production, and significant developmental delay of F1 through 96 hpf. Gene expression of all transcripts (*tgfb1a, slco3a1, slco2b1, and slco4a1*) studied were significantly decreased, except for *slco1d1* which was increased.

PFOA exposure resulted in a persistent decreased size, but this effect was eventually mitigated in adulthood. Chronic PFOA exposure resulted in the most severe reproductive effects compared to PFOS and PFNA.

**6.5 Environmental and Ecological Concerns**

PFASs typically exist in the environment as a mixture of different compounds. This makes analysis of the actual exposure and chemical uptake in organisms difficult. However, the phenotypic effects observed after each individual PFAS exposure could lead to a number of environmental and ecological concerns at both the organism and population level.

The possible relationships between the genes analyzed and phenotypic affects are represented in Figure 6.3. Each of the genes altered in multiple compounds at multiple

life stages (*ap1s1, tgfb1a, slco2b1, slco1d1, bdnf*) could be responsible for the changes in morphometric measurements, behavior endpoints, and reproduction endpoints observed. Additionally, each phenotypic effect itself could be detrimental to the fitness of the population, and could also result in effects in other endpoints observed.

Morphometric effects such as yolk sac size could result in changes in total body size, or in nutrient uptake or availability. This could make these fish more available for predators to find, as well as affect their affect reproductive success (Chapter 5) as previous studies indicate that female zebrafish prefer to mate with larger males (Skinner and Watt 2007). A reduction in body size could lead to a lower ability to catch prey. To compensate for body size, there could be alterations in behavior, such as hyperactivity and increased swimming locomotion. This increased swimming could increase the energy usage needed, leaving less available for reproduction, which could decrease the number and quality of offspring produced.

### 6.6 Overall conclusions

The overall summary of this dissertation is presented in figure 6.2. PFOS, PFNA, and PFOA all have detrimental effects on zebrafish that impact both individual and population fitness. Each compound has specific developmental periods and end points in which they are most effective at producing negative outcomes. PFOS appears to induce the greatest number of effects at the embryo-larval stages in terms of morphometrics and gene expression. PFNA severely affected behavior at the adult stage, and PFOA seems to be the most critical in terms of reproductive success. Therefore, each of these PFASs

have different toxicity profiles and should be analyzed separately when studying the effects on zebrafish and other teleost fish.

| | PFOA | PFOS | PFNA |
|---|---|---|---|
| Developmental (5 dpf) Morphometrics, gene expression | + | + + + | + |
| Juvenile (14 pf), morphometrics, swim activity, gene expression | + + | + | + + |
| Adult (90 dpf), morphometrics, swim activity, gene expression | + | + | + + + |
| Reproduction endpoints (6 months) | + + + | + | + + |

Figure 6.1. Ranking of affects for each PFAS at each time point analyzed relative to each other. "+" indicate relatively few toxic effects, "++" indicates moderate toxic effects, and "+++" indicates severe toxic effects. PFOS appears to have the most affects at the 5 dpf time point, whereas PFNA seems to be the most effective at the 90 dpf time and PFOA elicits the most effects when analyzing reproductive success.

| PFOS | 5 dpf | 14 dpf | Adult (embryo) | Adult (chronic) |
|---|---|---|---|---|
| **Morphometrics** | △ Total length, interoccular distance, yolk sac size | NS | NS | ♿ Total size |
| **Gene Expression** | △*calm3a, cdkn1a, cyp1a, flk, tgfb1a, tcf3a* ♿*slco2b1* | ♿*slco2b1, tcf3a* | △*slco2b1 (M,F), slco1d1 (M,F), tgfb1a (M)* | ♿*slco1d1 (M)* △*slco1d1(F), slco2b1(F), slco3a1 (F), tgfb1a (M, F)* |
| **Behavior** | | ♿Distance traveled, velocity, crossing frequency | △Aggression (M) | |
| **Reproductive effects** | | | | ♿Accelerated development |

Table 6.1 Summary of significantly altered endpoints after PFOS exposure at 5 dpf, 14 dpf, adults, and chronically exposed adults. ♿ represent a significantly increased endpoint, △ represent a significantly decreased endpoint compared to controls, $p < 0.05$.

| PFOA | 5 dpf | 14 dpf | Adult (embryo) | Adult (chronic) |
|---|---|---|---|---|
| **Morphometrics** | △ Total length<br>♿ Yolk sac size, interoccular distance | △ Total length | NS | △ Total size |
| **Gene Expression** | ♿ *tcf3a, ap1s1, slco2b1, cfos* | ♿ *slco2b1, tcf3a* | △ *slco1d1 (M,F)* | ♿ *slco1d1*<br>△ *tgfb1a, slco3a1, slco2b1, slco4a1* |
| **Behavior** | | ♿ Distance traveled | △ Time in light | |
| **Reproductive effects** | | | | △ Number of eggs, % embryo viability, delayed development |

Table 6.2 Summary of significantly altered endpoints after PFOA exposure at 5 dpf, 14 dpf, adults, and chronically exposed adults. ♿ represent a significantly increased endpoint, △ represent a significantly decreased endpoint compared to controls, $p < 0.05$.

| PFNA | 5 dpf | 14 dpf | Adult (embryo) | Adult (chronic) |
|---|---|---|---|---|
| **Morphometrics** | △ Total length<br>♿ Yolk sac size | ♿ Total length | NS | ♿ Total size |
| **Gene Expression** | ♿ *tcf3a, ap1s1, slco2b1, tgfb1a* | ♿ *slco2b1* | △ *slco1d1 (F), slco2b1 (M,F), tgfb1a (M)* | ♿ *slco1d1 (M), tgfb1a (M)*<br>△*tgfb1a (F), slco1d1 (F), slco3a1(M,F), slco2b1(M,F)* |
| **Behavior** | | ♿ Distance traveled, time in middle<br>△ Velocity, shrimp eaten | △ Distance traveled, time in middle, time immobile<br>♿ Velocity, time in light, aggression | |
| **Reproductive effects** | | | | △ % embryo viability, delayed development |

Table 6.3 Summary of significantly altered endpoints after PFNA exposure at 5 dpf, 14 dpf, adults, and chronically exposed adults. ♿ represent a significantly increased endpoint, △ represent a significantly decreased endpoint compared to controls, $p < 0.05$.

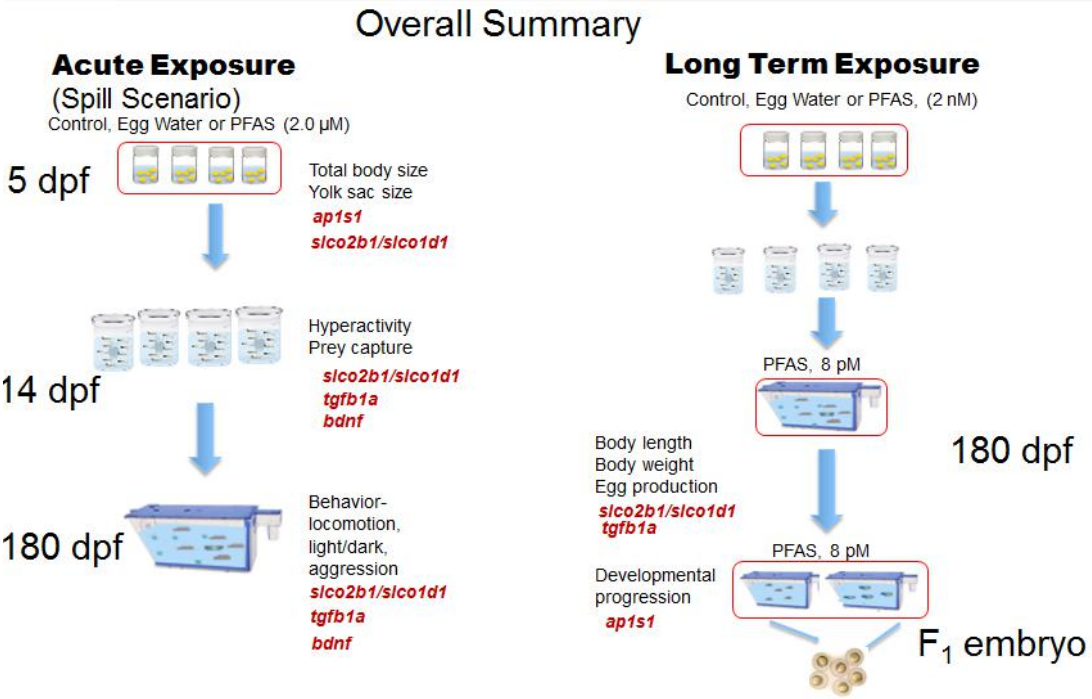


Figure 6.2. Overall Summary describing the two different exposure scenarios studies in this thesis. Each time point of analysis has the major effects observed as well as genes that were altered that could be playing a role in these phenotypic affects.

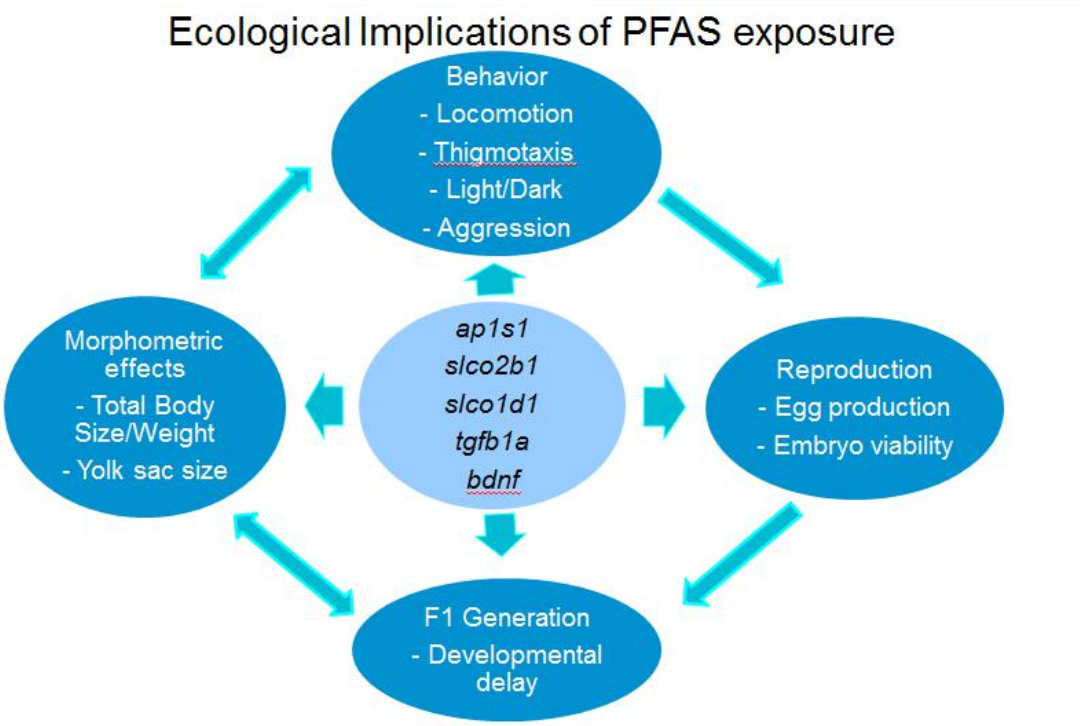


Figure 6.3. Ecological implications of PFAS exposure. Genes analyzed throughout these studies are presented in the middle, and each can be playing a role in multiple phenotypic endpoints observed. Additionally, changes in one type of endpoint observed could be having affected on other endpoints at various life stages.

## Future Areas of Research

This dissertation proved that three PFASs; PFOS, PFOA, and PFNA; each resulted in a unique toxicological profile in a zebrafish model. All three PFASs studied affected a number of endpoints including morphometric measurements, behavior, and gene expression. A variety of gene expression pathways were significantly altered, including those involved in embryonic development, protein transport, intercellular transport, and hormone signaling. While there are likely correlations between transcript alterations and phenotypic defects, the exact mechanism(s) of action for each compound is currently unknown.

For all PFASs analyzed, there appears to be many effects of the organic anion transporting polypeptides (oatps; *slco*). In zebrafish, these are responsible for the transport of many endogenous and exogenous compounds, mainly hormones, prostaglandins and related compounds (Popovic et al. 2014). OATps have also been shown to play a role in the elimination rates of PFASs, which vary between compound and sexes (Klaassen and Aleksunes 2010). Studies of *slco1d1* in zebrafish have found that PFOA acts as an inhibitor and PFOS a substrate of this transporter (Popovic et al. 2014). This leads to the question of whether either an inhibitor or a substrate is affecting the uptake of natural substrates. Cell culture substrate uptake assays can be performed to determine the functional outcomes of PFASs with oatps.

Additionally, there are a large number of oatps in zebrafish and they are found in a number of different organ systems. These transporters can have multiple overlapping substrates with low specificity. Analyzing multiple transporters will allow for classification of the interactions between transporters and allow identification of which group in particular are being affected by each PFAS.

PFOA and PFNA chronic exposures both resulted in reproductive defects. In addition to the *slco* transcripts, one pathway that was attributed to this was *tgfb1a*. These transcripts are involved in the regulation of oocyte maturation in zebrafish and were significantly affected in both PFAS treatments. PFOA and PFNA both had decreased embryo survival and delayed embryo development, but PFOA also resulted in decreased total egg production. It would be beneficial to more thoroughly understand the mechanism by which PFASs cause reproductive defects but further investigation the role of *tfb1a* or by identifying other possible pathways. This can help to predict and understand reproductive effects seen at higher vertebrates after PFAS exposure.

PFAS exposure, PFOS in particular, resulted in changes in yolk sac size. The yolk sac of zebrafish contains nutrients, lipids, and vitellogenin necessary for the fish to grow and survive until they are large enough to catch their own food. The changes in yolk sac size, although not seen in the F1 embryos, could play a role in the developmental delay observed. A decreased yolk sac (PFOS) could be due an irregular uptake of nutrients; however, with these fish being smaller in size, the nutrients are not being used for growth. A larger yolk sac (PFOA, PFNA) would be due to increased vitellogenin given to the

embryo from the mother, a lack of uptake in the embryo, or the larger size could be due to edema. Future studies of yolk sac content and uptake would provide an answer to this question.

PFNA embryonically exposed adults showed a great number of behavioral and anxiety driven modifications. One explanation for these changes was an increase in expression of the neurotrophic factor *bdnf*. However, *bdnf* expression was also increased in the males of the PFOS and PFOA exposure groups, but very few behavior changes were observed. Looking at the activity of the bdnf protein as well as activity and expression of the *bdnf* receptor would provide further insight to its role in behavior effects. Another explanation put forth to explain this change of behavior was an alteration in hormone circulation and transports as a function of *slco* and *tgfb1a* transcripts. These genes were analyzed from isolated liver tissue. Future studies could analyze organic anion transporting polypeptides in the brain such as *slco1c1*, as well as other neurotransmitters commonly associated with anxiety behaviors. This would determine if the effects observed are due to changes in hormone cycling and transport, or if there is a structural neurological deficit.

## Acknowledgement of Previous Publications

Chapter 3:

Jantzen CE, Annunziato KA, Bugel SM, Cooper KR (2016a) PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression. Aquatic toxicology 175:160-170 doi:10.1016/j.aquatox.2016.03.026

## References:

Abbott BD, Wolf CJ, Schmid JE, et al. (2007) Perfluorooctanoic acid induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha. Toxicological sciences : an official journal of the Society of Toxicology 98(2):571-81 doi:10.1093/toxsci/kfm110

Ahmad F, Richardson MK (2013) Exploratory behaviour in the open field test adapted for larval zebrafish: impact of environmental complexity. Behavioural processes 92:88-98 doi:10.1016/j.beproc.2012.10.014

Ahrens L, Yeung LW, Taniyasu S, Lam PK, Yamashita N (2011) Partitioning of perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS) and perfluorooctane sulfonamide (PFOSA) between water and sediment. Chemosphere 85(5):731-7 doi:10.1016/j.chemosphere.2011.06.046

Ankley GT, Kuehl DW, Kahl MD, et al. (2005) Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow (Pimephales promelas). Environmental toxicology and chemistry / SETAC 24(9):2316-24

Arvaniti OS, Stasinakis AS (2015) Review on the occurrence, fate and removal of perfluorinated compounds during wastewater treatment. The Science of the total environment 524-525:81-92 doi:10.1016/j.scitotenv.2015.04.023

Bao Y, Niu J, Xu Z, et al. (2014) Removal of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) from water by coagulation: Mechanisms and influencing factors. Journal of colloid and interface science 434:59-64 doi:10.1016/j.jcis.2014.07.041

Beanes SR, Dang C, Soo C, Ting K (2003) Skin repair and scar formation: the central role of TGF-beta. Expert reviews in molecular medicine 5(8):1-22 doi:doi:10.1017/S1462399403005817

Benninghoff AD, Bisson WH, Koch DC, Ehresman DJ, Kolluri SK, Williams DE (2011) Estrogen-like activity of perfluoroalkyl acids in vivo and interaction with human and rainbow trout estrogen receptors in vitro. Toxicological sciences : an official journal of the Society of Toxicology 120(1):42-58 doi:10.1093/toxsci/kfq379

Berthiaume J, Wallace KB (2002) Perfluorooctanoate, perflourooctanesulfonate, and N-ethyl perfluorooctanesulfonamido ethanol; peroxisome proliferation and mitochondrial biogenesis. Toxicology letters 129(1-2):23-32

Biegel LB, Hurtt ME, Frame SR, O'Connor JC, Cook JC (2001) Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicological sciences : an official journal of the Society of Toxicology 60(1):44-55

Blaser RE, Chadwick L, McGinnis GC (2010) Behavioral measures of anxiety in zebrafish (Danio rerio). Behavioural brain research 208(1):56-62 doi:10.1016/j.bbr.2009.11.009

Boehm M, Bonifacino JS (2001) Adaptins: The Final Recount. Molecular Biology of the Cell 12(10):2907-2920 doi:10.1091/mbc.12.10.2907

Brown DD (1997) The role of thyroid hormone in zebrafish and axolotl development. Proceedings of the National Academy of Sciences of the United States of America 94(24):13011-6

Bugel SM, Tanguay RL, Planchart A (2014) Zebrafish: A marvel of high-throughput biology for 21 century toxicology. Current environmental health reports 1(4):341-352 doi:10.1007/s40572-014-0029-5

Bugel SM, Wehmas LC, La Du JK, Tanguay RL (2016) Phenotype anchoring in zebrafish reveals a potential role for matrix metalloproteinases (MMPs) in tamoxifen's effects on skin epithelium. Toxicology and applied pharmacology 296:31-41 doi:10.1016/j.taap.2016.02.013

Buhrke T, Kruger E, Pevny S, Rossler M, Bitter K, Lampen A (2015) Perfluorooctanoic acid (PFOA) affects distinct molecular signalling pathways in human primary hepatocytes. Toxicology 333:53-62 doi:10.1016/j.tox.2015.04.004

Butenhoff JL, Kennedy GL, Jr., Hinderliter PM, et al. (2004) Pharmacokinetics of perfluorooctanoate in cynomolgus monkeys. Toxicological sciences : an official journal of the Society of Toxicology 82(2):394-406 doi:10.1093/toxsci/kfh302

Butt CM, Berger U, Bossi R, Tomy GT (2010) Levels and trends of poly- and perfluorinated compounds in the arctic environment. The Science of the total environment 408(15):2936-65 doi:10.1016/j.scitotenv.2010.03.015

Calafat AM, Wong LY, Kuklenyik Z, Reidy JA, Needham LL (2007) Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003-2004 and comparisons with NHANES 1999-2000. Environmental health perspectives 115(11):1596-602 doi:10.1289/ehp.10598

Carnevali O, Tosti L, Speciale C, Peng C, Zhu Y, Maradonna F (2010) DEHP impairs zebrafish reproduction by affecting critical factors in oogenesis. PloS one 5(4):e10201 doi:10.1371/journal.pone.0010201

Champagne DL, Hoefnagels CC, de Kloet RE, Richardson MK (2010) Translating rodent behavioral repertoire to zebrafish (Danio rerio): relevance for stress research. Behavioural brain research 214(2):332-42 doi:10.1016/j.bbr.2010.06.001

Chang SC, Thibodeaux JR, Eastvold ML, et al. (2008) Thyroid hormone status and pituitary function in adult rats given oral doses of perfluorooctanesulfonate (PFOS). Toxicology 243(3):330-9 doi:10.1016/j.tox.2007.10.014

Cheng J, Fujimura M, Zhao W, Wang W (2013) Neurobehavioral effects, c-Fos/Jun expression and tissue distribution in rat offspring prenatally co-exposed to MeHg and PFOA: PFOA impairs Hg retention. Chemosphere 91(6):758-64 doi:10.1016/j.chemosphere.2013.02.016

Conder JM, Hoke RA, De Wolf W, Russell MH, Buck RC (2008) Are PFCAs bioaccumulative? A critical review and comparison with regulatory criteria and persistent lipophilic compounds. Environmental science & technology 42(4):995-1003

Consoer DM, Hoffman AD, Fitzsimmons PN, Kosian PA, Nichols JW (2014) Toxicokinetics of perfluorooctanoate (PFOA) in rainbow trout (Oncorhynchus mykiss). Aquatic toxicology 156C:65-73 doi:10.1016/j.aquatox.2014.07.022

Coombs VN (2005) The Hydrodyanmics and Structural Mechanics of the Lateral Line System. Fish Physiology 23:103-139

Cooper K (2015) Personal Communication Rutgers University, New Brunswick NJ

Cornelis C, D'Hollander W, Roosens L, et al. (2012) First assessment of population exposure to perfluorinated compounds in Flanders, Belgium. Chemosphere 86(3):308-14 doi:10.1016/j.chemosphere.2011.10.034

Cui L, Zhou QF, Liao CY, Fu JJ, Jiang GB (2009) Studies on the toxicological effects of PFOA and PFOS on rats using histological observation and chemical analysis. Archives of environmental contamination and toxicology 56(2):338-49 doi:10.1007/s00244-008-9194-6

D'Hollander W, De Bruyn L, Hagenaars A, de Voogt P, Bervoets L (2014) Characterisation of perfluorooctane sulfonate (PFOS) in a terrestrial ecosystem near a fluorochemical plant in Flanders, Belgium. Environmental science and pollution research international 21(20):11856-66 doi:10.1007/s11356-013-2449-4

Das KP, Grey BE, Rosen MB, et al. (2015) Developmental toxicity of perfluorononanoic acid in mice. Reproductive toxicology 51:133-44 doi:10.1016/j.reprotox.2014.12.012

Davis KL, Aucoin MD, Larsen BS, Kaiser MA, Hartten AS (2007) Transport of ammonium perfluorooctanoate in environmental media near a fluoropolymer manufacturing facility. Chemosphere 67(10):2011-9 doi:10.1016/j.chemosphere.2006.11.049

De Silva AO, Mabury SA (2004) Isolating isomers of perfluorocarboxylates in polar bears (Ursus maritimus) from two geographical locations. Environmental science & technology 38(24):6538-45

de Vos MG, Huijbregts MA, van den Heuvel-Greve MJ, et al. (2008) Accumulation of perfluorooctane sulfonate (PFOS) in the food chain of the Western Scheldt estuary: Comparing field measurements with kinetic modeling. Chemosphere 70(10):1766-73 doi:10.1016/j.chemosphere.2007.08.038

Delinsky AD, Strynar MJ, Nakayama SF, et al. (2009) Determination of ten perfluorinated compounds in bluegill sunfish (Lepomis macrochirus) fillets. Environmental research 109(8):975-84 doi:10.1016/j.envres.2009.08.013

Den Broeder MJ, Kopylova VA, Kamminga LM, Legler J (2015) Zebrafish as a Model to Study the Role of Peroxisome Proliferating-Activated Receptors in Adipogenesis and Obesity. PPAR research 2015:358029 doi:10.1155/2015/358029

Deng J, Liu C, Yu L, Zhou B (2010) Chronic exposure to environmental levels of tribromophenol impairs zebrafish reproduction. Toxicology and applied pharmacology 243(1):87-95 doi:10.1016/j.taap.2009.11.016

Derynck R, Zhang YE (2003) Smad-dependent and Smad-independent pathways in TGF-[beta] family signalling. Nature 425(6958):577-584

DeWitt JC, Williams WC, Creech NJ, Luebke RW (2016) Suppression of antigen-specific antibody responses in mice exposed to perfluorooctanoic acid: Role of PPARalpha and T- and B-cell targeting. Journal of immunotoxicology 13(1):38-45 doi:10.3109/1547691X.2014.996682

Diekmann H, Anichtchik O, Fleming A, et al. (2009) Decreased BDNF levels are a major contributor to the embryonic phenotype of huntingtin knockdown zebrafish. The Journal of neuroscience : the official journal of the Society for Neuroscience 29(5):1343-9 doi:10.1523/JNEUROSCI.6039-08.2009

DRBC (2016) Contaminants of Emerging Concern in the Delaware River Basin. In: Commission DRB (ed). http://www.nj.gov/drbc/quality/reports/emerging/

Du G, Huang H, Hu J, et al. (2013) Endocrine-related effects of perfluorooctanoic acid (PFOA) in zebrafish, H295R steroidogenesis and receptor reporter gene assays. Chemosphere 91(8):1099-106 doi:10.1016/j.chemosphere.2013.01.012

Elcombe CR, Elcombe BM, Foster JR, Chang SC, Ehresman DJ, Butenhoff JL (2012) Hepatocellular hypertrophy and cell proliferation in Sprague-Dawley rats from dietary exposure to potassium perfluorooctanesulfonate results from increased expression of xenosensor nuclear receptors PPARalpha and CAR/PXR. Toxicology 293(1-3):16-29 doi:10.1016/j.tox.2011.12.014

Escher P, Wahli W (2000) Peroxisome proliferator-activated receptors: insight into multiple cellular functions. Mutation research 448(2):121-38

Fang C, Wu X, Huang Q, et al. (2012) PFOS elicits transcriptional responses of the ER, AHR and PPAR pathways in Oryzias melastigma in a stage-specific manner. Aquatic toxicology 106-107:9-19 doi:10.1016/j.aquatox.2011.10.009

Feng Y, Shi Z, Fang X, Xu M, Dai J (2009) Perfluorononanoic acid induces apoptosis involving the Fas death receptor signaling pathway in rat testis. Toxicology letters 190(2):224-30 doi:10.1016/j.toxlet.2009.07.020

Filgo AJ, Quist EM, Hoenerhoff MJ, Brix AE, Kissling GE, Fenton SE (2015) Perfluorooctanoic Acid (PFOA)-induced Liver Lesions in Two Strains of Mice Following Developmental Exposures: PPARalpha Is Not Required. Toxicologic pathology 43(4):558-68 doi:10.1177/0192623314558463

Gallen C, Baduel C, Lai FY, et al. (2014) Spatio-temporal assessment of perfluorinated compounds in the Brisbane River system, Australia: impact of a major flood event. Marine pollution bulletin 85(2):597-605 doi:10.1016/j.marpolbul.2014.02.014

Gasanov EV, Rafieva LM, Korzh VP (2015) BDNF-TrkB axis regulates migration of the lateral line primordium and modulates the maintenance of mechanoreceptor progenitors. PloS one 10(3):e0119711 doi:10.1371/journal.pone.0119711

Gerlai R (2010) Zebrafish antipredatory responses: a future for translational research? Behavioural brain research 207(2):223-31 doi:10.1016/j.bbr.2009.10.008

Gewurtz SB, Martin PA, Letcher RJ, et al. (2016) Spatio-temporal trends and monitoring design of perfluoroalkyl acids in the eggs of gull (Larid) species from across Canada and parts of the United States. The Science of the total environment 565:440-50 doi:10.1016/j.scitotenv.2016.04.149

Giesy JP, Kannan K (2001) Global distribution of perfluorooctane sulfonate in wildlife. Environmental science & technology 35(7):1339-42

Guruge KS, Yeung LW, Yamanaka N, et al. (2006) Gene expression profiles in rat liver treated with perfluorooctanoic acid (PFOA). Toxicological sciences : an official journal of the Society of Toxicology 89(1):93-107 doi:10.1093/toxsci/kfj011

Hagenaars A, Vergauwen L, De Coen W, Knapen D (2011) Structure-activity relationship assessment of four perfluorinated chemicals using a prolonged zebrafish early life stage test. Chemosphere 82(5):764-72 doi:10.1016/j.chemosphere.2010.10.076

Han X, Nabb DL, Russell MH, Kennedy GL, Rickard RW (2012) Renal elimination of perfluorocarboxylates (PFCAs). Chemical research in toxicology 25(1):35-46 doi:10.1021/tx200363w

Han X, Snow TA, Kemper RA, Jepson GW (2003) Binding of perfluorooctanoic acid to rat and human plasma proteins. Chemical research in toxicology 16(6):775-81 doi:10.1021/tx034005w

Han Z-X, Zhang M, Lv C-X (2011) Toxicokinetic behaviors and modes of perfluorooctane sulfonate (PFOS) and perfluorooctane acid (PFOA) on tilapia (Oreochromis niloticus). African Journal of Biotechnology 10(60):12943-12950

Hart K, Kannan K, Tao L, Takahashi S, Tanabe S (2008) Skipjack tuna as a bioindicator of contamination by perfluorinated compounds in the oceans. The Science of the total environment 403(1-3):215-21 doi:10.1016/j.scitotenv.2008.05.035

Hascoet M, Bourin M, Nic Dhonnchadha BA (2001) The mouse light-dark paradigm: a review. Progress in neuro-psychopharmacology & biological psychiatry 25(1):141-66

Hill AJ, Teraoka H, Heideman W, Peterson RE (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicological sciences : an official journal of the Society of Toxicology 86(1):6-19 doi:10.1093/toxsci/kfi110

Houde M, Bujas TA, Small J, et al. (2006) Biomagnification of perfluoroalkyl compounds in the bottlenose dolphin (Tursiops truncatus) food web. Environmental science & technology 40(13):4138-44

Houde M, De Silva AO, Muir DC, Letcher RJ (2011) Monitoring of perfluorinated compounds in aquatic biota: an updated review. Environmental science & technology 45(19):7962-73 doi:10.1021/es104326w

Houde M, Douville M, Despatie SP, De Silva AO, Spencer C (2013) Induction of gene responses in St. Lawrence River northern pike (Esox lucius) environmentally exposed to perfluorinated compounds. Chemosphere 92(9):1195-200 doi:10.1016/j.chemosphere.2013.01.099

Hu XC, Andrews DQ, Lindstrom AB, et al. (2016) Detection of Poly- and Perfluoroalkyl Substances (PFASs) in U.S. Drinking Water Linked to Industrial Sites, Military Fire Training Areas, and Wastewater Treatment Plants. Environmental Science & Technology Letters doi:10.1021/acs.estlett.6b00260

Ingman WV, Robertson SA (2008) Mammary gland development in transforming growth factor beta1 null mutant mice: systemic and epithelial effects. Biology of reproduction 79(4):711-7 doi:10.1095/biolreprod.107.067272

Innes KE, Wimsatt JH, Frisbee S, Ducatman AM (2014) Inverse association of colorectal cancer prevalence to serum levels of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in a large Appalachian population. BMC cancer 14:45 doi:10.1186/1471-2407-14-45

Jantzen CE, Annunziato KA, Bugel SM, Cooper KR (2016a) PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression. Aquatic toxicology 175:160-170 doi:10.1016/j.aquatox.2016.03.026

Jantzen CE, Annunziato KM, Bugel SM, Cooper KR (2016b) PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression. Aquatic toxicology 175:160-70 doi:10.1016/j.aquatox.2016.03.026

Jardine D, Litvak MK (2003) Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. Journal of Fish Biology 63(2):388-397 doi:10.1046/j.1095-8649.2003.00161.x

Jardine DL, M.K (2003) Direct yolk sac volume manipulation of zebrafish embryos and the relationship between offspring size and yolk sac volume. Journal of Fish Biology 63(2):388-397 doi:Journal of Fish Biology

Ji K, Kim Y, Oh S, Ahn B, Jo H, Choi K (2008) Toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid on freshwater macroinvertebrates (Daphnia magna and Moina macrocopa) and fish (Oryzias latipes). Environmental toxicology and chemistry / SETAC 27(10):2159-68 doi:10.1897/07-523.1

Jin W, Zhang J, Lou D, Chavkin C, Xu M (2002) C-fos-deficient mouse hippocampal CA3 pyramidal neurons exhibit both enhanced basal and kainic acid-induced excitability. Neuroscience letters 331(3):151-4

Johansson N, Eriksson P, Viberg H (2009) Neonatal exposure to PFOS and PFOA in mice results in changes in proteins which are important for neuronal growth and synaptogenesis in the developing brain. Toxicological sciences : an official journal of the Society of Toxicology 108(2):412-8 doi:10.1093/toxsci/kfp029

Kalueff AV, Gebhardt M, Stewart AM, et al. (2013) Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10(1):70-86 doi:10.1089/zeb.2012.0861

Kannan K, Newsted J, Halbrook RS, Giesy JP (2002) Perfluorooctanesulfonate and related fluorinated hydrocarbons in mink and river otters from the United States. Environmental science & technology 36(12):2566-71

Kannan K, Tao L, Sinclair E, Pastva SD, Jude DJ, Giesy JP (2005) Perfluorinated compounds in aquatic organisms at various trophic levels in a Great Lakes food chain. Archives of environmental contamination and toxicology 48(4):559-66 doi:10.1007/s00244-004-0133-x

Kantheti P, Qiao X, Diaz ME, et al. (1998) Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 21(1):111-22

Kato K, Wong L-Y, Jia LT, Kuklenyik Z, Calafat AM (2011) Trends in Exposure to Polyfluoroalkyl Chemicals in the U.S. Population: 1999−2008. Environmental science & technology 45(19):8037-8045 doi:10.1021/es1043613

Kim CH, Oda T, Itoh M, et al. (2000) Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. Nature 407(6806):913-6 doi:10.1038/35038097

Kim SK, Kannan K (2007) Perfluorinated acids in air, rain, snow, surface runoff, and lakes: relative importance of pathways to contamination of urban lakes. Environmental science & technology 41(24):8328-34

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Developmental dynamics : an official publication of the American Association of Anatomists 203(3):253-310 doi:10.1002/aja.1002030302

Klaassen CD, Aleksunes LM (2010) Xenobiotic, bile acid, and cholesterol transporters: function and regulation. Pharmacological reviews 62(1):1-96 doi:10.1124/pr.109.002014

Kohli G, Hu S, Clelland E, Di Muccio T, Rothenstein J, Peng C (2003) Cloning of transforming growth factor-beta 1 (TGF-beta 1) and its type II receptor from zebrafish ovary and role of TGF-beta 1 in oocyte maturation. Endocrinology 144(5):1931-41 doi:10.1210/en.2002-0126

Kudo N, Kawashima Y (2003) Toxicity and toxicokinetics of perfluorooctanoic acid in humans and animals. The Journal of toxicological sciences 28(2):49-57

Kudo N, Suzuki E, Katakura M, Ohmori K, Noshiro R, Kawashima Y (2001) Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. Chemico-biological interactions 134(2):203-16

Kumar KS, Zushi Y, Masunaga S, Gilligan M, Pride C, Sajwan K (2008) Perfluorinated organic compounds in sediment, aquatic animals including shark from Georgia coast, USA. Organohalogen Comp 70

Laan M, Richmond H, He C, Campbell RK (2002) Zebrafish as a model for vertebrate reproduction: characterization of the first functional zebrafish (Danio rerio) gonadotropin receptor. General and comparative endocrinology 125(3):349-64 doi:10.1006/gcen.2001.7738

Lambert WM, Xu CF, Neubert TA, Chao MV, Garabedian MJ, Jeanneteau FD (2013) Brain-derived neurotrophic factor signaling rewrites the glucocorticoid transcriptome via glucocorticoid receptor phosphorylation. Molecular and cellular biology 33(18):3700-14 doi:10.1128/MCB.00150-13

Lau C (2012) Perfluorinated compounds. Exs 101:47-86 doi:10.1007/978-3-7643-8340-4_3

Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J (2007) Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicological sciences : an official journal of the Society of Toxicology 99(2):366-94 doi:10.1093/toxsci/kfm128

Lau C, Thibodeaux JR, Hanson RG, et al. (2006) Effects of perfluorooctanoic acid exposure during pregnancy in the mouse. Toxicological sciences : an official journal of the Society of Toxicology 90(2):510-8 doi:10.1093/toxsci/kfj105

Lee JJ, Schultz IR (2010) Sex differences in the uptake and disposition of perfluorooctanoic acid in fathead minnows after oral dosing. Environmental science & technology 44(1):491-6 doi:10.1021/es901838y

Lehmler HJ (2005) Synthesis of environmentally relevant fluorinated surfactants--a review. Chemosphere 58(11):1471-96 doi:10.1016/j.chemosphere.2004.11.078

Li MH (2009) Toxicity of perfluorooctane sulfonate and perfluorooctanoic acid to plants and aquatic invertebrates. Environmental toxicology 24(1):95-101 doi:10.1002/tox.20396

Li MO, Flavell RA (2008) TGF-beta: a master of all T cell trades. Cell 134(3):392-404 doi:10.1016/j.cell.2008.07.025

Lieschke GJ, Currie PD (2007) Animal models of human disease: zebrafish swim into view. Nature reviews Genetics 8(5):353-67 doi:10.1038/nrg2091

Liew WC, Orban L (2014) Zebrafish sex: a complicated affair. Briefings in functional genomics 13(2):172-87 doi:10.1093/bfgp/elt041

Lindstrom AB, Strynar MJ, Libelo EL (2011) Polyfluorinated compounds: past, present, and future. Environmental science & technology 45(19):7954-61 doi:10.1021/es2011622

Liu C, Du Y, Zhou B (2007) Evaluation of estrogenic activities and mechanism of action of perfluorinated chemicals determined by vitellogenin induction in primary cultured tilapia hepatocytes. Aquatic toxicology 85(4):267-77 doi:10.1016/j.aquatox.2007.09.009

Liu W, Yang B, Wu L, et al. (2015) Involvement of NRF2 in Perfluorooctanoic Acid-Induced Testicular Damage in Male Mice. Biology of reproduction 93(2):41 doi:10.1095/biolreprod.115.128819

Liu Y, Wang J, Fang X, Zhang H, Dai J (2011) The thyroid-disrupting effects of long-term perfluorononanoate exposure on zebrafish (Danio rerio). Ecotoxicology 20(1):47-55 doi:10.1007/s10646-010-0555-3

Maack G, Segner, H. (2003) Morphological development of the gonads in zebrafish. Journal of Fish Biology 62(4):11

Mattsson A, Karrman A, Pinto R, Brunstrom B (2015) Metabolic Profiling of Chicken Embryos Exposed to Perfluorooctanoic Acid (PFOA) and Agonists to Peroxisome Proliferator-Activated Receptors. PloS one 10(12):e0143780 doi:10.1371/journal.pone.0143780

Miyake Y, Kobayashi T, Kameya T, Managaki S, Amagai T, Masunaga S (2014) Comparison study on observed and estimated concentrations of perfluorooctane sulfonate using a fate model in Tokyo Bay of Japan. Journal of environmental science and health Part A, Toxic/hazardous substances & environmental engineering 49(7):770-6 doi:10.1080/10934529.2014.882192

Montpetit A, Cote S, Brustein E, et al. (2008) Disruption of AP1S1, causing a novel neurocutaneous syndrome, perturbs development of the skin and spinal cord. PLoS genetics 4(12):e1000296 doi:10.1371/journal.pgen.1000296

Moody CA, Hebert GN, Strauss SH, Field JA (2003) Occurrence and persistence of perfluorooctanesulfonate and other perfluorinated surfactants in groundwater at a fire-training area at Wurtsmith Air Force Base, Michigan, USA. Journal of environmental monitoring : JEM 5(2):341-5

Mossner R, Daniel S, Albert D, et al. (2000) Serotonin transporter function is modulated by brain-derived neurotrophic factor (BDNF) but not nerve growth factor (NGF). Neurochemistry international 36(3):197-202

Muir M, Romalo G, Wolf L, Elger W, Schweikert HU (2004) Estrone sulfate is a major source of local estrogen formation in human bone. The Journal of clinical endocrinology and metabolism 89(9):4685-92 doi:10.1210/jc.2004-0049

Murakami M, Shinohara H, Takada H (2009) Evaluation of wastewater and street runoff as sources of perfluorinated surfactants (PFSs). Chemosphere 74(4):487-93 doi:10.1016/j.chemosphere.2008.10.018

Naile JE, Garrison AW, Avants JK, Washington JW (2016) Isomers/enantiomers of perfluorocarboxylic acids: Method development and detection in environmental samples. Chemosphere 144:1722-8 doi:10.1016/j.chemosphere.2015.10.075

Nakatsu F, Okada M, Mori F, et al. (2004) Defective function of GABA-containing synaptic vesicles in mice lacking the AP-3B clathrin adaptor. The Journal of cell biology 167(2):293-302 doi:10.1083/jcb.200405032

Nakayama SF, Strynar MJ, Reiner JL, Delinsky AD, Lindstrom AB (2010) Determination of perfluorinated compounds in the upper Mississippi river basin. Environmental science & technology 44(11):4103-9 doi:10.1021/es100382z

Nasiadka A, Clark MD (2012) Zebrafish Breeding in the Laboratory Environment. ILAR Journal 53(2):161-168 doi:10.1093/ilar.53.2.161

Norton WH, Stumpenhorst K, Faus-Kessler T, et al. (2011) Modulation of Fgfr1a signaling in zebrafish reveals a genetic basis for the aggression-boldness syndrome. The Journal of neuroscience : the official journal of the Society for Neuroscience 31(39):13796-807 doi:10.1523/JNEUROSCI.2892-11.2011

OECD. (2011) Test No. 212: Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages. OECD Publishing

Ohno H (2006) Physiological roles of clathrin adaptor AP complexes: lessons from mutant animals. Journal of biochemistry 139(6):943-8 doi:10.1093/jb/mvj120

Olsen GW, Hansen KJ, Stevenson LA, Burris JM, Mandel JH (2003) Human donor liver and serum concentrations of perfluorooctanesulfonate and other perfluorochemicals. Environmental science & technology 37(5):888-91

Onishchenko N, Fischer C, Wan Ibrahim WN, et al. (2011) Prenatal exposure to PFOS or PFOA alters motor function in mice in a sex-related manner. Neurotoxicity research 19(3):452-61 doi:10.1007/s12640-010-9200-4

Orn S, Andersson PL, Forlin L, Tysklind M, Norrgren L (1998) The impact on reproduction of an orally administered mixture of selected PCBs in zebrafish (Danio rerio). Archives of environmental contamination and toxicology 35(1):52-7

Paull GC, Filby AL, Giddins HG, Coe TS, Hamilton PB, Tyler CR (2010) Dominance hierarchies in zebrafish (Danio rerio) and their relationship with reproductive success. Zebrafish 7(1):109-17 doi:10.1089/zeb.2009.0618

Pavlidis M, Theodoridi A, Tsalafouta A (2015) Neuroendocrine regulation of the stress response in adult zebrafish, Danio rerio. Progress in neuro-psychopharmacology & biological psychiatry 60:121-31 doi:10.1016/j.pnpbp.2015.02.014

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29(9):e45

Popovic M, Zaja R, Fent K, Smital T (2014) Interaction of environmental contaminants with zebrafish organic anion transporting polypeptide, Oatp1d1 (Slco1d1). Toxicology and applied pharmacology 280(1):149-58 doi:10.1016/j.taap.2014.07.015

Post GB, Cohn PD, Cooper KR (2012) Perfluorooctanoic acid (PFOA), an emerging drinking water contaminant: a critical review of recent literature. Environmental research 116:93-117 doi:10.1016/j.envres.2012.03.007

Post GB, Louis JB, Lippincott RL, Procopio NA (2013) Occurrence of perfluorinated compounds in raw water from New Jersey public drinking water systems. Environmental science & technology 47(23):13266-75 doi:10.1021/es402884x

Power DM, Llewellyn L, Faustino M, et al. (2001) Thyroid hormones in growth and development of fish. Comparative biochemistry and physiology Toxicology & pharmacology : CBP 130(4):447-59

Reference Genome Group of the Gene Ontology C (2009) The Gene Ontology's Reference Genome Project: a unified framework for functional annotation across species. PLoS computational biology 5(7):e1000431 doi:10.1371/journal.pcbi.1000431

Renner R (2001) Growing concern over perfluorinated chemicals. Environmental science & technology 35(7):154A-160A

Rosen MB, Schmid JR, Corton JC, et al. (2010) Gene Expression Profiling in Wild-Type and PPARalpha-Null Mice Exposed to Perfluorooctane Sulfonate Reveals PPARalpha-Independent Effects. PPAR research 2010 doi:10.1155/2010/794739

Schiavone A, Corsolini S, Kannan K, et al. (2009) Perfluorinated contaminants in fur seal pups and penguin eggs from South Shetland, Antarctica. The Science of the total environment 407(12):3899-904 doi:10.1016/j.scitotenv.2008.12.058

Schnorr SJ, Steenbergen PJ, Richardson MK, Champagne DL (2012) Measuring thigmotaxis in larval zebrafish. Behavioural brain research 228(2):367-74 doi:10.1016/j.bbr.2011.12.016

Seacat AM, Thomford PJ, Hansen KJ, et al. (2003) Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. Toxicology 183(1-3):117-31

Seacat AM, Thomford PJ, Hansen KJ, Olsen GW, Case MT, Butenhoff JL (2002) Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. Toxicological sciences : an official journal of the Society of Toxicology 68(1):249-64

Serra EL, Medalha CC, Mattioli R (1999) Natural preference of zebrafish (Danio rerio) for a dark environment. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] 32(12):1551-3

Services MDoHaH (2013) Former Wurtsmith AFB Public Meeting Presentation by the Michigan Department of Community Health, January 24, 2013. http://www.michigan.gov, http://www.michigan.gov/documents/mdch/Wurtsmith_Air_Force_Base_Public_Mtg_MDCH__012413_compressed_410340_7.pdf

Seth A, Stemple DL, Barroso I (2013) The emerging use of zebrafish to model metabolic disease. Disease models & mechanisms 6(5):1080-8 doi:10.1242/dmm.011346

Sharma S, Coombs S, Patton P, Burt de Perera T (2009) The function of wall-following behaviors in the Mexican blind cavefish and a sighted relative, the Mexican tetra (Astyanax). Journal of comparative physiology A, Neuroethology, sensory, neural, and behavioral physiology 195(3):225-40 doi:10.1007/s00359-008-0400-9

Shi Y, Pan Y, Yang R, Wang Y, Cai Y (2010) Occurrence of perfluorinated compounds in fish from Qinghai-Tibetan Plateau. Environment international 36(1):46-50 doi:10.1016/j.envint.2009.09.005

Simon P, Dupuis R, Costentin J (1994) Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behavioural brain research 61(1):59-64

Sinclair E, Mayack DT, Roblee K, Yamashita N, Kannan K (2006) Occurrence of perfluoroalkyl surfactants in water, fish, and birds from New York State. Archives of environmental contamination and toxicology 50(3):398-410 doi:10.1007/s00244-005-1188-z

Skinner AMJ, Watt PJ (2007) Strategic egg allocation in the zebra fish, Danio rerio. Behavioral Ecology 18(5):905-909 doi:10.1093/beheco/arm059

Society AC, DC NMtW, Halden RU, American Chemical Society. Division of Environmental Chemistry., American Chemical Society. Contaminants of

emerging concern in the environment : ecological and human health considerations. In: ACS symposium series, Washington DC

New York, 2010. American Chemical Society ;

Distributed by Oxford University Press, p xiii, 606 p., 16 p. of plates

Spulber S, Kilian P, Wan Ibrahim WN, et al. (2014) PFOS induces behavioral alterations, including spontaneous hyperactivity that is corrected by dexamfetamine in zebrafish larvae. PloS one 9(4):e94227 doi:10.1371/journal.pone.0094227

Steenland K, Fletcher T, Savitz DA (2010) Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environmental health perspectives 118(8):1100-8 doi:10.1289/ehp.0901827

Steenland K, Zhao L, Winquist A (2015) A cohort incidence study of workers exposed to perfluorooctanoic acid (PFOA). Occupational and environmental medicine 72(5):373-80 doi:10.1136/oemed-2014-102364

Stephenson JF, Whitlock KE, Partridge JC (2011) Zebrafish preference for light or dark is dependent on ambient light levels and olfactory stimulation. Zebrafish 8(1):17-22 doi:10.1089/zeb.2010.0671

Svoboda M, Riha J, Wlcek K, Jaeger W, Thalhammer T (2011) Organic anion transporting polypeptides (OATPs): regulation of expression and function. Current drug metabolism 12(2):139-53

Tarpey PS, Stevens C, Teague J, et al. (2006) Mutations in the gene encoding the Sigma 2 subunit of the adaptor protein 1 complex, AP1S2, cause X-linked mental retardation. American journal of human genetics 79(6):1119-24 doi:10.1086/510137

Toms LM, Thompson J, Rotander A, et al. (2014) Decline in perfluorooctane sulfonate and perfluorooctanoate serum concentrations in an Australian population from 2002 to 2011. Environment international 71:74-80 doi:10.1016/j.envint.2014.05.019

Tomy GT, Tittlemier SA, Palace VP, et al. (2004) Biotransformation of N-ethyl perfluorooctanesulfonamide by rainbow trout (Onchorhynchus mykiss) liver microsomes. Environmental science & technology 38(3):758-62

Treit D, Fundytus M (1988) Thigmotaxis as a test for anxiolytic activity in rats. Pharmacology, biochemistry, and behavior 31(4):959-62

Uchida D, Yamashita M, Kitano T, Iguchi T (2002) Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. The Journal of experimental biology 205(Pt 6):711-8

Vassiliadou I, Costopoulou D, Ferderigou A, Leondiadis L (2010) Levels of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) in blood samples from different groups of adults living in Greece. Chemosphere 80(10):1199-206 doi:10.1016/j.chemosphere.2010.06.014

Verrecchia F, Mauviel A (2002) Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. The Journal of investigative dermatology 118(2):211-5 doi:10.1046/j.1523-1747.2002.01641.x

Walker MB, Kimmel CB (2007) A two-color acid-free cartilage and bone stain for zebrafish larvae. Biotechnic & histochemistry : official publication of the Biological Stain Commission 82(1):23-8 doi:10.1080/10520290701333558

Walz N, Muhlberger A, Pauli P (2015) A Human Open Field Test Reveals Thigmotaxis Related to Agoraphobic Fear. Biological psychiatry doi:10.1016/j.biopsych.2015.12.016

Wang J, Zhu X, Zhang X, et al. (2011) Disruption of zebrafish (Danio rerio) reproduction upon chronic exposure to TiO(2) nanoparticles. Chemosphere 83(4):461-7 doi:10.1016/j.chemosphere.2010.12.069

Wang L, Wang Y, Liang Y, et al. (2014) PFOS induced lipid metabolism disturbances in BALB/c mice through inhibition of low density lipoproteins excretion. Scientific reports 4:4582 doi:10.1038/srep04582

Wei Y, Liu Y, Wang J, Tao Y, Dai J (2008) Toxicogenomic analysis of the hepatic effects of perfluorooctanoic acid on rare minnows (Gobiocypris rarus). Toxicology and applied pharmacology 226(3):285-97 doi:10.1016/j.taap.2007.09.023

White SS, Fenton SE, Hines EP (2011) Endocrine disrupting properties of perfluorooctanoic acid. The Journal of steroid biochemistry and molecular biology 127(1-2):16-26 doi:10.1016/j.jsbmb.2011.03.011

Xing C, Gong B, Xue Y, et al. (2015) TGFbeta1a regulates zebrafish posterior lateral line formation via Smad5 mediated pathway. Journal of molecular cell biology 7(1):48-61 doi:10.1093/jmcb/mjv004

Yahia D, El-Nasser MA, Abedel-Latif M, et al. (2010) Effects of perfluorooctanoic acid (PFOA) exposure to pregnant mice on reproduction. The Journal of toxicological sciences 35(4):527-33

Yamada K, Nabeshima T (2003) Brain-derived neurotrophic factor/TrkB signaling in memory processes. Journal of pharmacological sciences 91(4):267-70

Yamashita N, Kannan K, Taniyasu S, Horii Y, Petrick G, Gamo T (2005) A global survey of perfluorinated acids in oceans. Marine pollution bulletin 51(8-12):658-68 doi:10.1016/j.marpolbul.2005.04.026

Yanase K, Herbert NA, Montgomery JC (2012) Disrupted flow sensing impairs hydrodynamic performance and increases the metabolic cost of swimming in the yellowtail kingfish, Seriola lalandi. The Journal of experimental biology 215(Pt 22):3944-54 doi:10.1242/jeb.073437

Yang CH, Glover KP, Han X (2010) Characterization of cellular uptake of perfluorooctanoate via organic anion-transporting polypeptide 1A2, organic anion transporter 4, and urate transporter 1 for their potential roles in mediating human renal reabsorption of perfluorocarboxylates. Toxicological sciences : an official journal of the Society of Toxicology 117(2):294-302 doi:10.1093/toxsci/kfq219

Yang Q, Abedi-Valugerdi M, Xie Y, et al. (2002) Potent suppression of the adaptive immune response in mice upon dietary exposure to the potent peroxisome proliferator, perfluorooctanoic acid. International immunopharmacology 2(2-3):389-97

Yoo H, Yamashita N, Taniyasu S, et al. (2009) Perfluoroalkyl acids in marine organisms from Lake Shihwa, Korea. Archives of environmental contamination and toxicology 57(3):552-60 doi:10.1007/s00244-008-9282-7

Zhang W, Liu Y, Zhang H, Dai J (2012) Proteomic analysis of male zebrafish livers chronically exposed to perfluorononanoic acid. Environment international 42:20-30 doi:http://dx.doi.org/10.1016/j.envint.2011.03.002

Zhang Y, Beesoon S, Zhu L, Martin JW (2013) Biomonitoring of perfluoroalkyl acids in human urine and estimates of biological half-life. Environmental science & technology 47(18):10619-27 doi:10.1021/es401905e

Zheng XM, Liu HL, Shi W, Wei S, Giesy JP, Yu HX (2011) Effects of perfluorinated compounds on development of zebrafish embryos. Environmental science and pollution research international 19(7):2498-505 doi:10.1007/s11356-012-0977-y