



# Developmental exposures to perfluorooctanesulfonic acid (PFOS) impact embryonic nutrition, pancreatic morphology, and adiposity in the zebrafish, *Danio rerio*<sup>☆</sup>



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## ABSTRACT

Perfluorooctanesulfonic acid (PFOS) is a persistent environmental contaminant previously found in consumer surfactants and industrial fire-fighting foams. PFOS has been widely implicated in metabolic dysfunction across the lifespan, including diabetes and obesity. However, the contributions of the embryonic environment to metabolic disease remain uncharacterized. This study seeks to identify perturbations in embryonic metabolism, pancreas development, and adiposity due to developmental and subchronic PFOS exposures and their persistence into later larval and juvenile periods. Zebrafish embryos were exposed to 16 or 32  $\mu$ M PFOS developmentally (1–5 days post fertilization; dpf) or subchronically (1–15 dpf). Embryonic fatty acid and macronutrient concentrations and expression of peroxisome proliferator-activated receptor (PPAR) isoforms were quantified in embryos. Pancreatic islet morphometry was assessed at 15 and 30 dpf, and adiposity and fish behavior were assessed at 15 dpf. Concentrations of lauric (C12:0) and myristic (C14:0) saturated fatty acids were increased by PFOS at 4 dpf, and PPAR gene expression was reduced. Incidence of aberrant islet morphologies, principal islet areas, and adiposity were increased in 15 dpf larvae and 30 dpf juvenile fish. Together, these data suggest that the embryonic period is a susceptible window of metabolic programming in response to PFOS exposures, and that these early exposures alone can have persisting effects later in the lifecourse.

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## 1. Introduction

Perfluorooctanesulfonic acid (PFOS) is an anthropogenic chemical used in the manufacture of surfactants used for household, food, and industrial applications, as well as in aqueous film forming foam (AFFF) (Annunziato et al., 2020). PFOS was voluntarily phased out of production in the United States in 2002, but is still utilized globally. PFOS is readily detected in global waters with concentrations in the ng/L– $\mu$ g/L range for surface waters and up into the mg/L range in some industrial waste waters (Chen et al., 2012; Oliaei et al., 2013; reviewed in Banzhaf et al., 2017; Nakayama et al.,

2005; Zareitalabad et al., 2013). Epidemiological evidence from the Nutritional Health and Examination Survey (NHANES) showed that PFOS was detected in >98% of blood samples, with average serum concentrations peaking at 30.4 ng/ml in 2000 (Calafat et al., 2007a). Concentrations of PFOS in U.S. serum have been steadily decreasing since phase out. American Red Cross serum PFOS concentrations decreased by 60% between 2001 and 2006 (Olsen et al., 2008), and concentrations for participants of the NHANES decreased by 35% between 1999 and 2004 (Calafat et al., 2007b) and another 76% by 2014 (Calafat et al., 2019; Dong et al., 2019). Despite these improvements, PFOS is still a major environmental concern due to its persistence biologically and in the environment, with a half-life ranging from 3.4 to 21.6 years in humans based upon severity of exposure (Fu et al., 2016; Li et al., 2018; Olsen et al., 2007; Zhang et al., 2013), and 8.4–86.8 days in fish (Consoer et al., 2016; Falk et al., 2015; Martin et al., 2003a, b). PFOS has been found

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in various human tissues, including the pancreas, liver, and adipose tissue (Maestri et al., 2006). Likewise, PFOS has been found in cord blood samples, demonstrating an ability to cross the placenta during pregnancy and inflict fetal exposures (Inoue et al., 2004).

PFOS exposures in humans have been widely associated with metabolic dysfunction (reviewed in Qi et al., 2020), defined as a suite of symptoms characterized by at least 3 pathologies: hyperglycemia, hypertension, elevated triglycerides, large waist circumference, and low HDL and high LDL cholesterol (Alberti et al., 2005). Prevalence of metabolic syndrome has been increasing in recent decades, estimated to occur in more than 1/3 of the United States population (Moore et al., 2017). Behavioral factors such as diet and exercise largely contribute to metabolic syndrome, but cannot alone predict disease trends and demographics. A growing number of studies have assessed the contributions of the environment to diabetes (reviewed in Dendup et al., 2018; Knip et al., 2005; Kolb and Martin, 2017), obesity (reviewed in Barness et al., 2007; Hill and Peters, 1998), and metabolic syndrome (Benyamin et al., 2007). Epidemiological studies have shown that human serum PFOS concentrations were positively associated with cholesterol, blood insulin, insulin resistance, and  $\beta$  cell dysfunction (Dong et al., 2019; Lin et al., 2009; Nelson et al., 2010; Liu et al., 2018), and these findings have also been supported by rodent models (Lv et al., 2013; Wan et al., 2014). The embryonic, fetal, and neonatal environments are some of the most dynamic and plastic windows of the lifespan, giving rise to potential windows of susceptibility to chemical insults. The developmental environment has been increasingly scrutinized for its contributions to the developmental origins of obesity, diabetes, and metabolic syndrome (reviewed in Gluckman and Hanson, 2004; Inadera, 2013; Janesick and Blumberg, 2011; Newbold et al., 2007). However, the majority of this research has been largely associative, and therefore there is more need for mechanistic and controlled studies to better understand these very complex relationships.

The pancreas is a master-regulator of metabolic function, including processes such as lipid, carbohydrate, and protein metabolism. The exocrine pancreas constitutes the majority of the organ mass, primarily responsible for secretion of digestive enzymes. The endocrine Islets of Langerhans, though a small portion of the total pancreas mass, are responsible for nutrient sensing and synthesis of hormones and peptides engaged in glucoregulatory function. Beta ( $\beta$ ) cells, comprising almost 80% of the islet mass, are the sole cells in the body responsible for insulin production and secretion. Therefore,  $\beta$  cells are the primary pathological targets of diabetic studies, and their size, architecture, and integrity are important for metabolic health. In addition to glucoregulatory function,  $\beta$  cells are very sensitive to other compounds such as saturated fatty acids, which can induce  $\beta$  cell toxicity and cell death (Oh et al., 2018; Nemezc et al., 2019). Numerous studies have found associations between xenobiotic exposures and abnormal pancreatic function or diabetes (Lee et al., 2011; Lind et al., 2014; reviewed in Fabricio et al., 2016; Fénichel and Chevalier, 2017). We have previously demonstrated that several environmental toxicants can disrupt pancreatic organogenesis in zebrafish, decreasing gene expression of pancreatic hormones and digestive enzymes and altering pancreas structure during the early lifespan (Brown et al., 2018; Jacobs et al., 2018; Sant et al., 2017, 2018b; Timme-Laragy et al., 2015). The persistence of these phenotypes and physiological consequences are of particular interest to better understand the relationship between chemical insults and metabolic syndrome.

We previously examined the relationship between embryonic PFOS exposures and pancreatic organogenesis (Sant et al., 2017). We found that PFOS exposures decreased islet area and overall pancreas size, while decreasing gene expression of numerous

digestive enzymes and glucoregulatory hormones in embryonic zebrafish. In a follow-up study, we also found that embryonic PFOS exposures impacted gene expression within the Peroxisome proliferator-activated receptor (PPAR) pathway—a major regulatory pathway involved in nutrient sensing, metabolism, and storage (Sant et al., 2018a). Though these studies collectively showed that PFOS exposures could impact early metabolic function, these endpoints were only experimentally assessed during the eleuther-oembryo (4 days post fertilization; dpf) period, prior to the onset of the first adipocytes between 8 and 10 dpf (Flynn et al., 2009). The Developmental Origins of Health and Disease (DOHaD) paradigm has been repeatedly applied to health studies where developmental exposures and nutritional deficits have produced lasting health consequences into adulthood, including metabolic outcomes such as diabetes, obesity, and cardiovascular disease (reviewed in Barker, 2007; Heindel et al., 2015). Therefore, our earlier studies demonstrating the deleterious effects on the pancreas due to PFOS exposures could have more lasting consequences throughout the lifespan. Here, we expand our observations to characterize the longitudinal and pathological changes associated with these early life PFOS exposures.

The goal of this study was to identify perturbations in embryonic metabolism, pancreas development, and adiposity due to developmental and subchronic PFOS exposures. This work expands upon previous work, examining pathological consequences of exposures during the mid-larval and juvenile periods in order to assess the persistence of the early effects to later in the lifecourse. We hypothesized that PFOS exposures would increase lipid concentrations in embryos and larvae, and that the previously observed decreases in pancreatic islet areas would persist into the larval and juvenile periods.

## 2. Methods

### 2.1. Chemicals & reagents

Heptadecafluorooctanesulfonic acid (synonym for perfluorooctanesulfonic acid; PFOS; Cat. #:77,283) and hydrogen chloride – methanol solution (Cat. #: 17,935) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), glycerol, glucose, cholesterol, methanolic hydrogen chloride, GC grade hexane (Cat. #H307), 4% para-formaldehyde, and potassium chloride were purchased from Fisher Scientific (Pittsburgh, PA). Stock solutions of 0 (100% DMSO), 160 mM, and 320 mM PFOS were prepared in DMSO for embryonic exposures, and stored in amber vials in a light-prohibitive container at room temperature. All experimental procedures were performed using the appropriate personal protective equipment, and all chemicals were disposed in strict accordance to local and federal regulations.

### 2.2. Zebrafish strains

Wildtype AB zebrafish were originally obtained from Boston Children's Hospital (Boston, MA). Transgenic *Tg(insulin:GFP)* fish were originally obtained as a heterozygous population on an AB background from Dr. Philip dilorio at the University of Massachusetts Medical School (Worcester, MA). Populations of the *Tg(insulin:GFP)* fish maintained in the Timme-Laragy laboratory were bred to homozygosity prior to the initiation of this study. The *Tg(insulin:GFP)* strain of zebrafish expresses green fluorescent protein in the insulin-producing  $\beta$  cells of the endocrine pancreas, allowing for direct visualization of  $\beta$  cells *in vivo*.

### 2.3. Zebrafish husbandry

Adult fish were maintained as a breeding population in the Timme-Laragy laboratory in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with approval and regular inspection from the University of Massachusetts Amherst Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3551-01). Adult fish were housed in an Aquaneering (San Diego, CA) automated zebrafish system, maintained at 28.5 °C in a 14 h light:10 h dark daily cycle. Breeding populations were housed in tanks containing approximately 30 females:15 males, and provided the recommended amount of GEMMA Micro 300 zebrafish diet (Skretting; Westbrook, ME; 59% protein/14% oil/14% ash) only daily in the AM. Embryos for experimentation were collected from breeding tanks <1 h post fertilization (hpf), washed gently, and transferred to clean 100 mm polystyrene petri dishes containing 0.3X Danieau's medium (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO<sub>4</sub>, 1.8 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.5 mM HEPES, pH 7.6) (Westerfield, 2000).

### 2.4. Exposures

#### 2.4.1. Embryonic exposures for biochemical analyses

Exposures to either 0 (0.01% v/v DMSO), 16, or 32 μM PFOS (matched volumes) PFOS were performed. These concentrations are supra-environmental, though concentrations may be consistent with industrial wastewater runoffs, and originally selected due to other studies in the zebrafish model (Chen et al., 2014; Zheng et al., 2012). These concentrations were previously utilized in our other studies and shown to impact pancreatic development and disrupt metabolic pathways including PPAR signaling while minimizing gross morphological defects (Sant et al., 2017; 2018a). The purpose of this study was to examine the persistence of our previously observed phenotypes, and therefore the same exposure concentrations were utilized. The highest concentration (64 μM PFOS) from our previous studies was not continued because of decreased survival to 15 and 30 dpf.

Wild-type (AB) embryos were microscopically examined at 3 hpf to confirm fertilization and viability (normal, symmetrical development into the blastula stage). Successful embryos were randomized and individually transferred to wells of 24-well polystyrene plates, and exposure media was prepared daily in 0.3X Danieau's medium prior to this transfer. One ml of the assigned exposure medium was added to each well containing an embryo. Exposure media was refreshed daily to model subchronic exposures throughout development, and embryos were maintained in a dedicated incubator at 28.5 °C. Experiments were repeated 3 times each for the biochemical assays and fatty acid analysis, each with 4–6 samples per exposure group each containing 15 pooled embryos.

At 4 dpf, larvae were rinsed thoroughly and transferred to 1.5 ml polypropylene microcentrifuge tubes. For protein, cholesterol, glucose, and triglyceride quantification, 15 larvae were pooled per sample, all remaining liquid was removed, and 160 μl of 0.05% Tween 20 was added to each tube. For fatty acid analysis, 20 larvae were pooled per sample and all liquid was removed from each tube. All samples were stored at –80 °C until analysis.

#### 2.4.2. Developmental and subchronic exposures for pancreatic morphology & adiposity assessments

Transgenic *Tg(insulin:GFP)* embryos and wild-type (AB) embryos were used for experiments measuring pancreatic islet morphology and adiposity, respectively. For both experiments, embryos were manually dechorionated at 1 dpf using watchmakers' forceps.

Embryos were separated into the 3 exposure concentrations (0, 16, and 32 μM PFOS), and each group was subdivided into two exposure paradigms (developmentally-exposed or subchronically exposed). Embryos were individually transferred to wells of 24-well polystyrene plates, and exposure media was refreshed daily as described in section 2.3.1. These waterborne exposures were maintained until 5 dpf, when larvae were individually transferred to borosilicate glass 100 ml beakers. For developmentally-exposed fish, larvae were transferred into fresh 0.3X Danieau's medium, and the exposures were discontinued. For subchronically-exposed fish, waterborne exposures continued and were refreshed daily until imaging timepoints at 15 and 30 dpf. All beakers were maintained at 50 ml media volumes, with 50% volume refreshed daily. Beginning at 5 dpf, larvae were provided with GEMMA 75 larval zebrafish diet (Skretting) and allowed to feed *ad libitum*. Larval survival was monitored for each experiment (through either 15 or 30 dpf) and was >80% for each exposure group.

#### 2.5. Assessment of larval protein, cholesterol, glucose, and triglyceride concentrations

Total larval concentrations of protein, cholesterol, glucose, and triglyceride were each quantified on pooled samples of 20 larvae (4 dpf) exposed to 0 (DMSO), 16, or 32 μM PFOS using commercially available colorimetric kits. Detailed methods are included in the Supplemental Information.

#### 2.6. Larval fatty acid analysis

Lipids were extracted from zebrafish samples according to the Folch method (Folch et al., 1957) and submitted to transmethylation of the fatty acids using hydrogen chloride in methanol, according to Park's method with slight modifications (Park et al., 2001). Total larval concentrations fatty acid species were each quantified on pooled samples of 20 larvae (4 dpf) exposed to 0 (DMSO), 16, or 32 μM PFOS. Samples were sonicated using a Branson SFX250 Sonifier equipped with a microtip extender and homogenates extracted chloroform/methanol mixture (2:1). Fatty acid methyl esters (FAMES) were prepared by 3 N methanolic hydrogen chloride. FAMES were analyzed on GC–MS–QP2010 SE (Shimadzu, Japan) using a SUPELLOWAX™ 10 column (100 m Å–0.25 mm i. d., 0.25 μm film thickness, Sigma Aldrich, USA). Helium was used as the carrier gas (inlet pressure, 210 kPa). The column oven temperature started at 50 °C, increased to 200 °C at 20 °C/min and then to 220 °C at 2 °C/min and finally held at 220 °C for 162.50 min. The injector and the detector were maintained at 250 °C. The fatty acids were identified by comparison of their retention times with the standards and confirmed by searching the National Institute of Standards and Technology (NIST) mass spectrum library. Data were collected from 4 to 5 replicates and reported as a percentage of the total fatty acids.

#### 2.7. Gene expression of peroxisome proliferator-activated receptors (PPARs)

RNA isolation, reverse transcription, and quantitative PCR were performed as previously described (Sant et al., 2017; 2018a). Briefly, RNA was isolated from 96 hpf larvae using the GeneJET RNA Purification Kit (Fisher Scientific), following manufacturer instructions. RNA concentrations were quantified using a BioDrop μLITE spectrophotometer (BioDrop; Cambridge, UK), and 1 μg of RNA was reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad; Hercules, CA). cDNA was diluted to working stocks of 0.25 ng/μl, and was stored at –20 °C until use. Quantitative PCR was performed using the CFX Connect Real-Time PCR Detection System

and iQ SYBR Green Supermix (Bio-Rad). Gene expression of *pparaa*, *pparbb*, and *pparg* were assessed using the  $\Delta\Delta\text{CT}$  model, utilizing the arithmetic mean of two housekeeping genes: *actb* and *b2m*. Primer sequences and  $T_m$  information are provided (Table S1). Each sample contained 10 pooled embryos, and 4–5 samples were collected per exposure.

## 2.8. Analysis of islet morphometry and growth during the mid-larval and juvenile stages

Morphology of the  $\beta$  cell cluster within the primary Islet of Langerhaans was quantified in *Tg(insulin:GFP)* larval and juvenile zebrafish using confocal microscopy. At the conclusion of the exposure period on 15 or 30 dpf (detailed in section 2.4.2), zebrafish were gently washed in fresh 0.3X Danieau's, anaesthetized, and low-magnification (20X) images were taken to measure fish length. Once anaesthetized and measured, fish were preserved in 4% paraformaldehyde. Preserved fish were stored in VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, Burlingame, CA) until imaging. Zebrafish were staged on glass slides and imaged laterally using a Nikon A1 Spectral Detector Confocal with FLIM Module at the University of Massachusetts Light Microscopy Core Facility located within the Institute for Applied Life Sciences. To obtain a 3-dimensional islet structure, z-stacks were obtained by taking photographs at 1.8  $\mu\text{m}$  steps.  $\beta$  cell volumes and sphericity were calculated using the Nikon NIS Elements Advanced Research software package. Larvae were obtained from 3 to 4 experimental replicates with 19–43 larvae per group at 15 dpf and 22–39 fish per group at 30 dpf.

## 2.9. Assessment of adiposity during the mid-larval period

Zebrafish adiposity was quantified in wild-type (AB) zebrafish using light microscopy. Briefly, larvae were exposed as described in section 2.4.2, and imaged for fish length and preserved in 4% paraformaldehyde as described in section 2.6. Preserved zebrafish larvae were stained with Oil Red O (ORO), a neutral-lipid stain, as described originally by Schlegel and Stainier (2006). Images of zebrafish were taken on an EVOS FL Auto Microscope (Thermo Fisher, Pittsburgh, PA) equipped with a color camera. Total lipid concentrations were regionalized by selecting the respective structures and quantifying red intensity. Larvae were obtained from 3 experimental replicates with 9–10 larvae per group.

## 2.10. Kinematic analysis of behavior

For behavioral assays, embryos were obtained from a mixture of Tübingen and AB strains (spontaneous swimming). Behavior was monitored using an IDT X-Stream 1440p PCIe 2.0 high speed camera (Integrated Design Tools, Inc., Pasadena, CA, USA) in a temperature-controlled setting (25 °C). Larvae were imaged from above and illuminated using a white light source from below. Larvae were transferred to a 24-well plate at 15 dpf and allowed to acclimate to lighting conditions for 2 min prior to imaging for 5 min. Recordings were obtained at a resolution of  $1312 \times 1312$  pixels with a framerate of 10 Hz and exposure time of 0.8 ms.

Kinematic analysis was performed using Marigold, custom zebrafish behavioral analysis software (Teicher et al., In preparation). Briefly, the software uses convolutional neural networks which have been trained using a novel dataset of manually labeled frames extracted from larval zebrafish behavioral recordings. The trained neural networks accurately track multiple keypoints along the rostral-caudal axis of larval zebrafish and distinguish the fish from other objects in the field of view. The keypoint coordinates are then used to derive further kinematic

parameters. A graphical user interface facilitates making manual corrections and interactively visualizing the data before exporting it to CSV files for downstream analysis. For spontaneous swimming at 15 dpf (15–24 larvae per treatment group, from 3 experimental replicates), kinematic parameters were computed using a point centered between the eyes. Swimming bouts were defined as beginning when a velocity greater than 3 mm/s was detected and ending when activity returned to a level below this threshold.

## 2.11. Data analysis

All data analyses were performed using IBM SPSS Statistics v25. Shapiro-Wilk tests for normality of data, and Levene's tests for equal variances were first used to determine appropriate statistical tests. One-way ANOVA (parametric) or Kruskal-Wallis tests (non-parametric) were used to assess statistically significant changes between exposure groups. These tests were paired with Tukey (parametric) or Mann-Whitney U (non-parametric) pair-wise tests. Chi-square tests were utilized to assess differences in incidence of islet structural morphologies. A confidence level of 95% ( $\alpha = 0.05$ ) was used for all analyses.

## 3. Results

### 3.1. Macronutrient composition

Total larval concentrations of protein, cholesterol, triglycerides, and glucose were quantified at 4 dpf (Fig. S1). Kruskal-Wallis tests found no overall significant changes in any concentrations, despite slightly decreasing trends for cholesterol, triglycerides, and glucose ( $p > 0.05$ ). Pairwise Mann-Whitney U tests also found no significant changes due to PFOS exposure in any parameter when compared to controls ( $p > 0.05$ ).

### 3.2. Fatty acid analysis

Embryonic concentrations of saturated (Fig. 1) and unsaturated (Fig. S2) fatty acids were quantified at 4 dpf. Dose-dependent increases were observed for the saturated acids lauric acid (C12:0) and myristic acid (C14:0), the two shortest chained fatty acids assessed ( $p < 0.05$ ). Though dose-dependent decreases of the most abundant fatty acids, palmitic acid and stearic acid, were observed, these trends were not statistically significant ( $p > 0.05$ ). No statistically significant changes of unsaturated fatty acid concentrations were observed ( $p > 0.05$ ; Fig. S2).

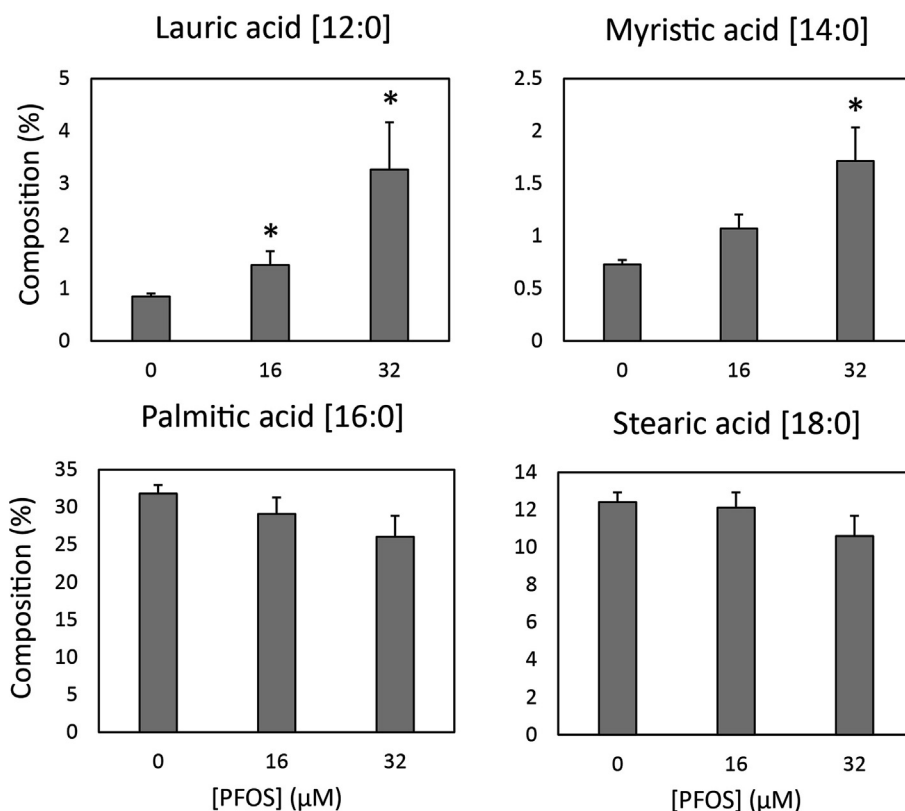
### 3.3. PPAR gene expression profiles

Expression of PPAR isoforms was assessed at 4 dpf (Fig. 2). Decreasing trends were observed for all (*pparaa*, *pparbb*, and *pparg*) PPAR isoforms, though Kruskal-Wallis tests found significant decreases for only *pparaa* and *pparg* ( $p < 0.05$ ). Pairwise Mann-Whitney U tests found significant decreases in *pparg* expression in embryos exposed to both 16 and 32  $\mu\text{M}$  PFOS compared to controls ( $p < 0.05$ ). Likewise, expression of *pparaa* was decreased in embryos exposed to 32  $\mu\text{M}$  PFOS compared to controls ( $p < 0.05$ ).

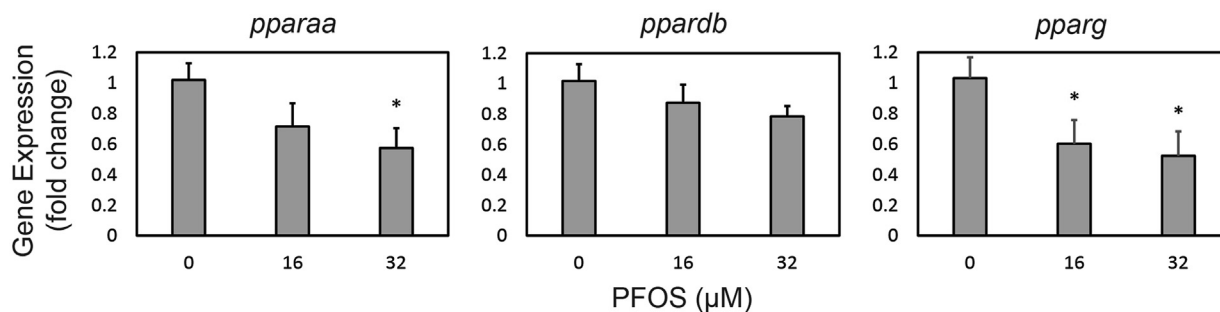
### 3.4. Islet morphometry and growth during the mid-larval (15 dpf) stage

Islet morphometry and fish growth were assessed in 15 dpf larvae following either developmental (exposed days 1–5 only) or subchronic (exposed until 15 dpf) PFOS exposures (Fig. 3). Different exposure paradigms were employed to observe whether the timing and duration of exposures may play a role in any aberrant islet





**Fig. 1.** Embryonic concentrations of saturated fatty acids are impacted by PFOS exposures. Concentrations of saturated fatty acids (C12, C14, C16, and C18) were quantified using gas chromatography. Dose-dependent increases of the two shortest chain fatty acids, lauric acid and myristic acid, were observed with increased PFOS exposures ( $p < 0.05$ ).  $n = 4-6$  pooled samples of 15 embryos per exposure group.

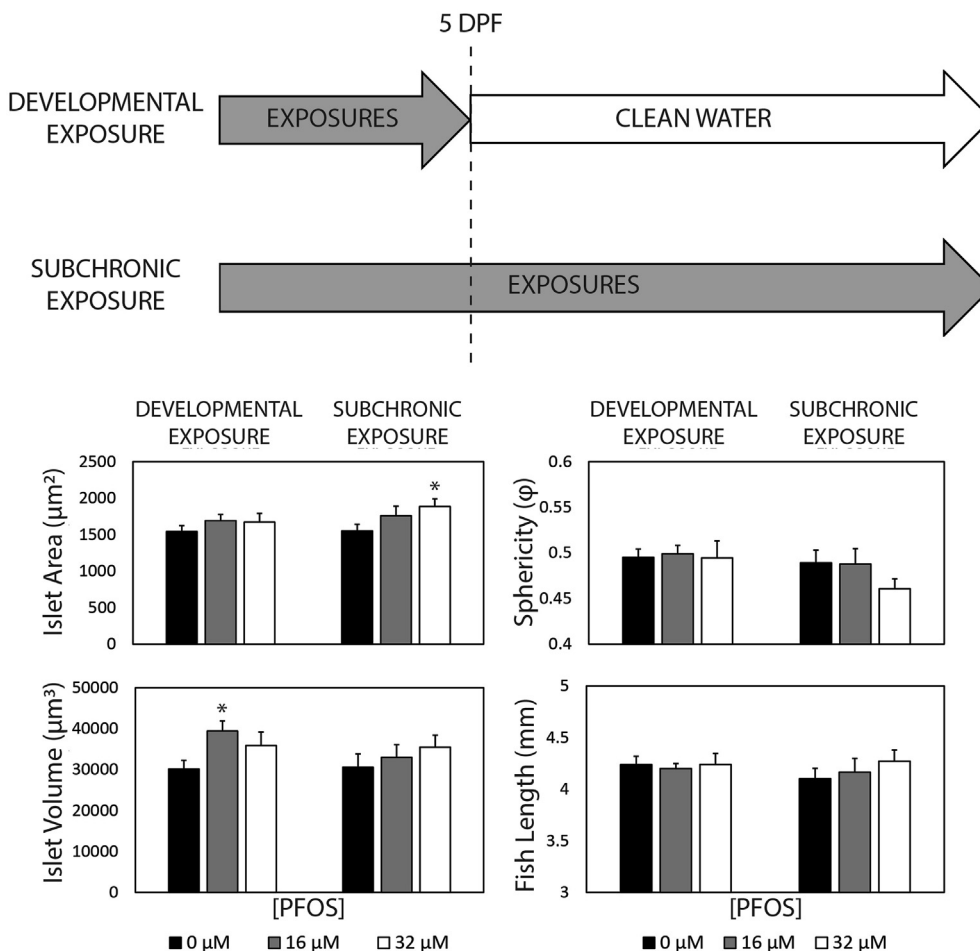


**Fig. 2.** Gene expression of PPAR isoforms is reduced by developmental PFOS exposures. Embryos were individually exposed to PFOS, and exposures were renewed daily to mimic exposure throughout development through to 4 dpf. Expression of *pparaa* was significantly reduced in embryos exposed to 32 μM PFOS, and expression of *pparg* was significantly reduced in all exposed embryos compared to controls. Asterisks (\*) denote statistically significant changes from respective controls ( $p < 0.05$ ).  $n = 4-5$  samples per exposure group, each containing 10 pooled embryos.

morphometry, because zebrafish are capable of cellular regeneration (Matsuda, 2018; Moss et al., 2009). No changes were observed due to exposures for islet sphericity nor fish length, and no differences were observed between the developmental and subchronic exposures for any exposure concentration ( $p > 0.05$ ). Islet area was increased in larvae subchronically exposed to 32 μM PFOS ( $p = 0.019$ ). Islet volume was increased in larvae developmentally exposed to 16 μM PFOS ( $p = 0.005$ ). Islet volume, islet area, and islet sphericity were not correlated with fish length ( $R^2 < 0.01$ ) at 15 dpf regardless of exposure concentration or paradigm.

### 3.5. Islet morphometry and growth during the early juvenile (30 dpf) stage

Overall growth and islet morphologies were characterized at 30 dpf in developmentally-exposed fish (Fig. 4). At 30 dpf, fish lengths did not significantly differ due to developmental PFOS exposures ( $p > 0.05$ ). Islet volume and islet area were positively correlated ( $R^2 = 0.873$ ) in control fish at 30 dpf, but they were only weakly correlated for PFOS-exposed fish ( $R^2 < 0.5$ ). To improve the model, islet volume and islet area were normalized to fish length. Normalized islet area was increased in fish exposed to 32 μM PFOS,



**Fig. 3.** Larval pancreatic islet morphometry are minimally impacted by developmental or subchronic PFOS exposures at 15 dpf. Islet area was increased in larvae subchronically exposed to 32 μM PFOS, and islet volume was increased in larvae developmentally exposed to 16 μM PFOS ( $p < 0.05$ ). Islet sphericity and fish length were not significantly impacted by exposures ( $p > 0.05$ ). No differences were observed due to exposure paradigm (developmental vs. subchronic exposures) for any PFOS exposure concentration ( $p > 0.05$ ).  $n = 19\text{--}43$  larvae per group from 3 to 4 experimental replicates.

but volume was decreased in all PFOS-exposed fish ( $p < 0.05$ ). For these reasons, sphericity was assessed, and was found to decrease with increasing PFOS concentration ( $p < 0.001$ ). To characterize the morphology of islets, we first examined the integrity of the primary islets and classified them as tight (normal) or loose (heterogeneous islet cell populations and/or intracellular gaps between  $\beta$  cells). The incidence of loose primary islets increased in fish exposed to 16 and 32 μM PFOS ( $p = 0.003$  and  $p = 0.027$ , respectively). Isolated cells and cell clusters distal from the primary islet were also observed, as we have previously observed due to PFOS exposures in embryos (Sant et al., 2017). These isolated cells and clusters occurred at higher incidence in fish exposed to 32 μM PFOS ( $p = 0.023$ ).

### 3.6. Assessment of adiposity and kinematics during the mid-larval (15 dpf) stage

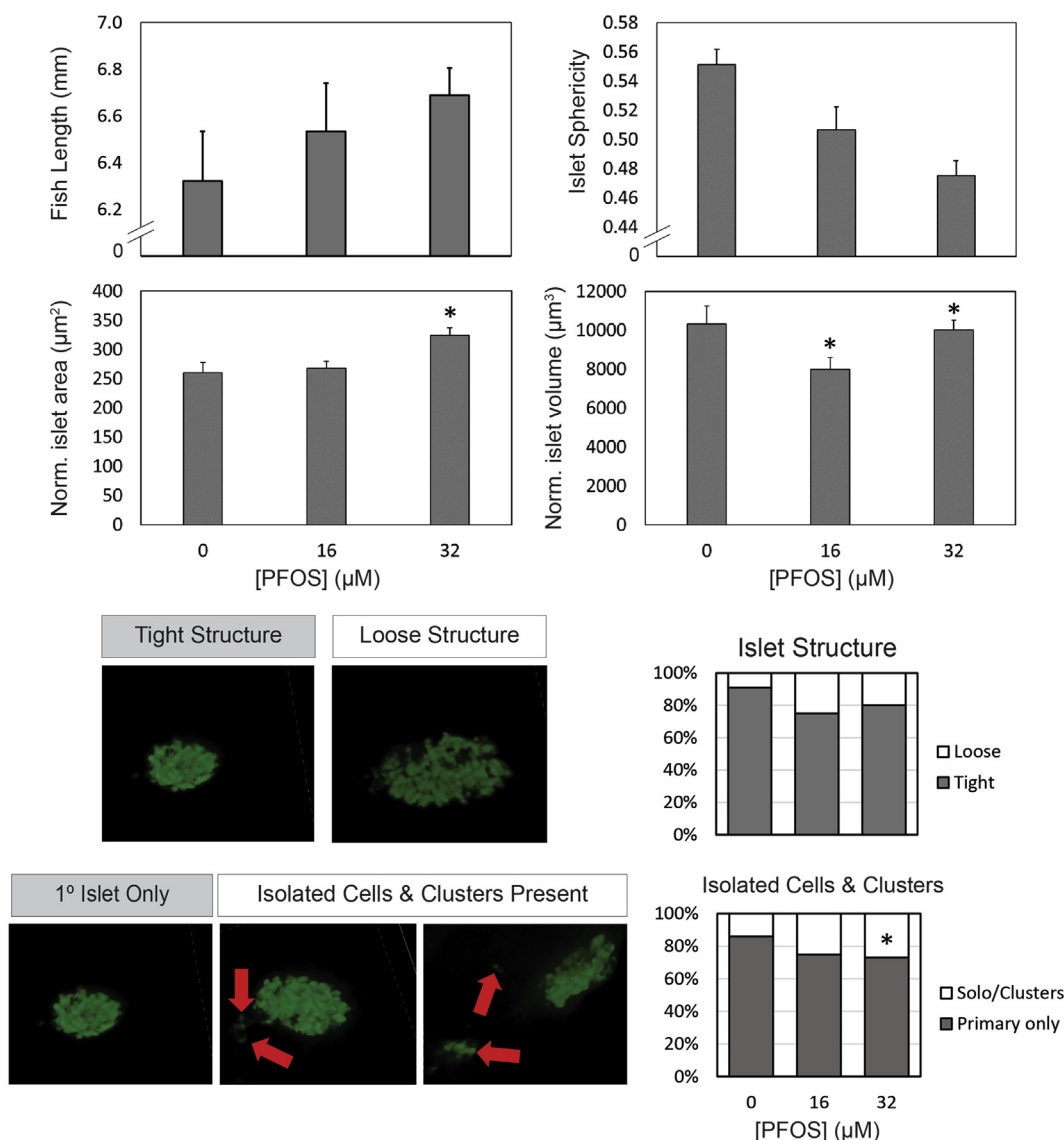
Lipid accumulation was spatially and quantitatively examined in 15 dpf larvae following developmental exposures to PFOS from 1 to 5 dpf using ORO (Fig. 5). ORO staining intensity was increased by 18% ( $p = 0.109$ ) and 23% ( $p = 0.012$ ) in larvae exposed to 16 and 32 μM PFOS, respectively. The number of distinct abdominal lipid droplets was quantified and was significantly elevated in larvae exposed to 16 ( $p = 0.001$ ) and 32 ( $p = 0.013$ ) μM PFOS. The total ORO staining area and fish length were not significantly impacted

by PFOS exposures. Fish length and ORO staining intensity were only weakly correlated ( $R^2 = 0.477$ ).

Larval spontaneous behavior was characterized at 15 dpf in developmentally-exposed fish to assess any potential neuro-behavioral effects from exposures and to identify any confounding hyper- or hypo-activity which may impact adiposity measures (Fig. S3). No significant changes in swimming behavior were observed due to exposures ( $p > 0.05$ ).

## 4. Discussion

Metabolic syndrome is a growing epidemic in the United States and worldwide. While rates of adult-onset diabetes have leveled in the United States, diabetes has been steadily increasing in juveniles and is exacerbated in ethnic minorities (Dabelea et al., 2014; Mayer-Davis et al., 2017; National Center for Chronic Disease Prevention and Health Promotion, 2017). To exacerbate this trend, young-onset type 2 diabetes is a more aggressive form of diabetes, with faster deterioration of  $\beta$  cell function, less effective therapeutic responses, and more severe complications (Magliano et al., 2020). Childhood and adolescent obesity follows the same increasing trend as juvenile diabetes, also exacerbated in ethnic minorities (Fryar et al., 2015; Hales et al., 2017). As the prevalence of juvenile diabetes and obesity continue to climb in youth, the rates of

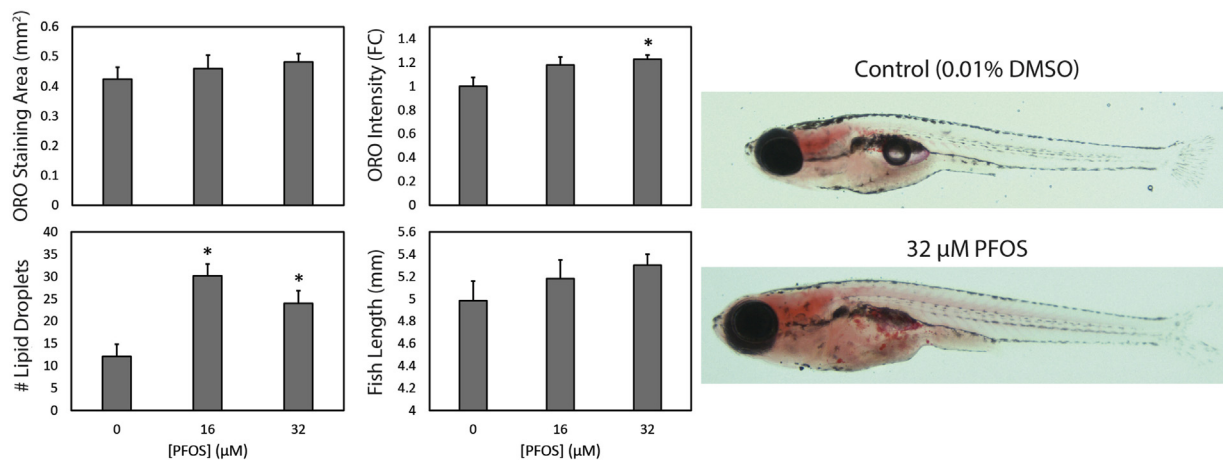


**Fig. 4.** PFOS alters pancreas primary islet morphology in 30 dpf fish. Fish length at 30 dpf fish was not significantly altered by developmental (1–5 dpf) PFOS exposure. Islet sphericity was decreased due to 32 μM PFOS exposure, and islet area (normalized to fish length) was increased ( $p < 0.05$ ). Normalized islet volume was significantly decreased by all PFOS exposures ( $p < 0.05$ ). The incidence of loose islet structures as well as the incidence of isolated cells and clusters were increased in exposed fish ( $p < 0.05$ ).  $n = 22–39$  fish per group from 3 experimental replicates.

metabolic syndrome would be expected to increase in adulthood following this generation. Numerous epidemiological studies have investigated the relationship between metabolic dysfunction and exposures to perfluoroalkyl substances with conflicting results (Cardenas et al., 2017; Conway et al., 2016; He et al., 2018; Lauritzen et al., 2018; Lind et al., 2014; Sun et al., 2018). However, the majority of these studies have been in adults and have been cross-sectional, excluding a more temporal approach. Here, we present our data examining the impacts of embryonic exposures to PFOS and resulting biochemical and pathological consequences during the later larval and juvenile periods in zebrafish. Though we did observe persisting changes, islet area and adiposity had reciprocal relationships with embryonic PFOS effects, suggesting that processes such as adaptation, catch-up growth, or recovery may occur following exposures.

In a previous study, we demonstrated that embryonic exposures to PFOS decreased islet size and sphericity (manifested embryonically as aberrant morphologies, including fragmented islets or

ectopic cells) in zebrafish embryos and early-stage larvae (Sant et al., 2017). These exposures were associated with decreased gene expression of pancreatic endocrine and exocrine function hormones and enzymes, including targets such as insulin, somatostatin, ghrelin, trypsin, and chymotrypsin (Sant et al., 2017). This work suggested that the pancreas is a sensitive target of environmental exposures, and that the embryonic period is a window of potential pancreatic susceptibility. However, these exposures were acute and ended at 4 dpf, and the persistence of these changes was unknown. Here, we found similar decreased islet sphericity in 30 dpf fish, demonstrating persistence into the juvenile period (Fig. 4). Though PFOS resulted in decreased islet area in embryos (4 dpf), islet sizes were, if anything, increased at 15 and 30 dpf (Figs. 3 and 4). This change could result from adaptation and potential “catch-up growth”, as is a common trend in studies investigating the developmental origins of health and disease (Barker, 2004; Hales and Ozanne, 2003). The relationship between early life “catch-up” growth and components of metabolic



**Fig. 5.** Developmental exposures to PFOS increase lipid accumulation in zebrafish at 15 dpf. Oil Red O stain (ORO) intensity and the number of distinct lipid droplets were increased in PFOS-exposed fish ( $p < 0.05$ ). Though fish length and the total staining area were marginally increased, these changes were not statistically significant ( $p > 0.05$ ).  $n = 22-41$  larvae per group from 3 experimental replicates.

syndrome are increasingly well-characterized (Bieswal et al., 2006; Huxley et al., 2000; Lin, 2018; Morrison et al., 2010; Ong et al., 2000; Ozanne and Hales, 2004). For prediabetes specifically, expansion of islet mass due to hyperglycemia is a common observation and can lead to diabetes progression (reviewed in Meier et al., 2008; Weir and Bonner-Weir, 2004). More intermediate experimental timepoints in paired samples are needed to identify the progression of changes during this dynamic window.

Changes in islet morphology due to PFOS exposures were more pronounced in 30 dpf juveniles than 15 dpf larvae (Figs. 3 and 4). Perhaps what is more profound is that these 30 dpf juveniles were only exposed to PFOS from 1 to 5 dpf, and then were not exposed for the remaining 25 days prior to measurement. And yet, the changes in islet area and sphericity were greater than those measured for subchronically-exposed (1–15 dpf) larvae. Because of the ability of zebrafish islets to regenerate (Matsuda, 2018; Moss et al., 2009), this significant finding suggests that there is an underlying change or programming of the  $\beta$  cells that occurs due to developmental PFOS exposures, which may influence islet size and function later in the lifecycle. With the recent gains in single-cell sequencing technologies, future work should investigate these early changes in  $\beta$  cells due to embryonic exposures, and longitudinally assess the persistence of these effects throughout the lifecycle.

We previously showed that embryonic PFOS exposures impacted gene expression in processes involved in adipogenesis and lipid homeostasis, namely within the PPAR signaling pathway (Sant et al., 2018a). Numerous studies have demonstrated relationships between PFOS exposures and activation of PPAR signaling, including moderate agonism of PPAR $\alpha$  due to exposures in various models and tissues (Cwinn et al., 2008; Jacobsen et al., 2018; Mylorie et al., 2020; Rosen et al., 2010; Takacs et al., 2007; Wolf et al., 2008). PPAR signaling is a tissue-specific nutrient-sensing pathway, stimulating processes such as glycolysis, adipogenesis, and fatty acid metabolism and synthesis. Unsaturated and, to some extent saturated fatty acids are known ligands for PPARs, stimulating processes such as lipid catabolism (PPAR $\alpha$ ) or storage (PPAR $\gamma$ ) (reviewed in Grygiel-Górniak, 2014; Varga et al., 2011). Because exposures to lipid such as saturated fatty acids are known to be toxic to  $\beta$  cells, it is possible that the previously published effect of embryonic PFOS exposures on  $\beta$  cells and islet morphology at 4 dpf could have also been indirectly related to embryonic fatty acid concentrations. In this study, we assessed whether embryonic

PFOS exposures did indeed impact fatty acid concentrations in our embryos that may lead to these developmental or potentially persisting phenotypes. We found that PFOS exposures increased concentrations of two medium- and long-chain fatty acids in a dose-dependent manner: lauric acid (C12:0) and myristic acid (C14:0) in embryos (Fig. 1). Of the quantifiable fatty acids in embryos, these were the two with the shortest carbon chains, and no other saturated or unsaturated fatty acids were impacted by PFOS exposures. Though long-chain unsaturated fatty acids are most closely associated with PPAR activation (reviewed in Echeverría et al., 2016), both lauric and myristic acid also can function as agonists and activators of PPAR $\gamma$  in cell culture (Kliwer et al., 1997; Liberato et al., 2012; Xu et al., 1999). Therefore, we would have expected the increased embryonic fatty acid concentrations to correspond with increased *pparg* gene expression. However, the reciprocal was observed, with decreased expression of both *pparaa* and *pparg* (Fig. 2). One possible explanation is that there are divergent tissue-specific responses or temporal responses in our PFOS-exposed zebrafish embryos that may not have been elucidated due to sampling whole embryos for both fatty acid analysis and gene expression. Another consideration is that PPAR gene expression could be decreasing as a negative feedback mechanism stimulated due to the increased concentrations of PPAR ligands, including PFOS. More mechanistic work co-investigating PFOS exposures and PPAR modulation (through functional agonists and antagonists) could provide mechanistic information to better understand this complex relationship.

In our previous study, embryonic PFOS exposures increased gene expression of lipid transporter apolipoprotein A1 (*apo1a*) and PPAR $\gamma$ -responsive fatty acid binding protein 1b1 (*fabp1b.1*), while decreasing expression of PPAR $\alpha$  target fatty acid binding protein 1a (*fabp1a*) (Laprairie et al., 2016; Sant et al., 2018a). In this study, gene expression of both *pparaa* and *pparg* was decreased in PFOS-exposed 4 dpf embryos by as much as 50% (Fig. 2). PPAR signaling plays a crucial role in the regulation and control of adipogenesis, especially PPAR $\gamma$  which is highly expressed in adipose tissues (Grimaldi, 2001; Rosen et al., 2000). Therefore, decreased expression of PPARs, especially *pparg*, would have been expected in larvae with fewer fat droplets and reduced lipid accumulation. However, the opposite was observed, and PFOS decreased *pparg* expression in embryos and increased adiposity in 15 dpf larvae. PPAR gene expression was measured in whole embryos at 4 dpf only, and therefore adipocyte-specific PPAR signaling may be more



informative of adiposity in larvae.

There is a growing body of evidence suggesting links between developmental exposures to environmental 'obesogens' and pediatric and adult obesity (reviewed in [Janesick and Blumberg, 2012](#); [La Merrill and Birnbaum, 2011](#)). We observed increased lipid droplet numbers and concentrations in 15 dpf larvae, despite only developmental (1–5 dpf) PFOS exposures ([Fig. 5](#)). These exposures occurred prior to the establishment of preadipocytes (~6 dpf) and adipocytes (8–10 dpf) in zebrafish, suggesting that early exposures alone are capable of increasing adipogenesis later during the larval period ([Flynn et al., 2009](#)). Though we have shown decreased PPAR gene expression due to exposures at 4 dpf, we did not assess the persistence of this decreased expression later into the larval period at 15 dpf. Future studies assessing the expression of PPARs and their targets longitudinally are required to shed light on the relationship between exposures and nutritional control and regulation of adipogenesis. However, a growing body of literature supports the relationship between PFOS exposures and adiposity. Other studies have demonstrated that PFOS exposures to murine-derived 3T3-L1 preadipocytes in cell culture increase adipocyte differentiation and increase triglyceride concentrations ([Watkins et al., 2015](#); [Xu et al., 2016](#)). Other studies have also shown increased lipid droplet sizes and concentrations in the liver of PFOS-exposed animals, especially when combined with a high-fat diet ([Du et al., 2009](#); [Marques et al., 2020](#); [Seacat et al., 2002](#); [Wang et al., 2014](#)). Here, we support the existing literature, showing that ORO staining intensity and the number of abdominal lipid droplets were significantly increased by PFOS exposure despite only a developmental exposure paradigm ([Fig. 5](#)). Therefore, these early developmental exposures prior to adipogenesis are sufficient to produce increased adiposity.

The relationship between exposures, overall growth, and adiposity require elucidation. Fish length and adiposity were poorly correlated at 15 dpf ( $R^2 = 0.477$ ), demonstrating that PFOS-induced adiposity occurs independent of overall larval growth ([Fig. 5](#)). These fat depots were primarily visceral, or located near the pancreas. The locations of these fat depots are consistent with other zebrafish studies, which found that the first preadipocytes and adipocytes occur near the pancreas ([Flynn et al., 2009](#); [Imrie and Sadler, 2010](#)). A study by Imrie et al. demonstrated that increased fish length corresponded to, and could potentially "accelerate", increased adipogenesis in zebrafish—more so than fish age ([Imrie and Sadler, 2010](#)). In this study, no statistically significant changes in fish length were observed due to exposure, though there was an increasing trend in mean fish length with increasing PFOS concentration in 30 dpf and subchronically exposed 15 dpf larvae. Thus, it is likely that the increased adiposity was in fact due to direct PFOS exposure.

This work presented several novel biochemical and pathological consequences due to developmental PFOS exposures including changes in embryonic fatty acid profiles and alterations to the mid-larval and juvenile-stage islet morphology. However, one major limitation of this work is the administration of larval diets. Because of the aquatic habitats, it is difficult to precisely measure the administration of powdered diets nor the amount of food consumed each day. For this reason, we administered *ad libitum* diets and we cannot provide accurate information about the relationship between food consumption, islet morphology, or adiposity. The use of a powdered diet (Gemma 75) is a strength of this study, because this feed is more highly regulated from a nutritional and contaminant perspective than typical larval live diets. Another limitation of this study is the inability to track social contributions to the growth and development of larvae and juvenile fish. Fish were individually housed in order to ensure congruent exposures and to minimize aggressive feeding behaviors, which was considered a strength of the study design. However, fish social hierarchy can play a major role in overall growth and

development ([Hesse and Thünken, 2014](#); [Mas-Muñoz et al., 2011](#); [Volkoff and Peter, 2006](#)), and therefore we cannot make any conclusions about the social consequences nor contributions to these phenotypes.

We observed no significant changes in spontaneous swimming behavior at 15 dpf due to developmental PFOS exposures ([Fig. S3](#)). There may have been changes in feeding behavior associated with chemical exposures which could lead to altered metabolic phenotypes, or perhaps more significant behavioral changes occurred more acutely during or immediately following the exposure window. Several kinematic studies in zebrafish embryos have previously found increased hyperactivity in PFOS-exposed zebrafish embryos and eleutherolarvae ([Gaballah et al., 2020](#); [Huang et al., 2010](#); [Khezri et al., 2017](#); [Menger et al., 2020](#); [Spulber et al., 2014](#)). In Menger et al. startle responses, burst activity, and swimming distance were impacted by PFOS exposure, but only at the highest concentration examined, which was more than 2X our highest concentration used in this study ([Menger et al., 2020](#)). Two studies by Jantzen et al. utilized developmental PFOS exposure paradigms and examined behaviors later in the lifecourse, similar to the methods used in this study. At 14 dpf, total distance traveled, crossing frequency, and velocity were increased by exposures to 2  $\mu\text{M}$  PFOS until 5 dpf ([Jantzen et al., 2016a](#)). However, at 6 months of age, these responses were ameliorated ([Jantzen et al., 2016b](#)).

A major limitation is the lack of data regarding the PFOS concentrations in the fish to compare body burdens between developmental and subchronic exposures. Several other studies have assessed the toxicokinetics of PFOS in zebrafish. In a study by [Chen et al. \(2016\)](#), the authors report that the plasma rate of uptake for PFOS is 774–1917 L/kg/day and the half-life is 27.7–34.7 days depending upon the concentration utilized ([Chen et al., 2016](#)). Another study measured the rate of PFOS uptake in zebrafish embryos, finding that the rate increased over the first 5 dpf and ultimately that exposures to 8  $\mu\text{M}$  PFOS (50% of our low concentration) accumulated 66.1 ng PFOS in 5 dpf larvae ([Huang et al., 2010](#)). Following a 6-day static exposure to 3.1  $\mu\text{M}$  PFOS in zebrafish embryos, [Gaballah et al. \(2020\)](#) reported larval tissue concentrations of approximately 60 pmol (approximately 30 ng) per larva ([Gaballah et al., 2020](#)). Another study using zebrafish embryos found that the uptake rate for PFOS in embryos was <1 pmol/h/embryo ([Vogs et al., 2019](#)). Based on this information, we can infer that the developmentally-exposed larvae in our study should have markedly reduced total PFOS body burden than those exposed subchronically due to decreased uptake rates and depuration time. However, we cannot assume that the elimination constants are comparable between groups, and the mobility of PFOS within tissues may differ based on exposure stage and timing. Therefore, additional controlled toxicokinetic studies are needed to better spatiotemporally contextualize our observed phenotypes with PFOS body burden.

In conclusion, this work shows that early life exposures to the toxicant PFOS may impact early nutrition and metabolism, namely fatty acid concentrations and PPAR signaling. Pancreatic islets, predominantly insulin-producing  $\beta$  cells, are larger in exposed 15 dpf larvae, regardless of developmental (1–5 dpf) or subchronic (1–15 dpf) exposures. These changes in size and aberrant morphology are exacerbated in developmentally-exposed 30 dpf juvenile fish, despite removal from exposures at 5 dpf. Adiposity is increased in developmentally-exposed 15 dpf larvae, with increased visceral fat depots evident with increased exposure concentrations. Ultimately, this work demonstrates that exposures during the embryonic period are sufficient to produce metabolic dysfunction that persists into the juvenile period, regardless of exposure duration. This research supports PFOS as a contaminant of

interest in the developmental origins of diabetes and obesity.

### Author statement

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### Declaration of competing interest

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

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2021.116644>.

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