

Perfluorobutanesulfonic Acid Disrupts Pancreatic Organogenesis and Regulation of Lipid Metabolism in the Zebrafish, *Danio rerio*

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ABSTRACT

Following the phase-out of highly persistent perfluorosulfonates in the United States from non-stick and stain-resistant products in the early 2000s, perfluorobutanesulfonic acid (PFBS) has replaced these compounds as a primary surfactant. Measurements of PFBS in environmental and human samples have been rising in recent years, raising concerns about potential negative health effects. We previously found that embryonic exposures to a related compound, perfluorooctanesulfonic acid (PFOS), decreased pancreas length and insulin-producing islet area in zebrafish embryos (*Danio rerio*). The objective of this study was to compare the effects of PFBS exposures on pancreatic organogenesis with our previous PFOS findings. Dechorionated zebrafish embryos from two different transgenic fish lines (*Tg[insulin:GFP]*, *Tg[ptf1a:GFP]*) were exposed to 0 (0.01% DMSO), 16, or 32 μ M PFBS daily beginning at 1 day post fertilization (dpf) until 4 and 7 dpf when they were examined using fluorescent microscopy for islet area and morphology, and exocrine pancreas length. PFBS-exposed embryos had significantly increased caudal fin deformities, delayed swim bladder inflation, and impaired yolk utilization. Incidence of fish with significantly stunted growth and truncated exocrine pancreas length was significantly increased, although these two effects occurred independently. Islet morphology revealed an increased incidence of severely hypomorphic islets (areas lower than the 1st percentile of controls) and an elevated occurrence of fragmented islets. RNA-Seq data (4 dpf) also identify disruptions in regulation of lipid homeostasis. Overall, this work demonstrates that PFBS exposure can perturb embryonic development, energy homeostasis, and pancreatic organogenesis.

Key words: PFAS; developmental toxicity; pancreatic beta cells; gene expression; lipid homeostasis.

Perfluorobutanesulfonic acid (PFBS) is an emerging toxicant of international interest, becoming increasingly concentrated in both the environment (Chen et al., 2017; Shao et al., 2016; Wang et al., 2016) and in biological samples (Glynn et al., 2012; Huang et al., 2018) due to increased manufacture and use. PFBS and its derivatives are used in the manufacture of surfactants, such as Scotchgard, metal plating, flame retardants, and fire-fighting foams (Wang et al., 2013). PFBS is very stable in the environment with an atmospheric half-life of > 100 days, and no evidence of

degradation in water (Taniyasu et al., 2013). It has a biological elimination half-life of approximately 1 month in adult human serum (Olsen et al., 2009), but it is unknown whether this would be similar in children or pregnant women. PFBS is a growing concern internationally, as its manufacture and use in both consumer and commercial grade products has rapidly increased worldwide. This increased utilization is due primarily to the voluntary phase-out of legacy compound perfluorooctanesulfonic acid (PFOS) in American products in the early 2000s. PFBS

has been widely utilized as the replacement chemical for PFOS-based surfactants mostly because it is believed to have decreased toxicity and bioaccumulation (Concawe Soil and Groundwater Taskforce, 2016). Compared with the 1 month half-life of PFBS in human tissues, legacy PFOS has a half-life of approximately 5.4 years (Olsen et al., 2007). Like PFBS, PFOS is very stable in the environment, and the half-life remains unknown due to its recalcitrance to biotransformation and photodegradation.

In a previous study, we examined the effects of PFOS exposures on pancreatic organogenesis and expression of hormones and peptides vital for pancreas function (Sant et al., 2017). We found that pancreata were significantly shorter in zebrafish larvae exposed to 32 μ M PFOS throughout embryonic development, and this finding was concordant with decreased gene expression of pancreatic digestive enzymes. Furthermore, PFOS-exposed larvae also had reduced insulin-producing β cell area, and decreased gene expression of the hormones governing glucoregulation. These data complement a growing body of literature associating PFOS exposures throughout the lifecourse with metabolic dysfunction in numerous populations (Lin et al., 2009; Lv et al., 2013; Nelson et al., 2010; Sun et al., 2018; Wan et al., 2014). Altogether, these data suggest that the developing pancreas is a sensitive target of PFOS toxicity, and could play a role in the developmental origins of metabolic diseases. However, the effects of PFBS have not yet been studied.

Pancreatic organogenesis is a dynamic process, beginning as early as the fourth week of human gestation (Edlund, 2002). First, the pancreas forms as two bud structures developing around the gut midline, arising from the endodermal germ layer. Soon after, these buds proliferate, rotate around the gut, and fuse to form the initial pancreatic structure. This structure is comprised of two key tissues: the exocrine pancreas, which synthesizes and releases digestive enzymes to aid in the breakdown of molecules to fuel metabolism, and the endocrine pancreas, comprised of the Islets of Langerhans which secrete glucoregulatory hormones including insulin. The endocrine function of the pancreas is evident at the embryonic fetal transition (roughly 8–10 weeks into gestation), but islet architecture continues to structurally and functionally mature until approximately 15 weeks into gestation (Meier et al., 2010; Nussey and Whitehead, 2001; Pan and Brissova, 2014). The exocrine pancreas acinar and ductal structures develop throughout gestation (weeks 11–37), though exocrine function initiates late in gestation (after 22 weeks) and matures primarily after the onset of exogenous feeding (postpartum) (McClean and Weaver, 1993; Pan and Brissova, 2014). These structures and developmental processes are well-conserved across vertebrate species, including in the zebrafish (Tiso et al., 2009; Ward et al., 2007). However, zebrafish dorsal and ventral buds contain distinct endocrine and exocrine populations, whereas mammalian buds contain mixed populations (Angelo et al., 2012; Tiso et al., 2009).

Though pancreas function is routinely examined in clinical and experimental studies, pancreas structure is often overlooked. However, aberrant phenotypes are found in a large percentage of the population and may play a critical role in metabolic disease. Abnormal islet architectures are often found in both animal and human studies, and are regularly associated with increased risk for hyperglycemia and Types 1 and 2 Diabetes mellitus (Bosco et al., 2010; Cabrera et al., 2006; Kilimnik et al., 2011; Kim et al., 2009). Several pancreas congenital defects have also been observed, including pancreas divisum (two pancreas ducts fail to fuse into one), ectopic pancreatic tissue (pancreatic cells develop in other organs), dorsal pancreas agenesis

(failure of the pancreas to grow, resulting in a short pancreas), and annular pancreas (pancreatic tissue encircling the duodenum, which may constrict the digestive tract). Though conditions such as an annular pancreas is rarely observed in adults, pancreas divisum and ectopic pancreas tissues are predicted to occur in nearly 10% of the population (Prasad et al., 2001; Varshney and Johnson, 1999; Vaughn et al., 1998). These prognoses can often be mild, so diagnoses are not commonly made unless patients present with conditions such as pancreatitis, impaired pancreatic function, or until autopsy. These malformations are found at higher rates amongst diabetic patients (Campbell-Thompson et al., 2012; Gilbeau et al., 1992; Hardt et al., 2000) but the causes are not well understood.

The objective of this study is to compare the toxicity of the emerging replacement surfactant PFBS to the legacy chemical PFOS in the developing pancreas. Because of its chemical properties, it is hypothesized that PFBS would result in fewer or milder aberrant pancreas phenotypes compared with PFOS. Herein, we use transgenic zebrafish models to visualize these processes *in vivo* throughout the developmental process, and use RNA-Seq to examine major pathways affected by these embryonic exposures. Ultimately, the goal of this research is to identify whether developmental exposures to PFBS, an emerging toxicant, may increase risk for metabolic syndrome throughout the lifecourse.

MATERIALS AND METHODS

Chemicals and reagents. Nonafluorobutane-1-sulfonic acid (PFBS) was purchased from Sigma-Aldrich (St. Louis, Missouri; 97% purity). Working stocks were prepared by diluting PFBS in dimethyl sulfoxide (DMSO), to 160 and 320 mM concentrations, and stored in amber glass vials at 4°C. All experimental procedures using PFBS were conducted using appropriate safety precautions.

Animals and husbandry. Transgenic *Tg(insulin:GFP)* (diIorio et al., 2002) and *Tg(ptf1a:GFP)* (Field et al., 2003) zebrafish were obtained as heterozygous populations from Dr Philip diIorio at the University of Massachusetts Medical School (Worcester, Massachusetts) and bred to homozygosity. *Tg(insulin:GFP)* zebrafish express GFP in β cells, which comprise the majority of Islet of Langerhans mass and secrete insulin. *Tg(ptf1a:GFP)* zebrafish express GFP throughout the exocrine pancreas, and also in the retina and hindbrain (Godinho et al., 2005; Lin et al., 2004). All animal use and care was conducted in strict accordance with protocols approved by the University of Massachusetts Amherst Institutional Animal Care and Use Committee, Animal Welfare Assurance Number A3551-01.

Exposures. To identify the dose-responses for survival, hatching, and swim bladder inflation, embryos were exposed to 0 (0.01% DMSO), 8.25, 82.5, 825, or 8250 μ M PFBS (2.5 μ g/ml–2.5 mg/ml) in 0.3 \times Danieau's medium beginning at 3 h post fertilization. Embryos were housed individually in the wells of 96-well plates containing 200 μ l of exposure medium, which was refreshed daily. Embryos were examined for viability following the OECD Fish Embryo Acute Toxicity Test guidelines (OECD, 2013), and examined under microscope for hatching at 3 dpf and swim bladder inflation at 4 dpf.

For all other experiments, embryos were exposed to 0 (DMSO control) 16 or 32 μ M PFBS beginning at 1 dpf, by adding 0.01% v/v of the working stocks to 0.3 \times Danieau's media. All exposures were performed in 100 mm polystyrene petri dishes, in a total

volume of 30 ml. Embryos were confirmed for proper stage and development at 1 dpf (Kimmel et al., 1995), prior to manual dechoriation using watchmakers' forceps and exposure. Exposures were refreshed daily until 7 dpf. Experiments were replicated 3–4 times on groups of 10–20 embryos per concentration. All concentrations were selected based on previous studies conducted with the C8 perfluorinated compound (PFOS), which was found to produce pancreas phenotypes investigated in this study. These PFOS concentrations were initially selected based on other zebrafish and cell culture studies (Chen et al., 2014; Wang et al., 2011; Xu et al., 2016; Zheng et al., 2012).

Microscopy. *Tg(insulin:GFP)* embryos and larvae were imaged at 4 and 7 days post fertilization (dpf) to observe morphogenesis of the primary islet, as previous performed in Sant et al. (2017). *Tg(ptf1a:GFP)* embryos and larvae were also imaged at 4 and 7 dpf to visualize development and posterior extension of the exocrine pancreas (Sant et al., 2016, 2017). All microscopy was performed on an EVOS FL Auto inverted epifluorescence microscope (Life Technologies, Pittsburgh, Pennsylvania) equipped with a GFP filter. Following exposures, embryos and larvae were thoroughly washed and transferred to petri dishes containing 2% v/v MS-222 for anesthesia (prepared as 4 mg/ml tricaine powder in water, pH buffered, and stored at -20°C until use). Embryos and larvae were mounted in 3% methylcellulose for imaging, and oriented for optimal pancreas imaging on their right lateral side. General morphometry and exocrine pancreas visualization was performed using $2\times$ and $4\times$ objectives, and islet imaging was done using a $10\times$ objective. Because images were acquired on an inverted microscope, images presented in these figures have been mirror-flipped (around the y-axis) to reflect actual orientation. Experiments were replicated 3–4 times on groups of 10–20 embryos per concentration.

RNA isolation, RNA-Seq library preparation, and sequencing. RNA isolation, library preparation, and sequencing were all performed at the University of Massachusetts Genomics Resource Laboratory (Amherst, Massachusetts). Briefly, zebrafish larvae (15 larvae pooled per sample) were collected at 4 dpf into FastPrep homogenization tubes containing TRIzol Reagent (Invitrogen, Carlsbad, California) and 0.1 mm RNase-free glass beads (Next Advance, Inc, Troy, New York). All contents were vortexed for 15 s and homogenized in a FastPrep-24 5 G bench-top homogenizer equipped with the QuickPrep adapter (MP Biomedicals, Santa Ana, California). The supernatant was transferred to a sterile tube, and total RNA was purified using the Direct-zol RNA MicroPrep kit with in-column DNase-I treatment following manufacturer instructions (Zymo Research, Irvine, California). RNA quantity was assessed using Qubit RNA BR Assay (Invitrogen), and quality on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Assay (Agilent Technologies, Santa Clara, California). Electropherogram results suggest that the total RNA was intact without degradation, and the RNA Integrity Numbers (RINs) ranged from 6.9 to 9.2.

Total RNA (input of 1 μg) was used to isolate poly(A) mRNA and prepare stranded libraries using the NEBNext Ultra-II Directional RNA Library Prep Kit for Illumina following manufacturer instructions (New England Biolabs, Ipswich, Massachusetts). Library quantity and quality were confirmed, and were of good quality without detection of primer or adapter dimerization. Libraries were diluted and normalized, pooled, denatured, and sequenced in the Illumina NextSeq 500 platform using the NextSeq 500/550 High Output v2 kit (75 cycles) with the 1×75 sequencing parameter using single-end reads.

Outputs were uploaded onto the Illumina BaseSpace Sequence Hub platform and annotated and assessed using the Illumina RNA-Seq Alignment and Cufflinks Assembly & DE apps. Gene ontology and pathway analysis were performed using LPath (<http://lpath.ncibi.org/>; Last accessed, October 4, 2018.) (Kim et al., 2012). Resulting data has been deposited into the Gene Expression Omnibus (GEO), and can be viewed using the accession number GSE114356.

Data analysis and statistics. Data analyses were performed using JMP Pro 13 (Cary, North Carolina). All data is presented as mean \pm SEM. Levene's tests were used to confirm non-parametric findings. Non-parametric Kruskal-Wallis tests were used to compare outcomes across PFBS concentrations, with Wilcoxon pairwise tests. Fisher exact tests for trend and pairwise relationships were used to compare survival and frequencies of defects and anomalies across exposure groups. Linear regression was used to assess independence of fish and pancreas length. A confidence level of 95% ($\alpha = .05$) was used. All sample sizes met or exceeded estimated Least Significant Number cutoffs to improve data rigor and reproducibility.

RESULTS

Larval Survival and Morphology

We previously demonstrated that 16 and 32 μM exposures to the C8 perfluorinated compound PFOS disrupt pancreatic organogenesis in the zebrafish (Sant et al., 2017). Here, we compare the toxicity of the increasingly utilized C4 compound PFBS on a 1:1 molar basis. Survival and embryonic morphology were examined in embryos exposed to 0 (0.01% v/v DMSO), 16 μM , or 32 μM PFBS (4.8 and 9.6 $\mu\text{g}/\text{ml}$, respectively). Whereas these exposure concentrations were several orders of magnitude lower than the estimated LC_{50} (1310 μM ; Probit analysis, 95% Confidence Interval = 881–3020 μM ; Supplementary Figure 1), they were only an order of magnitude lower when estimating individual embryonic load (7.5–15 \times ; 30 ml exposure media/10–20 embryos per dish). The exposure concentrations for which hatching and swim bladder inflation were significantly reduced (825 μM ; Supplementary Figure 1). Following exposures to either 16 or 32 μM PFBS, larval survival to 4 dpf was 100% (control), 96%, and 95%, respectively ($p > .05$).

To assess the impact of PFBS exposure on embryo growth, total anterior-posterior fish length was measured at both 4 and 7 dpf (Figure 1). Controls were statistically similar to historical data. At 4 dpf, there was a decreasing trend in fish length, though not statistically significant ($p > .05$). Embryos exposed to 16 ($3575.31 \pm 39.37 \mu\text{m}$) and 32 ($3499.01 \pm 58.40 \mu\text{M}$) PFBS had means indicating as a group that they were shorter than controls (3639.96 ± 19.30), but they exhibited highly skewed distributions of length. Notably, the incidence of stunted growth (< 10 th percentile of controls) was increased for embryos exposed to 16 ($p = .021$) and 32 ($p = .011$) μM PFBS compared with control embryos ($p = .015$ for trend). Furthermore, the incidence of severely stunted growth (< 1 st percentile for controls) was also significantly elevated for embryos exposed to both 16 ($p = .020$) and 32 ($p = .002$) μM PFBS ($p = .003$ for trend). Similar effects persisted at 7 dpf, although this was not statistically significant ($p > .05$).

Several morphological defects were observed at 4 dpf following PFBS exposures (Figure 2). Concordant with decreased fish length, increased incidence of 'blunt' (short, broad tail bud) and 'curly' tails was observed for embryos exposed to 16 ($p = .002$)

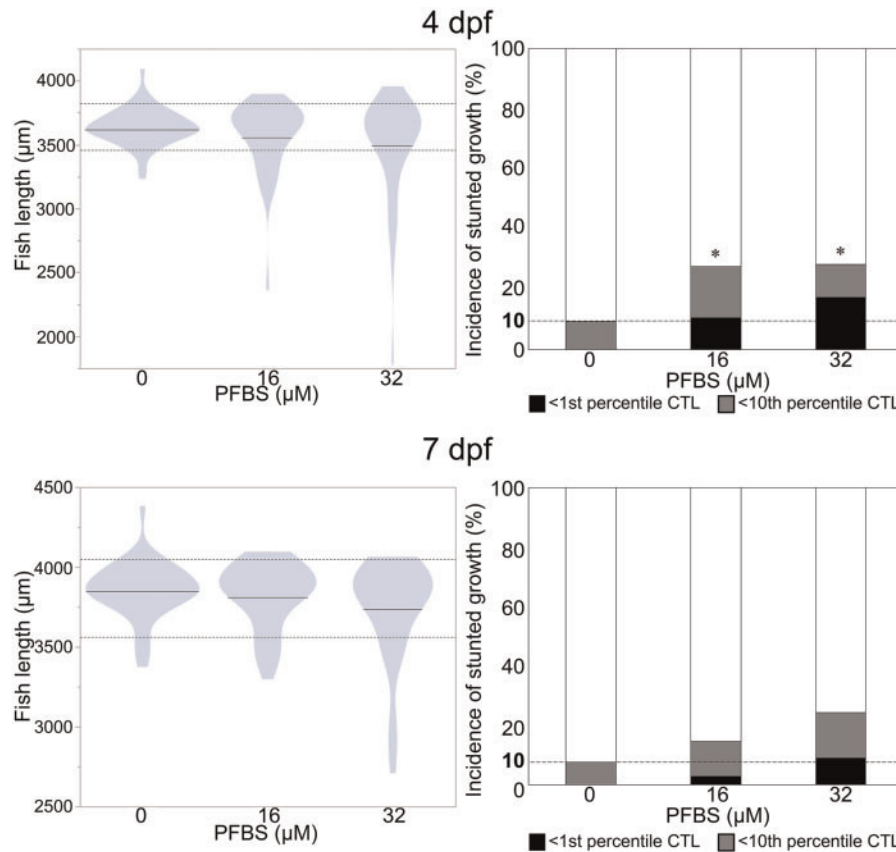


Figure 1. Embryonic PFBS exposures decrease embryonic growth at 4 and 7 dpf. Total rostral-caudal fish length was assessed following daily PFBS exposures beginning at 1 dpf. Controls in this study match historical data. Though mean fish length decreased with increasing PFBS exposure at 4 dpf, this change was not statistically significant. However, the incidence of fish with stunted growth (< 10th percentile of controls) and severely stunted growth (< 1st percentile of controls) was significantly increased with PFBS concentration. Though these trends were also observed at 7 dpf, they were not statistically significant. $N = 46$ – 53 embryos at 4 dpf, $N = 39$ – 45 embryos at 7 dpf, $\alpha = .05$.

and 32 ($p = .001$) μM PFBS. Embryos exposed to 16 μM PFBS had decreased incidence of swim bladder inflation ($p = .009$) and increased incidence of craniofacial malformations ($p = .028$). Though there were dose-dependent increasing trends for incidence of both pericardial edema and impaired yolk utilization, only embryos exposed to 32 μM PFBS had impaired yolk utilization ($p = .043$). Several of the defects co-manifested with other defects in embryos, but there were no consistent associations ($p > .05$).

Pancreas Extension

Posterior extension of the pancreas was measured at 4 and 7 dpf as previously described by Sant et al. (2017). Pancreas length was defined as the length of the pancreas from the primary islet stretching posteriorly, as visualized by GFP in *Tg(ptf1a:GFP)* embryos (Figure 3). PFBS exposures significantly decreased pancreas length at 4 dpf (Kruskal-Wallis $p = .012$). Embryos exposed to 16 μM ($333.98 \pm 13.42 \mu\text{m}$) PFBS had the most significantly decreased pancreas lengths compared with controls (379.91 ± 6.60 ; $p = .003$). Pancreas length means were not significantly decreased in embryos exposed to 32 μM PFBS (355.15 ± 17.08), though lengths were highly skewed. Incidence of shortened pancreata was increased in exposed embryos ($p = .020$), and several PFBS-exposed embryos had severely short (< 1st percentile of controls) pancreata. Changes to individual pancreas lengths were not correlated with decreased fish length ($R^2 = 0.4$) and pancreas length:fish length ratios significantly differed

between exposure groups ($p = .046$), indicating that decreased pancreas length is not dependent on decreased fish growth at 4 dpf. Occurrence of shortened pancreata and gross morphological defects were independent, suggesting that incidence of shortened pancreata was not correlated with presence of other embryonic malformations (χ^2 test of independence, $p > .05$). At 7 dpf, pancreas length and incidence of shortened pancreata were not significantly changed ($p > .05$).

Islet Development

Islet areas were measured at 4 dpf as previously described by Sant et al. (2016, 2017). Islets areas are a cross-sectional measure of islet size using *Tg(insulin:GFP)* embryos (Figure 4). No statistically significant differences were observed between control embryo islet areas (1075.50 ± 56.37) and embryos exposed to 16 (961.00 ± 59.21) or 32 (1018.84 ± 63.03) μM PFBS ($p > .05$). However, incidence of severely hypomorphic islets (areas lower than the 1st percentile of controls) and fragmented islets was significantly elevated with increased PFBS concentration ($p = .011$ and $p = .014$ for trends, respectively). These trends were also continued at 7 dpf, though at a lower incidence than 4 dpf.

Gene Expression

Gene expression was assessed in whole embryos exposed to DMSO, 16 μM PFBS, or 32 μM PFBS ($n = 3$ samples per group) (Figure 5). Following alignment and statistical analyses, significantly upregulated or downregulated genes were examined,

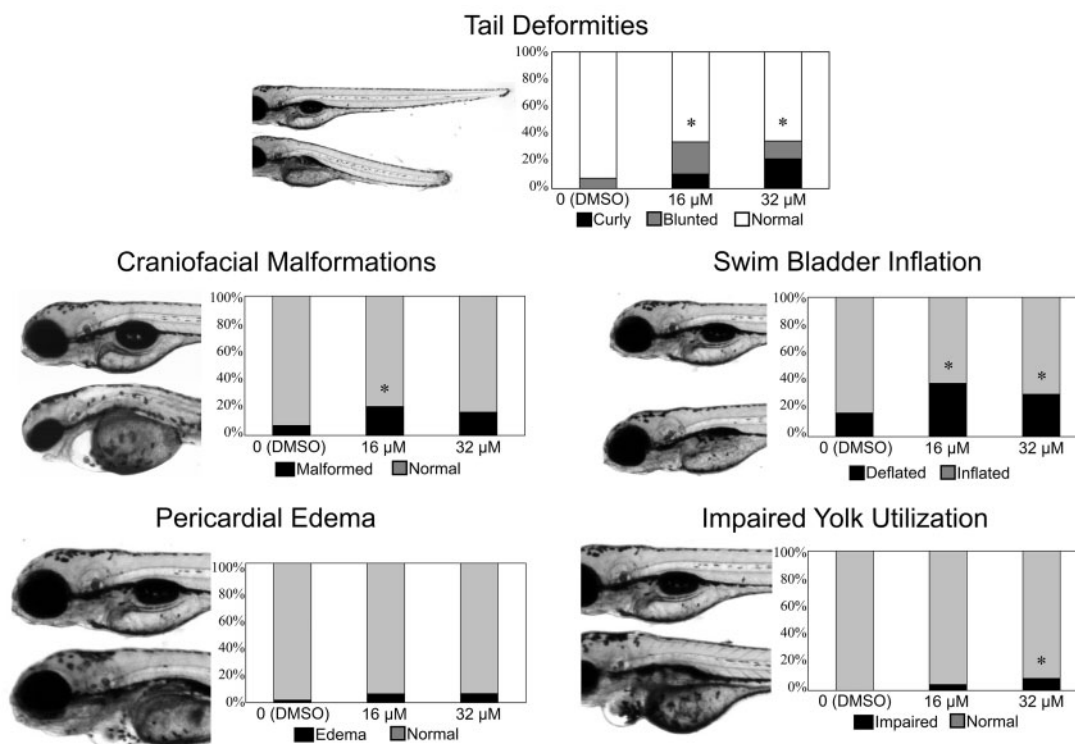


Figure 2. PFBS exposures increase the incidence of embryonic defects. Embryos were microscopically examined at 4 dpf to visualize developmental progress following daily PFBS exposures. The most common deformities observed were tail deformities, manifesting as ‘curly’ or ‘blunted’ tails ($p < .05$ for both PFBS concentrations). Increased incidence of craniofacial malformations and failure of the swim bladder to inflate was observed due to PFBS exposure, but only significant for 16 μ M exposures. Impaired yolk utilization (quantified by total yolk sac areas) followed an increasing trend, and was significantly increased due to 32 μ M PFBS exposure. $N = 46$ –53 embryos, $\alpha = .05$.

and compared across exposures ($FDR < 0.05$). Compared with controls, 38 genes were significantly changed by 16 μ M PFBS exposure, and 438 were changed following 32 μ M exposure. Twenty-five genes were significantly changed by both exposure concentrations (Supplementary Table 4)—two of which were significantly changed across all comparisons: *thyrotrophic embryonic factor a (tefa)* and *cytochrome P450, family 1, subfamily A (cyp1a)*. Because of the morphological changes observed, we more closely examined the expression of key genes involved in pancreas development and function (Table 1). None of the genes central for islet function were significantly affected by PFBS treatment, though most of the examined genes involved in exocrine pancreas function were upregulated, including *amylase alpha 2 A (amy2a)*, *carboxypeptidase A5 (cpa5)*, *chymotrypsinogen b1 (ctrb1)*, *trypsin (try)*, and *elastases ela2l and ela3l*. Gene expression of the pancreatic proteases *try* and *ctrb1* was more abundant (FPKM 380–505) than all other exocrine and endocrine pancreatic hormones, digestive peptides, and transcription factors (FPKM 3–204). Log ratios and q -values for all significantly changed genes are provided (Supplementary Tables 1–3). Genes significantly up- or downregulated by both PFBS concentrations compared with controls are listed in Supplementary Table 4. GO Biological Processes overrepresented ($p < .01$) by these genes included pathways involved in the response to hormone stimuli and signaling, response to lipid signaling, MAPK signaling, and glycolytic pathways.

LRpath was used to identify pathways significantly upregulated or downregulated by PFBS exposures in embryos using logistic regression (Sartor et al., 2009). Regression coefficients (direction and magnitude of change) were calculated for each KEGG pathway (<http://www.genome.jp/kegg/>), and pathway

hierarchies were determined using the KEGG database. Pathways significantly up- or downregulated ($p < .05$) were plotted to visualize functional clusters (Figure 5). Because of the large number of significantly changed metabolic pathways, subclasses were further distinguished using color. Pathways involved in lipid metabolism (blue circles) and xenobiotics metabolism (aqua circles) were significantly upregulated by both PFBS exposure concentrations, namely peroxisome proliferator-activated receptor (PPAR) signaling, steroid biosynthesis, and cytochrome P450 metabolic pathways. Other pathways were downregulated by both PFBS exposures, including the p53 signaling pathway and energy metabolism pathways such as glycolysis/gluconeogenesis and pentose phosphate pathways. All significantly changed pathways are provided (Supplementary Tables 5 and 6).

DISCUSSION

Perfluorinated compounds are a growing environmental health concern worldwide due to their persistence in both the environment and organisms. Whereas we know some of the health outcomes of the legacy perfluorinated compounds that are no longer being used in the United States (like PFOS), we know very little about the health consequences of exposure to the compounds replacing the longer chain PFAS congeners. The primary replacement for PFOS in the manufacture of consumer products has been PFBS, which has a C4 backbone rather than the C8 of PFOS. Though legacy compounds such as PFOS are still widely detected in environmental and biological samples, increased replacement in manufacturing worldwide prompts the necessity

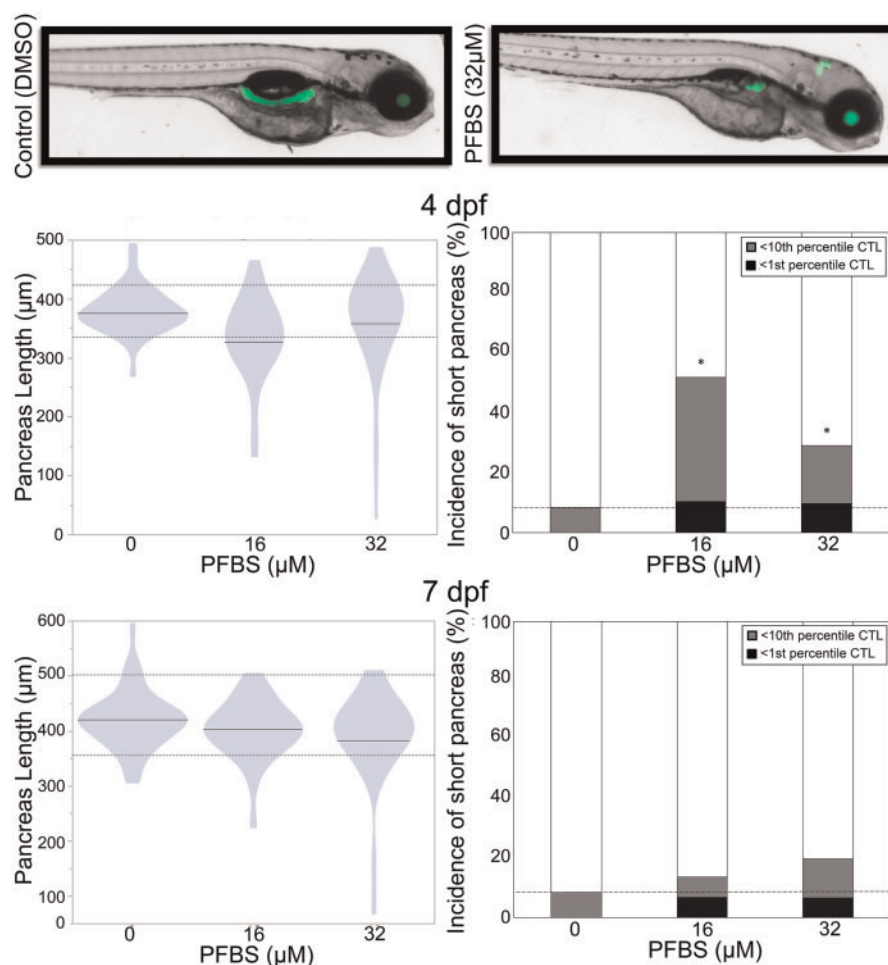


Figure 3. Embryonic exposures to PFBS increase the incidence of shortened pancreata at 4 and 7 dpf. Pancreas extension (islet to tail) was assessed in *Tg(ptf1a:GFP)* embryos exposed daily to PFBS. Current controls matched historical data. Though mean pancreas lengths were similar, the distribution of lengths was increasingly variable with increasing PFBS concentration. All embryos with pancreata shorter than the 10th percentile for controls were considered 'short.' Embryos exposed to both 16 and 32 μ M PFBS had increased incidence of shortened pancreata compared to controls ($p < .05$). To assess the degree of severity, embryos less than the minimum length for control embryos were considered 'severely shortened' (represented by the black portion of the stacked bar graph). For both 16 and 32 μ M PFBS exposures, approximately 10% of embryos had severely shortened pancreata. Though these trends were still observed at 7 dpf, these changes were not statistically significant. $N = 29\text{--}36$, $\alpha = .05$.

of investigation into these replacement compounds. Here, we examine the developmental toxicity of PFBS in comparison with our previous study that identified aberrant pancreas development in the zebrafish embryo when exposed to PFOS (Sant et al., 2017).

We have previously demonstrated that the Islets of Langerhans are highly sensitive targets of toxicant exposures (Brown et al., 2018; Jacobs et al., 2018; Sant et al., 2016, 2017; Timme-Laragy et al., 2015). In this study, we did not observe any significant changes in endocrine islet area, though we did observe increased incidence of islet morphological variants including fragmented islets. In our previous study, we observed significantly decreased islet area due to PFOS exposures and increased incidence of islet morphological variants (Sant et al., 2017). Increased islet dysmorphogenesis was observed in this study, namely increased incidence of severely hypomorphic and fragmented islets. Overall this data suggests that the Islets of Langerhans are still susceptible targets of embryonic PFBS exposures, but that PFBS may not be as deleterious as PFOS exposures when compared at a 1:1 molar basis.

In this study, pancreas lengths were slightly shorter at both 4 and 7 dpf, though not statistically significant at 7 dpf ($p > .05$). In our previous study, PFOS significantly decreased pancreas length at these concentrations, which suggests that PFOS may be more deleterious for pancreas organogenesis than PFBS. However, embryos exposed to PFBS followed significantly skewed distributions toward shortened pancreata. Those affected had severely shortened pancreata, falling well below current and historical control pancreas lengths. Some of these embryos had as much as an 80% reduction of pancreas length compared to the average control length, more severe than any observed reduction of length due to embryonic PFOS exposures. This data suggests that there might be a vulnerable subpopulation which may be especially susceptible to pancreas dysmorphogenesis, although the biological basis for this susceptibility is unknown.

Several congenital malformations of the pancreas have been observed in humans, some of which are phenotypically similar to what we have observed in the PFBS-exposed zebrafish larvae. Pancreatic dorsal agenesis (complete or partial) is a rare congenital malformation of the pancreas, in which the tail of the

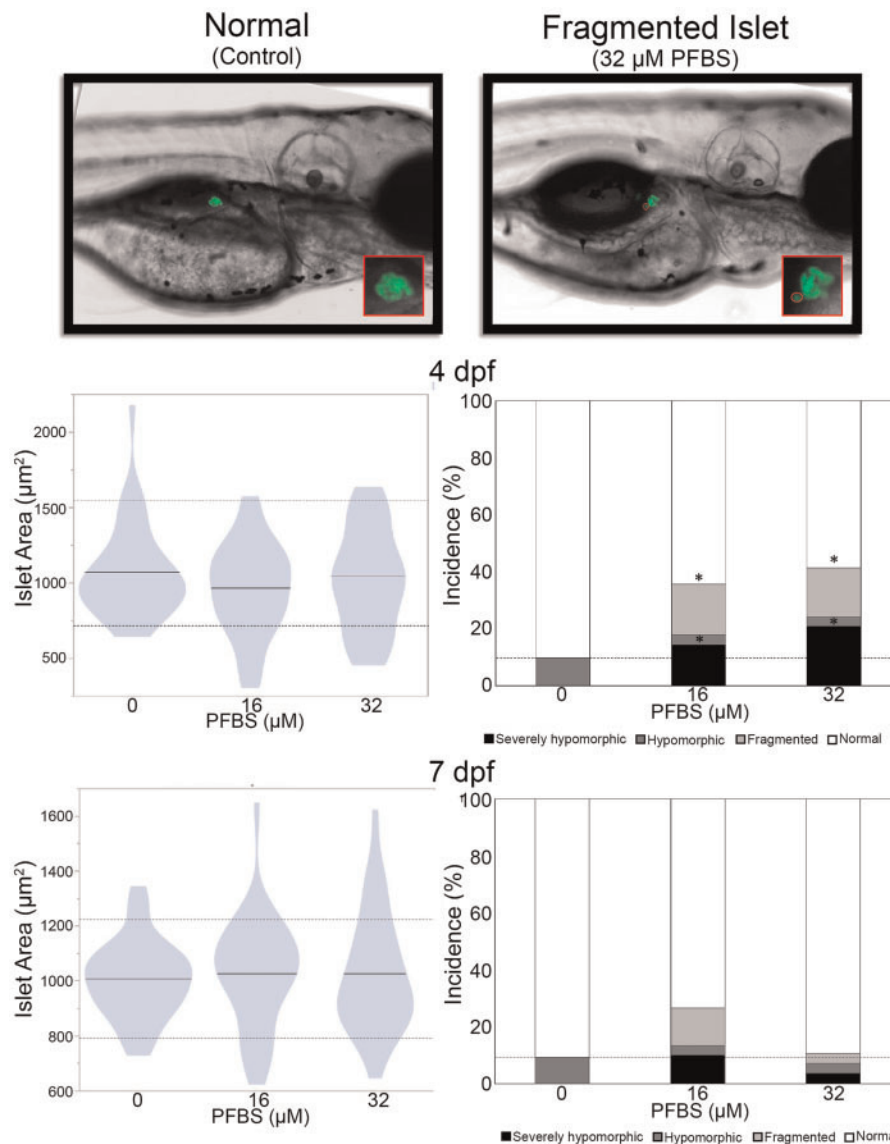


Figure 4. PFBS exposures increase incidence of islet morphological variants in embryos. Islet areas and morphology were assessed using fluorescence microscopy in *Tg(insulin:GFP)* embryos exposed daily to PFBS at 4 and 7 dpf. Current controls matched historical data. PFBS did not alter islet areas, as quantified by fluorescent areas. However, PFBS exposures did increase the incidence of severely hypomorphic and fragmented islets at 4 dpf. $N = 29\text{--}32$, $\alpha = .05$.

pancreas fails to form or is significantly impaired, resulting in a drastically shortened pancreas (Vaughn *et al.*, 1998). Complete agenesis is quite rare, being reported in the literature fewer than 100 times in the past 100 years (Cho *et al.*, 2013). However, a milder phenotype, pancreas hypoplasia, is characterized by a reduction of the total size of the pancreatic tail—the region of the human pancreas which contains the most concentrated islets (Cho *et al.*, 2013). Because screening for these phenotypes is not common practice, they often go unrecognized until relevant symptoms such as severe abdominal pain and/or pancreatitis, or onset of Type I Diabetes present (Cho *et al.*, 2013; Schnedl *et al.*, 2009). Because ultrasound-based elastography or tomography examination techniques are non-invasive, incorporation into standard screening procedures may help identify individuals with significantly increased susceptibility to diabetes and pancreatic diseases (Cho *et al.*, 2013; Ozturk and Yildirim, 2017). Incorporation of such screening into environmental epidemiology studies could also help identify the

relationship between pancreas malformations and chemical exposures within the human population.

We assessed fish length and pancreas morphology at both 4 and 7 dpf, and the incidence of aberrant morphologies present at 4 dpf was reduced at 7 dpf. Because changes to individual pancreas lengths were not correlated with decreased fish length, these pancreas effects are unlikely to occur because of a global developmental delay; however, it may be these earlier phenotypes are due to organ-specific developmental delays, or that these phenotypes may trigger an adaptive response aimed to compensate for decreased pancreatic growth. It has been repeatedly demonstrated that endocrine and exocrine pancreas cells can regenerate in the zebrafish model (Moss *et al.*, 2009; Prince *et al.*, 2017) and in mammals (reviewed in: [Bonner-Weir *et al.*, 2010; Murtaugh and Keefe, 2015]). Because embryos in these experiments were collectively exposed and not individually tracked, we cannot determine whether this amelioration of aberrant phenotypes occurred. In future work, individual

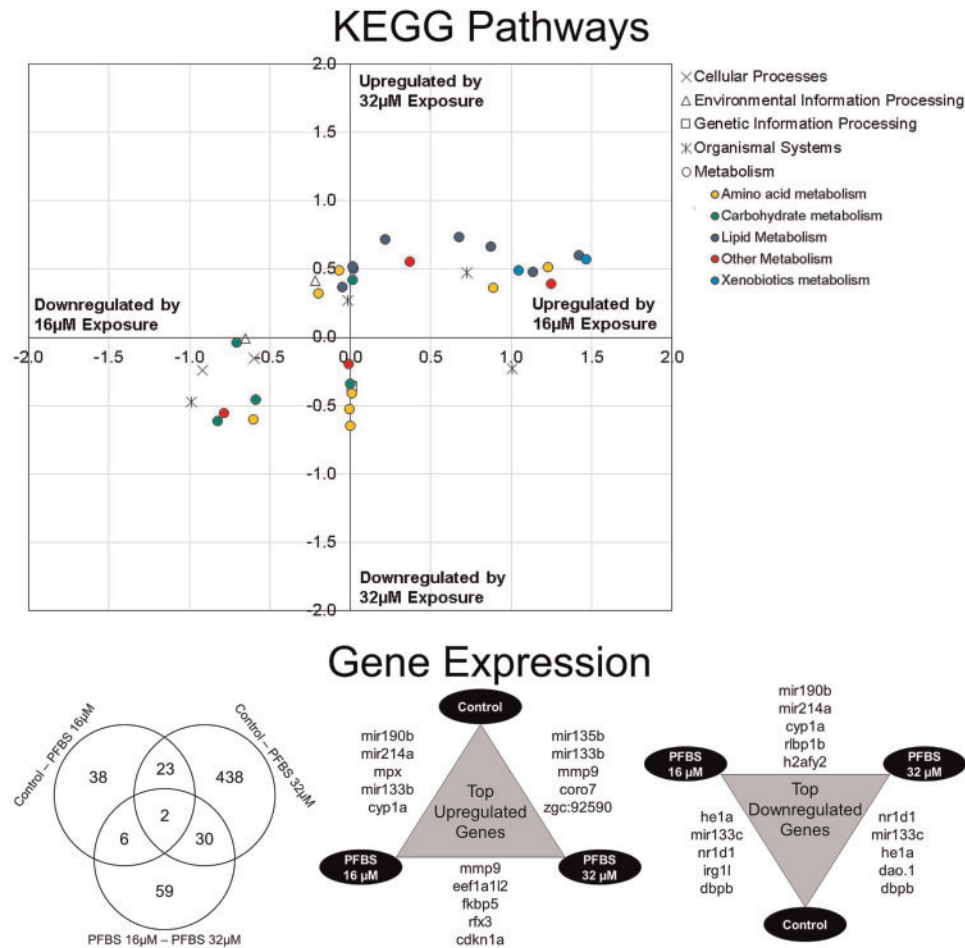


Figure 5. Embryonic PFBS exposures disrupt gene expression of metabolic targets. Expression of 38 and 438 genes were significantly altered by 16 and 32 µM PFBS exposures, respectively. Twenty-five total genes were significantly changed by both exposure concentrations, including 2 genes which were significantly changed across all group comparisons. The most significantly up- and downregulated genes by PFBS treatment are shown, and include several key genes involved in cell cycle and metabolic regulation. Pathways involved in lipid metabolism and xenobiotic metabolism were upregulated by both PFBS concentrations. Several key glucoregulatory pathways, including glycolysis/gluconeogenesis and the pentose phosphate pathway were downregulated by both PFBS exposure concentrations. $N = 3$ samples of 15 pooled embryos per exposure group, $FDR < 0.05$.

Table 1. Expression of Genes Involved in Pancreas Development and Function (Bold = Significant Change, $FDR < 0.05$)

	FPKM (% Change)		
	Control	PFBS (16µM)	PFBS (32 µM)
Endocrine pancreas genes			
<i>gcga</i>	6.45	6.63 (+3.5%)	6.59 (+1.4%)
<i>insa</i>	35.75	47.55 (+33%)	40.79 (+14.1%)
<i>nkx2.2a</i>	8.34	8.28 (−0.7%)	7.78 (−6.7%)
<i>pdx1</i>	3.97	3.86 (−2.1%)	4.44 (+11.7%)
<i>sst2</i>	13.27	13.18 (−0.7%)	16.34 (+22.3%)
Exocrine pancreas genes			
<i>amy2a</i>	20.53	27.10 (+32%)	30.70 (+49.5%)
<i>cpa5</i>	165.42	183.55 (+11%)	203.66 (+23.1%)
<i>ctrb</i>	407.31	458.25 (+12.5%)	504.95 (+24%)
<i>ela2l</i>	122.79	128.89 (+5%)	149.09 (+21.4%)
<i>ela3l</i>	71.51	77.17 (+9.4%)	109.14 (+53.7%)
<i>ptf1a</i>	2.69	2.95 (+9.4%)	2.93 (+7.2%)
<i>try</i>	380.04	424.61 (+12.5%)	442.64 (+16.5%)

Numbers presented are the Fragments per Kilobase of Transcript per Million mapped reads (FPKM).

tracking could be performed to determine correlation between early dysmorphogenesis and recovery at later timepoints.

A growing number of studies have demonstrated that perfluorinated compounds, including PFOS, can alter PPAR signaling pathways and lipid metabolism, including our previous work investigating zebrafish embryonic PFOS exposures at these concentrations (Sant et al., 2017, 2018). In this study, expression of 25 genes was significantly changed by both 16 and 32 µM PFBS exposure, including a number of cell cycle regulators, xenobiotic responders, and lipid and glucoregulatory genes (Supplementary Table 4). Examination of significantly changed pathways revealed several clusters of similar pathways being either up or downregulated by both exposures. A large cluster of pathways involved in lipid metabolism was upregulated by PFBS exposures, namely pathways involved in PPAR signaling and lipid biosynthesis. In this study, gene expression of *pparaa*, *pparab*, and *pparg* were not significantly altered by PFBS exposure. However, numerous PPAR targets were significantly altered by PFBS exposures, namely fatty acid binding proteins (*fabp2* and *fabp1b1*) and apolipoproteins (*apo1a*, *apo2*, and *apo4b*). Therefore, the changes in gene expression found in this study support previous works demonstrating the ability of PFBS

to perturb regulation of lipid metabolism (Bijland *et al.*, 2011; Gorrochategui *et al.*, 2014; Qi *et al.*, 2018).

We mined our RNA-Seq data to more closely examine gene expression of pancreas hormones, digestive peptides, and key transcription factors. Gene expression of insulin did not change due to PFBS exposure, which is concordant with islet area data. Other islet gene expression was also unchanged by treatment, which suggests that islet endocrine function may not be a primary target of embryonic PFBS exposures. Interestingly, other glucoregulatory pathways including glycolysis/gluconeogenesis were downregulated, suggesting that the glucoregulatory changes observed due to PFBS exposure are not mediated by the endocrine pancreas. However, expression of several exocrine pancreas digestive peptides was increased 17%–50% due to PFBS exposures, namely *amylase, alpha 2 A (amy2a)*, *carboxypeptidase A5 (cpa5)*, *chymotrypsinogen b1 (ctrb1)*, *trypsin (try)*, and *elastases ela2l* and *ela3l*. In our previous PFOS study, expressions of genes that encode for several of these enzymes were significantly decreased due to embryonic exposures. This data depicts a contradictory effect between PFOS and replacement compound PFBS. Though these digestive peptides are secreted primarily by acinar cells in the pancreas, the exocrine pancreas is a heterogeneous population of cell types. Therefore, it is possible that the secretory acinar cells are specifically stimulated by PFBS exposures. However, because these digestive enzymes are secreted in their mature forms from acinar cells, increased gene expression may be a compensatory mechanism to overcome exocrine insufficiency or deficient enzymatic function.

When comparing embryotoxicity of PFOS to PFBS, we concur with previously published findings in zebrafish that PFBS is less toxic than PFOS (Hagenaars *et al.*, 2011; Ulhaq *et al.*, 2013). Our study adds to a growing body of literature that aims to understand the potential health effects of PFAS chemicals. Uniquely, our findings demonstrate that PFBS can disrupt pancreatic organogenesis, and perturb expression of genes involved in lipid metabolism. Though PFBS increases endocrine and exocrine pancreas dysmorphogenesis, the overall impact is not as severe when compared to prior findings with embryonic PFOS exposures. However, the effects of PFBS exposures follow skewed distributions, suggesting that vulnerable subpopulations may be disproportionately sensitive to exposures during development.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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