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Developmental perfluorobutanesulfonic acid (PFBS) exposure alters exocrine pancreas function in zebrafish (*Danio rerio*)

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Original Article

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Abstract

Embryonic exposures to perfluorobutanesulfonic acid (PFBS) in zebrafish have been shown to impair pancreas growth. This study investigated the persistence of the truncated pancreas phenotype, functional impacts on digestive enzyme production, and the hypothesis that redox stress contributes to these outcomes. Transgenic ptf1a:GFP zebrafish were exposed to 0 (0.01% dimethyl sulfoxide (DMSO)), 16, and 32 μM PFBS from 24 to 96 h post-fertilization (hpf) and raised in clean water to 15 days post-fertilization (dpf). Confocal microscopy revealed the pancreata regained normal size, suggesting "catch-up" growth. To evaluate the importance of redox stress in this recovery and acinar cell function, wild-type or mutant zebrafish embryos with a mutation in the DNA-binding domain of the Nrf2a transcription factor were exposed to PFBS from 3 to 96 hpf and raised to 9 dpf in clean water. At 9 dpf, Nrf2a mutants had shorter exocrine pancreases than wild-types with the same treatment, indicating this phenotype likely involves redox stress. Expression of pancreatic proteases and Trypsin were examined to assess functional changes. Although protease gene expression did not differ significantly at 4 or 9 dpf, Trypsin levels decreased with increasing PFBS concentrations in wild-type larvae at 6 and 9 dpf. Lipid analysis showed no change in cholesterol but lower triglycerides in Nrf2a mutants. Assessment of glutathione utilization using monochlorobimane fluorescence indicated more robust antioxidant responses in wild-type fish. This study increases understanding of how Nrf2a modulates the response to PFBS exposure, impacts on exocrine pancreas function, and has implications for understanding "catch-up" growth in response to embryonic toxicant exposures.

Introduction

Per-and poly-fluoroalkyl substances (PFAS) are a class of persistent toxicants known to negatively affect organism growth and development, as demonstrated by epidemiological, *in vitro*, and *in vivo* studies. PFBS, a PFAS of rising concern, is a short chain (4-carbon) PFAS replacement for the long-chain (8-carbon) perfluorooctanesulfonic acid (PFOS). PFOS was used in commercial and industrial products for decades but was phased out of production in the United States in the 2000s. PFBS has replaced PFOS in many products, including metal plating, flame retardants, and firefighting foams.[1] It has a shorter half-life compared to PFOS (1 month vs ~5.4 years)[2] and therefore is thought to have lower toxicity and bioaccumulation.[3] However, PFBS is becoming concentrated in the environment.[4–7] PFBS is increasingly found in the human population, particularly in children; while 0.6% of the U.S. population over age 12 had detectable levels of PFBS in serum, the concentrations were notably higher (9%) in children ages 3-11 compared to adults.[8] Additionally, PFBS can cross the placenta and has been detected in pregnant women and infants,[9,10] with higher PFBS levels in maternal umbilical cords positively associated with measurable increases in waist circumference and body fat percentage in female children[11] suggesting altered metabolism. The prevalence, increasing concentration, and critical window of exposure to PFBS during early life raises concerns for later-life health consequences, as per the Developmental Origins of Health and Disease framework.

The pancreas regulates a variety of metabolic functions and is a sensitive target of environmental contaminants,[12,13] The endocrine pancreas contains the Islets of Langerhans, regulating blood glucose, while the exocrine pancreas provides most of the organ mass, synthesizing and releasing digestive enzymes required for metabolism[14] such as amylase, lipase, trypsin, chymotrypsin, and elastase.[15] Impaired exocrine pancreas function can lead to exocrine pancreatic insufficiency (EPI), resulting in inability to maintain normal digestion.[16] Developmental PFOS exposure in zebrafish reduces pancreas length and expression of pancreatic enzymes, including proteases at 4 days post-fertilization (dpf),[17] and reduces protease activity, Trypsin, and digestive protease gene expression at 6 dpf.[18] Developmental PFBS

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exposure increased incidence of the truncated pancreas phenotype characterized by failure of the pancreatic tail to fully extend and reduced expression of genes encoding pancreatic digestive enzymes at 4 dpf.[12] However, it is unknown what mechanisms contribute to these effects and whether or not they persist to later stages.

The pancreas is highly susceptible to oxidative stress due to its high metabolic activity and endogenous production of reactive oxygen species (ROS) while having low levels of antioxidant defenses.[19,20] Environmental contaminants, such as PFBS, can lead to an increase in oxidative stress, defined as an imbalance between ROS and antioxidant defense systems leading to a loss of redox signaling and control.[21–23] During embryonic development, perturbations in the complex intracellular redox environment at critical stages can alter progenitor cell fate, impacting proliferation, differentiation, and cell death. As such, the embryo has antioxidant defense systems that help to regulate and prevent damage from oxidation,[24] the most abundant of which is glutathione, a tripeptide made of glutamate, cysteine, and glycine.[25] Enzymes involved in glutathione synthesis can be upregulated by nuclear factor erythroid 2-related factor 2 (NFE2L2 or Nrf2),[26] a transcription factor that regulates the adaptive response to oxidative stress. Under oxidative conditions, Nrf2 translocates to the nucleus and increases expression of genes regulated by antioxidant response elements in the promoter regions, including many involved in glutathione synthesis and utilization.[27] PFBS is thought to induce oxidative stress through indirect mechanisms such as disruptions in mitochondrial function and lipid homeostasis that increase reactive oxygen species.[28–30]

Zebrafish are a powerful tool for identifying effects of environmental toxicants. Their rapid growth allows for effects of toxicants to be seen quickly,[31] and produce large clutches that have synchronized fertilization. Larval zebrafish are optically transparent[32] allowing for easy visualization of the pancreas through transgenic lines.[14] In zebrafish, development of the pancreas begins around 24 h post-fertilization (hpf) and has a similar cellular composition to a mammalian adult pancreas by 5 dpf.[14,33,34] An evolutionary genome-wide duplication in zebrafish led to two Nrf2 paralogs, Nrf2a and Nrf2b. While some functional redundancy exists, these paralogs largely have partitioned roles, with Nrf2a being most functionally similar to mammalian Nrf2 and acting as the primary response to oxidative stress.[35] Mutation of the Nrf2a pathway is not embryolethal and does not cause developmental impairment. However, when exposed to toxicants, embryos with impaired or mutated Nrf2a function can be more sensitive to toxicants, particularly those that are redox-active.[17]

This study examined the impact of PFBS on exocrine pancreas protease function and the role of Nrf2a in a zebrafish model. We hypothesized that impairment of the Nrf2a pathway would negatively affect development and function of the exocrine pancreas as well as increase oxidative stress.

Materials and methods

Chemicals

Perfluorobutanesulfonic acid potassium salt (PFBS, 97% Purity, Sigma–Aldrich Catalog #562629; St Louis, MO) was stored at 4°C. Dimethyl sulfoxide (DMSO, Fisher Scientific, Catalog #D126-1; Fairlawn, NJ) was stored at room temperature. Monochlorobimane (MCB, Catalog #M1381MP, ≥70% Purity), stored at −20°C, and Pierce™ BCA Protein Assay Kit (Catalog #A55864 and #A55865), stored at room temperature, were both purchased from Thermo Fisher Scientific (Waltham, MA).

Zebrafish strains

Tg(Nrf2a$^{+/+}$*;ptf1a:GFP)* and *Tg(Nrf2a*$^{fh318/fh318}$*;ptf1a:GFP)* zebrafish were used for these studies. Heterozygous fish with the Nrf2a$^{fh318/fh318}$ mutation were generated by the TILLING mutagenesis project (R01HD076585) and bred to homozygosity on an AB strain. Genotypes were confirmed using PCR (previously described in[36]). The *nrf2a*$^{fh318/fh318}$ genotype has a point mutation that produces a mutant amino acid sequence in the DNA-binding domain of the Nrf2a protein causing impaired transcriptional activity and is considered a loss-of-function mutation.[37] Adult homozygous wild-types and mutants were crossed with *Tg(ptf1a:GFP)* zebrafish, which express green fluorescent protein (GFP) in the exocrine pancreas as well as the retina and hindbrain (obtained from the University of Massachusetts Chan Medical School) ((Worcester, MA)[38]). All breeding adults were genotyped to confirm homozygosity through Transnetyx (Cordova, TN).

Zebrafish husbandry

Adult zebrafish (*Danio rerio*) were maintained in the Timme-Laragy aquatics facility under the guidance and approval of the University of Massachusetts Institutional Animal Care and Use Committee and housed on an automated zebrafish system (Danio Lab, Boston, MA) at 28.5°C, 14:10 h light:dark cycle. Zebrafish were fed GEMMA Micro 300 (Skretting, Westbrook, MA) twice daily. Fish were bred in 9L tanks, male to female ratio 1:2–3. Embryos used for experiments were collected at 1 hpf, rinsed with egg water, placed in 0.3X Danieau's medium (17 mM NaCl, 2 mM KCl, 0.12 mM $MgSO_4$, 1.8 mM $Ca(NO_3)_2$, 1.5 mM HEPES, pH 7.2) in polystyrene petri dishes, and maintained at a temperature of 28.5°C, 14:10 h light:dark cycle.

Exposures

Stock solutions of 0 (100% DMSO), 80, 160, and 320 mM PFBS prepared in 100% DMSO were stored at 4°C. Embryos were exposed at 3 hpf after confirming proper developmental stage[39] to PFBS concentrations of 0 (0.01% DMSO), 8, 16, or 32 µM PFBS by diluting stock solutions in 0.3X Danieau's medium. Exposures were conducted with 1 ml of exposure solution/embryo either individually in 24-well plates or in polystyrene petri dishes in group exposures. At 1 dpf, embryos were manually dechorionated, and daily solution changes were performed until 4 dpf when exposure was complete and fish were rinsed in clean Danieau's.

Fish grown to 6 dpf were grown in petri dishes in clean Danieau's from 4 to 6 dpf with a 50% water change performed at 5 dpf. Fish grown to 9 dpf, were grown in petri dishes from 4-5 dpf in 50% Danieau's/50% system water and fed a small amount of Gemma 75 (Skretting) before being placed into 0.8L or 1L tanks ($\leq n = 35$ fish/tank) at 5 dpf and maintained in clean water on a stand-alone Aquaneering rack, and fed Gemma 75 twice daily until 9 dpf. Fish were fasted overnight immediately before 9 dpf timepoint to minimize gut autofluorescence.

For 15 dpf endpoints, *Tg(ptf1a:GFP)* zebrafish were exposed to 0, 16, or 32 µM PFBS from 24 to 96 hpf with daily solution changes. Fish were grown from 4-5 dpf in clean Danieau's. At 5 dpf, fish were placed into 0.8L tanks ($\leq n = 30$ fish/tank) and maintained on a stand-alone Aquaneering rack in clean water and fed Gemma 75 twice daily until 15 dpf.

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Morphological analysis

Tg(Nrf2a$^{+/+}$;ptf1a:GFP) and *Tg(Nrf2a$^{fh318/fh318}$;ptf1a:GFP)* embryos were exposed individually in 24-well plates to PFBS from 3 to 96 hpf and imaged using an upright fluorescent microscope (FBS10 Fluorescence Biological Microscope, Kramer Scientific) with a monochromatic camera (Zeiss) at 4 or 9 dpf.

At 4 and 9 dpf, larvae were anesthetized using a 2% v/v MS-222 solution (tricaine mesylate, 4 mg/ml, pH buffered, stored at −20°C until thawed for use) and staged laterally on their left side in 3% methylcellulose. Full-body images were taken with a 20X objective and pancreas images at 100X using a GFP filter and brightfield. Larvae were fixed in 4% paraformaldehyde (PFA) overnight at 4°C. At 4 dpf, due to pigment inhibiting our ability to visualize the entire exocrine pancreas at this time point, fish were bleached as previously described after fixing and imaged.[40] All fish were stored in 25% glycerol at 4°C.

At 15 dpf, the larvae were fixed, stored in 25% glycerol at 4°C, and imaged on an inverted Nikon A1R confocal microscope at the Nikon Center of Excellence Light Microscopy Facility in the Institute of Applied Life Sciences at the University of Massachusetts Amherst.

For volume images, exocrine pancreata were imaged on an inverted Nikon A1R confocal microscope at the Nikon Center of Excellence Light Microscopy Facility in the Institute of Applied Life Sciences at the University of Massachusetts Amherst. Fish were staged laterally on their right side on MatTek dishes, and 3D images of the pancreas were taken using the 20X objective with a 0.75 numerical aperture. Z-stacks (1 μM step size; 1024 × 1024, 0.623 μM/pixel) were taken using resonant scanning with a laser power of 1.7 and a gain of 32 for all images. Images were denoised and exocrine pancreas volume measured as previously described.[18]

Gene expression

RNA isolation and cDNA prep

Tg(Nrf2a$^{+/+}$;ptf1a:GFP) and *Tg(Nrf2a$^{fh318/fh318}$;ptf1a:GFP)* embryos were exposed to PFBS from 3 to 96 hpf in petri dishes. At 4 and 9 dpf, fish were pooled into biological replicate groups of 7 fish each (3–5 individual replicate pools per treatment group) and stored in RNAlater (Invitrogen) at −80°C. RNA was isolated using a Zymo Direct-zol RNA Miniprep Kit (Catalog #R2053) per manufacturer instructions. RNA concentration was confirmed using a BioDrop μLite spectrophotometer. RNA was reverse transcribed using an iScript cDNA Synthesis Kit (BioRad), diluted 1:10 with nuclease free water, final concentration 100 ng/μL, and stored at −20°C.

qPCR

qPCR samples were prepared to a final volume of 20 μL consisting of 10 μL iQ SYBR Green Mastermix, 1 μL of a mixture of forward and reverse primers, 5 μL nuclease free water, and 4 μL cDNA. Primers used were previously optimized and sequences can be found in Supplementary Table S1. Analysis was performed in duplicate using a CFX Connect Real-Time PCR Detection System and CFX Manager software (BioRad). The following genes were analyzed: *trypsinogen (try), chymotrypsinogen B1 (ctrb1),* and *elastase A, (elaA)*. The housekeeping genes *β-actin (actb)* and *elongation factor 1Alpha (elf1a)* were analyzed and no differences in expression between treatment groups were observed. The geometric mean of *actb* and *elf1a* was used to calculate fold change of *try*, *ctrb1*, and *elaA* using the $\Delta\Delta C_T$ method.[41]

Protein extraction

Protein was quantified using the Pierce BCA Protein Assay (Thermo Scientific, Catalog #A55864) per manufacturer instructions. Protein was extracted from separate samples for the total cholesterol/triglyceride assays, and Trypsin western blot.

Trypsin western blot

SDS-PAGE

Zebrafish embryos were exposed to PFBS from 3 to 96 hpf in petri dishes. Larvae at 6 and 9 dpf were pooled ($n = 20$) and lysed in RIPA buffer (Thermo Scientific, Catalog #89900) containing protease inhibitor cocktail (Thermo Fisher, Catalog #7830). Samples were prepared in Laemmli sample buffer (BioRad) with 100 mM DL-dithiothreitol, boiled at 95°C for 10 min, and stored at −80°C until use. 25 μg of protein from each sample were resolved by SDS-PAGE in 12% Mini-PROTEAN TGX Stain-Free gels (BioRad) at 200 V for 30 min and transferred to nitrocellulose membranes (BioRad) at 100 V for 60 min.

Immunoblotting

Transfer membranes were rinsed in tris-buffered saline with Tween 20 (TBST) and blocked in TBST + 5% Milk at room temperature for 1 h. Membranes were incubated overnight with Trypsin primary polyclonal antibody (1:1000, Thermo Scientific, Catalog #PA5-106876) at 4°C and washed 4x in TBST. Membranes were incubated in HRP-conjugated mouse β-actin (1:20,000, Proteintech, Catalog #HRP-66009) and appropriate HRP-conjugate secondary antibody for 1 h at room temperature and washed 4x in TBST. Enhanced chemiluminescence (BioRad, Clarity Western ECL Substrate) was used to develop the immunoblots. Images were taken with a Syngene PXi gel imager and band intensity calculated using GeneTools software.

Total cholesterol and triglyceride assays

Zebrafish embryos were exposed to PFBS from 3 to 96 hpf in petri dishes. Larvae were pooled in groups of 20 at 4 dpf and 15 at 9 dpf into microcentrifuge tubes in 160 μL of 0.05% Tween 20 in RO water and immediately frozen at −80°C. Before use, fish were thawed and homogenized using a hand-held homogenizer. Total cholesterol and total triglycerides were measured through colorimetric assays using Infinity Cholesterol and Triglyceride Reagents (Thermo Scientific, Catalog #TR13421 and #TR22421) with 7-point (0–7.5 mg/ml cholesterol) and 6-point (0–10 μg/ml glycerol) standard curves, respectively. Protein was quantified using the Pierce BCA Protein Assay (Thermo Scientific, Catalog #A55864). Assays were read on a Cytation 3 (Biotek) plate reader using black 96-well plates. Total cholesterol and triglycerides were normalized to total protein content.

Monochlorobimane staining

Monochlorobimane (MCB) is a cell-permeable, non-fluorescent bimane dye that fluoresces when conjugated with GSH in a GST-dependent reaction and therefore serves as a proxy for reduced glutathione levels and conjugation activity in zebrafish.[42] Embryos were exposed to PFBS in 24-well plates from 3 to 96 hpf. MCB was diluted to 200 mM, aliquoted, and stored protected from light at −20°C. Fish were stained in 3 ml of 200 μM MCB solution in glass scintillation vials for 1 h in groups of ≤10 at 28.5°C, then rinsed with fresh Danieau's over ice as previously described.[42] Larvae were imaged using the EVOS FL Auto Microscope. Fluorescence

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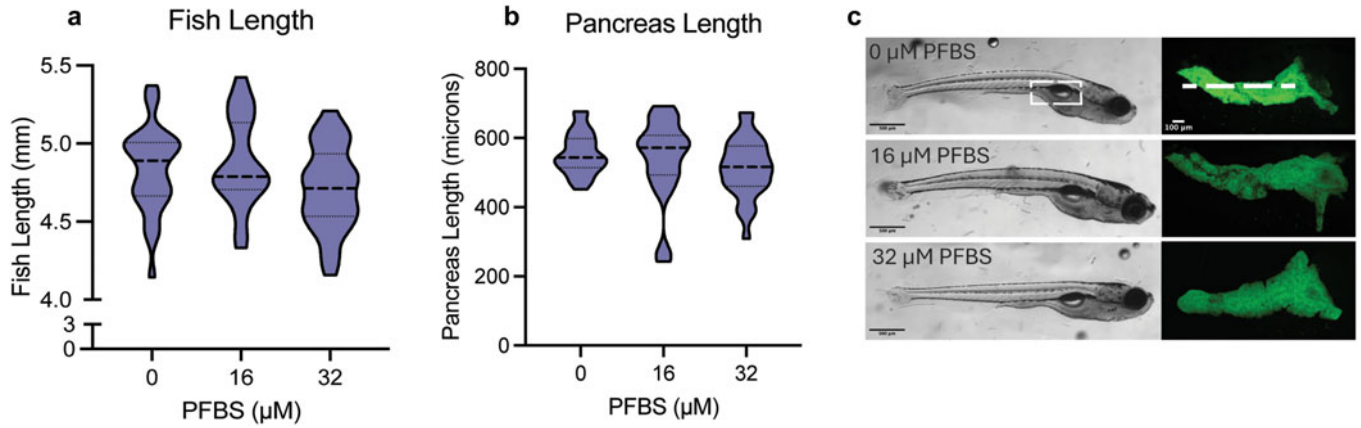


Figure 1. Larvae and pancreas length at 15 dpf following developmental PFBS exposure. *Tg(ptf1a:GFP)* zebrafish were exposed to PFBS from 24 to 96 hpf. Fish length and exocrine pancreas length were measured at 15 dpf. (a) Fish length ($n = 19–30$) with significance determined by one-way ANOVA with Tukey's multiple comparison test and (b) pancreas length ($n = 19–29$) with significance determined by Kruskal–Wallis test. (c) Representative images at 15 dpf.

intensity was measured in the exocrine pancreas, hindbrain, lens, gut, heart, and yolk.

Data analysis and statistics

Images were blinded and analyzed using ImageJ. Statistical tests were performed using GraphPad Prism 9 and 10. Data were assessed for normality and analyzed using one-way ANOVA or two-way ANOVA with Tukey's multiple comparison test. Graphs presented as mean and standard error of the mean.

Results

Effect of PFBS and Nrf2a on larval development and pancreas size at 4, 9, and 15 dpf

At 15 dpf, no significant differences in fish or pancreas length were observed with 16 and 32 μM PFBS compared to controls in *Tg(ptf1a:GFP)* fish (Figure 1). The absence of significant effects at this time point suggests catch-up growth following the truncated pancreas phenotype seen at 4 dpf.[12] Therefore, this study focused on 4 and 9 dpf to better understand the timing of catch-up growth, its functional impacts on acinar cell function, and Nrf2a as a possible mechanism of action.

At 4 dpf, immediately after exposure, Nrf2a mutant fish exposed to 16 μM PFBS showed significant reduction in fish length, measuring 3% shorter than mutant controls and wild-type fish exposed to the same PFBS concentration (Figure 2a). Pancreas length was significantly reduced in mutants exposed to 0, 16, and 32 μM PFBS compared to wild-types exposed to the same concentration (10%, 18%, and 10% shorter, respectively) (Figure 2b). At 9 dpf, in developmentally PFBS-exposed fish, mutants had significant increases in fish length (3%) with 16 μM PFBS when compared to wild-type fish also exposed to 16 μM PFBS (Figure 2c). However, pancreas length significantly decreased in mutants exposed to 8 μM PFBS compared to mutant controls (16%). Pancreas length significantly decreased in mutant fish exposed to 8, 16, and 32 μM PFBS when compared to wild-types exposed to the same PFBS concentration (17%, 9%, and 11% respectively) (Figure 2d), suggesting this pancreas phenotype likely involves redox stress.

To examine the full size of the exocrine compartment of the pancreas, volume was measured at 9 dpf using confocal microscopy. Mutant larvae exposed to 8 μM PFBS had significantly smaller pancreata; 32% smaller compared to mutant controls and 33% smaller than wild-type larvae also exposed to 8 μM PFBS. In contrast, mutant larvae exposed to 32 μM PFBS had significantly larger pancreata compared to wild-type fish exposed to 32 μM PFBS (32%) (Figure 3a). While these results differ from what was seen with pancreas length, both results indicate disruption of normal organ growth and suggest engagement of a compensatory mechanism with the loss of Nrf2a activity.

Gene expression at 4 and 9 dpf following developmental PFBS exposure

Expression of genes encoding for pancreatic proteases were assessed at 4 and 9 dpf to identify potential functional differences associated with organ growth. At both timepoints, there were no significant differences in gene expression for *ctrb1*, *elaA*, or *try*. However, at 4 dpf, there was a trend toward increased expression with increasing PFBS exposure of all three proteases in wild-type larvae (Figure 4), suggesting a compensatory response. Gene expression for Nrf2a mutant fish at 4 dpf showed an inverse u-shaped response. By 9 dpf, wild-type fish showed an inverse u-shaped response and Nrf2a mutant fish had less clear trends (Figure 4).

Trypsin protein expression is reduced at 6 and 9 dpf

Trypsin is an enzyme that is activated from trypsinogen, a precursor synthesized by acinar cells in the pancreas and secreted into the intestine.[34,43–45] We measured Trypsin protein content as a proxy for acinar cell function. At 6 dpf, after developmental PFBS exposure but before exogenous feeding, Trypsin was significantly lower in wild-type fish exposed to 8, 16, and 32 μM PFBS compared to wild-type controls (48%, 68%, and 65% respectively; Figure 5a and b). Mutant fish overall had less Trypsin than wild-types although not significantly different (wild-type control compared to Nrf2a mutant control $p = 0.07$). At 9 dpf, Trypsin decreased in wild-type fish with 8, 16, and 32 μM PFBS (10%, 35%, and 44% respectively). Wild-type fish exposed to 32 μM PFBS had significantly lower Trypsin than wild-type control fish (Figure 5c and d). This decrease in Trypsin was observed despite significant differences in *try* gene expression (Figure 4). Data from previous 5 figures is summarized in Figure 6.

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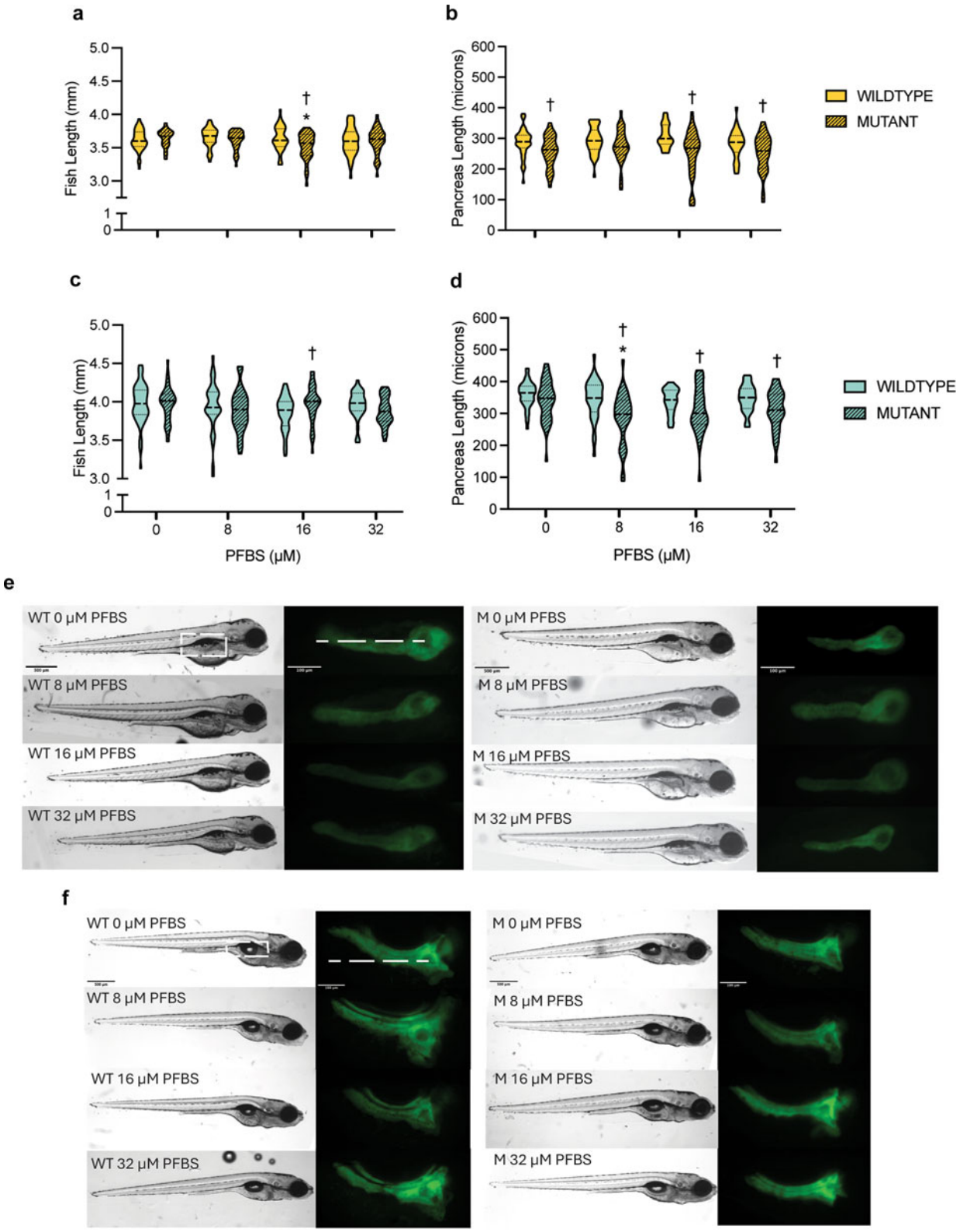


Figure 2. Larvae development and pancreas length at 4 and 9 dpf following developmental PFBS exposure. *Tg(Nrf2a$^{+/+}$;ptf1a:GFP)* and *Tg(Nrf2a$^{fh318/fh318}$;ptf1a:GFP)* zebrafish were exposed to PFBS from 3 to 96 hpf. Fish length and exocrine pancreas length were measured at 4 and 9 dpf. (a) 4 dpf fish length ($n = 34–63$) and (b) 4 dpf pancreas length ($n = 33–58$) with significance determined by two-way ANOVA with Tukey's multiple comparison test. Data compiled from 3 to 5 independent replicates. (c) 9 dpf fish length ($n = 35–50$) and (d) 9 dpf pancreas length ($n = 34–46$) with significance determined by two-way ANOVA with Tukey's multiple comparison test. Data compiled from 3 to 4 independent replicates. Significance within genotype indicated by $^*p \leq 0.05$ and significance between genotypes indicated by $\dagger p \leq 0.05$. Representative images at (*e*) 4 dpf and (*f*) 9 dpf.

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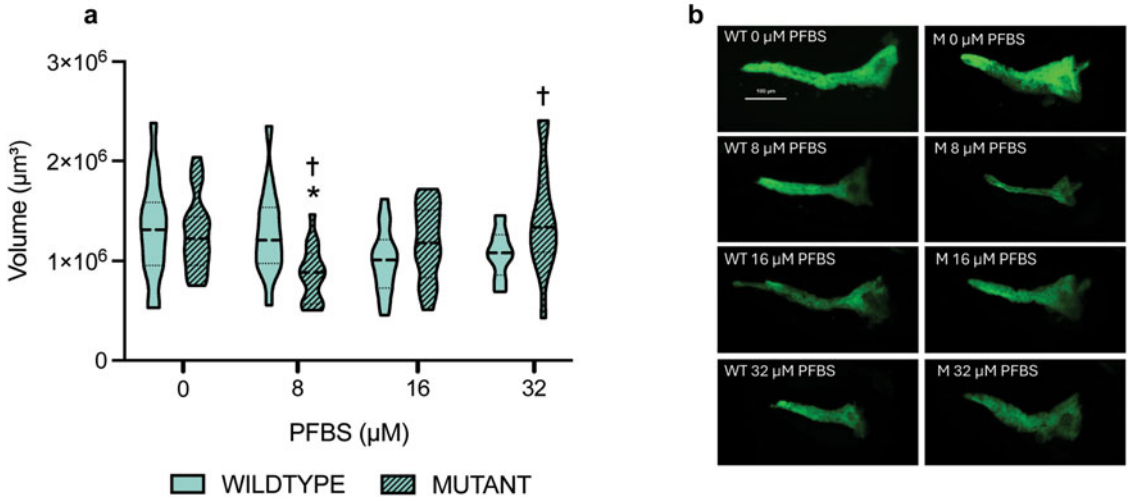


Figure 3. Pancreas volume 9 dpf following developmental PFBS exposure. A subset of *Tg(Nrf2a*$^{+/+}$*;ptf1a:GFP)* and *Tg(Nrf2a*$^{fh318/fh318}$*;ptf1a:GFP)* zebrafish imaged for morphology were fixed and imaged using confocal microscopy. (a) Pancreas volume ($n = 14$–16) at 9 dpf with significance determined by two-way ANOVA with Tukey's multiple comparison test. Data compiled from 3 to 4 independent replicates. (b) Representative images of exocrine pancreas; maximum intensity projection of z-stack.

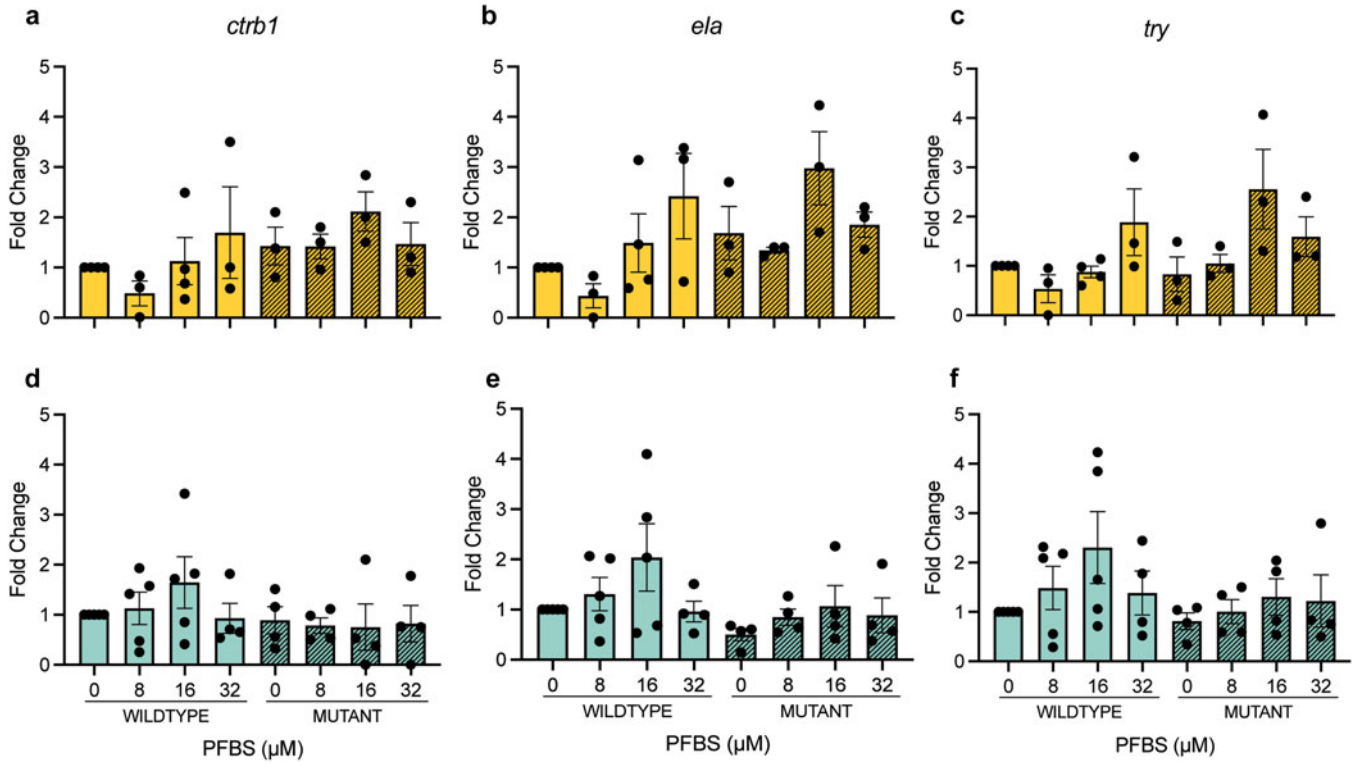


Figure 4. Gene expression at 4 and 9 dpf of *Tg(Nrf2a*$^{+/+}$*)* and *Tg(Nrf2a*$^{fh318/fh318}$*)* following developmental PFBS exposure. Gene expression at 4 dpf for (a) chymotrypsinogen B1 (*ctrb1*), (b) elastase (*elaA*), and (c) trypsinogen (*try*). Gene expression at 9 dpf for (d) chymotrypsinogen B1 (*ctrb1*), (e) elastase (*elaA*), and (f) trypsinogen (*try*). Fold change relative to WT DMSO calculated using geometric mean of *elf1a* and *β-actin* as housekeeping genes. Significance determined by two-way ANOVA. Bars represent ±SEM. $n = 3$–5 pools of 7 fish from 3 to 5 independent replicates.

Effect of PFBS and Nrf2a on total cholesterol and triglycerides at 4 and 9 dpf

Total cholesterol and triglyceride assays were used as an indirect, downstream measure of exocrine pancreas function. At 4 and 9 dpf, no significant differences in total cholesterol in zebrafish larvae within genotype or between genotypes were observed (Figure 7b and e). At 4 dpf, total triglycerides were significantly reduced in mutant controls compared to wild-type controls (43% lower; Figure 7c). At 9 dpf, total triglycerides were significantly reduced in mutant fish exposed to 16 µM PFBS compared to wild-type fish exposed to 16 µM PFBS (29% lower) and were significantly increased in mutant fish exposed to 32 µM PFBS compared to mutant control fish (49% higher; Figure 7f).

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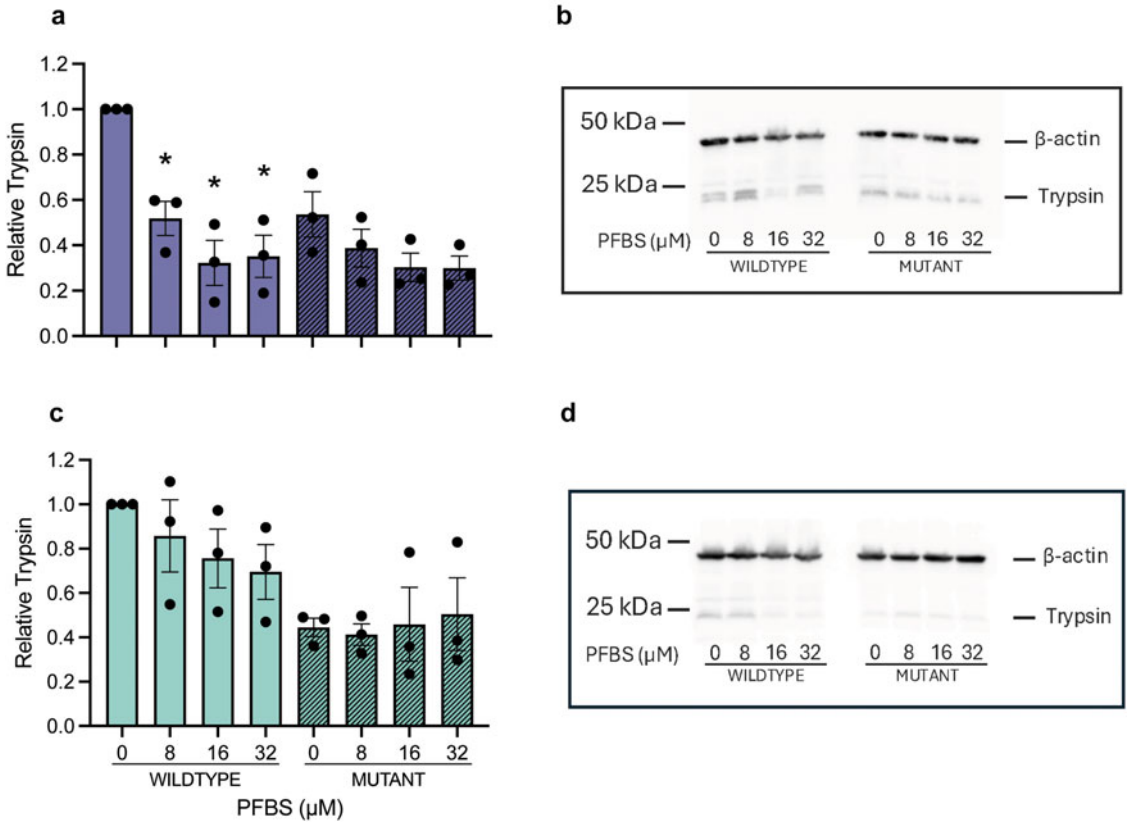


Figure 5. Trypsin protein expression at 6 and 9 dpf of *Tg(Nrf2a*$^{+/+}$*)* and *Tg(Nrf2a*$^{fh318/fh318}$*)* zebrafish following developmental PFBS exposure. Fish were PFBS from 3 to 96 hpf and grown in clean water until 6 and 9 dpf when they were pooled into groups of 20 for trypsin western blotting. (a) Quantification of (b) western blot for trypsin normalized to β-actin at 6 dpf. (c) Quantification of (d) western blot for trypsin normalized to β-actin at 9 dpf. Trypsin expected 24 kDa, β-actin expected 42 kDa. Significance determined by two-way ANOVA with Tukey's multiple comparisons test. Significance within genotype indicated by $^{*}p \leq 0.05$. Bars represent ±SEM.

Effect of PFBS and Nrf2a on glutathione utilization at 4 dpf

MCB staining served as a proxy for glutathione utilization at 4 dpf. In the pancreas, wild-type fish exposed to 16 μM PFBS had increased fluorescence compared to wild-type controls (32%), and mutant fish exposed to 8 μM PFBS had decreased fluorescence compared to mutant controls (39%; Figure 8a). In the hindbrain, there was a u-shaped trend in fluorescence in wild-type fish exposed to 8 and 32 μM PFBS with a significant increase in fluorescence (41% and 34% respectively) but no significant differences in fluorescence with 16 μM PFBS compared to wild-type controls. In the lens, wild-type fish had significant increases in fluorescence with 8, 16, and 32 μM PFBS compared to wild-type controls (28%, 24%, 41% respectively) but no significant differences in Nrf2a mutant fish exposed to PFBS (Figure 8c). In the gut, 8 μM PFBS caused a 49% increase in fluorescence in wild-type fish compared to wild-type controls, but no significant differences in Nrf2a mutant fish exposed to PFBS (Figure 8d). In the heart, wild-type fish had a u-shaped trend in fluorescence with an increase in fluorescence with exposure to 8 and 32 μM PFBS when compared to wild-type controls (44% and 33% respectively) but no significant differences in Nrf2a mutant fish exposed to PFBS (Figure 8e). In the yolk, wild-type fish exposed to 8 μM PFBS had a 59% increase in fluorescence compared to wild-type controls. Additionally, Nrf2a mutant fish exposed to 32 μM PFBS had a 23% decrease in fluorescence compared to mutant controls (Figure 8f). Overall, examining fluorescence in these six different organs suggest additional targets of PFBS exposure where redox disturbances have taken place that require changes in glutathione homeostasis.

Discussion

In this study we investigated how developmental PFBS exposure impacts exocrine pancreas function. PFOS, a long-chain PFAS, crosses the placenta and has been found in cord blood and amniotic fluid[46] and has been linked to metabolic dysfunction,[47,48] which can contribute to cardiovascular disease, diabetes mellitus, and obesity. Following the phase out of PFOS, a shorter-chain PFAS, PFBS became widely used. PFBS has been found to accumulate in children,[8] and has been detected in pregnant women, mothers, and infants, indicating its ability to pass the placenta.[9,10] Through this study, PFBS was shown to impact both exocrine pancreas size and function. In the absence of Nrf2a, pancreas size varies in length and volume. Additionally, Trypsin was found to decrease with increasing PFBS exposure in wild-type fish and Nrf2a mutant fish were found to have overall lower Trypsin compared to wild-type fish.

Previous work using *Tg(ptf1a:GFP)* zebrafish demonstrated that PFBS exposure increases incidence of the truncated pancreas phenotype at 4 dpf.[12] Here, we showed that by 15 dpf, there is no longer a significant reduction in pancreas length with developmental PFBS exposure, suggesting catch-up growth. Studies have linked catch-up growth to health concerns, including diabetes, cardiovascular disease, obesity, and metabolic syndrome.[49–53] The pancreas plays a major role in the development of a healthy

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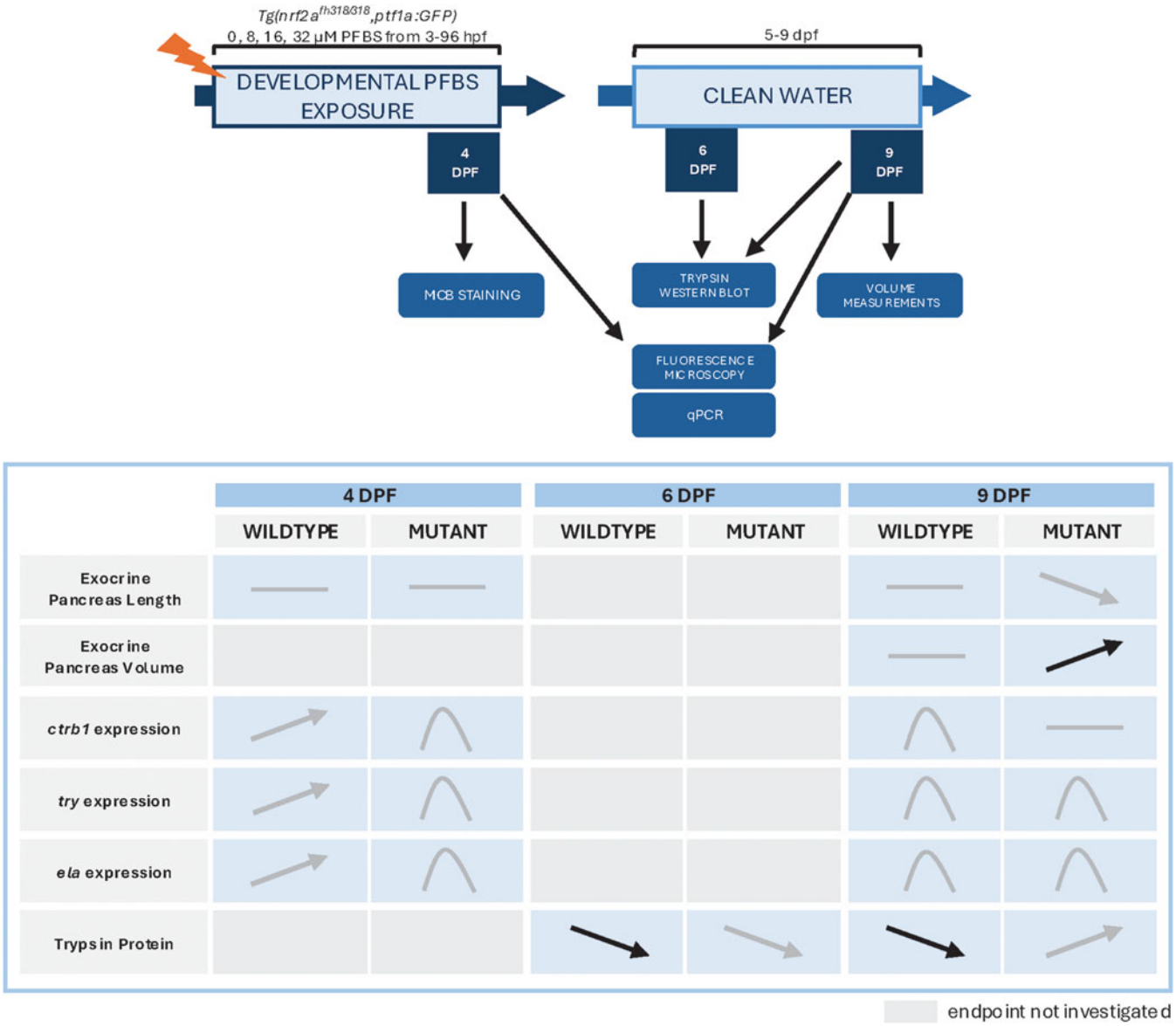


Figure 6. Summary of trends in Figures 1, 2, 3, 4 and 5. Black line indicates significance; gray line indicates trend. Made in powerpoint.

metabolism with endocrine beta cells regulating glucose levels through insulin secretion and exocrine cells supporting nutrient digestion through the production of digestive enzymes. Understanding how development is perturbed is important as it can impair these functions, contributing to metabolic dysfunction that may persist throughout the lifespan.

To better understand the mechanisms behind altered pancreas size, we assessed timepoints following developmental exposure and prior to the timepoint where catch-up growth was observed. Additionally, we examined modulation of PFBS impacts on exocrine pancreas growth and function by the Nrf2a pathway. We examined the effects of PFBS pancreatic tail extension and saw decreased pancreas length at 9 dpf in mutant fish with non-functional Nrf2a compared to wild-type fish, indicating this phenotype likely involves redox stress. Endoplasmic reticulum (ER) homeostasis is a major component in the development and function of the exocrine pancreas.[54] The unfolded protein response (UPR), an adaptive signaling network that senses ER stress, adjusts protein folding capacity, regulates metabolism, and is linked to both ER homeostasis and maintenance of oxidative stress.[54] UPR target and UPR-associated genes such as *herpud1*, *pdia2*, and *sdf2* have been shown previously to have altered expression in zebrafish at 96 hpf following exposure to 32 µM PFBS such as down-regulation of *herpud1* and upregulation of *pdia2* and *sdf2*.[12,55–60] These changes could suggest a dysregulated UPR response. During ER stress, PERK can phosphorylate Nrf2, linking UPR to redox homeostasis.[61,62] Loss of Nrf2a may therefore impair the UPR, causing the ER stress response to become overwhelmed as protein folding demand remains high, but removal of misfolded proteins is not occurring properly. Previous studies indicate that altered UPR function can have negative impacts on the exocrine pancreas.[63–65] Such disruptions could contribute to negative effects on pancreas development seen in this study.

To further assess pancreatic growth, we examined volume after PFBS exposure in wild-type and Nrf2a mutant zebrafish. This study showed increased volume in Nrf2a mutant fish with increasing PFBS concentrations compared to wild-type fish, which did not match the decrease in pancreas length seen between the

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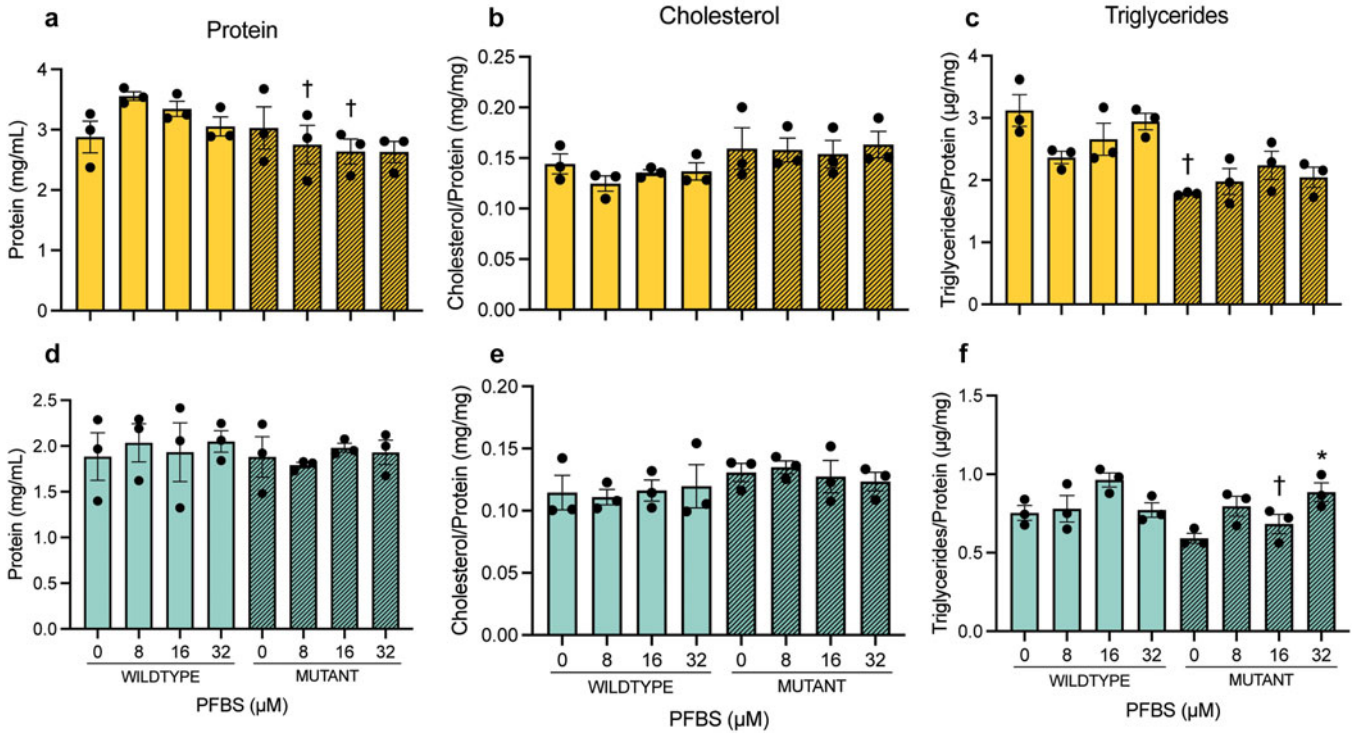


Figure 7. Total protein, cholesterol, and triglyceride quantification at 4 and 9 dpf of *Tg(Nrf2a$^{+/+}$)* and *Tg(Nrf2a$^{fh318/fh318}$)* following developmental PFBS exposure. (a) Protein, (b) cholesterol, and (c) triglycerides at 4 dpf. $n = 3$ pools of 20 fish from 3 independent experimental replicates. (d) Protein, (e) cholesterol, and (f) triglycerides at 9 dpf. $n = 3$ pools of 15 fish from 3 independent experimental replicates. Significance determined by two-way ANOVA with Tukey's multiple comparisons test. Significance within genotype indicated by $^{*}p \leq 0.05$ and significance between genotypes indicated by $^{\dagger}p \leq 0.05$. Bars represent ±SEM.

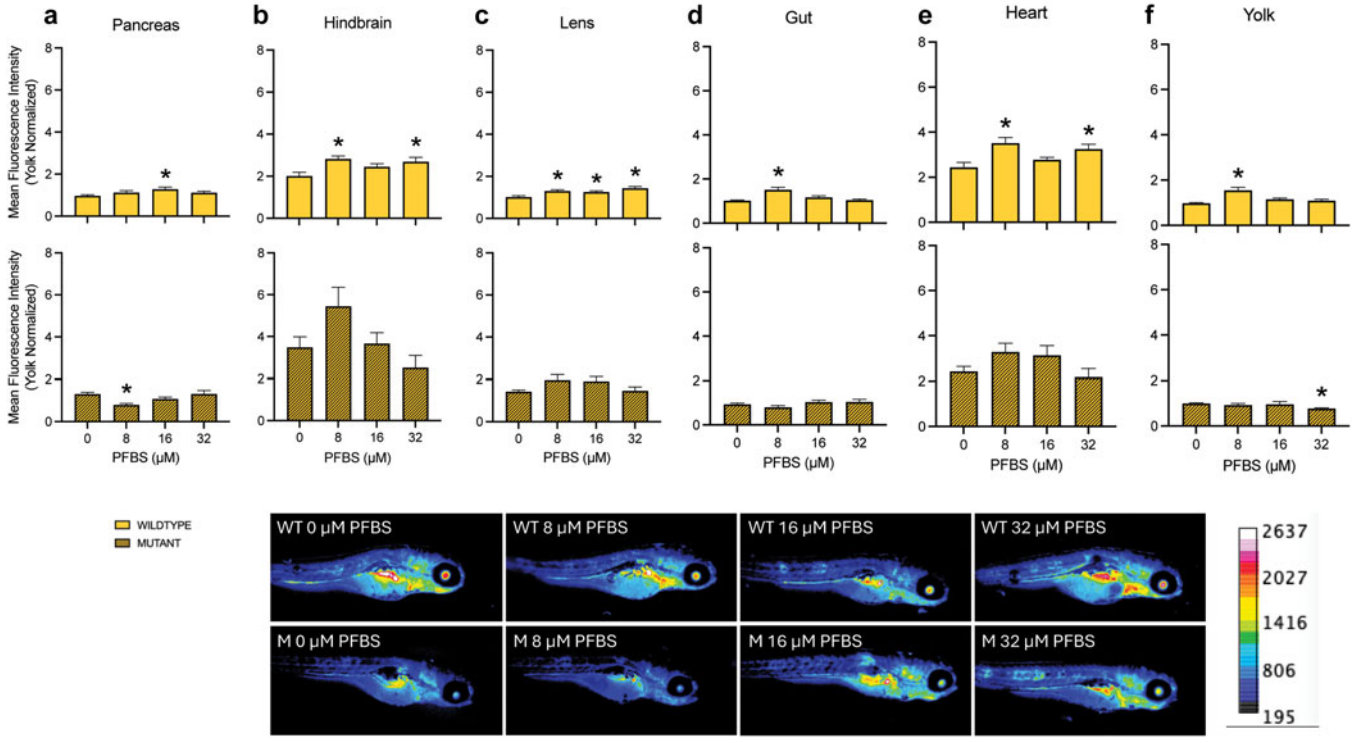


Figure 8. Mean fluorescence intensity for the (a) pancreas, (b) hindbrain, (c) lens, (d) gut, (e) heart, and (f) yolk normalized to the average yolk sac fluorescence of DMSO fish for each replicate at 4 dpf after developmental PFBS exposure. Significance was determined using a Kruskal–Wallis test ($^{*}p < 0.05$). Bars represent Mean ± SEM (WT $n = 35$–41 fish, *M* $n = 28$–29 fish).

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groups. This discrepancy suggests that PFBS alters exocrine pancreas structure in a Nrf2a-dependent manner, indicating changes in tissue organization rather than overall growth inhibition.

Alterations in pancreatic size raise questions about potential functional impacts. Lipases and proteases are two main enzymes secreted by the exocrine pancreas and aid in metabolism.[32,66,67] When secreted incorrectly health issues occur, such as EPI, which often occurs along with GI issues, bone disease, and diabetes.[68–71] Here, we evaluated pancreatic proteases as a proxy for exocrine pancreas function: they are the most highly produced digestive enzymes during development.[72–76] At the transcriptional level, we investigated three proteases; trypsinogen (*try),* which is secreted by the pancreas and is a precursor to Trypsin; chymotrypsinogen B1 (*ctrb1),* a digestive enzyme precursor primarily expressed in the pancreas and activated by Trypsin in the digestive tract, and elastase (*ela),* which is highly expressed in the pancreas and works with other dietary proteases to hydrolyze pancreatic proteases.[15] At 4 dpf, there were no significant differences in *ctrb1, ela,* or *try* including between wild-type and Nrf2a mutant fish indicating the loss of Nrf2a does not play a direct role in differences in gene expression of these proteases. The trend of increasing gene expression at 4 dpf in wild-type fish with exposure to increasing PFBS concentrations was consistent with previous studies with similar PFBS concentrations[12] and could suggest a compensatory mechanism to combat potential EPI. At 6 dpf, wild-type fish had significantly lower Trypsin across PFBS treatment groups compared to wild-type controls. In previous studies with PFOS exposure, similar impacts in Trypsin were seen at 6 dpf[18] and the expression of key pancreatic proteases were significantly reduced at 4 and 6 dpf,[17,18] indicating different responses to the longer carbon chain PFOS compared to the shorter carbon chain PFBS. At 9 dpf, there were no significant differences in *ctrb1, ela,* or *try*, nor significant differences between wild-type and Nrf2a mutant fish. However, the inverse u-shaped response observed in wild-type fish gene expression could indicate that compensatory mechanisms are being overwhelmed when exposed to 32 µM PFBS. Additionally, Trypsin decreased with increasing PFBS exposure in wild-type fish, indicating a functional issue. The significant decrease in Trypsin levels persisted from 6 to 9 dpf only in the highest PFBS-exposed wild-type groups, suggesting some catch-up in function of the exocrine pancreas.

Previous studies demonstrated exocrine zymogens, such as *try*, in zebrafish contain MARE or MARE-like sites in their regulatory regions which allows Mafk to bind and upregulate zymogens in the exocrine pancreas.[77] At 6 and 9 dpf, Nrf2a mutant larvae had lower Trypsin expression than wild-type larvae, although not significant. This could result from impaired Nrf2a function, which has been associated with lower zymogen activation and overall expression. The formation of the Nrf2a-Mafk heterodimer allows Mafk to bind to MARE and MARE-like sites and activate zymogens that are found in the exocrine pancreas including *try* and *tryl*.[77] Additionally, the lack of significant changes at the mRNA level alongside reduced protein abundance suggest post-transcriptional regulation. One potential mechanism is activation of the UPR, as oxidative stress can impair protein folding within the endoplasmic reticulum.[78] Specifically, the PERK-eIF2α arm of the UPR suppresses translation, which provides a possible mechanism for reduced protein abundance without significant transcriptional changes.[79–82] Together, the data gathered in this study related to gene expression and quantification of Trypsin suggest EPI.

The exocrine pancreas secretes lipases for the hydrolysis of triglycerides.[83] In zebrafish, the yolk sac is the only nutrient source until exogenous feeding starts around 5 dpf.[39] Yolk lipids are mostly phosphatidylcholine and cholesterol (reviewed in[84]). Total cholesterol and triglyceride assays were conducted in this study to investigate potential downstream effects of PFBS exposure on the exocrine pancreas. Our results showed no differences in total cholesterol at 4 or 9 dpf. Varying changes in total cholesterol have been seen in previous *in vitro* and *in vivo* studies with PFBS exposure, with no changes in some cell types but decreased cholesterol in rats.[85,86] Interestingly, there were only significant changes between wild-type and mutant control fish related to triglyceride content. This could be due to reduced yolk sac area seen between wild-type and mutant fish (Supplemental Figure 5). Additionally, wild-type fish had an inverse u-shaped dose response curve while Nrf2a mutant fish had a u-shaped dose–response curve. Triglyceride accumulation at mid-range doses in wild-type fish and at low and high doses in Nrf2a mutant fish may indicate PFBS-induced disruptions of lipid metabolism, including altered lipogenesis, lipolysis, or fatty acid oxidation, as PFAS exposure has been associated with dysregulated lipid homeostasis and metabolic pathways in zebrafish.[12,86] These differing dose–response patterns suggest that functional vs non-functional Nrf2a alters the metabolic response to PFBS exposure.

We showed previously that it is possible to spatially observe glutathione utilization in zebrafish.[37] PFAS are known to disrupt redox signaling[17,87–90] and reduced glutathione is crucial in mitigating oxidative stress and maintaining cellular homeostasis.[24,25,91,92] GSH is the most abundant endogenous antioxidant,[25] and this pathway relies heavily on a variety of enzymes to help with its production, recycling, and utilization, all of which can be upregulated by Nrf2.[25,93,94] We analyzed six larval regions: pancreas, gut, heart, hindbrain, lens, and yolk sac. There were more tissues with significant differences in glutathione utilization in wild-types than in Nrf2a mutants, suggesting a stronger antioxidant response to PFBS in larvae with a functional Nrf2a, whereas the limited changes observed in Nrf2a mutants may reflect a failure to respond to oxidative stress.

Overall, this study links altered exocrine pancreas size and function and provides insight towards mechanisms that might contribute to these phenotypes. Developmental PFBS exposure may contribute to pancreatic dysfunction through pathways involved in oxidative stress regulation, metabolic homeostasis, and exocrine pancreas development overall. The shortened pancreas phenotype seen in this study mirror human congenital malformations.[95,96] Understanding the impact of PFBS on the pancreas is important because of its potential implication for humans in development and throughout their lifespan. From a clinical perspective, type 1 and type 2 diabetes has been shown to be associated with EPI.[97] EPI can be described as alterations at any point in digestion that cause the pancreas to be unable to secrete enzymes to digest food properly[98] and is of concern as pancreatic secretion stimulation, production of digestive enzymes by the pancreas, and the ability for pancreatic juices to mix with food could all be impacted by these alterations.[99] PFAS exposure has been linked to diabetes and therefore could also contribute to EPI. It is important to understand this link early as EPI has been strongly linked to malnutrition despite a correct diet.[98] The prevalence of EPI is hard to determine because of difficulty with underdiagnosis and undertreatment.[98] Although this study does not explore a specific link between developmental PFBS exposure and EPI, understanding the effects of PFBS on exocrine pancreas function and what mechanisms are playing a role aid understanding of the greater impact of PFBS exposure on humans.

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This study provides insights into the importance of Nrf2a functionality during PFBS exposure. In the absence of functional Nrf2a, pancreas phenotypes vary, and there are trends of reduced Trypsin. Alternatively, with functional Nrf2a, more consistent exocrine pancreas phenotypes are seen. Despite a normal morphology, the negative effects of PFBS exposure are seen through altered gene expression for pancreatic proteases and reduced Trypsin with increasing PFBS concentrations, suggesting functional changes in the exocrine pancreas following developmental PFBS exposure. These results are consistent with the DOHaD paradigm that early-life toxicant disruptions can lead to later-life disease risk.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174426100683.

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Competing interests. None.

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of zebrafish (*Danio rerio*) and have been approved by the University of Massachusetts Institutional Animal Care and Use Committee.

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