Metabolomic Changes Following GenX and PFBS Exposure in Developing Zebrafish

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Keywords: GenX, PFBS, Zebrafish, Embryos, Metabolomics, Body Burden

ABSTRACT

Short chain per- and polyfluoroalkyl substances (PFAS), including hexafluoropropylene oxide dimer acid (GenX) and perfluorobutane sulfonate (PFBS), are replacement chemicals for environmentally persistent, long-chain PFAS. GenX and PFBS have been detected in surface and groundwater worldwide; however, few studies provide information on the metabolic effects or risks associated with their exposures. In this study, larval zebrafish were used as a model for investigating the toxicity of early-life exposure to either GenX or PFBS. Zebrafish were chronically exposed from 4 hours post-fertilization (hpf) to 6 days post-fertilization (dpf) to 150 μM GenX or 95.0 μM PFBS. Ultra-high-performance liquid chromatography paired with high resolution mass spectrometry was used to quantify uptake of PFAS into zebrafish larvae and perform targeted and untargeted metabolomics. Our results indicate that PFBS was 20.4% more readily absorbed into the zebrafish larvae compared to GenX. Additionally, PFBS exposure significantly altered 13 targeted metabolites and 21 metabolic pathways, while GenX exposure significantly altered 1 targeted metabolite and 17 metabolic pathways. In both the GenX and PFBS exposures, the greatest number of altered metabolic pathways occurred in the global amino acid pathways, including alterations in the carbohydrate, lipid, cofactors and vitamins, nucleotide, and xenobiotics metabolisms. Our results indicate that GenX and PFBS impact the zebrafish metabolome, with implications of altered growth and development and global metabolic dysregulation.

1. INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) have emerged as contaminants of concern due to their bioaccumulative properties and potential adverse effects on animal and human health. PFAS are widely used in a range of consumer products, including non-stick or stain/water-resistant products (Sunderland et al., 2019). PFAS are a broad class of chemicals containing at least one fully fluorinated carbon atom and a functional head group (Rayne and Forest, 2009; Wallington et al., 2021). Due to the strength of the carbon fluorine bond, PFAS are recalcitrant to most chemical and biological degradation processes and persist in the environment (Glüge et al., 2020; Kucharzyk et al., 2017). Exposure to PFAS is associated with adverse effects to the immune, reproductive, and endocrine systems and diseases, including cancers in both animal models and humans (Barbo et al., 2023; Podder et al., 2021; Sunderland et al., 2019).

Due to concerns about the potential health risks of PFAS exposure, long-chain PFAS (containing eight or more carbons in the perfluoroalkyl chain, including legacy perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS)) were voluntarily phased out of production in the early 2000s, resulting in increased production of replacement short-chain PFAS (containing less than seven carbons) (Janousek et al., 2019; Kabadi et al., 2020). Because of their shorter carbon chain length, these replacement chemicals were intended to be less persistent in the environment and are now used in industrial applications across the world (Bao et al., 2018; Wang et al., 2016). In 2022, the US Environmental Protection Agency (EPA) set health advisory levels for four PFAS compounds in drinking water: PFOA and PFOS (C_8) (0.004 ppt and 0.02 ppt, respectively) and their short chain replacement chemicals GenX (hexafluoropropylene oxide dimer acid (HFPO-DA)) (C_6) and perfluorobutane sulfonate (PFBS) (C_4) (10 and 2,000 ppt, respectively) (USEPA, 2022). In March of 2023, the EPA proposed maximum contaminant level (MCL) goals

for PFOA and PFOS in drinking water at 4 ppt each (USEPA, 2023). The MCL also proposed a Hazard Index, which sums the ratios of the water concentration to the level determined not to cause health effects for two short chain PFAS - GenX and PFBS – and two long chain PFAS - perfluorononanoic acid (PFNA) and perfluorohexane sulfonate (PFHxS) (USEPA, 2022; USEPA, 2023).

GenX and PFBS have been detected in surface and ground waters in many countries, with demonstrated bioaccumulation in nearby plants, animals, and humans (Blake et al., 2020; Taniyasu et al., 2003). The potential health effects of these replacement PFAS are largely unknown, but preliminary animal studies suggest that there may be an association between GenX and PFBS exposure and alterations to thyroid function, reproductive problems, low birth weight, and growth problems (Blake et al., 2020; Chen et al., 2018; Newsted et al., 2008). Zebrafish are widely used in toxicology research to study the effects of xenobiotic exposure on the vertebrate exposome, which reflects the biological outcomes of exposures (Howe et al., 2013). Due to their rapid ex-utero embryonic development and genomic similarity to humans, zebrafish are an excellent model organism for studying vertebrate embryonic development and health (Gaballah et al., 2020; Howe et al., 2013). Previous studies of early-life exposure of zebrafish to short-chain PFAS reveal alterations to exposed zebrafish such as abnormal behavior, altered growth and development, cardiac edema, and developmental neurotoxicity (Gebreab et al., 2020; Min et al., 2023; Rericha et al., 2022). As short-chain replacers for PFAS become more prevalent in the environment, it is essential to study biological systems that may be affected by exposure to these chemicals.

Incorporating "omics", such as genomics (the study of gene expression), transcriptomics (the study of tRNA), proteomics (the study of proteins), and metabolomics (the study of metabolites and pathways), into zebrafish studies provides molecular insight into physiological

93 alterations caused by chemical exposures (da Silva et al., 2021; Sukardi et al., 2010). Since
94 metabolites are products of cellular processes, metabolic alterations directly indicate physiological
95 and phenotypic alterations (da Silva et al., 2021; Lai et al., 2021). When applied to embryonic or
96 larval zebrafish, metabolomics provides important insight into the effects of exposures on growth
97 and development and molecular mechanisms for chemically induced toxicological endpoints (da
98 Silva et al., 2021; Kossack et al., 2023).

99 Previous metabolomic studies of zebrafish exposure to the legacy long-chain PFAS,
100 primarily PFOA and PFOS, have demonstrated alterations to the lipid, carbohydrate, and amino
101 acid metabolisms (Cheng et al., 2016; Du et al., 2016; Gebreab et al., 2020). These studies included
102 short-term and chronic exposure during several different life stages of zebrafish (embryos, larvae,
103 and adults) and compared metabolomic effects of PFOA, PFOS, and next-generation PFAS
104 (GenX, PFBS, PFO3TDA (perfluoro-3,6,9-trioxadecanoic acid)). One study exposed zebrafish
105 embryos to either GenX or PFOA at concentrations up to 100 mg L^{-1} and compared the embryonic
106 toxicity and metabolic alterations induced by exposure to PFOA or GenX, finding that exposures
107 to either chemical altered metabolites that are essential to liver function and development (Gebreab
108 et al., 2020). A second study of embryonic exposure to 10 mg L^{-1} PFBS indicated an induced stress
109 response, neurotoxicity, and altered folate biosynthesis and methionine metabolisms (Hu et al.,
110 2022). Both studies used very different exposure doses and examined different resulting metabolic
111 impacts, thus knowledge of short-chain PFAS uptake and the use of similar exposure levels are
112 needed to assess metabolomic alterations.

113 The objective of this study was to understand global metabolic alterations in developing
114 zebrafish resulting from exposure to the short-chain PFAS GenX and PFBS. Using targeted
115 analysis, GenX and PFBS uptake and body burden of exposed zebrafish larvae were quantified

and targeted metabolomics was performed to quantify levels of 33 amino acid metabolites to compare between exposed and non-exposed larvae. Using untargeted metabolomics, global metabolic alterations induced by GenX and PFBS exposure were discovered, which provided insight into the molecular mechanisms of toxicity in early-life zebrafish exposure to short-chain PFAS. The results of this study provide new information on short-chain PFAS uptake and metabolic alterations in exposed zebrafish larvae.

2. MATERIALS AND METHODS

2.1 Chemicals

All solutions were prepared using water purified by a Millipore Milli-Q® Reference purification system (18.2 MΩ.cm at 25°C and total organic content below 5 ppb). GenX (Ammonium perfluoro (2-methyl-3-oxahexanote), 95% purity) used for the exposure solutions was purchased from Manchester Organics (Runcorn, England). Dimethyl sulfoxide (DMSO) and PFBS (perfluorobutane sulfonate) for exposure solutions were obtained from MilliporeSigma (St. Louis, MO). Isotopically labeled perfluorinated compound internal standards for quantitation of GenX and PFBS were purchased from Wellington Laboratories (Guelph, Ontario, Canada). All solvents, including Ultra High-Performance Liquid Chromatography (UHPLC) grade acetonitrile and water (99.9%), were purchased from Fisher Scientific (Waltham, MA). Isotopically labeled metabolites (containing L-alanine, L-leucine, L-phenylalanine, L-tryptophan, and L-tyrosine) were obtained from Cambridge Isotopes (MSK-QC-KIT, Tewksbury, MA). Amino acid metabolite standards (A9906-10 mL) and other targeted metabolites (DL-3-Ureidoisobutyric acid, N-Acetylputrescine hydrochloride, γ-Aminobutyric acid, L-Asparagine, L-Pipecolic acid, L-Glutamine, 3-Ureidopropionic acid) were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Zebrafish Spawning

All maintenance and experimental procedures involving zebrafish (Danio rerio) were approved by the Brown University Institutional Animal Care and Use Committee (IACUC; 19-12-0003) and adhered to the National Institute of Health's "Guide for the Care and Use of Laboratory Animals." The zebrafish were kept in an aquatic habitat (Aquaneering Inc., San Diego, CA) with a 14:10 hour light-dark cycle (Westerfield, 2000) and water temperature control (28.5 ± 2°C), filtration, purification, automatic pH and conductivity stabilization, and ultraviolet (UV) irradiation disinfection.

The night before spawning, adult AB (wildtype) zebrafish were acclimated to their new tank (1.7 L specialized, gridded spawning tanks (Techniplast, USA)) conditions to facilitate timed reproduction. A transparent partition was used to separate sexes. The tank partition was removed within 2 hours of light cycle onset so that the zebrafish could spawn for 1 hour. Three independent spawning events were performed on separate days to acquire three biological replicates of each dosing experiment. Spawning occurred with separate parents on three different dates for each exposure compound (GenX, PFBS, or vehicle (egg water containing 0.1% DMSO) to ensure an adequate number and varied parenthood of embryos. All embryos were collected in 100 mm non-treated culture petri dishes containing fresh egg water (60 mg L^{-1} Instant Ocean Sea Salts; Aquarium Systems, Mentor, OF) after spawning. At 4 hpf, any embryos that did not show adequate cellular development by 4 manual screening for quality were discarded.

2.3 Exposure

Exposure Solutions: Aqueous stock solutions of PFBS (64 mM) and GenX (41.48 mM) were prepared and validated at the start of the study. Two exposure solutions were prepared by

diluting the stock solutions in fresh egg water in polypropylene tubes to obtain desired exposure concentrations of 150 µM (49.5 mg L^{-1}) for GenX and 100 µM (30.0 mg L^{-1}) for PFBS. Solutions were validated using targeted mass spectrometry (further described below) and the actual exposure solution concentrations were 150 µM (49.5 mg L^{-1}) for GenX and 95.0 µM (28.5 mg L^{-1}) for PFBS. For GenX, a previously published concentration between 100 and 200 µM demonstrated no physiological deformities or adverse effects in larval zebrafish at our desired timepoints (Gebreab et al., 2020); indeed, no physiological deformities or excess mortality were noted in our study following exposure to 150 µM GenX by 6 days post-fertilization (dpf). Likewise, the concentration selected for PFBS was also based on previously published work by Gaballah et al., who found that at 100 µM PFBS, exposed zebrafish had no physiological deformities or differences in locomotor activity in comparison with non-exposed groups (Gaballah et al., 2020). These exposure concentrations were verified not to produce physical deformities indicative of poor health, such as a bent spine or cardiac edema, by performing daily observations throughout the exposure period using a microscope.

Fish Exposure: At 4 hpf, we plated 3 fertilized embryos per well in three sterile 24-well plates (ThermoFisher, Waltham, MA). Each well was dosed with 2 mL of either GenX, PFBS, or egg water with 0.1% DMSO (vehicle control) (**Figure 1**). DMSO was chosen as the vehicle because PFBS was dissolved in DMSO to generate a stock solution. GenX is unstable in DMSO and soluble in water (Gaballah et al., 2020); therefore, we dissolved GenX in purified deionized water. A previous study compared exposure solutions containing PFBS dissolved in DMSO and GenX in deionized water at the same concentrations and found that exposures resulted in changes of similar magnitudes (Gaballah et al., 2020). The plates were then incubated at 28.5 ± 1˚C

incubator (Powers Scientific Inc., Pipersville, PA) from 4 hpf until 6 dpf. Fish were chronically exposed without replacement of the treatment solution.

All embryos were manually dechorionated at 24 hpf to ensure unprotected exposure to toxicants throughout the protocol and placed back into the incubator until collection at 6 dpf (Henn and Braunbeck, 2011). For each exposure, 6 dpf larvae were pooled into 6 polypropylene centrifuge tubes (2 mL) of n=10 larvae. Excess exposure solution was discarded from the larval pools, and then the pools were washed three times in laboratory-grade deionized water with all excess water removed between each round of washing. Samples were snap frozen in liquid nitrogen to be stored at -80˚C until ready for extraction. In total, 6 pools of 10 larvae were collected per biological replicate for each exposure group, resulting in 180 larvae per exposure group and 540 total larvae collected across all groups.

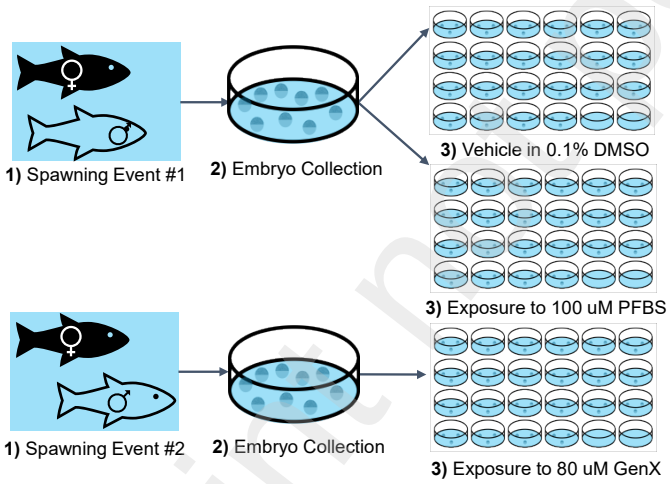

Figure 1: Experimental design for embryo spawning and exposure dosing for each exposure.

Sample Extraction: 30 µL of PFAS internal standard solution and 270 µL of acetonitrile containing an isotopically labeled metabolite internal standard solution were added to each sample. Samples were sonicated for 30 minutes until homogenized, and then all samples were centrifuged at 755 x *g* for 10 minutes using a Savant HSC-10K High Speed Centrifuge. For metabolomics, 200 µL of the supernatant was transferred into an amber LC/MS vial containing a 300 µL glass insert. For PFAS analysis, the remaining 100 µL of supernatant was diluted in acetonitrile and spiked with internal standard to comprise 10% of the total volume of the sample. These samples were transferred into 300 µL amber glass insert LC/MS vials. All samples were stored at 5˚C prior to analysis.

2.4 Analytical Methods

PFAS Analysis: Concentrations of GenX and PFBS were confirmed in the following solutions: (1) GenX and PFBS stock solutions, (2) diluted GenX and PFBS exposure solutions and matching vehicle (egg water containing 0.1% DMSO), and (3) zebrafish larval extracts. To quantify PFBS or GenX in each solution, samples were diluted with UHPLC-grade acetonitrile containing 10% isotope labeled internal standard.

GenX and PFBS concentrations were quantified based on a modified version of EPA Draft Method 1633 using a high resolution Thermo QExactive HF-X Orbitrap MS equipped with a Vanquish ultra-high-performance liquid chromatograph (UHPLC-Orbitrap-HRMS) (USEPA, 2021). Analyte separation was achieved by injecting 20 µL of each sample onto a Thermo Hypersil Gold Vanquish C18 column (100 mm X 2.1 mm x 1.9 µm). Mobile phases consisted of 2 mM ammonium acetate in 5% acetonitrile and 2 mM ammonium acetate in 100% acetonitrile. The MS acquisitions were performed with negative electrospray ionization (ESI), in full scan data-dependent (MS-ddMS^2) with an inclusion list for GenX, PFBS, and internal standards, and with

100-1500 m/z scan range. An HCD collision cell filled with N_2 gas (produced by a Peak Scientific Nitrogen Generator, Genius NM32LA) was used to perform MS2 fragmentation. Additional details on the chromatography, source settings, and MS data collection parameters are provided in the SI (**Table S1**). PFAS concentrations were quantified using one quantification ion and eight-point calibration curves with concentrations ranging from 0 to 20,000 ng/L for each compound. Compound confirmation was performed using retention time and two confirming ions, when available. The Limits of Detection (LODs) were 42.1 ng/L for GenX and 34.6 ng/L for PFBS, which were determined from seven injections of calibration standards and multiplying the standard deviation of these injections by 3.143 ($t_{0.99}$ at 6 degrees freedom) divided by the calibration curve slope.

Targeted and Untargeted Metabolomics: Both targeted and untargeted metabolomics were performed using the Thermo UHPLC-Orbitrap-HRMS. All samples were analyzed in triplicate using a randomized order in a single batch. All samples were analyzed using two chromatography methods, one with positive and negative ESI. For positive ESI (referred to as ESI+), a HILIC column (Thermo Syncronis HILIC 100 mm X 2.1 mm x 3 μm) was used. For negative ESI (referred to as ESI-), a C18 column (Thermo Hypersil Gold Vanquish, 100 mm X 2.1 mm x 1.9 μm) was used. Additional details on the chromatography, source settings, and dd-MS2 data collection parameters are provided in the SI (**Table S2**). For targeted metabolomics detected in the ESI+ method, a ten-point calibration curve ranging from 1.25 to 333 nM was used to quantify the metabolites in **Table S3**.

2.5 Untargeted Metabolomics – Data Analysis

XCalibur File Converter (ThermoFisher) was used to convert data files from .raw to .cdf format. R packages apLCMS and xMSanalyzer were used to produce m/z feature tables (Denison

and Nagy, 2003; Uppal et al., 2013), which contained each feature (representative of a metabolite) in a row and the m/z value, paired retention time (seconds), and unitless feature areas in columns. All features containing greater than 20% non-detection were removed. Each feature was then log2 transformed to normalize the data.

Statistical Analysis: An Analysis of Covariance (ANCOVA) test was performed for each metabolomics data set (targeted and untargeted) for each exposure (GenX or PFBS) to test for between-group variance vs. within-group variance and account for the varying birth dates and parenthood of the zebrafish collected. For targeted data, ANCOVA results with an F-value > 0.05 were considered to be significantly altered metabolites (Mishra et al., 2019). In untargeted metabolomics, a false discovery rate (FDR) of 20% was used to account for Type I errors in the comparisons (Kossack et al., 2023) and any feature above this threshold was used for pathway analysis.

Pathway Analysis: Pathway analysis was performed using MetaboAnalyst's Functional Analysis for zebrafish (Danio rerio (KEGG)) (Chong and Xia, 2018). Features were matched to metabolites with 5.0 ppm mass tolerance for ESI+ and ESI- data, including the feature F-value (equivalent to p-value in an ANCOVA) obtained from statistical analysis and retention time. The Mummichog algorithm was used with the default p-value cutoff of the top 10% of features, which resulted in the following p-value (F-value) cut-offs: ESI+ GenX 0.25; ESI- GenX 0.1; ESI+ PFBS 0.05; ESI- PFBS 0.15 (Li et al., 2013). Our results include pathways that contained least 3 metabolites matched to features. Alterations to global metabolisms were mapped using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000).

3. RESULTS

3.1 Body Burden

To establish uptake of PFBS and GenX, body burden per larva was determined for the six-day exposure period. GenX body burden in the exposure and vehicle groups was 26.6 ± 10.8 µg/embryo and 1.53 ± 0.495 µg/embryo, respectively (n=10, **Figure 2**). PFBS body burden in the exposure and vehicle groups was 19.3 ± 4.41 µg/embryo and 0.114 ± 0.0413 µg/embryo, respectively (n=10, **Figure 2**). Based on the exposure concentration in each exposure well (n=3 embryos), the bioconcentration factors, or the ratio of the concentration inside the zebrafish to the surrounding aqueous chemical concentration, were calculated to be 63.8 for PFBS and 50.6 for GenX (**Table S4**).

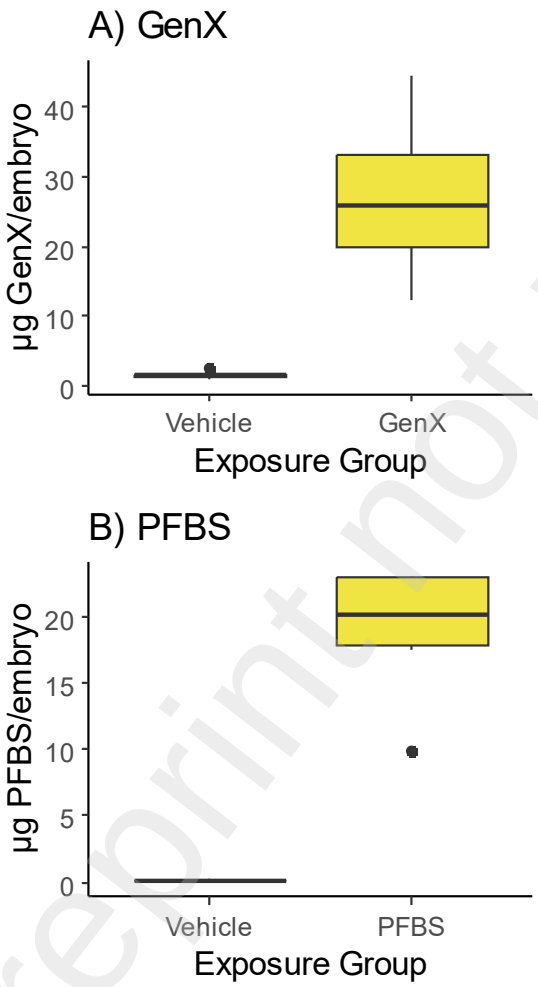

Figure 2: PFAS body burden of exposed to 100 µM PFBS or 80 µM GenX vs. vehicle groups. Each box plot represents 6 samples (each containing n=10 larvae) that were analyzed by isotope dilution mass spectrometry in triplicate.

3.2 Targeted Metabolic Pathway Analysis

In control and exposed larvae, 15 out of the 33 targeted metabolites were detected, including glutamic acid, isoleucine, L-alanine and B-alanine, L-citrulline, L-creatinine, L-tryptophan, methionine, phenylalanine, proline, sarcosine, serine, taurine, threonine, tyrosine, and valine (**Figure 3; Table S5**). Concentrations ranged from <0.1 µg/embryo to 11.1 µg/embryo across all samples and targeted metabolites.

In GenX exposed fish, one targeted metabolite, L-citrulline, was significantly decreased ($p < 0.05$) (**Figure 3; Table S5**). In PFBS exposed fish, 11 targeted metabolites (isoleucine, L-alanine and B-alanine, L-citrulline, L-tryptophan, methionine, phenylalanine, proline, sarcosine, serine, threonine, and tyrosine) were significantly decreased ($p < 0.05$), while two metabolites were significantly increased (L-creatinine and taurine) ($p < 0.05$).

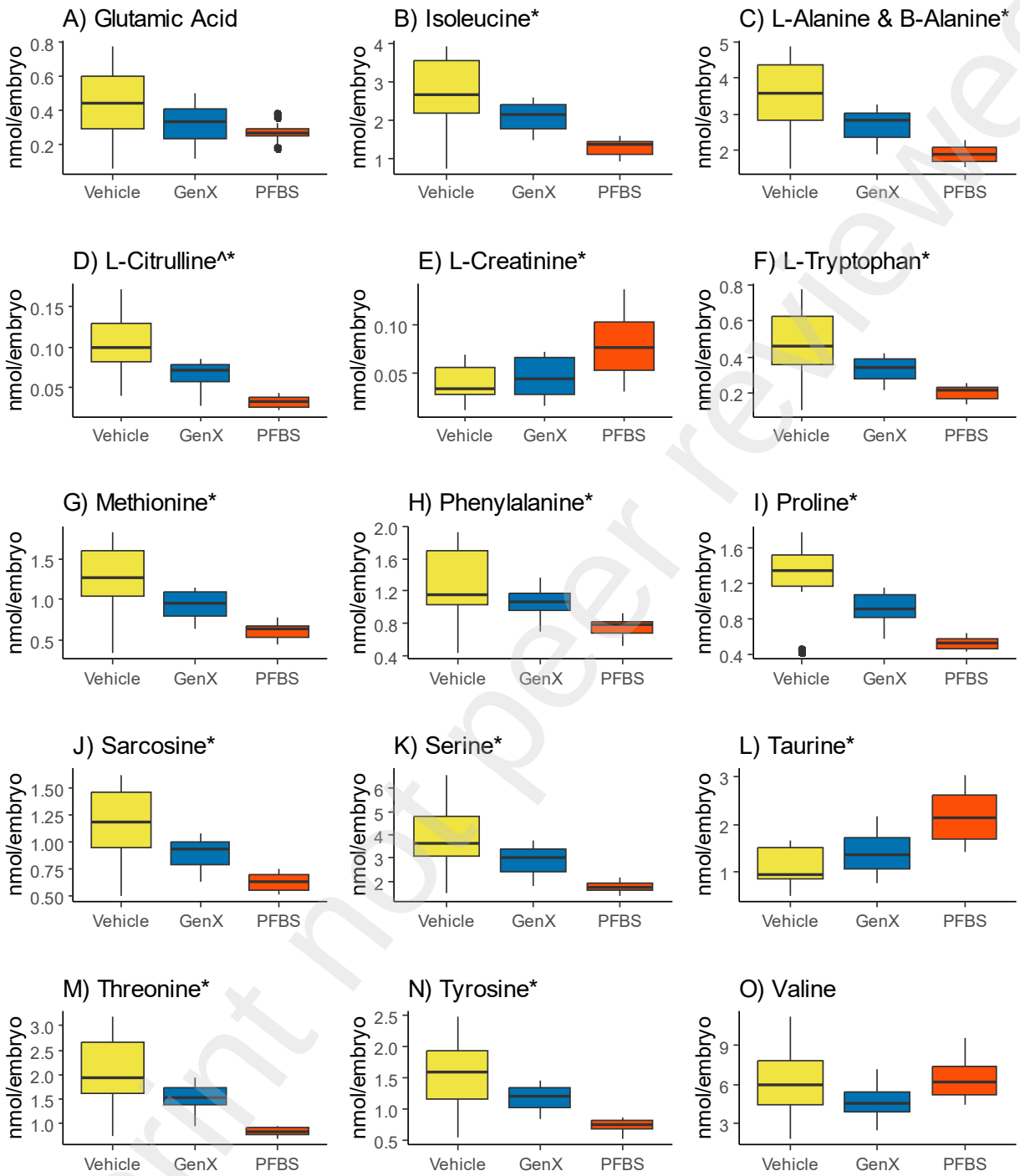

Figure 3: Targeted metabolites detected in GenX and PFBS exposed zebrafish embryos (each exposure group contained n = 6 groups of 10 larvae). * indicates significant alteration from the vehicle for PFBS exposure. ^ indicates significant alteration from the vehicle for GenX exposure.

3.3 Untargeted Metabolomics: Pathway Analysis

A metabolome-wide association study (MWAS) was performed to examine alterations in the metabolome due to GenX or PFBS exposure in embryonic zebrafish. In GenX exposed embryos, 831 of 4,554 detected features in the ESI- mode were significantly altered (FDR < 0.2), and 630 of 8,517 detected features in ESI+ mode were significantly altered (FDR < 0.2) (**Figure 4**). From the PFBS exposure, 596 of 4,518 detected features in ESI- mode were significantly altered (FDR < 0.2), and 1,897 of 8,532 detected features in ESI+ mode were significantly altered (FDR < 0.2) (**Figure 4**).

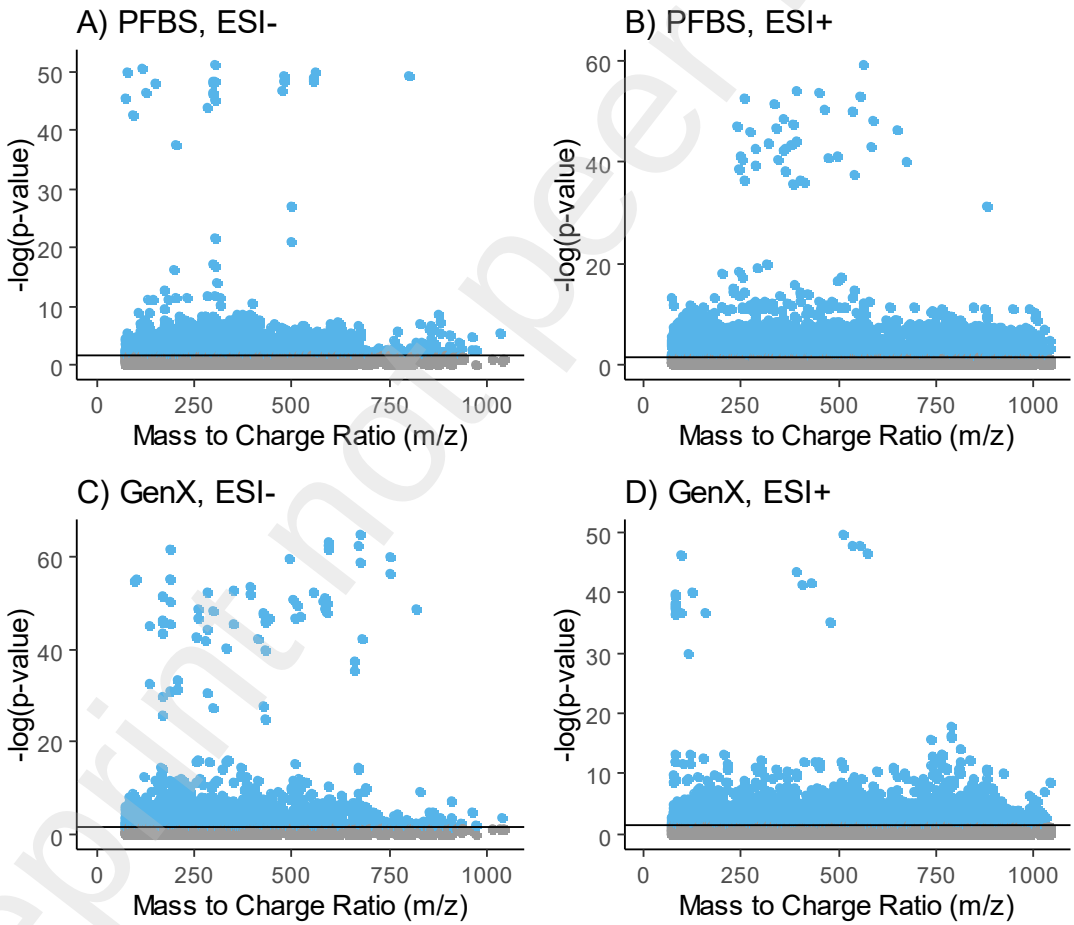

Figure 4: Manhattan plots for PFBS (a,b) and GenX (c,d) exposed larva, with ionization mode indicated as ESI+/-. Each dot represents an individual feature. The horizontal black line indicated on each graph represents a FDR= 0.2; all features (blue) above this line were significantly altered in the exposed groups in comparison to the vehicle group.

Features with -log10(p-value) above the 0.2 FDR threshold (**Figure 4**) were used in pathway analysis to identify enriched pathways. Across both exposures, enriched pathways were identified using 3 or more associated metabolites (features) for 42 metabolic pathways in total (**Figure 5**). For the GenX exposure group, 32 total pathways were enriched with 18 unique to ESI+ mode, 13 overlapping between ESI+/- modes, and 1 unique to ESI- mode (**Table S6**). In the PFBS exposure group, 34 total pathways were enriched with 5 unique to ESI+ mode, 21 overlapping between ESI+/- modes, and 8 unique to ESI- mode (**Table S6**).

In the GenX exposed zebrafish, 17 out of 32 pathways were significantly ($p > 0.05$) enriched in at least one ionization mode. In the PFBS exposed zebrafish, 21 out of 34 pathways were significantly enriched in at least one of the two ionization modes. Enriched pathways were grouped into nine different global metabolomic categories (**Table S6**): Amino acid metabolism, Carbohydrate metabolism, Energy metabolism, Genetic information processing, Glycan biosynthesis, Lipid metabolism, Metabolism of cofactors and vitamins, Nucleotide metabolism, and Xenobiotics metabolism. Across all ionization modes and exposures, the global amino acid metabolism contained the greatest number of enriched (15) and significantly enriched (14 out of 15 in at least one exposure group and ionization mode) pathways.

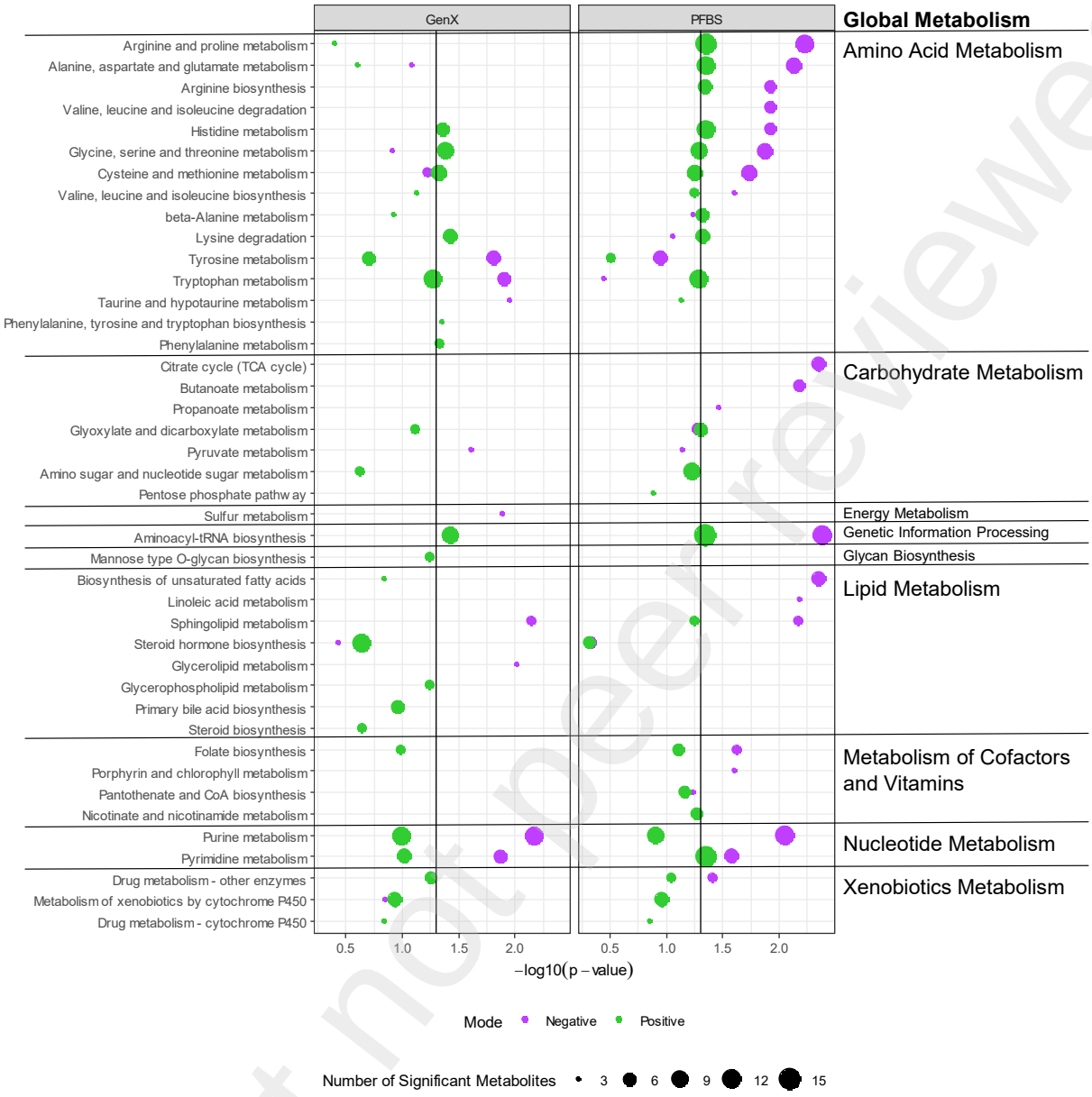

Figure 5: Enriched pathways from MetaboAnalyst pathway analysis, categorized by global metabolisms. The vertical black line represents -log10(0.05) (p-value = 0.05). Values to the right of the line on each plot represent significantly altered pathways (p-value > 0.05).

4. DISCUSSION

4.1 Body Burden

Overall, the body burden data demonstrate a high uptake of each compound within the embryos, as greater than 80% of the applied mass of each compound within exposure solutions was absorbed by the larvae. However, the difference in body burden between each compound provide insight into how readily each compound entered the zebrafish tissue. Despite a 73.7% higher GenX exposure dose than PFBS (mg L^{-1} comparison), GenX bioconcentration factor (BCF) was lower than PFBS BCF. Results for both compounds demonstrate BCFs within a comparable range to other known aqueous contaminants, with higher results indicating a more bioaccumulative substance (Wassenaar et al., 2020; Winston H. Hickox, 2000). It is important to note that BCF can vary greatly based on the population studied, and results from this study were used to determine the difference between GenX and PFBS uptake into the zebrafish. The discrepancy between exposure dose uptake and body burden suggests that PFBS more readily entered the tissue of exposed zebrafish larvae than GenX or that GenX is more readily excreted from the larvae.

Additionally, a comparison between body burdens and metabolic pathway alterations demonstrates that PFBS exposure resulted in a greater number of both significantly altered targeted metabolites and significantly altered pathways despite a lower exposure dose and body burden. The greater number of metabolic alterations observed in the PFBS group may indicate a higher toxicity of PFBS exposure as compared to GenX exposure.

4.2 Biological Significance of Impacted Pathways

GenX Exposure: Overall, GenX exposure demonstrated fewer impacted pathways or metabolites than PFBS exposure, with only one targeted metabolite being significantly altered

from the vehicle group, and 32 altered pathways, of which 17 were significantly altered (description of each of the significantly altered pathways can be found in **Table 1**). The significantly altered pathways in the GenX exposed zebrafish fell into all nine global metabolisms: Amino Acid Metabolism, Carbohydrate Metabolism, Energy Metabolism, Genetic Information Processing, Lipid Metabolism, Metabolism of Cofactors and Vitamins, Nucleotide Metabolism, and Xenobiotics Biodegradation and Metabolism (**Table S6**).

Table 1: Descriptions of significantly altered metabolic pathways, grouped into significantly altered metabolisms from the GenX exposure group only, both exposure groups, and the PFBS exposure group only.

Metabolic Pathway	Function	Reference
GenX		
Tryptophan Metabolism	Regulates energy homeostasis and aids in controlling inflammation, associated with aging.	(Sorgdrager et al., 2019)
Tyrosine Metabolism	Required for synthesis of proteins, used as an alternative energy source for cells. Used in the liver as a precursor to other metabolic processes.	(Nguyen et al., 2020)
Taurine and Hypotaurine Metabolism	Regulates processes in the central nervous system such as anti-oxidation and anti-inflammatory responses.	(Ichikawa et al., 2023)
Phenylalanine, Tyrosine, and Tryptophan Biosynthesis	Functions in the TCA cycle to help regulate energy expenditure and maintain energy homeostasis.	(Mishra et al., 2017)
Phenylalanine Metabolism	Functions in the TCA cycle to help regulate energy expenditure and maintain energy homeostasis. Acts as a precursor to the formation of important neurotransmitters.	(Mishra et al., 2017)
Pyruvate Metabolism	Involved in mitochondrial processes, end-product of glycolysis.	(Gray et al., 2014)
Sulfur Metabolism	Aids in utilizing sulfur-containing compounds, particularly the processing of cysteine and methionine amino acids.	(Griffith, 1987)
Glycerolipid Metabolism	Used to synthesize glycerolipids in the glycerolipid/free fatty acid cycle. Essential to the breakdown of lipids to maintain body temperature. Alterations can be associated with metabolic diseases such as metabolic syndrome.	(Prentki and Madiraju, 2008)
GenX and PFBS		
Cysteine and Methionine Metabolism	Regulation of metabolic processes, lipid metabolism, immune system, and digestion. Antioxidative effects through endogenous antioxidant enzyme activation and glutathione biosynthesis.	(Martínez et al., 2017)
Folate Biosynthesis	Involved in the synthesis of DNA, amino acids, and nucleotides. Alterations to this metabolism is associated with cellular damage, cardiovascular, and neurodegenerative disorders or diseases.	(Yao et al., 2022)
Glycine, Serine, and Threonine Metabolism	Required for synthesis of proteins, nucleic acids, and lipids. Biosynthesis of glycine and serine is essential to antioxidative properties of cells.	(Amelio et al., 2014)
Histidine Metabolism	Required for synthesis of proteins used in the small intestine and liver, incorporated into amino acids essential to muscle and brain tissue.	(Moro et al., 2020)
Lysine Degradation	Involved in neurometabolic and mitochondrial processes.	(Leandro and Houten, 2020)
Aminoacyl-tRNA Biosynthesis	Required for protein synthesis and the production of transfer RNAs. Function in the immune system as regulators and signaling molecules for immune cell development and regulators for biological processes in immune cells.	(Nie et al., 2019)
Purine Metabolism	Structural component of DNA and RNA, functional component of many metabolic processes. Disruptions to the purine metabolism is associated with dysregulation of the nervous system in humans.	(Ren et al., 2018)
Pyrimidine Metabolism	Essential to the synthesis of DNA and RNA, used for cell growth, development, signaling, and energy.	(Wang et al., 2021)
Sphingolipid Metabolism	Found in nervous tissue, sphingolipids regulate cell function and accumulate during periods of oxidative stress.	(Doccini et al., 2022)
PFBS		
Alanine, Aspartate and Glutamate Metabolism	Derived from the Citric Acid (TCA) Cycle and is a precursor for other amino acids and several important metabolic processes.	(Reitzer, 2004)

Arginine and Proline Metabolism	Proline acts as a precursor to arginine, proline metabolism acts as metabolic regulation pathway to account for deficiencies in associated pathways. Arginine and proline play roles in nitrogen cycling in organisms.	(Phang, 2017)
Arginine Biosynthesis	Precursor to other amino acids, plays a role in protein synthesis.	(Charlier and Glansdorff, 2004)
Beta-Alanine Metabolism	Involved in glycolysis through combination with histidine to form carnosine.	(Vaughan et al., 2014)
Biosynthesis of Unsaturated Fatty Acids	Formed in the liver for cell walls, membranes, and protein modification. Used for energy storage and a precursor to other metabolites.	(Beld et al., 2015)
Butanoate Metabolism	Involved in the biosynthesis of polysaccharides in the gut lining, and play an essential role in intestinal immune responses.	(Liu et al., 2022; Wang et al., 2020)
Citrate Cycle (TCA Cycle)	Main energy metabolism within organisms. Oxidizes fats, proteins, and carbohydrates to product energy.	(Martínez-Reyes and Chandel, 2020)
Glyoxylate and Dicarboxylate Metabolism	Utilizes fatty acids and acetate to produce energy as an alternative pathway to the TCA Cycle. Alterations associated with oxidative stress.	(Ahn et al., 2016)
Valine, Leucine, and Isoleucine Biosynthesis	Branched chain amino acids that play a role in the energy metabolism of zebrafish. Alterations can be associated with oxidative stress and organ damage.	(Meng et al., 2022)
Linoleic Acid Metabolism	Fatty acid metabolism to process dietary linoleic acid.	(Banni, 2002)
Porphyrin and Chlorophyll Metabolism	Essential to the formation of hemoglobin.	(Zhou et al., 2020)
Drug Metabolism – Other Enzymes	Enzymes that play a role in drug absorption and processing within the zebrafish.	(Hung et al., 2012)

From the significantly altered metabolisms resulting from GenX exposure, there are several common metabolic systems and biological processes that were affected by multiple metabolic pathways, including protein synthesis, cellular signaling, growth and development, antioxidative properties, energy processes, and immune and nervous system regulation. Disruptions to these pathways, individually or in combination with each other, could have effects on many organ systems and biological processes, such as response to oxidative stress or disease, liver functionality, and maintenance of metabolic processes through cellular development. In humans, alterations to several of the identified significantly altered pathways are associated with severe

diseases, including neurodegenerative diseases such as Alzheimer's and Parkinson's Diseases (purine metabolism), cancerous tumor growth (glycine, serine, and threonine metabolism), and Metabolic Syndrome (association with weight gain, Type 2 Diabetes, obesity (alanine, aspartate, and glutamate metabolism; cysteine and methionine metabolism; glycerolipid metabolism; glycine, serine, and threonine metabolism) (Amelio et al., 2014; Ansoleaga et al., 2015; Chen et al., 2022; Garcia-Esparcia et al., 2015; Li et al., 2020). A previous study of zebrafish embryonic and larval exposure to PFOS and other toxicants found that alterations to the alanine and proline metabolism indicated oxidative stress and imbalances in homeostasis and energy metabolisms (Ortiz-Villanueva et al., 2018). A different study discovered that alterations to the methionine metabolism had adverse effects on liver formation during zebrafish development (Liu et al., 2016). Our observed perturbations to metabolic pathways suggest metabolic dysregulation in the zebrafish embryos, with potential physiological implications ranging from liver development to tissue maintenance.

In addition to the functions listed in **Table 1**, several significantly altered pathways are associated with essential amino acids, or amino acids that must be consumed in animals' diets for proper development and maintenance of cells, tissues, and in bodily functions. From the list of impacted metabolic pathways in **Table 1**, the following are nutritionally essential amino acids: cysteine, histidine, lysine, methionine, threonine, tryptophan, and tyrosine (Watts and D'Abramo, 2021). Disruption of essential amino acid pathways for processing and utilizing amino acids in the body can have effects on growth, survival, reproduction, and overall health of the animal, which indicates that metabolic pathways essential to development and health of the zebrafish have been impacted by exposure to GenX in this study (Watts and D'Abramo, 2021). In combination with the impacts of altered metabolisms that affect cellular development and growth, protein synthesis

and muscular maintenance, and metabolic homeostasis, exposure to GenX during the embryonic and larval stages may have impacts on the proper development and growth of the zebrafish.

PFBS Exposure: Despite lower body burden in the PFBS exposure group, PFBS exposure resulted in a greater number of significantly altered pathways and targeted metabolites. Significant overlaps exist between significantly altered pathways from the GenX exposure group and PFBS exposure group; there were 12 significantly altered pathways uniquely in the PFBS exposure group in addition to the 9 shared with the GenX exposed zebrafish (**Table 1**). Significantly altered pathways for the PFBS exposed zebrafish (including overlap with the GenX exposed zebrafish) were in 8 global metabolisms: amino acid metabolism, carbohydrate metabolism, genetic information processing, lipid metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, and xenobiotics biodegradation and metabolism (**Table S6**). PFBS exposure resulted in 12 additional significantly altered targeted metabolites with the only overlap with the GenX exposure group being L-Citrulline, which was significantly downregulated in both exposures (**Table S5**).

Several metabolic pathways affected in the PFBS exposure group further contribute to Metabolic Syndrome, including alanine, aspartate, and glutamate metabolism, arginine biosynthesis, TCA Cycle, and valine, leucine, and isoleucine biosynthesis (Chen et al., 2022). Several metabolic pathway alterations overlap with significantly altered metabolites from targeted analysis, including alterations to levels of alanine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. There are also several additional functions impacted by the pathways that were altered uniquely in the PFBS exposure group, specifically the alterations observed in the energy/carbohydrate metabolism and its related amino acid pathways (TCA Cycle; glyoxylate and dicarboxylate metabolism; alanine, aspartate, and glutamate metabolism) and

effects on the lipid metabolism through use of fatty acids. Previous zebrafish toxicology studies indicate that alterations to the lipid (fatty acid) metabolism affects lipid creation and utilization in the liver, and disturbances to the carbohydrate metabolism may indicate insulin resistance, associated with diabetes (Du et al., 2016; Teng et al., 2018). In humans, diabetes can be a result of Metabolic Syndrome, so alterations to the amino acid metabolisms affected in both exposures as well as the alterations to the lipid metabolism observed in the PFBS exposure group may indicate chronic perturbations to organ functions and bodily regulation (Chen et al., 2022).

It is also important to consider the implications of these results for human health, especially if contamination of water with replacement PFAS compounds such as PFBS and GenX becomes more widespread. The primary route of exposure to PFAS is through consumption of contaminated food and drinking water, and studies show that consumption of freshwater fish contaminated with PFAS can cause a direct increase in blood serum levels of PFAS in humans (Barbo et al., 2023). Considering the high uptake of GenX and PFBS by zebrafish in this study, ingestion of these compounds could pose dangers to human health through an increase in blood serum levels of GenX and PFBS. Not only would consumption of contaminated fish pose risks to the consumer, but the effects observed in this study would likely be most relevant to *in utero* exposure to PFAS. Fetal exposure to PFAS during pregnancy can cause the fetus to be at higher risk for metabolic syndrome, which is consistent with metabolites altered in this study, as well as thyroid dysfunction and kidney disease (Blake and Fenton, 2020). Using zebrafish as a toxicological model for both fish uptake of novel PFAS as well as potential human health effects demonstrates that adverse health effects are possible in vertebrate populations exposed to GenX and PFBS.

4.3 Study Limitations

The single analyte exposure paradigm used in this study was different from environmentally relevant exposures to these chemicals. Across the US, freshwater fish are chronically exposed to combinations of PFAS compounds (concentrations depending on geographic location) at lower exposure doses than used in this study (Barbo et al., 2023). Even in the Cape Fear River, a site of known GenX contamination due to an upstream chemical plant, the maximum concentration of GenX in a water quality study was 4.56 µg L^{-1}, with an average of 630 ng L^{-1}, indicating that the 49.5 mg L^{-1} exposure dose used in this study was an order of magnitude higher than GenX concentrations found in surface waters across the US (Sun et al., 2016). Although our exposure doses may not be representative of chronic exposure levels, the results are relevant to embryonic or early life exposure to GenX or PFBS. Therefore, developmental metabolome alterations from this study could be detected to a lesser extent at lower exposure doses observed in aquatic environments.

This study also included several limiting factors that do not allow for further comparison or analysis of the toxicity and metabolic effects of GenX and PFBS. Firstly, the exposure doses used were based on mortality and/or physiological alterations that occurred due to exposure; however, the concentrations used in exposure were not equal, nor were there multiple concentrations to compare the level of toxicity of each compound to the embryos. For example, it would be useful to assess exposure doses for each compound leading to a similar magnitude or number of metabolic, as we observed a greater number of metabolic alterations in the PFBS exposure group with a different exposure dose. Additionally, this study focused only on the individual effects of each compound; future studies would ideally assess exposure to a mixture of PFBS and GenX as well as other short-chain PFAS. While there are limitations to this study, including unequal exposure doses and lack of multiple dosing concentrations or additional dosing

compounds, the study as a whole provides a basis for future studies that can provide additional insight into the toxicity of GenX and PFBS to zebrafish and other vertebrates.

CONCLUSIONS

Overall, this study provides an understanding of metabolic alterations induced by exposure to GenX or PFBS in zebrafish embryos. These exposures resulted in no physiological deformities or excess mortality of the zebrafish over a 6-day exposure period. The body burden assessment indicated that either PFBS is more readily absorbed in zebrafish larvae compared to GenX or GenX is more readily excreted from zebrafish larvae. Targeted and untargeted metabolomics were used to understand alterations to the zebrafish metabolome induced by exposure to GenX and PFBS, and PFBS exposure induced a greater number of significantly altered metabolites and metabolic pathways than GenX exposure. Altered metabolic pathways in both groups indicate altered responses to oxidative stress, dysregulation of energy homeostasis and growth-related metabolites, and perturbations to lipid creation in the liver. These results indicate that developmental exposure to GenX or PFBS may disrupt cellular development and metabolic regulation in multiple organ systems. Using zebrafish as a toxicological model, the results from this study demonstrate the metabolic effects of early-life exposure to GenX and PFBS. Further studies on combined effects of GenX, PFBS, and other short-chain replacement PFAS will provide greater detail on potential health effects of ingestion of these chemicals for both humans and other animals.

ACKNOWLEDGEMENTS

FD was supported by the Brown University School of Engineering DiMase Summer Internship Fellowship. KM was supported by NIEHS K01 ES035398 (PI) and R01 ES032386. The Thermo LC-Orbitrap MS was partially funded by NSF Major Research Instrumentation (MRI) award

CBET-1919870 to KDP (PI) and JSP (Co-I). SEP was supported by a Ruth L. Kirschstein Predoctoral Individual National Research Service Award (NRSA; F31HL156460) from the NHLBI and was previously supported by the Brown University Environmental Pathology Training Grant (T32ES007272-26). JSP was supported by a NIEHS Outstanding New Environmental Scientist (ONES) award (R01ES030109).

AUTHOR CONTRIBUTIONS

FD: Conceptualization, Investigation, Formal Analysis, Original Draft, Reviewing and Editing. SP: Investigation, Methodology, Reviewing and Editing. KDP: Conceptualization, Resources, Reviewing and Editing. JP: Methodology, Resources, Supervision, Reviewing and Editing. KEM: Conceptualization, Methodology, Validation, Data Curation, Supervision, Reviewing and Editing.

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