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## **Early life perfluorooctanesulphonic acid (PFOS) exposure impairs zebrafish organogenesis**

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1 **Abstract**

2 As a persistent organic contaminant, perfluorooctanesulphonic acid (PFOS) has been widely  
3 detected in the environment, wildlife, and humans. The present study **revealed** that zebrafish  
4 embryos exposed to 16  $\mu$ M PFOS during a sensitive window of 48-96 hour post-fertilization (hpf)  
5 disrupted **larval morphology** at 120 hpf. Malformed zebrafish larvae were characterized by  
6 uninflated swim bladder, less developed gut, and curved spine. Histological and ultrastructural  
7 examination of PFOS-exposed larvae showed structural alterations in swim bladder and gut.  
8 Whole genome microarray was used to identify the early transcripts dysregulated following  
9 exposure to 16  $\mu$ M PFOS at 96 hpf. In total, 1,278 transcripts were significantly misexpressed ( $p <$   
10  $0.05$ ) and 211 genes were changed at least two-fold upon PFOS exposure **in comparison to the**  
11 **vehicle exposed** control group. A PFOS-induced network of perturbed transcripts relating to swim  
12 bladder and gut development revealed that misexpression of genes were involved in organogenesis.  
13 Taken together, early life stage exposure to PFOS perturbs various molecular pathways potentially  
14 resulting in observed defects in swim bladder and gut development.

15

16 **Keywords:** Zebrafish embryo; perfluorooctanesulfonic acid; swim bladder; gut; developmental  
17 toxicity

18

## 1. Introduction

Perfluorinated compounds (PFCs) are a class of persistent contaminants widely used as surfactants, lubricants, adhesives, fire retardants, propellants, and medicines (Renzi et al., 2013)**Error! Reference source not found.** Perfluorooctanesulphonic acid (PFOS), an end product of the breakdown of multiple PFCs, is widely detected in wildlife, humans and the environment (Giesy and Kannan, 2001; Houde et al., 2006; Zhang et al., 2011b). Although PFOS is generally found at low levels in surface water, the chemical is characterized by high bioaccumulation and negligible elimination (Kannan et al., 2005a). As a consequence, higher concentrations of PFOS have been detected in a variety of fish species. For example, PFOS was detected in the liver of wild Gibel carp at levels of up to 9,031 µg/kg wet weight (Hoff et al., 2005) and average PFOS concentrations detected in fish tissue were 8850-fold greater than those measured in surface water (Sinclair et al., 2006). Additionally, high concentrations of PFOS were also detected in fish eggs (145–381ng/g) in lake whitefish from Michigan waters in the United States (Kannan et al., 2005b), which suggests oviparous transfer of this compound (Kannan et al., 2005b).

Zebrafish (*Danio rerio*) is a freshwater fish that is extensively used as a model organism for various research fields due to their small size, embryonic transparency, and rapid developmental cycle (Hill et al., 2005; Jang et al., 2013). The availability of its complete genome sequence enables the construction of zebrafish microarrays that permit global gene expression analysis (Pichler et al., 2003). Monitoring the expression of thousands of genes simultaneously through microarray analysis allows researchers to identify biological pathways perturbed by chemical exposure (Mathavan et al., 2005). For these exact same reasons, zebrafish have been used for studying PFOS toxicity (Huang et al., 2010; Shi et al., 2008). Zebrafish embryos exposed to 1-5 mg/l PFOS from 4-132 hpf exhibit spinal curvature, uninflated swim bladder, reduced hatching rates, and decreased blood flow and body length (Shi et al., 2008). PFOS-exposed zebrafish embryos are subject to increased cell death, muscle lesions, and abnormal swimming behaviors (Huang et al., 2010). Among various

47 | reported phenotypic changes, [we previously found alteration of gut and swim bladder from](#)  
48 | both acute and chronic PFOS exposure (Chen et al., 2013; Huang et al., 2010; Wang et al.,  
49 | 2011), yet the mechanisms that underlie these effects are not well understood. Previous  
50 | studies have reported widespread proteomic (Shi et al., 2009) and microRNA expression  
51 | (Zhang et al., 2011a) changes associated with acute PFOS exposure in embryonic zebrafish;  
52 | however, studies on transcriptional changes upon PFOS exposure are still lacking. In the  
53 | present study, we characterized gene expression changes induced by developmental  
54 | exposure to PFOS to identify signaling networks that may contribute to adverse  
55 | morphological outcomes.

56

## 57 **2. Materials and methods**

### 58 2.1. Fish husbandry and embryo collection

59 Wildtype (AB strain) zebrafish were raised and kept at standard laboratory conditions of  
60 28°C on a 14:10 dark/light photoperiod in a recirculation system according to standard  
61 zebrafish breeding protocols (Westerfield, 1993). Water supplied to the system was filtered  
62 by reverse osmosis (pH 7.0-7.5), and Instant Ocean® salt was added to the water to raise the  
63 conductivity to 450-1000 µS/cm (system water). The fish were fed three times daily with  
64 zebrafish diet (Zeigler, Aquatic Habitats, Apopka Florida) and a live artemia (Jiahong Feed  
65 Co., Tianjin, China). Zebrafish embryos were obtained from adults in tanks with a sex ratio  
66 of 1:1, and spawning was induced in the morning when the light was turned on. Embryos  
67 were collected within 0.5 h of spawning and rinsed in an embryo medium (EM: 0.137 M  
68 NaCl, 5.4 mM KCl, 0.25 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.0 mM MgSO<sub>4</sub>  
69 and 4.2 mM NaHCO<sub>3</sub>) (Westerfield, 1993). Fertilized embryos with normal morphology  
70 were staged under a dissecting microscope SMZ 1500 (Nikon, Japan) according to the  
71 standard methods (Kimmel et al., 1995).

72

### 73 2.2. PFOS stock solutions and exposure protocols

74 Perfluorooctanesulphonic acid (PFOS; CAS # 1763-23-1, purity >96%) was purchased from  
75 Sigma-Aldrich Chemical (St. Louis, MO, USA) and dissolved in 100% dimethyl sulfoxide  
76 (DMSO) to prepare PFOS stock solutions of 32 mM. A serial dilution was made in 100%  
77 DMSO that was 1,000 times more concentrated to allow for a 1:1,000 dilution with EM to  
78 create a serial dilution with a final DMSO concentration of 0.1%. The control also received  
79 0.1% DMSO (v/v in EM).

80

### 81 2.3. Sensitive exposure period screening

82 To determine which developmental stage is most sensitive to PFOS-induced malformations,  
83 embryos/larvae were waterborne exposed to PFOS (8, 16, 32 µM) in 6-well plates (20

84 embryos per well with 5 mL solution) from 0-48 hpf or 48-96 hpf. The chemical solution was  
85 not changed during the exposure window, and there were three biological repeats. At the end  
86 of each exposure period, the embryos or larvae were rinsed three times with EM and  
87 transferred to 96-well plates (1 embryo per well with 200  $\mu$ L solution) for continuous  
88 development until 120 hpf, where the incidence of various malformation was scored. The  
89 embryos in one repeat were from the same well in the 6-well-plates when they were exposed.

90

#### 91 2.4. Histological examination of the larval swim bladder and gut

92 For hematoxylin and eosin (HE) staining, embryos were exposed to 0.1% DMSO or 16  $\mu$ M  
93 PFOS from 48 to 96 hpf, rinsed three times with EM and continuously developed until 120  
94 hpf in EM. These larvae were fixed overnight with 4% [paraformaldehyde \(PFA\)](#) at 4°C, and  
95 then dehydrated in graded series of ethanol solutions prior to paraffin embedding. Embedded  
96 larvae were sectioned (5 $\mu$ m longitudinal sections) and stained with HE. Fifteen embryos  
97 were used for each treatment group. Images were obtained with a confocal microscope  
98 FV1000 (Olympus, Japan) and images were captured using a FITC filter.

99

#### 100 2.5. Transmission electron microscopic examination of the larval swim bladder and gut

101 Embryos were exposed to 0.1% DMSO or 16  $\mu$ M PFOS from 48 to 96 hpf then transferred to  
102 EM until 120 hpf. At 120 hpf, larvae were fixed in 2.5% glutaraldehyde at 4°C for 48 h,  
103 rinsed in 0.1M PBS, and then set in 1% osmium tetroxide at 37°C for 1 h. Larvae were then  
104 stained in 1% uranyl acetate at 37°C for 1 h. Samples were dehydrated through an ethanol  
105 series (50%, 75% and 100%), transferred to acetone, and embedded in pure resin prior to  
106 sectioning. The plastic blocks were sectioned transversely to obtain 1  $\mu$ m using a LKB2008  
107 instrument and ultrathin sections of interest were selected using light microscopy. The  
108 ultrathin sections of 80 nm made by a POWER TOME XL instrument were collected on  
109 200-mesh copper grids and stained with lead citrate for 10 min. The sections were analyzed  
110 with a Hitachi H-7500 transmission electron microscope (TEM).

111

## 112 2.6. NimbleGen microarray

113 Embryos at 48 hpf were exposed to 0.1% DMSO or 16  $\mu$ M PFOS for 48 h, and [RNAs were](#)  
114 [then extracted from](#) embryos at 96 hpf. There were six [biological](#) replicates [per treatment](#)  
115 [group with each replicate consisted of pooled tissue from 40 larvae. A total of 12 samples](#)  
116 [were assessed on a single chip that contains 12 individual arrays.](#) Total RNA was isolated  
117 with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. The  
118 quantity and quality of RNA were determined using the Nanodrop-1000 Spectrophotometer  
119 and gel electrophoresis. All RNA samples passed the concentration and quality requirements  
120 ( $A_{260}/A_{280} \geq 1.8$  and  $A_{260}/A_{230} \geq 1.8$ ). For microarray processing, 10  $\mu$ g of total RNA was  
121 reverse transcribed using SuperScriptIII and oligo primer (Invitrogen), and double stranded  
122 cDNA was synthesized and purified using a Qiagen MinElute PCR Purification spin column.  
123 Double stranded cDNA was labeled with Cy5 dNTP, and samples were then hybridized to  
124 12x135K zebrafish gene expression arrays (Roche Nimblegen, Madison, WI) and scanned  
125 using the Axon GenePix Pro 4200A scanner (Molecular Devices, Sunnyvale, CA) according  
126 to the manufacturer's instruction. The labeling, hybridizing, and scanning steps were  
127 finished at the IBEST DNA Sequencing Analysis Core of the University of Idaho, with the  
128 details stated in our previous study (Tal et al., 2012).

129

## 130 2.7. Microarray data processing and pathway design

131 The raw data were extracted, background subtracted, and quantile normalized (Bolstad et al.,  
132 2003) using NimbleScan v2.5 software. Gene calls were generated using the Robust  
133 Multichip Average (RMA) algorithm as previously described (Irizarry et al., 2003). Principal  
134 component analysis of all genes on array was used to evaluate if samples are outliers within  
135 each treatment group by correlation. Statistical analysis was performed using an unpaired  
136 t-test with 5% FDR in GeneSpring GX v10.0 (Agilent Technologies) to generate significant  
137 gene lists. Importing the statistically significant gene list into the Multi-Experiment Viewer



138 (MEV) produced a bi-hierarchical clustering heat map. Individual clusters were further  
139 analyzed with the Database for Annotation, Visualization and Integrated Discovery (DAVID  
140 (<http://david.abcc.ncifcrf.gov/home.jsp>) to determine common and unique functional  
141 pathways (Dennis Jr et al., 2003). A zebrafish nimblegen background and individual cluster  
142 gene lists were uploaded into DAVID using entrez gene identifiers. Functional annotation of  
143 clustering using levels 3, 4, and 5 of the gene ontology category of biological processes was  
144 applied to each gene list. Only biological processes receiving an enriched score greater than  
145 1 were noted on the bi-hierarchical clustering heat map. Zebrafish mRNA sequences on the  
146 microarray were blasted on the NCBI website to find the human orthologs with the highest  
147 blast score before subjecting them to Ingenuity Pathways Analysis (IPA, Ingenuity®  
148 Systems). The identified genes were mapped to corresponding gene objects in the Ingenuity  
149 Pathways Knowledge Base to generate networks, bio-functions, and canonical pathways.

150

## 151 2.8. Quantitative RT-PCR validation

152 Quantitative real time PCR (qRT-PCR) was used to confirm expression changes resulting  
153 from the microarray analysis. A subset of RNAs from the same samples used for the  
154 microarray analysis was used for qRT-PCR validation. cDNA was prepared from 5 µg of  
155 total RNA per group using a Prime Script® RT reagent Kit (Takara, Japan) following the  
156 manufacturer's instructions. qRT-PCR using gene-specific primers (Table 1, Sunny  
157 Biotechnology) was conducted on an Eppendorf Mastercycler® Realplex2. Gradient  
158 annealing temperature studies were initially completed to confirm the optimal annealing  
159 temperature for each primer set. The reaction mixtures included 10 µl power™ SYBR  
160 Green® supermix, 0.4 µl of each primer, 4.2 µl of ddH<sub>2</sub>O, and 5 µl of cDNA. The thermal  
161 cycle reaction was performed using standard procedures - 95°C for 30s, 40 cycles of 95°C  
162 for 5s and 60°C for 30s and the data were collected at the end of each extension step. The  
163 gene expression levels were measured in a total of three biological replicates per treatment  
164 group (n=3, with 40 embryos per replicate). For each biological replicate, three technical

165 | [repeats were used to reduce sampling error.](#) mRNA levels were calculated and normalized  
166 against housekeeping gene  $\beta$ -actin using the equation: fold change =  $2^{-\Delta\Delta CT}$  (Schmittgen and  
167 Livak, 2008)**Error! Reference source not found.** Gel electrophoresis and thermal  
168 denaturation (melt curve analysis) were used to confirm product specificity. To compare the  
169 results from the microarray and qRT-PCR, gene expression profiles were displayed as a fold  
170 change relative to the vehicle control group.

171

## 172 2. 9. Statistical analysis

173 Sigmoidal regression was used to generate the dose–response curves for  $EC_{50}$  calculation  
174 (Origin 8.0, OriginLab). For gene expression comparisons, an unpaired t-test with 5% FDR  
175 was performed (SPSS, Chicago, IL, USA). All data are reported as means  $\pm$  standard error  
176 (SEM) unless otherwise stated.

177

178 **3. Results**

179 3.1. PFOS exposure produces uninflated swim bladder and less developed gut

180 PFOS exposure during 48-96 hpf resulted in several distinct malformations including an  
181 uninflated swim bladder, less developed gut, and curved spine at 120 hpf (Fig. 1A-B).

182 Typically, malformed larvae presented with all three types of malformations together.

183 However, larvae developmentally exposed to PFOS from 8-48 hpf did not develop any  
184 obvious malformations, even at a concentration of 32  $\mu$ M (Fig.1A). All the embryos

185 survived during our experiment and no mortality occurred (data not shown). For embryos

186 exposed from 48-96 hpf, all malformations were scored at 96 and 120 hpf. At 96 hpf, 16  $\mu$ M

187 PFOS treated larvae appeared morphologically normal, while about 25% of 32  $\mu$ M group

188 larvae showed some malformations at this time point. At 120 hpf, both 16  $\mu$ M and 32  $\mu$ M

189 resulted approximately 100% malformation. Thus, 16  $\mu$ M dose was selected for gene

190 expression analyses.

191

192 3.2. PFOS induced histological alteration in swim bladder and gut section

193 Histologically, PFOS exposure altered the structures of swim bladder and gut relative to  
194 vehicle controls (Fig. 2). Compared to vehicle control larvae, the swim bladders in

195 PFOS-exposed larvae were smaller (uninflated), but still showed three distinct layers (Fig.

196 2C-D). However, the inner wall of the swim bladder cavity was less smooth with some caved

197 shapes (Fig. 2D). On the contrary, PFOS-exposed larvae showed a larger gut tube than the

198 control and displayed a non-uniform inner structure, which was shape uniform in the control

199 larvae (Fig. 2E-F).

200

201 When examined with TEM, the inner cells in PFOS-exposed larvae swim bladder showed

202 pyknosis and mitochondrial vacuole changes when compared with controls (Fig.3A-B). All

203 three layers of cells showed mild apoptosis such as nuclear shrinkage and nuclear envelope

204 gap expansion in PFOS-exposed larvae relative to controls (Fig. 3C vs. Fig. 3D). In the

205 middle yolk layer, the cytoplasm content was decreased, the mosaic-like structure was  
206 significantly reduced, the arrangement of collagen fibers was partly disordered, and mild  
207 edema was found (Fig. 3C, E vs. Fig. 3D, F). For the guts in the control group, the intestine  
208 mucosal epithelial cells were mainly column-shaped and closely connected, with oval nuclei,  
209 uniform chromatin, and abundant organelles of mitochondria and endoplasmic reticulum,  
210 and a surface arranged with rich, uniform microvilli and an intact basement membrane (Fig.  
211 3G, I). In comparison, the columnar epithelial cells in the PFOS-exposed larvae had partially  
212 pyknotic nuclei, increased heterochromatin, partial mitochondrial vacuolation, mild dilated  
213 endoplasmic reticulum, and uneven surface microvilli though the intercellular junctions in  
214 the PFOS-exposed larvae were closed and the basement membrane was intact (Fig. 3H, J).

215

### 216 | 3.3. PFOS exposure leads to differential gene expression at 96 hpf

217 To identify gene expression changes following PFOS exposure during development, global  
218 microarray analysis was conducted using RNA isolated at 96 hpf from larvae exposed to  
219 PFOS from 48-96 hpf. A principal component analysis of all genes on the array shows  
220 separation of the two treatment groups into distinct clusters with four outliers (circles, Fig.  
221 4A). The box plot of normalized data shows consistency in the interquartile range across the  
222 biological replicates without outliers (Fig. 4B). Statistical analysis of the differentially  
223 expressed transcripts was performed both with and without outliers (Table 2). In the analysis  
224 with all six repeats, 162 transcripts were significantly misexpressed ( $p < 0.05$ ) and 5  
225 transcripts were changed more than two-fold by PFOS as compared with the control. When  
226 removing the four outliers from the analysis, 1,278 transcripts were significantly  
227 misregulated ( $p < 0.05$ ) and 211 genes were changed at least two-fold by PFOS as compared  
228 with the control group (Table 2 and Fig. 5).

229

230 To validate the array data, nine transcripts involved in organogenesis or metabolic processes  
231 were selected for validation by qRT-PCR. In general, the comparison of mRNA abundance

232 determined by the microarray and qRT-PCR revealed similar trends for all examined  
233 transcripts (Fig. 6).

234

235 3.4. PFOS exposure resulted in the misexpression of organogenesis and developmental  
236 network related transcripts

237 Differentially expressed transcripts were analyzed for enriched biological processes (Table  
238 3). Genes significantly elevated by PFOS exposure were associated with nucleic and  
239 macromolecule metabolism, cell differentiation and proliferation, neuron differentiation and  
240 development, and voltage-gated channels (Table 3). In contrast, downregulated genes were  
241 associated with cellular protein metabolic processes, macromolecular complex assembly,  
242 protein-DNA complex assembly, and positive regulation of translation and multicellular  
243 organism growth (Table 3). We also used IPA to identify pathways that are significantly  
244 altered compared to the control using genes significantly changed in PFOS group. The top  
245 toxicity pathways perturbed by PFOS exposure were mechanisms of gene regulation by  
246 peroxisome proliferators via PPAR $\alpha$ , decreases of transmembrane potential of mitochondria  
247 and mitochondrial membrane, and cardiac necrosis/cell death (Supplemental Table 1).

248 Specific analysis of transcripts related to swim bladder and gut development were used to  
249 build a PFOS-perturbed network (Fig.7). A total of 16 transcripts were upregulated and are  
250 labeled in red (e.g., *xdh*, *ide*, *lrp*, *insr*, and *anxa5*). An additional 9 transcripts were  
251 downregulated by PFOS exposure and are labeled in green (e.g., *cyp19a1*, *brd8*, and  
252 *nkx2-1a*).

253

254 **4. Discussion**

255 In the present study, malformations induced by acute PFOS exposure during a sensitive  
256 window of 48-96 hpf included uninflated swim bladder, less developed gut, and bent spine.  
257 These observations were consistent with previous findings (Huang et al., 2010; Shi et al.,  
258 2008; Wang et al., 2011). Further histology and TEM analysis revealed detailed structural  
259 changes in swim bladder and gut associated with PFOS acute exposure. Transcriptional  
260 analysis identified several potential pathways and candidate genes involved in the PFOS  
261 perturbed organogenesis.

262

263 The selection of sensitive window revealed that embryos at the developmental window of  
264 48-96 hpf are more sensitive to PFOS exposure than those at 8 to 48 hpf as a dose of 16  $\mu$ M  
265 led to 100% malformation in embryos exposed to PFOS during 48-96 hpf yet a dose of 32  
266  $\mu$ M did not cause any malformation for embryos exposed between 8 to 48 hpf. One possible  
267 reason for the relative resistance to PFOS of embryos at earlier developmental stage could be  
268 due to slower PFOS absorption prior to 48 h and more rapid PFOS accumulation in embryos  
269 after 48 h as we have shown previously (Huang et al., 2010). Alternatively, candidate  
270 receptors that mediate PFOS-induced toxicity may not become evident till 48 hpf (Bardet et  
271 al., 2002). Future studies are necessary to identify the underlying cause for this different  
272 window sensitivity to PFOS exposure.

273

274 In the present study, whole genomic microarray analysis was used to identify transcripts that  
275 are differentially expressed by PFOS exposure. We observed that a total of 1,278 transcripts  
276 were significantly affected by PFOS exposure and the biological processes enriched included  
277 metabolic processes. These include nucleus, phosphate, macromolecule, cellular glucan and  
278 protein metabolism. The digestive system plays a critical role in metabolic processes  
279 (DeWitt and Kudsk, 1999) thus the perturbed metabolic process may result from malformed  
280 digestive organs (e.g., the zebrafish gut) upon PFOS exposure. The microarray findings we

281 [reported here](#) [are](#) consistent with the analysis of the [proteomic changes identified](#) (Shi et al.,  
282 2009) [following](#) developmental PFOS exposure (till 192 hpf) as energy metabolism and lipid  
283 transport/steroid metabolic process were also implicated in the latter study. [Our findings](#)  
284 [were also in good agreement with an earlier study in PFOS exposed carps where altered](#)  
285 [genes in the liver were mainly involved in energy metabolism, reproduction, and stress](#)  
286 [response \(Hagenaars et al., 2008\)](#). A PFOS-induced network of perturbed transcripts relating  
287 to swim bladder and gut development revealed misexpression of insulin-degrading enzyme  
288 (*ide*), cytochrome P450- family 19- subfamily a- polypeptide 1 (*cyp19a*) and NK2  
289 homeobox 1b (*nkx2-1b*), all these genes [are](#) involved in organogenesis (Donoghue et al.,  
290 2000; Lieb et al., 2006; Wendl et al., 2002). [Confirmation of alterations at the](#)  
291 [protein/enzyme level is the next step in the assessment, but is beyond the scope of this study.](#)

292

293 Similar to the mammalian lung (Spooner and Wessells, 1970), the zebrafish swim bladder  
294 arises from an outgrowth of the foregut endoderm, and is in close temporal and spatial  
295 proximity to the liver and pancreas (Field et al., 2003). Prenatal PFOS exposure affects lung  
296 development in perinatal rats (Grasty et al., 2005). In the present study, we observed altered  
297 structure and gene expression in swim bladder-related transcripts. The [observation](#) that swim  
298 bladder was one of the main targets for PFOS induced developmental toxicity in zebrafish  
299 also corroborates previous findings that liver and lung are two primary target organs of PFOS  
300 (Hagenaars et al., 2008; Luebker et al., 2005). Gene expression profiling in the liver and lung  
301 of PFOS-exposed mouse fetuses revealed that PFOS-dependent changes are primarily  
302 related to activation of PPAR $\alpha$  (Rosen et al., 2009). A similar mechanism was proposed for  
303 PFOS induced gene expression changes associated with lipid metabolism and cholesterol  
304 biosynthesis (Lau et al., 2007) and hepatomegaly changes in lymphoid organs (DeWitt et al.,  
305 2009). [PFOS has also been shown to affect peroxisomal fatty acid  \$\beta\$ -oxidation pathway by](#)  
306 [altering peroxisomal membrane permeability to allow fatty acid influx](#) (Hu et al., 2005).  
307 Although we did not observe significant alteration of PPAR $\alpha$  in the present study, [IPA](#)

308 [analysis revealed that mechanism of gene regulation by peroxisome proliferators via PPAR \$\alpha\$](#)   
309 [was the top 1 toxicity pathway perturbed by PFOS exposure \(Supplemental Table 1\). Further](#)  
310 [analysis of PPAR signaling identified](#) significant [expression](#) changes of [transcripts](#) related to  
311 canonical pathway of PPAR $\alpha$ /RXR $\alpha$  activation (Supplemental Fig. [1](#)). Future functional  
312 validation is necessary to uncover whether PPAR $\alpha$ -dependent signaling plays a [functional](#)  
313 role in PFOS induced morphological changes in zebrafish larvae.

314

315 The zebrafish gut is ventral to the swim bladder, and it forms in early somite stages (10-18  
316 hpf), giving rise to the organs of the digestive tract and its accessory organs such as liver and  
317 pancreas at the pharyngula and hatching stages (48-72 hpf) (Wallace et al., 2005; Wallace  
318 and Pack, 2003). PFOS exposure induced multiple structural abnormalities in the gut  
319 including nuclei pyknosis, heterochromatin, and uneven surface microvilli in the columnar  
320 epithelial cells. This is the first [study](#) to [report](#) abnormal gut morphology upon exposure to  
321 PFOS. More studies are needed to delineate mechanisms underlying gut abnormalities upon  
322 exposure to PFOS during embryonic development.

323

324 [It is known that oxidative stress can induce cellular damage and this form of cellular stress](#)  
325 [involves in many biological and pathological processes](#) (Carnevali et al., 2003; MacNee,  
326 2000). [Previous studies](#) indicated that oxidative [stress](#) plays an important role in  
327 developmental toxicity by PFOS [exposure](#) (Liu et al., 2009; Qian et al., 2010; Wei et al.,  
328 2008). [More recently, prenatal PFOS exposure in rats from gestation day 1 to day 21 induced](#)  
329 [significant induction of oxidative stress in postnatal pups, representing by increased](#)  
330 [malondialdehyde level, decreased glutathione content, and declined superoxide dismutase](#)  
331 [activity](#) (Chen et al., 2012). [In fish, reactive oxygen species \(ROS\)-induced oxidative stress](#)  
332 [is thought to contribute to abnormal development during embryogenesis \(Yamashita, 2003\).](#)  
333 PFOS exposure to embryonic zebrafish from 4 to 96 hpf caused hypergeneration of ROS,  
334 which in turn induced phase II detoxification enzymes and nuclear factor erythroid 2 related



335 factor 2 (*nrf2*) pathway against oxidative stress to protect oxidative damage (Shi and Zhou,  
336 2010). Findings in our study showed that oxidative stress and nrf2-mediated oxidative stress  
337 response signaling are significantly perturbed by PFOS exposure, e.g., mitogen-activated  
338 protein kinase 3 (*mapk3*), janus kinase 2 (*jak2*), and aldehyde dehydrogenase family 1  
339 member L2 (*aldh1l2*) were significantly up regulated. Together, these findings indicate that  
340 oxidative stress may play an important role in PFOS induced developmental toxicity.

341

342 In summary, our study demonstrates that early life stage exposure to PFOS perturbs zebrafish  
343 embryonic swim bladder and gut development. Early life stage exposure to PFOS perturbs  
344 numerous molecular pathways, collectively leading to the morphological defects observed in  
345 the swim bladder and gut of PFOS exposed larvae. \_

346

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352

353

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**Table 1. Primers used for qPCR expression validation.**

Target	Forward (F) and reverse (R) sequence	PCR (bp)
<i>β-actin</i>	F: AAGCAGGAGTACGATGAGTC R: TGGAGTCCTCAGATGCATTG	238
<i>ace2</i>	F: GGCCCTTTCACCTGACAAAGCT R: GCCTTCCCATACATGCAGACGC	184
<i>xdh</i>	F: AGGAGGTTGTGGAGCCTGCACT R: CCTCCACGGTTGTTACCGCACA	134
<i>dhx58</i>	F: TGC GTTACGGGCTGTTGACCA R: TCTTTGCGCACTTCCCGTCCA	118
<i>nkx2-1b</i>	F: GCTGGTACGGAACGAATCCTGAC R: TCAGTGGACCCATGCCTTTACCA	135
<i>tipin</i>	F: AA ACTGGGCCCATCGCCTGT R: TGGCATGTCCAACCGAATCCGT	121
<i>anxa5</i>	F: GAAGCCTCCAAGAAATAC R: GTCAAGCAAGTCCACCTC	158
<i>acta2</i>	F: ACCAAGTGGCTAAATACCC R: CAGTGCTTTCTTCGTCGTC	108
<i>cyp19a</i>	F:CTTCAGATTGGACTGGCTGCACAA R: TTCTCTGCGCTCAGCTCTCCA	180

472

473

474 **Table 2. Numbers of differentially expressed genes in PFOS-exposed larvae relative to**  
 475 **controls.**

476

	Analysis	All samples (n = 6)	No outliers* (n = 4)
p05, 5% FDR	T-test	162	1278
p05, 5% FDR, 2-FC	T-test	5	211
2-FC only	None	13	595

477 \*Outlier samples (Control 2, 5; PFOS 2, 5) were removed for statistical analysis and  
 478 calculation of fold-change

479

480 **Table 3. PFOS perturbed functional enrichment of biological process GO terms for up-**  
 481 **and down-regulated genes in dataset.**

Up-regulated term	Count	P-value	%
Regulation of nucleic metabolic process	79	0.0289	14.13
Negative regulation of cellular process	54	0.0511	9.66
Phosphate metabolic process	38	0.0708	6.80
Negative regulation of macromolecule metabolic process	28	0.0339	5.01
Macromolecular complex assembly	26	0.0441	4.65
Regulation of cell differentiation	22	0.0236	3.94
Cellular component morphogenesis	20	0.0208	3.58
Positive regulation of cell proliferation	19	0.0315	3.40
Neuron differentiation	18	0.0976	3.22
Neuron development	16	0.0495	2.86
Cell projection morphogenesis	13	0.0607	2.33
Blood vessel development	12	0.0824	2.15
Positive regulation of transferase activity	12	0.0468	2.15
Regulation of cell motion	10	0.0662	1.79
Voltage-gated channel	8	0.0987	1.43
Cellular response to hormone stimulus	8	0.0634	1.43
Nucleosome organization	6	0.0987	1.07
Cellular glucan metabolic process	5	0.0195	0.89
Insulin receptor signaling pathway	5	0.0195	0.89
Regulation of fibroblast proliferation	4	0.0724	0.72
Regulation of transporter activity	4	0.0608	0.72
Positive regulation of osteoblast differentiation	4	0.0362	0.72
Regulation of carbohydrate catabolic process	4	0.0127	0.72
Down-regulated term	Count	p Value	%



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Cellular protein metabolic process	57	0.0824	13.41
Macromolecular complex assembly	24	0.0069	5.65
Protein-DNA complex assembly	6	0.0317	1.41
Response to temperature stimulus	5	0.0886	1.18
Positive regulation of translation	3	0.0729	0.71
Positive regulation of multicellular organism growth	3	0.0590	0.71
Response to vitamin D	3	0.0525	0.71
Growth hormone secretion	3	0.0036	0.71

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483

484 **Figure Legends**

485 **Fig. 1.** Transient exposure to PFOS results in window-specific morphological effects. (A)  
486 PFOS exposure (0-32  $\mu$ M) induces morphological defects at 120 hpf. SB: swim bladder; BS:  
487 bent spine; USB: uninflated swim bladder; G: gut. (B) Embryos were exposed to 0-32  $\mu$ M  
488 PFOS or DMSO control from 0-48 or 48-96 hpf. Graph shows incidence of malformations at  
489 120 hpf. Data represent 3 biological repeats with 20 embryos per treatment group.

490

491 **Fig. 2.** PFOS induced histological alteration in swim bladder and gut section. Representative  
492 histological sections of the gut and swim bladder from DMSO control or 16  $\mu$ M PFOS  
493 exposed larvae at 120 hpf. There are 3 biological repeats with 5 embryos per treatment group.  
494 SB: swim bladder; USB: uninflated swim bladder; G: gut.

495

496 **Fig. 3.** PFOS induced ultrastructure alteration in swim bladder and gut section.  
497 Representative TEM images showing the swim bladder (A-F) and gut (G-J) region for  
498 DMSO control (A, C, E, G, I) and PFOS exposed larvae (B, D, F, H, J). The vertical (G, H)  
499 and transect (H, J) sections of the gut region are shown. There are 3 biological repeats with 5  
500 embryos per treatment group. Cf: collagen fibers; M: mitochondria; N: nucleus; Nm: nuclear  
501 envelope; Mv: microvilli.

502

503 **Fig. 4.** Principal components and normalized plot analyzed PFOS-perturbed genomic mRNA  
504 expression [in compare with the controls](#). (A) Principal components analysis of all genes on  
505 the array confirms that samples 2 and 5 are outliers within each treatment group. This  
506 analysis uses non-transformed data and shows variation among biological replicates.  
507 (B) Box plot of normalized data shows consistency in the interquartile range across  
508 biological replicates excluding the outliers.

509

510 **Fig. 5.** Hierarchical clustering analyzed PFOS-perturbed genomic mRNA expression [in](#)

511 | [compare with the controls](#). (A) It showed the changed transcripts at  $p < 0.05$  and (B) those  
512 | with at least two-fold [gene expression](#) changes between the control and PFOS exposed  
513 | embryos [when assessed](#) at 96 hpf. Values [represent](#)  $\text{Log}_2$  fold-changes ( $p < 0.05$  by T-test  
514 | with 5% FDR).

515

516 | **Fig. 6.** PFOS-misexpressed the mRNA expression of 8 genes [in compare with the controls](#).  
517 | qRT-PCR validation of PFOS-regulated transcripts in 96 hpf zebrafish. The mean fold  
518 | change relative to the controls for the microarray and qPCR are graphed for comparison. The  
519 | gene name (when known) or the sequence ID was listed for each transcript. Data are  
520 | representative of 3 biological replicates with 40 embryos per replicate.

521

522 | **Fig. 7.** PFOS-perturbed organogenesis and developmental network. It is constructed from  
523 | differentially regulated transcripts related to swim bladder and gut development. Red and  
524 | green shading indicate up- and down-regulated transcripts at 96 hpf relative to the baseline,  
525 | respectively. The intensity of shading indicates the magnitude of regulation.

526

527

**Supplemental data**

528

**Supplemental Table 1. PFOS perturbed top toxicity pathways**

Name	P-value	Ratio
Mechanism of gene regulation by peroxisome proliferators via PPAR $\alpha$	7.54E-05	11/95 (0.116)
Decreases transmembrane potential of mitochondria and mitochondrial membrane	1.18E-04	12/117 (0.103)
Cardiac hypertrophy	3.44E-04	23/368 (0.062)
Cardiac necrosis/cell death	1.91E-03	16/248 (0.065)
Decreases depolarization of mitochondria and mitochondrial membrane	2.13E-03	4/20 (0.2)

529

530

531 | **Supplemental Fig. 1** PFOS-perturbed the canonical pathway PPAR signaling. Genes  
532 include insulin receptor substrate (*irs*), achaete (*ac*), cAMP-dependent protein kinase (*pka*),  
533 protein kinase C (*pkc*), 5-AMP-activated protein kinase (*ampk*), janus kinase 2 (*jak2*),  
534 TGFbeta-recepte (*tgfbr*), mitogen-activated protein kinase (*erk1/2*), acetoin catabolism  
535 protein (*acox*) were upregulated and nuclear receptor coactivator (*ncoa*), aryl-hydrocarbon  
536 receptor-interacting protein (*xap2*), integrin, beta 5 (*itgb5*), and sarcoplasmic  
537 calcium-binding protein (*cbp*) were downregulated.