

## **SUPPLEMENTAL MATERIAL**

### **Assessment of unique behavioral, morphological, and molecular alterations in the comparative developmental toxicity profiles of PFOA, PFHxA, and PFBA using the zebrafish model system**

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**Table S1. Physico-chemical properties of test chemicals.**

**Table S2. Summary of behavioral data from studies that exposed developing zebrafish to PFOA, PFHxA, or PFBA.**

**Figure S1. Percent survival of developing zebrafish exposed to (A) PFOA, (B) PFHxA, or (C) PFBA at 24, 48, 72, 96, and 120 hours post fertilization (hpf).**

**Figure S2. Representative images of 72 hpf eleuthero-embryos with an embryonic exposure to 0, 4, 40, or 400 ppb PFOA.**

**Figure S3. Representative images of 72 hpf eleuthero-embryos with an embryonic exposure to 0, 4, 40, or 400 ppb PFHxA.**

**Figure S4. Representative images of 72 hpf eleuthero-embryos with an embryonic exposure to 0, 4, 40, or 400 ppb PFBA.**

**Figure S5. Venn diagram of the number of mapped genes changed in (A) PFOA, (B) PFHxA, and (C) PFBA treatment groups at 72 hpf following embryonic exposure.**

**Figure S6. ERBB signaling pathway alterations associated with 4 ppb PFOA treatment group.**

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**Table S1. Physico-chemical properties of test chemicals.**

<b>Chemical</b>	<b>Perfluorooctanoic acid</b>	<b>Perfluorohexanoic acid</b>	<b>Perfluorobutanoic acid</b>
<b>Acronym</b>	PFOA, C8	PFHxA, C6	PFBA, C4
<b>CAS Registry No.</b>	335-67-1	307-24-4	375-22-4
<b>Chemical Formula</b>	CF <sub>3</sub> (CF <sub>2</sub> ) <sub>6</sub> COOH	CF <sub>3</sub> (CF <sub>2</sub> ) <sub>4</sub> COOH	CF <sub>3</sub> (CF <sub>2</sub> ) <sub>2</sub> COOH
<b>Molecular Weight (g/mol)</b>	414.07	314.05	214.04
<b>Solubility in Water (at 25°C)</b>	9.5 x10 <sup>3</sup> mg/L	15,700 mg/L	2.14 x 10 <sup>3</sup> mg/L
<b>pKa</b>	2.8	-0.16	0.08

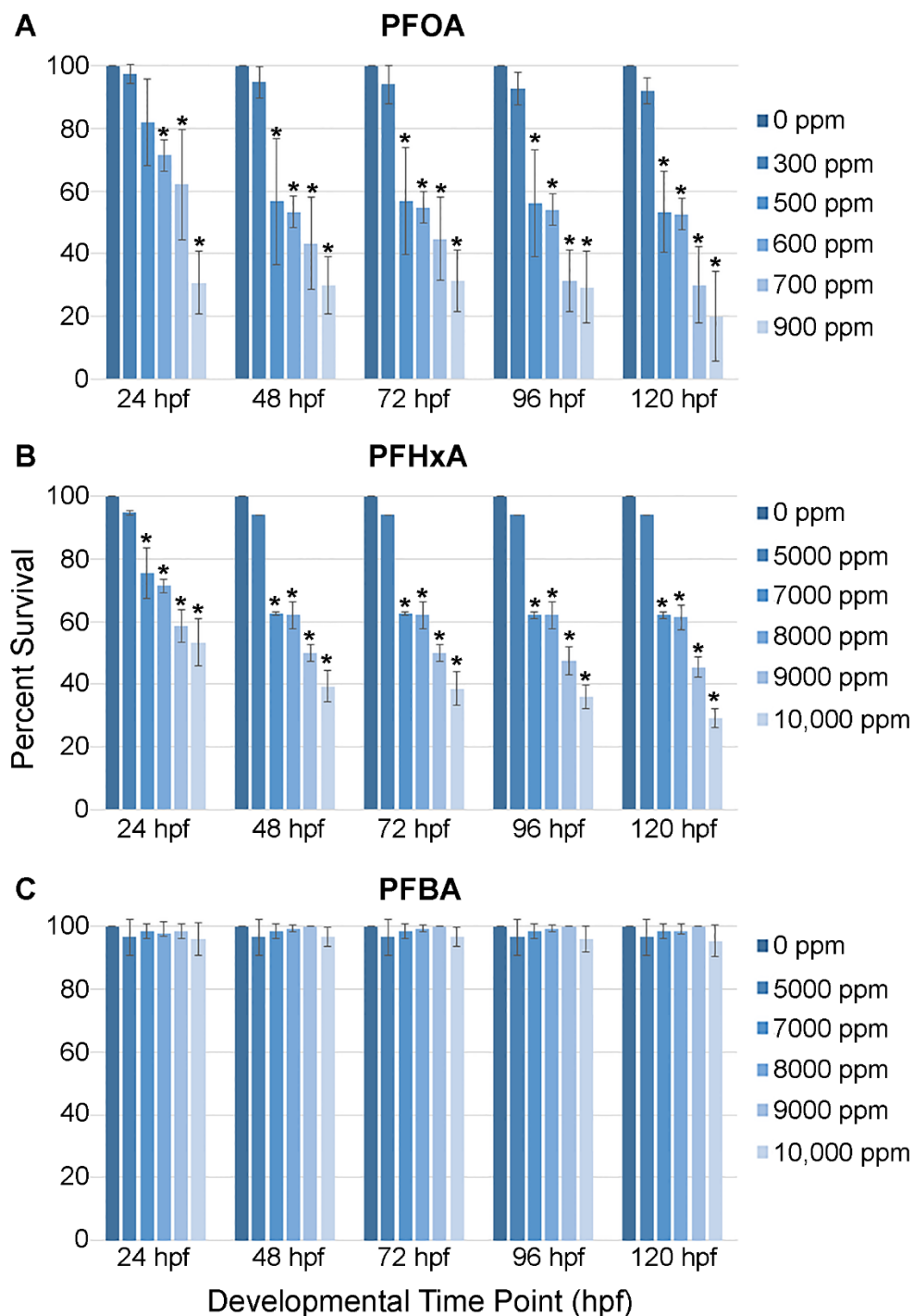
**Table S2. Summary of behavioral data from studies that exposed developing zebrafish to PFOA, PFHxA, or PFBA.**

<b>PFAA</b>	<b>Strain</b>	<b>Concentrations, Exposure Period</b>	<b>Age at assessment</b>	<b>Temperature</b>	<b>Well Plate</b>	<b>Light/dark protocol</b>	<b>Outcomes</b>	<b>Reference</b>
<b>PFOA</b>	AB	100 X higher concentration than human serum, 6-96 hpf	96 hpf	28 ± 1°C		10 min of light for acclimation; 10 min of light; 10 min of dark; 10 min of light	No effect on average swimming speed	Khezri et al. 2017 <sup>43</sup>
<b>PFOA</b>	Mixed wild type	1,822-33,125 ppb (4.4-80 µM), 1-144 hpf	144 hpf	26°C	96-well plate	20 min of dark for acclimation followed by 20 min light (L1: 10min then L2: 10 min) and 20 min dark (D1: 10 min then D2: 10 min)	No effect on locomotor activity	Gaballah et al. 2020 <sup>44</sup>
<b>PFOA</b>	AB	3,000-1,000,000 ppb, 0.5-144 hpf	144 hpf	26 ± 1°C	48-well plate	10 min of light for acclimation followed by two series of 10 min dark and 10 min light	Increased in activity in dark phases	Ulhaq et al. 2013b <sup>41</sup>
<b>PFOA</b>	Wild type	0.1-100,000 ppb, 1-144 hpf	144 hpf	26 ± 1°C	96-well plate	10 min of light for acclimation followed with 4 series of 5 min dark and 5 min light	4,968 and 62,110 ppb (12 and 150 µM) increased swimming distance in dark phase	Menger et al. 2020 <sup>40</sup>

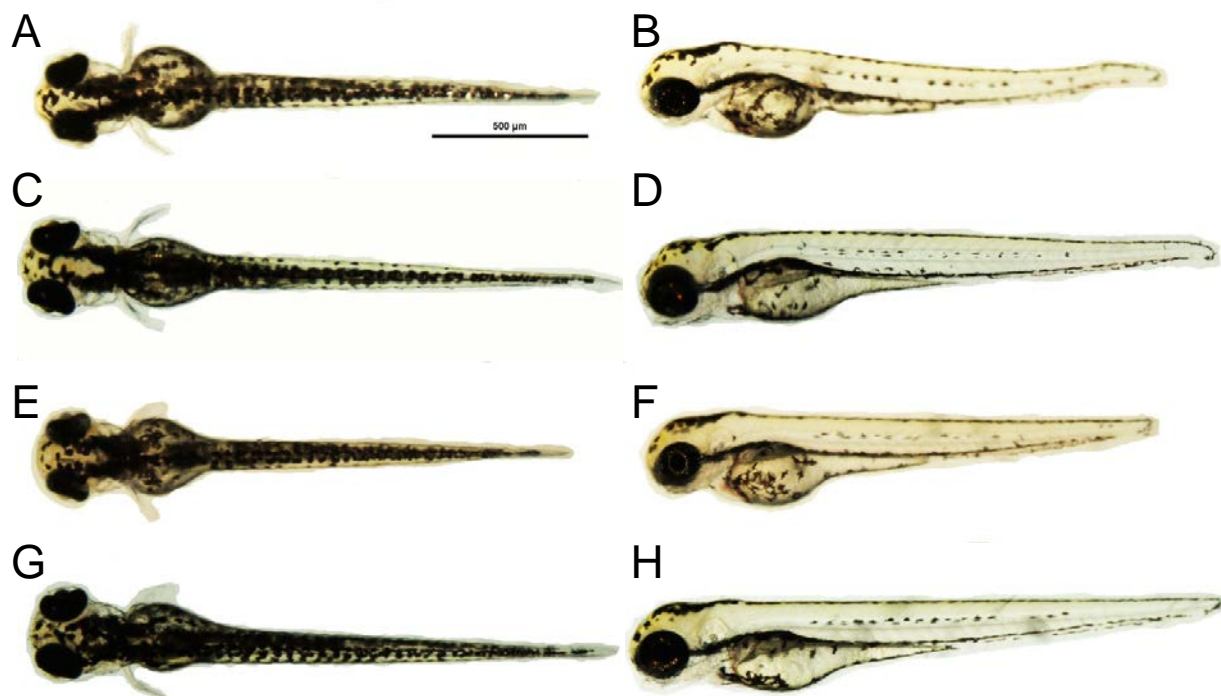
<b>PFOA</b>	5D	83-484 ppb (0.2–1.17 $\mu$ M), 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	414 ppb (1 $\mu$ M) caused hyperactivity in dark phase	Rericha et al. 2021 <sup>39</sup>
<b>PFOA</b>	AB	25 ppb, 2-30 hpf	120 hpf	28.5 $\pm$ 0.5°C	96-well plate	10 min of dark for acclimation, 10 min of alternating periods of dark and light for 60 min	Decreased total distance moved and average velocity	Yu et al, 2022 <sup>52</sup>
<b>PFOA</b>	AB Strain	10-1000 ppb at 2 -168 hpf, solutions renewed daily	168 hpf	28.5 $\pm$ 0.5 °C	96-well plate	10 min of dark for acclimation, 10 min of alternative periods of dark and light for 60 min	Decreased total distance and average velocity	Yu et al. 2021 <sup>42</sup>
<b>PFHxA</b>	Wild type (strain not specified)	0.1-100,000 ppb, 1-144 hpf	144 hpf	26 $\pm$ 1°C	96-well plate	10 min of light for acclimation followed with 4 series of 5 min dark and 5 min light	No significant effects observed	Menger et al. 2020 <sup>40</sup>
<b>PFHxA</b>	Mixed wild type	1,382-25,124 ppb (4.4-80 $\mu$ M), 1-144 hpf	144 hpf	26°C	96-well plate	20 min of dark for acclimation followed by 20 min light (L1: 10min	Hyperactivity in 1 <sup>st</sup> dark phase at 4,397-7,883 ppb (14-25.1 $\mu$ m) and 2 <sup>nd</sup>	Gaballah et al. 2020 <sup>44</sup>

						then L2: 10 min) and 20 min dark (D1: 10 min then D2: 10 min)	light phase at 7,883 ppb (25.1 $\mu$ M)	
<b>PFHxA</b>	5D	314-31,400 (1-100 $\mu$ M), 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark period, the last 6 min was used for analysis	785 and 5,150 ppb (2.5 and 16.4 $\mu$ M) caused hyperactivity	Rericha et al. 2021 <sup>39</sup>
<b>PFHxA</b>	AB	62.8-6,280 ppb (0.2-20 $\mu$ M), 3-120 hpf	14 dpf	26-27°C	24-well plate	30 min of dark	No changes in total distance moved and mean velocity	Annunziato et al. 2019 <sup>37</sup>
<b>PFHxA</b>	AB	480, 2,400, 12,000 ppb, 2-120 hpf	120 hpf	28°C	24-well plate	10 min acclimation, the 4 cycles of 5 light, 5 min dark, and 5 min light	Only the 480 ppb caused increase in swimming activity	Guo et al., 2021 <sup>45</sup>
<b>PFBA</b>	5D	214-21,400 ppb (1-100 $\mu$ M), 6-120 hpf	120 hpf	28°C	96-well plate	24 min and consisted of 4 cycles of a 3 min light period and 3 min dark	535, 1,391, 7491, and 15,839, and 21,404 ppb (2.5, 16.5, 35, 74, and	Rericha et al. 2021 <sup>39</sup>

						period, the last 6 min was used for analysis	100 $\mu$ M) caused hyperactivity in light and dark phases	
<b>PFBA</b>	AB	10,000-3,000,000 ppb, 0.5-144 hpf	144 hpf	$26 \pm 1$ °C	48-well plate	10 min of light for acclimation followed by two series of 10 min dark and 10 min light	Increased activity in dark phases	Ulhaq et al. 2013b <sup>41</sup>

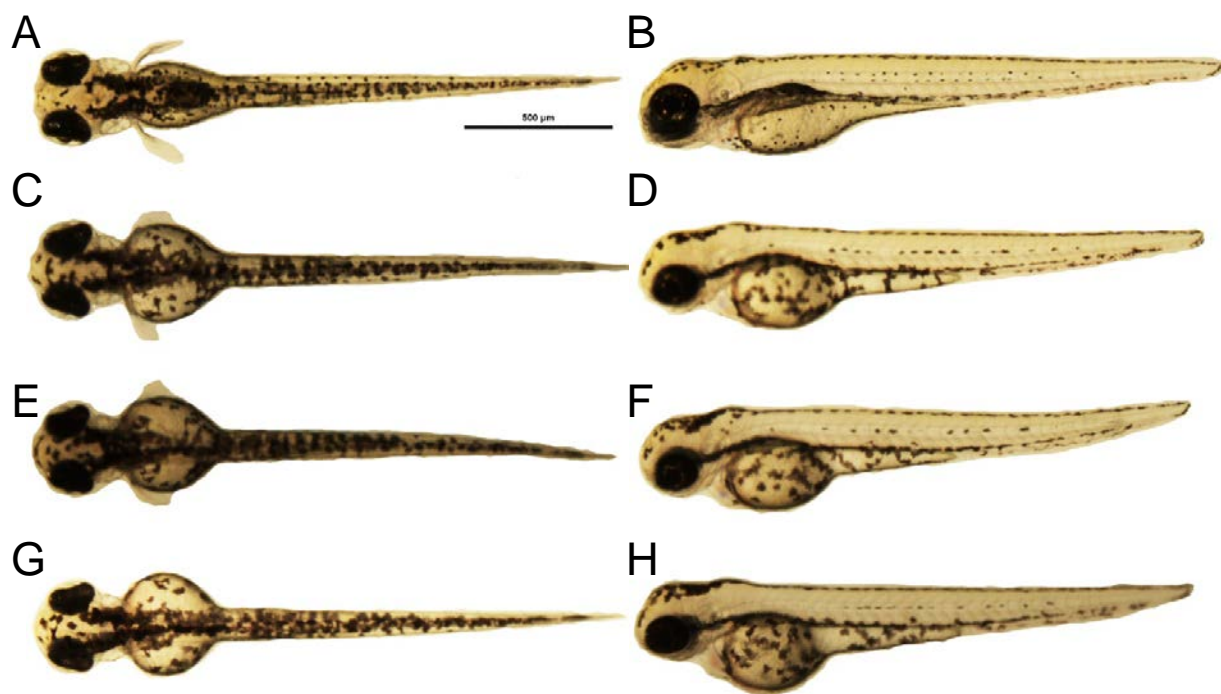


**Figure S1. Percent survival of developing zebrafish exposed to (A) PFOA, (B) PFHxA, or (C) PFBA at 24, 48, 72, 96, and 120 hours post fertilization (hpf).** Exposures were initiated at 1 hpf. N=3 with 50 subsamples per treatment in each biological replicate. Error bars are standard deviation. \*p<0.05 compared to the control treatment group at each developmental time point.

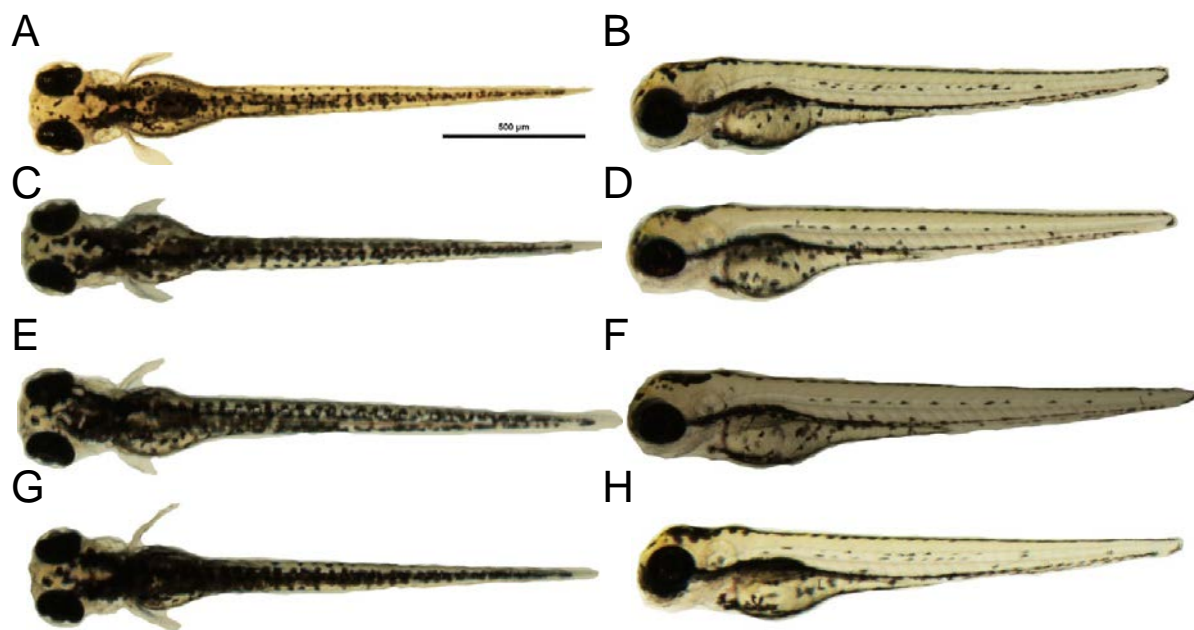


**Figure S2. Representative images of 72 hpf eleuthero-embryos with an embryonic exposure to (A and B) 0, (C and D) 4, (E and F) 40, or (G and H) 400 ppb PFOA. Scale bar = 500  $\mu\text{M}$ .**

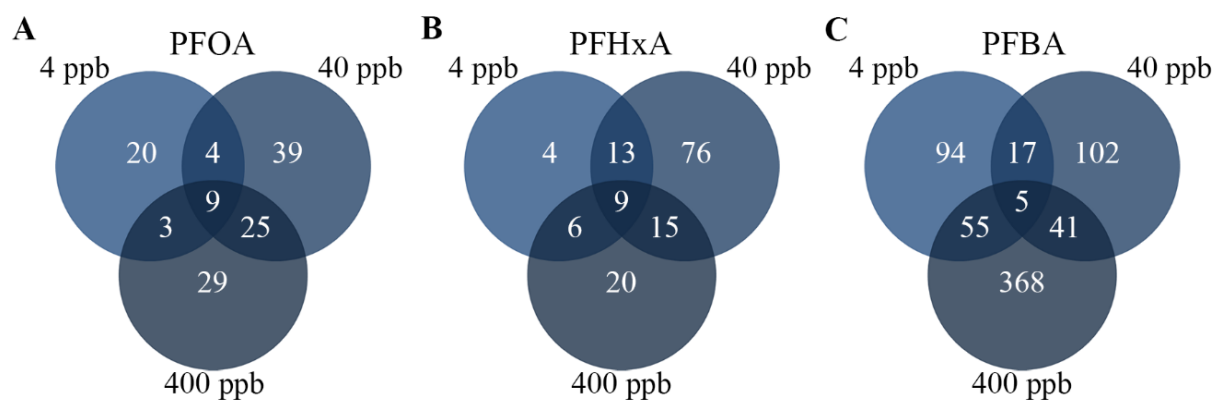




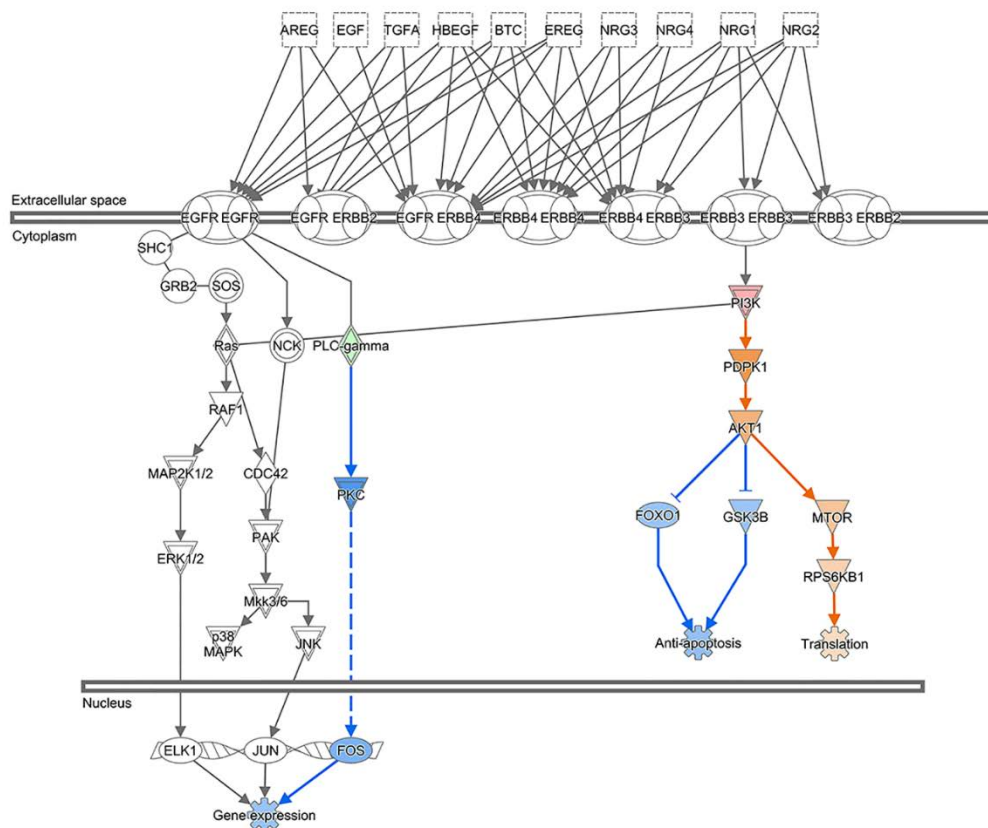
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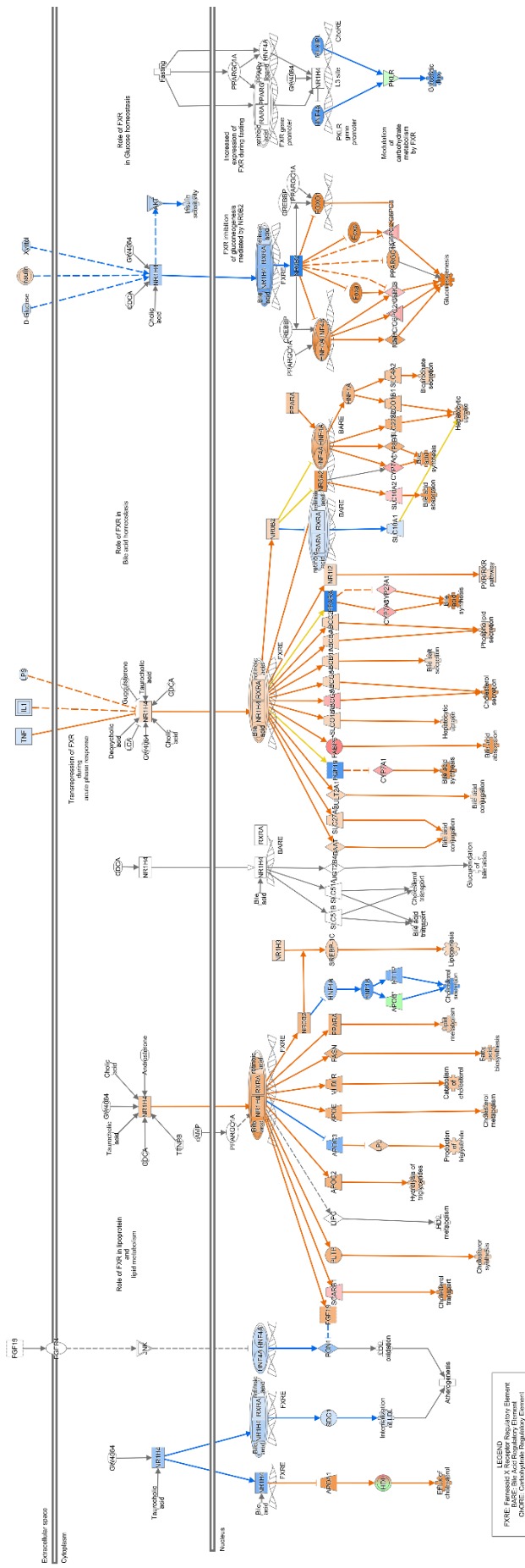
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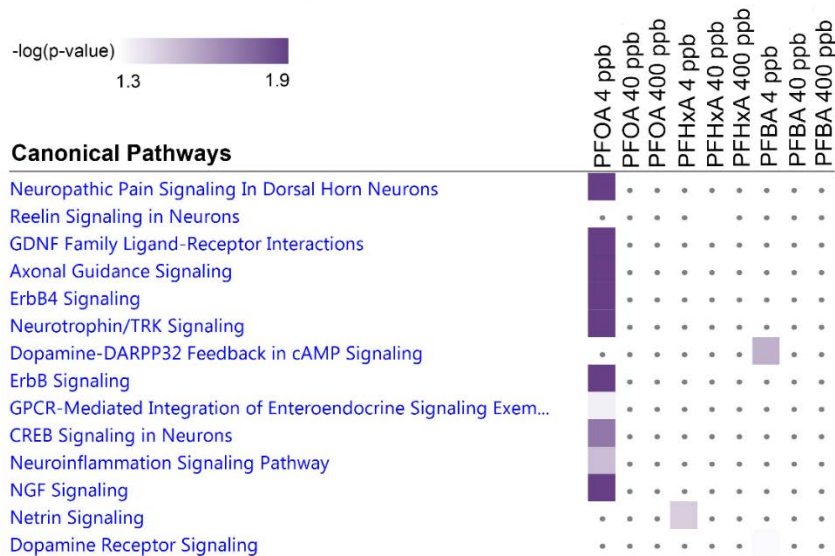
**Figure S5: Venn diagram of the number of mapped genes changed in (A) PFOA, (B) PFHxA, and (C) PFBA treatment groups at 72 hpf following embryonic exposure.**



**Figure S6. ERBB signaling pathway alterations associated with 4 ppb PFOA treatment group.** Triangle indicates kinase, oval shape indicates transcription regulator, and diamond shape indicates enzyme. Red indicates upregulation, while green indicates down regulation. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates a direct interaction and dashed line indicates an indirect interaction. A dotted line indicates that it is predicted but not confirmed in literature.



**Figure S7. Multiple FXR pathways are activated and/or inhibited in zebrafish exposed to 400 ppb PFBA during embryogenesis.** Rectangles represent ligand-dependent nuclear receptor, hexagons represent function, ovals represent transcription regulator, and circles represent other molecules. Red indicates upregulation of a molecule and green indicates down regulation. Blue indicates predicted inhibition and orange indicates predicted activation. Lines with arrows indicate activation and lines without arrows indicate inhibition. A solid line indicates a direct interaction, and a dashed line indicates an indirect interaction. A dotted line indicates that the association is predicated but not confirmed in literature.



**Figure S8. Comparison analysis showing canonical pathways related to neurotransmitter and nervous system signaling following embryonic exposure to PFOA, PFHxA, or PFBA.** Purple color indicates statistical significance ( $-\log(p\text{-value}) = 1.3$ ). Intensity of color correspond to  $-\log(p\text{-value})$ .