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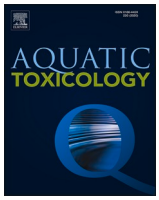

Developmental exposure to a POPs mixture or PFOS increased body weight and reduced swimming ability but had no effect on reproduction or behavior in zebrafish adults

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ABSTRACT

Complex mixtures of persistent organic pollutants (POPs) are regularly detected in the environment and animal tissues. Often these chemicals are associated with latent effects following early-life exposures, following the developmental origin of health and disease paradigm. We investigated the long-term effects of a human relevant mixture of 29 POPs on adult zebrafish following a developmental exposure, in addition to a single PFOS exposure for comparison, as it was the compound with the highest concentration within the mixture. Zebrafish embryos were exposed from 6 to 96 h post fertilization to x10 and x70 the level of POP mixture or PFOS (0.55 and 3.83 μM) found in human blood before being transferred to clean water. We measured growth, swimming performance, and reproductive output at different life stages. In addition, we assessed anxiety behavior of the adults and their offspring, as well as performing a transcriptomic analysis on the adult zebrafish brain, as the POP mixture and PFOS concentrations used are known to affect larval behavior. Exposure to POP mixture and PFOS reduced swimming performance and increased length and weight, compared to controls. No effect of developmental exposure was observed on reproductive output or anxiety behavior. Additionally, RNA-seq did not reveal pathways related to anxiety although pathways related to synapse biology were affected at the x10 PFOS level. Furthermore, pathway analysis of the brain transcriptome of adults exposed as larvae to the low concentration of PFOS revealed enrichment in pathways such as calcium, MAPK, and GABA signaling, all of which are important for learning and memory. Based on our results we can conclude that some effects on the endpoints measured were apparent, but if these effects lead to adversities at population levels remains elusive.

1. Introduction

Persistent organic pollutants (POPs) are omnipresent in the environment leading to humans and wildlife experiencing a near continuous exposure to these chemicals (WHO/UNEP 2012). POPs include many chemicals with anthropologic origins such as polychlorinated biphenyls (PCBs), pesticides such as dichlorodiphenyltrichloroethane, brominated flame retardants including polybrominated diphenyl ethers (PBDEs), dioxins, and *per-* and poly-fluoroalkylated substances (UNEP 2005). POPs are found in numerous past and present products, such as plasticizers, pharmaceuticals, pesticides, and industrial chemicals. Levels of POPs are increasing in the environment due to the consequences of human activity and their lipophilicity and persistency makes them very potent for bioaccumulation and biomagnification (Ritter et al., 1998).

Many POPs are endocrine disrupting chemicals (EDCs) defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (EC 2019). Since hormone balance is of particular importance during early development, early-life exposure to EDCs are expected to increase the susceptibility to disease in later-life, following the developmental origins of health and disease (DOHaD) hypothesis (Hanson and Gluckman 2014). Indeed, early-life exposure to EDCs are associated with later-life effects on cardiovascular, metabolic, and reproductive function, as well as being associated

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with the development of obesity, diabetes, and cancer in humans and experimental models (Zhang and Ho 2011; Barouki et al., 2012). Furthermore, the DOHaD proposes that exposure to environmental stressors early in life can produce changes to the genome or epigenome leading to adverse effects in the offspring of individuals during their life leading to transgenerational effects (Guerrero-Bosagna et al., 2010). For example, exposure of pregnant female rats to a PCB mixture led to increased body weight and lineage-specific effects of exposure were found for serum progesterone and estradiol in the F2 and F3 generations that were not observed in the directly exposed F1 offspring (Mennigen et al., 2018).

The zebrafish is a widely used vertebrate model to study the effects of chemical exposure (e.g. Huang et al., 2010; Lyche et al., 2011). Zebrafish have many advantages such as small size, external fertilization, and embryonic transparency that facilitates early chemical exposure and visualization of exposure effects. Additionally, large clutch sizes and a short generation time of 3 – 4 months allows for the evaluation of chemical effects over multiple generations. Lastly, the availability of genomic and bioinformatic resources enables the investigation of mechanisms of action (Hill et al., 2005).

POPs are part of complex mixtures in the environment, yet many toxicological studies are based on single compound exposures. Such studies fail to detect possible additive, synergistic, or antagonistic interactions within mixtures. For example, zebrafish exposed to a mixture of EDCs had lower egg production even if each compound was present in the mixture at a concentration which on its own would not produce an observable effect (Thrupp et al., 2018). Such interactions are poorly studied with POPs, which can have effects on multiple endpoints including survival, swimming performance, growth, sex ratios, reproduction, anxiety-like behaviors, and multi-generation effects on offspring survival and behavior (McCarthy et al., 2003; Nourizadeh-Lillabadi et al., 2009; Lyche et al., 2011; Xia et al., 2014; Vignet et al., 2015; Horri et al., 2018; Alfonso et al., 2019). In addition, there is no information on developmental exposures and transgenerational effects with environmentally relevant POP mixtures.

In this study we use zebrafish to explore the long-term impacts of early developmental exposure (6 – 96 hpf) to an environmentally relevant mixture of 29 POPs, or a single PFOS exposure. A single PFOS exposure was chosen for comparison due to it being the most abundant constituent of the POP mixture and we have previously found it is responsible for behavioral effects seen in larvae exposed to our POP mixture (Khezri et al., 2017). The POP mixture is based on the average levels of chemicals found in human blood of the Scandinavian population (Berntsen et al., 2017), but we previously found the levels within larval zebrafish tissues following developmental exposure are also similar to the concentrations of chemicals detected in fish from Norwegian lakes (Nourizadeh-Lillabadi et al., 2009). In continuation of our previous research where early life exposure of zebrafish larvae (F0, 6 – 96 hpf) to the POP mixture or single PFOS caused hyperactive behavior and changes to the transcriptome (Christou et al., 2020), we hypothesize that exposure will also produce adverse long-term health effects in adults (F0) and their progeny (F1). We investigated the effect of developmental chemical exposure on different key life-traits such as survival, growth, swimming ability, reproduction, sex ratio, and adult anxiety-like behavior. In addition, because of the previously reported results on anxiety-like behavior in exposed larvae (Christou et al., 2020), we also performed transcriptomic analysis on adult zebrafish brains to explore for long-term effects of early exposure. Finally, we assessed F1 offspring behavior to assess whether multigenerational effects following exposure exist.

2. Materials and methods

The study was approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences (NMBU) and the Norwegian Food Safety Authority (application ID: FOTS 13,094). It was conducted in strict accordance with The Norwegian Regulation on Animal Experimentation at the Section for Experimental Biomedicine, NMBU-Faculty of Veterinary Medicine, in Oslo, Norway.

2.1. Fish maintenance and breeding

AB wild-type (AB) were kept at 28 ± 1 °C under a 14:10 light/dark photoperiod. Animal care was performed in accordance with lab protocols (see supplementary material). For embryo production, adults were placed in breeding tanks in the afternoon. The next morning the separator was removed as soon as the lights turned on (08:00) and embryos were collected an hour later. Embryos were maintained in sterile embryo media (60 μg/mL Instant Ocean® sea salts) until the time of exposure.

2.2. POPs mixture and chemicals

Dimethyl sulfoxide (DMSO, >99.7%, CAS number 67–68–5) and PFOS (≥ 98%, CAS number 2795–29–3) were purchased from Sigma-Aldrich. The composition of the POPs mixture is described in Table 1, and further details of its preparation can be found in Berntsen et al. (2017). Stock solutions of POPs and PFOS were prepared in DMSO and were stored at −20 °C until use.

2.3. Solutions preparation

For exposure experiments, two concentrations of the POP mixture were used. The low concentration was equal to the levels of chemicals that are 10 times higher than what is found in average Scandinavian

Table 1
Composition and concentration of chemicals in the POP mixture.

Chemicals	Nominal concentration of stock solution (μM)	Nominal concentration of stock solution (mg/ml)
PFASs		
PFOA	10,923	4.523
PFOS	54,801	29.425
PFDA	962	0.495
PFNA	1723	0.800
PFHxS	7873	3.450
PFUnDA	990	0.560
BFRs		
BDE-47	18	0.009
BDE-99	7	0.004
BDE-100	3	0.003
BDE-153	1	0.00
BDE-154	3	0.002
BDE-209	11	0.011
HBCD	38	0.025
PCBs		
PCB 28	50	0.013
PCB 52	34	0.010
PCB 101	24	0.008
PCB 118	196	0.064
PCB 138	615	0.222
PCB 153	1003	0.362
PCB 180	490	0.194
Other organochlorines		
p,p-DDE	1578	0.502
HCB	410	0.117
α-chlordane	26	0.011
Oxy-chlordane	51	0.022
Trans-nonachlor	92	0.041
α-HCH	20	0.006
β-HCH	182	0.053
γ-HCH (lindane)	20	0.006
Dieldrin	63	0.024

human blood levels and the high concentration corresponds to levels 70 times higher (exposures will be referred to as POP10 and POP70 from here on). Working solutions of the POP mixture were prepared on the day of the experiment by diluting the stock solution (1,000,000x) in sterile embryo media and adjusting the concentration of DMSO to 0.1%. Concentrations of the POPs mixture were based on previous work done in our group where POP10 was the highest concentration with no observable behavioral effects in larvae and POP70 was the lowest concentration with observable effects (Khezri et al., 2017). The concentrations of PFOS were based on the nominal concentration found in the POP mixture and corresponded to 0.3 mg/L (0.55 μM, will be referred from now as PFOS10) for the low concentration and 2.06 mg/L (3.83 μM, will be referred to as PFOS70) for the high concentration. PFOS working solutions were prepared on the day of the experiments by diluting the stock solution (54.8 mM) in sterile embryo media and adjusting the DMSO concentration to 0.1%. We previously measured the concentrations of chemicals found within larvae exposed to POP70 and PFOS70 from 6 – 96 hpf. We detected 24/29 of the compounds in the POP mixture in the larvae whereas concentrations within the media were around 1–35% the nominal concentrations at 96 hpf when the compound was detectable (Christou et al., 2020).

2.4. Larval exposures and maintenance of experimental populations

For the establishment of the F0 generation, five populations of larvae were produced per replicate with four independent biological replicates produced in total. Each population consisted of 300 fertilized eggs. The control population consisted of eggs exposed only to the solvent (0.1% DMSO) and treated larvae were exposed to either POP10, POP70, PFOS10 or PFOS70. Similar to previous studies (e.g. Huang et al., 2010; Parsons et al., 2019), due to space limitation and the size and duration of the experiments, it was not possible to include a non-solvent control. However, DMSO is a common solvent in toxicology, the concentration we used is often applied in zebrafish studies (Huang et al., 2010; Parsons et al., 2019), and it is expected to be rapidly cleared from the body (Layman and Jacob 1985). Eggs and larvae were kept in exposure media from 6 – 96 h post fertilization (hpf) in petri dishes with 60 mL exposure media (size 150 mm x 15 mm, Sigma-Aldrich, Norway AS). After 96 hpf the larvae were transferred to 1 L beakers (VWR®) with clean system water with a stocking density of 150 larvae/L and 90% daily renewal of water. At 15 days post fertilization (dpf) the larvae were transferred to a ZebTEC Stand Alone system (Tecniplast S.p.A, Italy) until the termination of the experiments when fish were 15 months old (mo). Larvae and adult fish were kept in 8 L tanks. The initial stocking density of larvae was 37.5 individuals/L and at 50 dpf the populations were divided to reach a final stocking density of 8 individuals/L. For the duration of the experiments the photoperiod was kept on a 14:10 hr light/dark cycle, pH at 7 – 7.5, conductivity at 500 – 550 μS/cm and temperature at 28 – 28.5 °C. Oxygen saturation levels were > 95% and there was 100% water recirculation rate per hour with a 15% daily renewal rate. Larvae were fed 3 times daily with artemia (Sep-art Artemia, Ocean Nutrition, Belgium) and were gradually introduced to dry feed of different sizes according to the manufacturer's instructions (ZebraFeed, Sparos, Portugal). Adult fish from each condition and replicate were used for all performed subsequent tests.

2.5. Survival and growth

Survival of experimental populations was monitored daily until 150 dpf. After this age mortality was below 10%. Random samples of 15 – 20 individuals/population were taken at 5, 15, 30, 60, 90 and 120 dpf for the evaluation of growth rate. At 5 and 15 dpf, larvae were placed under a stereomicroscope and from 30 dpf onwards fish were placed under a camera mounted on a tripod. Fish were anesthetized and photographed for the measurement of standard length in mm (SL, tpsDig v2.30, Rohlf (2005)). Fish taken for growth rate estimation were returned to their respective tanks. One-way analysis of variance (ANOVA) followed by Dunnett's test with the Control population as a reference group was performed to test the effect of chemical exposure on fish growth in each sampling day and G-test was applied with a significance level of 0.05 to test whether chemical exposure had an effect on survival rates.

2.6. Behavioral test adults

At 7 mo, adult zebrafish were submitted to a novel tank diving test and recorded using the Ethovision XT13 software (Noldus Information Technology, The Netherlands). Two females and two males per condition and replicate were subjected to the behavioral test with a total of 40 fish per sex and condition (Control, POP10, POP70, PFOS10, PFOS70). Fish were immediately introduced to a 1.5 L tank (trapezoid tank Aquatic Habitats, Apopka, Florida, USA, size in cm: height 15.2 × width 7.1 × length 27.9 at the top and 22.5 at bottom) and their swimming pattern was recorded for 5 min. Recording started immediately after the transfer. The camera used was able to capture two tanks in one frame. Tanks were divided by a separator to ensure individuals could not see one another. Between trials, the tanks were rinsed, and water renewed to remove waterborne pheromones. Cumulative duration (seconds), distance moved (cm), and mean velocity (cm/s) were calculated for each fish in two predefined zones, the bottom zone (the bottom half of the tank) and the top zone (top half of the tank). Additionally, the number of zone crossings was calculated (Cachat et al., 2010).

Behavioral data were analyzed using linear mixed effect (LME) models with the dependent variables tested were either cumulative time in bottom zone (seconds), cumulative distance moved (cm), mean velocity (cm/s), and number of crossings between zones, with condition (5 levels) and sex (2 levels) as categorical independent variables, and replicate as a random effect (ee supplementary materials for additional information).

2.7. Behavioral tests of F1 larvae

2.7.1. Tests involving no re-exposures to PFOS or POP mixture

To test whether early life exposure of F0 generation had a multigenerational effect on the behavioral outcome of F1 generation, larvae were submitted to a light/dark transition test and a thigmotaxis assay according to Christou et al. (2020). Fertilized embryos derived from adult zebrafish of the F0 generation (belonging to Control, POP10, POP70, PFOS10 and PFOS70 populations) were transferred into clear polystyrene 96 well plates (Nunc™ MicroWell™) with one embryo per well from 6 hpf until the time of testing at 96 – 100 hpf (between 9:00 – 13:00) in 200 μL of sterile embryo media. For the thigmotaxis assay, embryos were placed in 24 well plates (Corning® Primaria™) with one embryo per well in 1 mL sterile embryo media. For the thigmotaxis assay two controls were used, one for the POPs mixture F1 larvae and one for the PFOS treatment respectively, as the plate layout meant that each group could not be equally represented on each row and column without the addition of extra controls. All groups were spread equally on each row and column to avoid bias based on position during behavioral testing. For the light-dark transition test, each well plate included 10 embryos per condition and was repeated 4 times, one for each replicate of the F0 generation. For the thigmotaxis assay, each well plate contained 3 embryos per condition with 3 well plates per replicate. The experiment was also repeated 4 times.

2.7.2. Tests involving re-exposures to PFOS or POP mixture

To test whether prior exposure history had an adaptation effect on the behavioral response of F1 larvae, embryos derived from F0 adults of Control, POP70 and PFOS70 conditions were re-exposed to either a control medium (0.1% DMSO), a POP10, or a POP70 exposure medium for embryos originating from Control and POP70 adults or control medium, PFOS10 and PFOS70 exposure medium for embryos originating from Control and PFOS70 adults. The behavioral outcome was evaluated

with the light-dark transition test described above. Two well plates were included in each replicate, one containing embryos originating from F0 Control and F0 POP70 adults and another with embryos from F0 Control and F0 PFOS70 adults. Each well plate included 16 embryos per condition and the experiment was performed 4 times.

Behavioral assays were performed in a ViewPoint® Zebrabox and its tracking software (ViewPoint Life Sciences, Lyon, France). Behavioral tests were conducted between 9:00 – 13:00 in 96 – 100 hpf zebrafish larvae. For information about the behavioral parameters tested and technical information of the behavioral apparatus see supplementary material.

For data analysis, the dependent variable for the light-dark transition test was either the cumulative time spent active (seconds), the cumulative distance travelled (mm), or average swimming speed (calculated as the cumulated distance travelled/cumulated time spent active). For the thigmotaxis assay, the variable used was the percent of the total distance moved in the outer zone. Both for the light-dark transition and the thigmotaxis test only the behavior during the dark period was analyzed. For the re-exposure experiments, models also tested the interaction between exposure and history. Exposure was either Control, POP10 or POP70 for the POP experiment or Control, PFOS10 and PFOS70 for the PFOS experiment. History described the origin of F1 larvae and was either Control, POP70 or PFOS70. A significant interaction indicates that F1 larvae derived from POP70 or PFOS70 adults responded to the chemical exposure in a different manner than their F1 Control counterparts.

2.8. Reproductive tests adults

Reproductive tests were performed when zebrafish were 6 mo. Reproductive tests were performed on 3 of the 4 replicates. The experimental protocol was based on Uusi-Heikkila et al. (2010). Briefly, female and male fish from control and exposed populations were kept in separate tanks for one week prior to the start of the reproductive tests. All fish were anesthetized and measured for standard length (SL), to make sure there were no statistical differences in length between the tested populations, as there is a positive correlation between size and reproductive output (Uusi-Heikkila et al., 2010). After one week, fish were placed in breeding tanks for 5 days with a ratio of 1 female to 2 males per tank. Seven breeding pairs were set for each condition. Each morning eggs were collected from each tank and pooled together for each condition. The number of fertilized and unfertilized eggs was counted using ImageJ (v1.51k, https://imagej.nih.gov/ij/) and the fertilization rate was calculated. Afterwards, 100 fertilized eggs from each condition were placed in a petri dish. These eggs were monitored daily for mortality at 24 hpf and hatching rates at 72 hpf. The procedure was repeated for each of the five days, and the number of breeding pairs that laid eggs was also recorded. At the end of each reproductive trial, fish were euthanized and weighed both for total and gonadal weight. The gonadosomatic index (GSI = [gonad weight / total tissue weight] × 100) was calculated for each sex.

One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test relative to control was performed to test the effect of early chemical exposure on the gonadosomatic index of female and male zebrafish. Fertilization rate and average number of fertilized and unfertilized eggs per day were normalized based on the number of breeding pairs on each day. We used LME models and generalized least square models to assess fertilization rate, the mean number of fertilized and unfertilized eggs, mortality at 24 hpf, and hatching at 72 hpf. Further details can be found in the supplementary material.

2.9. Swimming tests adults

Swimming performance was tested by estimating the sustained critical swimming speed (U_{crit}) in three males from each replicate (n = 12 males/condition) beginning at 10 months of age (tests started in Jan 2019). For the U_{crit} experiment, individual males were subjected to a stepwise increment in swimming velocity of 2.7 body lengths/s every 39 min until exhaustion (when the fish was unable to swim). Critical swimming speed was calculated using the equation described in Brett (1964). A more detailed description of the setup can be found in the supplementary material. In total, 60 males were assessed over a 25-week period. LME models were used to assess swimming activity with U_{crit} (BL/s) as the dependent variable, with condition (5 levels) as a categorical independent variable, and length or weight as continuous independent variables, and replicate as a random effect. Further details can be found in the supplementary material.

2.10. Brain tissue sampling and transcriptome analysis

Whole brain tissue from 2 females and 2 males per condition and replicate (total 8 females and 8 males per condition) were collected after euthanasia of adult fish in MS-222. Brain tissue was collected individually in Eppendorf® tubes (2 mL), snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction for high-throughput sequencing analysis. Details on RNA purification, the analytical pipeline (SnakePipes, Bhardwaj et al., 2019), and the sequencing analysis can be found in the supplementary material. A principal component analysis was performed on all the expressed genes that had a log 2 ≥ 0 expression in all groups and sex separately (Control F, Control M, POP10 F, POP10 M, POP70 F, POP70 M, PFOS10 F, PFOS10 M, PFOS70 F, PFOS M) using ClustVis, a web tool for visualizing clustering of multivariate data (Metsalu and Vilo 2015). PCA scores were loaded to R (version 3.6.1) and biplots of principal components were designed with "ggplot2" library, while the stat_ellipse argument within the "ggplot2" library was used to compute 95% confidence ellipses to test whether there was a clear separation of different groups and sex. The transcriptomic data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE162503 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162503). Differentially expressed genes were imported in Webgestalt (Liao et al., 2019) for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis and gene ontology (GO) analysis to explore affected pathways. Only pathways that had a p-value < 0.05 were considered significantly enriched.

2.11. Final sampling

All remaining fish (N = 54 – 71/condition and replicate) were euthanized with an excess of MS-222 at the end of the experiment at 15 months. All fish were individually measured for length (mm) and weight (mg) and internally inspected for sex determination. Condition factor indices (K) were calculated for each fish using their weight and length measurements (K = [weight × 100]/length^3) (Jones et al., 1999). A chi square test was performed (JMP PRO v15.0, SAS Institute Inc.) to test whether there was a difference in sex ratio between conditions. G-test was applied with a significance level of 0.05 to test differences in final survival rates between conditions.

2.12. Statistics

When presenting data, we have used least square means to compare groups that have been analysed using LME. For adult behavior (Fig. 1), the arithmetic mean is presented as there were no significant group differences identified by LME.

3. Results

3.1. Survival and growth

No significant differences were observed in length between control and exposed populations on any of the sampling days up to 120 dpf

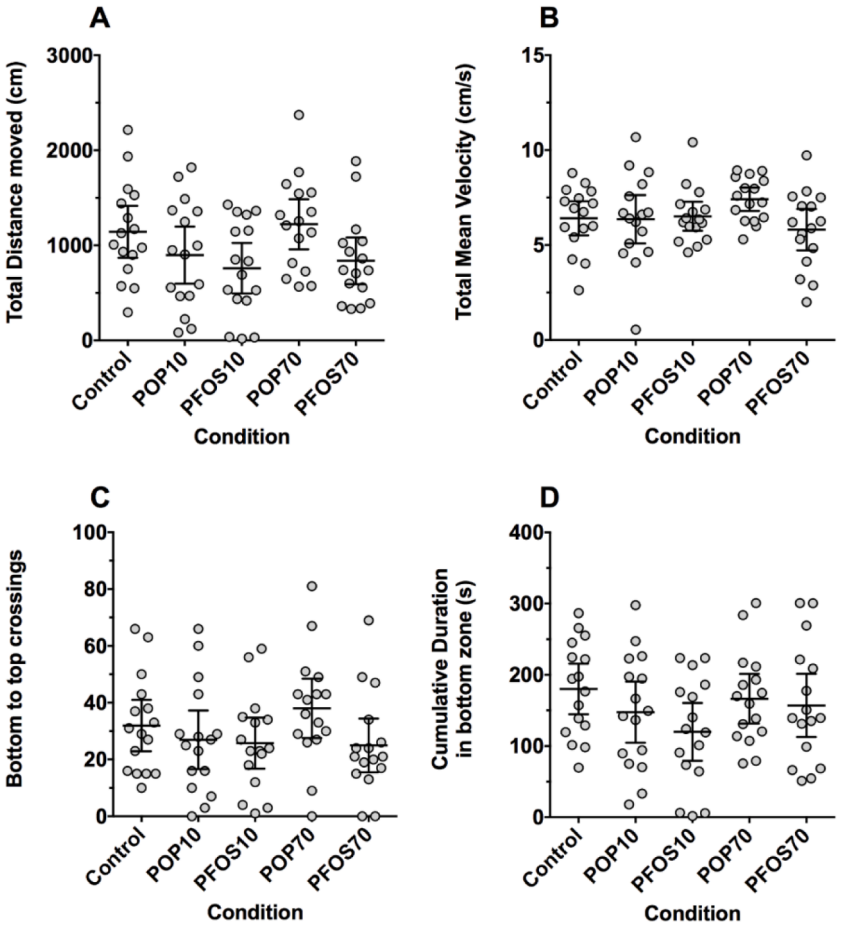

Fig. 1. Behavioral responses of adult zebrafish following the novel tank diving test, A) Total distance moved, B) Total mean velocity, C) Bottom to top crossings and D) Cumulative duration in bottom zone. Data shown are means ± 95% CI, $n = 16$ individuals/condition, age = 7 mo.

(Supplementary material Figure S1). Furthermore, no differences were observed on the survival rates at 150 dpf (Supplementary material Figure S2).

3.2. Behavioral test adults

All details of the statistical results are summarized in Supplementary material Table S1. No significant differences were observed between Control and exposed groups for the time spent in bottom zone, mean velocity (cm/sec), or number of crossings between the two zones. Here, the null model was selected for all three tested variables using the AICc meaning that the variation in our results could not be explained by any of our independent variables i.e. group and sex. A significant effect of group was observed in cumulative duration in the bottom zone ($p = 0.03$). Here POP10, PFOS10, and PFOS70 tended to spend less time in the bottom zone compared to controls, but post-hoc analysis with Control as a reference group failed to return significant pairwise differences (Fig. 1).

3.3. Behavioral tests of F1 larvae

All details of the statistical results are summarized in Supplementary material Table S1.

3.3.1. Tests involving no re-exposures to PFOS or POP mixture

No behavioral effects were observed in F1 larvae submitted to the light/dark transition test or the thigmotaxis assay in contrast to the F0 directly exposed larvae, where the exposure to chemicals caused higher activity during the light/dark transition test and increased thigmotactic behavior (Christou et al., 2020). The null model explained all the variation observed for cumulative distance moved, total time spent active, swimming speed, and percent of total distance moved in the outer zone (Figure S6, Supplementary material).

3.3.2. Tests involving re-exposures to PFOS or POP mixture

Re-exposure to PFOS10 and PFOS70 had a significant effect on all variables tested in the light/dark transition test for larvae originating from F0 Control and PFOS70 adults but with no tendency of interaction with history. All groups reacted with a dose-dependent increase of their responses to PFOS re-exposure (Fig. 2A-C).

Exposure to the POP mixture revealed an interaction between exposure and history for total time spent active however post-hoc analyses revealed no significant differences between individual groups. All groups reacted with a dose-dependent increase of their responses to POP mixture re-exposure (Fig. 2D-F), particularly swimming speed, as previously reported in naïve larvae (F0) in our earlier study (Christou et al., 2020).

3.4. Reproductive tests

Fertilization rate, mean number of fertilized and unfertilized eggs, mortality at 24 hpf, and hatching at 72 hpf did not present significant differences between control and treated populations (Fig. 3A). The number of fertilized and unfertilized eggs reduced gradually from day 1 to day 5 of the reproductive test (Fig. 3B, C). The fertilization rate however was relatively consistent during the 5 days of the reproductive test. Additionally, some conditions e.g. Control's group fertilization rate (Fig. 3A) were characterized by large variation as shown with the

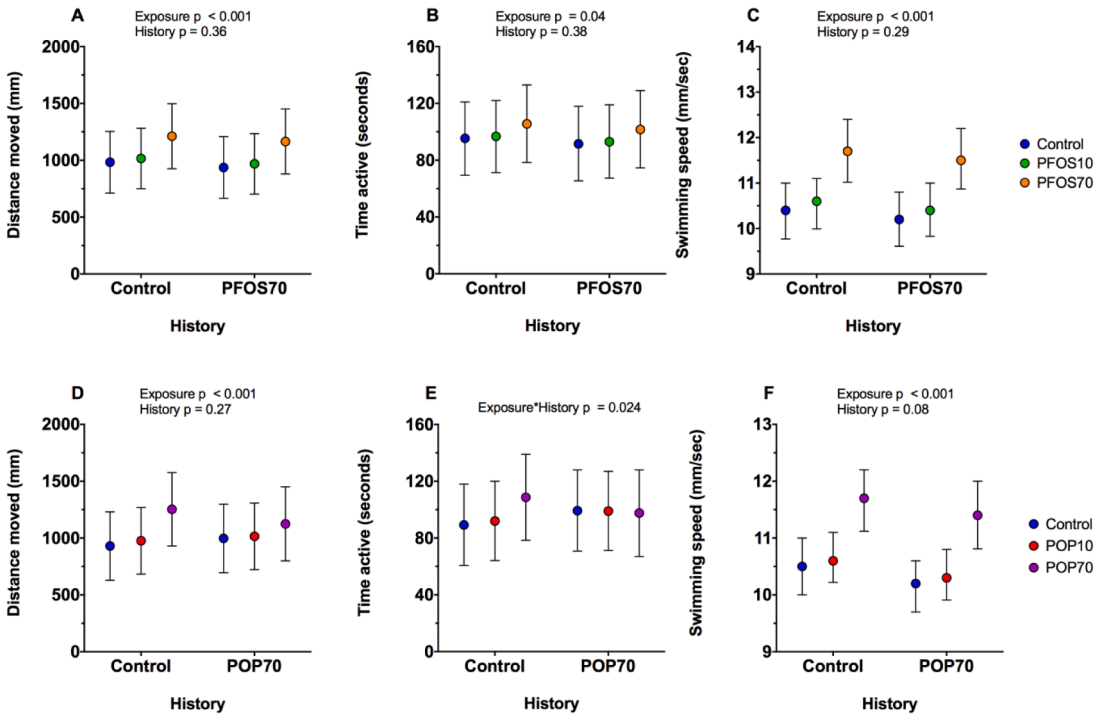

Fig. 2. Behavioral responses of F1 larvae originating from Control and PFOS70 F0 adults (A-C), and from Control and POP70 F0 adults (D-F). (A, D) Distance moved. (B, E) Time spent active. (C, F) Swimming speed. Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Asterisk indicates an effect of re-exposure within different history groups (for the significant interaction model only). For panels A, B, C, D and F the exposure effect is 0 < x10 < x70. N = 58 – 64 individuals/group, age = 96 – 100 hpf. In panel E, although there was a significant interaction, post-hoc analyses revealed no significant differences between individual groups, rather there was a weak positive trend with exposure in controls, but a weak negative trend in larvae from parents exposed to POP70.

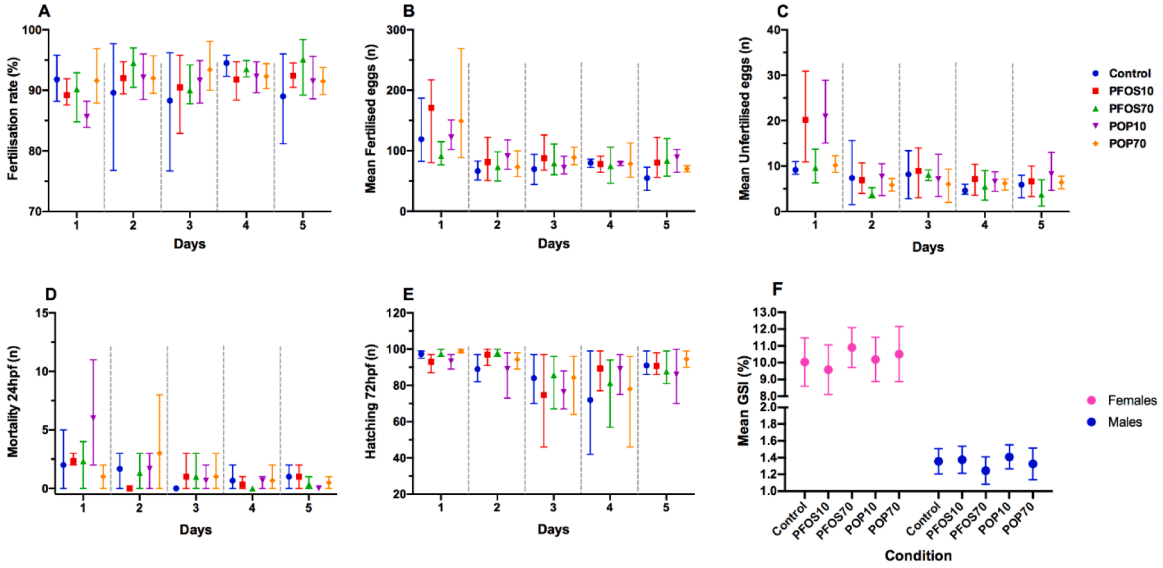

Fig. 3. Reproductive variables obtained from the reproductive tests performed on adult zebrafish. A) Fertilization rate B) Mean number of fertilized eggs C) Mean number of unfertilized eggs D) Mortality of embryos at 24hpf E) Hatching at 72hpf and F) gonadosomatic index of females and males. Data shown are A-F Least square means ± 95% CI. n = 21 females and males/condition, age = 6 mo.

confidence intervals hence the lack of statistical differences between groups. No statistical differences where observed on the GSI of males and females of different conditions (Fig. 3F).

3.5. Swimming tests

All details of the statistical results are summarized in Supplementary material Table S1.

Critical swimming speed, the speed when the fish cannot keep their position in the swim tunnel and fatigue sets in (Brett 1964) (Body length/second, BL/s) was significantly affected by early life exposure (p < 0.001). When compared to Control, the critical swimming speed of POP10, PFOS10, and POP70 individuals was significantly lower (Fig. 4). Body size had no effect on U_{crit} (Table S1).

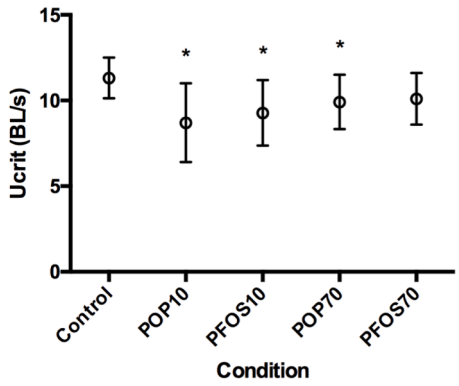

Fig. 4. Reduced swimming speed in adult fish exposed as larvae to POP10, POP70, PFOS10 and PFOS70. Values represent least square means ± 95% CI relative to body length/second (BL/s). Asterisks indicate statistical differences relative to control ($p < 0.05$). $n = 12$ individuals/condition, age = 10 – 16 mo.

3.6. RNA sequencing expression results on adult brains

Principal component analysis (PCA) revealed no clustering of samples coming from different conditions and sex so all samples were pooled together irrespective of their sex (data not shown). Additionally, PCA analysis revealed a consistent outlier in Control male group therefore this sample was excluded from further analyses (data not shown).

Deseq2 analysis revealed that PFOS10 was the group with the highest number of differentially expressed genes (DEG) with 466 genes (463 upregulated). The rest of the conditions had a very low number of DEG, 2 genes (1 upregulated), 5 genes (5 upregulated), 13 genes (12 upregulated) for PFOS70, POP10, and POP70 respectively. Principal component analysis was subsequently performed on all measured genes in the 5 conditions (Fig. 5). The first principal component explained 24.6% and the second 10.6% of the variance. We did not observe any separation along either of the principal components indicating no effects in the global transcriptome of the exposure groups.

3.7. Webgestalt pathway analysis

DEGs were imported to Webgestalt (Liao et al., 2019) using a custom reference list of all measured genes for gene enrichment analysis. An overview of all pathways from GO and KEGG analysis are presented in Tables 2 & 3. Only PFOS10 and POP70 had an adequate number of DEGs for pathway analysis. PFOS10 had the most enriched pathways since it presented with the highest number of DEGs (463 genes). GO analysis of biological functions revealed multiple pathways in synaptic or post-synaptic transmission and signaling pathways along with transmembrane transport pathways (Table 2). KEGG analysis revealed enrichment in pathways such as mitogen-activated protein kinases (MAPK), apelin, calcium, ErbB (epidermal growth factor receptors), Wnt and adipocytokine signaling (Table 3). For the POP70 group, GO analysis revealed pathways involved in transcription, metabolic and biosynthetic processes, and immune system response (Table 2). KEGG pathway analysis failed to return significantly enriched pathways (Table 3).

3.8. Final sampling

All details of the statistical results are summarized in Supplementary material Table S1.

No significant differences were observed in the sex ratio and survival rate at the end of the experiments (Supplementary material Figure S5). Model testing revealed no interaction between group and sex for all dependent variables. Sex had a significant effect on weight, length and *K* with females always having greater values than males. We also observed differences between groups. Specifically, the POP10 and PFOS70 population had greater weight compared to control, in both females and males. The PFOS70 population was also larger in terms of length. Condition factor was determined to be significantly greater in the POP10 population compared to control (Fig. 6).

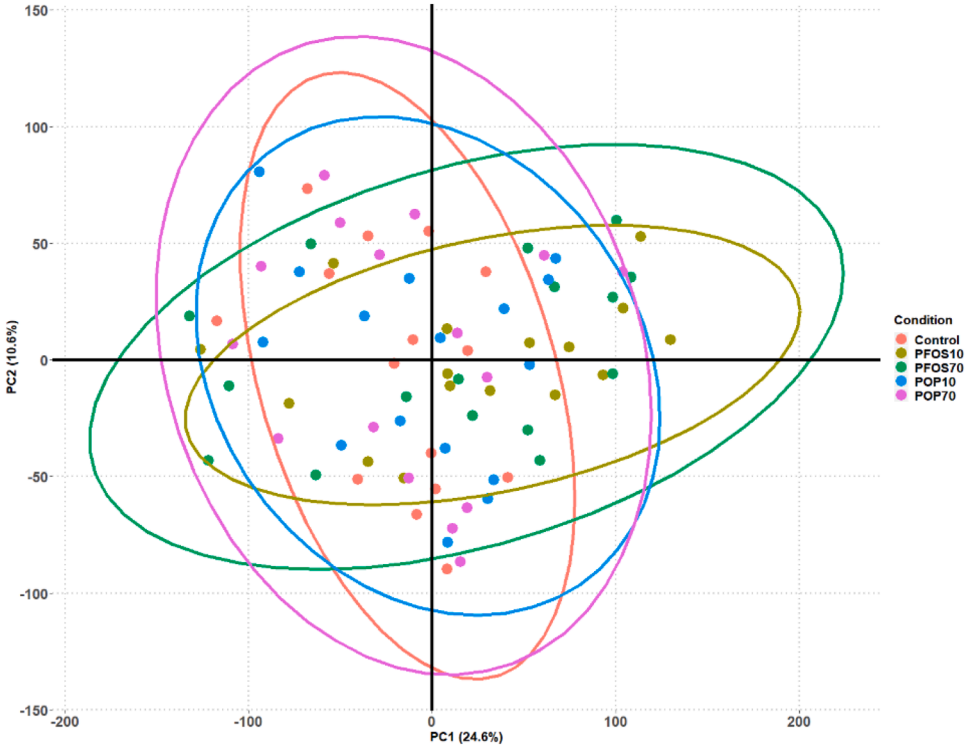

Fig. 5. Principal component analysis of all adult groups of all genes expressed in each group. Circles around each condition represent 95% confidence ellipses. Percent explained by each principal component is presented in parenthesis of x and y axes.

Table 2
GO analysis of canonical pathways involved in biological processes for the adults exposed as larvae in PFOS10 or POP70, FDR = false discovery rate.

ID	Name	#Genes in pathway	#DEGs in pathway	p-value	FDR
PFOS10					
GO:0007268	chemical synaptic transmission	166	17	3.12E-07	0.000337
GO:0098916	anterograde trans-synaptic signaling	166	17	3.12E-07	0.000337
GO:0099537	trans-synaptic signaling	167	17	3.41E-07	0.000337
GO:0099536	synaptic signaling	168	17	3.71E-07	0.000337
GO:0051932	synaptic transmission, GABAergic	6	4	4.29E-06	0.003113
GO:0034220	ion transmembrane transport	401	25	6.93E-06	0.004193
GO:0030001	metal ion transport	219	17	1.44E-05	0.007266
GO:0007214	gamma-aminobutyric acid signaling pathway	8	4	1.93E-05	0.007266
GO:0055085	transmembrane transport	566	30	1.99E-05	0.007266
GO:0098660	inorganic ion transmembrane transport	272	19	2E-05	0.007266
POP70					
GO:0006357	regulation of transcription by RNA polymerase II	566	5	0.000165	0.23612
GO:0006366	transcription by RNA polymerase II	596	5	0.000211	0.23612
GO:0080090	regulation of primary metabolic process	1585	7	0.000274	0.23612
GO:0031323	regulation of cellular metabolic process	1611	7	0.000305	0.23612
GO:0031326	regulation of cellular biosynthetic process	1106	6	0.000377	0.23612
GO:0009889	regulation of biosynthetic process	1115	6	0.000395	0.23612
GO:0019222	regulation of metabolic process	1712	7	0.000455	0.23612
GO:0050778	positive regulation of immune response	38	2	0.00076	0.3162
GO:0002252	immune effector process	42	2	0.000928	0.3162
GO:0050776	regulation of immune response	50	2	0.001315	0.3162

Table 3
KEGG analysis of canonical pathways for the adults exposed as larvae in PFOS10, FDR = false discovery rate.

ID	Name	#Genes in pathway	#DEGs in pathway	p-value	FDR
PFOS10					
dre04010	MAPK signaling pathway	308	23	2.6E-06	0.000416
dre04371	Apelin signaling pathway	145	13	7.75E-05	0.006199
dre04020	Calcium signaling pathway	173	13	0.000457	0.024363
dre04012	ErbB signaling pathway	96	9	0.000745	0.0298
dre04080	Neuroactive ligand-receptor interaction	206	13	0.002314	0.074049
dre04144	Endocytosis	264	15	0.003009	0.08025
dre04310	Wnt signaling pathway	145	10	0.003971	0.090761
dre04912	GnRH signaling pathway	103	8	0.00482	0.096395
dre04920	Adipocytokine signaling pathway	69	6	0.008347	0.14839
dre04914	Progesterone-mediated oocyte maturation	98	7	0.01281	0.20495

4. Discussion

We addressed a knowledge gap by investigating the developmental origins of adult health and disease in zebrafish. We observed effects of developmental exposure to POPs or PFOS on adult swimming performance and body size parameters whereas effects on the brain transcriptome were only found following developmental exposure to PFOS. However, developmental exposure had no effect on reproduction or anxiety-like behavior, or larval behavior in the F1 generation. These results suggest developmental exposure can have long-lasting effects on key life-traits, but subtle differences exist between single compound exposures and related mixtures.

We observed latent effects on body size parameters, with developmental exposure to the POP mixture and PFOS increasing body size at 15 months of age. Interestingly, both POP10 and PFOS70 had significantly higher body weights and lengths (only PFOS70) than controls. A similar increase in body mass was recorded in 5 mo zebrafish exposed to a mixture of POPs through feeding accompanied with changes in pathways involved in endocrine signaling and weight homeostasis (Nourizadeh-Lillabadi et al., 2009; Lyche et al., 2011). This increased weight gain may be explained by the obesogenic effects of many POPs via disruption of the PPAR signaling pathway which is involved in lipid biological processes such as metabolism, transport and homeostasis (Darbre 2017). Condition factor was only significantly increased in POP10 individuals due to the relationship between weight and length, since only weight was elevated; this led to an increase in condition factor, whereas in PFOS70 adults, weight was affected by exposure only

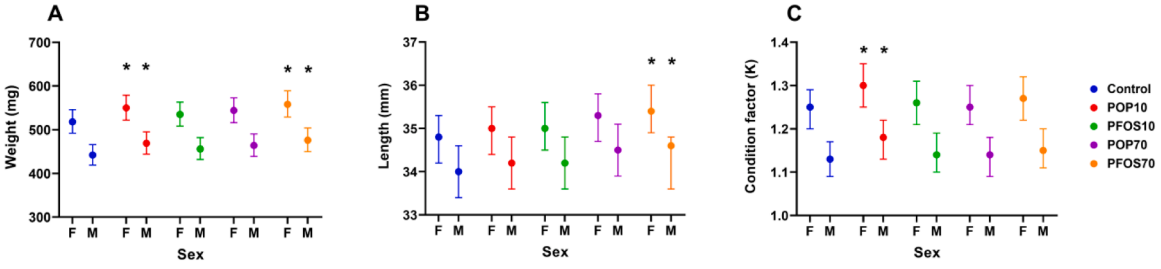

Fig. 6. Final weight, length and condition factor (K) of each condition for females (F) and males (M). Statistics are from linear mixed effect models. Data shown are least square means ± 95% CI. Asterisk indicate significant differences compared to control with $p < 0.05$. Females $N = 131 – 149$ individuals/condition, Males $N = 95 – 115$ individuals/condition, age = 15 mo.

based on its allometric relationship with length.

We found both the POP mixture and PFOS significantly lowered U_{crit} values. This agrees with a study by Xia et al. (2014) where exposure of adult topmouth gudgeon (*Pseudorasbora parva*) to PFOS (8 and 32 mg/L) for 96 h led to a decrease in U_{crit}. Previous work following developmental exposure to crude oil also found a significant reduction in the swimming speed of adult zebrafish (Hicken et al., 2011). This was associated with subtle changes in heart shape, that is important for heart function and swimming performance (Farrell 2002). Acute exposure to PFOS (4 and 16 mg/L) has also been found to affect the development and function of the heart in the marine medaka (4 −10 dpf) (Huang et al., 2011). We did not investigate whether there were changes in heart shape, however, we previously found developmental exposure to the POPs mixture or PFOS resulted in significant changes in gene expression in larvae related to cardiovascular disease such as atherosclerotic lesions, cardiomyopathy, hypertrophy, effect on diastolic function, and cardiac contraction (Christou et al., 2020). Therefore, future work should investigate heart function following developmental exposure to POPs.

We found no effect of developmental exposure on anxiety-like behavior in adults. This was unexpected, as we previously found increased levels of thigmotaxis in larvae exposed to POP70 and PFOS70 (Christou et al., 2020) which is a measure of anxiety (Schnörr et al., 2012). The lack of an effect later in life might suggest that the POPs in our study only affect early developmental stages, which have been shown to be more sensitive in chemical exposures (Makri et al., 2004), whereas detoxification during the growing phase might account for the lack of effects. Another study using the novel tank test concluded that chemical exposure leads to higher anxiety levels as shown by zebrafish showing a reduction in the amount of time spent in the top area of the tank. However, this study exposed zebrafish chronically to a chemical mixture of polycyclic aromatic hydrocarbons through the diet for 6 months (Vignet et al., 2014).

Pathway analysis of transcriptomic results agrees with the lack of effects on anxiety-like behavior of adult zebrafish since we did not observe enriched pathways that might induce anxiety such as the corticotrophin-releasing hormone pathway (Timpl et al., 1998). Multiple pathways relating to synaptic transmission and signaling were observed, however, in the brains of PFOS10 adults that might be related to other behavioral endpoints. KEGG analysis of DEGs revealed enrichment of the calcium signaling pathway (Supplementary material Figure S7). All genes in this pathway showed upregulation (Table 4). Alteration of calcium signaling pathway can further affect downstream signaling pathways. One of the pathways directly affected by changes in the calcium signaling pathways was the MAPK signaling pathway (Supplementary material Figure S8) which is involved in many of the cellular processes such as proliferation, differentiation and apoptosis. Changes in both signaling pathways have also been observed in the brains of 6 mo zebrafish after developmental exposure (4 – 24 hpf) to non-lethal doses of PCB126 (0.3 and 1.2 nM) (Aluru et al., 2017). MAPK signaling pathway is involved in brain development and has been suggested to play a role in synaptic plasticity, learning and memory, and depression-like behaviors (Thomas and Huganir 2004; Jeanneteau and Deinhardt 2011; Wefers et al., 2012).

Another pathway that is suggested to be involved in learning and memory, but also depression-like and anxiolytic effects in pathological conditions, is the gamma aminobutyric acid (GABA) signaling pathway. The GABA signaling pathway was enriched in the brains of PFOS10 adults and an upregulation of genes encoding GABA receptors was observed (Table 4) (Collinson et al., 2002; Liu et al., 2007). A study has suggested an association between GABAA receptor and cognitive and spatial memory of rats exposed to pesticides (Godinho et al., 2016). We did not observe any behavioral alterations suggesting elevated anxiety in zebrafish subjected to the novel tank diving test but additional behavioral tests could be more informative, such as the T-maze test, to evaluate the effects of early life exposure on learning and memory

Table 4
Lists of DEGs involved in each significant pathway in PFOS10 adult brains.

Pathway	Fold change	Description
Calcium signaling pathway		
adcy1a	3.2	adenylate cyclase 1a
camk2b	2.3	calcium/calmodulin dependent protein kinase II beta
chrm2a	4.9	cholinergic receptor, muscarinic 2a
erbb4b	2.4	erb-b2 receptor tyrosine kinase 4b
gna11a	2.5	guanine nucleotide binding protein (G protein), alpha 11a (Gq class)
grm5b	3.7	glutamate receptor, metabotropic 5b
ppp3r1a	2.5	protein phosphatase 3, regulatory subunit B, alpha a
Prkacaa	2.3	protein kinase, cAMP-dependent, catalytic, alpha, genome duplicate a
Prkcg	2.8	protein kinase C, gamma
si:ch73–374l24.1	2.6	si:ch73–374l24.1
slc8a1b	2.1	solute carrier family 8 (sodium/calcium exchanger), member 1b
slc8a3	2.2	solute carrier family 8 (sodium/calcium exchanger), member 3
slc8a4a	2.8	solute carrier family 8 (sodium/calcium exchanger), member 4a
MAPK signaling pathway		
akt3a	1.7	v-akt murine thymoma viral oncogene homolog 3a
arrb1	2.3	arrestin, beta 1
cacnb3a	2.9	calcium channel, voltage-dependent, beta 3a
cacng4b	3.8	calcium channel, voltage-dependent, gamma subunit 4b
dusp4	1.7	dual specificity phosphatase 4
erbb4b	2.4	erb-b2 receptor tyrosine kinase 4b
igf1ra	2.3	insulin-like growth factor 1a receptor
map2k4a	2.5	mitogen-activated protein kinase 4a
mapk10	2.1	mitogen-activated protein kinase 10
mapk8b	2.7	mitogen-activated protein kinase 8b
mapk8ip2	3	mitogen-activated protein kinase 8 interacting protein 2
nf1b	2.7	neurofibromin 1b
nr4a1	3.5	nuclear receptor subfamily 4, group A, member 1
ntrk2a	2	neurotrophic tyrosine kinase, receptor, type 2a
ppm1aa	2.3	protein phosphatase, Mg2+/Mn2+ dependent, 1Aa
ppp3r1a	2.5	protein phosphatase 3, regulatory subunit B, alpha a
Prkacaa	2.3	protein kinase, cAMP-dependent, catalytic, alpha, genome duplicate a
Prkcg	2.8	protein kinase C, gamma
rasgrf2b	4	Ras protein-specific guanine nucleotide-releasing factor 2b
si:ch73–374l24.1	2.6	si:ch73–374l24.1
taok2b	2.5	TAO kinase 2b
tgfbr1b	2.6	transforming growth factor, beta receptor 1 b
traf2b	1.6	Tnf receptor-associated factor 2b
synaptic transmission, GABAergic		
gabra1	2.6	gamma-aminobutyric acid (GABA) A receptor, alpha 1
gabra2a	3.7	gamma-aminobutyric acid type A receptor alpha2 subunit a
gabra4	2	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 4
npas4a	3.3	neuronal PAS domain protein 4a

(Bailey et al., 2015).

We found no multigenerational effect in larvae, either on basal behavior or in response to a second developmental exposure. Multigenerational effects have previously been seen in F1 zebrafish larvae in terms of higher swimming speed in a light/dark assay derived from parents that were exposed chronically to PFOS at three time periods 1 – 20, 21 – 120, or 1 – 120 dpf, or 5 months continuously. Behavioral changes were highly correlated with residues of PFOS in F1 embryos (Wang et al., 2011; Chen et al., 2013). Lack of effect in F1 larvae in our study may be due to shorter exposure periods that might have facilitated clearance of the chemical burden from the body of adults, thus no maternal transfer of chemicals in the developing eggs.

No effects of early life exposure on survival rates were evident in 5 and 15 mo zebrafish in this study. Reduced survival was observed in zebrafish only between 10 and 20 dpf when fed with an environmentally relevant mixture of POPs containing PCBs, PBDEs and organochlorine pesticides (Nourizadeh-Lillabadi et al., 2009). In contrast, no effect on acute or late mortality was observed in zebrafish fed with an environmentally relevant mixture containing 22 PCB congeners and 7 PBDE congeners (Horri et al., 2018) suggesting that zebrafish may be particularly sensitive to chemical stress during early life stages and that different routes, composition of exposures, and/or duration might affect the outcome.

We found no effect of developmental exposure to the POP mixture or PFOS on the sex ratio or reproduction. In contrast, zebrafish exposure to PFOS for 5 months resulted in a female dominant sex ratio whereas exposure to a POP mixture led to a male dominance in the exposed groups compared to control (Nourizadeh-Lillabadi et al., 2009; Wang et al., 2011). Since zebrafish do not have highly differentiated sex chromosomes the mechanisms involved in sex determination and how this is affected by chemical exposures are still unclear. Studies investigating effects of PCBs, PBDEs and organochlorine pesticides on the reproductive output have shown effects on fertilization rate but also number of eggs produced, hatching success, survival and gonadosomatic index of females and males (Johnson et al. (2013) and references therein). However, these positive results are generally observed in studies that use chronic exposures or exposed adults immediately prior to the reproductive tests (Johnson et al. (2013) and references therein).

Concentration differences were observed in most of the variables that were affected by early chemical exposure. Weight was only affected at the highest concentration of PFOS whereas it was affected at the low concentration of the POPs mixture. This might suggest a possible synergistic effect of PFOS with other compounds in the mixture. The lack of an effect at the higher concentration of the POP mixture might imply a shift to an antagonistic relationship due to possible oversaturation of cellular binding sites (Vandenberg et al., 2012). Transcriptomic analysis of adult brains also responded in a non-monotonic manner where the PFOS10 group had the highest number of DEGs with 466 genes. Interestingly the PFOS10 group had the most DEGs following transcriptomic analysis in larvae from our previous study (Christou et al., 2020). In contrast to the gene profile of PFOS10 in adult brains, which was mainly characterized by upregulation of genes (463 genes), in larvae there was a downregulation of 96% of the total number of DEGs. The higher number of DEGs in the lower concentrations than in the high concentrations suggest that the mechanisms of action (MoA) might be different and can be attributed to the non-monotonic effects of toxicants. Acute non-monotonic effects of toxicants have been previously demonstrated (Birnbaum 2012), but this is one of the few studies that underline the non-monotonic effects of toxicants in a DOHaD scenario (Aluru et al., 2017). Additionally, these observations point to the necessity of sampling at different time points and different tissues for a more thorough evaluation of MoA of chemical exposure.

Although the POP mixture is designed for humans, the sum of PCBs, PBDEs and OCPs in the low and high exposure used here are comparable to the concentrations reported in fish from Norwegian lakes (Nourizadeh-Lillabadi et al., 2009). For instance, we measured a sum of 37 ng/g lipid of PCBs in whole larvae (based on the premise that 5% of a larvae is lipid (Hachicho et al., 2015), one larvae weighs 0.45 mg (Falcinelli et al., 2015) and the concentration of PCBs found in an individual larvae (Christou et al., 2020)), whereas values of between 794 and 5240 ng/g lipid in the liver have been reported in wild fish in Norway (Nourizadeh-Lillabadi et al., 2009). Therefore, our results also have relevance for wildlife. Examination of swimming abilities is emerging as an effective method to evaluate the effects of chemical exposure in fish. During this study we observed that the critical swimming speed of adult fish was significantly reduced that might hinder the survivability of individuals making them prone to predation or unable to acquire food which in consequence might affect the population size (Hammer 1995). Furthermore, it is not clear whether an increased condition factor or weight can be considered an unfavorable outcome for a wildlife population. However, potential changes of behavior for larger fish may include unsuitable timing for migration, inappropriate seasonal behavior such as an increased appetite during winter, and/or higher activity leading to higher metabolic demands (Meador 2011).

In conclusion, early developmental exposure to an environmentally relevant POP mixture or PFOS alone led to some effects on adult zebrafish physiology, but an absence of effects in their offspring. This might mean that adults have the ability to detoxify once they are removed from the chemical exposure and that the effects are reversible. Effects on the weight of adult fish exposed as larvae to POPs and PFOS might indicate an obesogenic effect of persistent organic pollutants as these have been reported before (Yang et al., 2017).

Author contributions

Maria Christou: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, **Erik Ropstad:** Funding acquisition, Supervision, Writing – review & editing. **Stephen Brown:** Data curation, Investigation, Methodology. **Jorke H. Kamstra:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. **Thomas W. K. Fraser:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105882.

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