

## Embryonic exposure to PFAS causes long-term, compound-specific behavioral alterations in zebrafish

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### ABSTRACT

*Per-* and polyfluoroalkyl substances (PFAS) are commonly used as surfactants and coatings for industrial processes and consumer products. These compounds have been increasingly detected in drinking water and human tissue, and concern over their potential effects on health and development is growing. However, relatively little data are available for their potential impacts on neurodevelopment and the degree to which different compounds within this class may differ from one another in their neurotoxicity. The present study examined the neurobehavioral toxicology of two representative compounds in a zebrafish model. Zebrafish embryos were exposed to 0.1–100 μM perfluorooctanoic acid (PFOA) or 0.01–1.0 μM perfluorooctanesulfonic acid (PFOS) from 5 to 122 h post-fertilization. These concentrations were below threshold for producing increased lethality or overt dysmorphologies, and PFOA was tolerated at a concentration 100× higher than PFOS. Fish were maintained to adulthood, with behavioral assessments at 6 days, 3 months (adolescence) and 8 months of age (adulthood). Both PFOA and PFOS caused behavioral changes in zebrafish, but PFOS and PFOA produced strikingly different phenotypes. PFOA was associated with increased larval motility in the dark (100 μM), and enhanced diving responses in adolescence (100 μM) but not adulthood. PFOS was associated with a reversed light-dark response in the larval motility test (0.1–1 μM), whereby the fish were more active in the light than the dark. PFOS also caused time-dependent changes in locomotor activity in the novel tank test during adolescence (0.1–1.0 μM) and an overall pattern of hypoactivity in adulthood at the lowest concentration (0.01 μM). Additionally, the lowest concentration of PFOS (0.01 μM) reduced acoustic startle magnitude in adolescence, but not adulthood. These data suggest that PFOS and PFOA both produce neurobehavioral toxicity, but these effects are quite distinct from one another.

### 1. Introduction

*Per-* and polyfluoroalkyl substances (PFAS) are widely used industrial additives which are increasingly viewed as environmental contaminants of concern. These chemicals have been broadly used as surfactants, coatings and flame retardants for industrial processes and consumer products (Glüge et al., 2020; Vestergren et al., 2008). These include many distinct classes of chemicals, including carboxylic acids such as perfluorooctanoic acid (PFOA) and sulfonic acids such as perfluorosulfonic acid (PFOS). PFAS are stable, making them chemicals of choice for products which need to be heat, flame or water resistant (Glüge et al., 2020). For example, firefighting foams and coated cookware/food packaging frequently contain PFAS (Dahlbom et al., 2022; Seltenrich, 2020). However, their resistance to degradation also makes

these so called “forever chemicals” highly persistent in the environment and causes them to bioaccumulate. Recent water testing has shown that PFAS water pollution exists broadly across the US, and that the degree of water pollution varies considerably from location to location (Andrews and Naidenko, 2020). Food and house dust contamination have also been documented (Schildroth et al., 2022; Trudel et al., 2008; Vestergren et al., 2008) allowing for multiple possible routes of human exposure.

Recent epidemiological data indicates that human exposure to PFAS is common and potentially harmful to human health. PFAS has been detected in diverse human samples, including blood, serum, and breast milk (Graber et al., 2019; Oh et al., 2021; Zheng et al., 2021). PFAS exposure is associated with impairments in maternal reproductive health and with adverse outcomes on the growth, immune systems and

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cognitive or behavioral health of exposed children (Anderko and Pennea, 2020; Harris et al., 2021; Vuong et al., 2021). However, these conclusions are somewhat complex, given that thousands of PFAS exist, and exposures are likely to include a mixture of compounds with varying characteristics, and at various concentrations. While human studies suggest that PFAS may be harmful to neurological development, additional toxicological data are needed to assess the relevance of individual compounds to such effects.

Animal studies provide additional evidence that PFAS are toxic in the developing nervous system, without the causality limitations which impact human association studies. For example, existing studies have documented PFAS-induced changes in neurotransmission in mice, frogs, and nematodes, including alterations of monoaminergic, glutamatergic and cholinergic signaling (Foguth et al., 2019; Foguth et al., 2020; Grønnestad et al., 2021; Sammi et al., 2019). Additionally, animal models, particularly zebrafish, have shown that PFAS neurotoxicity can be behaviorally revelant, producing deficits in motor function, motility and photomotor responses at the larval stage (Menger et al., 2020; Rericha et al., 2021) leading to potentially persistent locomotor and affective effects into adulthood (Jantzen et al., 2016a). However, existing data does show that larval assays may not adequately predict effects in adulthood. For example, Jantzen et al. (2016a) found reduced locomotor responses, reduced light-avoidance, and enhanced "aggressive" reactions to a mirror in adults embryonically treated with perfluorononanoic acid (PFNA), but not PFOS or PFOA. By contrast, Menger et al. (2020) observed larval behavioral effects in all three compounds, and Rericha et al. (2021) observed effects with PFOS and PFNA, but not PFOA. Recently, Christou et al., 2021 found that embryonic PFOS exposure reduced larval swimming activity but did not produce apparent behavioral alterations when these fish aged into adulthood. This generally suggests that the effects of PFAS exposures may be relatively compound specific, and that such effects are not necessarily persistent to adulthood. What remains unclear is the degree to which neurological effects significantly shift or attenuate over the course of early development, and when in this process such changes occur.

The current study used zebrafish as the model organism to allow the relative neurotoxicities and developmental trajectories of PFAS-induced neurobehavioral effects to be assessed. Zebrafish are valuable models for early developmental and aging research due to their fecundity, accessible behavioral characteristics, genetic tractability, and cellular/physiological homology with humans and other vertebrates (Bailey et al., 2014). Zebrafish share 71.4% of their genes with humans, and 84% of those genes are known to be associated with human disease have a zebrafish counterpart (Howe et al., 2013). Additionally, many organs and tissues belonging to zebrafish share molecular, physiological, and anatomical similarities with humans, making them an excellent model organism (Dubinska-Magiera et al., 2016). Zebrafish models are cost- and space-effective due to their small size and can be reasonably maintained for the bulk of their lifespan for longitudinal analysis.

The current study examined the developmental and behavioral effects that two representative compounds, the carboxylic acid compound PFOA and the sulfonic acid compound PFOS, have on developing zebrafish. Dosimetry pilots were performed to determine the maximum tolerable levels for zebrafish embryos, and doses were selected to fall below the threshold for increased lethality or dysmorphology. Embryos were exposed to these compounds through their housing water over the first 5 days after fertilization (5–120 h post fertilization, hpf), and behavioral testing was performed at three stages of development: larval (144hpf), adolescent (3 months) and adult (7–8 months). Behavioral batteries included tests of locomotor, sensory, and affective functions.

## 2. Materials and methods

### 2.1. Animals and animal care

Wild-type zebrafish (AB) were bred in-house in the Levin Lab at Duke

University. They were housed in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) on a 14:10 h light/dark cycle. The pH remained at 6.8–7.0 and at a temperature of 27–29 °C. System water contained 5 g of instant ocean salt/10 L, 0.34 g of neutral regulator/10 L, and 1 g of alkaline regulator/10 L (Seachem Laboratories, Madison, GA, USA) to set the appropriate pH. Sodium bicarbonate was used to return the pH to these ranges if weekly tests identified a pH below this range. Yolk-stage embryos and larvae were maintained in an incubator, and fish were transferred to 3 L tanks in a recirculating system at 6dpf. Juvenile fish were fed three times daily with Golden Pearl active spheres (Brine Shrimp Direct, Ogden, UT, USA), and transitioned to an adult diet from 1 to 3 months of age. Adult fish were fed twice daily (morning and afternoon) with brine shrimp (Brine Shrimp Direct, Ogden, UT, USA), and their diet was supplemented with Gemma Micro 300 pellets (Skretting USA, Tooele, UT, USA) at noon each day. At 3 months of age, surviving fish were rehoused to achieve a density of <5 fish per liter of water (8–15 per 3 L tank), based on numbers available per tank (i.e. tanks with >15 fish were split into equally sized groups of 8–11 individuals). All testing was conducted at room temperature and between the hours of 0900–1600 h. The PFOA and PFOS effects were assessed in separate studies. Within the egg batches all doses of the relevant compound and the vehicle control were included. Within each study the same control and exposed zebrafish were repeatedly tested at the different ages. The adult fish were sexed by external morphology. All available fish were tested in each behavioral test. The protocols for this project were approved by the Duke University Animal Care and Use Committee.

### 2.2. Breeding, exposures and chemical preparation

Tanks containing multiple male and female fish were bred to create a heterogeneous sample of fertilized eggs. These eggs were rinsed with a 0.001% bleach solution and then twice with clean system water. The eggs were then sorted under a dissecting microscope to identify successfully fertilized eggs, and viable embryos were randomly placed into glass petri dishes at a density of 40 embryos per 40 ml of system water. Two replicate breedings were used to produce the PFOS cohorts and two replicate breedings were used to produce the PFOA cohorts, with a target N of >30 individuals total reaching adulthood. PFOS and PFOA exposure began at 5 h after fertilization. Throughout the exposure period, embryos/larvae were housed in an incubator (28 °C) maintained on a 14:10 light:dark cycle. Concentrations of PFOS and PFOA were chosen based on preliminary pilots which detected the lowest concentration leading to increased lethality or dysmorphogenesis (1 mM PFOA, 10 μM PFOS). Concentration ranges approaching this threshold were then established with 1-log unit separation between treatments. PFOA groups were exposed as embryos to 0.1 μM, 1 μM, 10 μM, or 100 μM of PFOA in a 0.1% DMSO vehicle. PFOS groups were exposed as embryos to 0.01 μM, 0.1 μM, or 1.0 μM of PFOS in a 0.1% DMSO vehicle. Tolerability thresholds were generally consistent with previous studies and are consistent with the observation that PFOA is tolerated at  $\geq 10\times$  higher concentrations than PFOS (Ulhaq et al., 2013; Ye et al., 2009). Controls were exposed to the 0.1% DMSO vehicle alone. DMSO has been found to have no effect on survival, morphology or larval behavior at this concentration, and has been shown to have no interaction with PFOS (Christou et al., 2020). These solutions were replaced daily until 120hpf. Among viable embryos, mortality and dysmorphology rates were observed and these rates did not systematically differ across replicates and treatment groups (control range 5–14%; overall range 1–22%, no dose-dependency apparent). As is typical in zebrafish, significant attrition was observed between the larval stage and adulthood (control range across replicates 30–48%, overall range across replicates and groups 25–53%) but this was not observed to differ systematically across treatment conditions.

Testing occurred at 6 days (144hpf) post-fertilization for larval effects, 3 months post-fertilization for adolescent effects, and 7–8 months

for adult effects. Fish completed each test only once per age (larval, adolescent or adulthood) and the two replicate cohorts were equally represented, as available due to attrition, within each dataset. Testing times were chosen to observe differences in neurobehavioral toxicity across developmental maturation.

### 2.3. Larval motility

Larval testing consisted of a light-dark motility test. On 6dpf, larvae were individually placed into a well on a 96 well plate, containing 0.5 ml of freshly-made system water. One row ( $n = 12$ ) per plate was occupied by each treatment and two plates ( $2 \times 12$  individuals) run per replicate cohort (48 individuals total). The location of the row/treatment was varied for each replicate plate. Testing then occurred between 1000 and 1230 h, preceded by a one-hour acclimation period in a dark incubator (28 °C). The room was lit during transfer from the dark incubator to the testing chamber (~240 lx). For testing, the 96-well plate was transferred to the DanioVision Observation Chamber (Noldus Information Technology, Wageningen, The Netherlands) for a 50 min testing session, during which Ethovision XT software (version 14, Noldus, Wageningen, The Netherlands) analyzed the motion of each animal. The testing session consisted of five, 10-min phases which shifted between dark (0% light output) and lit (100% light output, ~100 lx) conditions (dark-light-dark-light-dark). Motility was measured as the distance moved (in cm) per 10 min lighting phase. Additionally, a stimulation index was calculated to determine the change in locomotor output attributable to the change in lighting conditions (dark activity level – light activity level). In control fish, the stimulation score is positive, indicating that larvae are more active in the dark than the light. This testing was performed only once, at 144hpf.

### 2.4. Novel tank dive test

The novel tank dive test measures zebrafish locomotor and anxiety-like behavior in an unfamiliar environment for 5 min, as in our established protocol (Glazer et al., 2018). The novel tank diving test has been validated behaviorally and pharmacologically as an index of anxiety-like behavior in zebrafish placed in a novel environment and reversible by anxiolytic drug treatment (Bencan et al., 2009; Levin et al., 2007) and has become widely used in the field (Glazer et al., 2018). Fish were individually placed into 1.5 L plastic tanks filled with system water with a tank depth of 10 cm [22.9 cm long on the bottom, 27.9 cm  $\times$  6 cm at the top, 15.2 cm tall, 15.9 cm on the diagonal side, and 5.1 cm wide]. Novel tank testing occurred in a room location without immediate overhead lighting and an opaque white sheet was used to prevent direct lighting by the ceiling fixtures (“shaded tanks”, ~180 lx). Locomotor speed (cm/min) and vertical position in the tank (cm from the floor) data were collected and separated into a time-series of five, 1-min bins. Vertical position from the floor represents an anxiety-like response; remaining closer to the bottom of the tank quantifies the magnitude of anxiety-like behavior. This test was performed twice, once at 3 months of age, and once at 7–8 months of age).

### 2.5. Tap startle test

The tap startle test gauged sensorimotor startle responses and their habituation in zebrafish, and this was performed using an established protocol (Glazer et al., 2018). The test apparatus consists of eight clear acrylic cylindrical arenas (5.7 cm in diameter) arranged in two rows of four, set upon a flat white 23  $\times$  29 cm surface with visual barriers to prevent the fish from seeing one another. Underneath each arena was a 24 V DC push solenoid that could fire a pin into the bottom of the well to generate a startling “tap” sound. Fish were individually placed into each arena in 40 ml of system water. After placement into each well, the tapping program was initiated and taps were delivered at 1-min intervals following a 30s delay period. As in the novel tank, testing

occurred in a room location without immediate overhead lighting and an opaque white sheet was used to prevent direct lighting by the ceiling fixtures (“shaded tanks”, ~180 lx). Startle responses and locomotor behavior were measured using measurements of the total distance moved (in cm) by each fish in the 5 s immediately before and immediately after each tap. Startle magnitudes, or the level of post-tap activity attributable to the acoustic stimulus, were then calculated by determining the difference between pre- and post-tap activity scores (post – pre). This test was performed twice, once at 3 months of age, and once at 7–8 months of age.

### 2.6. Shoaling social approach

Social approach was determined using an established protocol with uniform social stimuli (Glazer et al., 2018). Prior to testing, fish were individually placed in 1.5 L tanks for 30 min, to facilitate social approach. Following this isolation period, these fish were individually placed into one lane of a rectangular partitioned experimental tank (30 cm long, 6 cm wide, filled to 10 cm deep). This lane was bordered with clear Plexiglas on both ends and black Plexiglas on the side walls. The partitioned tank was backlit from underneath (~340 lx) with a light box (Huion Technology, Shenzhen, China) and each transparent end faced a flat computer screen. After starting the video recording, the trial began with a 2-min habituation phase, during which the two screens showed control ovoid shapes. At 2 min, a video of a shoal of swimming zebrafish played on one of the two screens for 5 min. The video player was anchored on the left screen, from the experimenter’s view, so videos were presented on the same end of the testing apparatus for all fish. As fish were allowed two minutes to freely swim prior to the video presentation, fish varied in their distance from the video screen and their relative orientation to it when the video began. Locomotor activity through the session was measured as distance moved (in cm) across seven, 1-min time bins. Side preference was measured as the average distance of the fish from the screen-adjacent side of the tank (in cm) during the baseline phase and during the video presentation. Social approach was then measured as the change in side preference attributable to the presentation of the video (no video – video). This test was performed only once, at 7–8 months of age.

### 2.7. Predator avoidance test

Predator cue avoidance and fleeing were determined using an established protocol with artificial aversive stimuli (Glazer et al., 2018). For this test, the partitioned tank and 2-screen setup were used as in the shoaling social approach test, with the exception that non-cue conditions featured a blank white screen instead of a screen with control shapes. Within the test session, fish were exposed to alternating 1-min periods with either a blank screen (no cue) or a video with an aversive dot (predator cue), which grows from very small to very large. This is a 2-dimensional representation of a large object moving towards the viewer. In zebrafish, this stimulus can trigger fleeing behavior and avoidance of the adjacent side of the enclosure. Predator cues were delivered at one of two speeds: slow (duration of 1–23 cm increase = 4-s), or fast (duration of 1–23 cm increase = 1-s). The first two stimulus presentations were at the slow speed (min 2 and 4) and the second two were at the fast speed (min 6 and 8). As fish were allowed one minute to freely swim prior to the first cue presentation, fish varied in their distance from the screen and their relative orientation to it when the video began. Locomotor activity through the session was measured as distance moved (in cm) across nine, 1-min time bins. Side preference was measured as the average distance of the fish from the screen-adjacent side of the tank (in cm) during the baseline (no cue) phases and during the predator cue presentations. To take in account the initial position of the fish before the onset of the visual stimulus, fleeing responses were measured as the change in side preference attributable to the presentation of the video (cue – no cue). This test was performed only once, at

7–8 months of age.

## 2.8. Statistical analysis

All data was analyzed using SPSS v.27 (IBM Corp.). Each analysis began with a mixed-factorial or univariate analysis of variance (ANOVA), as relevant to the number of independent variables. Mixed-factorial ANOVAs (Type III sums of squares) addressed repeated measures aspects of the data (e.g. 1 min-block), stimulus repetition (e.g. tap in sequence) or cue condition (e.g. cue present/absent, fast or slow predator cue). Treatment served as the primary between-subjects variable, and sex and replicate cohort were included as covariates. For clarity, main effects are reported only in the absence of a higher order interaction. Post hoc analyses were performed with Dunnett's correction for multiple testing, which compared all treatment groups with controls. Significance was set at  $p < 0.05$  for all analysis of variance (ANOVA) and follow-up testing. Given that fish identity was not maintained across repetitions of the same test, each age was initially treated as an independent outcome. However, as interactions with age may be relevant for interpretation, follow-up analyses on repeated tests (novel tank and tap test) were performed with age as a between-subjects variable to allow age x treatment interactions to be identified. These are presented separately. Inclusion criteria included a >96% quality scores on Ethovision subject tracking (<4% of frames had no subject found). Outliers associated with technical problems with data acquisition were identified and removed and normality was assessed using the Explore function in SPSS.

## 3. Results

### 3.1. Larval motility: 6 days post-fertilization

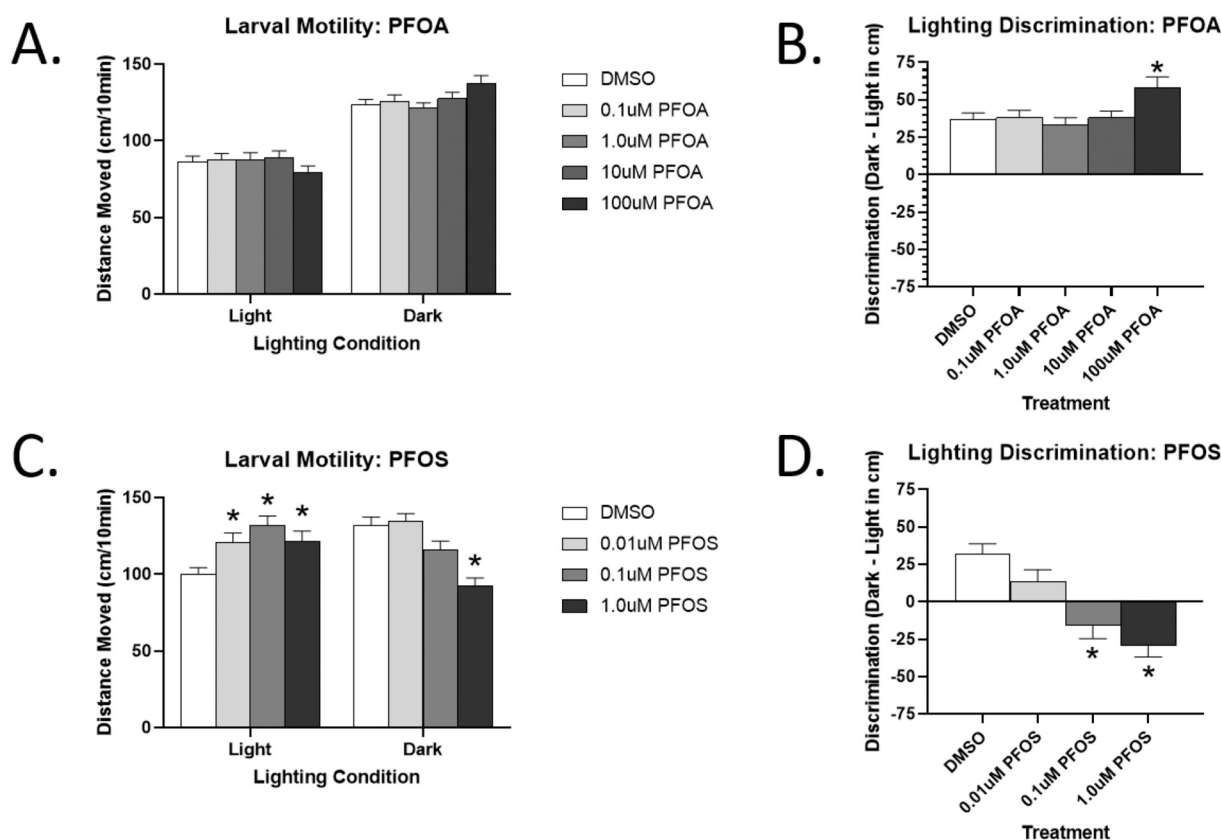
Both PFOS and PFOA displayed neurobehavioral effects in the larval motility test (Fig. 1), and these effects were detected as changes in distance moved (Fig. 1a/c) and light-dark discrimination (Fig. 1b/d).

For PFOA, analyses detected a light (present/absent) x treatment interaction,  $F(4, 223) = 3.61, p < 0.05$ , for distance moved. Post hoc testing observed that no PFOA groups differed from controls during specific lighting phases (Fig. 1a). However, univariate analysis of variance further showed a main effect of treatment on discrimination scores (dark distance moved – light distance moved),  $F(4, 224) = 3.51, p < 0.05$ . Fish exposed to 100uM PFOA showed a larger increase in activity due to the light turning off relative to controls ( $p < 0.05$ ) (Fig. 1b).

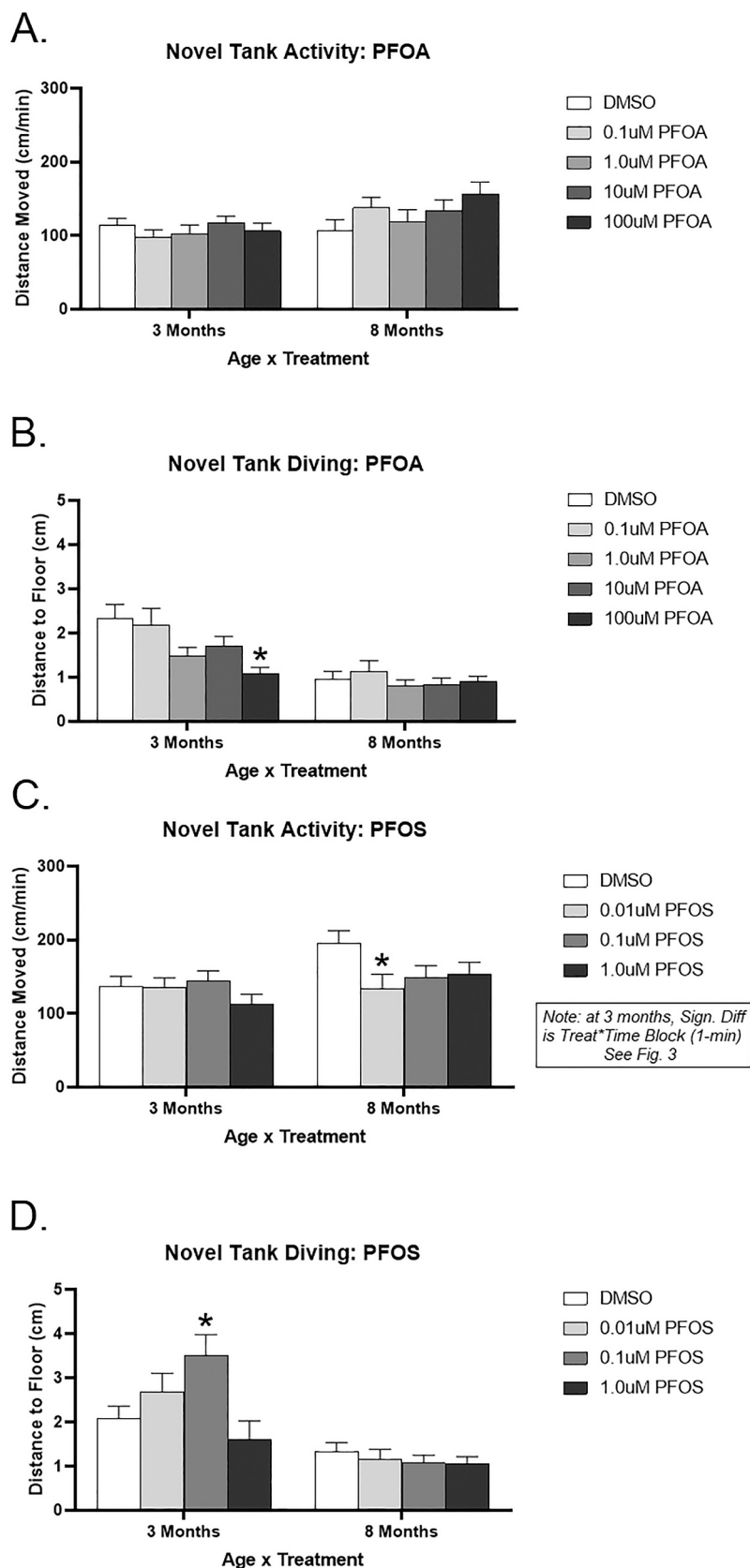
For PFOS, analyses detected a light x treatment interaction,  $F(1, 185) = 2.70, p < 0.05$ , for distance moved. Post hoc testing observed that fish with 0.01–1.0uM PFOS exposure were more active than controls under lit conditions ( $p < 0.05$ ), and fish with 1.0uM PFOS exposure were less active under dark conditions ( $p < 0.05$ ) (Fig. 1c). Univariate analysis of variance further showed a main effect of treatment on discrimination scores,  $F(3, 185) = 12.73, p < 0.05$ . Fish exposed to 0.1–1.0uM PFOS showed negative discrimination scores (more active in the light than the dark), and were significantly lower relative to controls ( $p < 0.05$ ) (Fig. 1d).

### 3.2. Novel tank dive test: 3 and 8 months of age

Both PFOS and PFOA displayed neurobehavioral effects in the novel tank test (Fig. 2), and these effects were detected as changes in distance



**Fig. 1.** Larval Motility Analyses. Larval Motility expressed as distance moved (cm/10 min) (A/C) and locomotor stimulation in the dark (dark – light in cm/10 min) (B/D). Data are expressed as mean  $\pm$  SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). (A) PFOA induced hyperactivity in the dark, but not the light, at 100uM. (B) PFOA caused an increase in locomotor stimulation in the dark at 100uM. (C) PFOS led to hyperactivity in the light (0.1–1.0uM) and hypoactivity in the dark (1.0uM). (D) PFOS reversed the typical pattern of dark-induced stimulation, reaching significance for the 0.1–1.0uM groups.



**Fig. 2.** Novel Tank Effects by Age. Locomotor activity is expressed as distance moved (cm/min) (A/C) and the anxiety-like diving response as distance from the floor (cm) (B/D). Data are expressed as mean  $\pm$  SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). (A) PFOA did not alter locomotor activity at 3 or 8 months of age. (B) PFOA caused a reduction in distance from the floor at 3 months (100uM) but not 8 months of age. (C) PFOS led to a main effect at 8 months, leading to hypoactivity which only reached significance in the 0.01uM group. No main effect was detected at 3 months, but a time\*treatment interaction at this age is shown in Fig. 3. (D) PFOS caused an increase in exploration away from the floor (0.1uM) at 3 months, but not 8 months.

moved (Fig. 2a/c) and/or distance from the floor (diving response) (Fig. 2b/d).

For PFOA, adolescent analyses detected a main effect of time for distance moved,  $F(4, 460) = 6.37, p < 0.05$ . No effects of treatment or relevant interactions were detected (Fig. 2a). In adulthood, a main effect of time was observed for distance moved,  $F(4, 564) = 5.24, p < 0.05$ , but no effects of treatment or relevant interactions were detected. For distance from the floor, adolescent analyses observed main effects of time,  $F(4, 460) = 3.97, p < 0.05$ , and treatment,  $F(4, 115) = 3.87, p < 0.05$ , on distance moved. Post hocs observed that fish exposed to 100uM PFOA remained closer to the floor relative to controls in adolescence ( $p < 0.05$ ) (Fig. 2b). In adulthood, a main effect of time was observed,  $F(4, 564) = 3.37, p < 0.05$ , but no effects of treatment or relevant interactions.

For PFOS, adolescent analyses observed a treatment x time (1-min block) interaction,  $F(12, 380) = 3.56, p < 0.05$ , on distance moved. Post hoc analyses observed that fish exposed to 0.1uM PFOS were hyperactive compared to controls in the first minute of the session ( $p < 0.05$ ) and fish exposed to 1.0uM PFOS were hypoactive compared to controls in the final minute of the session ( $p < 0.05$ ) (Fig. 2c). In adulthood, a main effect of treatment was observed,  $F(3, 117) = 3.57, p < 0.05$ , whereby fish exposed to 0.01uM PFOS were hypoactive relative to controls ( $p < 0.05$ ) (Fig. 2c). For distance from the floor, adolescent analyses showed a main effect of treatment, whereby fish exposed to 0.1uM PFOS swam further from the floor relative to controls ( $p < 0.05$ ) (Fig. 2d). In adulthood, a treatment by time interaction was observed,  $F(12, 468) = 1.80, p < 0.05$ . However, no PFOS-treated groups significantly differed from controls in post hoc testing (Fig. 3).

### 3.3. Tap startle test: 3 and 8 months of age

PFOS exposure, but not PFOA, caused neurobehavioral effects in the tap startle test (Fig. 4a/b), and these effects were measured as distance moved in the 5 s prior to the tap (pre-tap), the 5 s following the tap (post-tap) and as a difference between pre- and post-tap activity (startle).

For PFOA, adolescent analyses detected a main effect of tap (x10) for pre-tap,  $F(9, 639) = 6.40, p < 0.05$ , and startle magnitude,  $F(9, 639) = 3.06, p < 0.05$ , as well as a tap x treatment interaction for post-tap measurements,  $F(36, 639) = 1.62, p < 0.05$ . Post hoc analysis of the tap x treatment interaction for post-tap activity observed no significant differences relative to controls. No effects of treatment or relevant interactions were detected (Fig. 4a). In adulthood, a main effect of tap was observed for pre-tap activity,  $F(9, 1251) = 4.66, p < 0.05$ , but no other effects or interactions were detected (Fig. 4a).

For PFOS, adolescent analyses detected a main effect of tap (x10) for post-tap activity,  $F(9, 945) = 2.92, p < 0.05$ , as well as a tap x treatment interaction for pre-tap,  $F(27, 936) = 1.70, p < 0.05$ , and a main effect of treatment for startle magnitude,  $F(3, 104) = 2.97, p < 0.05$ . Post hoc

analysis of the tap x treatment interaction for pre-tap activity observed only one difference, whereby the 0.01uM PFOS group had lower pre-tap activity prior to the second tap relative to controls ( $p < 0.05$ ) (not shown). Post hoc analysis of the main effect of treatment on startle magnitude showed that fish exposed to 0.01uM PFOS showed reduced startle magnitudes relative to controls (Fig. 4b). In adulthood, main effects of tap were observed for pre-tap activity,  $F(9, 1017) = 11.04, p < 0.05$ , and startle magnitude,  $F(9, 1017) = 6.73, p < 0.05$ , but no other effects or interactions were detected (Fig. 4b).

### 3.4. Shoaling social approach: 8 months of age

Neither PFOS nor PFOA exposure led to neurobehavioral effects in the shoaling social approach test (Fig. 5a-c). Behavioral measures included locomotor activity measured as distance moved (in cm) and social approach, measured as the distance from the screen prior to the onset of the video (no video), during the video (video) and as the change in position due to the video (no video - video).

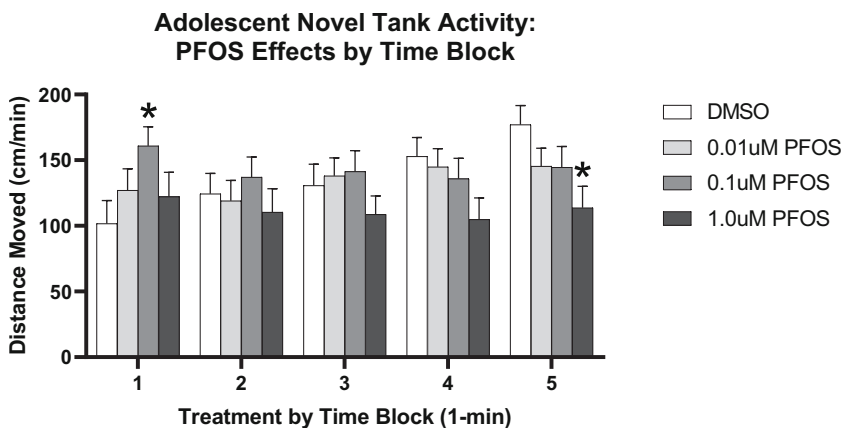
For PFOA, analyses detected a main effect of time for distance moved,  $F(6, 708) = 4.81, p < 0.05$ , (Fig. 5a). Additionally a main effect of cue condition (video/no video) was observed on distance from the screen,  $F(1, 118) = 6.66, p < 0.05$ , (Fig. 5b) and a main effect of sex on approach score,  $F(1, 118) = 4.03, p < 0.05$ , whereby females showed greater approach scores than males ( $p < 0.05$ ). No main effects of treatment or relevant interactions were observed (Fig. 4b). For PFOS, a main effect of cue condition (video/no video) was observed on distance from the screen,  $F(1, 86) = 4.21, p < 0.05$ , but no effects of treatment or relevant interactions reached significance (Fig. 5c/d).

### 3.5. Predator avoidance: 8 months of age

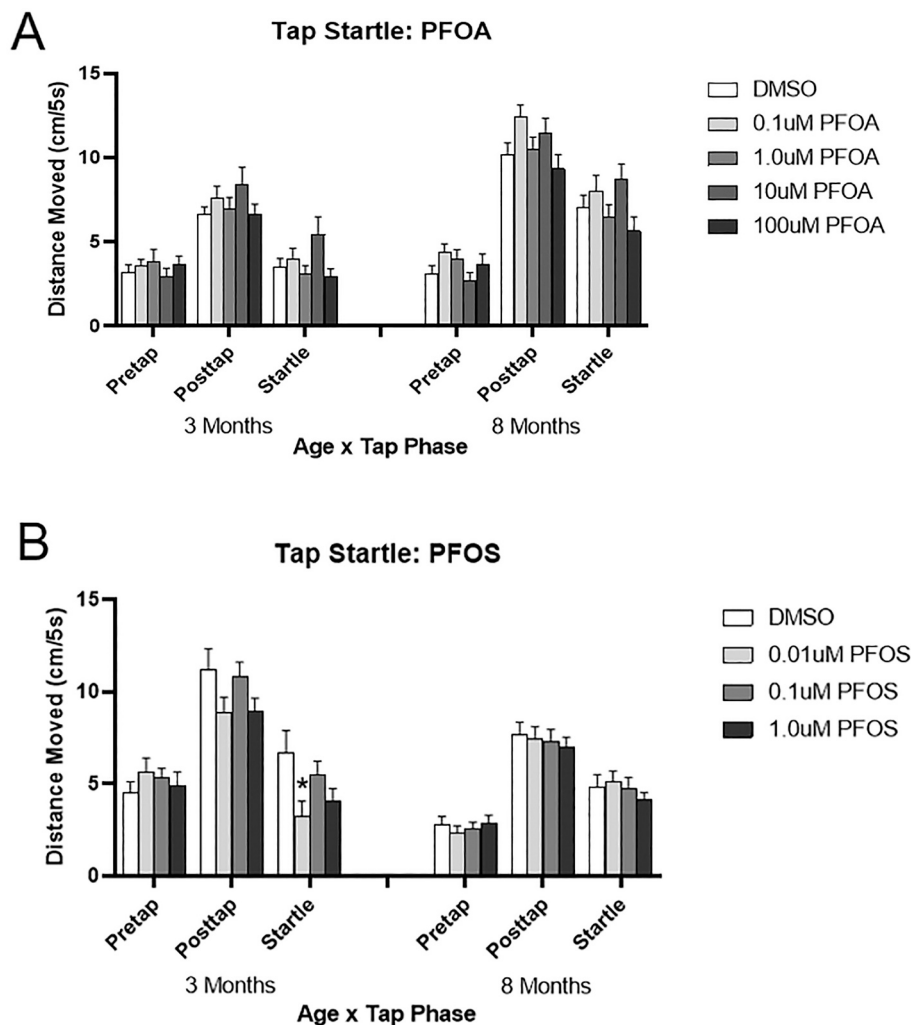
Neither PFOS nor PFOA exposure led to neurobehavioral effects in the predator avoidance test (Fig. 6a-c). Behavioral measures included locomotor activity measured as distance moved (in cm) and social approach, measured as the distance from the screen prior to the onset of the predator cue (no cue), during the cue presentations (cue) and as the change in position due to the video (flee, cue - no cue).

For PFOA, analyses detected a main effect of treatment for distance moved,  $F(4, 119) = 2.64, p < 0.05$ , (Fig. 6a), but no post hoc tests reached significance. A main effect of treatment was observed on distance from the screen,  $F(4, 119) = 3.36, p < 0.05$ , whereby fish in the 100uM group swam closer to the screen than controls regardless of cue presentation ( $p < 0.05$ ) (Fig. 6b). Additionally a main effect of cue condition (cue/no cue),  $F(1, 119) = 17.35, p < 0.05$ , as was cue speed (slow/fast),  $F(1, 119) = 5.34, p < 0.05$  on distance from the screen and (Fig. 6b) and sex on fleeing score,  $F(1, 119) = 4.03, p < 0.05$ , whereby females showed greater fleeing scores than males ( $p < 0.05$ ).

For PFOS, analyses detected treatment,  $F(3, 109) = 6.17, p < 0.05$ ,



**Fig. 3.** PFOS Adolescent Effects on Novel Tank Activity. Locomotor activity is expressed as distance moved (cm/min). Data are expressed as mean  $\pm$  SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). PFOS altered the time course of locomotor activity at 3 months in a dose-dependent manner. At the middle concentration (0.1uM), fish were hyperactive relative to controls in time block 1, but failed to increase activity across the session, as controls do. At the higher concentration (1.0uM), fish begin at a comparable level of activity, but fail to increase activity across the session, leading to a significant difference vs controls in time block 5.



**Fig. 4.** Tap Startle Effects by Age. Locomotion and startle movement is expressed as distance moved (cm/5 s) across the 5 s before (left) and after (middle) the tap, and as the difference between these scores (post – pre), interpreted as startle magnitude (right). Data are expressed as mean  $\pm$  SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). (A) PFOA did not alter locomotion or startle magnitude at 3 or 8 months of age. (B) The lowest concentration of PFOS led to reduced startle magnitudes at 3 months of age, although pre and post tap treatment effects did not reach significance. No treatment effects were observed at 8 months of age.

whereby fish in the 0.01 and 1.0  $\mu$ M groups were hypoactive compared to controls ( $p < 0.05$ ) (Fig. 6a). Main effects of time,  $F(8, 872) = 2.83, p < 0.05$ , (Fig. 5a), and sex  $F(1, 109) = 9.61, p < 0.05$ , whereby males were more active than females ( $p < 0.05$ ), were also detected. For distance from the screen, there were main effects cue presentation (present/absent),  $F(1, 109) = 29.43, p < 0.05$ , and interactions of cue speed and cue presentation,  $F(1, 109) = 8.03, p < 0.05$ . No effects of treatment or relevant interaction were observed for distance from the screen or the magnitude of the fleeing response (Fig. 6c).

### 3.6. Age effects on novel tank and tap startle

Within the followup analyses, multiple main effects of age and/or relevant interactions were observed. Main effects of age are shown in the supplementary figures (Suppl. Fig. 1/2). A general age-effect was noted across both PFOA and PFOS studies, whereby locomotor activity increases from adolescence to adulthood, and the diving response is enhanced across the same time period. Age-effects for the tap-startle response were not consistent across the two studies. A full summary of age-based interactions and treatment effects are reported below.

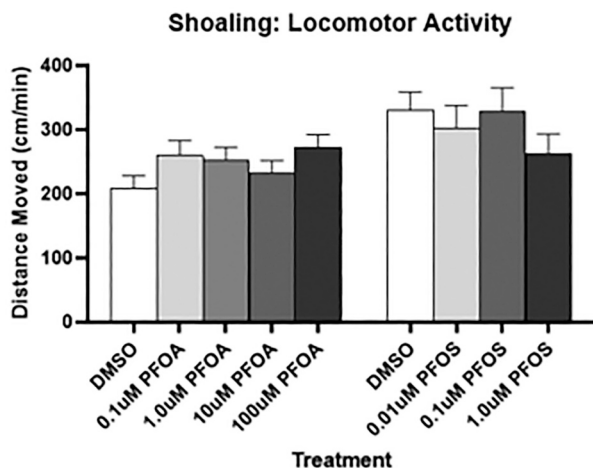
For PFOA, novel tank analyses detected a main effect of age for distance moved,  $F(1, 259) = 7.37, p < 0.05$ , and a time x age interaction,  $F(4, 1036) = 2.96, p < 0.05$ . No main effects of treatment or relevant interactions were observed. With respect to age, adult fish were more active than adolescents ( $p < 0.05$ ) (Suppl. Fig. S1a). For distance from the floor, a main effect of age was observed,  $F(1, 259) = 37.91, p < 0.05$ ,

as was a time x age interaction,  $F(4, 1036) = 2.85, p < 0.05$ . A main effect of treatment was also observed,  $F(1, 259) = 3.90, p < 0.05$ , whereby the 100 $\mu$ M PFOA group remained closer to the floor than controls regardless of age (*not shown*). With respect to age, adult fish remained closer to the floor than adolescents ( $p < 0.05$ ) (Suppl. Fig. S1b).

For PFOS, novel tank analyses detected a main effect of age on distance moved,  $F(1, 217) = 4.97, p < 0.05$ , a time x age interaction,  $F(4, 868) = 2.44, p < 0.05$ , a time x treatment interaction,  $F(12, 868) = 2.21, p < 0.05$ , and a time x age x treatment interaction,  $F(12, 868) = 1.91, p < 0.05$ . With respect to age, adult fish were more active than adolescents ( $p < 0.05$ ) (Suppl. Fig. S1a). For distance from the floor, a main effect of age was observed,  $F(1, 217) = 39.73, p < 0.05$ , as was a main effect of treatment,  $F(3, 217) = 3.67, p < 0.05$ , an age x treatment interaction,  $F(3, 217) = 4.15, p < 0.05$ , and a time x age x treatment interaction,  $F(12, 868) = 2.85, p < 0.05$ . With respect to age, adult fish remained closer to the floor than adolescents ( $p < 0.05$ ) (Suppl. Fig. S1b).

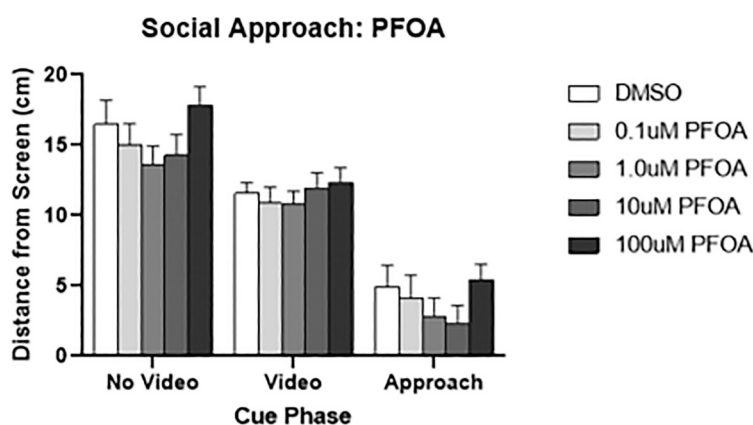
For PFOA, tap test analyses detected a marginal effect of treatment for post-tap activity,  $F(4, 213) = 2.41, p = 0.050$ , a main effect of age,  $F(1, 213) = 43.82, p < 0.05$ , and interactions of tap x treatment,  $F(36, 1917) = 1.45, p < 0.05$  and tap x age,  $F(9, 1917) = 6.98, p < 0.05$ . With respect to the main effect of age, adults showed higher post-tap activity than adolescents (Suppl. Fig. s2a). With respect to the main effect of treatment, no PFOA-treated groups significantly differed from controls. For startle magnitude, a tap x age interaction was detected,  $F(9, 1917) = 5.23, p < 0.05$ , as were main effects of treatment,  $F(4, 213) = 2.68, p <$

A.

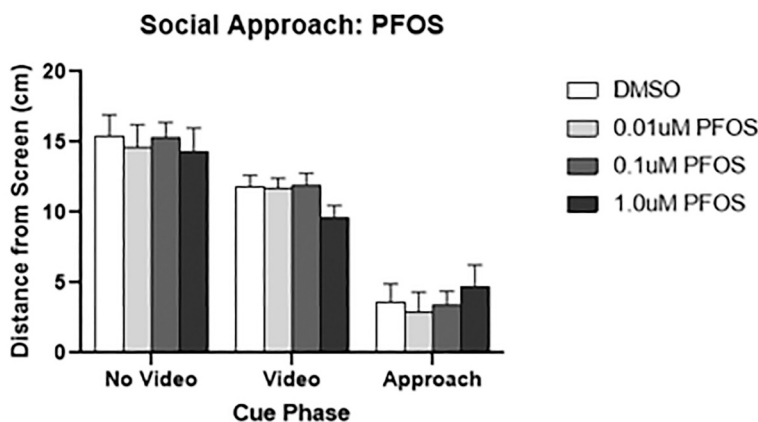


**Fig. 5.** Shoaling Performance Summary. Locomotion and social approach is expressed as distance moved (cm/min) (A) and distance from the screen-adjacent side of the tank across the 2 min before (left, no video) and the 5 min during (middle, video) the social video was presented, and as the difference between these scores (no video - video), interpreted as social approach (right) (B/C). Data are expressed as mean  $\pm$  SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). (A) Neither PFOA nor PFOS altered locomotion in the shoaling test at 3 or 8 months of age. (B) PFOA exposure did not significantly alter distance from the screen in the shoaling test at 8 months of age. (C) PFOS exposure did not significantly alter distance from the screen in the shoaling test at 8 months of age.

B.



C.



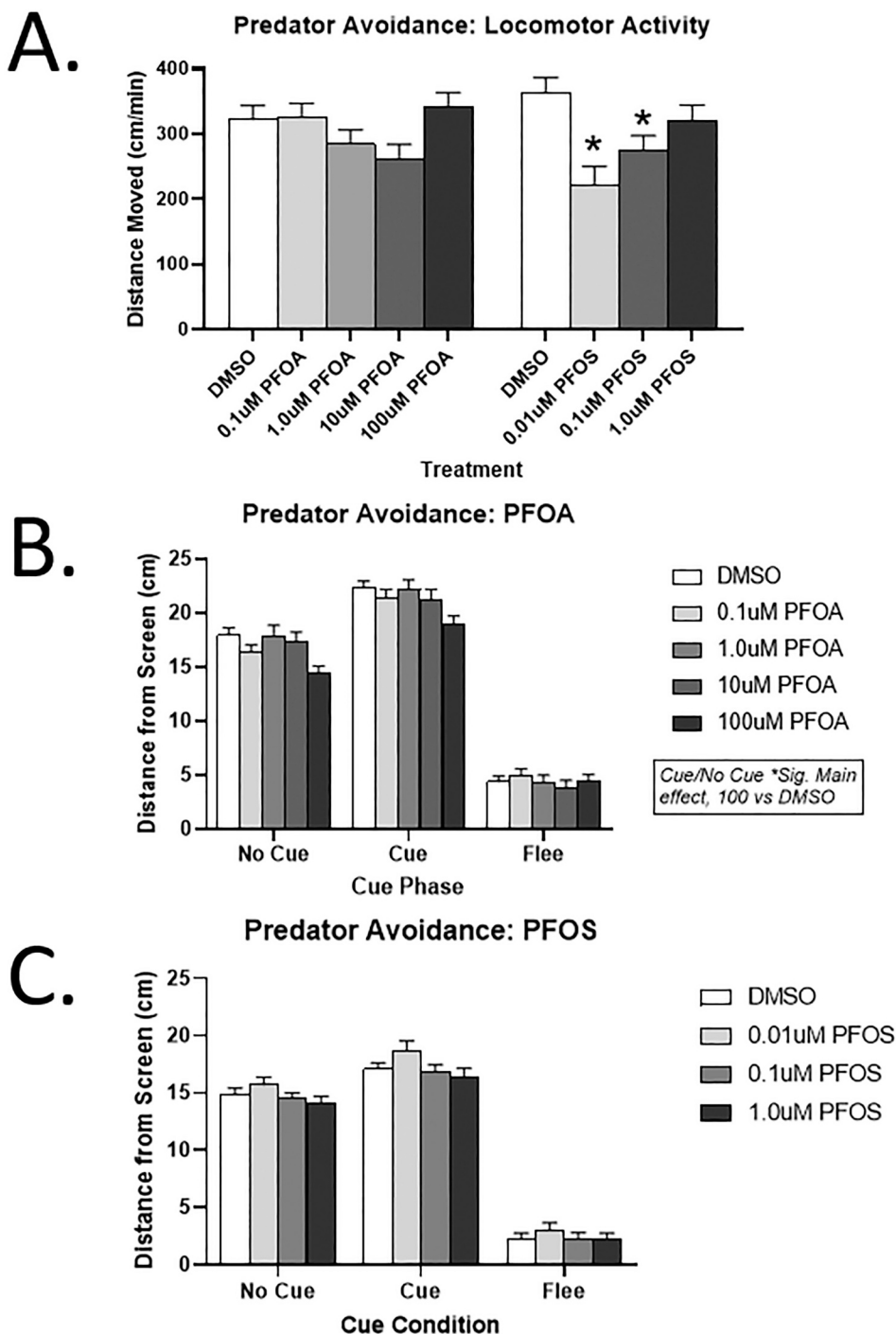
0.05, and age,  $F(1, 213) = 39.82, p < 0.05$ . With respect to age, adults showed higher startle magnitudes than adolescents ( $p < 0.05$ ) (Suppl. Fig. s2a). With respect to the main effect of treatment, no PFOA-treated groups significantly differed from controls.

For PFOS, tap test analyses detected a tap  $\times$  age  $\times$  treatment interaction for pre-tap activity,  $F(27, 1971) = 1.72, p < 0.05$ , as well as a main effect of age,  $F(1, 219) = 41.73, p < 0.05$ . With respect to the main effect of age, adults showed lower post-tap activity than adolescents (Suppl. Fig. s2b). For post-tap activity, a main effect of age was also detected,  $F(1, 213) = 39.82, p < 0.05$ , whereby adults showed lower post-tap activity than adolescents ( $p < 0.05$ ) (Suppl. Fig. s2b). For startle magnitude, a tap  $\times$  age interaction was detected,  $F(9, 1971) = 5.38, p <$

0.05, and no age effects were detected (Suppl. Fig. s2b).

#### 4. Discussion

The current study was conducted to assess the neurobehavioral effects of embryonic PFAS exposures, with particular attention to the developmental persistence and the relative similarity of phenotypes produced by two representative compounds. Comparisons focused on dose-effect functions of the carboxylic acid PFAS compound PFOA, and the sulfonic acid PFAS compound PFOS in larval, adolescent and adult animals. With respect to dosimetry, preliminary pilots observed that PFOA was tolerated by zebrafish embryos at a higher concentration than



**Fig. 6.** Predator Avoidance Performance Summary. Locomotion and cue avoidance is expressed as distance moved (cm/min) (A) and distance from the screen-adjacent side of the tank during the 1 min before (left, no cue) and the 1 min during (middle, cue) the predator cue was presented, and as the difference between these scores (cue – no cue), interpreted as fleeing (right) (B/C). Data are expressed as mean ± SEM. Asterisks (\*) indicate significance versus controls ( $p < 0.05$ ). (A) Neither PFOA nor PFOS altered locomotion in the predator avoidance test at 3 or 8 months of age. (B) PFOA exposure led fish to remain closer to the screen (100uM), however, this did not differ between cue and non-cue phases of the session, or alter the magnitude of the fleeing response. (C) PFOS exposure did not significantly alter distance from the screen in the predator avoidance test at 8 months of age.

those of PFOS, which is consistent with prior analyses (Ulhaq et al., 2013; Ye et al., 2009), and exposures in the main experiment followed these concentrations.

Following embryonic exposures, both compounds caused neurobehavioral effects, but those effects were compound-specific. PFOA (100uM) elevated locomotor activity under stimulating dark conditions, while PFOS dose-dependently reversed classic dark-stimulation and light-inhibition responses. The distinctiveness of these two compounds was also observed at later points in development. PFOA (100uM) caused a dose-dependent enhancement of the diving response in adolescence, while PFOS impaired the diving response at this age. Further, PFOS caused age-dependent alterations in locomotor activity in adolescence and adulthood, and a reduction of the startle response in adolescence

only. Overall these data indicate that PFOA and PFOS exposures cause distinct phenotypes and that these effects may be observable well after (3+ months) the end of the exposure period. However, they also indicate that these effects can weaken or shift across maturation, with many aspects of the phenotype attenuating by adulthood (8 months). Similar outcomes of embryonic exposure of zebrafish to PFOS exposure causing readily discernable effects on locomotor behavior in larvae with much diminished effects later in life has also been seen recently by Christou et al., 2021.

The behavioral battery used in this study examined several key aspects of neurobehavioral function, including locomotor activity, sensory responsiveness, and affective function. Short-term toxicity testing evaluated locomotor and affective functions in larval fish 24 h after the end

of the exposure period. This testing showed that neither PFOA nor PFOS induced overall motor dysfunction at tolerable levels of exposure. Rather, they altered lighting-based responsiveness in compound-specific ways. These data supplement prior work examining larval motility, spontaneous movements or photomotor effects during early stages of development following PFOA/PFOS exposures (Huang et al., 2010; Jantzen et al., 2016b; Rericha et al., 2021; Spulber et al., 2014; Ulhaq et al., 2013; Yu et al., 2021).

The highest concentration of PFOA enhanced activity in the dark due to an enhanced pattern of dark-induced stimulation. This finding is consistent with a previous report (Menger et al., 2020) which observed that PFOA produced hyperactivity in the dark (1.2 or 150 $\mu$ M) but not the light. However, effects on the larval motility test remain somewhat mixed, with Yu et al. (2021) reporting hypoactivity rather than hyperactivity in the dark at 7 days of age (24 nM–2.4 $\mu$ M) and Rericha et al. (2021) reporting no effects of PFOA at 1 or 5 days of age (0.6 $\mu$ M). The latter study is in agreement with the present study though, as similar concentrations failed to produce locomotor effects in the present study as well. PFOS, by contrast, dose-dependently reversed of the light-dark response. This too is consistent with the work of Menger et al. (2020), who reported that 4.3 $\mu$ M PFOS reduced distance moved in the dark and enhanced burst swimming behavior in the light, and with the work of Ulhaq et al. (2013) which showed that PFOS dose-dependently disrupts the light-dark response. Spulber et al. (2014) did not find a similar disruption in a conceptually similar test at comparable concentrations (0.2 $\mu$ M), but did observe an overall reduction in locomotion at concentrations higher than those used in this experiment (2 $\mu$ M). At yet higher concentrations, Huang et al. (2010) and Christou et al. (2020) observed general increases in swimming speed (3.8–8 $\mu$ M), with Christou et al. (2020) additionally reporting changes in spatial patterns of exploration at concentrations as low as 0.5 $\mu$ M. Khezri et al. (2017) noted that at much lower concentrations (0.99–54.8 nM), such a speed-enhancing effect was relatively unique to PFOS, without being observed for five other PFAS, including PFOA. Prior studies also indicate that the larval effects of PFOS are timing dependent (2010). The present data complements existing knowledge on the potential neurotoxicity of PFAS compounds in the embryonic zebrafish, and the distinctiveness of differing PFAS representatives, but also highlights that the literature on those effects remains mixed as to the nature of related phenotypes. Some of this disagreement may stem from differences in the timing, duration and levels of exposure, which varied considerably across studies, as well as the methodologies employed to measure those effects. Prior analyses indicate that seemingly minor differences in husbandry, handling and testing design can alter the nature of neurobehavioral toxicity, as is the case with bisphenol A, which can produce either hyper- or hypo-activity depending on the methodology selected (Fraser et al., 2017). Similar analyses for PFAS are needed so that discrepancies between studies can be reasonably clarified. Additionally, future studies are needed to determine the detailed toxicokinetics of these compounds will shed additional light on their differential functional effects.

As these animals continued to develop, additional patterns of neurobehavioral impairment were noted. In a prior study, Jantzen et al. (2016a) found an array of neurobehavioral effects in adults embryonically treated with perfluorononanoic acid (PFNA), but found that PFOS or PFOA failed to cause deficits that persisted into adulthood. The present evaluation of PFOS and PFOA provides some support for this, as adult fish in this experiment generally showed minimal, if any, impairment relative to controls. However, this experiment suggests that the failure of effects to persist into adulthood does not indicate that they fail to persist across earlier portions of development. Adolescent testing detected neurobehavioral effects on each of the tests used, with PFOA and PFOS causing distinct patterns of neurobehavioral toxicity. PFOA dose dependently enhanced the diving response in adolescent fish, while PFOS nonmonotonically reduced the diving response. PFOS was also associated with flatter time-effect functions across the locomotor portion of the test, leading the moderate (0.1 $\mu$ M) and the higher

concentrations (1.0 $\mu$ M) to differ from controls at the beginning and end of the session, respectively. PFOS was further associated with reduced startle sensitivity in adolescence, at the lowest concentration used (0.01 $\mu$ M). By adulthood, only one of these effects remained, an alteration in locomotor activity among PFOS-exposed fish, and this effect changed in both dosimetry and character across that developmental period. Rather than showing impaired locomotor slopes, placed in a similar activity ranges, at moderate to higher concentrations, adults showed generalized hypoactivity which only reached statistical significance at the lowest concentration (0.01 $\mu$ M). The stark difference in outcome from adolescence to adulthood suggests that while PFAS effects can be persistent to some degree, it is likely that processes of neuroplasticity and neurodevelopment substantially alter their nature and severity. In the cases of PFOS and PFOA, these processes appear capable of attenuating many of these effects outright.

In the clinical literature, there is some documentation of attenuation of certain symptoms in the transition from adolescence to adulthood, as is often the case with hyperactivity symptoms in young patients with ADHD (Sudre et al., 2018). It is known that neural circuits undergo dramatic restructuring during adolescence, highlighted by broad patterns of synaptic restructuring and pruning which play out developmentally and in response to experience (Dow-Edwards et al., 2019). It may be hypothesized that similar effects may mediate the attenuation and/or transformation of behavioral phenotypes across maturation, although it should be noted that similar patterns have not been well documented in zebrafish to date. Future work will be necessary to more convincingly identify which processes mediate this remission, and how they may be leveraged to protect against longer-term consequences of early life exposures.

One of the primary comparisons the present study aimed to address is the similarity, or in this case, dissimilarity, of PFOA and PFOS for disrupting neurobehavioral function long after developmental exposure. These two compounds represent subclasses of PFAS chemicals which are used for similar purposes, and are often found together in environmental mixtures. With respect to their neurobehavioral toxicity, however, they share few similarities. Indeed, there are multiple examples in the present data where outcomes are targeted by one compound, but not the other, or where these compounds exert opposing effects on the same outcomes. As noted previously, this is in agreement with larvae-focused studies in zebrafish (Menger et al., 2020; Rericha et al., 2021). Prior studies with rodents have found similar differentiation on some outcomes (Onishchenko et al., 2011), but not others (Johansson et al., 2008) suggesting this differentiation may be translationally relevant to some degree. Preclinical models demonstrate cellular and neurophysiological alterations which may underlie neurotoxicity risks for PFAS compounds (Brown-Leung and Cannon, 2022; Wang et al., 2019), including enhanced oxidative stress (Chen et al., 2014), disrupted calcium homeostasis (Brown-Leung and Cannon, 2022), induction of apoptosis (Dong et al., 2015; Long et al., 2013), altered neurotransmission (Foguth et al., 2019; Foguth et al., 2020; Long et al., 2013; Slotkin et al., 2008) and alterations of pathways regulating synaptogenesis and other neurodevelopmental processes (Johansson et al., 2009). Some of these effects have been shown to differ substantially between PFAS chemicals (Slotkin et al., 2008), although more research is needed to clarify which outcomes are likely to differ across varying exposures, and which may be more generally represented by PFAS as a whole. In any case, the present findings suggest that some caution should be taken when performing risk assessments of PFAS as a whole, as associations across compounds with dissimilar or conflicting effects could under- or misrepresent those risks. The identification of persisting neurobehavioral impairments in the current study provides a functional anchor for future studies to track back neurochemical, cellular and molecular mechanisms for these impairments.

Taken more broadly, the present study complements previous zebrafish work indicating that while PFAS may be viewed as potentially neurotoxic, this group of compounds is by no means uniform. A recent

study by [Truong et al. \(2022\)](#) screened 139 PFAS compounds for developmental toxicity using a comparable dosing schedule as that in the present study (6-120hpf). Of these 139 compounds, only 35% of them were found to produce morphological or behavioral effects, assayed through embryonic or larval versions of the photomotor test at sublethal levels of exposure. Of those which impacted a given test, the nature of the effect also varied widely across compounds. While this certainly indicates uncertainty about the toxicity of PFAS as a whole, [Gaballah et al. \(2020\)](#) suggests that clusters of PFAS share phenotypes which could allow adverse outcomes to be correlated with chemical properties, perhaps allowing advances in toxicity detection and characterization to clarify these issues.

It should be noted that the concentrations used in this study were scaled to the species in question rather than a specific environmental condition, as basing them on a tolerability threshold will allow these data to be more readily compared with other model species with differing sensitivity to PFAS. As a result, these concentrations exceed regulatory limits for surface waters ([Garnick et al., 2021](#)) and levels observed in water systems with documented contamination. For example, water samples in Wilmington, NC, USA ([Pétre et al., 2022](#)) and multiple water systems in Nevada ([Bai and Son, 2021](#)) observed maximum PFAS loads of 350 and 2234 ng/l respectively. For comparison, the lowest concentrations tested in this study were equivalent to 41.4µg/l of PFOA and 5µg/L PFOS. So, these data should not be taken as expected consequences of current contamination on freshwater fish. Rather, these data should be viewed as a representation of the inconsistencies between different PFAS representatives, and evidence for PFAS' potential to contribute to neurological dysfunction in vulnerable populations like developing offspring alongside other risk factors.

In summary, the present study suggests that PFAS are developmentally neurotoxic and induce measurable neurobehavioral dysfunction, but that those consequences are distinct across two compounds from different PFAS subclasses, and across periods of development. PFOA and PFOS produced distinct profiles of impairment in each instance where effects were detected, and those profiles generally became attenuated by the maturity. Of the two compounds, PFOS impacted a greater number of features. Based on these findings, it is clear that more data is needed on the developmental regulation of neurotoxicity, particularly with respect to PFAS. Further, the stark differences between representative compounds emphasize the need for comparative analyses of PFAS between and within subclasses, to determine whether representative compounds adequately predict differences in their toxicity and resulting symptomatology. However, this work also shows that zebrafish are an effective and valuable model organism for showing the neurobehavioral significance of PFAS exposures, and their short and long-term effects across the lifespan.

#### 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.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ntt.2023.107165>.

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