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Effects of pre-fertilization exposure to perfluorooctanoic acid on mortality and behaviour of the European whitefish (*Coregonus lavaretus) larvae.*

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Abstract

Due to extensive production and use, worldwide distribution of perfluorooctanoic acid (PFOA) or other related compounds has been investigated in ecotoxicology since the early 80's. Since they are non-reactive and highly persistent, these compounds are easily distributed in the environment and have been found in environmental matrices and wildlife at a wide range of ecosystems, thus causing growing environmental concern. In addition to this, studies have demonstrated bioaccumulation potential of PFASs in humans and other animals, and the toxicological effects in exposed biota are currently well established. Since chemical contaminants are continuously discharged into water bodies, aquatic organisms such as the European whitefish (*Coregonus lavaretus*) are more susceptible to contamination and have been used as study organisms in ecotoxicological research. In addition, external fertilization of whitefish makes their gametes highly vulnerable to environmental stressors during fertilization, and during embryonic and larvae stages, and any disruption in early developmental processes can have long term impacts at the individual and population levels. Along with morphological development, animal behaviour is of great ecological and evolutionary importance and provides additional information on animal welfare. Thus, integrating behavioural endpoints in ecotoxicological research helps to better understand how animal movement and behaviour can be directly related to neuronal effects of environmental stressors including, pharmaceuticals or industrial chemicals. As such, in the present study my main purpose was to assess if PFOA exposure of whitefish gametes during fertilization, at concentrations of 0.01 mg/L and 10 mg/L, would impact early developmental stages, such as embryo mortality and hatching time, and subsequently morphometric and behavioural parameters at the larvae stage, including body size, swimming and behavioural performance. My conclusions are that different concentrations of PFOA have several detrimental effects on numerous biological responses of whitefish throughout different life stages. The lowest concentration of PFOA induced premature hatching and significantly reduced body weight at the larvae stage. Whereas embryos exposed to the highest concentration of PFOA showed a significantly increased body length. My findings indicate that chemical contamination can affect early developmental processes of fish, however, the results were only marginally significant. In addition, these effects are often very complex, and the lack of more significant results could be due to limitations that should be considered in future studies. Nonetheless, changes in early developmental processes can shape the quality, quantity, and fitness of offspring. Moreover, such disturbances in the normal development of a species are expected to have long-term detrimental effects in other biological events that are important for offspring survival including predation avoidance, feeding behaviours, reproduction, or migration.

Keywords: whitefish, embryo, larvae, growth, perfluorooctanoic acid, animal behaviour, tracking software, ecology, ecotoxicology.

Abbreviations

ANOVA	Analysis of variance.
BCF	Bioconcentration factor.
CNS	Central nervous system.
EFSA	European Food Safety Authority.
EQS_{bioto}	Environmental quality standard for biota
FTOHs	Fluorotelomer alcohols.
LMM	Linear Mixed Models.
LOQ	Limit of quantification.
PFASs	Per- and polyfluoroalkyl substances.
PFHXs	Perfluorohexane sulfonic acid.
PFNA	Perfluorononanoic acid.
PFOA	Perfluorooctanoic acid.
PFOS	Perfluorooctane sulfonic acid.
PFOSF	Perfluorooctane sulfonamide.
POP	Persistent organic pollutant.
SCHEER	Scientific Committee on Health, Environmental and Emerging Risks.
TWI	Tolerable weekly intake.

Contents

1 Introduction

Perfluorooctanoic acid or PFOA is a persistent organic pollutant (POP) and belongs to a larger family of per- and polyfluoroalkyl substances (PFASs) that are extensively manufactured for industrial purposes since the early 40's (Glüge et al., 2020). PFOA is extensively produced and commonly used to make non-stick coatings in cookware (Teflon), waterproof textiles, or firefighting foams (Teaf et al., 2019). The molecular structure of PFOA includes a hydrophilic carboxylate functional group and a hydrophobic alkyl chain with seven carbon atoms where all C-H bonds are replaced with carbon-fluor bonds (C-F), and such bonds make this molecule highly resistant to heat or other chemical reactions (Giesy et al., 2006) (**Figure 1**). The properties that give PFOA high chemical and thermal stability, water/oil resistance, and surfactant properties, make them highly persistent and bio accumulative, since they are able to resist many processes of chemical degradation (Kissa, 2001). Therefore, their potential toxic effects on human and environmental health are causing growing concern worldwide.

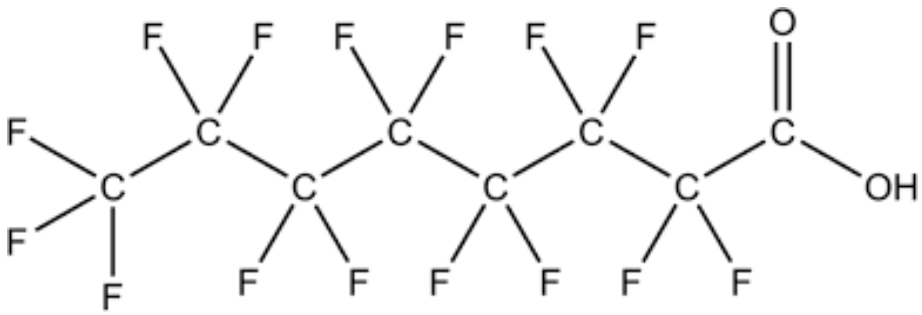


Figure 1. Chemical structure of PFOA (Macheka-Tendenguwo et al., 2018).

Since environmental contaminants such as PFOA are continuously discharged into water bodies, from direct and indirect sources, aquatic organisms such as the European whitefish (*Coregonus lavaretus*) are more susceptible to contamination and have been used as study organisms in multiple studies (Keinänen et al., 2003; Arola et al., 2017; Karjalainen et al., 2021; Yaripour et al., 2021). Because of its high persistence and toxicity to biota, PFOA is found in many fish species in variable concentrations across Europe (Schuetze et al., 2010; Noorlander et al.,

2011; Squadrone et al., 2015; Giari et al., 2023). In the Baltic Sea and in Finnish freshwater bodies thirteen fish species, including whitefish, had previously detected PFASs concentrations ranging from 1.43 ng g^{-1} to 33.1 ng g^{-1} (Kumar et al., 2022). Among food products that are potential pathways for human exposure, fishes are highly concerning since they are extremely vulnerable to chemical contamination in water bodies (Mazzoni et al., 2019). In Nordic countries, where fishes are central part of the population's diet, several fish species have been studied for the detection of PFASs, and by comparison with existing safety thresholds, were found to be one of the major sources of exposure in human populations (Berger et al., 2009; Kowalczyk et al., 2020; Kumar et al., 2022). Current environmental quality standards for biota (EQS_{biota}) for the sum of PFASs were proposed in 2022, by the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER), which endorsed a $QS_{biota,\ sec\ pois}$ (secondary poisoning) of 22.3 µg.kg_{ww}^{-1} for fish and 6.2 µg.kg_{ww}^{-1} for bivalves, as well as a $QS_{biota,\ hh}$ (human health) of 0.077 µg.kg^{-1} and a $QS_{dw,\ hh}$ (drinking water, human health) of 4.4 ng.L^{-1} (SCHEER, 2022).

European whitefish is an environmentally relevant species and was selected as a study organism based on its widespread distribution, commercial and economic value, and biological features. Because whitefish is abundant in Finnish lakes, and it's regularly caught by local and professional fishermen, it has been cause of concern and, in 2019, the Finnish Environment institute classified whitefish as an endangered species in Finland (Hyvärinen et al., 2019). Whitefish is frequently captured in both professional and recreational fishing and is farmed in commercial aquaculture (Nielsen et al., 2020; Turenhout et al., 2023). The spawning usually occurs in shallow waters in autumn, by external fertilization, and the incubation period lasts for around 6 months until late April or early May, when hatching starts (Ikonen, 1980). External fertilization makes gametes highly vulnerable to xenobiotic contamination during fertilization and consequently during early development, especially at the embryonic and larvae stage (Von Westernhagen, 1988; Hutchinson et al., 1998). Therefore, since they produce a large number of gametes, in the present study whitefish was also a suitable model organism for the applied full-factorial breeding design, which has a high statistical power, to study offspring fitness throughout early life stages. Moreover, fish and mammals share many physiological characteristics which makes toxicological findings in fish studies often conveyable to other vertebrates, including humans (Rinkwitz et al., 2011). Despite a high number of recent studies

that provide information on accumulation in fish, specific patterns of contamination in particular species remains unclear. Assessing the impacts of such contaminants in aquatic environments relies on the development of integrated ecotoxicological methodologies. Thus, along with morphological development, animal behaviour is of great ecological and evolutionary importance and provides additional information on animal welfare. As a response to external stimulus, including environmental contaminants, fish can express anxiety or stress-related behaviour such as freezing behaviour, aggressive responses, disruption of feeding or mating behaviours, or other unusual behaviours that might be indicators of compromised welfare (Galhardo & Oliveira, 2009). In farming systems such as commercial aquaculture sea cages, the use of video tracking software can evaluate fish welfare by detecting unusual behaviours on a continuous real-time basis, and potentially sending warnings to farm operators about environmental stressors (Pinkiewicz et al., 2011).

Since PFOA and other related compounds have revealed to cause detrimental impacts in biological factors of aquatic organisms, more studies are of great importance for the ecological risk assessment of these compounds. In this study, my main objective was to assess the effects of environmentally relevant concentrations of PFOA toxicity in embryonic and larval stages of aquatic organisms using European whitefish (Coregonus Lavaretus) as a model organism. Lethal and sublethal toxicity at early life stages is of great importance since at this stage organisms are especially sensitive to contaminants and risk assessments often include endpoints such as hatching rate and success. In addition to these I also evaluated the swimming performance and behaviour patterns at the larval stage.

2 Per- and polyfluoroalkyl substances (PFASs)

Perfluoroalkyl substances or PFASs are organic acids formed by a long-fluorinated carbon chain with a length that can range from four to thirteen carbon atoms, with a carboxylate or sulfonate terminal group, and are emerging environmental contaminants of anthropogenic origin widely spread in humans and wildlife (Conder et al., 2008). Due to these and some other physical-chemical properties, PFASs are widely used for applications such as surface coating agents, grease resistant coatings in food packaging, cleaning agents or stain- resistant coatings

in textiles (Glüge et al., 2020). In addition, PFASs' properties make them favourable and accessible choices for industries to use in products such as surfactants, repellents, emulsifiers, and dispersants for many industrial purposes (Kissa, 2001). Since they are non-reactive and highly persistent with a high capacity for long-range transport, PFASs are easily distributed through air or water and have been found to bioaccumulate in wildlife at a wide range of ecosystems including unpopulated places such as the Arctic region (Butt et al., 2010; Pickard et al., 2018). PFOA is also indirectly discharged to the environment since many precursors such as fluorotelomer alcohols (FTOHs) or perfluorooctane sulfonamide (PFOSF) based chemicals have been confirmed to degrade to PFOA due to abiotic processes (Giesy et al., 2006). In fact, it is possible that atmospheric transport of these precursors, is one of the indirect sources of PFASs into the Arctic or other pristine ecosystems, since they are highly volatile and can undergo oxidation processes in environments with low NOx: HO_2 ratios (Giesy et al., 2006; Pickard et al., 2018). For this reason, snowmelt can then be an additional source of PFASs into freshwater ecosystems. PFOS and PFOA are the most prevalent in the environment and thus many studies have been conducted regarding these two congeners both in humans and other animals.

Human exposure to PFOA arises mainly through water consumption, food (primarily fish meat, seafood, eggs, fruits, vegetables, and related products), but also possibly through air inhalation or dust ingestion (EFSA, 2020). In human serum, perfluorooctane sulfonic acid (PFOS) is the most prominent PFAS found in adults, followed by PFOA, and even though there is a decreasing trend since 2000, alternative PFASs have been increasingly detected instead (EFSA, 2020). Many studies have proposed that, unlike other POPs which are mainly lipophilic, PFOA and other PFASs are proteinophilic with a high binding affinity to proteins, and thus organ tissues rich in proteins, such as liver or blood, are the main repositories for PFASs in most animals (Han et al., 2003; Conder et al., 2008). In 2018, the European Food Safety Authority (EFSA) set a tolerable weekly intake (TWI) of 6 ng kg^{-1} body weight $week^{-1}$ for PFOA (EFSA, 2018). Based on toxicokinetics of PFASs and corresponding effects, and observed levels in humans or other animals, in 2020, EFSA established a group TWI of 4.4 ng kg^{-1} body weight $week^{-1}$ for the sum of PFOA, perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHXs), and PFOS (EFSA, 2020). The choice of these four PFASs was based on the fact that these are the ones that are often observed in humans at higher levels, and because they show similar accumulation

patterns and share toxicokinetic properties. Still in 2020, the EU Drinking Water Directive 2020/2184 of the European Parliament established limit concentrations of total PFASs of 0.5 µg/L and to 0.1 µg/L for the sum of PFASs of highest concern, to guarantee human safety regarding exposure from drinking water consumption (EU, 2020). Mean exposure to PFOA ranges from 0.1 to 0.6 ng/kg of body weight per day for the lower bound estimation, and from 3.0 to 29 ng/kg of body weight per day for the upper bound estimation (EFSA, 2020). Human exposure to high levels of PFOA is linked to reduced immune responses, complications in foetal development, damage to nervous tissue, endocrine disruption, and cancer (Heuvel et al., 1991; Cui et al., 2009; K. Li et al., 2017; D. Liu et al., 2023). In several animal studies, PFOA induces liver peroxisome proliferation and mitochondrial proliferation, leading to hepatic disorders (Perkins et al., 2004; Lau et al., 2006; Loveless et al., 2006), and in humans PFOA has been linked to increased levels of alanine aminotransferase which is one possible indicator of liver damage (Gleason et al., 2015).

Since PFASs are resistant to environmental transformation processes their half-lives can range from years to decades leading to different adverse effects, and in humans PFOA has a long half-life of around 2 to 4 years (Giesy et al., 2006; Olsen et al., 2007; Y. Li et al., 2018). Half-life of PFASs change according to the functional end-group, chain length, molecular structure (linear vs branched), the exposed species or inter-individual variations (e.g. age or sex) (Conder et al., 2008; Y. Li et al., 2022; Dawson et al., 2023). Due to many driving factors, the fate of these substances is highly complex and further insight is needed to better understand toxicokinetics, bioaccumulation, and biomagnification potential of PFASs.

3 Behavioural assays in ecotoxicology

In ecological risk assessment it is fundamental to understand how movement and behaviour can be directly related to neuronal effects of environmental stressors including, pharmaceuticals or industrial chemicals, on animals (Galhardo & Oliveira, 2009). In multiple fields of research such as behavioural ecology, ecotoxicology, ethology, evolutionary ecology, or others, this understanding can be achieved with controlled laboratory studies coupled with video tracking analysis that relies in automatic methods to extract behavioural data from video or pictures with high accuracy (Mönck et al., 2018).

Since direct observation can be time-consuming, biased and difficult to replicate, automated tracking allows researchers to extract larger and more detailed datasets of animal movement. Experimental trials in laboratory settings usually take place in pre-defined arenas and model organisms such as zebrafish or whitefish, are often used (AlZu'bi et al. 2015). By video tracking fish movements, we can collect both short-time and long-time footage of spatial position and specific behavioural patterns in a feasible, faster, and non-invasive way (Rodriguez et al., 2018). However, reconstructing accurate behavioural information over time to address specific research questions can be a difficult and time-consuming task, especially when we collect multiple high-quality video datasets that require automated software applications to reduce manual analysis and human errors that are often associated with it (Sridhar et al., 2019). As animal positions change through time and space parameters like image resolution, noise, illumination, blurriness, light reflections on water, camera motions, and other issues can highly decrease video quality and thus hamper effective tracking results (Chiara & Kim, 2023). Researchers must therefore familiarize themselves with these variables and learn how to adjust them to achieve better results.

Many innovative tracking technologies were recently designed and are rapidly improving in precision and robustness, to best suit particular experimental conditions, study organisms, or camera setups (Ray & Stopfer, 2022). Each software follows a specific tracking pipeline, and every step of this pipeline has their own functionalities. Differences in algorithm, detection and segmentation methods, user-friendliness, cost, robustness, and many other features make these programs tailored for the specific processing tasks that experimental conditions demand (Panadeiro et al., 2021). Commercial tools often provide more features and statistical outputs but can be highly costly, thus depending on the research focus, free software tools are likely capable of providing the information of interest. Open-source tracking software are commonly available for researchers to use and, with their own differences, have been successful at specific tasks. Such tasks are for example handling multiple organisms and/or multiple arenas simultaneously (Rodriguez et al., 2018), and exhibiting functionalities such as data analysis tools with multiple behavioural metrics (Chiara & Kim, 2023), automated programs that control sensors and actuators in behavioural mazes (Aguiar et al., 2007), or even allowing the researcher to test and develop a custom processing pipeline in order to apply the best tracking

solution for their specific problems (Mönck et al., 2018) – some of the aforementioned tools have more than one of these functionalities. Data collection in more complex and realistic natural environments, which are often heterogenous, require more complex and robust automated extraction methods such as classical pose estimation (Nath et al., 2019), or a combination of classical and neural network-based image processing methods (Ray & Stopfer, 2022). Regardless, most software allow researchers to analyse large datasets accurately thus reducing cost and time of completing such tasks.

In short, detecting fish movement to extract different analytical parameters that can be linked to anthropogenic environmental stressors it is of great importance in fields like ecotoxicology that studies the hazardous effects of chemicals in the environment.

4 Objectives

In this study, my main objective was to assess the effects of environmentally relevant concentrations of PFOA toxicity in embryonic and larval stages of aquatic organisms using European whitefish (Coregonus Lavaretus) as a model organism. In addition to these I also evaluated the swimming performance and behaviour patterns at the larval stage. I performed a full-factorial breeding design in which male and female gametes where exposed to two different PFOA concentrations. I tested whether exposure to PFOA during fertilization could affect offspring survival and hatching time, body growth, and behavioural and swimming performance of whitefish. The applied full-factorial experimental design has previously shown to provide high statistical power identifying biologically significant variations and capturing interaction effects between multiple factors (Kekäläinen et al., 2018). Control and exposed embryos were continuously evaluated for multiple endpoints during the embryonic and larvae stage. Endpoints included mortality rate, hatching time, total body length (cm), total body weight (mg), total distance moved (cm), average velocity (cm/s) and acceleration (cm/s^2), time spent in four different zones (s), and average mobility (%). Thereby my scientific hypotheses were that exposure to different PFOA concentrations would affect (i) embryo survival; (ii) early-stage development or hatching time; (iii) exploratory behaviour at the larvae stage; (iv) swimming

performance. My hypotheses are that these parameters would be negatively affected by PFOA exposure. This study also intended to demonstrate how an automated tracking system can be a valuable tool to analyse behavioural patterns of fish in controlled laboratory settings in ecotoxicology or ecological risk assessment research. Finally, I considered possible limitations and future developments of this study.

5 Material and Methods

5.1 Gamete collection

Parental fish of the species (*Coregonus Lavaretus*) were acquired from a breeding stock of the River Koitajoki population maintained at the Saimaa Fisheries Research and Aquaculture Station of the Natural Resource Institute Finland (Luke), located in Enonkoski, Finland. On November 22nd of 2021 eight males and five females of the breeding stock were haphazardly sampled and reared under strict safety and hygiene protocols. Gametes of the selected fish were stripped and stored on ice in air-filled plastic zipper bags (milt) or plastic containers (eggs). The collected gametes were later transported to one of the laboratories of the University of Eastern Finland in Joensuu, where the artificial fertilizations took place later in the same day. Handling of experimental fish, sample collection and animal experimentation were conducted in accordance with the license by the Finnish Animal Experiment Board (ESAVI -4934-2021).

5.2 Pre-fertilization and PFOA exposure

To evaluate the toxicity of PFOA whitefish gametes were exposed to PFOA concentrations of 0, 0.01, and 10 mg/L during fertilization. Artificial fertilizations were performed with the gametes of 5 females and 8 males in all possible family combinations to create a total of 40 full-sib families and all the fertilizations were replicated twice (**Figure 2a**). Therefore, the experimental design included a total of 240 fertilization batches (5 females × 8 males × 3 treatments × 2 replicates) and after fertilization the mean number of eggs per family was 97.7 (SD = 17.4). Fertilizations

were carried out at a temperature of 4° C in which initially eggs were placed in 90 mm plastic Petri dishes and then 3 µL of milt was directly added on the eggs with a micropipette. Immediately after, 40 mL of one of the three exposure mediums was added to the corresponding Petri dishes of each treatment, and lastly the Petri dishes were carefully shaken for 3 seconds to allow fertilization of eggs. The exposure mediums for each treatment were as follows: non-chlorinated tap water (Control or Treatment 0), non-chlorinated tap water with a PFOA concentration of 0.01 mg/L (Treatment 1), and non-chlorinated tap water with a PFOA concentration of 10 mg/L (Treatment 2). The exposure lasted for one hour and after that the medium was replaced with 40 mL of pure tap water at 4ºC. The fertilized eggs of each family were randomly distributed into separated incubation boxes placed in tanks with a volume of 600 L, two tanks for each treatment, filled with non-chlorinated tap water at 4° C (**Figure 2a**). The incubation period lasted until all the eggs had been hatched and all the performance tests were completed in May 2022. All the experiments were conducted at the laboratories of the Department of Environmental and Biological Sciences at the University of Eastern Finland in Joensuu.

The figure below visually demonstrates the experimental design in which whitefish gametes where exposed to three different concentrations of PFOA (**a**), and later tested for behavioural (**b**) and swimming performance (**c**).

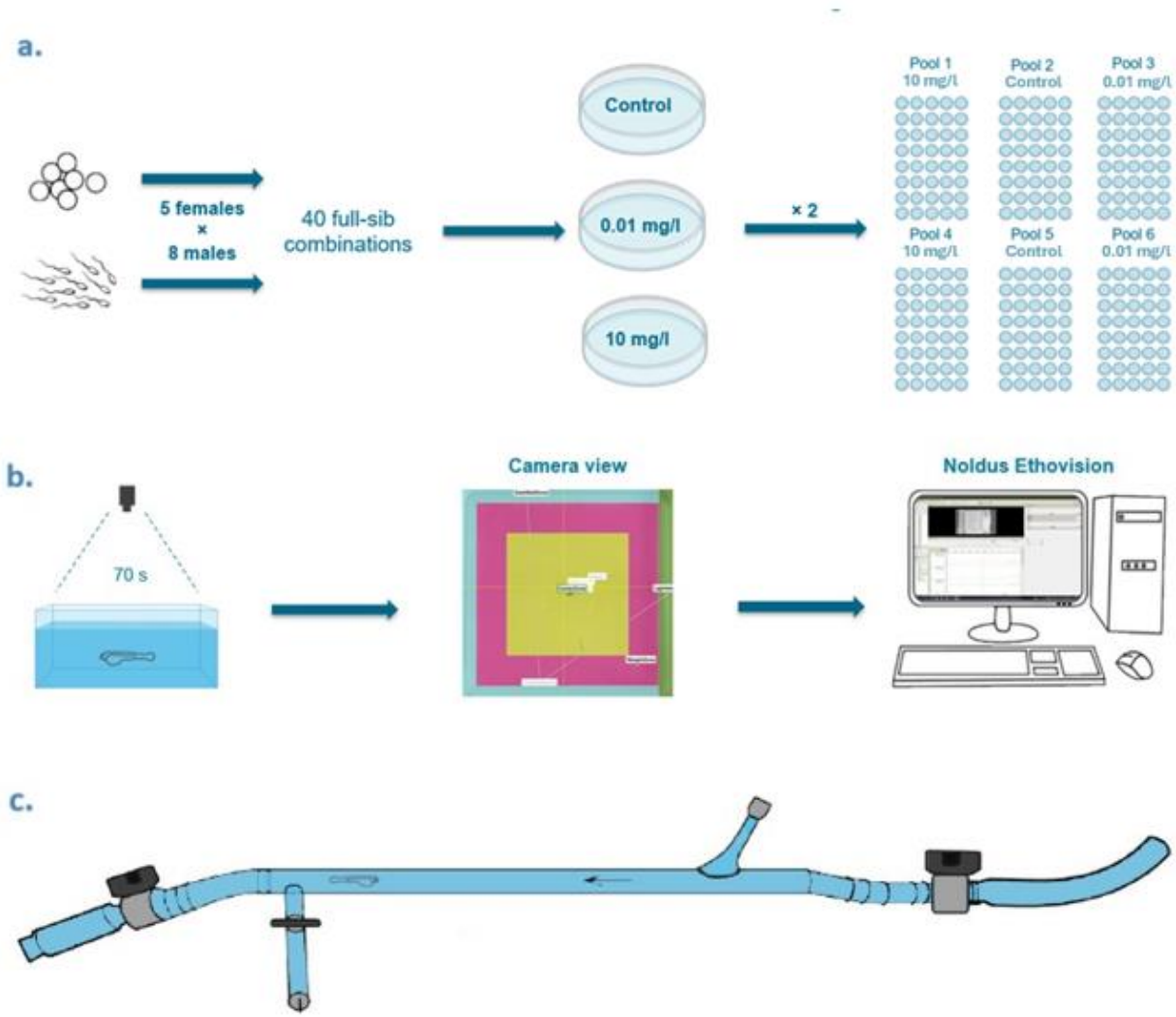


Figure 2. Illustration of the main parts of the experimental design. Artificial fertilization and exposure of whitefish gametes (a), video recording for behavioural analysis with Noldus Ethovision tracking software (b), and swimming performance tests with a swimming tube system with a constant gravity-driven water flow (Kekäläinen et al., 2010; Yaripour, 2022) (c).

5.3 Hatching and survival rate

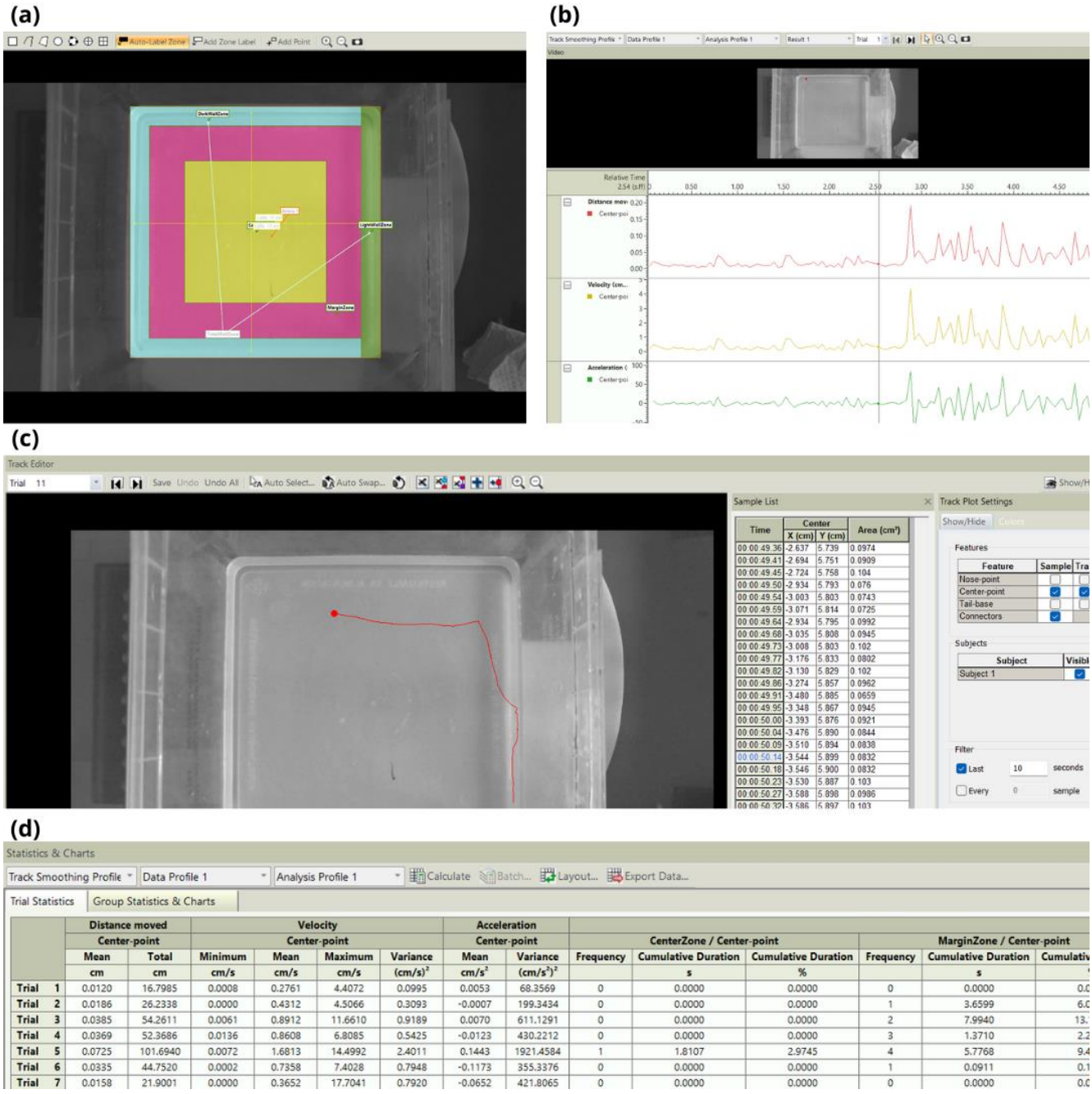
During the incubation period, water was kept at 4° C and room temperature at 10° C. During the winter the room was kept in dark conditions and from 20th of March 2022 onwards lights were switched on with a photoperiod simulation cycle of 13h hours of light and 11 hours of dark. On the 8th of March 2022, water temperature was gradually raised to 6° C to imitate the arrival of

spring and to accelerate hatching. Throughout the incubation period dead eggs and hatching events were counted, first on a weekly basis, and later, on a daily basis, for hatching and mortality rate analysis. For each individual box, we counted the number of hatched eggs until a total of 10 hatching events and after this, we recorded the day when all the eggs in that same box were hatched. Throughout the incubation period dead embryos and larvae were removed from the incubation boxes.

5.4 Behavioural tests

Behavioural tests consisted of 634 behavioural trials each following the same procedure: starting from the first box, one larvae was randomly chosen with a plastic pipette and carefully placed in the middle of a squared plastic container (170 × 170 mm) filled to a total volume of 40 mL of tap water at 4° C (**Figure 2b**). To capture videos of individual larvae behaviour over the entire arena we used a Logitech c615 HD webcam. Immediately after placement 70 seconds of swimming behaviour were recorded by the camera positioned above the container (**Figure 2b**). From each one of the 240 male-female combinations (40 families × 3 treatments × 2 replicates), three larvae were randomly selected and individually recorded for further behavioural analysis. However, in family replicates with high mortality rate less than 3 larvae were recorded. The recordings were performed throughout the day during light time, in a room at constant temperature, constant light conditions, and without any noise interferences. At the end of each individual test, the tested larvae were euthanized with an overdose of the anaesthetic MS-222. For posterior total body length and body weight measurements euthanized larvae were preserved in small Eppendorf tubes filled with a solution of 70% ethanol and 1% neutralized formalin. Recordings were analysed offline using the Noldus Ethovision tracking software (XT 16, Netherlands) to study total distance moved, average swim speed, and average mobility (**Figure 3**). In addition, with the tracking software a virtual grid was generated dividing the evaluation arena into different areas to study time spent in each area expressed both in seconds and frequency (**Figure 3a**). Ethovision then provides data output in a widespread format that can be later exported as an Excel spread sheet, which makes further analysis significantly easier (**Figure**

3d). Video tracking was performed on a HP Pavilion laptop running Windows 11 with an AMD Ryzen 5 5600H CPU and 8GB RAM.



		Distance moved		Velocity				Acceleration		CenterZone / Center-point			MarginZone / Center-point		
		Center-point		Center-point				Center-point							
		Mean	Total	Minimum	Mean	Maximum	Variance	Mean	Variance	Frequency	Cumulative Duration	Cumulative Duration	Frequency	Cumulative Duration	Cumulativ
		cm	cm	cm/s	cm/s	cm/s	(cm/s)²	cm/s²	(cm/s²)²		s	%		s	
Trial	1	0.0120	16.7985	0.0008	0.2761	4.4072	0.0995	0.0053	68.3569	0	0.0000	0.0000	0	0.0000	0.0
Trial	2	0.0186	26.2338	0.0000	0.4312	4.5066	0.3093	-0.0007	199.3434	0	0.0000	0.0000	1	3.6599	6.0
Trial	3	0.0385	54.2611	0.0061	0.8912	11.6610	0.9189	0.0070	611.1291	0	0.0000	0.0000	2	7.9940	13.
Trial	4	0.0369	52.3686	0.0136	0.8608	6.8085	0.5425	-0.0123	430.2212	0	0.0000	0.0000	3	1.3710	2.2
Trial	5	0.0725	101.6940	0.0072	1.6813	14.4992	2.4011	0.1443	1921.4584	1	1.8107	2.9745	4	5.7768	9.4
Trial	6	0.0335	44.7520	0.0002	0.7358	7.4028	0.7948	-0.1173	355.3376	0	0.0000	0.0000	1	0.0911	0.1
Trial	7	0.0158	21.9001	0.0000	0.3652	17.7041	0.7920	-0.0652	421.8065	0	0.0000	0.0000	0	0.0000	0.0

Figure 3. Main view of some of the features of the tracking software Ethovision. (a) arena settings, colours indicate different tracking areas within the arena; (b) integrated visualization; (c) track editor, red line indicates fish trajectory; and (d) statistical data output.

5.5 Swimming performance

Swimming performance tests were conducted using a swimming tube system with a constant gravity-driven water flow of 5,4 cm/s (**Figure 2c**) (Huuskonen et al., 2009). From each of the 240 male-female combinations (40 combinations × 3 treatments × 2 replicates), three larvae were randomly selected and placed individually in the swimming tube with stagnant water. Each larvae was placed in the swimming system and, after the water flow was activated, the larvae was enforced to swim against the current. Time was recorded until larvae reached fatigue or, more precisely, until they drifted against the rear end of the tube and didn't initiate swimming activity within five seconds. After each swimming performance test the tested larvae were immediately euthanized with an overdose of MS-222. For posterior body mass and length measurements, they were preserved in small Eppendorfs containing a solution of 70% ethanol and 1% neutralized formalin.

5.6 Statistical Analysis

To statistically analyse behavioural parameters, a multivariate statistical technique called principal component analysis (PCA) was carried out. Due to the large sample of variables related to behaviour this method allowed for a significant data reduction. PCA extracts important information from a dataset and allows for quantitative changes in data by calculating new variables, or principal components (PC), based on the covariation between the initial variables (Abdi & Williams, 2010). Whenever eigenvalues were greater than 1 this meant that principal components could explain and express a significant proportion of the variation in those same variables (**Table 1**). After principal component extraction, all the behavioural dependent variables can be explained by three underlying factors and these three components explained 97.7% of the total variance (**Table 1**).

Table 1 Total variance explained, and principal components extracted from the original data.

	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
Component	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	2,877	41,105	41,105	2,877	41,105	41,105	2,837	40,536	40,536
2	2,661	38,021	79,126	2,661	38,021	79,126	2,001	28,584	69,119
3	1,299	18,559	97,685	1,299	18,559	97,685	2,000	28,565	97,685
4	,160	2,281	99,966						
5	,002	,031	99,997						
6	,000	,003	100,000						
7	2,530E-13	3,614E-12	100,000						

Extraction Method: Principal Component Analysis.

By interpreting the rotated component matrix (**Table 2**), we can estimate the correlations between variables and corresponding components. The rotated components, or loadings, can be interpreted based on the positive or negative loadings of each one of the original variables. In principal component 1, total distance moved, mean velocity, and mean mobility have high positive loadings which means that all these variables have a strong positive correlation with PC1, and this component seems to describe overall activity or exploration rates of larvae. In the second principal component, time spent in the dark zone has a strong negative loading whereas the time spent in the lighter zone of the arena has a strong positive loading and thus PC2 seems to measure the propensity of larvae to reach lighter or darker zones, also known as phototaxis. Lastly, time spent at the centre has a strong positive loading and time spent at the walls of the arena has a strong negative loading, and this makes up for the third component, PC3, which describes the propensity of larvae to stay at the edges of the arena or at centre.

Table 2 Rotated Component Matrix.

	Component		
	1	2	3
TotalDistance	,985	,051	,048
Velocity_mean	,985	,053	,045
CenterMargin	,047	-,170	,984
LightWallZone	,042	,984	-,168
TotalWallZone	-,044	,169	-,984
Mobility_mean	,944	-,005	,018
DarkZone	-,038	-,984	,171

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.

Effects of PFOA treatments, male, female, male-female interactions, and day on embryo mortality, hatching time, offspring body size (length and weight), and offspring behavioural and swimming performance were tested using linear mixed models (LMM) and ANOVA in R. Dependent variables were subjected to graphical and numerical normality tests. In the models, treatment and day were treated as fixed factors, while male, female, and male-female interactions were treated as random factors. Every model assumption was verified with corresponding Q-Q plots and residual plots and statistical significance was set at $P < 0.05$. Pairwise comparisons were conducted between treatments using Tukey post hoc test. The statistical analysis was conducted using the lmerTest package in R (version 4.3.1).

6 Results

6.1 Effects of PFOA exposure in survival and hatching time

Embryo mortality was affected by male ($P < 0.001$), and by male:female interaction ($P = 0.004$), but not by female ($P = 0.058$) and PFOA exposure ($P = 0.3374$) (**Table 3**). Hatching time of

embryos was affected by PFOA exposure (P < 0.001), male (P < 0.001), and female (P < 0.001), but not by male:female interaction (P = 1.000) (**Table 3**).

Table 3. Effects of random and fixed factors on embryo mortality and hatching time.

Effects	**Embryo mortality**			**Hatching time**		
Fixed	*F*-value	d.f.	*P*-value	*F*-value	d.f.	*P*-value
Treatment	1.0926	2	0.3374	16.549	2	**< 0.001**
Random	χ^2	d.f.	*P*-value	χ^2	d.f.	*P*-value
(1|Male)	56.053	1	**<0.001**	28.148	1	**< 0.001**
(1|Female)	3.594	1	0.058	12.960	1	**< 0.001**
(1|Male: Female)	8.404	1	**0.004**	0.000	1	1.000

Pairwise comparisons revealed that hatching time was significantly faster for embryos exposed to the lowest concentration of PFOA (0.01 mg/L), in comparison to control and eggs exposed to the highest concentration of PFOA (10 mg/L) (**Table 4** and **Figure 4**).

Table 4. Pairwise comparisons for hatching time with Tukey's post hoc test.

	Pairwise Comparisons		
	Control – 0.01 mg/L	Control – 10 mg/L	0.01 mg/L – 10 mg/L
p-value	**< 0.001**	0.4751	**< 0.001**
t.ratio	5.471	1.666	-4.271

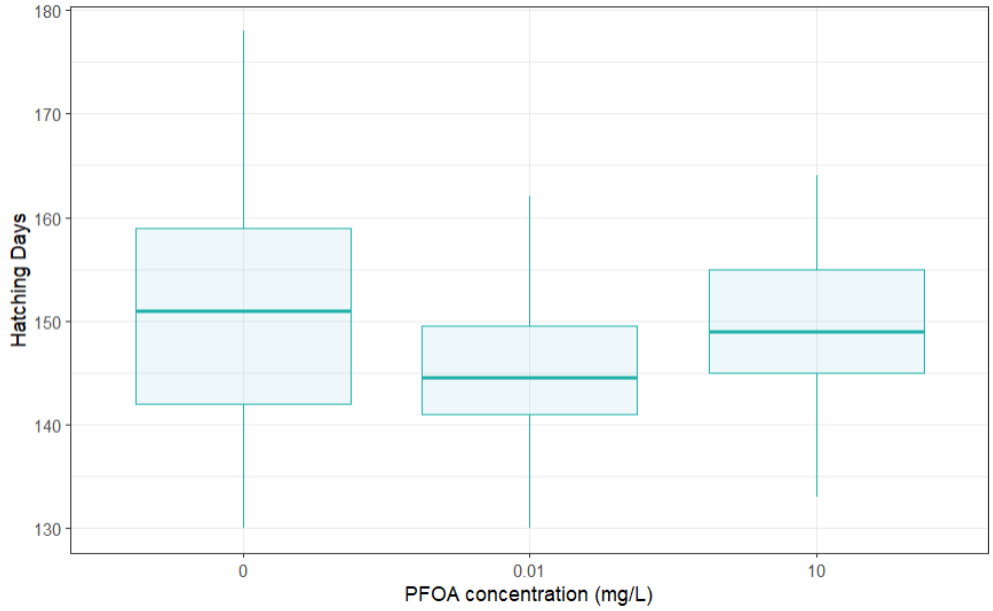


Figure 2. Average days of hatching time for the three different treatments.

6.2 Behavioural performance

Behaviour of larvae exposed to PFOA concentrations of 0.01 mg/L, and 10 mg/L did not show significant changes when compared to control (**Table 5**).

Table 5. Effects of random and fixed factors on all principal components (PC) related to behavioural parameters.

Effects	**PC1**			**PC2**			**PC3**		
Fixed	*F*-value	d.f.	*P*-value	*F*-value	d.f.	*P*-value	*F*-value	d.f.	*P*-value
Treatment	0.6321	2	0.5318	0.2115	2	0.8094	0.6037	2	0.5471
Day	7.0057	1	**0.01**	0.1754	1	0.6776	1.6357	1	0.2049
Random	χ^2	d.f.	*P*-value	χ^2	d.f.	*P*-value	χ^2	d.f.	*P*-value
(1|Male)	0.4589	1	0.4981	0.0480	1	0.8267	0.1134	1	0.7363
(1|Female)	1.0918	1	0.2961	0.2599	1	0.6102	2.7735	1	0.0958
(1|Male: Female)	0.000	1	1.000	0.000	1	1.000	0.0028	1	0.9579

6.3 Swimming performance

Swimming performance of larvae was affected by PFOA exposure (P = 0.0099), male (P = 0.0013), and female (P < 0.001), while the effects of day (P= 0.4792) and male: female interaction (P = 1.000) were not statistically significant (**Table 6**).

Table 6. Effects of random and fixed factors on swimming performance.

Effects		**Time**	
Fixed	*F*-value	*d.f*	*P* - value
Treatment	4.6528	2	**0.0099**
Day	0.5012	1	0.4792
Random	χ^2	d.f.	*P*-value
Male.ID	10.357	1	**0.0013**
Female.ID	10.946	1	**< 0.001**
Male.ID: Female.ID	0.000	1	1.000

Pairwise comparisons revealed that larvae exposed to the highest concentration of PFOA swam significantly longer than the ones exposed to the lowest concentration. Larvae from control did not show any significant differences when compared to the other two treatments (**Table 7** and **Figure 5**).

Table 7. Pairwise comparison for swimming time with Tukey's post hoc test.

	Pairwise Comparisons		
	Control – 0.01 mg/L	Control – 10 mg/L	0.01 mg/L – 10 mg/L
p-value	0.5771	0.1212	**0.0079**
t-ratio	1.000	-1.968	-2.999

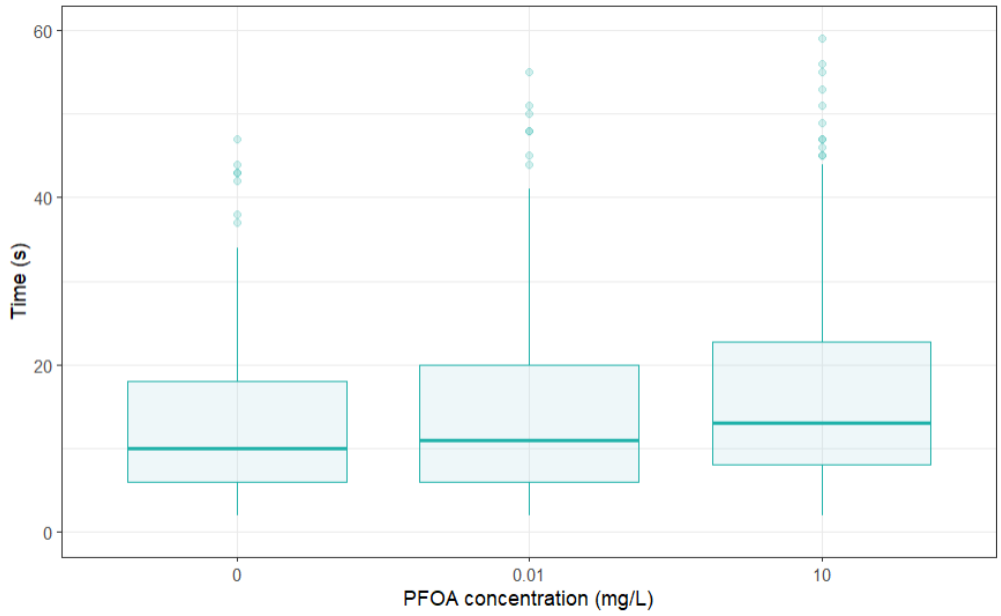


Figure 5. Average time of swimming performance before reaching fatigue.

6.4 Length and weight of tested larvae

In larvae tested for behavioural performance, body length was significantly affected by treatment (P < 0.001), male (P = 0.027) and female (P < 0.001), but not by day (P = 0.7102) and male: female interaction (P = 0.5621). Body weight was significantly affected by day (P < 0.001) and female (P < 0.001) but was not significantly affected by treatment (P = 0.3128), male (P = 1.000) and male: female interactions (P = 1.000) (**Table 8**).

Table 8. Effects of random and fixed factors on body length and weight in behavioural tests.

Effects	**Length**			**Weight**		
Fixed	*F*-value	d.f.	*P*-value	*F*-value	d.f.	*P*-value
Treatment	13.078	2	**< 0.001**	1.1643	2	0.3128
Day	0.1383	1	0.7102	135.87	1	**< 0.001**
Random	χ^2	d.f.	*P*-value	χ^2	d.f.	*P*-value
(1|Male)	4.9128	1	**0.027**	0.000	1	1.000
(1|Female)	23.185	1	**< 0.001**	11.293	1	**< 0.001**
(1|Male: Female)	0.3361	1	0.5621	0.000	1	1.000

Pairwise comparison showed significant increase in length of larvae exposed to the highest concentration of PFOA, in comparison with the ones exposed to the lowest concentration and control. (**Table 9** and **Figure 6a**).

Table 9. Pairwise comparisons for length in behavioural studies.

	Pairwise Comparisons		
	Control – 0.01 mg/L	Control – 10 mg/L	0.01 mg/L – 10 mg/L
p-value	0.5526	**< 0.001**	**< 0.001**
t.ratio	-1.039	-4.841	-3.822

In larvae tested for swimming performance tests, body length was influenced by PFOA exposure (P = 0.026), day (P < 0.001), male (P = 0.049), and female (P < 0.001), while the effect of male: female interaction (P = 0.4472) was not statistically significant. Body weight was affected by PFOA exposure (P = 0.0038), day (P < 0.001), male (P < 0.001), and female (P < 0.001), while the effect of male: female interactions (P = 0.8232) was not statistically significant (**Table 10**).

Table 10. Effects of random and fixed factors on body weight and body length in swimming tests.

Effects	**Length**			**Weight**		
Fixed	*F*-value	d.f.	*P*-value	*F*-value	d.f.	*P*-value
Treatment	3.6596	2	**0.026**	5.6398	2	**0.0038**
Day	77.244	1	**< 0.001**	163.65	1	**< 0.001**
Random	χ^2	d.f.	*P*-value	χ^2	d.f.	*P*-value
Male.ID	3.883	1	**0.049**	22.336	1	**< 0.001**
Female.ID	33.414	1	**< 0.001**	22.755	1	**< 0.001**
Male.ID: Female.ID	0.578	1	0.4472	0.0499	1	0.8232

Pairwise comparisons revealed that larvae exposed to the highest concentration were significantly longer when compared to the ones exposed to the lowest concentration. However, larvae from control did not show any significant differences when compared to the other two treatments (**Table 11** and **Figure 6c**). Body weight was significantly lower in larvae exposed to the lowest concentration, when compared to control and exposure to the highest concentration. Whereas larvae exposed to the highest concentration were not significantly different from control (**Table 11** and **Figure 6d**).

Table 11. Pairwise comparisons for length and weight in swimming tests.

	Pairwise Comparisons					
	Control – 0.01 mg/L		Control – 10 mg/L		0.01 mg/L – 10 mg/L	
	p-value	t.ratio	*p*-value	t.ratio	*p*-value	t.ratio
Length	0.6329	0.912	0.1973	-1.723	**0.0216**	-2.664
Weight	**0.0096**	2.940	0.9939	0.106	**0.0121**	-2.861

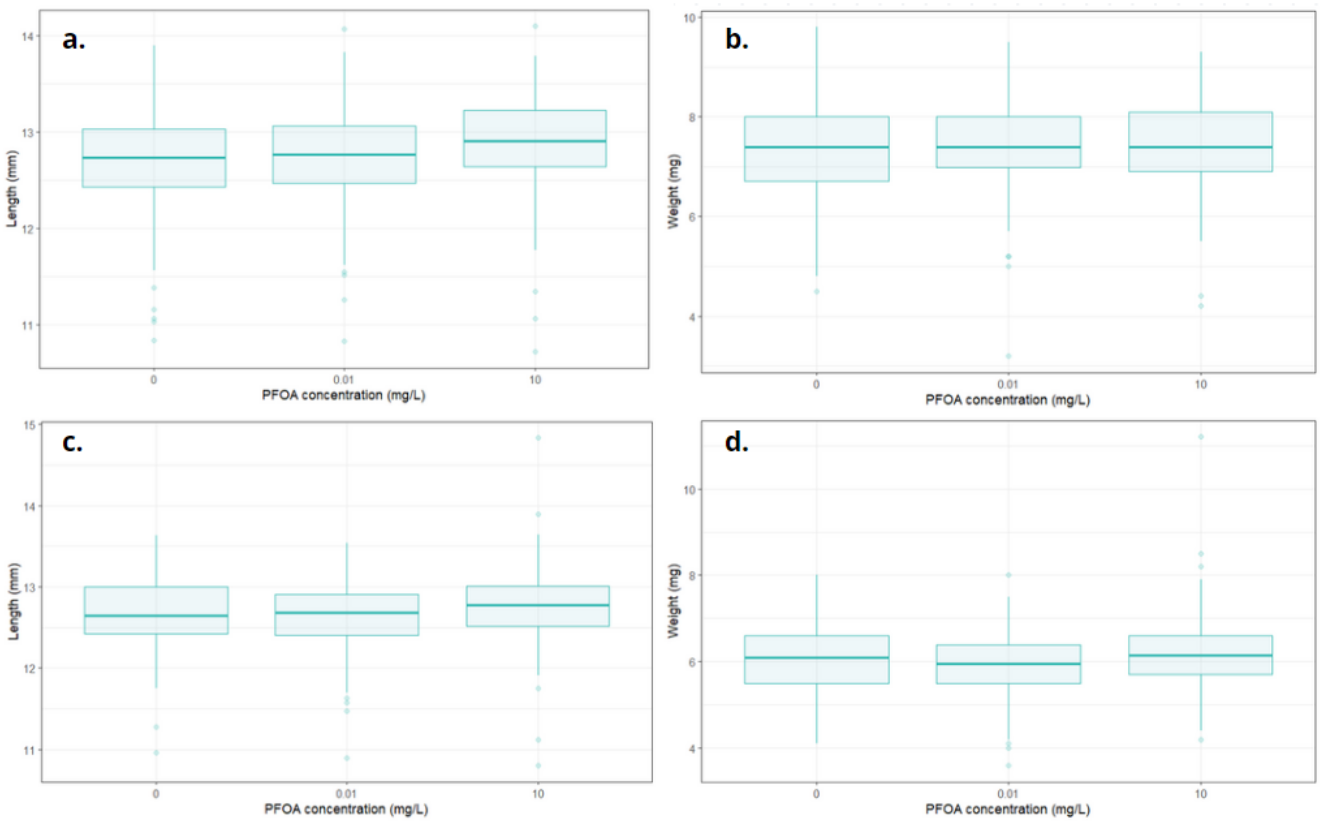


Figure 6. Average length of tested larvae in behavioural (a) and swimming tests (c), and average body weight in behavioural (b) and swimming tests (d).

7 Discussion

The present results indicate that embryos exposed to the lowest concentration of PFOA (0.01 mg/L) were significantly premature with a smaller body mass whereas embryos exposed to the highest concentration resulted in an overall increase in body length and swimming performance.

Embryo survival had no significant differences between treatments. Mortality of embryos was statistically analysed with dead embryos percentage which didn't consider if treatments had any impact on how fast these embryos died. This could possibly be a limitation since the mortality rates were only related to the total percentage of dead embryos in each box and not relating the rate or speed in which each specific embryo died. To get around this issue and in order to study survival rates the most common approach is to perform the log-rank test that, based on the

fraction of eggs living for a specific amount of time after exposure, calculates if there are significant differences between survival curves of each treatment (Bewick et al., 2004; Meinelt et al., 2010). In my understanding future experiments should take this into consideration to achieve more accurate conclusions, and to perform this, the time between the beginning of treatment and the occurrence of death should be recorded.

To my knowledge, no other study has found correlations between PFASs exposure and premature hatching in fish. However, accelerated hatching has been documented in fish exposed to other environmental contaminants during early development. In a similar experiment in which whitefish sperm was exposed to different concentrations of nanoplastics before fertilization, premature hatching was also found at the highest concentrations along with reduced body mass and diminished swimming performance (Yaripour et al., 2021). These results are further exemplified in studies where fish embryos were exposed to heavy metals (Ismail & Yusof, 2011; Nagamatsu et al., 2021), tributyltin (Liang et al., 2017), titanium dioxide (TiO_2) (Samaee et al., 2015), polynuclear aromatic hydrocarbons (Carls et al., 1999), and glyphosate (Z. Liu et al., 2022). After fertilization, a protective barrier between the egg and the chorion, known as perivitelline space, is formed after water uptake from the embryo (Blaxter, 1969). The perivitelline space along with the chorion not only constitute a line of defence against mechanical or chemical injury until hatching, but also control the osmotic influx of water and electrolytes into the embryo, due to changes of the perivitelline fluid osmolarity (Laale, 1980; Eddy & Talbot, 1985). For this reason, the egg is highly permeable and chemicals that penetrate the egg along with water can change the chorionic membrane physical properties, leading to pore clogging or disturbances in ions, gas exchange, or nutrients exchange, inducing premature hatching (Jurgelėnė et al., 2021; Pérez Atehortúa et al., 2023). In addition, damage of the protective barriers, or chorionic deterioration, can make them more fragile and lead to earlier tearing of the membranes inducing premature hatching (Small & Wolters, 2003; Jezierska et al., 2009).

Given that PFOA has a high binding affinity to proteins, one possible cause of structural changes of the chorionic membrane could be the interaction of PFOA with the different number of glycoproteins, or choriogenins, that constitute the protective membrane (Ulhaq et al., 2013; Pérez Atehortúa et al., 2023). Previous studies have found correlations between premature

hatching of fish embryos and decreased oxygen exchange or cell hypoxia, adsorption of chemicals to the chorion surface, increased muscle movements or vibrations, or increased respiration rates (Leung & Bulkley, 1979; Jezierska et al., 2009; Samaee et al., 2015).

The evidence presented thus far supports the idea that premature hatching has detrimental effects on the development of fish at different embryonic stages, and premature larvae can exhibit deformities, growth reduction, or other physiological anomalies. For this reason, along with the mortality and hatching recordings, studies have also incorporated other daily observations during the incubation and hatching period including death after hatching, body deformities, lack of swimming or twitches, yolk sac malformations, craniofacial or cardiovascular anomalies, spinal curvature, or others (Von Westernhagen, 1988; Carls et al., 1999; Bartlett et al., 2021). However, given the high number of eggs and larvae in the present study this would present to be an extremely laborious task. In contrast to this findings, evidence of delayed hatching after embryonic exposure has also been found (Jezierska et al., 2009). Nevertheless, it becomes clear that early developmental processes of fish are highly vulnerable to chemical contamination, processes that can shape the quality and quantity of offspring. Moreover, disturbances in the normal development of a species are expected to have detrimental effects in other biological events that are synchronized with ecological cues such as seasons or food availability, including reproduction or migration (Roland Billard & Bernard Breton., 1978).

In the present study, the lowest concentration of PFOA (0,01 mg/L) induced premature hatching unlike the highest concentration (10 mg/L) that didn't have any significant effects on hatching time. Environmental concentrations of PFOA have been measured in multiple studies across Europe which have reported concentrations at the ng/L range (Kunacheva et al., 2012; Antonopoulou et al., 2024). For this reason, PFOA concentrations that are found to be lethal or damaging to fish and other aquatic organisms in most studies are usually far above the concentrations present in environmental matrices. However, toxicity studies have found lethal or sub-lethal effects of PFASs exposure at lower concentrations approaching the environmentally relevant ones (Gebreab et al., 2022).

Bioaccumulation and biomagnification potential of PFASs is highly dependent on their physical-chemical properties such as the fluorinated carbon chain length, structure, functional group, hydrophobicity, log P, or octanol-water partition coefficients (k_{ow}) (Gebreab et al., 2022;

Liang et al., 2022, 2023). Increased potential for bioaccumulation and biomagnification has been linked with longer chain PFASs with more than eight carbon atoms, with sulfonates rather than carboxylates, or increased bioaccumulation factors for linear isomers rather than branched ones (Martin et al., 2003; Conder et al., 2008; Ulhaq et al., 2013; Menger et al., 2020). Along with chemical properties, studies have shown dependencies between PFASs toxicity and intraspecific variations between individual including age, sex, stage-dependent toxicity of hepatic development (liver and hepatic enzymes), or overall developmental stage of other target organs (Gebreab et al., 2020; Y. Li et al., 2022; Dawson et al., 2023). The relationship between fish habitat and diet and the level of exposure to PFASs has also been study focus. For instance, some studies have shown higher concentrations in benthic fish suggesting that sediments and pore water are also important sources of PFASs uptake (Langberg et al., 2020). Furthermore, it should be considered that longer term exposures even at lower concentrations could potentially be highly toxic for organisms and thus biomagnification and evolutionary studies are of utmost importance for the protection of exposed organisms. PFAS concentrations that are environmentally relevant have been associated with increased bioaccumulation factors which indicates that these concentrations can gradually accumulate in aquatic organisms leading to multigenerational toxicity (Du et al., 2009; Jantzen et al., 2017). Therefore, despite the global reduction of PFOA concentrations found in environmental matrices over the years, due to strict restrictions on their manufacture and use, low levels of PFOA can still affect biological responses and thus remain of great ecological concern.

Reduced body mass of larvae exposed to the lowest concentration of PFOA are consistent with the differences found in hatching times since premature hatching could explain subsequent weight differences, since when hatching occurs, the larvae's body mass can be directly correlated with the developmental stage at which the embryo is currently at. Disturbances such as accelerated hatching can impact the viability of larvae which results in smaller, immature offspring, and consequent reduced survival fitness (Jezierska et al., 2009).

Body length results were more inconclusive as for the two different performance experiments the findings were slightly different. Since larvae exposed to the highest concentration of PFOA (treatment 2) and tested for swimming performance were longer than control but not significantly different, we don't have sufficient evidence to suggest that treatment 2 had a

significant effect on growth when compared to control. Therefore, since larvae exposed to treatment 1 were also not significantly different from control, it is important to be cautious in drawing any conclusions regarding the observed differences between treatment 1 and treatment 2, as they may not me meaningful and any observed differences could be due to random variation rather than a true effect of the treatment itself. Nonetheless, in larvae tested for behavioural tests, body length was significantly increased at the highest concentration of PFOA exposure when compared to both control and treatment 1. A study where zebrafish embryos at 3 hpf were exposed to different PFASs during five days, perfluorononanonanoic acid (PFNA; C:9) has shown to result in significant increase of body length after 14 dpf (Jantzen et al., 2016). Similarly, exposure to perfluorohexane (PFHxS) during 5 dpf also resulted in significant increase in body length of zebrafish larvae (Annunziato et al., 2019). To my knowledge the mechanisms that lead to increased body length after exposure to PFASs are not yet understood, however it is possible that the observed increased length is resulting from the stimulation of biochemical pathways related to fish growth or energy allocation. Qiang et al. (2016) reported possible correlations between increased growth of zebrafish larvae with a faster absorption of the yolk sac, after exposure to environmentally relevant concentrations of carbamazepine(Qiang et al., 2016). During the early stages of development, fish embryos and larvae obtain their nutritional requirements from the yolk sac, thus an accelerated absorption of the yolk could indicate an increase in energy demand (Blaxter, 1969). In turn, the increased consumption of nutrients provided by the yolk sac could lead to abnormal intensification of larvae growth. In fact, previous studies have found correlations between larger yolk-sacs and reduced body length in newly hatched larvae after exposure to cadmium, indicating a slower yolk absorption, thus it is possible that PFOA could have an opposite effect on larvae growth (Rombough & Garside, 1982).

In the present study exposure to the highest concentration of PFOA (10 mg/L) has resulted in a significant increase in body length growth. Low levels of environmental contaminants have previously caused stimulatory effects in many different taxa, a process generally named as hormesis or growth hormesis (Calabrese & Baldwin, 2001). However, one of the main foundations of the hormesis theory is that sublethal concentrations may induce inhibition of several biological responses, but conversely, lower concentrations could induce stimulation of these responses (Stebbing, 1982). The present results have shown overall growth stimulation at

the highest concentration which is not consistent with the hormesis theory. The concept of hormesis is not yet fully understood and it could be a transient phenomenon, thus a wider range of exposure concentrations can result in different dose-response curves which could help to better understand how PFOA inhibits or, in this case, stimulates biological responses in fish (Stebbing, 1982; Calabrese & Baldwin, 2001).

In addition to this, body length of larvae tested for behavioural performance was significantly increased at the highest concentration but surprisingly there was no significant differences in body weight of the same larvae. A previous study, in which whitefish embryos were exposed to aged nanoplastic particles, has interestingly demonstrated similar findings as body length was significantly increased whether significant changes in body weight were not observed, which indicates that exposed larvae were slender then normal (Yaripour et al., 2022). Even though it is not clear what could be the biological significance for this, Yaripour et al. suggested that, in order to prevent accelerated hatching (which in this study did occur at the lowest concentration), larvae trade-off energy allocation towards growth rather than to internal processes that regulate hatching time, such as body movements or respiration rates. However, additional research is necessary do determine which mechanisms of action of PFOA cause an increase of body length at the concentration of 10 mg/L in the present study. While generally PFASs concentrations can be directly correlated with fish weight and length, both positive and inverse correlations have been reported and should be more closely investigated.

Swimming performance of larvae was evaluated by recording the amount of time each larvae could swim against a current before reaching fatigue. Similarly to the previous length results in larvae tested for swimming performance, swimming performance of larvae exposed to the highest concentration (treatment 2) was longer than control but not significantly different. Thus, since not only there were no significant differences between control and treatment 2, but also between treatment 1 and control, the observed differences between treatment 1 and treatment 2, may not me meaningful and any observed differences could be due to random variation rather than a true effect of the treatment itself. Nonetheless, based on published literature, increased swimming performance or hyperactivity has been previously documented in fish exposed to PFOA or other related compounds (Jantzen, Annunziato, Bugel, et al., 2016; Annunziato et al., 2019; Menger et al., 2020). In some of these studies, exposure to PFOA

concentrations of 2, 12 and 150 µM during embryonic stage, which are relatively high concentrations closer to the ones we used in this study (2 µM = 0,83 mg/L, 12 µM = 4,97 mg/L, and 150 µM = 62 mg/L), also resulted in a significant hyper swimming activity (Jantzen, Annunziato, Bugel, et al., 2016; Menger et al., 2020). In addition to PFOA induced hyperactivity, the same studies also found this to be true for embryonic exposures to 6:2-fluorotelomersulfonic acid (6:2 FTSA) or perfluoroheptanoic acid (PFHpA), at concentrations of 180 µM and 89 µM respectively. Since, previous exposures to lower concentrations of PFOA, which are closer to the environmentally relevant ones, have also resulted in increased swimming activity of fish larvae, we could consider PFOA to be mainly inducing hyperactivity (Rericha et al., 2021; Wasel et al., 2022). To explain behavioural and morphometric changes in larvae exposed to different PFASs, Jantzen et al. also incorporated in the study analysis of multiple genes expression and found an increase in the c-fos transcription factor complex (Jantzen, Annunziato, Bugel, et al., 2016). The c-fos transcript regulates neuronal excitability in the CNS and is involved in stress response situations, thus the increase expression of this gene could explain hyper swimming activity (Buhrke et al., 2015). In studies with other model organisms including human hepatocytes and zebrafish embryos, increased c-fos expression was also found after exposure to PFOA (Buhrke et al., 2015; Wang et al., 2022). Interestingly, both hyper and hypoactivity have been documented and conflicting findings could be a result of different experimental setups or different exposure concentrations. Swimming performance is an important behavioural trait and exerts influence on many biological activities including preying, predator avoidance, or migratory behaviour, and thus impacts survival or growth of fish after hatching (Yaripour et al., 2021). Disruptions on the swimming ability of fish larvae can in turn affect individual survival fitness and overall population dynamics (Fuiman & Cowan, 2003).

Just like swimming performance other behavioural parameters are important factors that contribute to the survival fitness of fish, as well as subsequent growth and reproduction. Exposure to anthropogenic contaminants can have unforeseen effects on social, ecological, and evolutionary behaviours of aquatic organisms, and such behaviours are important regulators of aquatic ecosystems function and composition (Fuiman & Cowan, 2003). For this reason, many behavioural endpoints can also be incorporated as markers of the toxicological effects of pollutants in various ecosystems. Other behavioural parameters evaluated in this study had no

significant differences between treatments. Conclusions related to behavioural endpoints that were not found in this study may be explained by differences in the applied methodology. In addition to this, the larvae assessed for behavioural parameters in the present study were recorded during one minute after being placed in the arena without any previous acclimation period. Based on published scientific literature, behavioural effects of PFASs exposure are usually examined or automatically recorded during an average of ½ or 1 hour, never less than 5 minutes, and most of these studies incorporated an acclimation period before recordings started (Xia et al., 2015; Jantzen, Annunziato, & Cooper, 2016; Jantzen, Annunziato, Bugel, et al., 2016; Annunziato et al., 2019; Menger et al., 2020; Sana et al., 2021; Wang et al., 2022). Some studies restricted recordings between 1-4 pm since the natural circadian rhythm can affect overall swimming behaviour of fish larvae (MacPhail et al., 2009), however in the present study recordings were obtained throughout the day. However, previous studies have demonstrated that locomotor behaviour (Sana et al., 2021), aggressive behaviours , or response to stimulus (Hamed et al., 2024) are disrupted by PFOA or other related compounds. Most behavioural studies have incorporated other external stimulus or biological natural cues to better represent the natural complex environment. For example, touch response or flight response of fish larvae is directly correlated with predator avoidance behaviour and can be recreated in laboratory settings by touching larvae at resting state and analysing how long it takes until the larvae escapes (Qiang et al., 2016). Test of dark and light preference of fish, or scototaxis, can also be used as a measure of anxiety or boldness (Jantzen, Annunziato, & Cooper, 2016), and even though we specified areas in the arena with shadows or lighter areas, the presence of these were merely due to the normal lighting of the room and a more apparent division between dark and light areas could result in more conclusions. Scototaxis or light stimulation, in which fish larvae are exposed to sudden light flashes after periods of darkness (Qiang et al., 2016), or even behavioural recording at alternating periods of darkness and light (Menger et al., 2020), are also effective experiments to assess any behavioural disruptions. An additional indicator of stress or anxiety responses is the measurement of time that larvae spend swimming at the edges of the arena, also called thigmotaxis or wallpreference behaviour, which is a common avoidance behaviour usually as a way to find shelter or protection from predators (Ahmad & Richardson, 2013). PFOA-based studies with zebrafish found a relationship between exposure to PFOA and

fish anxiety behaviour such as tendency to spend most of the time occupying the bottom or the edges of the tanks (Adedara et al., 2022). In 2015, AlZu'bi et al., also found this to be true for fish subjected to common invasive procedures such as fin clipping, transponder tagging, or injection of chemicals, in which individual zebrafish reported abnormal behaviour after the procedures (Alzu'bi et al., 2015). Zebrafish embryos exposed to tributyltin, not only demonstrated accelerated hatching but showed impaired behaviour including reduced swimming distances and reduced overall activity, changes that have been linked to disruptions of the central nervous system (CNS) caused by tributyltin (Liang et al., 2017). This further illustrates that differences in toxicokinetics findings may be related to multiple factors including varying concentrations, lighting conditions, static exposure or daily renewal, experimental temperature, and exposed species.

Bioaccumulation of chemicals in a living organism is species-specific, and the mechanisms of PFOA accumulation of biomagnification is not yet understood. Chronic exposure (42 days) to PFOA in 2-9 days old amphipods has resulted in significant effects in growth, reproduction, and survival rate, whereas exposure to fathead minnows (21 days) during embryonic and larval stage had no significant effects in hatching, survival, and growth (Bartlett et al., 2021). The study was conducted with 9 different concentrations ranging from 0.01 to 100 mg/L of PFOA which highly exceeds the concentration scope of this study and study organisms represent two freshwater species that naturally inhabit similar habitats. This is a consequence of the complexity of living organisms which have specific bioaccumulation potentials, specific biotransformation processes in tissues, and protein-binding affinities that may vary for each compound and between species (Liang et al., 2023). Among common PFASs, perfluorooctanesulfonic acid (PFOS) is predominately found at the highest concentrations, often above the EQS_{biota}, in studies where multiple fish species are investigated whereas PFOA is often found under the limit of quantification (LOQ) (Kannan et al., 2005; Berger et al., 2009; Butt et al., 2010; Mazzoni et al., 2019; Kumar et al., 2022; Giari et al., 2023). Multiple studies have reported different results relating detected PFASs concentrations in different fish species, but it is important to note that the scope of some of these studies often don't include biomagnification parameters such as ecological characteristics or fish (habitat, diet, or trophic level) or toxicokinetics (target cells and organs). For instance, measurements of PFAS levels in the Arctic regions often detect PFOA at the highest

concentrations in comparison to other related compounds. Nevertheless, in arctic biota PFOA is often undetected or in very low concentrations (Butt et al., 2010). Low concentrations of PFOA, in comparison with other PFASs, such as PFOS, is inconsistent with its abundant presence in humans and environmental matrices (Kannan et al., 2005; K. Li et al., 2017; Antonopoulou et al., 2024). This could mean that unlike other PFASs, PFOA doesn't bioaccumulate as much in biota, as shown by many studies, and human exposure could be explained by other routes such as drinking water or dust inhalation (Cerveny et al., 2018; Mazzoni et al., 2019). For instance, based on the concentration of PFOS in water samples and on biota samples, studies have suggested a PFOS bioconcentration factor (BCF) in the order of thousands, whereas for PFOA, and despite the higher occurrence in water samples, the calculated BCF for PFOA was around 1000-fold lower than PFOS, indicating a low bioconcentration potential of PFOA (Martin et al., 2003; Kannan et al., 2005; Giesy et al., 2006). While in humans estimated half-lives can reach several years, especially for long-chain PFASs such as PFOA, due to interactions with molecules intervenient in absorption processes, in other living organisms the rates of elimination are much shorter (Dawson et al., 2023). Nonetheless, considering that many toxicity studies have found correlations between PFASs and multiple morphometric, physiological, or behavioural disorders in fish at early life stages, it is clear that these chemicals can interact with aquatic organisms especially at the most sensitive embryonic and larval stages. Since PFASs have a proteinophilic nature, studies have found higher accumulation in fish eggs (Kannan et al., 2005; Peng et al., 2010), and this could be due to the high binding affinity of these chemicals to vitellogenin, a phospolipoglycoprotein present in the liver (Raine et al., 2021). Vitellogenin is transported through blood into oocytes and thus maternal transfer can be an additional pathway of PFASs accumulation in eggs along with exposure through the surrounding environmental matrices (Lubzens et al., 2010). A previous study with radiolabelled PFOA not only found high absorption of PFOA to the chorion of pre-vitellogenic oocytes, as well as in the yolk but only after vitellogenesis, which corroborates the hypothesis that incorporation of PFASs inside the oocyte is most likely due to maternal transfer during vitellogenesis (Ulhaq et al., 2013). For this reason, PFASs concentration eggs to liver ratio is frequently used to assess if there is maternal transfer of PFASs to eggs (Peng et al., 2010). During early developmental stages the embryo gets its nutritional supplies from the egg yolk provided by the maternal oocyte (Lubzens et al., 2010), therefore any maternal deposition of

PFASs during oogenesis could lead to growth or neural developmental disorders. This further demonstrates that even lower concentrations of PFASs can affect the highly vulnerable early stages of fish, not only via direct exposure of these contaminants present in the surrounding environment, but also through maternal contribution in fish where bioaccumulation in females is observed.

Conclusions

As a consequence of their extensive use for commercial and industrial purposes, and their high environmental persistency, PFASs are being transported through water or air into multiple ecosystems. This study confirms that the presence of PFOA or other related compounds in environmental matrices can lead to detrimental effects in fish at the most vulnerable early developmental stages. The results indicate that accelerated hatching time and consequent decreased body weight could be caused by interactions of PFOA with the embryo membrane, when exposed to lower concentrations closer to the environmentally relevant ones. On the other hand, exposure to higher concentrations inducing increased body length and hyperactivity can be caused by disruptions in biochemical processes that regulate growth or neuronal processes.

Due to the toxicokinetics complexity of these compounds, integrating relevant endpoints including malformations at hatching, other behavioural parameters, or gene expressions can lead to more significant results, as well as including a broader range of exposure concentrations. Although PFOA bioaccumulation potential in biota is not high when compared to other related compounds such as PFOS, PFOA can still induce changes in the normal development of embryos and larvae, even at lower concentrations as demonstrated in this study. Furthermore, it should be considered that in the present study the performed exposure was acute, and that long term exposures even at lower concentrations could potentially be highly toxic for organisms and thus, in the future, biomagnification and evolutionary studies are of utmost importance.

Future studies are of great ecological importance not only to understand the underlying mechanisms that induce early developmental changes in fish, but also to closely monitor their occurrence in the environment, wildlife, and in humans.

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