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Sub-acute exposure of male guppies (*Poecilia reticulata*) to environmentally relevant concentrations of PFOA and GenX induces significant changes in the testis transcriptome and reproductive traits

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ABSTRACT

Poly- and perfluoroalkyl substances (PFAS) are frequently detected in the environment and are linked to adverse reproductive health outcomes in humans. Although legacy PFAS have been phased out due to their toxicity, alternative PFAS are increasingly used despite the fact that information on their toxic effects on reproductive traits is particularly scarce. Here, we exposed male guppies (*Poecilia reticulata*) for a short period (21 days) to an environmentally realistic concentration (1 ppb) of PFOA, a legacy PFAS, and its replacement compound, GenX, to assess their impact on reproductive traits and gene expression. Exposure to PFAS did not impair survival but instead caused sublethal effects. Overall, PFAS exposure caused changes in male sexual behaviour and had detrimental effects on sperm motility. Sublethal variations were also seen at the transcriptional level, with the modulation of genes involved in immune regulation, spermatogenesis, and oxidative stress. We also observed bioaccumulation of PFAS, which was higher for PFOA than for GenX. Our results offer a comprehensive comparison of these two PFAS and shed light on the toxicity of a newly emerging alternative to legacy PFAS. It is therefore evident that even at low concentrations and with short exposure, PFAS can have subtle yet significant effects on behaviour, fertility, and immunity. These findings underscore the potential ramifications of pollution under natural conditions and their impact on fish populations.

1. Introduction

Poly- and perfluoroalkyl substances (PFAS) are a large family of synthetic chemicals that are recognized as one of the world's major man-made pollutants. They are commonly released into the environment as a consequence of various industrial processes (e.g., they are used in water resistance proofing, food wrapping material, and non-stick cookware). Due to their widespread use and chemical stability in the environment, PFAS accumulate in surface water, groundwater, rain water, soils, and sediments (Muir et al., 2019; Muir and Miaz, 2021) and ultimately in living organisms (Ahmadireskety et al., 2021; Banzhaf et al., 2017; Brusseau et al., 2020; Burkhard, 2021; Campo et al., 2016; Ghisi et al., 2019; Podder et al., 2021).

For some of the most historically used PFAS (those known as 'legacy' PFAS), such as long-chain perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), toxicological data have been accumulating in the past two decades, particularly data on humans. Negative effects of PFAS exposure in humans include endocrine disruption, reproductive impairments, developmental and thyroid dysfunctions, immune and liver diseases, metabolic disorders, and cancer (Fenton et al., 2021). Exposure to PFAS has been linked to impairment in male and female reproductive health, with negative effects on ejaculate quantity and quality and consequently on fertility (Di Nisio and Foresta, 2019; Sun et al., 2023). Negative effects on ejaculate parameters and fertility are commonly due to the activity of PFAS as endocrine disruptors, but direct effects of PFAS not mediated by endocrine pathways

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have also been demonstrated (Šabović et al., 2020). Even though the phenotypic consequences on male fertility have been established, at least in humans, the changes at the molecular level in the relevant tissue, the testes, have been poorly studied.

Despite the well-established negative health consequences of PFAS exposure, to date, few compounds have been internationally regulated (Brennan et al., 2021), and limits in drinking waters and food commodities have been recommended but only for some PFAS (EU, Directive (EU), 2020; Epa, 2019; EFSA Panel on Contaminants in the Food Chain (EFSA CONTAM Panel) et al., 2020), and the presence of these substances in lakes, rivers, groundwater, and coastal waters is monitored. For these reasons, some of the most used legacy PFAS have been gradually phased out in some countries (EU, Regulation (EU), 2019), in favour of newly emerging PFAS (Ruan et al., 2022). This new generation of fluorinated derivatives are short-chain or branched compounds with lower chemical and biological stability and are deemed to be potentially less hazardous, cumulative, and toxic (Conder et al., 2008). However, as these compounds are relatively new, toxicological data are scarce and not encouraging; indeed, newly emerging PFAS seem to induce toxicity like legacy PFOS and PFOA (Jane et al., 2022). Additionally, how these novel PFAS accumulate, transform, and behave in the environment is largely unknown, and recent studies have shown that they may be even more pervasive and transportable than long-chain PFAS in the aquatic environment (Li et al., 2020).

One of the most important emerging substitutes for PFOA is the ammonium salt of 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy) propanoic acid, commonly known as GenX (trade name), which has recently been added to the list of 'Substances of Very High Concern' by the European Chemicals Agency. Since 2010, GenX has been widely used, and it is now frequently found in river waters downstream of fluorochemical plants at concentrations ranging from 0.1 to 36 μg/L (Brandsma et al., 2019; Gebbink et al., 2017; Pan et al., 2017; Joerss et al., 2020; de Kort et al., 2019), as well as in surface and drinking waters in China, the United States, and Europe (Gebbink et al., 2017; Fang et al., 2021; Pan et al., 2018). Alarming studies have suggested that GenX is resistant to abiotic and biotic degradation and highly mobile in the environment (Wang et al., 2015; Yan et al., 2020). In animal models, studies have reported that the toxicity of GenX might be even greater than that of PFOA and PFOS. For instance, GenX is hepatotoxic and carcinogenic in rats (Gomis et al., 2018; Caverly Rae et al., 2015), induces toxicity in zebrafish embryos (Gebreab et al., 2020), and disrupts thyroid hormones in animal models (Coperchini et al., 2021). There is also some evidence indicating that GenX affects reproduction (Chambers et al., 2021). For example, in zebrafish, GenX causes estrogen disruption similarly to PFOA by altering the balance of sex hormones (Xin et al., 2019a).

As the scientific interest in PFAS and their toxic effects grows, studies are still focused on humans and rodents, and little is known about the effects of PFAS on different animal models. Specifically, our knowledge of the effects of PFAS on such a key life-history trait as reproduction remains limited in non-mammalian animals, especially in aquatic organisms. This is surprising, as understanding the effects of PFAS exposure in these organisms, such as freshwater fish, is especially relevant because freshwater ecosystems are more easily and directly contaminated by pollution due to wastewater plants and biomagnification effects in the food web. Aquatic organisms can be exposed through a variety of routes, including food ingestion, uptake via gills, and absorption through the skin. This may add a further threat to aquatic ecosystems, which are already particularly fragile in terms of biodiversity loss and population abundance due to other concomitant factors.

Here, we tested the effect of GenX and PFOA on the phenotypic expression of reproductive traits and the gene expression in the testes of a freshwater fish, the guppy (*Poecilia reticulata*). Guppies are small, sexually dimorphic, live-bearing freshwater fish, whose reproductive traits and mating dynamics have long been studied. We focussed on males to parallel what is known in humans, namely that PFAS specifically affect ejaculate traits, and because of their well-studied characteristics, and defined endpoints, related to reproduction. They display alternative behavioural reproductive tactics (see below), so-called ornaments, and have easily assessable proxies for fertility. Association between sexual behaviour, colouration, sperm traits, and reproductive fitness have already been established, so these traits can be used as reliable proxies for fertility and fecundity and for overall reproductive fitness, in terms of successfully obtaining a mating (pre-mating success) and siring offspring (post-mating success). For example, males have higher reproductive fitness when they are more attractive to females (especially regarding their orange colouration) and have more and faster sperm (Devigili et al., 2015; Evans and Pilastro, 2011; Boschetto et al., 2011). Guppies have been used for many reproductive toxicology studies (Li et al., 2019; Saaristo et al., 2021), also because of their reproductive behaviour that represents relevant endpoints in this field (Söffker and Tyler, 2012; Bertram et al., 2015; Bertram et al., 2018; Tomkins et al., 2017). Male guppies can employ two alternative mating tactics, either courting females by performing courtship displays (sigmoid displays) (Houde, 1997) or forcing copulations by thrusting their modified anal fin (the gonopodium) into the female's genital pore, a behaviour called gonopodial thrust (Liley, 1966). *P. reticulata* is also a promising model for whole-transcriptional studies with increasing genomic and transcriptomic resources available (Fraser et al., 2011; Saaristo et al., 2017; Sharma et al., 2014). We exposed adult males to ecologically relevant concentrations of PFOA (legacy PFAS), GenX (newly emerging PFAS), or a control for 3 weeks to recreate a sub-acute exposure to the pollutants. We then assessed several reproductive traits at pre- and post-mating levels (including attractiveness, sexual behaviour, and sperm traits) and performed transcriptomic analysis to study the changes in gene expression in the testes associated with PFAS exposure.

2. Methods

2.1. Experimental overview

Fish were exposed for 21 days in a static system to ecologically relevant concentrations of PFOA, GenX, or a control. No solvents were used (more details are provided in Section 2.2). At the end of the exposure, we assessed a range of reproductive traits in males, including colouration, sexual behaviour, and sperm characteristics. After that, the fish were sacrificed to measure gene expression in the testes, the bioconcentration level (in the whole fish), and the gonadosomatic index. Water was sampled for chemical analysis at the beginning, after one week, and at the end of the exposure (days 0, 7, and 21, respectively) to ensure the effectiveness of our treatment and to confirm that chemical levels were maintained at our target concentration throughout the exposure period. This experiment was conducted according to the Italian legal requirements and was approved by the Ethics Committee of the University of Padova (permit no. 1059/2020-PR).

2.2. Chemicals

Perfluorooctanoic acid (PFOA 95 %; CAS number: 335–67-1) was purchased from Sigma-Aldrich (Milan, Italy), and ammonium perfluoro (2-methyl-3-oxahexanoate) (GenX or HFPO-DA 100 %; CAS number: 620–37-80–3) was purchased from Synquest Laboratories (Alachua, FL, USA). For each compound, a stock solution and a 1000X working solution were prepared. Stock solutions (1000 mg/L; 1000 ppm) were prepared in deionized water, as solvents such as DMSO have been shown to interfere with the analysis of PFAS toxicity (Polverino et al., 2019; Gasparini et al., 2019). The working solution (1 mg/L; 1 ppm) was obtained by diluting the stock solution and was subsequently used to spike the water. Both stock and working solutions were stored at + 4 °C in plastic bottles. Tricaine methanesulphonate (MS-222) was purchased from Sigma-Aldrich. All solvents employed for the LC-MS/MS analysis

were of liquid chromatography–mass spectrometry (LC-MS) grade: methanol, ammonium acetate, formic acid, hydrochloric acid, and sodium hydroxide were purchased from Sigma-Aldrich, while ultrapure water was freshly produced in-house on each day of analysis (Millipore, Milano, Italy). The pure standard of $^{13}C_4$-perfluorooctanoic acid (MPFOA; CAS number: 960315–48-4) was purchased from Wellington Laboratories (Guelph, Ontario, Canada).

2.3. Fish maintenance and PFAS treatment

The fish used in this experiment were descendants of wild-caught individuals from the Lower Tacarigua River in Trinidad. They were maintained in large stock tanks (with an approximately equal sex ratio) at a temperature of 26 ± 1 °C on a 12:12 h light:dark regime and fed a mixed diet of dry food and *Artemia salina* nauplii twice a day. The experimental tanks were rectangular plastic tanks sold for fish rearing (41 x 26 x 30 cm) filled with 15 L of freshwater and maintained at the same temperature, photoperiod, and feeding regime as the stock populations. To obtain a final concentration of 1 μg/L of PFOA or GenX (2.41 and 3.03 nanomolar, respectively) in the experimental tanks, 15 mL of PFOA or GenX stock solution (1 mg/L) was added to each experimental tank; the same amount of water was added to the control tanks. No filter was used in the experimental tanks to ensure that the chemical levels were maintained as appropriate. Each tank was equipped with a plastic plant for shelter, and a printed photograph of gravel was positioned underneath the tank to give the fish the impression of a normal gravel bottom while avoiding the problem of using real gravel, which would have interfered with cleaning and water exchange operations. For logistical reasons, the experiment was conducted in 5 blocks (3 tanks, one for each treatment, per block), each starting two weeks after the previous one. A total of 150 males (6–8 months old) were used in the experiment (50 males in each treatment). In each experimental tank, 10 adult males and 5 adult females were added the day after the experimental tanks were set up; females were not considered in this project, but their presence was needed to keep males sexually active and ensure normal production of sperm (Bozynski and Liley, 2003; Gasparini et al., 2009). During the exposure period, we established a routine of weekly cleaning along with 50 % water replacement; the water re-added was either control water or water with a 1 μg/L concentration of PFOA or GenX, according to the treatment group. Survival was monitored at least twice a day throughout the experiment.

2.4. Body size and colouration

At the end of the exposure period, males were weighed (in mg) and photographed to assess male body size (standard length, in mm) and male colouration (amount of orange spots, as a percentage of the body area). Details on how these measures were taken are given in the Supporting Information. Even though males were randomly assigned to either one of the three experimental groups, body size and colouration were also assessed before the start of exposure to ensure that there were no differences among groups. As expected, no significant differences in these biometric measurements were detected among groups (all $P > 0.40$). At the end of the experiment, a subsample of males ($n = 120$) was sacrificed, weighed, and dissected to obtain testis weight and calculate the gonadosomatic index (GSI = (gonad weight/body weight) × 100 %).

2.5. Sexual behaviour

Sexual behaviour was assessed in 25 males per treatment using an established behavioural assay (for an extensive description, see (Houde, 1997; Polverino et al., 2019). Sexual behaviour measured in guppies has already been used in ecotoxicology studies, showing effects of pharmaceutical pollutants on behaviour (e.g., (Bertram et al., 2015; Bertram et al., 2018; Tomkins et al., 2017)). Behavioural trials took place between 08:00 and 12:00, which corresponds to the peak of sexual activity in this species (Houde, 1997). An 8 L (29 x 39 x 32 cm) observation tank was used, in which an unreceptive female from the stock population (not experimental) was placed and allowed to settle for at least one hour. The use of unreceptive (pregnant) females is important as they do not respond to males' sexual attentions, providing a standardized behavioural response for the males. Females of similar size (standard length) were chosen across different trials. An experimental male was placed in the observation tank and allowed to settle for five minutes, or until he started showing sexual interest in the female (i.e., following the female or engaging in sexual activity), before starting data collection. The ID of each fish was re-coded (without references to the treatment) before the fish was tested so that the behavioural tests were conducted blind to the identity and treatment of the fish. Male sexual behaviour was observed for 15 min and recorded as the number of courtship displays (sigmoid displays: the male arches his body in a characteristic s-shaped posture and quivers in front of the female) and the number of forced mating attempts (gonopodial thrusts: the male approaches the female from behind and attempts to copulate without prior courtship or female cooperation). The total number of mating attempts was calculated as the sum of courtship displays and forced mating attempts. Male sexual interest was measured as the time (in seconds) that the male spent actively following the female within two body lengths.

2.6. Sperm traits

Sperm traits were assessed in the afternoon, after the behavioural assay, following established protocols (for details, see (Gasparini et al., 2019)). These traits were also assessed blind to the treatment, as for the behavioural tests. Each male was anaesthetized in MS-222, and sperm was collected in a physiological solution (0.9 % NaCl) after applying gentle pressure on the male's abdomen. Several aspects of sperm production and quality were considered: (i) sperm swimming performance, (ii) sperm viability, and (iii) sperm number. Sperm performance was assessed using a computer-assisted sperm analysis (CASA) sperm tracker (CEROS, Hamilton-Thorne Research, Beverly, MA, USA) after activating the sperm with 150 mM KCl. Sperm performance includes sperm swimming velocity, which is positively correlated with competitive fertilization success (Boschetto et al., 2011). Sperm velocity measurements were based on an average of 170.02 ± 3.63 SE sperm tracks per male. Sperm number and sperm viability were assessed using an automated cell counter (Luna-FL Dual Fluorescence Sperm Cell Counter, Logos Biosystems) after the sperm was diluted to an appropriate concentration following the manufacturer's instructions. Sperm viability was assessed by dying sperm with a membrane-permeant nucleic acid stain (acridine orange), which labelled live sperm in green, and a membrane-impermeant stain (propidium iodide), which labelled dead or damaged sperm in red.

2.7. RNA-seq library preparation and sequencing

Testes ($n = 36$) were collected and stored in 200 μL of RNA/DNA Shield (Zymo Research, CA, Irvine, USA) at −80 °C until processing. Total RNA was isolated using the Direct-zol RNA MicroPrep kit (Zymo Research), following the manufacturer's instructions; total RNA concentration was then determined using a Qubit RNA BR (Broad-Range) kit in a Qubit 4.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA quality was assessed using a 4150 TapeStation System (Agilent Technologies, Waldbronn, Germany). All samples had an RNA integrity number > 7. Equal amounts of RNA from 4 testes collected from fish of the same experimental group were pooled; a total of 9 RNA pools were obtained (i.e., 3 pools per experimental group). Nine tagged RNA-seq libraries were prepared using the Illumina TruSeq Stranded mRNA kit and sequenced on an Illumina NovaSeq 6000 instrument at the NGS Sequencing Core (Padova, Italy) following a 100 bp paired-end approach.

2.8. Chemical analysis

Until being processed, water and fish samples were stored at + 4 and −20 °C, respectively. GenX and PFOA concentrations in water and fish were measured using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Details about the LC-MS/MS analysis are presented in the Supporting Information.

2.9. Statistical methods

2.9.1. Survival

Kaplan–Meier survival curves were compared among groups using the log-rank (Mantel–Cox) test and visualized using GraphPad Prism 8.0 (GraphPad, USA).

2.9.2. Morphological, behavioural, and sperm traits

Traits across the three treatment groups were compared using ANOVAs. Data were checked for normality (Shapiro–Wilk normality test) and homogeneity of variance. Log transformation was used where appropriate (see Results). When violations of a normal distribution and/or homogeneity of variance were not removed by the log transformation, the corresponding nonparametric test was used (Kruskal–Wallis test). CASA provides three measures of sperm velocity (VAP, VCL, and VSL), which, in guppies, are highly correlated (in the present study, the correlation coefficients ranged between 0.69 and 0.98 and were all highly significant, $P < 0.001$, $n = 62$). Following an established procedure (e.g., (Devigili et al., 2016)), we obtained from these three measures a composite measure of sperm velocity using principal component analysis (PCA). We extracted one factor that explained 87.3 % of the variance (factor loadings: VAP = 0.984, VSL = 0.952, VCL = 0.861) and that was used in the subsequent analyses (positive PCA values correspond to faster sperm, whereas negative values correspond to slower sperm). Sperm viability (the number of live sperm cells divided by the total sperm count per male) was analysed using a generalized linear model, with a binomial error distribution and a logit link function. Statistical analyses were performed using SPSS 29.

2.9.3. RNA-seq data

Initial quality control was performed using FastQC software version 0.11.9 (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data, (n.d.). https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed October 12, 2023)). Read trimming and adapter removal were performed using Trimmomatic (version 0.39) with default parameters (Bolger et al., 2014). Reads shorter than 36 bp were excluded from the analysis. We used STAR (version 2.5.3a) in two-pass mode (Dobin and Gingeras, 2015) to count the reads mapping against the guppy reference genome (GCA_000633615.2) using the following parameters: maximum number of allowed mismatches = 8, maximum number of loci the read was allowed to map to = 10. Reads trimmed and cleaned as described above were then mapped against the guppy reference genome using Kallisto (version 0.46.2), a program that differs from other read mappers in that it rapidly maps reads using pseudoalignment, while preserving accuracy (Bray et al., 2016). Details about the differential expression (DE) analysis, and the following gene ontology (GO) enrichment and gene set enrichment analysis (GSEA), are presented in the Supporting Information.

Considering that the guppy reference genome has been assembled starting with female sequences only, we performed an *ad hoc* DE analysis to search for differentially expressed genes (DEGs) among male-specific genes. To this end, we performed the same analysis as above, but we mapped the reads against the 118 putative male-specific transcripts assembled in (Cattelan et al., 2020).

3. Results

3.1. Survival

Exposure to PFOA and GenX did not significantly affect male survival, with very similar rates of survival (i.e., control: 94 %; GenX: 90 %; PFOA: 86 %) across the three experimental groups ($P = 0.4065$, chi-square = 1.8, df = 2; Figure S1).

3.2. Chemical analysis

Concentration of PFOA and GenX were measured in water at days 0, 7, and 21 in fish after 21 days of exposure. Our untended treatment setup was confirmed by water analysis, as the concentrations of PFOA and GenX in water were close to the nominal concentrations throughout the exposure period (Figure S2). The average concentrations at the end of the experiment were 0.94 ± 0.07 μg/L for the PFOA nominal value of 1 μg/L and 0.90 ± 0.04 μg/L for the GenX nominal value of 1 μg/L. The chemical analysis conducted on whole-body fish revealed that the average concentrations of PFOA and GenX at the end of the experiment were 2.22 ± 0.41 μg/kg and 0.84 ± 0.36 μg/kg, respectively (Fig. 1). Based on these measured concentrations, we calculated a bioconcentration factor (BCF) of 2.36 for PFOA and 0.93 for GenX.

The concentration of PFOA and GenX in water (and fish) collected from the control tanks at the end of the exposure was below the limit of detection, indicating that there was no evidence of any release of PFOA or GenX from the tanks.

3.3. Male colouration, body size, and GSI

After the treatment, none of the male traits was significantly affected by the treatment: body size (SL): $F = 1.474$, df = 2,132, $P = 0.233$; body weight (BW, log-transformed): $F = 0.035$, df = 279, $P = 0.966$; relative area of orange: $H = 0.434$, $n_1 = 3$, $n_2 = 135$, $P = 0.805$ (Kruskal–Wallis test); GSI: $F = 0.554$, df = 270, $P = 0.577$. Descriptive statistics for these traits are reported in the Supplementary Information (Table S1).

3.4. Sexual behaviour

Male sexual behaviour was significantly affected by the treatment, but only in the GenX group. The overall sexual activity (time following

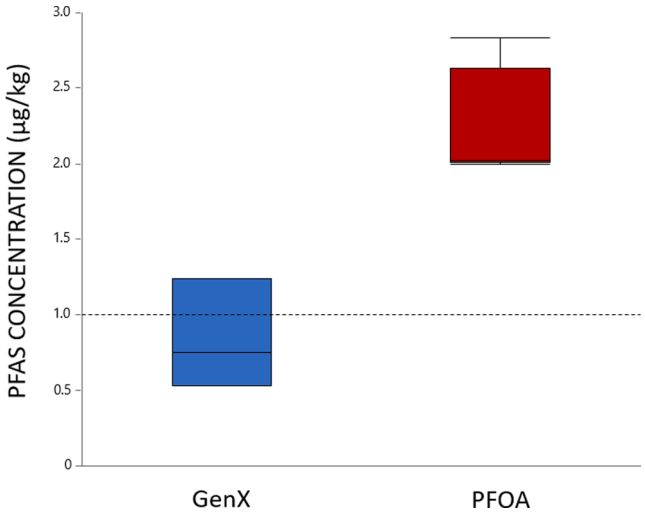

Fig. 1. PFAS bioconcentration. PFOA and GenX concentrations in the whole-body fish at day 21. Results are expressed as box-and-whisker plot (median and quartiles). The dashed line indicates the concentration of PFAS in the water. GenX; n = 3; PFOA, n = 4. Each sample is constituted by a pool of whole body fish (approximately 2 g in total).

the female and the total number of mating attempts) did not vary across groups (time following the female: H = 4.228, n_1 = 3, n_2 = 68, P = 0.121, Kruskal–Wallis test; total mating attempts: H = 2.283, n_1 = 3, n_2 = 68, P = 0.319). However, GenX males performed significantly more courtship displays than control males both in absolute terms (H = 8.128, n_1 = 3, n_2 = 68, P = 0.017, Kruskal–Wallis test; post-hoc difference CRTL-GenX: H = 15.304, P = 0.021; CTRL-PFOA: H = 3.150, P = 1.00; Fig. 2) and in relation to the total number of mating attempts (H = 12.984, n_1 = 3, n_2 = 61, P = 0.002, Kruskal–Wallis test; post-hoc difference CRTL-GenX: H = 19.00, P = 0.001; CTRL-PFOA: H = 5.831, P = 0.876). Descriptive statistics are reported in Supplementary Table S1.

3.5. Sperm traits

Treatment with GenX and PFOA significantly reduced sperm swimming velocity compared to the control (H = 12.767, n_1 = 3, n_2 = 66, P = 0.002, Kruskal–Wallis test; post-hoc difference CRTL-GenX: H = 19.955, P = 0.002; CTRL-PFOA: H = 14.682, P = 0.034; Fig. 3), whereas there was no effect on sperm production (H = 1.355, n_1 = 3, n_2 = 67, P = 0.508) and sperm viability (Wald chi-square = 0.745, df = 2, P = 0.698). Descriptive statistics are reported in Supplementary Table S1.

3.6. Transcriptome differential expression analysis

A total of 236,385,339 paired-end reads were sequenced, with an average of 26.26 million reads per sample. Raw reads were mapped against the guppy reference genome, and the overall mapping percentage was above 98 % for all the samples. The number of raw reads passing the filters and the number of filtered reads mapping to the guppy genome are provided in Table S2. The differential expression (DE) analysis identified a total of 86 genes with significantly different mRNA levels between control and GenX-treated fish. As to PFOA, the total number of DEGs, compared to controls, was 107. The two lists of significant DEGs (Table S3) were intersected to obtain DEGs shared (i.e., 43) by the two treatments (Fig. 4a and 4b). Of the 118 male-specific genes, 2 genes were significantly downregulated by PFOA (Table S3). The annotation available for these PFOA-regulated male-specific genes does not provide clues on their function (i.e., they are uncharacterized loci). As for GenX, no significant male-specific DEGs were found.

Three out of the 43 shared DEGs between GenX and PFOA were downregulated by both chemicals. The most significant one was *jumonji and AT-rich interaction domain containing 2* (JARID2B; LFC_{GenX} = -0.92, LFC_{PFOA} = -0.81), which is known to regulate gonad differentiation and

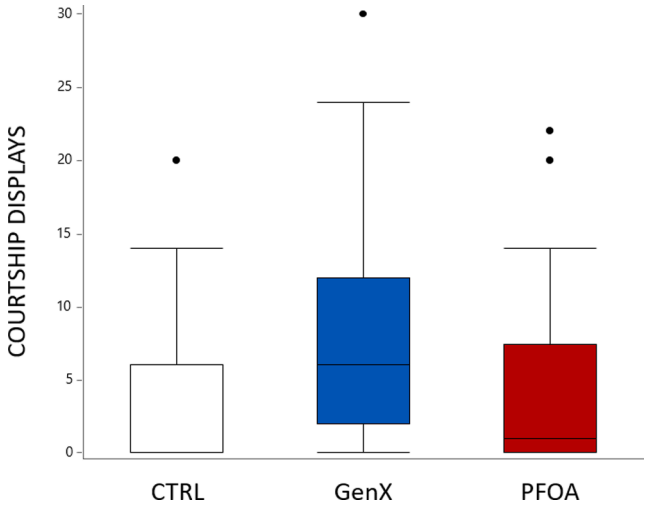

Fig. 2. Courtship displays. Box-and-whisker plot (median and quartiles) showing the number of courtship displays in the three experimental groups: CTR, n = 23; GenX, n = 23; PFOA, n = 22. Points represent outliers.

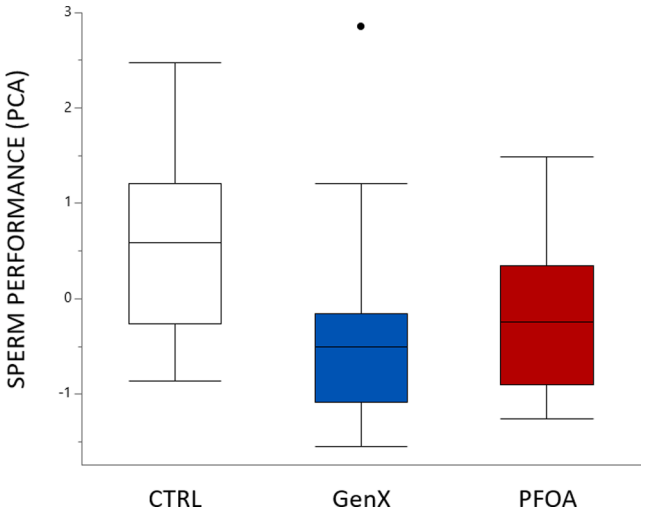

Fig. 3. Sperm performance. Box plot (median and quartiles) showing PCA of sperm performance (details in the text) assessed in the three experimental groups: CTRL, n = 22; GenX, n = 22; PFOA, n = 22. Points represent outliers.

embryonic development. Among the shared upregulated DEGs (Fig. 4a), we found apoptosis-associated genes, such as *caspase recruitment domain-containing protein 9* (CARD9; LFC_{GenX} = +1.62, LFC_{PFOA} = +1.49), *TNFα-induced protein 2* (TNFAIP2; LFC_{GenX} = +1.85, LFC_{PFOA} = +1.43), and *protein-glutamine gamma-glutamyltransferase 2-like* (TGM2-like; LFC_{GenX} = +1.59, LFC_{PFOA} = +1.84). Several immune-related genes were also upregulated, such as *cathepsin S* (CTSS; LFC_{GenX} = +1.49, LFC_{PFOA} = +1.24), *complement C1s subcomponent like* (C1S-like; LFC_{GenX} = +0.66, LFC_{PFOA} = +0.75), and the ones coding for the MHC class II molecules: *H-2 class II histocompatibility antigen, A-U alpha chain-like* (H2-Aa; LFC_{GenX} = +1.07, LFC_{PFOA} = +1.57), *H-2 class II histocompatibility antigen, E-S beta chain-like* (H2-Eb1; LFC_{GenX} = +1.17, LFC_{PFOA} = +1.45), *HLA class II histocompatibility antigen,* and *DP alpha 1 chain-like* (HLA-DPA1; LFC_{GenX} = +1.02, LFC_{PFOA} = +1.05). An upregulated gene supporting inflammation was *granulins-like* (LFC_{GenX} = +1.54, LFC_{PFOA} = +3.38). *Apolipoprotein Eb-like* (APOEB), a gene of cholesterol biosynthesis (including sex hormones), was significantly upregulated (LFC_{GenX} = +1.04, LFC_{PFOA} = +1.99). Likewise, the expression of genes constituting the blood–testis barrier, such as *claudin-4 like* (CLDN4-like; LFC_{GenX} = +2.30, LFC_{PFOA} = +2.17) and *connexin 32.3* (CX32.3; LFC_{GenX} = +4.29, LFC_{PFOA} = +5.40), appeared to be promoted by PFAS. Interestingly, also *glutathione peroxidase 1-like* (GPX1B) was upregulated by both GenX (LFC = +0.98) and PFOA (LFC = +0.87). This relevant overlapping between the transcriptional effects of the two treatments was confirmed by the functional analyses (Figs. 5 and 6, Tables S4 and S5). Both the GO enrichment and the GSEA yielded very similar results for the two treatments, especially for the GO terms related to the immune response. Also, some GO terms related to chemotaxis were enriched among both significant GenX and significant PFOA DEGs, although in this case, the genes regulated by the treatments were not identical. GenX significantly upregulated *C-X-C motif chemokine receptor 4* (CXCR4; LFC = +1.53), *permeability factor 2-like* (CXCL19; LFC = +3.01), and *C–C motif chemokine receptor 9* (CCR9; LFC =+1.96). PFOA upregulated *C–C chemokine receptor type 3-like* (CCR3; LFC = +5.10) and *chemokine (C–C motif) receptor 12a* (CCR12A; LFC = +1.30). Furthermore, several genes exerting their role in the extracellular matrix (ECM), which is implicated in the regulation of spermatogenesis and hormone synthesis, were modulated by both GenX and PFOA, such as *fibronectin-like* (FN1A; LFC_{GenX} = +1.36, LFC_{PFOA} = +1.75), *collagen VII alpha 1 chain* (COL7A1; LFC_{GenX} = -1.98, LFC_{PFOA} = -1.36), *periaxin* (LFC_{GenX} = -0.72, LFC_{PFOA} = -0.77), and other genes mentioned above (e.g., *granulins* and APOE).

As to the transcriptional changes triggered by GenX only (Table S3),

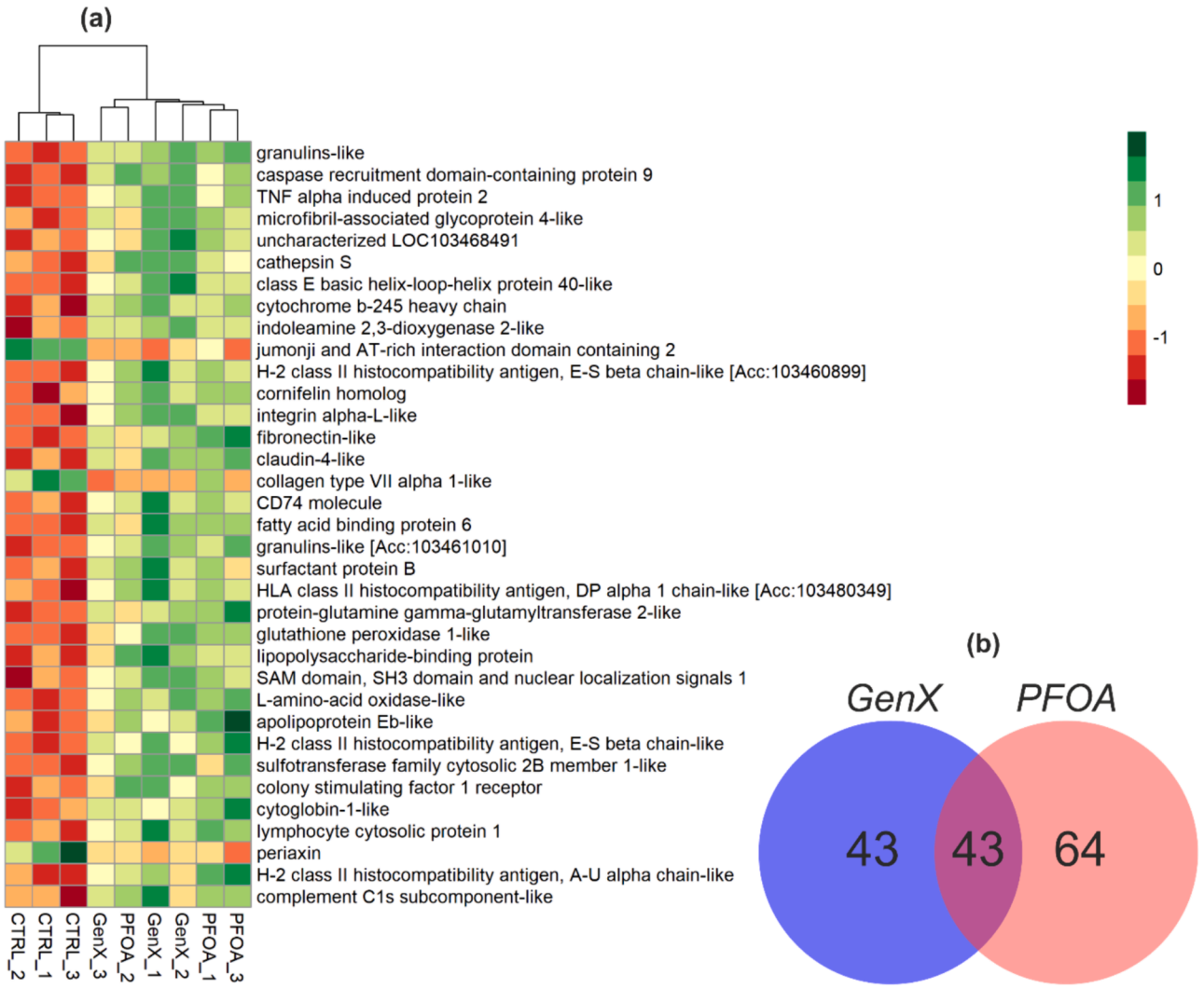

Fig. 4. Differentially expressed genes (DEGs). Heatmap reporting the normalized expression levels (expressed in $\log_2$ of estimated counts from the original kallisto quantification) of shared DEGs (**a**). Venn diagram highlighting the number of DEGs shared by GenX and PFOA treatments (**b**). Uncharacterized proteins (n = 9) have been removed.

a large number of DEGs play immune functions, such as *CD276 antigen-like*, *interferon regulatory factor 1* (IRF1; LFC = +1.00) and *7* (IRF7; LFC = -1.28), *TNF superfamily member 12* (TNFSF12; LFC = -0.86), *fish-egg lectin-like* (FEL; LFC = +2.47), *interferon-induced GTP-binding protein Mx-like* (MX1; LFC = -1.81), *SH2 domain-containing protein 1A-like* (SH2D1A; LFC = +1.83), *coronin-1A-like* (CORO1A; LFC = +0.95), and *interferon alpha-inducible protein 27-like protein 2B* (IFI27L2B; LFC = -1.82). Several DEGs were involved in inflammatory processes, such as *collagenase 3-like* (MMP13; LFC = +4.23), and antioxidant activity, such as *negative regulator of reactive oxygen species* (NRROS; LFC = +1.12). Interestingly, *glutamate decarboxylase 1-like* (GAD1) and *serotransferrin* (TF), which are possibly involved in fish reproduction and sperm quality, were both downregulated with an LFC value of -0.75 and -0.65, respectively. A further gene regulated by GenX and involved in sperm quality, particularly in motility, was *calmodulin 2* (CALM2; LFC = +0.76).

Regarding the transcriptional changes induced solely by PFOA treatment (Table S3), the data obtained demonstrated the activation of genes of the complement classical pathway, i.e., *complement C1q subcomponent subunit A*-like (C1QA; LFC = +0.88), *complement receptor type 1-like* (CR1; LFC = +0.64), *complement receptor type 1*-like, and *complement C1q subcomponent subunit B*-like (C1QB; $LFC_{PFOAvsCTRL}$ = +0.75). Genes involved in lipid binding and metabolism were also significantly upregulated, such as *apolipoprotein C1* (APOC1; LFC = +3.58), *large neutral amino acids transporter small subunit 4-like* (SLC43A3; LFC = +0.78), and *galactosylceramide sulfotransferase-like* (GAL3ST1 or CST; LFC = +2.05). Moreover, some genes that function in response to calcium were modulated by PFOA, e.g., *parvalbumin beta-like* (PVALB5; LFC = +1.24), *protein S100-A1-like* (S100A1; LFC = +0.89), and *polycystic kidney disease 2-like 1 protein* (PKD2L1; LFC = -0.97). Finally, PFOA significantly induced the expression of neurohormone *relaxin-3-like* (RLN3; LFC = +0.77) and *metallothionein 2* (MT2; LFC = +1.34), which plays a role in cellular defence against reactive oxygen species. Interestingly, PFOA also induced the expression of *cytochrome P450 3A40-like* (CYP3A40; LFC = +0.80), which has an estrogenic modulation in male fish.

4. Discussion

In the present study, using a vertebrate model species, *Poecilia reticulata*, we investigated the impact of a short exposure to PFAS at ecologically relevant concentrations on a range of fitness-related reproductive traits and the associated changes in testes' gene expression. Specifically, we looked at two PFAS, one legacy PFAS (PFOA) and one newly emerging PFAS (GenX). Overall, our work demonstrates that both PFAS can be detected in the fish and have effects on reproduction at the phenotypic and molecular levels, but with compound-specific

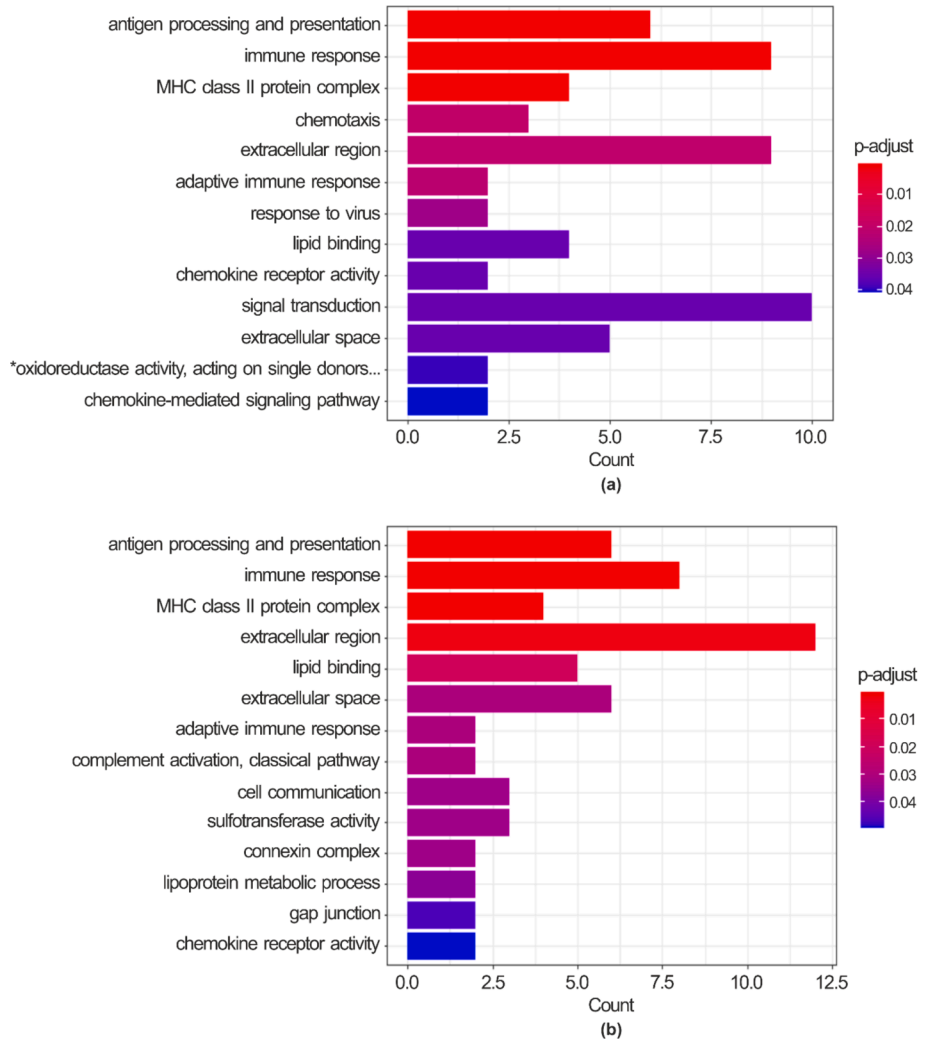

Fig. 5. Gene Ontology (GO) enrichment analysis of genes significantly regulated by GenX (**a**) and PFOA (**b**). The X-axis reports the number of genes representing each GO term. The colour gradient corresponds to the level of significance that is adjusted with the false discovery rate method. *oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen.

effects. Our study contributes significantly to the understanding of PFAS pollution in aquatic environments with three main elements. First, our findings indicate that even a short exposure can affect fertility and, specifically, reduce sperm velocity. Second, ecologically relevant concentrations of PFAS may have farther-reaching implications for aquatic organisms than previously thought, as we showed significant gene expression changes in the testes, and these effects can reverberate for a long time after the exposure has ended. Third, worldwide pollution by PFAS continues, with compounds demonstrated to be toxic (such as PFOA) replaced by new compounds, whose toxicity is still unknown (such as GenX), and our study shows that these new chemicals can be as detrimental to fertility as the old ones. Surprisingly, PFAS reproductive toxicity has scarcely been studied in fish; however, it is extremely relevant, as pollution by PFAS impacts aquatic organisms more than other organisms and freshwater aquatic ecosystems are already fragile and particularly vulnerable to pollutants. Although some work (in vivo) has been performed in fish, the experiments did not focus on reproduction (Sun et al., 2023; Wasel et al., 2022; Gaballah et al., 2020; Adedara et al., 2022), which is recognized as one of the primary targets of PFAS pollution (see Introduction), or used concentrations of PFAS exceeding ecologically relevant ones (Miranda et al., 2020; Kang et al., 2019; Godfrey et al., 2019; Lee et al., 2017). Previous studies showed that PFAS exposure induces endocrine-disrupting effects in *Oryzias latipes*; such as decreased fertility and fecundity, and dysregulation of key genes controlling reproduction (e.g., vitellogenin) (Kang et al., 2019; Godfrey et al., 2019; Lee et al., 2017). Although these studies contribute to the understanding of PFAS toxicity, their results should be interpreted with caution and are difficult to compare with ours, as we used ecologically relevant concentrations similar to those that may be experienced by fish in their natural environment. Indeed, the concentration of PFAS we used (1 μg/L) is similar to the concentration that can be experienced by freshwater fishes in surface waters of highly polluted areas, such as the Yangtze River (China), where the sum of PFAS is in the range of 0.1 to 2 μg/L (Hua et al., 2023), or some surface waters in Northern Italy, where the sum of PFAS reaches 10 μg/L (Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto, ARPAV; data collected in 2023). It is worth noting that, while PFAS concentrations in global surface waters may reach several hundred micrograms per liter (Sims et al., 2022), these levels are typically found in heavily polluted waters (such as those near industrial discharges of PFAS). In contrast, in typical freshwater ecosystems, PFAS concentrations usually range from 0 to 1 μg/L.

Below, we will discuss the main results grouped into three categories: survival and effects on body size (Section 4.1); bioconcentration (Section 4.2); reproductive traits, including sperm traits and sexual behaviour (Section 4.3); and changes in gene expression (Section 4.4).

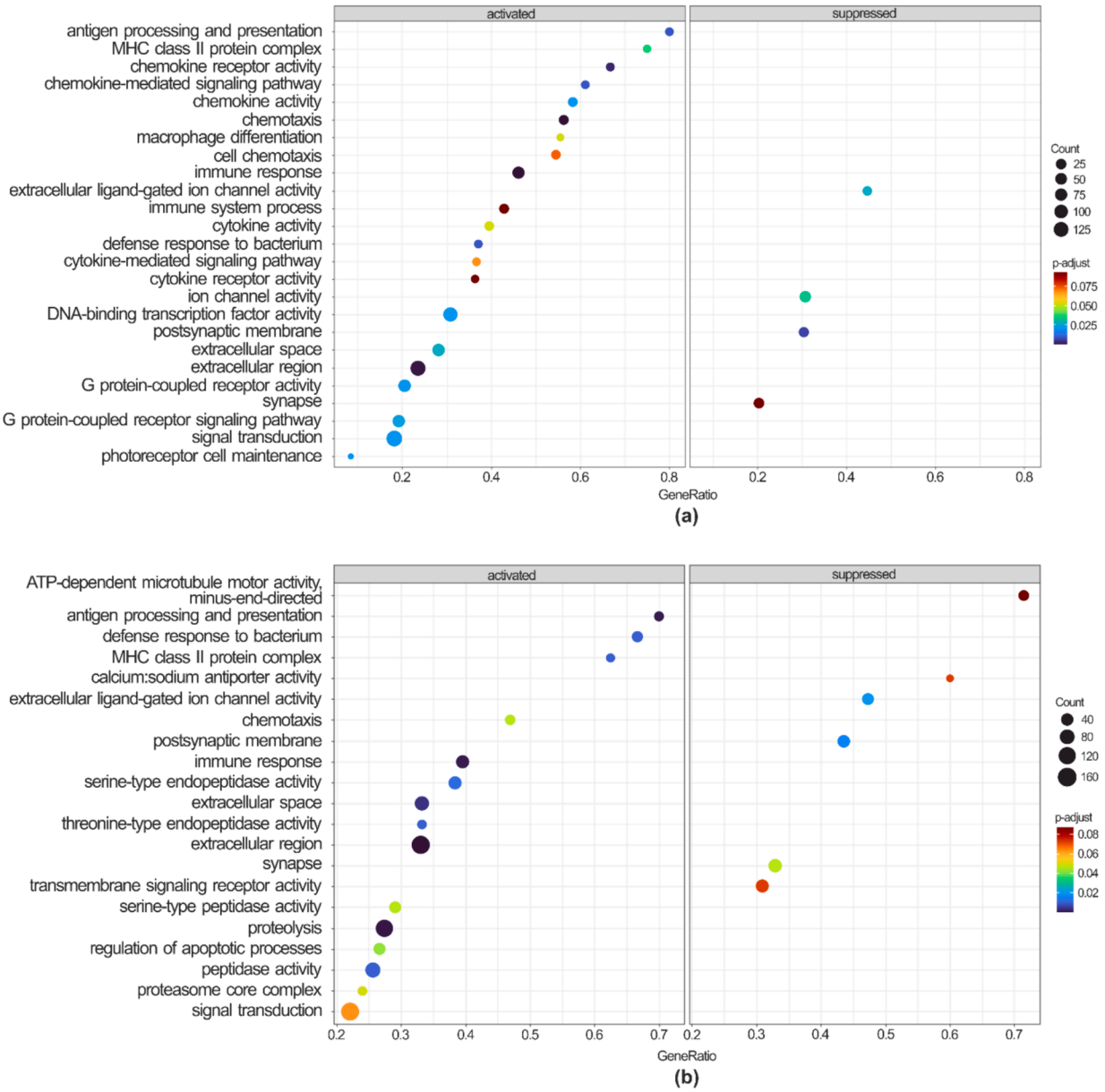

Fig. 6. Gene Set Enrichment Analysis: GenX vs CTRL **(a)** and PFOA vs CTRL **(b)**. The dot size represents the number of genes belonging to each GO term. The colour gradient is related to the level of significance that is adjusted with the Benjamini-Hochberg method. The box on the left collects activated pathways, while the box on the right the suppressed ones.

4.1. Survival and body size

Exposure to PFOA and GenX did not induce any significant mortality effects. This confirms that our exposure level, in terms of concentration and duration, was, as we expected, sublethal. Body size and weight were likewise not affected by PFAS exposure, suggesting that any effect on reproductive traits is unlikely to be mediated by overall health conditions, which should have been associated with a decrease in body weight or body size (as in males of this species exposed to a restricted diet (Devigili et al., 2013; Gasparini et al., 2013)).

4.2. Bioconcentration

The bioconcentration potential of PFAS grows with increasing molecular chain length (Gomis et al., 2018) and the octanol/water partition coefficient (K_{ow}), which is estimated to be higher for PFOA than for GenX (Martz et al., 2019; Hopkins et al., 2018). Therefore, PFOA was expected to bioconcentrate more than GenX. In measuring the toxicity potency of chemicals, it is extremely important to consider their bioconcentration. Toxic endpoints at specific concentrations are commonly used to measure the toxicity potency of PFAS, thus allowing us to compare and rank them for their ability to cause a specific toxic effect. However, this approach might result in misleading conclusions on PFAS toxicity potency and the hazard posed to human health and/or the environment. Indeed, differences in toxicokinetics have to be taken into account, since the relationship between administered dose and toxic response is in part also defined by the a substance's absorption, distribution, metabolism, and elimination, which determine the bioconcentration level. In the present study, only PFOA bioconcentrates, but its detrimental effects at the level of sperm velocity were lower than those of GenX. Thus, even though PFOA had a higher whole-body concentration than GenX (about two-fold), it was less toxic than GenX. We suggest that, based on the reproductive effects we observed in male guppies (i.e., effects on sexual behaviour and sperm velocity; see below),

the GenX toxicity potency might be higher than that of PFOA. It is worthy of note that previous studies conducted in rats and fish demonstrated that legacy and alternative PFAS do not differ much in potency when toxicokinetics are examined (Gomis et al., 2018; Satbhai et al., 2022). To further confirm that GenX is more toxic at the reproductive level than PFOA, additional aspects should be taken into account, such as a tissue-specific bioconcentration (i.e., a potentially preferential bioconcentration of GenX or PFOA in a specific tissue). This aspect is also relevant when considering the transcriptional effects occurring in target tissues, as they are expected to increase with increasing bioconcentration.

As a general comment, the PFOA whole-body BCF calculated in the present study is lower compared to the mean values so far reported in fish, but it falls within a range of values similar to BCFs previously observed (Burkhard, 2021). It's worth mentioning that PFOA, unlike other long-chain PFAS such as PFNA and PFDA, generally exhibits low BCFs or BAFs (bioaccumulation factors), and similar to those reported for short-chain PFAS. In addition, BCFs/BAFs vary from species to species depending on body composition, size, and exposure concentration (Lewis et al., 2022). The low BCF that we have observed can be in part attributed also to the controlled short-time exposure. Higher BCFs are typically expected following longer exposures or when the exposure occurs in the natural setting, potentially leading to higher BAFs. Indeed, BAF is the equivalent of BCF but in the field and it reflects all the possible exposure routes in the environment, e.g., dietary, water, and contact with sediments (Burkhard, 2021).

4.3. Reproductive traits

Sexual activity was similar across all treatments, but GenX-exposed males showed a shift in the preferred mating tactic used, relying more on courtship displays (both in absolute and relative terms) than on sneaky (forced) copulation attempts. This may have important fitness repercussions. To successfully reproduce, a male has to find a female, judge her quality in terms of reproductive return, and carefully invest in her to obtain a mating. This last aspect often involves expensive courtship behaviours that are traded off against other energy-demanding activities, including the pursuit of obtaining other matings and basic housekeeping activities, such as foraging, escaping predators, and fighting pathogens. Making a strategic allocation of resources among different tasks, or among pre- and post-mating stages (i.e., investing in obtaining a mating or investing at the gamete level), is therefore essential for maximizing reproductive fitness in any species (Edward and Chapman, 2011). A short exposure, such as that in our study, to chemicals (other ones than we used), drugs, and temperature has been reported to disrupt male mating behaviour in the guppy and cause a change in male sexual behaviour (e.g., (Breedveld et al., 2023; Fursdon et al., 2019)). Because guppies can choose among alternative mating tactics at any time, with different energy requirements and fitness returns (Evans and Pilastro, 2011; Rios-Cardenas and Morris, 2011), this species is ideal for exploring subtle resource and energy allocation due to sublethal stress, such as that caused by pollution. Interestingly, studies on guppies usually report a decrease in courtship behaviour in favour of the sneaky mating tactic when exposed to pollutants such as trenbolone (Bertram et al., 2015; Tomkins et al., 2017) or fluoxetine (Fursdon et al., 2019) for a similar period to that used in our study. One possibility is that the increase in the use of courtship displays we found is maladaptive, as behavioural tests were conducted with unreceptive females that did not respond to males due to their status. Under these conditions, males are expected not to court females, as increasing efforts in courtship displays would not lead to an increase in mating success because the females are unreceptive (Guevara-Fiore et al., 2010), so this change may be wasted energy. What this specific result means in terms of reproductive outcome or what pathways are affected in the cognitive processes that lead to this result will require further investigation. One possibility is that GenX damages the olfactory system so that the males cannot properly recognize the females' status anymore. A male's perception of female receptivity is indeed mediated not only by how the female responds to courtship but also by intraspecific chemical communication revealing female receptivity via olfactory cues (Guevara-Fiore et al., 2009). Overall, this unexpected finding for GenX underlines once more the importance of considering behaviour in addition to other endpoints when evaluating the impact of pollutants on organisms, especially because exposure to ecologically relevant concentrations may cause cryptic effects and because behaviour is particularly sensitive to even low levels of pollution (Bertram et al., 2022).

Sperm performance was negatively affected by both PFOA and GenX. Exposure to PFAS may directly affect mature (stored) sperm and/or interfere with the last stages of sperm maturation. The direct effect of PFAS on sperm quality could be attributable to PFAS entering the testes and causing direct detrimental effects on mature sperm. This hypothesis is corroborated by an interesting recent finding in humans, showing that PFAS can enter the testes and accumulate in the seminal fluid, decreasing sperm quality (Di Nisio et al., 2019). PFAS interfere with cell function through the alteration of plasma-membrane characteristics due to their similarity to fatty acids (Qiao et al., 2019). A study on in vitro exposure of ejaculate to PFAS (specifically PFOA) has recently confirmed that, in humans, PFAS accumulate in sperm membranes and can have a direct negative effect on sperm motility due to the impairment of metabolic performance (Šabović et al., 2020). Whether PFAS accumulate in the seminal fluid of guppies is unknown at the moment, but it may explain the results we have found. Another, not mutually exclusive, explanation is that PFAS can affect maturing sperm during the last stages of spermatogenesis. Similarly to other endocrine disruptors, such as BPA, PFAS are likely to impede sperm maturation, which may result in reduced sperm quality (Lahnsteiner et al., 2005). In addition, Sertoli cells are very sensitive to xenobiotics, and some chemicals, such as 4-octylphenol, have been shown to induce apoptosis of these cells, which are essential during spermatogenesis for their role in sperm nutrition and other functions, with negative consequences for sperm motility (Zhang et al., 2019). In our study, too, PFAS may have interfered with the function of Sertoli cells, causing a decline in sperm motility. The other sperm parameters we considered, namely the number of sperm cells produced, sperm viability, and GSI, were not affected by PFAS. This may indicate that PFAS act directly on mature sperm (as discussed above) but also that our short exposure has not caused the typical endocrine disruptive effects on sperm production, as we have exposed fish for less than a full spermatogenetic cycle (in this species about 36 days (Billard et al., 1969). It is therefore possible that a longer exposure will reveal negative effects on sperm production, but this requires an *ad hoc* study.

Whatever the mechanism underlying the damage to sperm performance caused by a short exposure to PFAS, the biological effect is clear and highlights that even a short exposure to PFAS can directly impair fertility. Indeed, decreased sperm velocity has consequences for fertility in competitive and non-competitive scenarios. The guppy is one of the vertebrates with the highest level of multiple paternity (Neff et al., 2008), so males constantly face high-risk and intense sperm competition (the competition among sperm from two or more males to fertilize a certain set of eggs) (Parker, 1970). Even a slight decrease in sperm velocity translates into lower reproductive success in this species (Boschetto et al., 2011), so the effect of PFAS exposure on sperm performance may be critical. Despite the attention to the effects of PFAS on reproductive traits, only a few studies, as far as we know, are looking at the effects of PFAS on sperm production in aquatic organisms (Wang et al., 2011; Wei et al., 2007), so there is a need to investigate this in other species to understand whether our findings can be generalized.

Overall, exposed fish did not show obvious signs of stress (no effect on weight, body size, colouration, or sexual activity) but did show changes in mating behaviour and sperm quality, suggesting that the effects of GenX and PFOA may at first glance be cryptic, but with important effects for fertility.

4.4. Gene expression

The number of genes whose expression was affected by the legacy compound PFOA was slightly greater than the number of genes affected by its alternative, GenX. However, a considerable number of DEGs were shared between the two treatments, indicating that GenX and PFOA, at the concentrations used, share several molecular targets. This was further confirmed by the functional analyses conducted on the complete lists of DEGs shared by GenX and PFOA, which highlighted the significant enrichment of very similar terms (e.g., those related to the immune response, inflammation, lipid binding, the extracellular matrix, and apoptosis).

4.4.1. Immune system

Fish exposed to GenX and PFOA showed a significant alteration in the expression of key genes involved in the immune response. The immune system is important in the testes, as they are characterized by the so-called 'immune privilege', a mechanism of increased tolerance to cells expressing antigens, such as spermatozoa (Chakradhar, 2018; Fijak and Meinhardt, 2006). Several PFAS commonly found in the environment, including the emerging ones, have mainly been reported to induce immunosuppression (Zhang et al., 2023). However, in the present study, we observed a transcriptional modulation that suggests an immunoenhancement rather than an immunosuppression.

Several MHC class II genes, such as H2-Aa, H2-Eb1, and HLA-DPA1, were upregulated. CTSS, too, was upregulated. The function of CTSS in teleosts, especially its immune role, is largely unknown, but in mammals it is highly expressed in immune cells, where it plays key roles in immune responses against microbial infection, such as antigen presentation (Hsing and Rudensky, 2005). The upregulation of these genes might indicate that PFAS activate antigen-presenting cells in the testes of guppy, ultimately resulting in a stimulation of the immune system. As testicular interstitial MHC class II-positive cells also contribute to the immunologically privileged microenvironment, there may be a potential immune modulatory effect of GenX and PFOA exposure on fish. A similar hypothesis has previously been postulated to explain the changes observed in mouse testes after chronic exposure to di-(2-ethylhexyl) phthalate. In that study, MHC class II-positive areas in the testicular interstitium of DEHP-exposed mice were significantly increased compared with the control group as evaluated by the immunohistochemical method (Kitaoka et al., 2013). Further actors of the immune response, so-called complement subcomponents, were particularly upregulated by PFOA. Complement activation by PFOA has previously been reported in the liver of mice exposed to PFOA (Botelho et al., 2015) and in that of the Medaka chronically exposed to low and environmental concentrations of a known endocrine-disrupting compound, bisphenol A (BPA) (Qiu et al., 2016). Pathogens, damaged cells, and toxic compounds activate the immune system, which in turn triggers inflammation. Therefore, in the present study, the observed upregulation of several genes involved in inflammatory pathways is likely a consequence of the immune system activation discussed above. BPA, too, has been demonstrated to induce an inflammatory state in rare minnow (*Gobiocypris rarus*) Sertoli cells (Tao et al., 2019). Nonetheless, among the adverse effects that have so far been attributed to PFAS, an alteration in the inflammatory state has often been reported (Zhang et al., 2023; Dunder et al., 2023), especially in rat Sertoli cells (Wan et al., 2020). Interestingly, an increase in MMP13 has been observed following exposure to both GenX and PFOA, which is considered a hallmark of the transition from acute to chronic inflammation in fish (Pedersen et al., 2015). A pro-inflammatory state in PFOA-exposed guppies is also suggested by the downregulation of *ceramide-1-phosphate transfer protein* (CPTP), a gene coding for a protein that regulates sphingolipid homeostasis in ways that impact programmed cell death and inflammation. Indeed, CTPT downregulation has been linked to inflammation (Gao et al., 2021). Our study is not the only one reporting an immunoenhancement by PFAS (Ehrlich et al., 2023). In several mouse tissues (liver, lung, kidney), PFOS is responsible for the activation of the innate immune system (Wang et al., 2021). Likewise, in cultured peripheral blood leukocytes of bottlenose dolphins (*Tursiops truncatus*), PFOS induces proinflammatory interferon-gamma, probably leading to a state of chronic immune activation (Soloff et al., 2017). In the digestive gland of clams (*Ruditapes philippinarum*) exposed to a low environmental concentration of C6O4, an emerging PFAS, several genes involved in the immune response were constantly upregulated following 7 and 21 days of treatment (Bernardini et al., 2021). Furthermore, it is important to emphasize that due to the immune privilege, the transcriptional regulation of immune-related genes induced by PFAS might have an even more relevant impact in the testes than in other tissues and organs. The immunotoxicity of PFAS has scarcely been studied in fish, but the present findings turn the spotlight on the effects that these compounds might exert in the testes, a critical tissue for reproduction and fertility and finely regulated at the immune level.

4.4.2. Spermatogenesis and sexual differentiation

GenX and PFOA modulated the expression of several genes of the extracellular matrix (ECM), which is fundamental to the transport of biologically active substances needed for the communication between different cellular components in the testes, as well as for the regulation of spermatogenesis and hormone production (Ungefroren et al., 1995). Some ECM genes modulated by both PFAS were granulins and APOEB. Granulins, known to regulate inflammation, wound healing, and tissue growth (Campbell et al., 2021), were among the top DEGs in both PFAS treatments. Interestingly, it has been reported that in rat brains, granulin is a sex steroid-inducible gene involved in sexual differentiation (Suzuki and Nishiahara, 2002) and that its expression might be modulated by endocrine-disrupting compounds such as phthalates and adipate esters (Lee et al., 2006). In rats, these chemicals may affect the sexual differentiation of the brain, resulting in a decrease in sex-specific behaviour in adulthood (Lee et al., 2006). A further gene putatively involved in sexual differentiation and modulated by PFAS was APOEB (an orthologue of the human APOE), a lipoprotein involved in cholesterol biosynthesis, including sex hormones. Notably, in the present study, APOEB is the most significantly upregulated gene by PFOA. It has recently been demonstrated in ricefield eels (*Monopterus albus*) that APOEB is expressed in gonadal tissue with peak values at the early intersexual stage, which is potentially involved in the initiation of sex change (Fan et al., 2022). Well-known endocrine disruptors impair APOEB mRNA and/or protein expression. APOEB mRNA expression was upregulated by BPA and 17α-ethinyl estradiol in zebrafish larvae and adult testes (Lam et al., 2011; Porseryd et al., 2018) and in the liver of males of the rare minnow (*Gobiocypris rarus*) (Zhang et al., 2021). In Syrian hamsters exposed to tributyltin, APOE induction was linked to spermatogenic defects and postulated to represent a potential mechanism of male infertility (Kanimozhi et al., 2014). Noteworthily, besides APOEB, in the testes of fish exposed to PFOA only, the expression of a further apoprotein, APOC1, was greatly enhanced (among the top 10 DEGs). This apoprotein has pleiotropic effects in lipid metabolism, mostly of cholesterol, as a precursor of steroid hormones. To the best of our knowledge, a specific role of this gene in testes has never been reported, neither in mammals nor in fish. However, it is now well established, both in vitro and in vivo, that several PFAS, including PFOA and, to a lesser extent, GenX, can interfere with lipid metabolic homeostasis in fish (Sun et al., 2023; Wen et al., 2020), even at environmentally relevant concentrations (Haimbaugh et al., 2022). The testicular expression of a further gene involved in lipid metabolism, GAL3ST1 (or CST), was significantly upregulated by PFOA. This gene is essential for spermatogenesis (Suzuki et al., 2010) and has therefore implications for male infertility (Zhang et al., 2005). In particular, GAL3ST1 plays a role in the synthesis of seminolipids, unique sulfated glycerogalactolipids that in mammalian testes represent more than 90 % of the glycolipids (Ishizuka, 1997). CST activation might be part of the molecular response involved in the PFOA-induced sublethal effects on the testes.

As to the downregulated genes, we found periaxin and COL7A1, both coding for proteins in the ECM. Periaxin encodes a protein of the peripheral nervous system, and it was downregulated by GenX and PFOA. Nerves in the ECM of bony fish testes play a key role in regulating various functions, including spermatogenesis (Uribe et al., 2014). COL7A1 was particularly downregulated in GenX-exposed guppies; together with other collagen types, COL7A1 is expressed by Sertoli cells and supports spermatogenesis and testis function. Specific to GenX was the mild downregulation of GAD1, the rate-limiting enzyme in gamma-aminobutyric acid (GABA) biosynthesis. GABA is a neurotransmitter that is also present in the testes of humans (Ritta et al., 1998) and fishes (Biggs et al., 2013). This is not surprising considering that, when comparing the human brain and testis, numerous common molecular features are evident, including similar gene expression, receptors, and signalling pathways (Matos et al., 2021). In the rat testis, both GAD1 and GAD2 are expressed, supporting the presence of an intra-testicular GABAergic system and a possible role for GABA in regulating steroid biosynthesis, and thus sex hormones, by Leydig cells (Geigerseder et al., 2003). Again, in rats, GABA activity via GABA-A receptors is involved in Leydig cell proliferation and testosterone production (Geigerseder et al., 2004). In particular, an increased GABA activity suppresses the proliferation of spermatogonial stem cells and thus spermatogenesis (Du et al., 2013). In humans, incubation with GABA increased the percentage of spermatozoa exhibiting hyperactivated motility, demonstrating the role of GABA in sperm motility (Ritta et al., 1998). In fish testes, few studies have been conducted so far, but GABAergic signalling has been postulated to be important for teleost testicular development (Lee et al., 2017). This, combined with the evidence from mammals, suggests that GABA might also affect sperm motility in fish. This hypothesis is further supported by the results obtained in the present study, particularly the consistency between the GAD1 downregulation and the decreased sperm velocity observed in GenX-exposed guppies.

Although sex determination in *P. reticulata* is under strong genetic control by the sex chromosomes XX and XY (Karayucel et al., 2006), it is worth noting that the expression of JARID2B, supposed to suppress ovary-determining genes and then promote male phenotypes, was decreased in PFAS-exposed fish. This evidence supports the hypothesis that the target of the PFAS of the present study possesses an endocrine-disrupting activity. JARID2B is involved in histone methylation, an epigenetic mechanism that is the target of several endocrine disruptor compounds (Carnevali et al., 2018). There is only one study focussing on this gene in a fish species, the Nile tilapia (*Oreochromis niloticus*), in which sex determination is genotypic but can be affected by temperature only during a certain time interval (the thermosensitive period) (Zhou et al., 2022). The aforementioned study showed that JARID2B is expressed in the gonads of both sexes during the thermosensitive period, and its expression pattern exhibits a sexual dimorphism that is maintained into adulthood. These and other lines of evidence suggest that JARID2B is involved in the early development and differentiation of the gonads and that it may also be important for maintaining the adult sexual phenotype.

4.4.3. Oxidative stress

The exposure to GenX and PFOA induced mild oxidative stress in guppy testes. This is demonstrated by the significant upregulation of some genes responding to the production of reactive oxygen species (ROS), such as GPX1B. GPX1B exhibits a similar function to that of GPX1A (Liu et al., 2016), i.e., they are both key antioxidant enzymes. Contrasting findings have been reported in association with PFAS exposure (Collí-Dulá et al., 2016; Bonato et al., 2020). Other genes involved in the antioxidant response were also upregulated: NRROS (by GenX) and MT2 (by PFOA). NRROS negatively regulates ROS as it directly interacts with the NADPH oxidase mediating the oxidative burst, NOX2 (encoded by CYBB). Interestingly, NOX2 was upregulated by PFOA, leading to an increased ROS production. Very recently, in pancreatic β-cell, NOX2 has been reported to have a key role in PFOS-induced oxidative stress (Elumalai et al., 2023), as previously demonstrated for polycyclic aromatic hydrocarbons (Smith et al., 2019). In the case of MT2, metallothioneins are the main proteins involved in the cellular defence against heavy metals and ROS, and its mRNA induction might represent a response to PFOA-induced oxidative stress. Lastly, GenX induced the downregulation of a gene coding for a serotransferrin, a protein in fish seminal plasma that protects spermatozoa from oxidative damage (Li et al., 2010) and that is correlated with sperm quality (Wojtczak et al., 2007; Xin et al., 2019b).

4.4.4. Apoptosis

Apoptosis is a well-known mechanism of quality control in the testis, but increased apoptosis may impair sperm production, eventually decreasing male fertility. Therefore, germ sperm apoptosis has been considered a good proxy for male reproductive output in mammals (Asadi et al., 2021; Rajender, 2012) and fish (Fan et al., 2021). PFAS have previously been reported to increase apoptosis in rodents by modulating the expression of key factors in this process, such as FAS, FAS ligand, and caspases 3 and 9 (Qu et al., 2016; Eggert et al., 2019). Although the mRNA levels of these central effectors and regulators of apoptosis were not affected by GenX and PFOA, other genes were significantly upregulated, namely CARD9, TNFAIP2, and TGM2-like. TNFAIP2 acts as a negative regulator of NF-κB activity, and in mice it was postulated to play an important role in spermatogenesis as well (Wolf et al., 1994). CARD9 inhibits apoptosis (Li et al., 2019), while TGM2 has a pro-apoptotic role, although this is still controversial (Cho et al., 2010). The putative role of the aforementioned genes in fish testes has never been elucidated, so we can only suggest, by similarity to other species and tissues, that GenX and PFOA induce subtle effects on apoptosis regulation, at least following the short exposure and the low environmental concentrations used in this study.

5. Conclusions

Our findings shed new light on the detrimental effects of PFAS on reproduction and provide new data on the negative effects of GenX, a newly emerging, and increasingly used, PFAS. Fish exposed to GenX showed a change in the male mating tactic and a decrease in sperm velocity. Despite the PFOA bioconcentration levels in fish being considerably higher than those of GenX, the impact of PFOA on the reproductive traits reported in the present study is lower, as PFOA-exposed fish have the same reduction in sperm performance, but there is no significant effect on male sexual behaviour. This evidence suggests that the newly emerging PFAS have a higher toxicity potential than the legacy ones, even at ecologically relevant concentrations and with short exposure. Sublethal effects were also appreciated at the transcriptional level (gene expression in the testes), with the modulation of some genes, most of them taking part in immune regulation, spermatogenesis, and sexual differentiation. The perturbed expression of genes involved in spermatogenesis might be the early evidence of an impairment in sperm production (sperm count), which we have not found at the phenotypic level, most probably because the duration of the exposure was less than a full spermatogenetic cycle. Our findings may therefore have broader implications that extend beyond the results of our data on reproductive phenotypic traits, as PFAS consequences might be even more negative when tested after a full spermatogenetic cycle or after a longer exposure. Overall, this might evoke PFOA and GenX endocrine-disrupting effects in this species.

In conclusion, our study showed that both reproductive phenotypic traits and gene expression are sensitive endpoints, revealing PFAS toxicity at low but environmentally relevant concentrations in guppies. The detrimental effects we found underscore the significance of our findings, particularly in light of the anticipated increase in environmental exposure to alternative PFAS in the future. Our results provide compelling evidence suggesting that the toxicity potential of GenX may be higher than that of PFOA, at least in the species used here, despite

GenX being regarded as less toxic.

CRediT authorship contribution statement

C Gasparini: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **S Iori:** Writing – review & editing, Investigation. **E Pietropoli:** Writing – review & editing, Methodology, Investigation, Formal analysis. **M Bonato:** Writing – review & editing, Investigation. **M Giantin:** Writing – review & editing. **A Barbarossa:** Writing – review & editing, Methodology, Investigation, Conceptualization. **A Bardhi:** Writing – review & editing, Methodology, Investigation. **A Pilastro:** Writing – review & editing, Formal analysis. **M Dacasto:** Writing – review & editing, Conceptualization. **M Pauletto:** Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis.

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

Sequencing data have been deposited in GeneBank under BioProject accession PRJNA843058.

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Appendix A. Supplementary data

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