Aquatic Toxicology 175 (2016) 269–276

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Zebrafish reproductive toxicity induced by chronic perfluorononanoate exposure

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ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form 4 April 2016
Accepted 4 April 2016
Available online 5 April 2016

Keywords:
Perfluoroalkyl acids
Zebrafish
Reproductive toxicity
VTG

ABSTRACT

Perfluoroalkyl acids (PFAAs) are a group of anthropogenic compounds that have been widely used in consumer products for over 50 years. One of the most dominant PFAAs is perfluorononanoate (PFNA), a compound detected ubiquitously in aquatic ecosystems. While PFNA is suspected of being an endocrine disruptor, the mechanisms behind PFNA-induced reproductive disorders are poorly understood. The aim of this study was to investigate the reproduction-related effects and possible mechanisms of PFNA on adult zebrafish (*Danio rerio*) following 180 days of exposure at different concentrations (0.01, 0.1, 1 mg/L). PFNA concentration in the gonads of zebrafish was tested by HPLC–MS/MS after chronic exposure to study possible inconsistent accumulation between the genders. The results showed that the accumulation of PFNA in the male gonads was almost one-fold higher than that in the female gonads, indicating a possible higher PFAA gonad burden for male zebrafish. Significant reductions in the male gonadosomatic index (GSI) and female egg production were observed. In addition, the decreased 72 h hatching rate displayed an evident dosage effect, indicating that maternal exposure to PFNA might impair offspring developmental success. To investigate how PFNA exposure affects the hypothalamic-pituitary-gonadal-liver axis (HPGL axis), the transcriptional levels of genes were measured by real-time PCR. The disrupted expression of genes, such as ERα, ERβ, FSHR, LHR, StAR, and 17βHSD, indicated the possible interference of PFNA on the HPGL axis function and sex hormone synthesis. Furthermore, testosterone (T) and estradiol (E_2) levels in serum and VTG content in the liver were detected to clarify the influences of PFNA on sex hormone levels. Except for the increase in serum estrogen levels, as an estrogen analogue, PFNA also induced the synthesis of biomarker protein vitellogenin (VTG) in the adult male liver. The results of this study indicate that chronic exposure to PFNA can lead to dysfunction in the HPGL axis and sex hormone synthesis and cause adverse effects on fish reproduction.

1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluorinated chemicals consisting of high-energy carbon-fluorine (C—F) bonds (Hekster et al., 2003). With their unique characteristics, PFAAs have been widely used over the past several decades in industrial and domestic products, ranging from surfactants and emulsifiers to textiles and paper products (Lemal, 2004; Renner, 2001, 2004). This has led to the continuous global detection of PFAAs in environmental matrices, including aquatic systems (Giesy and Kannan, 2002). These chemicals, which do not exist naturally, are non-biodegradable and persistent, resulting in widespread environmental detection and pollution (Yakata et al., 2003). Recently, concerns have arisen about the possible health impacts of exposure to perfluorononanoate (PFNA), a perfluorinated alkyl acid containing nine carbon atoms, due to the detection of increased PFNA levels in the environment as well as in human and wildlife tissue (Keller et al., 2005). PFNA concentrations have been found at higher levels than perfluorooctanoate (PFOA) in a variety of fish species (Houde et al., 2006; Kallenborn, 2006; Li et al., 2008; Martin et al., 2004a; Martin et al., 2004b). For example, in the serum of fish from Gaobeidian Lake in China, the concentration of PFNA ranged from 0.114 to 1.18 ng/mL, while PFOA ranged from 0.108 to 0.669 ng/mL (Li et al., 2008). As a result, PFNA has become a dominant PFAA in fish-eating marine mammals (PFNA: 236 ± 25 ng/g; PFOA: 10 ± 2 ng/g; perfluorodecanoic acid (PFDA): 89 ± 9 ng/g in polar bear liver) (Hart et al., 2009; Houde et al., 2005; Ishibashi et al., 2008; Smithwick et al., 2005).

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http://dx.doi.org/10.1016/j.aquatox.2016.04.005
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Studies on model organisms support the hypothesis that PFAAs may exhibit estrogen-like activity. For teleosts, PFAA exposure can affect sex steroid hormone levels, increase hepatic vitellogenin (VTG) expression, disrupt gonad development and even lead to bisexual gonads (Ankley et al., 2005a,b; Benninghoff et al., 2011; Du et al., 2009; Oakes et al., 2005; Wei et al., 2007). Evidence for estrogen-like activity of some PFAAs has also been shown *in vitro* (Ishibashi et al., 2007; Liu et al., 2007; Maras et al., 2006). However, although these studies examined PFOS and PFOA, little research has been done on PFNA.

Evidences in vertebrates have shown that PFAAs can affect the hypothalamic-pituitary-gonadal (HPG) axis (Liu et al., 2009b; Liu et al., 2011). In fish, the HPG axis is commonly referred to as the hypothalamic-pituitary-gonadal-liver axis (HPGL axis) because many egg-yolk and chorionic proteins (*e.g.* VTG and choriogenin) are synthesized heterologously in fish livers, which are necessary for oocyte growth and development in females (Arukwe and Goksoyr, 2003). In addition, previous studies have shown that genes regulating steroidogenesis are important target sites for endocrine disrupting chemicals (EDCs). For example, after exposure to EDCs, such as bisphenol A (BPA), ketoconazole, and vinclozolin, genes in the HPG axis, including ERα, ERβ, FSHR, LHR, StAR, CYP11A, and 17βHSD, were found to be significantly up or down regulated (Rhee et al., 2011; Villeneuve et al., 2007; Walker and Gore, 2011). Due to their short reproductive cycle and facile material, zebrafish are considered appropriate models for testing EDCs, and have been used previously for investigating the effects of EDCs, such as PFOA and PFOS (Ankley and Johnson, 2004; Liu et al., 2011). However, existing studies on the reproductive toxicity of PFNA are limited and no study has assessed the potential endocrine disrupting effect of PFNA on fish following long-term exposure.

The objectives of this study were to investigate the adverse effects of chronic PFNA exposure on zebrafish. Male and female adult zebrafish were exposed to PFNA at concentrations of 0.01, 0.1 and 1.0 mg/L for 180 days under a flow-through system. PFNA concentrations in gonadal tissue were measured to assess accumulative levels. Toxicological endpoints were tested, including histological alterations of gonads as well as reproductive and developmental success (*i.e.* gonadosomatic index (GSI) of both genders, female egg production, fertilization rate, hatching rate, and abnormality rate of the F1 generation). The VTG content in liver and sex steroid hormones (testosterone (T) and estradiol (E_2)) in serum were measured. Finally, transcriptional profiles of a suite of functionally relevant genes associated with the synthesis of sex hormones and the HPGL axis were investigated. Based on previous research on long-chain PFAAs (Liu et al., 2011), significant interference on VTG content, sex steroid hormone levels, and HPGL axis-related gene expressions were expected, and could lead to adverse effects on reproduction. The results obtained in this study will be helpful in clarifying the mechanism of PFNA estrogenic activity and evaluating the potential long-term ecological risks of PFNA on aquatic organisms.

2. Materials and methods

2.1. Materials

The PFNA was purchased from Sigma Aldrich (CAS number 375-95-1, 97% purity, St. Louis, MO, USA). The PFNA physiochemical data are given in Table S1 (Supplementary material). Solvent-free stock solutions of PFNA were prepared by dissolving crystals in water with stirring. Three stock solutions of 30, 300, and 3000 mg/L were used to span the desired range of target solutions in exposure water.

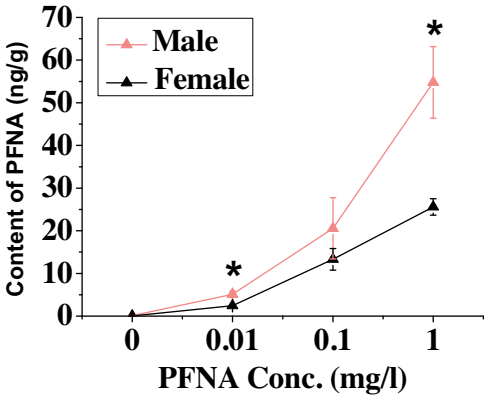

Fig. 1. PFNA content in gonads of zebrafish (n = 6 for each group). Error bars indicate standard errors, and * $p < 0.05$ indicates significant differences between sexes.

2.2. Animals and treatment

Five-month-old zebrafish ($n = 480$) (wild-type, *Tuebingen* strain) were separated by sex and randomly assigned to nominal PFNA concentrations of 0 (control), 0.01, 0.1, and 1.0 mg/L (0, 22, 215, and 2150 nM, respectively) for 180 days using a flow-through exposure system (ISO7346-3) with a flow velocity of 30 mL/min. All zebrafish were fed twice a day with live brine shrimp. During the 180 days of exposure, all fish were held under the same photoperiodic conditions of 16-h light: 8-h dark, and water temperature of 25–27 °C (pH 8.1–8.3). Six pairs of male and female fish from the same dose groups were selected randomly to count egg production, fertilization rate, hatching rate, and abnormality rate every week. After exposure, all fish were ice-bath anesthetized for sampling. The body weight and gonad weight of fish were measured. Gonads were surgically removed after blood was taken from the tail fin using a glass capillary; a portion was accurately weighed to analyze PFNA accumulation in the gonad and the remainder was immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. The GSI was calculated according to the formula (organosomatic index = organ weight × 100/body weight) (Bharti and Banerjee, 2013).

2.3. PFNA accumulation in gonads

Concentrations of PFNA in gonad samples from males ($n = 12$) and females ($n = 6$) of each group were quantified using high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) following the process described in our previous study (Zhang et al., 2012). Briefly, gonads were extracted with 5 mL of acetonitrile (ACN) in a 15 mL polypropylene (PP) tube. All tubes were then placed on a mechanical shaker for 20 min followed by centrifugation at 3000*g* for 10 min. The top layers, which contained PFNA (analytes and internal standards), were transferred into new PP tubes. The extraction procedure was repeated and a final solution of 10 mL of acetonitrile was combined and concentrated to 0.5 mL under nitrogen gas at 40 °C. After the addition of 0.5 mL of MeOH, the final solution was diluted into 10 mL Milli-Q water for SPE cleanup. All samples were then extracted using Oasis WAX cartridges (Oasis1HLB; 150 mg, 6 cc; Waters, USA). The cartridges were pre-equilibrated by the addition of a sequence of 4 mL of 0.1% NH_4OH in MeOH, 4 mL of MeOH, and 4 mL of water at a rate of 1 drop per second. Samples (11 mL) were then passed through the cartridges at a rate of 1 drop per second. After loading all samples, cartridges were rinsed with 5 mL of Milli-Q water and then washed with 4 mL of 25 mM acetate buffer solution (pH 4). Any water remaining in the cartridges was removed by centrifugation

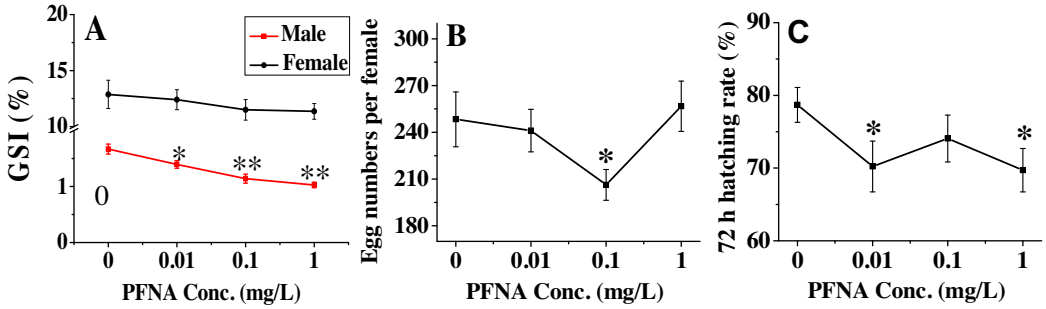

Fig. 2. GSI (A), egg numbers of parent zebrafish (B), and 72 h hatching rate of larvae after parent zebrafish were exposed to 0, 0.01, 0.1, and 1 mg/L PFNA for 180 days (C). The GSI results are means of 30 individual fish, and the 72 h hatching rate is the mean value of 17 weeks (eggs from six pairs of zebrafish). Error bars indicate standard errors, and * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between the control and exposure groups.

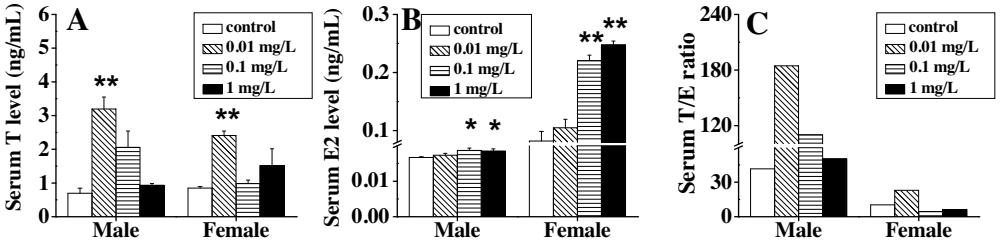

Fig. 3. Levels of T (A), E_2 (B), and T/E ratio (C) in serum of zebrafish (n = 3, pooled sample from 20 individuals at each group). Error bars indicate standard errors, and * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between the control and exposure groups.

at 3000 rpm for 2 min, and PFNA was eluted by 4 mL of 0.1% NH_4OH in MeOH and then concentrated to 1 mL under a stream of nitrogen.

The instrumental chromatographic setup consisted of a P680 binary gradient pump, an UltiMate 3000 autosampler and a Chromeleon 6.70 chromatography workstation (Dionex, USA). Mass spectra were collected using an API 3200 triple quadrupole tandem mass spectrometer, fitted with an electrospray ionization source and operated in negative ionization mode. Quantification using these transitions was performed using Analyst 1.4.1 software. Chromatographic separations were carried out on an Acclaim 120C18 column (4.6 × 150 mm, 3 μm, Dionex, USA), with a binary gradient. Methanol (A) and 50 mM ammonium acetate (NH_4Ac) (B) were employed as mobile phases. The flow rate was 1 mL/min and the injection volume was 10 μL. The elution gradient was: 0–4 min, from 28 to 5% B linearly; 4–7 min, 5% B; 7–10 min, 28% B.

A calibration curve was prepared from a series of concentrations (0, 10, 50, 100, 500, 1000, 5000, 20000, and 50000 pg/mL), and standard deviations were less than 20%. Blanks and recoveries were assessed following the same procedure as described above with each group of extractions. The blanks were all below the limit of quantification (LOQ). Quality control is given in the Supplementary material (Table S2). The native standard was spiked into samples and analyzed. The concentrations of PFNA in the experimental samples were not corrected for their corresponding recoveries.

2.4. Testosterone (T) and estradiol (E_2) levels in serum

For determination of T and E_2 levels in serum, plasma from the males and females ($n = 60$, respectively) of each group was taken from the tail fin using a glass capillary at room temperature. The glass capillary was preconditioned by heparin. After transfer into the PP tubes, the plasma was centrifuged at 3000*g* for 5 min to obtain serum. Both T and E_2 were determined (three parallel) by radioimmunoassay using commercial kits (Beijing North Institute of Biotechnology, China), as per the manufacturer's protocols (Yamamoto et al., 1984).

2.5. VTG content in liver

The VTG content in male livers was measured using zebrafish *(Danio rerio)* vitellogenin enzyme-linked immunosorbent assay (ELISA) kits (Biosense Laboratories AS, Bergen, Norway), following the manufacturer's instructions (Bakos et al., 2013). Briefly, diluted standards and samples were added to remaining wells and incubated for 1 h, followed by the addition of detecting antibody, 1 h incubation, addition of secondary antibody, 1 h incubation, addition of substrate solution, and 30 min incubation. We then added 2 M H_2SO_4 to stop the reaction. The absorbance was read with a Gen5 microplate reader (BioTek, Vermont, USA) at 492 nm. The vitellogenin concentration of each individual was calculated with non-specific binding correction and regression analysis, performed by log-log transformation of the data according the manufacturer's recommendations.

2.6. Quantitative real-time PCR assays

Total tissue RNA was extracted from six frozen tissue (brain, liver or gonad) samples for each group, respectively, using a MiniPrep RNeasy Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. In brief, 30 mg of tissue was homogenized in Buffer RLT and applied to the RNeasy Mini spin column. Then, after the addition of ethanol, total RNA was bound to the membrane and eluted in RNase-free water. The concentration was measured by absorbance at 260 nm using a UV1240 spectrophotometer (Shimadzu, Japan). Purity was assessed by determining the A_{260}/A_{280} ratio. cDNA was then synthesized *via* reverse transcription (RT) using an oligo-$(dT)_{15}$ primer and M-MuLV reverse transcriptase (Promega, Madison, USA) in accordance with the manufacturer's recommendations.

Real-time PCR reactions were performed with the Stratagene Mx3000P q-PCR system (Stratagene, USA). A SYBR Green PCR Master Mix reagent kit (Tiangen, Beijing, China) was used for quantification of gene expression, according to the manufacturer's instructions. Zebrafish-specific primers were designed for VTG and

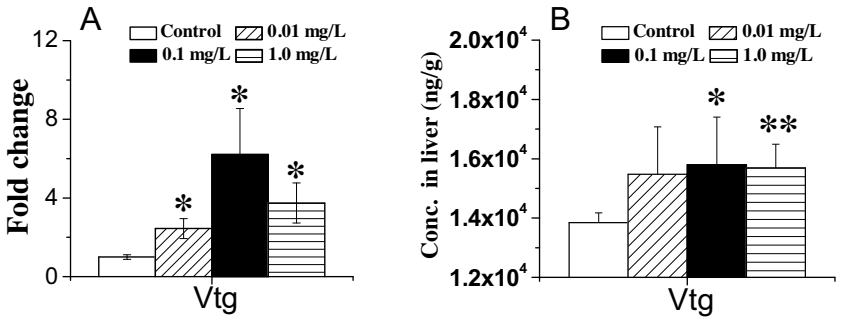

Fig. 4. Transcriptional expression of the VTG gene (A) and concentration of VTG (B) in the liver of male zebrafish (n = 6 for individuals at each group). Error bars indicate standard errors, and * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between the control and exposure groups.

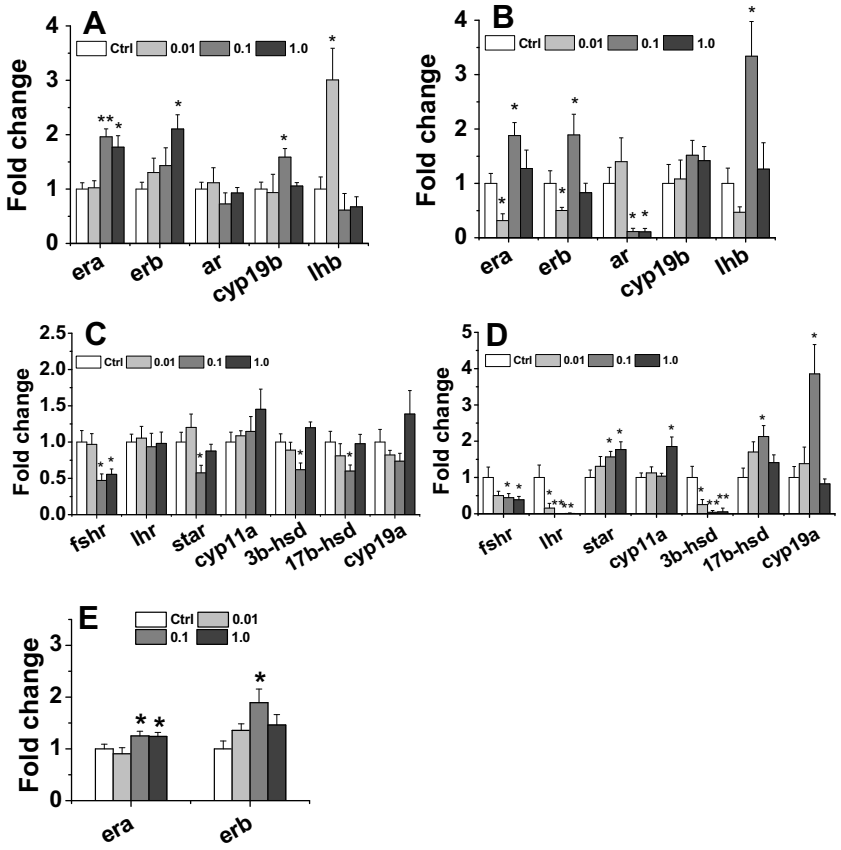

Fig. 5. Differential transcriptional expression of genes in female brain (A), male brain (B), female gonad (C), male gonad (D) and male liver (E), (n = 6 for each group). Error bars indicate standard errors, and * $p < 0.05$ and ** $p < 0.01$ indicate significant differences between the control and exposure groups.

genes involved in the HPGL axis, such as ERα, StAR, and 17β-HSD, using Primer Premier 6.0 software (Supplementary material). The housekeeping gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control. The differences in efficiencies of amplification between the target genes and HPRT were all less than 5%. The PCR amplification procedure was: 95 °C for 2 min, followed by 40 cycles at 94 °C for 10 s, 58 °C for 15 s and 68 °C for 15 s. Quantification of the transcripts was performed using the $2^{-D\Delta Ct}$ method as described in previous studies (Arocho et al., 2006; Huang et al., 2007; Wang et al., 2007).

2.7. Statistical analysis

Raw data were analyzed using SPSS for Windows 17.0 Software (SPSS, Inc., Chicago, IL, USA) and presented as means with standard errors (mean ± SE). When data met the assumptions of normal distribution and equal variance required for the application of parametric tests, differences between the control and treatment groups were determined using one-way analysis of variance (ANOVA) followed by the Fisher LSD post hoc test. When data failed to meet the assumptions, the Mann-Whitney rank sum test was employed to determine differences between the control group and each PFNA treatment group. The *p*-values were adjusted for multiple tests using Bonferroni's corrections. Results were considered statistically significant at $p < 0.05$.

3. Results

3.1. PFNA concentration in gonads

The PFNA concentrations in the gonads of both genders were quantified by HPLC–MS/MS. The results showed that PFNA concen-

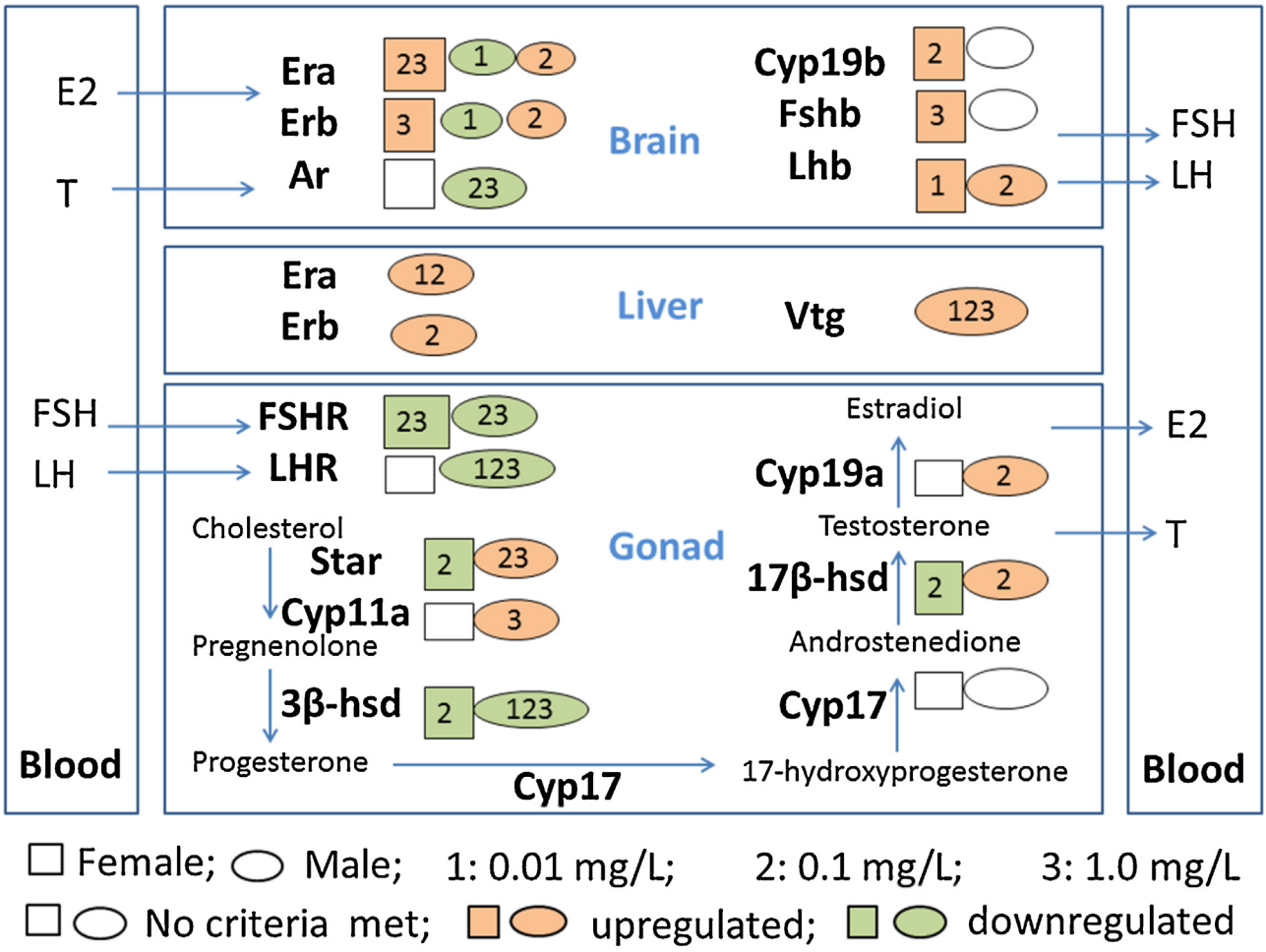

Fig. 6. Schematic diagram of changes in gene expression in the HPGL axis compared with that in the control groups. Females are represented by squares; males are represented by ovals. Numbers 1, 2, and 3 in squares or ovals represent different exposure groups. White means no significant change, yellow means upregulated, and green means downregulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tration exhibited a significant dose-dependent increase, and had a higher concentration in the spermary of male zebrafish than that in the ovary of females at a ratio of about 2:1 (Fig. 1 and Table S3). The differences between sexes were statistically significant in the 0.01 and 1.0 mg/L exposure groups ($p < 0.05$).

3.2. Effects of PFNA on GSI, egg production and hatching rate

To understand the adverse effects of PFNA on zebrafish, the GSI of both genders, egg production, and hatching rate were investigated (Fig. 2). Compared with the respective control groups, the GSI in males was remarkably reduced in all PFNA exposure groups ($p < 0.05$), but no significant changes were observed in females (Fig. 2A). The egg numbers per fish were remarkably reduced in the 0.1 mg/L PFNA exposure group ($p < 0.05$) compared with the control group (Fig. 2B). The hatching rate was detected at different times (48, 72 and 96 h). Among all three time points, a reduction in the 72 h hatching rate was statistically significant in the 0.01 and 1.0 mg/L PFNA-treated groups, suggesting that PFNA exposure of parent zebrafish could delay the embryonic hatching period of offspring at this time point (Fig. 2C).

3.3. T and E_2 levels in serum

After exposure to different concentrations of PFNA, the effects on testosterone (T) and estradiol (E_2) in male and female zebrafish, respectively, were measured (Fig. 3). Compared with the control groups, a consistent trend in the levels of T and E_2 were observed in both genders. The T levels increased significantly in the 0.01 mg/L exposure group, while the E_2 levels increased significantly in the 0.1 and 1.0 mg/L exposure groups (Fig. 3A and B). The T/E ratio also showed a similar trend in the three exposure groups of each gender, with the highest ratio occurring in the 0.01 mg/L group, followed by the 0.1 mg/L group, and then the 1 mg/L group (Fig. 3C).

3.4. VTG content and gene expression in male liver

In the male liver, the transcriptional expression (mRNA) of VTG increased 2-fold in the 0.01 mg/L group, 6-fold in the 0.1 mg/L group, and 4-fold in the 1.0 mg/L group ($p < 0.05$, Fig. 4). Furthermore, PFNA exposure caused a significant increase in the VTG protein in the 0.1 and 1.0 mg/L groups compared with that of the control group.

3.5. Gene transcription in the HPGL axis in male and female fish

We analyzed the transcript profiles of genes by quantitative real-time PCR (Fig. 5). Compared with the corresponding control groups, for genes in the brain, the expression of Erα and Erβ were significantly increased in females from the 0.1 and 1 mg/L groups and in males in the 0.1 mg/L group, but were decreased in males in the 0.01 mg/L group. Furthermore, Ar was significantly down-

regulated in males in the 0.1 and 1 mg/L groups, whereas Cyp19b and FSHβ were upregulated in females in the 0.1 and 1 mg/L group, respectively (Fig. 5A and B). In addition, LHβ were upregulated in females in the 0.01 mg/L group and in males in the 0.1 mg/L group (Fig. 5A and B). Changes in gene expression in the gonads were much more disrupted (Fig. 5C and D). For females, StAR, 17β-hsd, Fshr, and 3β-hsd showed a significant decrease in the 0.1 mg/L group, while no significant changes were observed for Lhr, Cyp11a, and Cyp19a in female gonads, or in Cyp17 for both genders (Fig. 5C). Fshr, Lhr, and 3β-hsd were significantly downregulated in males, whereas StAR, Cyp11a, 17β-hsd, and Cyp19a increased in males compared with that in the control group (Fig. 5D). For genes in the liver, especially the expression of ERα and ERβ, significant increases were only detected in males from the 0.01 and 0.1 mg/L group (Fig. 5E), while no significant changes were observed for them in female (data not shown) (Fig. 6).

4. Discussion

Consistent with other studies (Hagenaars et al., 2008; Wang et al., 2011), the concentrations of PFNA in male gonads were much higher than those in females, indicating a higher gonad burden of PFNA for males. Previous research has hypothesized that sex specific accumulation of PFOS may result from higher excretion rates in female zebrafish through spawning rather than higher accumulation rates in males (Sharpe et al., 2010). In addition, PFAA levels are positively correlated with protein content rather than lipid content, especially specific protein families (*e.g.* fatty acid-binding proteins or plasma albumin) (Hoff et al., 2003; Labadie and Chevreuil, 2011). Previous studies have shown that PFOS can be transferred from the liver to eggs as a protein-PFOS complex during the spawning period (Holmstrom and Berger, 2008). As zebrafish are distinguished by an unusual breeding cycle that occurs every five days and lasts for approximately one year, our results were possibly related to the high protein content in female eggs, with protein-bound PFNA eliminated from the body during frequent female fish breeding recirculation.

The decrease in the GSI was consistent with that found in swordtail fish (*Xiphophorus helleri*) exposed to PFOS for 21 days, and in zebrafish exposed to PFOS for 70 days (Du et al., 2009; Han and Fang, 2010). Moreover, in the present study, the male gonads showed more sensitivity to PFNA than that of females. The differences in gonad weight change following exposure to PFAAs need further exploration. According to the metabolic cost hypothesis, the energy drain imposed by PFOA could lead to the suppression of non-vital processes such as reproduction (Rowe et al., 2001). Along with the decrease in egg production, our results suggest that long-term exposure to PFNA at this concentration (0.1 mg/L) had significant inhibitory effects on female fecundity. The decrease in the 72 h hatching rate also demonstrated the negative effects of PFNA on zebrafish reproduction. However, how PFNA affects embryo development, such as heart function, and whether it is attributable to larval malformation is unknown and the underlying mechanism requires further exploration.

The increased E_2 levels in zebrafish following PFNA exposure were in agreement with other related studies (Liu et al., 2009a,b). However, the T levels found here were consistent with some (Feng et al., 2009; Zhao et al., 2014), but not all animal studies. Research on male rats found that exposure to high concentrations of PFOA and perfluorododecanoic acid of varying chain lengths could decrease T levels *in vivo* and *in vitro* (Shi et al., 2009; Shi et al., 2007; Zhao et al., 2010). Also, PFOS and PFOA are suspected endocrine disruptors, with effects on sex hormone levels resulting in lower T and higher E_2 levels (Joensen et al., 2013). In the present study, T levels in serum significantly increased in the male group exposed to 0.01 mg/L PFNA, and showed a decreasing trend in the 1 mg/L group, similar to results obtained in PFNA-exposed male rats (Feng et al., 2009). This phenomenon might indicate an adaptation to low concentration PFNA exposure, which may lead to an increase in T levels instead of the decrease observed when exposed to high concentrations of PFAAs. Our research indicates that the toxic effect of PFNA in zebrafish involves sex hormone disorders. Disturbing the balance between T and E_2 can affect reproduction, sexual development, and gametogenesis (Folmar et al., 1996). Reported alternative mechanisms of sex hormone disorders include replacement of E_2 and T to combine with sex hormone-binding globulin, interference with sex hormone synthesis, inhibition of steroidogenic enzyme activity, and weak estrogenic activity (Benninghoff et al., 2011; Jones et al., 2003). Nevertheless, how PFNA contributes to sex hormone disorders still needs further investigation.

Vitellogenin (VTG) is a liver-derived yolk-protein precursor in all oviparous teleosts (Hansen et al., 1998). Once synthesized, VTG is released into the blood stream and transported to the ovary, where it is incorporated by growing oocytes, thereby providing nutrients for the development of the new organism (Arukwe and Goksoyr, 2003). In the presence of substances that affect endocrine function, like p,p′-DDE, nonylphenol, and letrozole, males can also express the VTG gene (Monteiro et al., 2015; Wu et al., 2014). Previous results obtained in rare minnow (*Gobiocypris rarus*) in our laboratory demonstrated the ability of PFOA to affect reproduction by causing an increase in VTG, suggesting a PFOA estrogen-like potency (Wei et al., 2007). In the present study, significant increases in hepatic VTG levels and serum estrogen levels were found in males. In addition, for the first time, an estrogenic action of PFNA was also highlighted, along with its ability to interfere with fish reproduction and metabolism.

In the present study, the hepatic mRNA expressions of Era and Erb in male zebrafish were significantly increased with estrogen levels following exposure to 0.1 mg/L PFNA ($p < 0.05$). This supported the idea that PFNA estrogenicity may be mediated through estrogen receptors. Consistent with these findings, previous studies have reported estrogenic effects of certain PFASs *in vitro* and *in vivo*, although it is not known whether any of these chemicals interact directly with estrogen receptors (Blair et al., 2000; Matthews et al., 2000; Olsen et al., 2005).

The major sites of sexual hormonogenesis, the gonads, synthesize T and E_2 under the control of the HPGL axis (Liu et al., 2011). In turn, the sex hormones act on the brain and pituitary to regulate reproductive processes and control vitellogenesis and yolk formation in the liver (Busby et al., 2010; Levi et al., 2009). The *de novo* biosynthesis of sex hormones begins with the rate-limiting transportation of cholesterol into the mitochondria, mediated by the steroidogenic acute regulatory protein (StAR). Cholesterol is then converted to testosterone through the action of various enzymes, including Cyp11a1, 3β-HSD, Cyp17a, and 17β-HSD, step by step. Testosterone is then finally converted to estradiol-17β by aromatase (Cyp19a) (Levi et al., 2009; Miller and Bose, 2011).

In our study, for males exposed to 0.1 and 1 mg/L PFNA, the expression of genes played an important role in sex hormone synthesis. For example, StAR, Cyp11a, and 17β-HSD were increased, indicating positive effects on testosterone synthesis. Moreover, the significant upregulation of Cyp19a was also observed in male gonads, consistent with the results obtained in thick-lip grey mullet (*Chelon labrosus*) exposed to PFOS (Bilbao et al., 2010). Changes in aromatase activity can influence the level and balance of estrogen in zebrafish (Fenske and Segner, 2004; Uchida et al., 2004), and the upregulation of Cyp19a in the present study may have increased the conversion of T to E_2, resulting in the increase in serum E_2 levels in male zebrafish. Unlike that observed in males, most measured genes in female gonads were downregulated, which might be due to the estrogen-like potency of PFNA as an endocrine disrupter.

With the increase in E_2 levels in females, the negative regulation of E_2 synthesis might have initiated the downregulation of related genes in female gonads.

In summary, our results demonstrated that chronic PFNA exposure led to a decrease in male GSI and female fertility and an increase in serum E_2 levels in both genders. PFNA was less bio-accumulative in female gonads than that in male gonads. Furthermore, PFNA interfered with HPGL axis function and sex hormone synthesis by disturbing the expression of genes in the HPGL axis, which led to estrogenic effects such as significantly increased VTG content in males and increased E_2 levels in both genders. The results of this study provide a basis for research on the potential risks of this ubiquitous and persistent contaminant in aquatic ecosystems worldwide.

Acknowledgements

This project was sponsored by the National Basic Research Program of China (973; Grant: 2013CB945204) and the National Natural Science Foundation of China (31320103915).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.04.005.

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