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Endocrine disruption effects of long-term exposure to perfluorodecanoic acid (PFDA) and perfluorotridecanoic acid (PFTrDA) in zebrafish (*Danio rerio*) and related mechanisms

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HIGHLIGHTS

- PFDA and PFTrDA disrupted sex hormone balance through alteration of steroidogenesis.
- Long-chain PFAAs modulated hormone and genes of the HPG axis in a sex-dependent way.
- Significant up-regulation of *vtg1* gene was observed in male fish exposed to PFDA.

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ABSTRACT

Perfluoroalkyl acids (PFAAs) have been frequently detected in both the environment and biota, however the endocrine disruption potentials and underlying mechanism of long-chain PFAAs have not yet been fully understood in fish. In the present study, the effects of perfluorodecanoic acid (PFDA) and perfluorotridecanoic acid (PFTrDA) on sex steroid hormones and expression of mRNA of selected genes in hypothalamic–pituitary–gonad (HPG) axis were evaluated after 120 d exposure of zebrafish. In addition, production of sex hormones and transcription of steroidogenic genes were measured after *in vitro* exposure of H295R cells for 48 h. Exposure to PFTrDA resulted in reduced production of testosterone (T) along with lesser expression of *CYP17A* mRNA in H295R cells. In zebrafish, significant up-regulation of *vtg1* was observed in males exposed to PFDA, whereas down-regulation was observed in females exposed to PFTrDA. In male zebrafish, concentrations of 17β-estradiol (E2) were significantly increased at 0.01 mg L^{-1} PFTrDA. Significant increases in ratios of E2/T and E2/11-ketotestosterone (11-KT) were observed in male zebrafish after exposure to PFDA or PFTrDA, indicating estrogenic potentials of these compounds. The results of this study showed that long-term exposure to PFDA or PFTrDA could modulate sex steroid hormone production and related gene transcription of the HPG axis in a sex-dependent manner. Consequences of endocrine disruptions in reproduction performances of the fish warrant further investigation.

1. Introduction

Perfluoroalkyl acids (PFAAs) have been used in surfactants, lubricants, fire fighting foams, and indoor application over the past 50 years (Key et al., 1997). The production of perfluorooctyl sulfonyl fluoride, a major precursor of perfluorooctane sulfonic acid (PFOS), was voluntarily phased out in 2002, however many other PFAAs are still in production (Prevedouros et al., 2006). Among PFAAs, perfluoroalkyl sulfonic acids (PFSAs) and perfluoroalkyl carboxylic acids (PFCAs) have received great attention due to their persistence in the environment (Naile et al., 2010), bioaccumulation

Abbreviations: dpf, day post fertilization; *E2*, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; FTOHs, fluorotelomer alcohols; HPG axis, hypothalamic–pituitary–gonad axis; PCR, polymerase chain reaction; PFAAs, perfluoroalkyl acids; PFCAs, perfluoroalkyl carboxylic acids; PFDA, perfluorodecanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid; PFSAs, perfluoroalkyl sulfonic acids; PFTrDA, perfluorotridecanoic acid; PFUnDA, Perfluoroundecanoic acid; POPs, persistent organic pollutants; T, testosterone; *Vtg*, vitellogenin; 11-KT, 11-ketotestosterone.

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potential (Martin et al., 2003), and possible adverse effects on humans and wildlife (Lau et al., 2007). Consequently, PFOS, one of the most important PFAAs, has been added to the list of persistent organic pollutants (POPs) under the Stockholm Convention on POPs, (2009).

PFAAs have been detected in aquatic environment, wildlife, and humans worldwide. Previous studies have reported significant decreasing trends for some PFSAs and perfluorooctane sulfonamide, but continuously increasing trend for PFCAs (Hart et al., 2008; Ahrens et al., 2009; Holmström et al., 2010). For example, PFCAs concentrations measured in the eggs of peregrine falcon in the southwest Sweden have increased between 1974 and 2007, while those of PFOS leveled off after the mid 1980s (Holmström et al., 2010). Perfluorodecanoic acid (PFDA, C_{10} PFCA), one of the long-chain PFCAs, has been detected at concentrations of 0.23–15.4 ng L^{-1} in sediment samples collected from west coast of Korea (Naile et al., 2010), 3.74–160 ng L^{-1} in the Conasauga River in USA nearby carpet industry (Konwick et al., 2008), and 0.13–0.66 ng L^{-1} from Baiyangdian lake water in China (Shi et al., 2012). In Australia, median concentrations of 1.2 ng L^{-1} of PFDA and 0.1 ng L^{-1} of perfluorotridecanoic acid (PFTrDA, C_{13} PFCA) were detected in Parramatta river (Thompson et al., 2011).

Most toxicity studies of PFAAs have concentrated on PFOS and perfluorooctanoic acid (PFOA), hence limited information is available on the toxicological effects and risk of PFCAs with longer chains than PFOA. Regarding toxic effects of PFDA, investigations have been generally limited to lethal effects following acute exposures (Ding et al., 2012; Hoke et al., 2012). An exception is perfluorononanoic acid (PFNA, C_9 PFCA) which was reported to induce thyroid-disrupting effects and trans-generational effects by long-term sub-lethal exposure (Liu et al., 2011). Since half-lives and uptake rates tended to increase with increasing perfluoroalkyl chain length until the length reached 13 carbons (Martin et al., 2003), studies on the sub-lethal consequences of long-term exposure to long-chain PFCAs in aquatic organisms are needed.

Endocrine disrupting properties of PFCAs are of growing concern. For example, PFOA exposure in fish can alter circulating sex steroid hormone levels, induce expression of estrogen-responsive genes and *vitellogenin* (*vtg*) gene in mature male, and even lead to decrease in overall egg production in reproductive female (Oakes et al., 2004; Wei et al., 2007). In addition, PFOA, PFNA, PFDA, and perfluoroundecanoic acid (PFUnDA, C_{11} PFCA) can weakly bind to estrogen receptor *in vitro*, and are reported to be potent inducers of *vtg* in juvenile rainbow trout (Benninghoff et al., 2011). Fluorotelomer alcohols (FTOHs), which are widely used for manufacturing PFCAs, can alter plasma sex hormone and gene transcription in the hypothalamic–pituitary–gonad (HPG) axis and consequently impair reproductive success of zebrafish (Liu et al., 2009, 2010). These lines of evidences indicate the estrogenic activities of PFCAs, and also suggest possibilities of similar adverse effects of long-chain PFCAs.

In the present study, we chose PFDA and PFTrDA, and investigated their endocrine disrupting effects and their underlying mechanisms using both cell line and a fish. In the *in vitro* H295R assay, production of the steroid hormones (17β-estradiol (E2) and testosterone (T)) and transcription of the genes involved in steroidogenic pathways were examined. In the *in vivo* fish assay, effects on levels of sex steroid hormones, mRNA expressions of important genes in HPG axis, and survival and growth effects in zebrafish (*Danio rerio*) were investigated after long-term 120 d exposure. This study will help better understand the effects of chronic exposure to PFDA and PFTrDA on endocrine disruption potentials in zebrafish and related mechanisms.

2. Materials and methods

2.1. Chemicals

PFDA (CAS No. 335-72-6, 98% purity) and PFTrDA (CAS No. 72629-94-8, 97% purity) were purchased from Sigma–Aldrich (St. Louis, MO, USA). For H295R cell bioassay, each compound was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the exposure medium was less than 0.1% (v/v). Since these chemicals are water soluble, no solvent was used for preparation of fish test solutions.

2.2. H295R cell culture and exposure

The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC # CRL-2128, ATCC, Manasas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere as described by Kim et al. (2012). Viability was checked with an MTT bioassay, and non-cytotoxic doses (>80% of survival) were determined for evaluation of effects on hormone production and steroidogenic gene transcription. When determined to affect steroid hormone production, the mechanisms of steroidogenic effect were investigated by measuring changes in gene transcription of the steroidogenic pathways in H295R cells. For this purpose, H295R cells were first seeded into 24-well plates at a concentration of 3×10^5 cells mL^{-1} in 1 mL of medium per well. After 24 h, cells were exposed to PFDA (0, 0.1, 1, 10, or 100 mg L^{-1}) or PFTrDA (0, 0.05, 0.5, 5, or 50 mg L^{-1}) for 48 h. Forskolin (0.1, 1, or 10 μM) was used as a positive control for sex hormone change. Culture medium and remaining cells were used for hormone measurements and gene transcription analysis, respectively. Hormones were measured by competitive enzyme-linked immunosorbent assay (ELISA), and transcriptions of genes related to steroidogenesis were quantified by real-time polymerase chain reaction (PCR). (For details, see the Supporting Information).

2.3. Fish culture and exposure

Male and female adult zebrafish (>3 mo old) were acclimated for 2 weeks in tanks filled with the conditioned water prepared with sodium bicarbonate, calcium sulfate, and sea salt in distilled water, at 25 ± 1 °C under a photoperiod of 16:8 h light/dark. Then four male and six female fish were placed in a spawning aquarium (20 L filled in 15 L exposure water) and were mated. *Artemia* nauplii (<24 h after hatching) were fed *ad libitum* twice daily. Water quality parameters, including pH, conductivity, temperature, and dissolved oxygen were measured routinely.

Fertilized eggs produced from adult zebrafish pairs during the previous 24 h period were collected and were used for subsequent exposure assay. Four replicates with 10 eggs each were exposed to various concentrations of PFDA or PFTrDA (0, 0.01, 0.1, 1, or 10 mg L^{-1}). Test concentrations were prepared in conditioned water via sonication and nominal concentrations were used throughout the study. The exposure concentrations were determined based on the preliminary 96 h acute toxicity test using larval fish (11 day post fertilization (dpf)). Exposure was initiated in 50 mL beakers until hatching, and dead embryos were removed as soon as possible. Newly hatched larvae were then transferred to 250 mL beakers and observed for survival for additional three weeks. On 8 dpf, fish were begun to be fed with *Chironomus* sp. four-times per day. Half of test solution was changed three times per week and water quality parameters were checked at every water change. On 21 dpf, juvenile fish were transferred to 1 L beakers for the additional observation and then transferred to 3 L

beakers from 60 dpf to end of experiment. From 21 dpf until the conclusion of the exposure, fish were fed with *Artemia* nauplii *ad libitum* twice per day. Mortalities at larvae, juvenile, and adult stages were observed during the exposure period. All surviving fish were euthanized on 120 dpf, and body length and weight were measured. For hormone and gene transcription analysis, three male and female fish per each replicate tank were randomly sampled from four replicates. Transcriptions of 22 genes were measured as well as one housekeeping gene (*β-actin*) which is reported to be stably expressed following chemical treatment (McCurley and Callard, 2008; Table S1). Full names of the determined genes are shown in Table S2. (For details, see Supporting Information).

2.4. Statistical analysis

No observed effective concentration (NOEC) was calculated using Fisher's exact test in ToxStat® ver 3.5 (West, Cheyenne, WY, USA). Normality and homogeneity of each variance were tested by the Shapiro–Wilk test and Levene's test, respectively, using SPSS 18.0 K for Windows® (SPSS, Chicago, IL, USA). To determine significant differences between control and exposure groups, *t*-test, one-way analysis of variance (ANOVA) with Dunnett's test or Kruskal–Wallis test were performed using SPSS 18.0 K. Linear regression analysis was performed to evaluate linear trend using SPSS 18.0 K. To identify the genes that may explain the changes in sex steroid hormones, Spearman correlation analysis was performed using SAS (Version 9.2, SAS Institute, Cary, NC, USA). *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Hormone production and mRNA expression in H295R cells

Concentrations of E2 were slightly increased in cells exposed to PFTrDA, however statistical significances were not observed (Fig. 1A). Concentrations of T were significantly decreased in cells exposed to 50 mg L^{-1} PFTrDA (Fig. 1B), and the ratio between E2 and T (E2/T ratio) was significantly greater at 50 mg L^{-1} PFTrDA (Fig. 1C). However, PFDA, at the experimental range tested in the present study, did not result in significant effects on E2, T, or E2/T ratio (Fig. 1).

Exposure to PFTrDA affected transcription of the genes involved in steroidogenesis (Fig. 2). After 48 h exposure, mRNA expression of *CYP11A1* and *CYP17A* genes were significantly down-regulated in H295R cells exposed to ⩾0.5 mg L^{-1} PFTrDA (Fig. 2B and C). A small down-regulation of *CYP11B2* mRNA was observed (Fig. 2D, $\beta = -0.514$, $p = 0.050$), but the effect was not statistically significant.

3.2. Effects on survival and growth in zebrafish

Survival of the larval (NOEC: 0.01 mg L^{-1} PFDA and 0.1 mg L^{-1} PFTrDA), juvenile (NOEC: 0.01 mg L^{-1} PFDA and 0.1 mg L^{-1} PFTrDA), and adult fish (NOEC: 1 mg L^{-1} PFDA and 0.01 mg L^{-1} PFTrDA) exhibited significant decrease in dose-response manner ($p < 0.05$, Table S3). In juvenile fish (34 dpf) and adult fish (120 dpf), the growth of test organisms including length and wet weight exhibited no significant difference compared to control. Condition factor of 120 dpf adult fish showed significant increase by PFTrDA exposure (Table S4).

3.3. Effects on hormone concentrations of zebrafish following long-term exposure

Concentrations of E2 were significantly increased in male fish exposed to 0.01 mg L^{-1} PFTrDA ($p < 0.05$, Fig. 3A). Both E2/T and E2/11-KT ratios were significantly increased at 1 mg L^{-1} PFDA and 0.01 mg L^{-1} PFTrDA in male fish ($p < 0.05$, Fig. 3D and E).

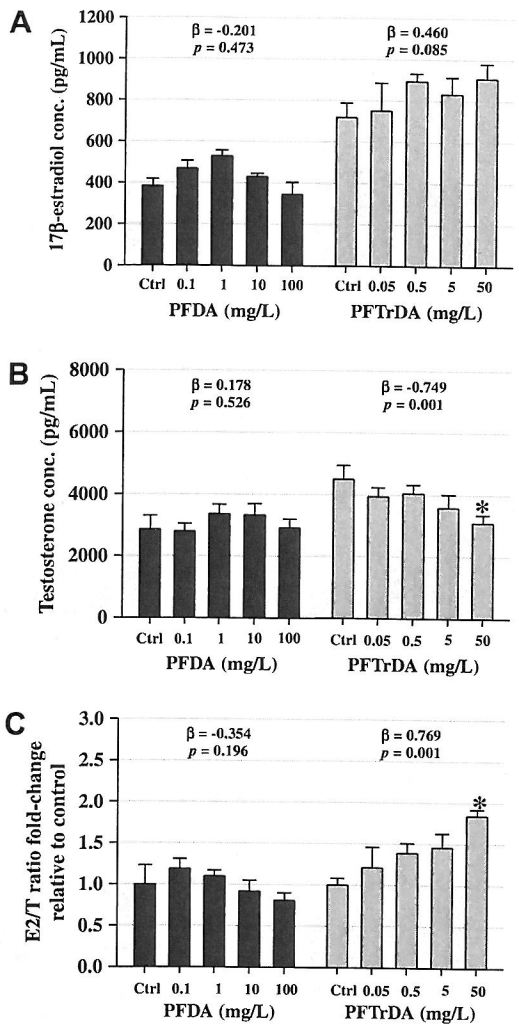

Fig. 1. Effects of PFDA and PFTrDA on (A) 17β-estradiol (E2) hormone concentration, (B) testosterone (T) hormone concentration, and (C) E2/T ratio in H295R cells. The results are shown as mean ± standard error of three replicates. Asterisk (*) indicates a significant difference from the control ($p < 0.05$). β = Slope, $p = p$ for trend.

3.4. Transcriptional change of HPG genes in zebrafish following long-term exposure

Profiles of mRNA expression of the genes involved in the HPG axis and liver were affected by exposure to PFDA or PFTrDA. In male fish brain, mRNA expressions of *cyp19b*, *erα*, and *erβ2* were significantly increased by exposure to 1 mg L^{-1} PFDA (Table S5 and Fig. S2). In female fish brain, transcription levels of *fshβ* were significantly up-regulated with a dose-dependent manner (Table S5 and Fig. S2). Following exposure to a sublethal level of PFTrDA exposure, i.e., 0.01 mg L^{-1}, expression of *gnrhr4* mRNA was significantly up-regulated in male brain (Table S6 and Fig. S3). Transcription levels of *cyp19a* gene in male gonad were significantly up-regulated at 1 mg L^{-1} PFDA (Table S5 and Fig. S2). In addition, mRNA expression of *hmgrb* mRNA in female gonad was significantly up-regulated by exposure to 0.01 mg L^{-1} PFTrDA (Table S6 and Fig. S3). Expression of *vtg1* mRNA in male liver exposed to 1 mg L^{-1} PFDA was significantly increased

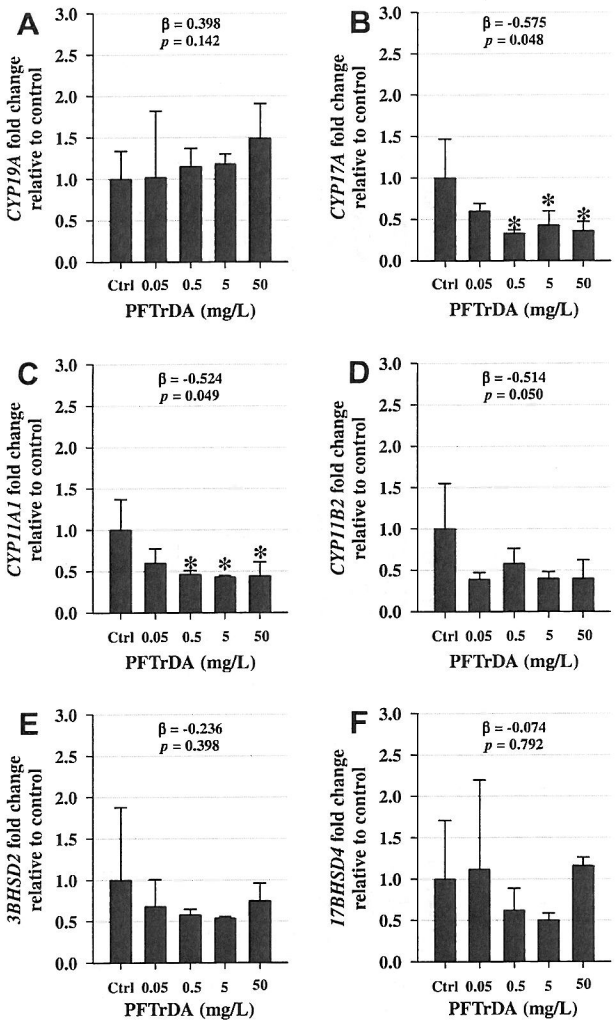

Fig. 2. Effects of PFDA and PFTrDA on expressions of (A) *CYP19A*, (B) *CYP17A*, (C) *CYP11A1*, (D) *CYP11B2*, (E) *3βHSD2*, and (F) *17βHSD4* genes in H295R cells. Values represent the mean ± standard error of three replicates. Asterisk (*) indicates a significant difference between exposure groups and the corresponding control ($p < 0.05$). β = Slope, $p = p$ for trend.

(Fig. 4). In female fish, transcription levels of *vtg1* were significantly decreased with a concentration-dependent manner after exposure to 0.01 mg L^{-1} PFTrDA (Fig. 4).

The relationship between gene transcription and sex steroid hormone concentration in male fish following exposure to 0.01 mg L^{-1} PFTrDA was summarized in Table S7. Expressions of *cyp19b*, *gnrhr4*, or *star* mRNAs were significantly correlated with E2 concentration and E2/T ratio. In addition, T concentration was highly associated with expressions of *gnrhr2*, *lhβ*, *ar*, or *17βhsd* mRNAs. Expressions of *gnrhr4*, *er2β*, or *cyp11a* mRNAs were significantly associated with 11-KT concentration and E2/11-KT ratio.

4. Discussion

Significantly increased E2/T and E2/11-KT ratios that were observed following long-term exposure to PFDA or PFTrDA in male zebrafish (Fig. 3) clearly shows estrogenic potential of long-chain PFCAs. Significant increase of E2/T ratio in male fish, e.g., following exposure to 0.01 mg L^{-1} PFTrDA, might be due to increased E2 concentration along with the increased transcription of *cyp19b* and *gnrhr4* genes (Table S7). In addition, significant increase of E2/11-KT ratio could be due to decrease of 11-KT concentration accompanied by reduced transcriptions of *gnrhr4*, *er2β*, and *cyp11a* genes. Measurement of sex steroid hormones has been suggested to be one of the most integrative and functional endpoints for understanding the effects of a chemical on reproduction in zebrafish (Ma et al., 2012). Significant increase of E2 and decrease of T concentrations have been previously reported in zebrafish exposed to 8:2 FTOH (Liu et al., 2010; Rosenmai et al., 2013). Exposure to PFNA in male rats significantly increased concentrations of E2 and decreased concentrations of T (Feng et al., 2009). These results suggest that PFAAs including long-chain PFCAs have a potential to alter sex hormone balance. Disturbing the balance of sex hormones could affect sexual development, gametogenesis, and reproduction in some fish species (Folmar et al., 1996; Shang et al., 2006). Therefore the ratios of E2/T or E2/11-KT can be used as sensitive biomarkers of abnormal sex hormones status in fish.

Alteration of vtg in both male and female fish (Fig. 4) also supports endocrine disruption potential of these PFCAs. Vtg is known to be produced in response to stimulation by estrogenic chemicals binding to specific ERs. The up-regulation of *vtg* gene transcription in male fish (Fig. 4) supports estrogenic potentials of these compounds. This observation is consistent with previous studies in male zebrafish exposed to 6:2 FTOH (Liu et al., 2009) and 8:2 FTOH (Liu et al., 2010), and male medaka exposed to 6:2 FTOH and 8:2 FTOH (Ishibashi et al., 2008). Significant increase of *vtg* expression in male rare minnows was observed following 14 and 28 days of exposure to 3, 10, or 30 mg L^{-1} PFOA (Wei et al., 2007). Plasma vtg concentrations in male common carp significantly increased in a concentration-dependent manner following 4 day exposure to $\geqslant$5 mg L^{-1} PFOA (Kim et al., 2010). On the other hand, significant down-regulation of *vtg1* gene in the female fish suggests different mechanisms of endocrine disruption by sex. Down-regulation of *vtg1* gene was coincided with apparently decreasing trend of E2 following exposure to PFTrDA (Fig. 3A). *Vtg* gene transcription is dependent on E2 concentration, and down-regulation of *vtg* gene is regarded as consequence of lower E2 concentration in fish (Rempel et al., 2006). Since vtg is a key protein for egg production in female fish, reduction in vtg protein could cause reduced egg quality and retarded vitellogenesis of the ovaries, and eventually lead to impaired reproduction. Previous studies showed that chemical exposure (e.g. prochloraz and fenarimol) resulted in decreased plasma vtg and E2 concentrations accompanied by decreased fecundity in fathead minnow (Ankley et al., 2005). Exposure of adult zebrafish to 8:2 FTOH also resulted in decreased egg production along with down-regulation of *vtg1* gene transcriptions (Liu et al., 2010). Although measurement of individual level endpoints such as fertility and egg production were not made in the present study, sex dependent alterations in vtg in the fish could be linked to adverse effects of PFDA and PFTrDA in zebrafish.

The changes in sex hormones, e.g., significant increase of E2, could in part be explained by up-regulation of *cyp19b* gene in brain and *cyp19a* gene in gonad in male zebrafish exposed to PFTrDA. Similar changes in *cyp19a and cyp19b* genes were also noted following exposure to PFDA (Tables S5 and S6, Figs. S2 and S3). Aromatase (cyp19) catalyzes the conversion of androgen to estrogen in the steroidogenesis, and the mRNA level of *cyp19* is well correlated with activity of aromatase enzyme (Trant et al., 2001). Liu et al. (2009) also showed that significant increase of E2 levels in zebrafish exposed to 6:2 FTOH was accompanied by up-regulation of *cyp19b* gene.

Our observation of altered plasma sex hormone concentrations in the fish also can be supported by changes in transcriptions of steroidogenic genes in H295R cells. While the observation in the human adrenal cell line may have limited value in elucidating the mechanisms of endocrine disruption in fish, H295R cells have been frequently used and were compared with those of *in vivo* fish

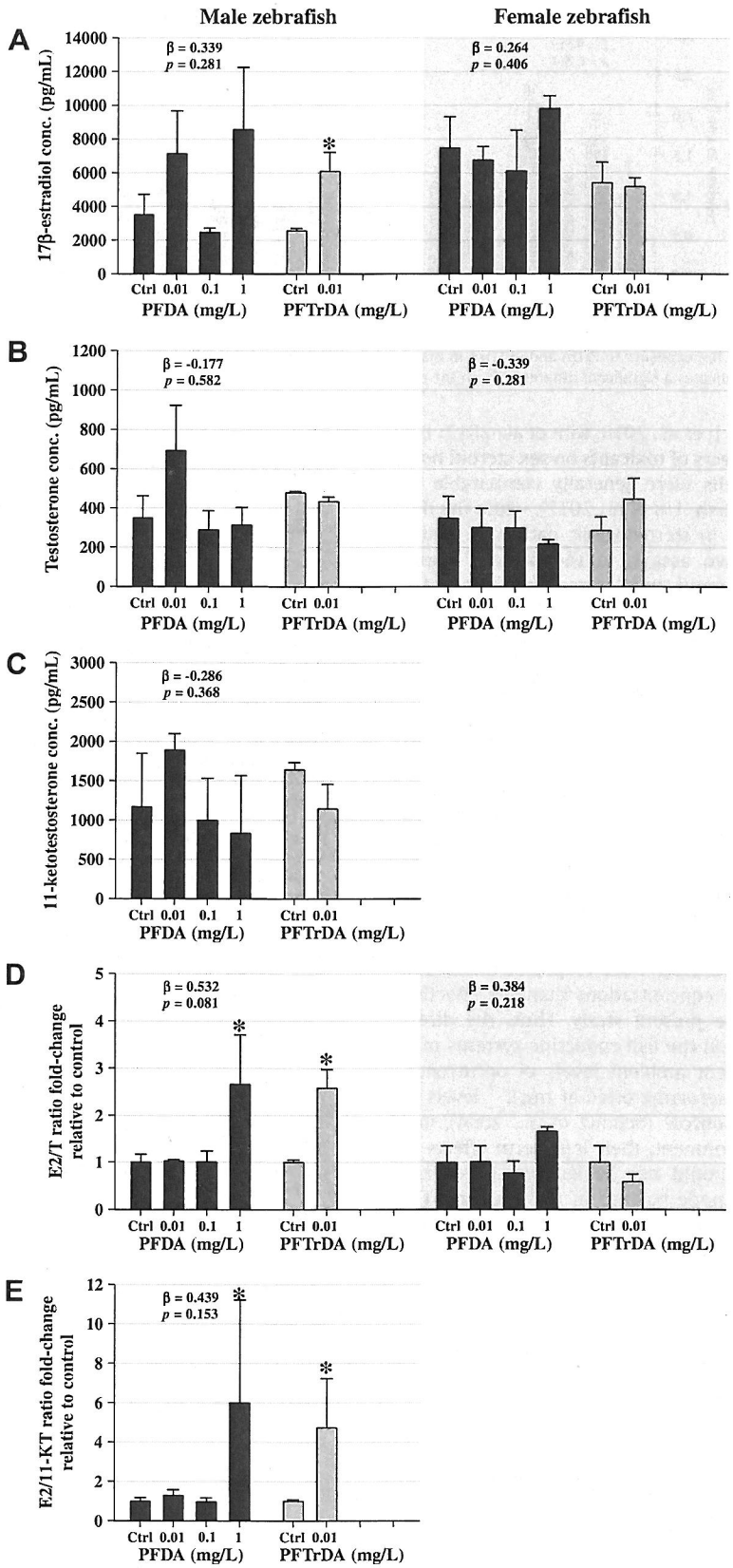

Fig. 3. Sex steroid hormones in male and female zebrafish exposed to PFDA and PFTrDA for 120 days. (A) 17β-estradiol (E2) hormone concentration, (B) testosterone (T) hormone concentration, (C) 11-ketotestosterone (11-KT) hormone concentration, (D) E2/T ratio, and (E) E2/11-KT ratio. The results are shown as mean ± standard error of three replicates for four tanks. Asterisk (*) indicates a significant difference from the control ($p < 0.05$). β = Slope, $p = p$ for trend.

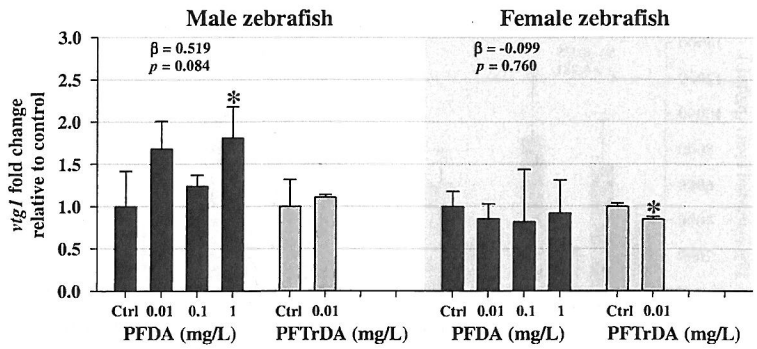

Fig. 4. Transcriptional levels of *vtg1* by the exposure to PFDA and PFTrDA in male and female zebrafish for 120 d. The results are shown as mean ± standard error of three replicates for four tanks. Asterisk (*) indicates a significant difference from the control ($p < 0.05$). β = Slope, $p = p$ for trend.

investigations (Han et al., 2010; Ji et al., 2010; Kim et al., 2012; Liu et al., 2012; Ma et al., 2012). Effects of toxicants on sex steroid hormone production in H295R cells were generally comparable to *in vivo* assays using male zebrafish (Liu et al., 2012), while the details of transcriptional changes in steroidogenic pathways somewhat differed between the two assays. In the present study, greater production of E2 and significantly lesser production of T were observed in H295R cells after exposure to PFTrDA, and this is consistent with the results of male zebrafish. The differences in gene transcription between H295R cells and fish might be due to different regulation in steroidogenic process and/or different sensitivities to endocrine disruptors.

In summary, the present study showed that exposure to PFDA or PFTrDA could modulate sex hormone balance of fish through alteration of steroidogenesis. Changes in sex hormone balance and *vtg1* level were somewhat sex dependent. While detailed mechanisms of sex dependent responses remain unknown, transcriptional level responses which were different by sex could partly explain these observations. To our knowledge, this study is the first report which links the transcriptions of genes of HPG axis to hormonal changes in fish by long-term waterborne exposure to long-chain PFAAs. PFDA and PFTrDA have been reported to occur in ambient waters at much lower concentrations than the effective concentrations observed in the present study. Thus, the direct damages by PFDA and PFTrDA on the fish endocrine systems may not be expected at their current ambient levels of occurrence. However, since PFAAs can be occurring often at mg L^{-1} levels in waters near industrial point sources (Schultz et al., 2004), and are persistent in aquatic environment, their long-term effects at lower water concentrations should not be ignored. Given the importance of sex steroid hormone balance in reproduction, the consequences of steroidogenic disturbance due to environmentally relevant concentrations deserve further investigation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.01.080.

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