Science of the Total Environment 665 (2019) 995–1002

Contents lists available at ScienceDirect

Science of the Total Environment

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

In vivo actions of Bisphenol F on the reproductive neuroendocrine system after long-term exposure in zebrafish☆

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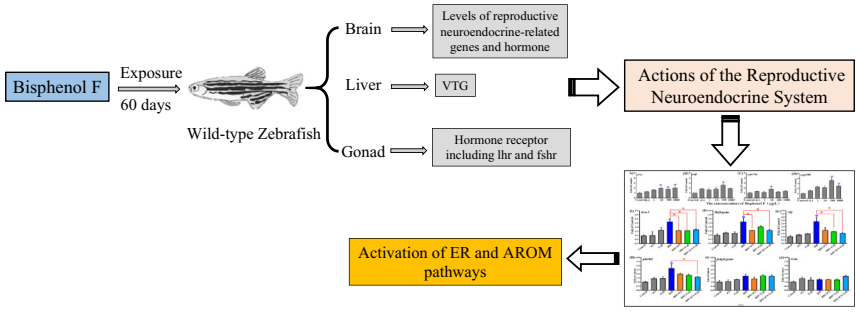
HIGHLIGHTS

- Low levels of BPF led to increased expression of reproductive neuroendocrine-related genes.
- BPF increased hormone levels of ACTH, GnRH, LH, FSH in brain and VTG in liver of zebrafish.
- BPF affects reproductive neuroendocrine system through the ER and AROM pathways.
- BPF and BPS induced similar toxic and reproductive neuroendocrine effects to those of BPA.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 November 2018
Received in revised form 6 February 2019
Accepted 10 February 2019
Available online 14 February 2019

Editor: Henner Hollert

Keywords:
Bisphenol F
Reproductive neuroendocrine system
Estrogen receptor
Enzyme aromatase pathways
Zebrafish

ABSTRACT

Although Bisphenol F (BPF), a bisphenol A (BPA) analogue with a similar chemical structure to that of BPA, is widely used in commercial products, little is known about its potential toxic effects on the reproductive neuroendocrine system *in vivo*. The present study aimed to comprehensively evaluate the effects of BPF on the reproductive neuroendocrine system in zebrafish and to assess the potential mechanisms underlying its association with estrogen receptor (ER) and aromatase (AROM) pathways. Long-term exposure to environmentally relevant and low levels of BPF led to increased expression of reproductive neuroendocrine-related genes (*kiss1*, *kiss1r*, *gnrh3*, *lhβ*, and *fshβ*) in the zebrafish brain, as well as increased levels of adrenocorticotropic, gonadotropin-releasing, luteinizing, and follicle-stimulating hormones in the zebrafish brain and vitellogenin in the zebrafish liver. In addition, these effects were associated with an increase in *erα*, *erβ*, *cyp19a*, and *cyp19b* activity. Meanwhile, ER and AROM antagonists, alone or in combination, significantly attenuated the stimulation of *kiss1*, *lhβ*, *vtg*, and *gnrh3* expression, thereby suggesting that chronic BPF exposure affects the regulation of the reproductive neuroendocrine system through activation of the ER and AROM pathways. Moreover, since BPF and bisphenol S induced toxic and reproductive neuroendocrine effects similar to those of BPA, the current accepted usage of BPA and its analogs should be reconsidered in the future.

☆ The authors declare no competing financial interest.

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https://doi.org/10.1016/j.scitotenv.2019.02.154
0048-9697/

1. Introduction

Bisphenol F (BPF) is widely used as a replacement for bisphenol A (BPA) because of BPA's wide range of adverse effects on human health. Bisphenol F is mainly applied in epoxy resins and coatings, but also has uses in industry such as for grout, industrial floors, and structural adhesives owing to its durability (Fiege et al., 2000). As it has broad applications, BPF has been detected at concentrations ranging from 0.1 to 180 ng/L in surface water (Fromme et al., 2002), 22 to 123 ng/L in sewage effluent (Song et al., 2014), at concentrations of 7300 ng/kg in sediment (Yang et al., 2014), and at concentrations as high as 0.054 mg/g in indoor dust in the United States (Liao et al., 2012a). Moreover, BPF has been detected in many personal care products (Liao and Kannan, 2014), paper products (Liao et al., 2012b), and beverages, and even in food products like dairy, fats, oils, cereals, and cereal products (Tzatzarakis et al., 2017), as well as in seafood at concentrations >23 ng/g in 95th percentile samples collected in Albany, New York (Liao and Kannan, 2013). More seriously, BPF was detected in 55% of urine samples collected from adult volunteers in Atlanta, Georgia, during 2009–2012, with a median concentration of 80 ng/L (Zhou et al., 2014). Taken together, these results show that BPF has emerged as a ubiquitous global environmental contaminant. Moreover, BPA biomonitoring results showed that children are sensitive to BPA and have greater exposure to it than do adults (Karzi et al., 2018; Tzatzarakis et al., 2015). This suggests that bisphenol exposure may be worse than anticipated, especially in children.

However, based on the available data, BPF is not as safe a substitute as was first assumed, as it shows several toxic effects similar to those presented by BPA (Eladak et al., 2015). First, similar morphological abnormalities, including cardiac edema, spinal malformation, and craniofacial abnormalities, are induced by BPF exposure with a potency comparable to that of BPA (Moreman et al., 2017). Moreover, according to a systematic review of 17 studies that tested BPA and its analogs using the same assays, the average estrogenic hormonal potency of BPF compared to that of BPA is 1.07 ± 1.20; consequently, BPF may pose the same or a more serious endocrine risk to humans and ecosystems as BPA (Rochester and Bolden, 2015). Our previous study showed that the *in vivo* effects of BPF on immune disturbance in teleosts are comparable to those of BPA (Qiu et al., 2018a, 2018b). Furthermore, other studies have demonstrated that BPF has distinct effects on the antioxidant system (Park et al., 2018; Michałowicz et al., 2015), reproductive system (Stroheker et al., 2003), and dopamine-serotonin systems (Castro et al., 2015), as well as exhibiting genotoxicity, DNA damage effects (Audebert et al., 2010), and anti-androgenic activity (Cabaton et al., 2009), both *in vivo* and *in vitro*, that are similar to the effects of BPA. Therefore, such alternatives should be comprehensively evaluated for potential toxicity before they are considered as safer replacements.

Endocrine disrupting chemicals can potentially act along the hypothalamic-pituitary-gonadal (HPG) axis and lead to endocrine disruption (Diamanti-Kandarakis et al., 2009). Gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide critical to the HPG axis, is responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland (Abraham et al., 2010). Bisphenols, including BPA and bisphenol S (BPS), elicit weak estrogenic activity, alter GnRH release, and interfere with neuroendocrine function, as reported in previous studies (Qiu et al., 2016). The neuroendocrine system plays an important role in regulating endocrine function; however, few studies have explored the potential neuroendocrine toxicity of the xenoestrogen BPF based specifically on long-term exposure under an environmentally relevant scenario. Considering that BPF and BPA have similar chemical structures and toxic effects, we hypothesized that BPF may have potentially toxic effects on the reproductive neuroendocrine system.

A few studies have reported that BPF has estrogenic potency comparable to that of BPA based on its binding affinity for estrogen receptors (ERs) (Le Fol et al., 2017). Estrogen receptors are abundant in hypothalamic cells and play a role in the regulation of neuroendocrine functions (MacLusky et al., 1979). Moreover, the aromatase (AROM) pathway, which converts androgens into estrogens in the final step of estrogen biosynthesis, and its modulation alter the rate of estrogen production and disturb the endocrine system (Cheshenko et al., 2008). The effects of BPF, BPA, and BPS are thought to be exerted *via* ERs and the AROM pathway through the induction of *ers* and *cyp19* gene expression (Yang et al., 2018a; Cheshenko et al., 2008). Furthermore, *cyp19a1b* expression most likely relies on ER expression in the developing zebrafish brain, suggesting that brain AROM might be regulated by ERs during fish development (Mouriec et al., 2009). Only a few studies have investigated the mechanisms involved in ER and AROM signaling under BPF exposure. The objective of this study was to use zebrafish to determine the impact of BPF on the reproductive neuroendocrine system after long-term exposure to environmentally relevant BPF levels and to reveal the possible pathways mediating potential neuroendocrine functions.

2. Materials and methods

2.1. Chemicals and animals

BPF (CAS Number 620-92-8, purity > 98%), BPS (CAS Number 80-09-1, purity > 98%) and BPA (CAS number 80-05-7, purity > 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BPF, BPS and BPA were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions (10 g/L, each) and stored at 4 °C. The final working solutions of BPS and BPA contained <0.05% DMSO. All chemicals in this study were of analytical grade.

2.2. Fish maintenance and experimental design

Juvenile wild-type zebrafish (AB, one month old), were maintained in a light incubator conditions at 28 ± 0.5 °C. The incubator was at a 14-hour-light:10-hour-dark photoperiod and the fish were fed twice daily with brine shrimp (Artemia nauplii). All protocols and procedures were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Southern University of Science and Technology.

Ten individuals were randomly distributed into 5 L glass tanks containing 0.1, 1, 10, 100, and 1000 μg/L BPF (3 replicates for per concentration; 10 fishes per replicate), respectively. The aqueous control received dechlorinated tap water only, and the vehicle control group received 0.05% DMSO (v/v). The exposure solutions in each tank were completely refreshed every 24 h. After 60 d exposure, the female/male ratio of the zebrafish has been recorded according to the dissection results. Then, the brain, liver, and gonad were removed from each fish and snap-frozen in liquid nitrogen and stored at −80 °C.The gonad of the female zebrafish were weighted before stored at −80 °C. Later, we compared the effects among BPF, BPS and BPA on reproductive neuroendocrine system using the outcome concentration from the current study. Fish were exposed to 100 μg/L BPF, 100 μg/L BPS and 100 μg/L BPA for 60 days. This is the dose of BPF that had consistent effects on endocrine hormone levels and reproductive-related gene expression. Animals were challenged for 60 days and then sampled.

The final set of experiments explored potential signal transduction pathways of estrogen receptors (ERs) and aromatase (AROM), which might regulate the actions of BPF on reproductive neuroendocrine system. The mRNA levels of *erα*, *erβ*, *cyp19a* and *cyp19b* genes have been analyzed in the fish liver after BPF exposure for 60 days. Furthermore, the same age zebrafish (10 fishes per treatment group; n = 3 replicate experiments) were exposed for 60 days to 100 μg/L BPF and in the presence or absence of the ER antagonist ICI 182780 (ICI, 1 μM), or the AROM inhibitor fadrozole hydrochloride (FAD, 1 μM). The concentrations of these antagonist inhibitors are according to previous work in zebrafish by Kinch and coworkers (Kinch et al., 2015). Then, the mRNA levels of

effects on *kisspeptin 1 (kiss1)*, *lhβ*, *vitellogenin (vtg)*, *gnrh3*, *fshβ*, and *synaptic vesicle protein-2 (sv2)* were measured for antagonists' experiment. The tested chemicals deviated <15% from the nominal concentrations according to our pervious study (Yang et al., 2018b), thus, the exposure BPF, BPS and BPA had not degraded to an appreciable level under the exposure conditions.

2.3. Growth parameters

The survival rate, and female/male ratio of the fish were recorded after 60 days of BPF exposure. The gonadosomatic index (GSI) was also calculated for female fish according to the following formula: $GSI = \frac{gonad\ \ weight}{body\ \ weight} \times 100\%$.

2.4. Biochemical assays

As the limitation on the sample size of zebrafish pituitary gland, brain samples were chosen to analyze pituitary hormones according to the previous study (Ji et al., 2013; Han et al., 2011). Brain samples were homogenized in ice-cold homogenization buffer (PBS, pH = 7.0). The homogenate was centrifuged at 12,000 ×*g* for 20 min at 4 °C and supernatant was collected for analysis. LH and FSH in fish brain were measured using a Fish ELISA kit (Nanjing Jiancheng Bioengineering Institute). GnRH in brain was measured using a Fish ELISA kit (Shanghai jinma biotechnology co. LTD). Liver samples were homogenized in ice-cold PBS and centrifuged at 12,000 ×*g* for 20 min at 4 °C, then the supernatant was collected for Vitellogenin (VTG) analysis by a Fish ELISA kit (Shanghai yili biotechnology co. LTD). The present study did not evaluate the differences of tested parameters between male and female in brain and liver of zebrafish. Antibody specific for fish was precoated onto a 96-well microplate. The standard curve was constructed in parallel whenever the samples were tested. Each sample for ELISAs were run in triplicates. For ELISA analysis, the intra-assay coefficients of variance (CV) was <10% and inter-assay CV was <12%.

2.5. RNA isolation, reverse-transcription, and quantitative polymerase chain reaction

Total RNA was extracted from brain, female gonad and male gonad samples using the RNAprep pure kit (Tiangen Biotech, China) and stored at −80 °C. Reverse-transcriptase reactions were performed on 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit. Real-time quantitative PCR (qPCR) was performed using the FastFire qPCR PreMix (SYBR Green) kit (Tiangen Biotech, China) by CFX Connect System (Bio-Rad, Singapore) with 96-well plates. Each sample for qPCR were run in duplicate. Quantitative PCR conditions were set as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Melting curves were generated to ensure specific amplification, thereby yielding a single product. The reference gene used in present study was Ribosomal protein L13A (*rpl13a*) according to Tang et al. (2007). The sequences of primers are listed in Table S1 (Supporting information). A Ct-based relative quantification with efficiency correction normalizing to *rpl13a* was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.6. Statistical analysis

Data are shown as means ± standard error of means (SEM). Statistical analyses were performed using IBM SPSS statistic 22.0 (IBM Corp., Armonk, NY, USA). After homogeneity of variances and normality of the data, the intergroup differences of the results were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's test. A $p < 0.05$ was considered to be statistically significant. For tested parameters in this study, there was no significant difference between a blank control group (no DMSO) and the vehicle control group (DMSO), and thus blank control group was set as the control group.

3. Results

3.1. Female rate and gonadosomatic index

The survival rate of zebrafish was not significantly altered during all the exposure experiment. After 60 days exposure, the female rate of juvenile zebrafish was ranged from 42.9% to 64.3%, and has no significant difference between treatment groups and control (Tukey's test, $p < 0.05$), as shown in Fig. 1. Moreover, GSI of the female was slightly increased after chronic BPF exposure, however, still has no significant difference between treatment groups and control.

3.2. Effects of BPF on expression of reproductive neuroendocrine-related genes

To evaluate the impact of BPF exposure on the zebrafish, expression levels of several reproductive neuroendocrine-related genes were analyzed using brain samples. As shown in Fig. 2, the results shown that mRNA levels of *kiss1*, *kiss1r*, and *gnrh3* were significantly induced in brain after a 60-d exposure to BPF. *kiss1* was significantly increased following exposure to 100 and 1000 μg/L compared to the controls (Tukey's test, $p < 0.05$). *kiss1r* was significantly increased following exposure to 10 and 1000 μg/L compared to the controls (Tukey's test, $p < 0.05$). Also, *gnrh3* was significantly induced at the concentration of 1000 μg/L compared to the control group. Unlike *kiss1* and *kiss1r*, *kiss2* and *kiss2r* expression showed no significant change in response to any concentration of BPF. We included sv2 analysis because of our earlier work showing that this marker for synaptic transmission is expressed on GnRH3 neurons during embryonic development (Zhao et al., 2014; Qiu et al., 2016) and *sv2c* mRNA levels showed no significant difference among the treatment groups and control.

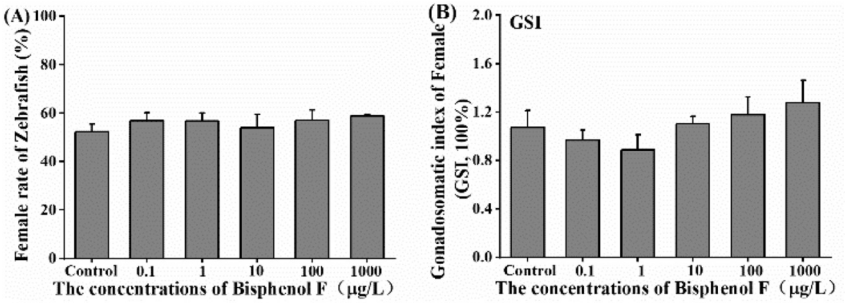

Fig. 1. Effects of Bisphenol F (BPF) on female rate of zebrafish (A), and gonadosomatic index (GSI) of female zebrafish (B) after 60 days exposure. Data are shown as means ± standard error of means (SEM) relative to the control (n = 3). * represents significant differences compared to control (*Tukey's test*, *ANOVA*, $p < 0.05$).

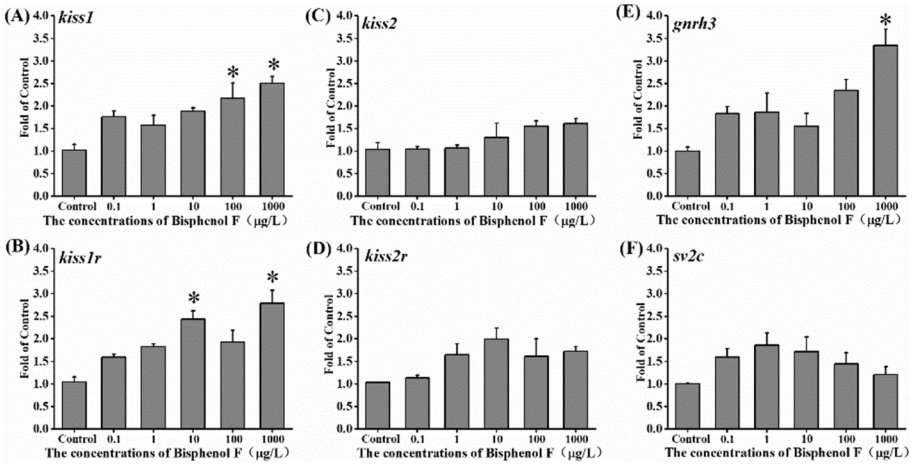

Fig. 2. Effects of Bisphenol F (BPF) on expression of reproductive neuroendocrine-related genes including *kiss1* (A), *kiss1r* (B), *kiss2* (C), *kiss2r* (D), *gnrh3* (E) and *sv2c* (F) in zebrafish after 60 days exposure. The present study did not evaluate the differences of above tested parameters between male and female zebrafish. Data are shown as means ± standard error of means (SEM) relative to the control (n = 3). * represents significant differences compared to control (Tukey's test, ANOVA, $p < 0.05$).

3.3. Effects of BPF on expression of endocrine hormone levels

As showed in the Fig. 3, the endocrine hormone including LH and FSH were significantly increased in brain of zebrafish following exposure to BPF for 60 days. The LH content levels were significantly increased at the concentration of 10 and 1000 μg/L compared to the control (Fig. 3A, Tukey's test, $p < 0.05$). The FSH content levels in the brain homogenates presented a significant increase in 10 μg/L compared to the control (Fig. 3B, Tukey's test, $p < 0.05$). Moreover, in order to confirm the effects of the endocrine hormones, we also analyzed the mRNA levels of *lhr* and *fshr* in the female or male gonad. As showed in the Fig. 3C and D, mRNA levels of *lhr* and *fshr* were significantly increased in the female and male gonad after BPF exposure. *Lhr* was significantly increased on exposure to 100 and 1000 μg/L in the female zebrafish and 1000 μg/L in the male zebrafish (Tukey's test, $p < 0.05$). *fshr* was significantly increased on exposure to 10 μg/L in the female zebrafish and 10, 100, 1000 μg/L in the male zebrafish (Tukey's test, $p < 0.05$).

Besides, other hormones including GnRH in brain, VTG in liver have been analyzed in present study. As shown in Fig. 4, GnRH, and VTG content and *vtg* gene were increased after BPF exposure. Specifically, 10, 100, and 1000 μg/L induced GnRH content (Fig. 4A); all the treatment groups induced VTG content (Fig. 4B) and 10, 100, and 1000 μg/L

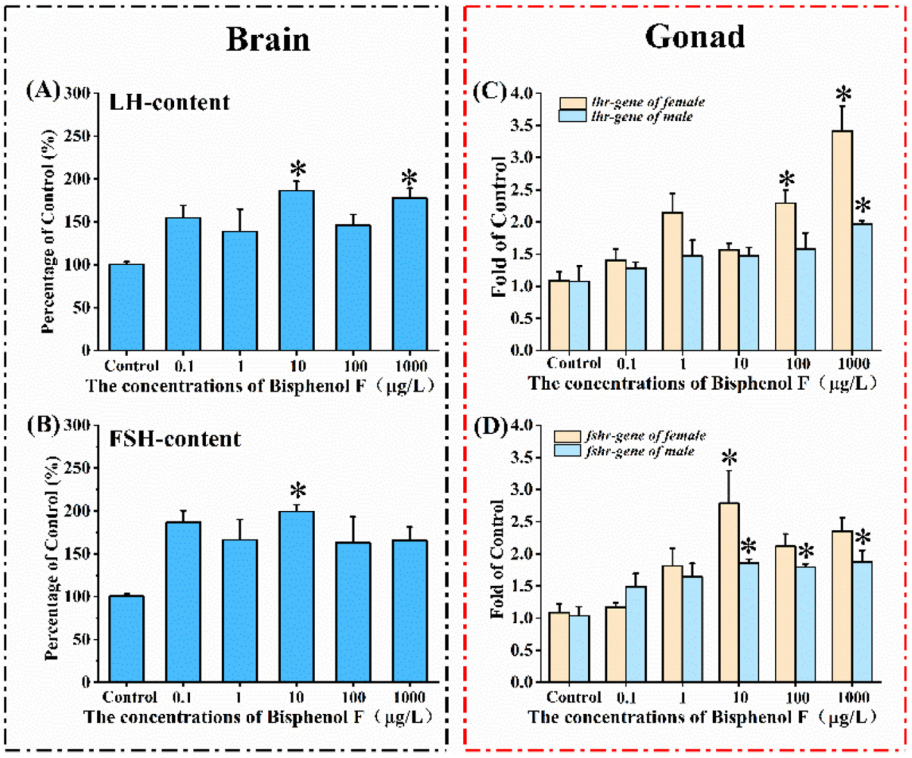

Fig. 3. Effects of Bisphenol F (BPF) on endocrine hormone levels in zebrafish after 60 days exposure. A–B: The protein level of LH and FSH analyzed in the brain; The present study did not evaluate the differences of LH and FSH protein levels between male and female zebrafish in the brain. C–D: The mRNA levels of *lhr* and *fshr* analyzed in the female or male gonad, respectively. Data are shown as means ± standard error of means (SEM) relative to the control (n = 3). The LH content in the control group was 7.6 ± 0.23 U/g protein. The FSH content in the control group was 23.4 ± 0.68 U/g protein. * represents significant differences compared to control (Tukey's test, ANOVA, $p < 0.05$).

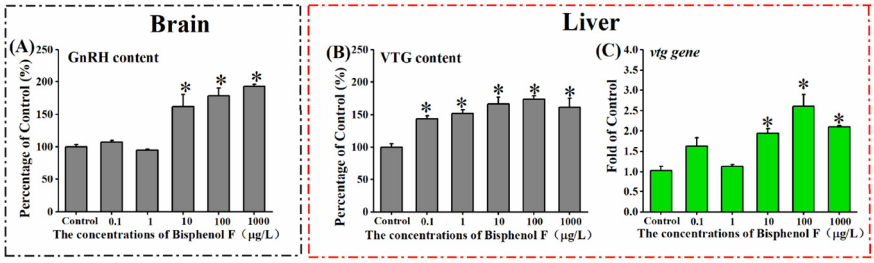

Fig. 4. Effects of Bisphenol F (BPF) on the protein level of GnRH in brain (A), VTG in liver (B) and mRNA levels of *vtg* in liver (C) of zebrafish after 60 days exposure. The present study did not evaluate the differences of above tested parameters between male and female zebrafish. Data are shown as means ± standard error of means (SEM) relative to the control (n = 3). The GnRH content in the control group was 73.2 ± 2.44 ng/g protein. The VTG content in the control group was 15.4 ± 0.81 mg/g protein. *represents significant differences compared to control (Tukey's test, ANOVA, $p < 0.05$).

induced *vtg* gene expression (Fig. 4C), as compared to controls (Tukey's test, $p < 0.05$).

3.4. Effects of estrogen receptors (ERs) and aromatase B pathways

We also investigated estrogen receptors and AROM pathways which are known to ultimately affect reproductive functions. The mRNA levels of *erα* were significantly increased in response to 10, 100, and 1000 μg/L BPF exposure, while *erβ* was significantly increased in response to 100 μg/L (Fig. 5A–B, Tukey's test, $p < 0.05$). Also, the mRNA levels of *cyp19a* and *cyp19b* have been also induced after BPF exposure (Fig. 5C–D). In order to further investigate possible involvement of estrogen receptors and aromatase B pathways, we used the ER antagonist ICI 182780 (ICI) and the AROM inhibitor fadrozole (FAD) to coexposure with the BPF exposure respectively. The survival rate of zebrafish was not significantly altered in the receptor inhibition experiment. As shown in Fig. 5E–H, 100 μg/L BPF exposure significantly increased gene expression of *kiss1*, *gnrh3* in brain and *vtg* in liver. ICI significantly attenuated the stimulatory actions of BPF on gene expression of *kiss1* (Fig. 5E) (Tukey's test, $p < 0.05$), but not *vtg* and *gnrh3* (Fig. 5F, G). Similarly, FAD significantly attenuated the stimulatory actions of kiss1 and *vtg* (Fig. 5E, F) (Tukey's test, $p < 0.05$), but not *gnrh3* (Fig. 5G). Both ICI and FAD together significantly attenuated the stimulatory actions of *kiss1*, *vtg* and *gnrh3* (Fig. 5E–G) (Tukey's test, $p < 0.05$).

3.5. Bisphenol analogues have similar effects on the expression of reproductive neuroendocrine-related genes

To evaluate the impact of the three bisphenol analogues chronic exposure on the zebrafish, the concentration of 100 μg/L BPA and BPS was chosen to compare the effects with the same concentration of BPF. As shown in Fig. 6A, the sex ratio and the GSI of female fish had no significant difference among BPA, BPS and BPF exposure. The reproductive neuroendocrine-related genes including *kiss1*, *kiss1r*, *kiss2*, and *gnrh3*

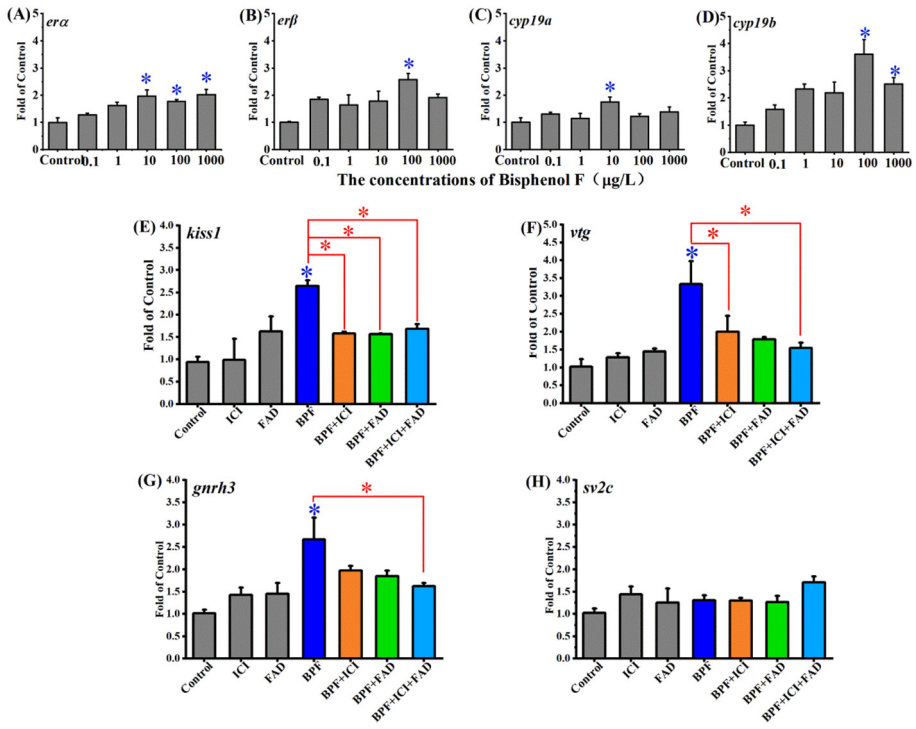

Fig. 5. Effects of estrogen receptors (ERs) and aromatase B pathways after chronic Bisphenol F (BPF) exposure in zebrafish. A–D: Effects of Bisphenol F (BPF) on gene expression of *erα* (A), *erβ* (B), *cyp19a* (C) and *cyp19b* (D) in zebrafish brain after 60 days exposure. * represents significant difference compared to control at $p < 0.05$ (ANOVA, Tukey's test). E–H: Effects on *kiss1* (E, brain), *vtg* (F, liver), *gnrh3* (G, brain), and *sv2c* (H, brain) mRNA levels following exposure to BPF (100 μg/L), a mixture of BPF (100 μg/L) and ER antagonist ICI 182780 (ICI, 1 μM), a mixture of BPF (100 μg/L) and the aromatase B inhibitor fadrozole hydrochloride (FAD, 1 μM) and a mixture of BPF (100 μg/L), ICI (1 μM) and FAD (1 μM) in zebrafish. Blue asterisks indicate represents significant differences compared to control at $p < 0.05$ and red asterisks indicate represents significant differences compared to 100 μg/L BPF treatment at $p < 0.05$ (ANOVA, Tukey's test). Data in the figure are shown as means ± standard error of means (SEM) relative to the control (n = 3).

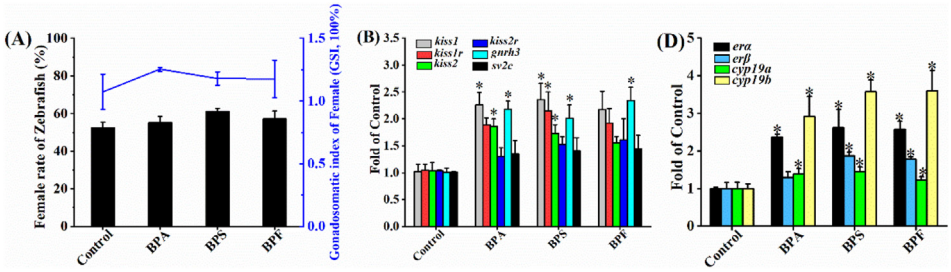

Fig. 6. Bisphenol analogues have similar effects on the expression of reproductive neuroendocrine-related genes. A: Effects of bisphenol A (BPA, 100 μg/L), bisphenol S (BPS, 100 μg/L) and Bisphenol F (BPF, 100 μg/L) on the female rate and the GSI of female in zebrafish after 60 days exposure. B: Effects of BPA, BPS, BPF at 100 μg/L on reproductive neuroendocrine-related genes including *kiss1*, *kiss1r*, *kiss2*, *kiss2r*, *gnrh3* and *sv2* in zebrafish brain after 60 days exposure. C: Effects of BPA, BPS, BPF at 100 μg/L on estrogen receptors (ERs) and aromatase B related genes including *erα*, *erβ*, *cyp19a* and *cyp19b* in zebrafish brain after 60 days exposure. * represents significant difference compared to control at $p < 0.05$ (ANOVA, Tukey's test). Data in the figure are shown as means ± standard error of means (SEM) relative to the control (n = 3).

were significantly increased in response to BPA, BPS and BPF exposure as shown in Fig. 6B. Together with BPA, BPS and BPF showed similar induction on *erα*, *erβ*, *cyp19a* and *cyp19b* gene expression (Fig. 6C), further providing a strong evidence that Bisphenol analogues have similar effects on reproductive neuroendocrine system.

4. Discussion

Consistent with a previous study on embryonic zebrafish, we found that environmental levels of BPF altered many aspects of the reproductive neuroendocrine system in adult zebrafish, including inducing the expression of reproductive neuroendocrine-related genes and the increase in endocrine hormone levels (Qiu et al., 2016). The correlation between the significant induction in most of the examined endocrine endpoints (Table S2) suggests that long-term exposure to environmentally relevant levels of BPF may have a comprehensive impact on the function of the reproductive neuroendocrine system in teleosts (Yang et al., 2017). Our results indicate that the effects of BPF on endocrine hormone levels included a non-monotonic response since intermediate BPF levels did not seem to lead to an increase in FSH and LH levels. These observations are consistent with previous findings that numerous chemicals present non-monotonic changes after exposure to low physiological concentrations (Renieri et al., 2017; Docea et al., 2018; Iwanowicz et al., 2014). In addition, all the effects were associated with increased *erα*, *erβ*, *cyp19a*, and *cyp19b* activity. Meanwhile, ER and AROM antagonists, alone and in combination, significantly attenuated *kiss1*, *vtg*, and *gnrh3* upregulation, suggesting that chronic BPF exposure affects the regulation of the reproductive neuroendocrine system through activation of the ER and AROM B pathways. This is consistent with previous observations that the ER and brain AROM B pathways were affected by BPA, BPS, and BPF *in vitro* using zebrafish hepatic reporter cell lines and *in vivo* using transgenic *cyp19a1b-gfp* zebrafish (Le Fol et al., 2017).

The BPF concentrations applied in this study were chosen according to documented environmental BPA concentrations as there are few reports on BPF environmental concentrations. The documented concentrations of BPA ranged from 0.317 to 1275 ng/L in water treatment plants (Muhamad et al., 2016) to concentrations of >1000 μg/L in landfill leachate and sewage treatment effluents (Crain et al., 2007; Kang et al., 2007). Additionally, BPF concentrations as high as 2850 ng/L were found in the Tamagawa River in Japan (Yamazaki et al., 2015). Thus, in the present study, zebrafish were exposed to a wide range of environmentally relevant BPF concentrations (0.1, 1, 10, 100, and 1000 μg/L) to comprehensively determine the effects on the reproductive neuroendocrine system. Some parameters, including VTG activity, were particularly affected at concentrations as low as 0.1 μg/L, lower than environmentally relevant BPF levels. This indicates that the fish endocrine system is sensitive to waterborne toxicant exposure, especially long-term exposure to low levels of toxins. In addition, the degradation efficiencies of BPF in seawater are reported to be similar to those of BPA (Danzl et al., 2009); combined with their increased usage, the chronic impacts of BPF on aquatic organisms should not be underestimated. In addition, given that BPF enhanced the expression of some, but not all, reproductive neuroendocrine-related parameters, we postulate that at least some of the selective actions of these endocrine disruptors were induced after BPF exposure.

Chronic exposure to BPF induced the expression of *kiss1* and *kiss1r*, along with altered GnRH secretion, suggesting that two months of critical exposure to BPF had significant effects on the Kiss/GnRH system. This result is in line with that of a previous study indicating that endocrine disrupting chemicals, including BPA and BPS, stimulate Kiss/GnRH system-related gene expression in teleosts (Qiu et al., 2016; Faheem et al., 2019). In mammals, Kiss can have both direct and indirect effects on the stimulation of neuronal GnRH activity (Pielecka-Fortuna et al., 2008). However, the functional relationship between Kiss and GnRH-expressing neurons in fish remains unclear. Previous work on cichlids showed that Kiss receptors are expressed on GnRH-expressing neurons, thereby predicting the potential for direct interactions (Parhar et al., 2004). However, Kiss receptor expression has not been detected in GnRH1-, GnRH2-, or GnRH3-expressing neurons in medaka (Kanda et al., 2013), and Kiss treatment acts indirectly through synaptic communication to stimulate electrical activity in GnRH3-expressing neurons (Zhao and Wayne, 2012). In the present study, there was a very high correlation between *kiss1* and *gnrh3* expression ($R = 0.527$, $p < 0.05$, Table S2), suggesting the effects of BPF on the levels of *GnRH* mRNA and GnRH secretion may be mediated by kisspeptin stimulation in zebrafish (Zhao et al., 2014).

Notably, BPF exposure altered the expression levels of *kiss1* and *kiss1r*, but not *kiss2* or *kiss2r*. In zebrafish, there is inconsistency in physiology that indicates a role for *kiss1*, but not *kiss2*, in stimulating the development of GnRH3-expressing neurons in the embryo and electrical activity in the adult (Zhao et al., 2014). Since *kiss1*-expressing neurons are located in the habenula whereas *kiss2*-expressing neurons are located in the hypothalamus, this suggests that *kiss2* is the dominant regulator of the hypothalamic-pituitary axis (Servili et al., 2011; Ogawa et al., 2012). However, our study showed that *kiss1*, but not *kiss2*, mediated the BPF-induced effects on the reproductive neuroendocrine system in zebrafish, providing further strong evidence for the important role of *kiss1* in regulating the hypothalamic-anterior pituitary axis in this teleost.

Moreover, the gonadotropins LH and FSH, important hormones in the HPG axis, were induced after chronic BPF exposure. As FSH and LH are known to regulate gonad development in fish (Yoshiura et al., 1997), the effects of environmentally relevant levels of BPF on the reproductive neuroendocrine system were likely exerted through the HPG axis, including the induction of the Kiss/GnRH system, stimulation of FSH and LH secretion, and enhancement of *lhr* and *fshr* expression in both the female and male gonads. These results were similar to those of

an earlier study showing that BPA exposure increased the mRNA levels of reproductive neuroendocrine-related genes in the brain/pituitary of hermaphroditic fish (*Kryptolebias marmoratus*) (Rhee et al., 2011). These results revealed the potential of BPF exposure to ultimately interfere with the development and reproduction of the gonads in fish. Furthermore, both animal and *in vitro* studies have supported the conclusion that endocrine disrupting chemicals have gender-specific effects (Sifakis et al., 2017). As shown in the present study, the induction of *lhr* and *fshr* mRNA levels in the female gonads was clearer than in those of the male, which also suggests that female zebrafish might be more sensitive to hormone alterations following BPF exposure (Galea et al., 1995).

The present study revealed that BPF affected not only the Kiss/GnRH system, but also the levels of other proteins such as VTG. Vitellogenin is an egg yolk precursor protein that in vertebrates is used as a biomarker to evaluate endocrine disruption in response to environmental estrogens (Hansen et al., 1998). Earlier work in juvenile Atlantic salmon (*Salmo salar*) showed that exposure to xenoestrogens, including BPA, lindane, and 4-nonylphenol, induces vitellogenin expression (Arukwe et al., 2000). In the present study, the increased GnRH and VTG content indicated that long-term exposure to low concentrations of BPF can lead to endocrine hormone disorders in fish, further suggesting that BPF may potentially have a function similar to that of xenoestrogens. In our study, hormone and reproductive neuroendocrine-related gene levels were analyzed in whole brain samples instead of in the pituitary gland or adenohypophysis; thus, it was difficult to pinpoint which hormone-producing tissue was affected. However, most of these parameters were significantly induced, suggesting that BPF exposure had a comprehensive effect on the reproductive neuroendocrine system in the zebrafish brain.

Many xenoestrogenic endocrine disrupting compounds (EDCs), including BPA and BPS, adversely impact estrogen signaling by interacting with two ERs, namely ERα and ERβ (Shanle and Xu, 2011). In this study, exposure to BPF increased *erα* and *erβ* mRNA levels, consistent with a previous study showing that BPF could efficiently activate both *erα* and *erβ* in *in vitro* bioassays using the MELN cell line (Molina-Molina et al., 2013). Combined with an ER antagonist, it could significantly attenuate the stimulatory actions of BPF on *kiss1* expression. Our results revealed that BPF affects the reproductive neuroendocrine system, at least in part, *via* the ER signaling pathway (Kuiper et al., 1998). We also observed that BPF was effective at upregulating the mRNA levels of the AROM genes *cyp19a* and *cyp19b* in the zebrafish brain, and that an AROM antagonist attenuated the stimulatory actions of BPF on *kiss1* and *vtg*; this suggests that AROM pathway activity also partly mediated the actions of BPF on the reproductive neuroendocrine system. The AROM pathway is central to estrogen synthesis and was reported to be activated after exposure to estrogenic chemicals, including E2, EE2, and BPA, in adult male zebrafish (Kallivretaki et al., 2006). Furthermore, the functional interaction between the ER and AROM signaling pathways could serve as an important regulatory link between the endocrine systems in fish (Strobl-Mazzulla et al., 2008). The inhibitors ICI and FAD together significantly attenuated the stimulatory actions of BPF on the expression of all the tested genes which had shown significant induction, revealing a complementary interaction between ER- and AROM-activated pathways in response to chronic exposure to xenoestrogenic EDCs (Kishida and Callard, 2001).

In conclusion, long-term exposure to environmentally relevant and low levels of BPF led to increased expression of reproductive neuroendocrine-related genes (*kiss1*, *kiss1r*, and *gnrh3*) and increased the levels of GnRH, LH, and FSH in the zebrafish brain, as well as VTG in the zebrafish liver; this further suggests that BPF may potentially function in a manner similar to xenoestrogen. In addition, these effects were associated with increased *erα*, *erβ*, *cyp19a*, and *cyp19b* activity. Meanwhile, ER and AROM antagonists, alone and in combination, significantly attenuated the BPF-induced stimulation of *kiss1*, *vtg*, and *gnrh*3, suggesting that chronic BPF exposure affects the regulation of the reproductive neuroendocrine system through activation of the ER and AROM pathways. Moreover, as BPF and BPS induced toxic and reproductive neuroendocrine effects similar to those of BPA, normal usage of BPA and its alternatives should be reconsidered in the future.

Acknowledgment

This work was sponsored by National Key R&D Program of China (2018YFC0406504), National Natural Science Foundation of China (Grants 21707064), State Key Laboratory of Marine Environmental Science, the MEL Visiting Fellowship Program, State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Guangdong Provincial Key Laboratory of Soil and Groundwater Pollution Control (No. 2017B030301012), Shenzhen Municipal Science and Technology Innovation Committee through project Shenzhen Key Laboratory of Soil and Groundwater Pollution Control (NO. ZDSY20150831141712549), Shenzhen Science and Technology Innovation Committee (KQTD20160226195840 22, 201803023000902), Southern University of Science and Technology (Grant No. G01296001), and the Leading Talents of Guangdong Province Program (Chunmiao Zheng).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.154.

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