ENDOCRINE DISRUPTION AND REPRODUCTION IMPAIRMENT IN ZEBRAFISH AFTER LONG-TERM EXPOSURE TO DE-71

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Abstract: The objective of the present study was to investigate the impact of polybrominated diphenyl ethers (PBDEs) on fish reproduction over 2 generations. Zebrafish (Danio rerio) embryos (F0) were exposed to low concentrations (3 μg/L, 10 μg/L, and 30 μg/L) of the PBDE mixture DE-71 until they were sexually mature, and steroid hormone production, expression of genes involved in steroidogenesis, gonadal development, and gamete characteristics were examined. Exposure of female zebrafish to DE-71 resulted in lower estradiol production and downregulation of cytochrome P450 aromatase mRNA. In males, exposure to DE-71 resulted in greater testosterone production and greater cytochrome P450 c17 α-hydroxylase,17,20-1ase mRNA expression. Moreover, hepatic vitellogenin mRNA and estrogenic receptor β gene transcription were downregulated in females and males. Expression of the follicle-stimulating hormone β gene in the pituitary was upregulated, and the expression of luteinizing hormone β was downregulated in both sexes. Histological examination showed inhibition of oocyte maturation in females and retarded spermatiation in males. The average number of eggs (F1) produced was also reduced. Additionally, exposure of F0 embryos to DE-71 did not result in developmental toxicity, whereas delayed hatching, reduced survival, and decreased growth were observed in the F1 embryos derived from parent fish exposed to DE-71. Therefore, long-term exposure to low concentrations of PBDEs in zebrafish could cause reproductive impairment, suggesting that PBDEs might have significant adverse effects on fish population in the highly PBDEs-contaminated aquatic environment. Environ Toxicol Chem 2014;33:1354–1362. © 2014 SETAC

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INTRODUCTION

Polybrominated diphenyl ethers (PBDEs) are additive flame retardants that have been used extensively in furniture, plastics, carpets, textiles, and electronic devices [1]. During the last decade, PBDEs have attracted much attention because of their persistent bioaccumulation properties and the ecological and health risks they pose to humans and animals. There is great concern about the potentially adverse effects of PBDEs, especially thyroid endocrine disruption, developmental neurotoxicity, and teratogenicity [1–4]. Studies have also indicated that exposure to PBDEs can affect steroid hormone levels and reproductive performance in vertebrates; for example, PBDEs adversely affected sperm production, sex steroid production, and sexual development in rats [5–8]. Moreover, exposure of American kestrels to environmentally relevant concentrations of DE-71 resulted in a decrease in courtship behaviors and reproductive success [9–11].

Only limited information on the potential reproductive toxicity of PBDEs in fish species is currently available. It has been reported that oral administration of BDE-47 to fathead minnows (Pimephales promelas) resulted in selective toxicity against sexually mature males via a reduction in mature sperm production [12] and that waterborne exposure of zebrafish to DE-71 caused a reduction in egg production [13,14]. In addition, in zebrafish, long-term exposure to BDE-209 resulted in reduced fecundity [15], and exposure to DE-71 caused disruption of steroid hormone synthesis [16].

In fish, reproduction is regulated via coordinated interaction of the hypothalamic–pituitary–gonadal (HPG) axis and the liver [17], and it involves certain hormones such as the follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), and estradiol (E2), which regulate gametogenesis and maturation to control the reproductive process [18,19]. Theoretically, endocrine-disrupting chemicals that can affect the concentration of these hormones may affect fish reproductive functions.

It is generally recognized that fish in their early life stages are more sensitive than adult fish to the effects of environmental chemicals, because the former are undergoing critical developmental processes [20]. However, the toxicological effects of developmental exposure may not be evident until the individual reaches reproductive maturity, which means that long-term studies are required to assess the health implications of early developmental exposure to contaminants [16]. Although several studies have reported the effects of PBDE exposure in fish, none of them have studied whether long-term exposure to low concentrations of PBDEs causes endocrine disruption and whether this in turn causes reproduction impairment. The present study sought to bridge this knowledge gap by investigating reproductive and developmental parameters in zebrafish embryos that were exposed to PBDEs until they reached sexual maturity. We used zebrafish because this species has been shown to be an appropriate model for investigating the biological effects of endocrine-disrupting chemicals [21]. The PBDE mixture used was DE-71, because it contains most of the lower brominated congeners that are found in the environment and in biota, such as BDE-47, -99, -100, -153, and-154, as its major constituents [22,23]; the results should therefore generate some insight into the action of PBDEs in the natural environment.
We studied (1) the developmental toxicity of DE-71 in zebrafish embryos and reproductive potential and gamete parameters of the parental fish, (2) sex hormone levels in the plasma, (3) transcriptional profiles of genes related to steroidogenesis and gametogenesis in adult zebrafish, (4) histological changes during gonadal development, and (5) developmental toxicity in the offspring.

MATERIALS AND METHODS

Chemicals

The PBDE mixture (DE-71; purity > 99.9%) was obtained from Wellington Laboratory and was dissolved in dimethyl sulfoxide (DMSO). We purchased MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) from Sigma-Aldrich. The T and E2 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cayman Chemical. Trizol reagent and the SYBR® Green PCR kit were purchased from Invitrogen and Toyobo, respectively. All other chemicals used were of analytical grade.

Fish culture and PBDE exposure

Adult zebrafish (AB strain) culture and embryo exposure were performed using a previously described method [24]. Briefly, the embryos that had developed normally and reached the blastula stage (2 h postfertilization [hpf]) were selected for the experiments. The embryos were randomly distributed into glass beakers containing 500 mL of DE-71 solution of different concentrations (0 μg/L, 3 μg/L, 10 μg/L, and 30 μg/L). The concentrations were selected based on the results of an experiment to detect the highest concentration of DE-71 that did not cause acute developmental toxicity. The control group received 0.003% (v/v) DMSO. There were 3 replicates for each exposure concentration, with each beaker containing 100 embryos. At 10 d postfertilization (dpf), the larvae were transferred into 20-L tanks containing the same concentration of DE-71. At 120 dpf, 6 males and 6 females were randomly selected from each control and exposure tank. The fish were paired, and eggs in each tank were collected daily. The total numbers of eggs produced in 3 consecutive weeks were summed and recorded. On the final day, 15 randomly selected eggs from each tank were fixed in Bouin’s solution for 24 h and then transferred to 70% ethanol. After dehydration in ethanol, the tissues were embedded in paraffin wax, and the sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E) staining.

Evaluation of gamete parameters

During the final 3 wk of the experiment, sexually mature male and female zebrafish were paired, and eggs were collected and counted according to previously described methods [25]. Eggs that had been collected from the mating pairs were counted. On the final day, 15 randomly selected eggs from each tank were used to determine the egg diameter and total protein content. The egg diameter was evaluated using an Olympus IX71 microscope (Olympus America) with a digital camera, and the image was examined in Image Pro Plus 6.0 software [25]. The total protein content in eggs was determined by the Bradford method, using bovine serum albumin (Sigma) as a standard.

Developmental toxicity in F0 and F1 embryos/larvae

To determine the developmental toxicity in the F0 embryos/larvae, the hatching rate was recorded at 4 dpf and 5 dpf, and malformation, growth, and survival were observed and recorded at 5 dpf. To study F1 embryos/larvae, 100 randomly selected fertilized eggs from each tank were collected and separately cultured in glass dishes containing fresh water without DE-71. Hatching was recorded at 4 dpf and 5 dpf, and survival, malformation (spinal curvature), and body length were recorded after 5 dpf.

Histological examination

After 141 d of exposure of F0 fish to DE-71, the gonadal tissues of female and male zebrafish were fixed in Bouin’s solution for 24 h and then transferred to 70% ethanol. After dehydration in ethanol, the tissues were embedded in paraffin wax, and the sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E) staining.

Figure 1. Diagram of the experimental design used to expose zebrafish (Danio rerio) to DE-71. hpf = hours postfertilization; dpf = days postfertilization.
with hematoxylin and eosin. The stained tissues were examined under a light microscope (×200; Zeiss Axioskop; Carl Zeiss). Quantitative staging of the ovaries and testes was performed as described by Shang et al. [26] and Liu et al. [25]. Briefly, for male fish, 10 lobules were randomly selected for examination, and 10 sections from each lobule were examined for each fish. The number of sections showing distinct stages of development was recorded: for spermatocyte development, the stages were spermatogonia, spermatocytes, and spermatids [27]. For female fish, oocytes were examined and classified into 4 developmental stages: oocyte/ooogonia, previtellogenetic, vitellogenic, and pre-vitellogenic oocytes [27,28]. The proportion of each cell type at each stage was expressed as a percentage of the total number of follicles in the section examined.

Sex hormone measurement

Blood samples were collected as described by Liu et al. [25]. Briefly, blood was collected from the caudal vein of each fish of the F0 generation, and blood samples from 6 fish of the same sex were pooled as 1 replicate. The blood samples were centrifuged at 3500 g for 2 min at 4 °C, and the supernatant (males 10 μL; females 7.5 μL) was collected and stored at −80 °C until analysis. Before ELISA could be performed, free steroids were extracted from the samples by using a previously reported method [29]. Briefly, each blood sample was diluted to 400 μL with Milli-Q water in clean glass tubes, 2 mL diethyl ether was added to each glass and vortexed, and then the solution was centrifuged at 4 °C for 10 min. The supernatants were removed and transferred to a clean tube, and 2 mL of diethyl ether was added for another extraction. The collected supernatants were evaporated to dryness with nitrogen and stored at −80 °C. The dried extracts were resuspended in 110 μL ELISA buffer. The T and E2 contents in adult zebrafish were measured with ELISA kits (Cayman Chemical Company; detection limits 6 pg/mL for T and 19 pg/mL for E2).

Quantitative real-time polymerase chain reaction assay

The liver, gonad, and brain of the F0 generation were collected and preserved in Trizol reagent at extraction, puriﬁed RNA in each sample was veriﬁed by determining the A260/A280 ratio and by conﬁrming the purity of 1.0 μg RNA using 1% agarose–formaldehyde gel electrophoresis with ethidium bromide staining. The puriﬁed RNA was used immediately for reverse transcription (RT) or stored at −80 °C until analysis.

Synthesis of ﬁrst-strand cDNA was performed using a PrimeScript® RT Reagent Kit (TaKaRa) following the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (PCR) was conducted on an ABI 7300 System (Perkin-Elmer Applied Biosystem) using the SYBR® Green PCR kit (Toyobo). The primer sequences of the selected genes were obtained using the online Primer 3 program (http://frodo.wi.mit.edu/) and are listed in Table 1. The thermal cycle was set at 95 °C for 2 min; this was followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The ribosomal protein L8 gene (rpl8) was used as an internal control, because mRNA expression of this gene in both male and female tissues was not affected after DE-71 exposure in the present study (data not shown). The mRNA expression level of each target gene was normalized to the mRNA content of its reference gene, and changes in the mRNA expression of the relevant genes were analyzed by using the 2−ΔΔCt method.

Statistical analysis

The normality of the data and the homogeneity of variances were analyzed with the Kolmogorov–Smirnov test and Levene’s test, respectively. Logarithmic transformation was performed if the Kolmogorov–Smirnov test failed, and the data were checked again for homogeneity of variances. When the data satisfied the assumption of homogeneity of variances, the differences between the variables were evaluated by one-way analysis of variance (ANOVA), followed by Tukey’s test. A p value of <0.05 was considered statistically significant. All values were expressed as the mean ± standard error (SEM).

Table 1. Primer sequences used in the study

| Gene     | Sequence of the primer (5’–3’) | Genbank accession no. |
|----------|--------------------------------|-----------------------|
| rpl8     | Forward: TTGTTGGTGGTTGCTGGTTGCTGGTTGCTGGT | NM_200713             |
|          | Reverse: GATGCTCAACAGGTCTCAT             |                       |
| FSHβ     | Forward: GTGCAGACTATCTGTTGACA            | AY424303              |
|          | Reverse: AGCTCTCCCTGTTGTTGTTG            |                       |
| LHβ      | Forward: GAGACGTTATCGTGTTGAAAAA          | NM_205622             |
|          | Reverse: AACAGTCGGGGCAGGTAT              |                       |
| CYP19a    | Forward: CTGAAAGGGCTACGGAGACAA           | AF226620              |
|          | Reverse: TGGTGGATTGTTGTCTGAG             |                       |
| 17βHSD   | Forward: AAATAAGGGGCGCTTGAGGA            | NM_205584             |
|          | Reverse: TACACGCTCTTGTGTCTCCAGT          |                       |
| CYP17    | Forward: GACATGCTCTCCCGGACATC            | AY2831362             |
|          | Reverse: GCAATGCTGTGGTTTTC              |                       |
| CYP19b    | Forward: GGCATGCTCTCAGGAGATGAC           | AY780257              |
|          | Reverse: CAGTGGTCTCGAGGTTTCCCA           |                       |
| ERα      | Forward: TAGGAAAGCAACACGAA               | NM_152959             |
|          | Reverse: GTGATGGAGGTGAGGTTTT             |                       |
| ERβ      | Forward: TAGATGCTGGGCGGAAG               | AJ414566              |
|          | Reverse: TATCCAGCCACGACAGATT             |                       |
| VTG1     | Forward: TCCATTGCTGAAAACGCAA             | AF406784              |
|          | Reverse: TGGATCTACGCACACTCTCA            |                       |

rpl8 = ribosomal protein L8; FSHβ = follicle-stimulating hormone β; LHβ = luteinizing hormone β; CYP19a = cytochrome P450 aromatase A; 17βHSD = 17β-hydroxysteroid dehydrogenase; CYP17 = cytochrome P450 c17α-hydroxylase, 17,20-1ase; CYP19b = cytochrome P450 aromatase B; ERα = estrogenic receptor α; ERβ = estrogenic receptor β; VTG1 = vitellogenin.
RESULTS

Developmental toxicity in the F0 and F1 generations

Exposure of the F0 generation to DE-71 did not significantly affect hatching, malformation, growth, or survival (Table 2). With regard to the F1 generation, more than 84.0% of the control embryos hatched successfully at 4 dpf (Table 2), whereas the hatching rate was significantly reduced in the F1 embryos from the 10-µg/L and 30-µg/L DE-71 treatment groups (Table 2). The hatching rate was restored to levels similar to control levels at 5 dpf (>92%). The malformation rate, survival rate, and growth (body length) were recorded at 5 dpf. A trend of increase in the malformation rate was observed in the F1 larvae compared with the control, but the difference was not significant (Table 2). Likewise, the survival rates were significantly decreased in the F1 larvae derived from parents exposed to 10 µg/L and 30 µg/L of DE-71 (Table 2). Growth (body length) of the F1 larvae was significantly inhibited in all the exposure groups relative to the control (Table 2).

After 141 d of exposure of the F0 fish, there were no effects on growth in females or males exposed to any concentration of DE-71. The gonadal somatic index did not change in females but was significantly increased in males in a concentration-dependent manner in all exposure groups (Table 3). In females, exposure to 30 µg/L DE-71 significantly increased the brain somatic index (Table 3). In males, significantly increased brain somatic index was observed in the groups exposed to higher concentrations of DE-71 (10 µg/L and 30 µg/L) (Table 3). However, none of the concentrations caused any significant changes in the hepatic somatic index (Table 3).

Egg production and gamete parameters

The total cumulative egg production was significantly reduced by 32.1% in adults exposed to 30 µg/L DE-71 (Figure 2A). The total protein content of eggs was also significantly decreased by 47.4% in adults exposed to 30 µg/L DE-71, and the decrease was concentration dependent (Figure 2B). The egg diameter did not change significantly in response to DE-71 exposure (data not shown).

Gonadal development

In the F0 females, histological examination showed that the percentages of oocyte/oogonia and previtellogenic did not significantly change after DE-71 exposure (Figure 3A). The percentage of vitellogenic was significantly increased by 57.0% and 67.1% in the adults exposed to 3 µg/L and 10 µg/L DE-71, respectively (Figure 3A). However, the percentage of prevulatory oocytes was significantly decreased by 56.3% in the 10-µg/L DE-71 group (Figure 3A).

Table 2. Effects of DE-71 on zebrafish developmental parametersa

| Fish | DE-71 (µg/L) | Hatching rate (%)b | Survival rate (%)b | Malformation rate (%)b | Body length (mm)c |
|------|-------------|-------------------|-------------------|-----------------------|------------------|
|      | 0           | 4 d               | 5 d               | (5)                   | (5)              |
| F0   | 0           | 94.0±2.08         | 95.0±1.33         | 93.3±2.33             | 1.33±0.33        | 3.81±0.03        |
|      | 3           | 89.3±1.33         | 90.7±1.76         | 87.7±1.76             | 1.67±0.67        | 3.78±0.03        |
|      | 10          | 88.7±1.33         | 89.8±1.76         | 86.3±1.67             | 2.67±0.67        | 3.80±0.01        |
|      | 30          | 90.0±0.58         | 92.2±1.80         | 89.7±1.20             | 2.67±0.67        | 3.79±0.02        |
| F1   | 0           | 84.0±3.05         | 98.8±1.32         | 96.6±0.68             | 2.33±0.67        | 3.95±0.02        |
|      | 3           | 88.8±1.87         | 97.3±1.67         | 94.5±0.67             | 2.67±0.33        | 3.76±0.03**      |
|      | 10          | 73.2±1.49**       | 95.7±1.73         | 92.0±1.55*             | 3.67±0.33        | 3.71±0.04**      |
|      | 30          | 7.76±1.76**       | 92.3±2.30         | 85.3±2.02**            | 4.00±0.56        | 3.62±0.04**      |

Table 3. Growth and somatic index in F0 fish after exposure to DE-71 for 141 d

| DE-71 (µg/L) | Female | Male |
|-------------|--------|------|
|             |        |      |
| 0 µg/L      | 3.67±0.04 | 3.77±0.04 |
| 3 µg/L      | 3.71±0.05 | 3.77±0.03 |
| 10 µg/L     | 3.77±0.03 | 3.72±0.03 |
| 30 µg/L     | 3.72±0.03 | 3.66±0.02 |
|             | 0 µg/L  | 3 µg/L  |
| 0 µg/L      | 3.72±0.03 | 3.66±0.02 |
| 3 µg/L      | 3.72±0.03 | 3.66±0.02 |
| 10 µg/L     | 3.72±0.03 | 3.72±0.03 |
| 30 µg/L     | 3.72±0.03 | 3.72±0.03 |

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| DE-71 (µg/L) | Female | Male |
|-------------|--------|------|
|             |        |      |
| 0 µg/L      | 3.67±0.04 | 3.77±0.04 |
| 3 µg/L      | 3.71±0.05 | 3.77±0.03 |
| 10 µg/L     | 3.77±0.03 | 3.72±0.03 |
| 30 µg/L     | 3.72±0.03 | 3.66±0.02 |
|             | 0 µg/L  | 3 µg/L  |
| 0 µg/L      | 3.72±0.03 | 3.66±0.02 |
| 3 µg/L      | 3.72±0.03 | 3.66±0.02 |
| 10 µg/L     | 3.72±0.03 | 3.72±0.03 |
| 30 µg/L     | 3.72±0.03 | 3.72±0.03 |
In the F0 males, there was no significant difference in the percentage of spermatogonia in all DE-71 exposure groups and the controls (Figure 3B), whereas a significant increase in the percentage of spermatocytes was observed in the 30-μg/L DE-71 group (54.9%; Figure 3B). However, the percentage of spermatids was significantly reduced by 50.9% after exposure to 30 μg/L DE-71 compared with the control (Figure 3B).

Plasma sex hormones

The sex hormone levels in both males and females of the F0 fish were altered on exposure to DE-71 (Figure 4). In females, exposure to 3 μg/L, 10 μg/L, and 30 μg/L DE-71 significantly reduced the plasma E2 level by 60.4%, 37.7%, and 47.0%, respectively, whereas the plasma testosterone level did not change (Figure 4A). In males, the concentration of T was significantly increased by 53.7% after exposure to 30 μg/L DE-71, but the concentration of E2 did not significantly change (Figure 4B).

Gene transcription

The mRNA expression of selected genes expressed in the HPG axis and liver in the F0 fish was examined (Table 4). In the brain, exposure to DE-71 caused significant upregulation of FSHβ gene transcription in females exposed to 3 μg/L, 10 μg/L, and 30 μg/L DE-71 (Table 4). In male fish, mRNA expression of FSHβ was significantly upregulated after 3 μg/L, 10 μg/L, and 30 μg/L DE-71 treatment (Table 4). A marked downregulation of LHβ gene transcription was observed in both females and males exposed to DE-71 (Table 4). Expression of cytochrome P450 aromatase B (CYP19b) mRNA was significantly downregulated in both females and males in the 30-μg/L DE-71 group relative to the control group.

In the ovary, the expression of cytochrome P450 c17 α-hydroxylase,17,20-lase (CYP17) and 17β-hydroxysteroid dehydrogenase (17β-HSD) did not change after DE-71 exposure, whereas a marked downregulation of cytochrome P450 aromatase A (CYP19a) gene expression was observed in a concentration-dependent manner (Table 4). In the testes, expression of CYP17 mRNA was upregulated in the 30-μg/L DE-71 group (Table 4). No significant difference was observed in 17β-HSD gene expression between the experimental and control groups. The transcription of CYP19A was significantly downregulated after exposure to 10 μg/L and 30 μg/L DE-71 (Table 4).
In the liver, mRNA expression of the hepatic vitellogenin (VTG1), estrogenic receptor α (ERα), and estrogenic receptor β (ERβ) genes was examined (Figure 3). In females, VTG1 gene transcription was significantly downregulated after exposure to 3 μg/L, 10 μg/L, and 30 μg/L DE-71 (Table 4). In males, the expression of VTG1 was also significantly downregulated in all exposure groups (Table 4). Estrogenic receptor α gene transcription was not significantly altered in either female or male zebrafish exposed to DE-71 (Table 4). However, expression of ERβ was significantly downregulated in females exposed to 30 μg/L DE-71. Moreover, in males, ERβ gene transcription was significantly downregulated in the 3-μg/L, 10-μg/L, and 30-μg/L groups (Table 4).

**DISCUSSION**

The present study demonstrates that long-term exposure of zebrafish to PBDEs altered plasma sex hormone levels as well as the transcription of genes involved in the steroidogenesis pathway. These results support the findings of a recent study in which zebrafish were exposed to PBDEs [16]. Several in vitro studies on human adrenocortical carcinoma cells (H295R cell line) also showed that exposure to PBDEs can interfere with steroidogenesis genes [31–34]. These results suggest that the steroidogenic pathway could be a target for endocrine disruption of PBDEs and might contribute to reproductive toxicity.

In female fish, the plasma E2 level was significantly reduced, but not the plasma T level. This result is consistent with a previous study showing decreased serum E2 and unaltered T levels in female zebrafish upon exposure to DE-71 [16]. This observation also indicates that PBDEs act as an endocrine disruptor of sex hormones. Although the mechanism for the decrease in E2 is not well understood, it possibly involves the role of CYP19, which is the terminal enzyme in the steroidogenic pathway that converts testosterone into E2 [35]. Our findings also indicate this involvement, because we found that expression of both Cyp19a and Cyp19b in the brain and ovary was significantly downregulated. Thus, the downregulation of CYP19 gene expression could explain the decrease in the production of E2 in the presence of PBDEs.

**Table 4. Gene expression in F0 adult zebrafish after exposure to DE-71 (0 μg/L, 3 μg/L, 10 μg/L, and 30 μg/L) for 141 d**

| Sex    | DE-71 (μg/L) | FSHβ | LHβ | Cyp19β | Cyp17β | 17βHSDβ | Cyp19α | E2α | ERβ | VTG1 |
|--------|--------------|------|-----|--------|--------|---------|--------|-----|-----|------|
| Female | 0            | 1.09±0.20 | 1.00±0.07 | 1.05±0.17 | 1.05±0.16 | 1.07±0.21 | 1.16±0.26 | 1.03±0.12 | 1.08±0.19 | 1.00±0.07 |
|        | 3             | 1.67±0.24 | 0.10±0.04** | 0.98±0.16 | 1.07±0.12 | 1.37±0.24 | 0.50±0.08** | 0.98±0.16 | 0.60±0.07 | 0.31±0.04** |
|        | 10             | 1.86±0.31 | 0.23±0.11** | 0.81±0.06 | 0.97±0.05 | 1.28±0.25 | 0.37±0.08** | 0.87±0.07 | 0.58±0.09 | 0.31±0.15** |
|        | 30             | 2.16±0.20‘ | 0.20±0.10** | 0.50±0.05‘ | 0.98±0.08 | 1.28±0.25 | 0.34±0.08** | 0.94±0.09 | 0.49±0.12‘ | 0.29±0.10‘ |
| Male   | 0             | 1.02±0.11 | 1.01±0.13 | 1.02±0.09 | 1.00±0.03 | 1.07±0.21 | 1.00±0.03 | 1.02±0.15 | 1.02±0.15 | 1.02±0.13 |
|        | 3             | 1.83±0.11** | 0.38±0.13** | 1.03±0.13 | 1.17±0.18 | 1.23±0.26 | 1.02±0.13 | 1.09±0.24 | 0.29±0.11** | 0.54±0.19 |
|        | 10             | 1.93±0.20‘ | 0.16±0.05‘ | 0.72±0.09 | 0.92±0.11 | 1.43±0.28 | 0.50±0.12‘ | 0.90±0.11 | 0.38±0.09‘ | 0.30±0.13‘ |
|        | 30             | 1.73±0.15** | 0.14±0.04** | 0.58±0.16‘ | 1.51±0.17‘ | 1.12±0.21 | 0.59±0.11‘ | 0.71±0.03 | 0.33±0.08** | 0.25±0.07** |

All results are expressed as mean ± standard error of the mean for 3 replicate samples (2 fish counted as one replicate).

**Note:**

- **FSHβ** = follicle-stimulating hormone β; **LHβ** = luteinizing hormone β; **Cyp19β** = cytochrome P450 aromatase B; **CYP17** = cytochrome P450 c17α-hydroxylase, 17,20-lyase; **17βHSD = 17β-hydroxysteroid dehydrogenase**; **Cyp19α = cytochrome P450 aromatase A**; **E2α = estrogenic receptor α; ERβ = estrogenic receptor β; VTG = vitellogenin.**
In male zebrafish, the concentrations of T were significantly increased, whereas production of E2 was not significantly affected. This observation again is consistent with a previous study on long-term exposure of zebrafish to DE-71 [16]. Stoker et al. [36] reported increased testosterone concentrations in DE-71-exposed adult male rats. It is possible that this increase is related to the expression of CYP17, which encodes enzymes that participate in the synthesis of testosterone. In keeping with this, the findings of the present study also indicate that the greater production of T in males might be partially the result of upregulation of mRNA expression of the CYP17 gene. Moreover, in males, mRNA expression of Cyp19a and Cyp19b was also significantly downregulated, which may lead to less testosterone converted to E2. In the present study, we observed that the Cyp19 genes are the most sensitive and markedly affected genes in the steroidogenesis pathway, which may suggest that Cyp19 is a potential target of PBDEs. It has been shown that some PBDE and PBDE metabolites had inhibitory effects on CYP19 gene expression or CYP19 activity in vitro [33,37].

In the present study, VTG1 gene transcription was downregulated in both males and females. Because VTG gene transcription is dependent on E2 concentration, the observed downregulation of VTG in females could be regarded as a consequence of lower E2 concentration. Hepatic vitellogenin is essential for vitellogenesis, oocyte maturation, and yolk biosynthesis in fish; therefore, downregulation of VTG probably contributed to the reduction in egg quality. In our study, we found downregulation of VTG1 gene transcription and a reduction in preovulatory oocytes, which indicate retarded development of ovaries. Decreased plasma VTG concentrations and fecundity also have been reported for fathead minnows exposed to other chemicals (e.g., prochloraz, fenarimol, 7b-trenbolone, 17α-trenbolone, and fadrozole) [38,39]. Because VTG is key to egg production in female fish, reduction in this lipoprotein could, theoretically, cause lower egg quality and poor vitellogenesis of the ovaries and serve as an indicator of impairment of reproductive success. In the present study, we also observed a reduction in egg production in fish after long-term exposure to DE-71, so it is possible that DE-71-induced downregulation of VTG1 transcription contributed to the reduced fecundity in females. However, a previous study showed that the VTG gene was strongly inhibited in the liver of male zebrafish exposed to DE-71 but not in females [16].

Histological examination showed a significant decrease in the production of mature oocytes and spermatozoa in DE-71-treated female and male zebrafish, respectively. In agreement with this finding, Muirhead et al. [12] found that BDE-47 treatment caused a significant decrease in mature spermatozoa in fathead minnows. Growth and maturation of the developing gonad and maintenance of gametogenesis are regulated by several endocrine and paracrine factors. Among them, the gonadotropins FSH and LH secreted by the pituitary have a major impact on the regulation of gametogenesis [40,41]. In females, the growth stage (vitellogenesis) is primarily under the control of FSH [42], whereas the oocyte maturation phase occurs primarily under the regulation of LH [40]. Therefore, in the present study, upregulation of FSHβ transcription in females suggests promotion of gametogenesis, whereas downregulation of LHβ gene transcription suggests retardation of oocyte maturation. Histological examination revealed a decrease in preovulatory oocytes, which presumably might be related to the marked downregulation of LH gene expression. Decrease in the production of mature oocytes resulted in a decrease in the number of eggs produced, which suggests that DE-71 exposure adversely affects ovarian gametogenesis and induces reproductive toxicity.

In male fish, FSH plays a regulatory role during the early stages of spermatogenesis, whereas LH is involved mainly in the final stages (regulation of spermiation) of maturation [41,43]. In the present study, mRNA expression of the FSHβ gene was upregulated, whereas LHβ gene transcription was strongly downregulated in male fish. The marked decrease in mRNA expression of the LH gene could result in reduced LH concentration, which may delay the final stage of maturation. Delay of the spermatid stage was further observed on histological examination, and this might have contributed to the increase in gonadal somatic index observed in the present study. In agreement with our results, upregulation of the FSHβ gene was also reported in male and female zebrafish after long-term exposure to DE-71 [16] as well as other endocrine disruptors (e.g., chlorophenol) [29], and LH gene expression was reported to be significantly downregulated in both females and males [16].

With regard to developmental toxicity, DE-71 exposure did not affect F0 fish; in the F1 embryos, however, DE-71 exposure resulted in delayed hatching and growth retardation. This observation is consistent with our previous study on long-term exposure of zebrafish to DE-71 [44]. Similarly, reduced hatching rates were found in zebrafish after maternal exposure to BDE-209 [15]. In keeping with these findings, it has been reported that PBDEs can be transferred from exposed adult fish to eggs [15,44,45]. In the present study, the discrepancy in the findings between offspring and their parents might be the result of the different levels of DE-71 in F0 and F1 embryos, which might have directly affected the earliest life stages of fish exposed to PBDEs. Our results confirm that offspring in the developmental stages were more sensitive than their parents who were exposed to PBDE [15,44].

The results of the present study also showed that PBDE exposure led to decreased egg production and egg protein content in females as well as alteration of gonadal development. Reduced egg protein content was also found in zebrafish after exposure to low concentrations of DE-71 [14]. Fish eggs supply all the nutritional requirements for embryonic development. Thus, the reduced protein content after DE-71 exposure might have affected the nutritional content of the eggs, thereby contributing to the decrease in the hatching rate and reproductive success of zebrafish. In the present study, decreased survival rates and growth retardation were observed in the offspring from parent pairs exposed to DE-71. These results further suggest that maternal exposure to DE-71 can impair the reproductive success of offspring. The reduced egg protein after DE-71 exposure has the potential to adversely influence embryonic development and hatching success in fish. Moreover, it should be noted that the decrease in gamete quality and quantity will impair fish reproduction, which might in turn affect species fitness [46].

In summary, our results demonstrate that embryonic exposure of zebrafish to PBDEs disrupted the reproductive success of the adults. Polybrominated diphenyl ethers could affect the production of sex hormones by changing the expression of several key steroidogenic genes, which in turn affect gamete parameters and adversely impair reproductive success in the offspring. It should be noted that exposure concentrations were relatively higher than those measured from environmental water samples [47]. However, environmental exposure to PBDEs with other toxicants (e.g., heavy metals) is prevalent at high concentrations, such as in rivers around e-waste
areas, and bioaccumulation in wild animals occurs [48]. Therefore, future research on environmental risk assessment may be necessary to elucidate the long-term effects on reproduction in fish exposed to PBDEs with other toxicants.

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