Chronic exposure to environmental levels of tribromophenol impairs zebrafish reproduction

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A B S T R A C T

Tribromophenol (2,4,6-TBP) is ubiquitously found in aquatic environments and biota. In this study, we exposed zebrafish embryos (F0; 2nd days post-fertilization, dpf) to environmental concentration (0.3 μg/L) and a higher concentration (3.0 μg/L) of TBP and assessed the impact of chronic exposure (120 dpf) on reproduction. TBP exposure did not cause a significant increase in the malformation and reduction in the survival in the F0-generation fish. After TBP exposure, the plasma testosterone and estradiol levels significantly increased in males and decreased in females. The transcription of steroidogenic genes (3β-HSD, 17β-HSD, CYP17, CYP19A, CYP19B) was significantly upregulated in the brain and testes in males and downregulated in the brain and ovary in females. TBP exposure significantly downregulated and upregulated the expression of VTG in the liver of female and male fish, respectively. Meanwhile, TBP exposure altered the sex ratio toward a male-dominant state. The F1-generation larvae exhibited increased malformation, reduced survival, and retarded growth, suggesting that TBP in the aquatic environment has significant adverse effects on fish population.

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Introduction

The sustained use of brominated flame retardants (BFRs), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), and various other phenolic compounds has resulted in global environmental contamination. Owing to their environmental persistence, bioaccumulative properties, and potential toxicity in animals and humans, BFR contamination has become a matter of great concern. Indeed, most of the recent studies on the toxicity of BFRs have focused on the effects of PBDEs, TBBPA, and HBCD (reviewed by de Wit, 2002; Law et al., 2008). Utilized as flame retardants, bromophenols have been consistently used in textiles, polyurethanes, plastics, epoxy resins, and paper manufacturing, as well as additives or intermediates for the yield of other flame retardants (Weil and Levchik, 2004). In addition, bromophenols are formed by the biodegradation of other pollutants such as brominated benzenes and some PBDEs (Bergman, 1990); bromophenols may also be generated as byproducts of the photochemical degradation of TBBPA in water (Thomsen et al., 2002) or the decomposition of plastics (Thomsen et al., 2001). Among bromophenols, 2,4,6-tribromophenol (TBP) is the most widely produced brominated phenol. The production volume of TBP in 2001 was estimated to be approximately 2500 ***tons/year in Japan and 9500 tons/year worldwide (IUCLID, 2003). TBP has been used as a pesticide for controlling insects, fungi, and bacteria and is a key component of wood preservatives. Furthermore, TBP has been used as flame retardant, and is a synthetic intermediate of most of the important BFRs; for example, TBP is generated by photolytic degradation of TBBPA (Eriksson and Jakobsson, 1998), which is the most abundant BFR. However, brominated phenols are generally not readily biodegradable and tend to persist in the environment (Nichkova et al., 2008).

2,4-Dibromophenol and TBP are the major constituents of the total bromophenol content in environmental samples (Chung et al., 2003). TBP has been detected in surface water (Blythe et al., 2006; Polo et al., 2006; López et al., 2009), drinking water, waste-treatment-facility effluent, sludge, and soil samples (Howe et al., 2005; Gutiérrez et al., 2008). For instance, the concentration of TBP ranged from 1 to 6 μg/L in German Bight (Reneke et al., 2006) and 0.378 to 20.2 ng/L in Korea (Sim et al., 2009). The maximum reported concentrations in surface fresh water were 0.3 μg/L for TBP in Saitama Prefecture in Japan (IUCLID, 2003). TBP in marine sediments ranged from 1.6 to 9 μg/kg dry weight in Korea (Sim et al., 2009), while high content was detected in surficial sediments in Rhone estuary France (26–3690 μg/kg dry weight) (Vetter and Janussen, 2005). In previous studies, TBP was commonly detected in a variety of marine organisms, including marine fish, crustaceans, mollusks, algae, and polychaetes; for example, TBP has been detected in the mussels and blubber of hooded seal (Cystophora cristata) (Vetter and Janussen, 2005). The TBP content in edible marine animals such as mollusks, crustaceans, and marine fish was found to be 198 μg/kg, 2360 μg/kg, and 39 μg/kg dry weight, respectively (Chung et al., 2003). Furthermore, TBP has
been detected in human urine (Nichkova and Marco, 2006), and relatively high TBP concentrations have been found in human serum samples (Thomsen et al., 2002). In human milk, the TBP concentration was as high as 110 ng/g lipid weight (Ohta et al., 2004). The highest reported concentration of TBP in the contaminated wood materials used in food industry is 2000 μg/L (Nichkova et al., 2008).

There is limited information on TBP toxicity and its effects on humans and the environment. Previous studies on the effects of this chemical revealed that TBP causes developmental neurotoxicity, embryotoxicity, and fetotoxicity in rats (Lyubimov et al., 1998). In addition, it is a potent competitor of the thyroid hormone (thyroxine, T4), which was indicated by the results of the in vitro TTR-binding assay (Legler and Brouwer, 2003; Hamers et al. 2006; Suzuki et al., 2008); it showed weak estrogen-like activity in the human breast cancer cell-line MCF-7 (Olsen et al., 2002); TBP caused an induction of aromatase activity in human adrenocortical (H295R) cell line (Cantón et al., 2005), and it induces neuroblastoma cell differentiation (Ríos et al., 2003) and disturbs cellular Ca2+ signaling in neuroendocrine cells (PC12) (Hassenniéver et al., 2006). Exposure to TBP has also been shown to affect the development of zebrafish embryos (Kammann et al., 2006).

Recently, bromophenols have attracted considerable attention because of their extremely low taste threshold even at subnanogram concentrations in aqueous solutions (Malleret et al., 2003; López et al., 2009) and evidence that they can be quite toxic to aquatic organisms (Gribble, 1996; Howe et al., 2005). Owing to their lipophilicity, these compounds can bioaccumulate in the food chain (Whitefield et al., 1998, 1999; Chung et al., 2003; Vetter and Janussen, 2005). In the light of the potential endocrine-disrupting activity of these compounds, there is very little information on the ecotoxicological effects of bromophenols. Therefore, in the present study, we exposed zebrafish embryos to the environmental concentration of TBP (0.3 μg/L) and investigated the effect of long-term (partial life cycle) TBP exposure on fish reproduction. Several toxicological endpoints were examined, including malformation, growth, survival, reproductive output, plasma levels of sex hormones (testosterone and estradiol), and vitellogenin (VTG) gene transcription. We also evaluated the expression of steroidogenic genes such as those involved in testosterone synthesis, including 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), and cytochrome P450 17α-hydroxylase/C17-20 lyase (CYP17) in the gonads, and the genes involved in estradiol synthesis, such as cytochrome P450 aromatase (CYP 19A in the gonads and CYP19B in the brain).

**Materials and methods**

**Reagents.** 2,4,6-Tribromophenol (purity >98%) was purchased from Sigma (St. Louis, MO, USA); TBP solution was prepared using HPLC-grade dimethyl sulfoxide (DMSO). All other chemicals used in the present study were of analytical grade.

**Zebrafish maintenance.** Adult 18-week-old zebrafish (Danio rerio) (AB strain) were maintained at 28 ± 0.5 °C in charcoal-filtered recirculating aerated tap water; the fish were fed freshly hatched Artemia nauplii twice daily and flake food (Tetra, Germany) once daily. Zebrafish maintenance and embryo collection were performed according to the protocol described by Deng et al. (2009). Briefly, zebrafish were maintained in a semi-static system that received charcoal-dechlorinated tap water with a constant temperature of 28 ± 0.5 °C. The photoperiod was adjusted to a 14:10 h (light:dark) cycle. The larvae and fry were fed newly hatched Artemia nauplii and commercial food (Tetra, Germany).

**Embryo exposure and experimental design.** Fertilized eggs were examined under a dissecting microscope and those that showed normal development until 2 days post-fertilization (dpf) were transferred into glass beakers (approximately 300 mL). These eggs were subsequently placed in a water bath at 28 °C. Next, approximately 150 embryos (F0) were exposed to 0.3 μg/L and 3.0 μg/L of TBP, and the same number of embryos was used as controls, which were not exposed to TBP. There were 4 replicate tanks for the control and for each TBP concentration, i.e., 0.3 μg/L and 3.0 μg/L, respectively. Both the control and exposure groups were received 0.01% (vol/vol) DMSO. The F0 embryos were observed daily under a dissecting microscope, and the hatching rate, malformation, and survival rate were recorded. Subsequently, the larvae were transferred to 1-L beakers containing the same concentrations of TBP, and half of the water in each beaker was replaced daily, and TBP was added during each replacement to keep constant throughout the exposure. After 10 dpf, 100 normal larvae from each group were randomly chosen and transferred into a 30-L glass tank containing 20 L of TBP solution. After 120 dpf, the genders of all the fish were determined by examining the macroscopic appearance of the gonads.

**Sex-hormone measurement.** After TBP exposure, the fish were anesthetized with 0.03% tricaine (MS-222) and their lengths and weights were recorded. Blood was collected from the caudal vein of each fish, and blood samples from 2 fish of the same sex were pooled as 1 replicate (approximately 8 μL). The blood samples were centrifuged at 3500×g for 5 min at 4 °C, and the supernatant was collected and stored at −80 °C. The sex hormones in the plasma samples were detected by using the testosterone enzyme immunoassay (EIA) Kit (detection limit, 6 pg/ml) and estradiol EIA Kit (detection limit, 19 pg/ml) obtained from Cayman Chemical (Ann Arbor, MI, USA) in accordance with the manufacturer's instructions and with slight modifications in the method proposed by Liu et al. (2009). The intra- and inter-assay coefficients of variance (CV) for measurements of 17β-estradiol and testosterone were determined to be <10%. We used 4 replicates for the control and exposure groups (two fish of the same sex per replicate). After blood collection, the gonad, the liver, and the brain were dissected, weighed, and stored in liquid nitrogen for further analysis.

**RNA extraction and quantitative real-time polymerase chain reaction assay.** RNA sample preparation, first-strand cDNA synthesis, and quantitative real-time polymerase chain reaction (q-RT-PCR) assay were performed according to previously described protocols (Deng et al., 2009). The primer sequences of selected genes (18S rRNA, 3β-HSD, 17β-HSD, CYP17, CYP19A, CYP19B, and VTG) were obtained by using the Primer 3 program (http://frodo.wi.mit.edu/). The 18S rRNA gene is a housekeeping gene that is ubiquitously used as an internal control in real-time PCR studies on the effects of estrogens in fishes (Tang et al., 2007) or as a suitable reference gene for zebrafish tissue analysis (Filby and Tyler, 2007). In the experimental conditions used in this study, the 18S rRNA gene is ubiquitously expressed, and the expression profile of this gene does not vary under experimental conditions in the present study (data not shown). The primer sequences of the selected genes are shown in Table 1. The thermal cycle for q-RT-PCR procedure was as follows: 95 °C for 3 min, followed by 39–42 cycles of 95 °C for 20 s, 60 °C for 15 s, and 72 °C for 1 min (Chromo 4 Four-Color Real-Time System; Bio-Rad). The expression of the selected genes was measured in triplicate, and the analysis was repeated 3 times on an ABI 7300 System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The Ct value (the first cycle at which the fluorescence signal is significantly distinguishable from the background) for each PCR reaction was determined. The expression profile of the target gene was normalized to the corresponding 18S rRNA mRNA content ($\Delta \Delta \text{Ct} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{18S rRNA}}$), and the average $\Delta \text{Ct}$ value of the target gene was determined ($\Delta \text{Ct} = \text{Ct}_{\text{exposure}} - \text{Ct}_{\text{control}}$). The fold change in transcript abundance was calculated using the formula $2^{-\Delta \Delta \text{Ct}}$. 

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Reproductive endpoints. After exposure the adult (F₀) until 120 dpf, a subset of sexually mature female and male fish was separated and paired according to the method described by Liu et al. (2009). Eggs were collected for 3 successive weeks. The fecundity was calculated as eggs/female per day based on the cumulative number of eggs from a given treatment group (Ankley et al., 2005). In the F₁ generation, the numbers of eggs that were spawned, fertilized, and hatched; the number of larvae that exhibited malformation; and the survival rate were evaluated at 10 dpf. The body length of the larvae was measured according to Shi et al. (2008).

Statistical analysis. The normality of data was tested by using the Kolmogorov–Smirnov test. If necessary, the data was log-transformed to approximate normality. One-way analysis of variance (ANOVA) and Tukey’s multiple-range test were used to determine the significance differences between the control group and each exposure group. Sex ratio was verified by performing the Chi-square test (χ² test). All data were expressed as mean±SEM. The significance level for all the statistical analyses was set at P<0.05.

Results

Growth and somatic indices of the F₀ generation

No delayed hatching or malformation was observed in the F₀ fish (data not shown). We observed a significant increase in the body length and weight of both male and female fish that were exposed to 0.3 μg/L TBP, in comparison with the corresponding values for the individuals in the control group (Table 2). The condition factors (K-factor = weight (g)/length (cm)³ × 100) in the male fish exposed to 0.3 μg/L and 3.0 μg/L of TBP were higher than that in the control group; in the case of female fish, the K-factor for the fish exposed to 3.0 μg/L TBP was higher than that for the control (Table 2). Exposure to 0.3 μg/L of TBP did not cause any significant differences in the brainsomatic index (BSI) in both males and females. However, exposure to 3.0 μg/L TBP resulted in a significant increase in the BSI in both male and female fish (Table 2). Exposure to TBP did not affect the hepatisomatic index (HSI) in males and females. However, the gonadsomatic index (GSI) was significantly higher in the males exposed to 0.3 μg/L of TBP, while there were no significant differences between the GSI values of the female fish that were exposed to 0.3 μg/L and 3.0 μg/L TBP (Table 2).

Sex ratio of the F₀ fish

The male/female ratio in all the groups was analyzed by the χ²-test (P<0.05), and the number of males was significantly higher (Fig. 1). The sex ratio of males to females in the control group was 42:38, while that of the fish exposed to 0.3 μg/L and 3.0 μg/L of TBP was 61:19 and 65:15, respectively.

Fecundity of the F₀ female fish

The egg production in the control group was 119.7±4.7 eggs/female per day. However, in comparison with the egg production in the control group, the fecundity in the fish exposed to 0.3 μg/L and 3.0 μg/L of TBP showed a significant decreasing trend (104.8±12.1 eggs/female per day and 96.7±9.3 eggs/female per day, respectively) (Fig. 2).

Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence of the primer (5'-3')</th>
<th>Gene bank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Forward-ttggctaggctgttccgtgg</td>
<td>Reverse-gggtgcacagggctgtcat</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>Forward-agggccag cacgtccagcag</td>
<td>Reverse-caggtgcacaggttcttcct</td>
</tr>
<tr>
<td>17β-HSD</td>
<td>Forward-aataggggctgtgtagagata</td>
<td>Reverse-tcagctcgccttgctcag</td>
</tr>
<tr>
<td>CYP17</td>
<td>Forward-gaacgtctgtggaacatct</td>
<td>Reverse-gctaacgtctgtggtcact</td>
</tr>
<tr>
<td>CYP19A</td>
<td>Forward-gcttatgagggagttag</td>
<td>Reverse-gttggcttgcatgcggtgcctc</td>
</tr>
<tr>
<td>CYP19B</td>
<td>Forward-agaagccccagggctggtta</td>
<td>Reverse-gttggcttgctagcggaggtgtgc</td>
</tr>
<tr>
<td>VTG</td>
<td>Forward-aggcctctggaggtcgtctra</td>
<td>Reverse-gttgccaggatcccccaggt</td>
</tr>
</tbody>
</table>

K-factor: condition factor calculated as (weight/length³) × 100. BSI = brain weight×100/body weight; HIS = liver weight×100/body weight; GSI = gonad weight×100/body weight.

⁎ The values are mean±SEM of three replicates (six fish per replicate). *P<0.05, **P<0.01 and ***P<0.001 indicate significant difference between exposure groups and the corresponding control group.

Table 2

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>3.95±0.05</td>
<td>4.30±0.08*</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>0.46±0.02</td>
<td>0.61±0.05*</td>
</tr>
<tr>
<td>K-factor*</td>
<td>1.28±0.01</td>
<td>1.48±0.02***</td>
</tr>
<tr>
<td>BSI (100%)*</td>
<td>1.02±0.15</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td>HIS (100%)*</td>
<td>1.21±0.02</td>
<td>1.34±0.19</td>
</tr>
<tr>
<td>GSI (100%)*</td>
<td>1.42±0.06</td>
<td>1.86±0.07*</td>
</tr>
</tbody>
</table>

K-factor: condition factor calculated as (weight/length³) × 100. BSI = brain weight×100/body weight; HIS = liver weight×100/body weight; GSI = gonad weight×100/body weight.

⁎ The values are mean±SEM of three replicates (six fish per replicate). *P<0.05, **P<0.01 and ***P<0.001 indicate significant difference between exposure groups and the corresponding control group.
respectively (Fig. 3A). In the male difference between exposure groups and the corresponding control.

Plasma concentrations of sex hormones

Based on one-way ANOVA, in comparison with the testosterone levels in the control fish, the levels in the female fish exposed to 0.3 μg/L and 3.0 μg/L of TBP were reduced by 16% and 27%, respectively (Fig. 3A). In the male fish, the plasma concentration in the 0.3 μg/L and 3.0 μg/L TBP-exposure groups increased by 6% and 16%, respectively (Fig. 3A). For estradiol levels, the plasma concentration in the female fish exposed to 0.3 μg/L and 3.0 μg/L of TBP reduced by 14% and 22%, respectively (Fig. 3B). The levels in the 0.3 μg/L and 3.0 μg/L TBP-exposure male fish significantly increased by 7% and 24%, respectively (Fig. 3B).

Gene-expression profile

In the females exposed to 3.0 μg/L TBP, the expression of 3β-HSD and CYP17 in the ovary was significantly downregulated (4.0- and 2.3-fold downregulation, respectively) (Figs. 4A, B). The expression of 17β-HSD in the fish exposed to 0.3 μg/L and 3.0 μg/L TBP was significantly downregulated (4.8-fold and 13.7-fold inhibition, respectively) (Fig. 4C). The transcription of CYP19A in the fish exposed to 3.0 μg/L TBP was significantly downregulated (1.6-fold inhibition) (Fig. 4D). In the brains of the fish exposed to 3.0 μg/L of TBP, the expression of CYP19B showed a significant reduction (1.3-fold inhibition) (Fig. 4E). The VTG transcription in the liver of fish exposed to 0.3 μg/L and 3.0 μg/L of TBP was significantly reduced (2.7-fold and 2.5-fold inhibition, respectively) (Fig. 4F).

In the males exposed to 3.0 μg/L of TBP, the transcription of the 3β-HSD, 17β-HSD, and CYP17 genes in the testes was significantly increased (4.0-fold, 1.9-fold, and 1.7-fold increase, respectively) (Figs. 5A–C), while there were no significant changes in transcription levels in the males exposed to 0.3 μg/L of TBP. Meanwhile, in the fish exposed to 0.3 μg/L and 3.0 μg/L of TBP, the expression of CYP19A in the testes showed a 1.7-fold and 1.9-fold increase, respectively (Fig. 5D). The CYP19B transcription in the brains of fish exposed to 3 μg/L of TBP was significantly higher (2.1-fold) (Fig. 5E). The expression of VTG in the liver of fish exposed to 3.0 μg/L of TBP was significantly upregulated (2.8-fold) (Fig. 5F).

F1 generation toxicological endpoints

In the F1 generation, there were no significant differences in the hatching rates of F1 fish (Table 3). The malformation rate in the fish exposed to 0.3 μg/L and 3.0 μg/L TBP were significantly higher (Table 3). The survival rate in the fish decreased significantly in the fish exposed to 0.3 μg/L and 3.0 μg/L of TBP (Table 3). TBP exposure caused significant inhibition of growth in the F1 larvae (Table 3).

Discussion

The findings of our study suggest that chronic TPB exposure decreased the fecundity and altered the sex ratio in the F0 generation, and increased embryonic malformation, mortality, and growth inhibition in the F1 generation. The exposure concentration used in this study (0.3 μg/L) has been reported to be prevalent in the aquatic environment (Grove et al., 1985; IUCLID, 2003; Reineke et al., 2006). The results of our study suggest that the environmental concentrations of TPB may have an adverse impact on fish reproduction in aquatic environment.

TBP exposure significantly altered the plasma levels of sex hormones and clearly caused endocrine disturbances in zebrafish. Indeed, very limited information is available on reproductive toxicity of bromophenols, while several studies have been conducted on other brominated flame retardants (BFRs). For example, oral exposure to the PBDE congener 2,2′,4,4′-tetrabromodiphenyl ether (PBDE-47) impaired reproductive activity in fathead minnow (Muirhead et al., 2006). Exposure to low doses of 2,2′,4,4′,5-pentabromodiphenyl ether (PBDE-99) during critical periods of development permanently impairs spermatogenesis in adult rat offspring (Kuriyama et al., 2005) and PBDE-99 have also been shown to interfere with sexual development and sexually dimorphic behaviors, both of which are mediated by hormones produced in endocrine glands (Lilienthal et al., 2006).
In fish, estradiol produced by the developing follicle stimulates the liver to produce the yolk protein vitellogenin (VTG, which is essential for vitellogenesis) and induces oocyte maturation and yolk biosynthesis; the yolk is sequestered and processed by the developing oocyte to provide nutrients for the offspring (Brion et al., 2004). The observed decrease in plasma estradiol concentration after TBP exposure might be partly responsible for the reduced expression of the VTG gene in the liver of female fish. The downregulation of the

Fig. 4. The transcription levels of genes in the ovaries (A, B, C, D), brains (E), and livers (F) of adult F0 female fish after exposure to 0, 0.3 μg/L and 3.0 μg/L of TBP until 120 dpf. We analyzed 3 replicates comprising 6 individual fish for each TBP concentration. All the data are exhibited as mean±SEM. *P<0.05, **P<0.01, and ***P<0.001 indicate marked difference between the exposure group and the solvent-control group.
VTG gene may reduce egg quality and retard the development of the ovary. In a study on female fathead minnows (*Pimephales promelas*), laboratory toxicity tests with an aromatase inhibitor (fadrozole) were performed over a period of 21 days; the results of this study revealed that alterations in plasma VTG levels reduced the fecundity of the minnows, thereby leading to a decline in the population of minnows.

**Fig. 5.** The expression profiles of the genes expressed in the testes (A, B, C, D), brains (E) and livers (F) of *F₀* adult male fish after exposure to 0, 0.3 μg/L and 3.0 μg/L of TBP until 120 dpf. We analyzed 3 replicates comprising 6 individual fish for each TBP concentration. All the data are expressed as mean ± SEM. *P*<0.05 indicates marked difference between the exposure group and the solvent-control group.
The results of these studies suggest that changes in the aromatase activity can influence the sex differentiation during the developmental period in zebrafish. In our study, decreased expression of the CYP19 aromatase gene and reduced estrogen synthesis were observed in females. Therefore, we suggest that the decreased gonadal CYP19A mRNA expression after TBP exposure inhibited estradiol synthesis, which in turn influenced the sex differentiation of the gonads.

Although there was no significant increase in the malformation and mortality in the F0 generation (data not shown), the fish in the F1 generation produced lower numbers of eggs, showed abnormal embryos, and showed increased mortality. Therefore, maternal exposure to TBP may have an adverse impact on the offspring and may lead to a decline in the population. Although we were unable to measure the TBP content in the eggs, a previous study has shown maternal transfer of TBP to the eggs of adult female zebrafish exposed to TBP (Nyholm et al., 2008). In fish, parental exposure to environmental concentrations of other toxicants such as diethylstilbestrol (Zhong et al., 2005), 17α-ethynylestradiol (EE2) (Zha et al., 2008), triphenyltin (Zhang et al., 2008), perfluorooctanesulfonate (PFOS) (Du et al., 2009), and TCDD (King Heiden et al., 2009) also caused significant adverse effects in the offspring. These studies suggest that in aquatic environments, long-term exposure to low concentrations of toxicants can affect the fecundity and reproductive success of the offspring; these findings highlight the importance of life-cycle tests for environmental risk assessment.

Although the underlying mechanisms by which TBP exposure affect steroidalogenetic gene expressions remain unknown, previous studies have demonstrated that steroidalogenetic factor 1 (SF-1) is highly expressed in steroidalogenetic tissues and that this controls expression of the genes involved in steroidalogenesis, including those encoding various steroidalogenetic enzymes (reviewed by Val et al., 2003). SF-1, a member of the nuclear receptor superfamily that was initially identified by its capacity to interact with and activate the promoters of steroidalogenetic enzymes, has emerged as an essential regulator in the mechanisms underlying sex determination, adrenal and gonadal development, reproductive function, and steroidalogenesis (Parker et al., 2002; Val et al., 2003; Hoivik et al., 2010). A recent study showed that atrazine upregulates Cyp19a1 gene transcription and alters the sex ratio in zebrafish under environmental conditions, and that the effects on Cyp19a1 are mediated by regulation of downstream SF-1 receptors (Suzawa and Ingraham, 2008). Given the central role that SF-1 plays in steroidalogenesis, future studies that investigate how SF-1 modulates the expression of these steroidalogenetic genes in fish exposed to bromophenols will provide new insights into the underlying mechanisms.

The present study showed that environmental levels of TBP affected F0 male and female steroidalogenesis and gender ratio, and the health and survival of the F1 generation. Reproductive hormones, controlled by the various genes involved in steroidalogenesis, are of vital importance during the early critical stages of embryonic development, sex differentiation, sex development, and thus population viability. There is now unequivocal evidence showing that endocrine-disrupting chemicals can have long-term effects on reproduction and subsequent population development in natural fish populations (Kidd et al., 2007). A well-known example is ethinylestradiol (EE2) in the lower ng/L range in surface water—concentrations that can disrupt normal developmental/reproductive processes, leading to long-term impacts on fish reproduction and populations (Jobling et al., 1998). It is important to note that the number of females is the primary limiting factor in determining the reproductive output of a population (Shang et al., 2006). Notably, the modulating hormones and genes involved in sex steroid synthesis are highly conserved, and thus the TBP-induced impairment of reproduction observed in zebrafish may also occur in other fish species. It should be noted that the TBP concentrations examined in this study are environmentally relevant, suggesting that the reduced capacity to produce viable offspring may occur in wild fish.

Table 3

<table>
<thead>
<tr>
<th>Concentration (μg/L)</th>
<th>Hatching rate (%)</th>
<th>Malformation (%)</th>
<th>Survival (%)</th>
<th>Body length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>89.26 ± 2.73</td>
<td>2.67 ± 0.67</td>
<td>82.08 ± 1.29</td>
<td>4.12 ± 0.02</td>
</tr>
<tr>
<td>0.3</td>
<td>84.79 ± 1.46</td>
<td>6.33 ± 0.88*</td>
<td>68.89 ± 1.92*</td>
<td>3.78 ± 0.03**</td>
</tr>
<tr>
<td>3.0</td>
<td>79.29 ± 5.96</td>
<td>8.90 ± 0.49**</td>
<td>63.41 ± 2.29**</td>
<td>3.73 ± 0.01***</td>
</tr>
</tbody>
</table>

Each concentration has three replicates (10 larvae per replicate). All the results were exhibited as means ± SEM. *P<0.05, **P<0.01 and ***P<0.001 show significant difference between exposure group and the solvent control group.

(Miller et al., 2007). Thus, the TBP-induced downregulation of VGT transcription should contribute to the reduced fecundity in females.

Steroidalogenetic genes such as 3β-HSD, 17β-HSD, and CYP17 encode enzymes that participate in the synthesis of testosterone. CYP19 is the terminal enzyme in the steroidalogenetic pathway, which converts testosterone into estradiol, and the mRNA level of CYP19 is well correlated with the aromatase activity (Trant et al., 2001). In our study, the expression of the 3β-HSD, 17β-HSD, CYP17, and CYP19 genes was downregulated in females. Ankley et al. (2002) conducted studies on female fathead minnows and reported that a 21-day exposure to fadrozole decreased CYP19B aromatase activity and reduced the plasma concentrations of estradiol. Other chemicals such as prochloraz and fenarimol also inhibited steroidalogenesis and the expression of the CYP19 aromatase gene in female fathead minnows (Ankley et al., 2005). In vertebrate steroidalogenesis, steroidalogenetic enzymes are critical for the production of androgens; therefore, the downregulation of the expression of the genes coding for these enzymes can lower the efficiency of testosterone and estradiol synthesis. In contrast, in males, exposure to TBP increased the levels of testosterone, implying that the expression of 3β-HSD, 17β-HSD, and CYP19 was upregulated. In the brain, the expression of the CYP19B gene was upregulated, and the increased CYP19 gene expression may have accelerated the formation of estradiol from testosterone (Fenske and Segner, 2004; Cheshenko et al., 2008). The increased mRNA levels of both CYP19A and CYP19B in the testes and brain can be attributed to the increased synthesis of estradiol from testosterone. Since testosterone is the precursor of estrogens in steroidalogenesis, changes in the androgen production, metabolism, and sensitivity can significantly alter sexual differentiation and female phenotype (Staub and De Beer, 1997). The different responses of steroidalogenetic gene expression to TBP exposure in males and females appears to be gender dependent and remains to be elucidated. In addition to the effect of TBP on steroidalogenetic gene expression, the mechanism underlying this effect needs to be elucidated by further investigations on the action of antagonists of androgen or estrogen receptor (s).

This is the first report to describe that the sex ratio in zebrafish is male biased in response to TBP exposure, and this observation suggests that long-term exposure to TBP affects sex differentiation and sex determination in zebrafish. Indeed, teleost populations exposed to estrogenic compounds showed skewing of the sex ratio in favor of females (Metcalfe et al., 2001; Lange et al., 2001; Nash et al., 2004; Zhong et al., 2005; Zha et al., 2008). The changes in sex ratios to female-biased types are known to be caused by the transformation of the testis to ovaries under the direct influence of environmental estrogen during early gonadal development (Lange et al., 2001; Nash et al., 2004). Therefore, the balance between estrogens and androgens is crucial for sex differentiation in fish; the inhibition of aromatase suppresses estrogen formation, thereby disturbing the normal sex ratio and resulting in a male-dominant population. In a previous study, female zebrafish larvae that were exposed to an aromatase inhibitor (fadrozole) showed gonadal masculinization (Uchida et al., 2004; Fenske and Segner, 2004), and the aromatase inhibitor suppressed the CYP19 gene expression in the gonads of these genetically masculinized females. This finding shows the association between the CYP19 aromatase enzyme activity and the differential regulation of CYP19A and CYP19B expression.
In summary, in the present study, we demonstrated that chronic exposure to environmental concentrations of TBP during the embryo stage (Fe) disrupted the reproductive physiology of the adults in zebrafish. Exposure to TBP altered the expression of steroidogenic genes and thus affected the levels of sex hormones, thereby resulting in reduced VTG gene expression and contributing to reduced fecundity in females. The results of our study highlight the potential endocrine-disrupting activity of bromophenols, which interfere with steroidogenesis in fish. Maternal exposure to TBP adversely affected offspring survival, which further emphasizes the significance of life-cycle tests for environmental risk assessment. Such endocrine disruption may lead to long-term reproductive impairments, which may in turn affect species fitness in the aquatic environment.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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