



Effects of tris (2-butoxyethyl) phosphate (TBOEP) on endocrine axes during development of early life stages of zebrafish (*Danio rerio*)



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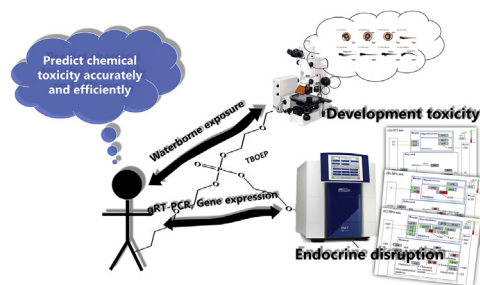
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HIGHLIGHTS

- Using two-step linear regression analysis (LRA) to predict chronic toxicity.
- Investigating the feedback pathway of endocrine axes in zebrafish embryos/larvae.
- Linking adverse effects on phenotypic to molecular effects along endocrine axes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 11 July 2015

Received in revised form 11 October 2015

Accepted 12 October 2015

Available online 11 November 2015

Handling editor: David C. Volz

Keywords:

Organophosphate flame retardant

Endocrine axes

qRT-PCR

Endocrine disruptor

Environmentally relevant

ABSTRACT

Due to phasing out of additive flame retardants such as polybrominated diphenyl ethers (PBDEs), Tris (2-butoxyethyl) phosphate (TBOEP) is widely used as a substitute. TBOEP is ubiquitous in the environment and has been measured at concentrations of micrograms per liter ($\mu\text{g L}^{-1}$) in surface waters and wastewater. Information on potential adverse effects on development of aquatic organisms caused by exposure to environmentally relevant concentrations of TBOEP is limited, especially for effects that may be caused through impairment of endocrine-modulated homeostasis. Therefore, this study was conducted to determine effects of TBOEP on ontogeny and transcription profiles of genes along the hypothalamus–pituitary–thyroidal (HPT), hypothalamus–pituitary–adrenal (HPA), and hypothalamus–pituitary–gonadal (HPG) axes in embryos/larvae of zebrafish (*Danio rerio*). Exposure to TBOEP ($2\text{--}5,000 \mu\text{g L}^{-1}$) from 3 h post-fertilization (hpf) to 120 hpf induced developmental malformations in zebrafish with a LC_{50} of $288.54 \mu\text{g L}^{-1}$ at both 96 hpf and 120 hpf. The predicted no observed effect concentration (PNOEC) was $2.40 \mu\text{g L}^{-1}$. Exposure to 2, 20, or $200 \mu\text{g TBOEP L}^{-1}$ altered expression of genes involved in three major molecular pathways in a concentration-dependent manner after 120 hpf. TBOEP caused lesser expression of some genes involved in synthesis of hormones, such as (*pomc* and *fsh β*) as well as upregulating expression of some genes coding for receptors (*thr*, *tshr*, *gr*, *mr*, *er* and *ar*) in zebrafish larvae. These changes at the molecular level could result in alterations of endocrine function, which could result in edema or deformity and ultimately death.

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1. Introduction

Commercial polybrominated diphenyl ethers (PBDEs) such as pentaBDE, a brominated flame retardant (BFR), were formerly used, but due to concerns about their persistence, potential for bioaccumulation and toxicity, were phased out in European and North American markets in 2004–2006 (Stapleton et al., 2012; McGee et al., 2013). To remain in compliance with safety standards for suppression of fire, such as those described in California's Technical Bulletin 117 (TB117) (State of California, 2000), organophosphate esters (OPEs) have been used as alternatives for pentaBDE. Tris (2-butoxyethyl) phosphate (TBOEP), an organophosphate ester, is increasingly used as a substitute for PBDEs in a number of applications and products (McGee et al., 2012). Furthermore, TBOEP is used as a plasticizer in various products such as floor polish, varnish, textiles, plastics, foams, furniture and electronic equipment (Marklund et al., 2003). It is estimated that current global production of TBOEP ranges between 5000 and 6000 tons per year (Liu et al., 2012b).

TBOEP is an additive organophosphate flame retardant (OPFR) and is not chemically bonded into final products. Thus, it can be released to the surrounding environment (Rodriguez et al., 2006). Because replacements for PBDEs, such as OPFRs, are used in household furniture and baby products, TBOEP has been detected in indoor air and house dust. For example, TBOEP was the most abundant contaminant in air and dust from classrooms in Norway, and concentrations were greater than those of PBDE, with an average concentration of 12.90 ng m⁻³ and 87,200 ng g⁻¹, respectively (McGee et al., 2012; Cequier et al., 2014). Moreover, because of its physical–chemical properties, such as polarity, relative stability and water-solubility (Supplement Information Table S1), which are different from traditional lipophilic-hydrophobic chemicals, TBOEP has been frequently detected in wastewater, wastewater treatment plant (WWTPs) effluent, surface water, ground water, sediments, drinking water and even human milk (Fries and Puttmann, 2003; Marklund et al., 2003, 2005; Andresen, 2006; Rodriguez et al., 2006; Reemtsma et al., 2008; Stapleton et al., 2009; Sundkvist et al., 2010). For example, concentrations of TBOEP in water from Albano lake in Italy, ranged from 10 to 127 ng L⁻¹. Mean concentrations of TBOEP in influents and effluents of municipal wastewater treatment plants in Sweden were 35 µg L⁻¹ and 30 µg L⁻¹, respectively, which indicates its recalcitrance to wastewater treatment processes (Marklund et al., 2005). TBOEP can infiltrate ground water and has been detected in bank filtrate of the Oder River, Germany, with an average concentration of 0.20 µg L⁻¹. In China, it has been reported that concentrations of TBOEP in sediment ranged from 1.00 to 5.00 mg kg⁻¹ dm (dry mass) and was the most abundant chemical in Tai Lake (Ch: Taihu) (Cao et al., 2012), as well as in drinking water in Nanjing, China, with a mean concentration of 70.10 ng L⁻¹ (range: 24.10–151 ng L⁻¹) (Li et al., 2014). TBOEP was also detected in human breast milk at concentrations of 63 ng g⁻¹ lipid mass (lm). Based on this value a baby of 5 kg mass could potentially take up significant amounts of OPEs (64 ng OPEs kg⁻¹) from an adult of 70 kg via breast feeding (Sundkvist et al., 2010). In addition, recently, several studies have detected TBOEP in wildlife, where it was the predominant OPFR. For example, concentrations of TBOEP ranged from 0.07 to 3.50 ng g⁻¹ wet mass (wm) in Lake Trout (McGoldrick et al., 2014), and 8.10–13.40 ng g⁻¹ wm in albumen and fat of eggs of the herring gull (*Larus argentatus*), respectively (Greaves and Letcher, 2014).

Disruption of the endocrine system can cause adverse effects including edema, developmental deformities or lethality. There are several mechanisms by which endocrine disrupting compounds can affect normal homeostasis. For example, previous studies of the endocrine disrupting compound 2,2',4,4'-tetrabromodiphenyl

ether (BDE-47) and its metabolites (–OH/–OMe BDE-47) have shown it can mimic the sex hormone, 17β-estradiol (E2) (Liu et al., 2015). During *in vitro* assays, OPEs have been found to cause receptor-mediated, disruption of synthesis of steroids and effect transcription of specific proteins transcriptional (Liu et al., 2012b; Kojima et al., 2013). However, there are few studies of effects of TBOEP on endocrine functions in aquatic organisms.

The hypothalamic–pituitary–thyroidal (HPT), hypothalamic–pituitary–adrenal (HPA), and hypothalamic–pituitary–gonadal (HPG) axes are responsible for regulating hormone dynamics by coordinating their synthesis, secretion, transport, and metabolism. Through these regulatory mechanisms they control reproductive, thyroid, and adrenal endocrine systems (Cyr and Eales, 1996; McGonnell and Fowkes, 2006; Zoeller et al., 2007). Moreover, several chemicals, such as prochloraz and propylthiouracil, have been reported to affect cross-talk among the HPT, HPA, and HPG axes of fish (Liu et al., 2011). Chemical-induced changes along these axes are also likely to lead to changes in whole endocrine pathways to further integrate bodily functions (Swapna and Senthilkumaran, 2007; Denver, 2009; Zheng et al., 2012). In this study, effects of TBOEP on several apical endpoints, including edema, hatchability, larvae length and curvature of the spine, were studied in embryos of zebrafish. Relationships between adverse effects of TBOEP on early life-stages of zebrafish and molecular mechanism of toxicity were studied by measuring changes in expression of critical genes along the HPT, HPA, and HPG axes.

2. Material and methods

2.1. Chemicals and reagents

TBOEP was purchased from Dr. Ehrenstorfer GmbH (A German abbreviation for *Gesellschaft Mit Beschränkter Haftung*) (95.8%, Augsburg, Germany). A stock solution of TBOEP was prepared in dimethyl sulfoxide (DMSO, Nanjing Chemical Reagent Co., Ltd, Nanjing, China), stored at –20 °C and diluted with embryo rearing water (60 mg L⁻¹ instant ocean salts in aerated distilled water) to final concentrations immediately before use. The final concentration of solvent (DMSO) in test solutions did not exceed 0.1%. RNAlater, RNA Stabilization Reagents, RNeasy Mini Kit, and Omniscript RT Kit were purchased from Qiagen (Hilden, Germany). SYBR Green Real time PCR Master Mix Plus Kit was obtained from Toyobo (Toyobo Co., Ltd, Tokyo, Japan).

2.2. Animals and protocol for exposure to TBOEP

Adult zebrafish (4-months old, AB wild-type) were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and maintained in a semiautomatic system (Zhongkehai Recycling Water Aquaculture System Co., Ltd, Qindao, China) with treated tap water, which contained no measurable ammonia, chlorine or chloramines, and was disinfected with UV light. Fish were exposed under a 14/10 h light/dark photoperiod. All procedures involving zebrafish were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing University for laboratory animal use. Culture and breeding of fish was performed according to the common OECD protocol for fishes (OECD, 1992). Briefly, fish were fed fairy shrimp (Tianjin Red Sun Aquaculture Co., Ltd., Tianjin, China) three times a day (breakfast, lunch and dinner). Nylon nets were placed at the bottom of tanks to isolate eggs and adult zebrafish. All fertilized embryos were examined under a stereo microscope (Nikon, Tokyo, Japan) and healthy embryos were selected for inclusion in the study. Embryos were observed 3 h post fertilization (hpf) and then used in subsequent experiments.

Based on environmentally relevant concentrations (surface water 127 ng L⁻¹ and wastewater up to 35 µg L⁻¹, respectively) and log K_{ow} (3.75) of TBOEP; concentrations were 0, 2, 20, 200, 2,000, and 5000 µg L⁻¹. 20 embryos were randomly selected and placed in each well of 6-well cell culture plates (Corning Inc. Steuben, New York, USA). Glass vessels were used to minimize adsorption, although TBOEP is relative hydrophilic (Water solubility at 25 °C, 27,000 µg L⁻¹, indicated in support information Table S1). Each well contained 10 mL TBOEP in triplicate. Exposures were conducted in an incubator that was used to maintain a stable environment over the course of the experiment (photoperiod: 14/10 h light/dark; static; temperature: 25 ± 2 °C). The experiment was terminated at 120 hpf. To avoid evaporation of the test solution, plates were covered with breathable membrane (Excel scientific, Inc., USA, aeraseal sterile). Concentrations of TBOEP in exposure media were confirmed by use of LC-MS/MS according to previously described methods (Han et al., 2014; Li et al., 2014). During the 120 h exposure, no extensive depletion of TBOEP (<20%) was observed.

Evaluation of morphological alterations of zebrafish embryos was conducted at several stages of development (12, 24, 36, 48, 54, 60, 72, 96 and 120 hpf) by use of a multipurpose light microscope (Nikon AZ100, Tokyo, Japan). Toxicological endpoints (Table S2) included developmental delay at 24 hpf; structural malformations at 72 or 96 hpf, and body lengths measured after hatching at 120 hpf (Zheng et al., 2012). Embryos that became coagulated during hatching and larvae that died after hatching were considered as mortalities. Malformations of the spine included scoliosis and abnormal lateral curvature. Each exposure experiment was replicated three times independently.

2.3. RNA isolation and qRT-PCR

Expressions of genes in embryos exposed to 0, 2, 20, or 200 µg TBOEP L⁻¹ were quantified. Larvae collected at 120 hpf were randomly sampled at termination of the experiment and stored in RNAlater solution (Qiagen Co., Ltd, Germany) at -20 °C until RNA isolation. Total RNA was isolated by use of the RNeasy Mini Kit (Qiagen Co., Ltd, Germany). Quantification and verification of RNA were performed as previously reported (Zheng et al., 2012). Transcription of genes involved in the HPT, HPA and HPG axes was quantified by qRT-PCR as described previously (Liu et al., 2012a). Briefly, Omniscript RT Kit (Qiagen Co., Ltd) was used to synthesize cDNA following the manufacturers' instructions. qRT-PCR was performed using the Applied Biosystems Stepone Plus Real-time PCR System (Applied Biosystems Inc. Foster city, CA, USA). The on-line Primer 3 program (<http://frodo.wi.mit.edu>) was used to design primers for selected genes. Primer sequences for qRT-PCR are listed in the Supporting Information (Table S3). Conditions of qRT-PCR reactions were as follows: initial denaturation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Melting curves were derived to confirm that amplification of cDNA resulted in a single product. Expression of mRNA for each target gene was standardized to the housekeeping gene 18s rRNA, and changes of mRNA expression of related genes were analyzed by the 2^{-ΔΔCt} method. Each replicate sample containing 20 larvae was measured in triplicate.

2.4. Statistical analyses

IBM SPSS statistics 19 (SPSS Inc., Chicago, Ltd, USA) was used for statistical analyses. The probit model was used to calculate LC₅₀ values for endpoints at different developmental stages of zebrafish. A two-step linear regression analysis (LRA) was used to extrapolate predicted no observed effect concentration (PNOEC)

(Equation (1)).

$$\text{Probit } P = \alpha + \beta \log(C) + \delta/T \quad (1)$$

The first regression process was to determination LC₅₀ values for each duration of exposure. Although the first linear regression can be carried out by various methods such as probit or logit, (Giesy and Graney, 1989; Mayer et al., 1994; Slaughter et al., 2007) in this study, the used probit method was used in this step. The second regression involved LC₅₀/5 (5 as a safety factor, which had been suggested previously (Slaughter et al., 2007)) as the dependent variable and versus reciprocal of time as independent variable. The latter regression gave a related NOEC over infinite time (Giesy and Graney, 1989; Slaughter et al., 2007). The Kolmogorov-Smirnov test was used to evaluate data for normality and a Levene's test was used to analyze homogeneity of variance. Statistically significant differences among groups were determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. A value of *P* < 0.05 was considered statistically significant.

3. Results

3.1. Developmental toxicity of TBOEP in zebrafish embryos/larvae

Exposure of zebrafish embryos/larvae to TBOEP from 3 to 120 hpf resulted in significant concentration-dependent mortalities as well as impairment of development (Figs. 1 and 2). LC₅₀ values decreased gradually in a time-dependent manner until 96 hpf reaching a value of 288.54 µg L⁻¹ and remaining constant between 96 hpf to 120 hpf (Fig. 1). Coagulated and hatch delay of embryos were observed in each experimental group at 24 hpf. For example, mortality of embryos/larvae was significantly greater (25%, 53%) when exposed to the two greatest concentrations of TBOEP (2000 or 5000 µg L⁻¹). Meanwhile, hatching was significantly delayed in embryos exposed to 2000 or 5000 µg TBOEP L⁻¹ relative to that of controls (Fig. 2A, B and C). Larvae exposed to 2000 or 5000 µg TBOEP L⁻¹ exhibited a significantly greater prevalence of curvature of the spine after 72 hpf (Fig. 2E). Exposure to 5000 µg TBOEP L⁻¹ resulted in a significant increase in prevalence of edema at 120 hpf (Fig. 2G). Furthermore, larvae that survived exposure to 5000 µg TBOEP L⁻¹ (3346 ± 28 µm) were significantly shorter than control fish (4103 ± 54 µm) at 120 hpf. Embryos in the control group hatched successfully (92%) and larvae

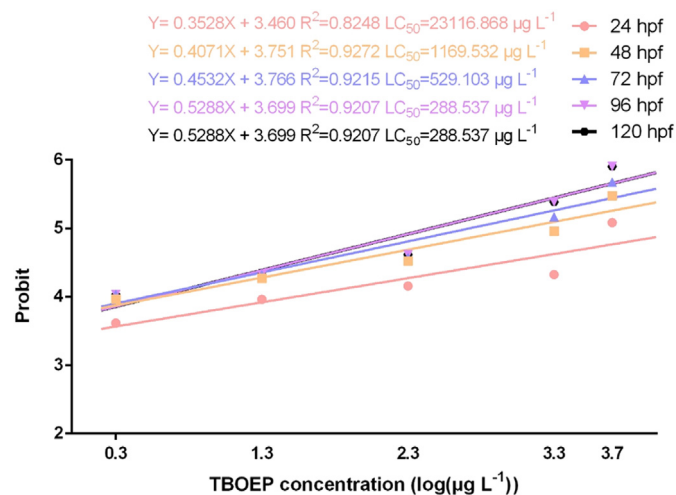


Fig. 1. Linear regression of various concentrations (2, 20, 200, 2000 and 5000 µg L⁻¹ TBOEP) against lethal probability of each concentrations. The x-axis represents concentrations of TBOEP, presented on a logarithmic scale, the y-axis represents the probability of lethality.

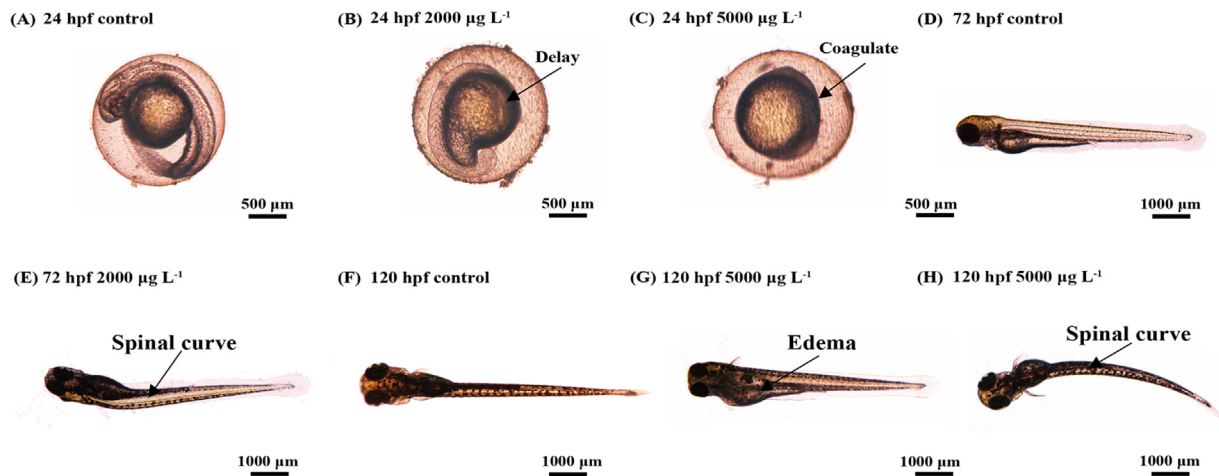


Fig. 2. Representative optical images of morphology at several development stages of zebrafish embryos/larvae. (A) Normal development of embryos (0.01% DMSO, 24 hpf). (B) Delayed development of embryos ($2000 \mu\text{g L}^{-1}$ TBOEP, 24 hpf). (C) Delayed development of embryos ($5000 \mu\text{g L}^{-1}$ TBOEP, 24 hpf). (D) Normally hatched larvae (0.01% DMSO, 72 hpf). (E) Malformed development of larvae ($2000 \mu\text{g L}^{-1}$ TBOEP, 72 hpf). (F) Normal development of larvae (0.01% DMSO, 120 hpf). (G) Edematous development of Larvae ($5000 \mu\text{g L}^{-1}$ TBOEP, 120 hpf). (H) Malformed development of larvae ($5000 \mu\text{g L}^{-1}$ TBOEP, 120 hpf).

had symmetrical pectoral fins on both sides and a linear body after 120 hpf.

The LRA method for determining the threshold/incipient LCx (the concentration that results in a particular mortality in the test relative to the control) was used as a threshold basis to extrapolate chronic toxicity (Fig. S1) (Giesy and Graney, 1989; Mayer et al., 1994). The PNOEC value in this experiment was $2.40 \mu\text{g L}^{-1}$.

3.2. Expression of genes along HPT, HPA and HPG axes

There were statistically significant, concentration-dependent changes in profiles of transcription of certain genes along the HPT, HPA and HPG axes in zebrafish embryos exposed to TBOEP (Figs. 3 and 4 and Fig. S2; Tables S4, S5 and S6). Magnitudes of effects were inversely proportional to concentrations of TBOEP with greater fold-changes at lesser concentrations. Expressions of genes associated with the pituitary and thyroid along the HPT axis were significantly up-regulated with maximum effects at $2 \mu\text{g TBOEP L}^{-1}$. There was no significant effect on expression of *trh*, which is associated with the hypothalamus, after being exposed to any of the concentrations of TBOEP (Figs. 3A and 4A). Thyroid hormone receptors α and β (*thra* and *thrβ*) and thyroglobulin (*tg*) were significantly up-regulated by 1.65-, 1.80-, and 1.97-fold, respectively when exposed to $2 \mu\text{g TBOEP L}^{-1}$. Meanwhile, thyroid stimulating hormone receptor (*tshr*) was significantly up-regulated by 2.24- and 1.97-fold when exposed to $2 \mu\text{g L}^{-1}$ or $20 \mu\text{g L}^{-1}$ TBOEP, respectively. Transcription of Na^+/I^- -synthetic (*nis*) was significantly down-regulated by 4.16-fold after exposure to $200 \mu\text{g TBOEP L}^{-1}$.

Along the HPA axis, TBOEP altered expression of a number of genes in a concentration-dependent manner (Figs. 3B and 4B). Exposure to $2 \mu\text{g TBOEP L}^{-1}$ significantly up-regulated expressions of corticotrophin-releasing hormone receptor 2 (*crhr2*), glucocorticoid receptor (*gr*) and mineralocorticoid receptor (*mr*) by 2.18-, 2.14- and 1.78-fold, respectively. In contrast, expression of pro-opiomelanocortin (*pomc*) was significantly down-regulated by 1.72- and 2.08-fold, respectively when exposed to 20 or $200 \mu\text{g TBOEP L}^{-1}$. TBOEP also caused down-regulation of several genes encoding for adrenal steroidogenic enzymes, such as cholesterol side-chain cleavage enzyme (*cyp11a*) and estrogen metabolic enzyme (*cyp17*). Specifically, expression of *cyp11a* was significantly reduced by 1.75- and 1.89-fold when exposed to 2 or $200 \mu\text{g TBOEP L}^{-1}$, respectively, and expression of *cyp17*

was significantly down-regulated by 2.13-fold when exposed to $20 \mu\text{g TBOEP L}^{-1}$.

TBOEP also caused concentration-dependent changes in expression of genes along the HPG axis (Figs. 3C and 4C). After exposure to $2 \mu\text{g TBOEP L}^{-1}$, gonadotropin-releasing hormone receptors (*gnrhr1*, *gnrhr2*, and *gnrhr4*) were significantly up-regulated by 1.73-, 1.52-, and 1.94-fold, respectively. Estrogen receptor 2b (*er2b*) was significantly up-regulated by 1.87-fold when exposed to $2 \mu\text{g TBOEP L}^{-1}$, and androgen receptor (*ar*) was also significantly up-regulated by 2.04-fold when exposed to $2 \mu\text{g TBOEP L}^{-1}$, respectively. When exposed to 2 or $20 \mu\text{g TBOEP L}^{-1}$, expression of 17 beta-hydroxysteroid dehydrogenase (*17βhsd*) was significantly up-regulated by 2.88- and 2.72-fold, respectively. However, expression of follicle-stimulating hormoneβ (*fshβ*) was down-regulated by 2.94-fold after exposure to $20 \mu\text{g TBOEP L}^{-1}$.

4. Discussion

The LC_{50} at 120 hpf and PNOEC value were $288.54 \mu\text{g L}^{-1}$, $2.40 \mu\text{g L}^{-1}$, respectively. The LC_{50} observed in this study was significantly different than the LC_{50} observed during a previous study of effects of TBOEP on zebrafish at 120 hpf ($10, 700 \mu\text{g L}^{-1}$) (Han et al., 2014). Differences between LC_{50} values observed during the two studies might be due to differences in ranges of observation time and magnitude of exposures. In this study, embryos were collected and morphological differences were observed at 3 to 120 hpf (blastula period stage) (peer viewing at 4–120 hpf). This time period was adopted because cleavage and rapid cellular division are initiated during the blastula stage (Braunbeck and Lammer, 2006). During in data processing of data, the two methods to calculate LC_{50} values use of the probability and s-shaped curve resulted in different values. The probability unit method had a larger coefficient of determination (R^2 : 0.8248–0.9272) than did the s-shaped curve (0.3196–0.6587). The PNOEC determined by use of the LAR method was particularly useful in assessment of risk because it was able to predict chronic no-effect concentrations from acute data (Mayer et al., 1994), which aided in minimizing the number of animals used and costs associated with more labor-intensive and time-consuming chronic tests. Concentrations of TBOEP observed in aquatic systems are comparable or greater than values of PNOEC calculated in this study. For example, TBOEP measured in effluent from wastewater treatment plants in Sweden exceeded the PNOEC by $10 \times$ ($30 \mu\text{g L}^{-1}$), ground wa-

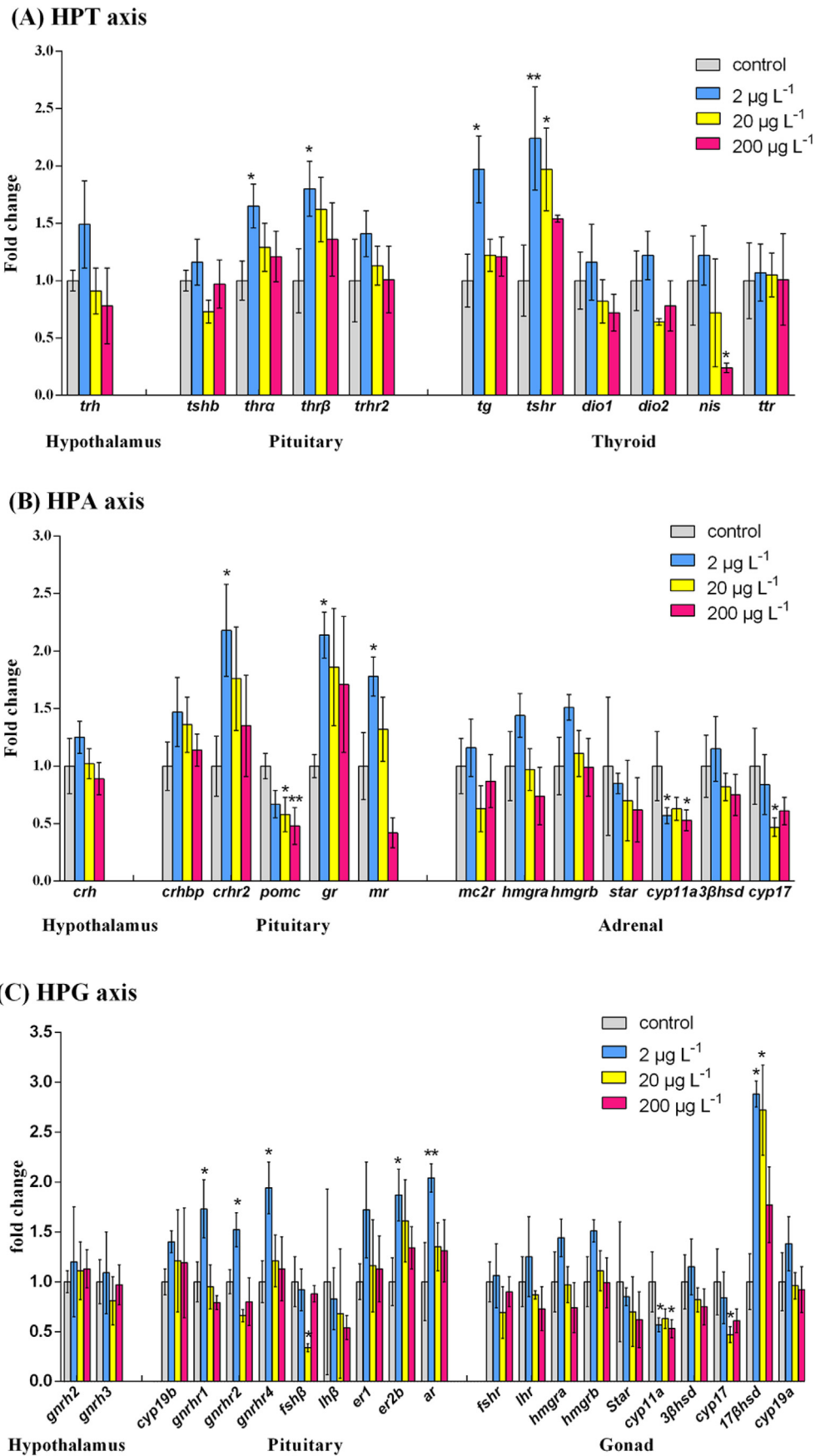


Fig. 3. Expression of genes along the hypothalamus–pituitary–thyroidal (HPT), hypothalamus–pituitary–adrenal (HPA), and hypothalamus–pituitary–gonadal (HPG) axes. (A) HPT axis, (B) HPA axis, and (C) HPG axis, determined by qRT-PCR at 120 hpf following 2, 20 or 200 µg L⁻¹ TBOEP exposure. Results were expressed as means ± SD of three replicates. **p* < 0.05 and ***p* < 0.01 indicate significant differences in exposure groups compared with control group.

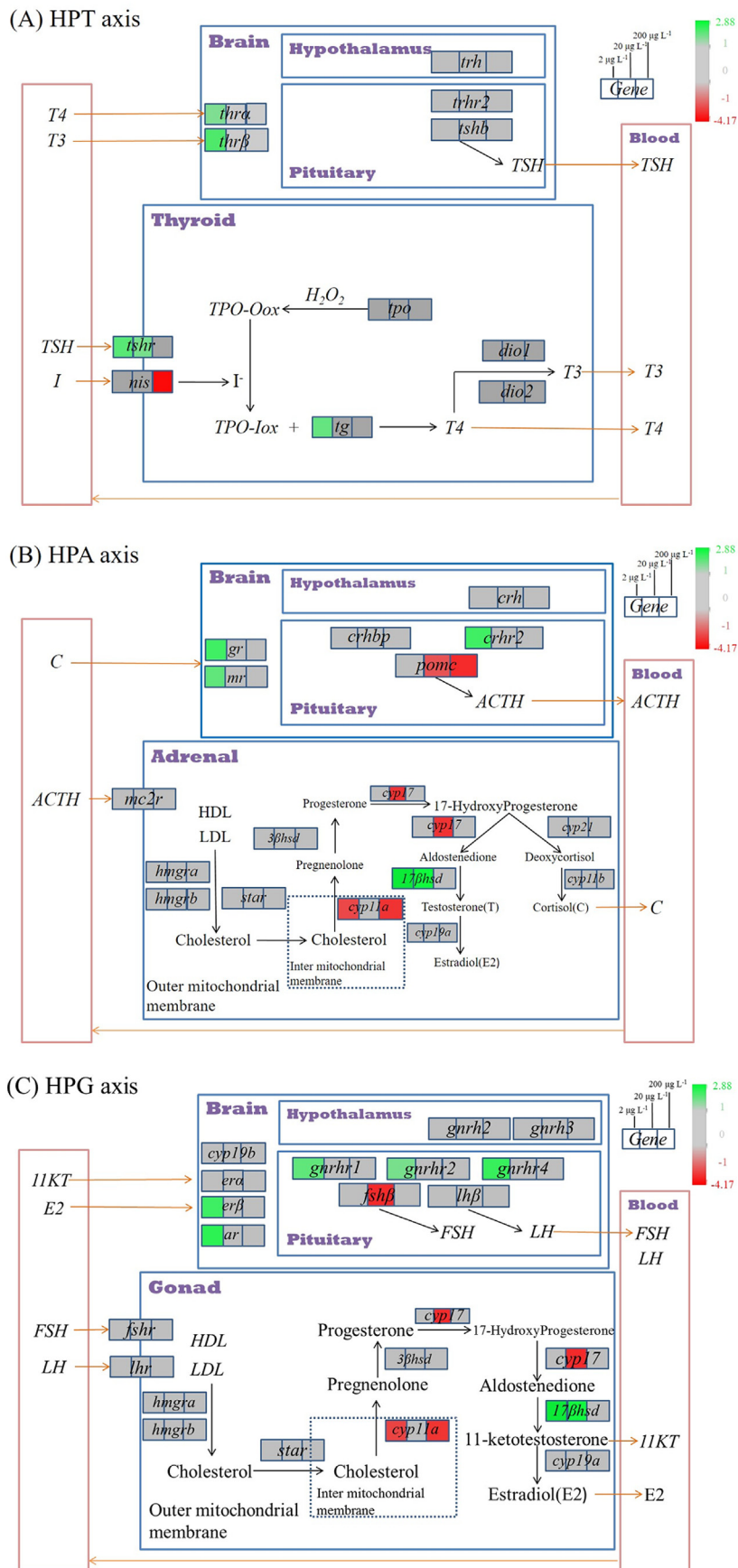


Fig. 4. Concentration-dependent effects and proposed toxicity pathways along the three axes ((A) HPT axis, (B) HPA axis and (C) HPG axis) of zebrafish larvae exposed to 2, 20 and 200 µg L⁻¹ TBOEP.

ter from the Oder River in Germany contained comparable concentrations (154–410 ng L⁻¹) to the PNOEC (2.40 µg L⁻¹) (Fries and Puttmann, 2003; Marklund et al., 2005). Furthermore, ΣOP (Total OP, levels of 12 OPEs) concentration (2100 ng L⁻¹) in water from the Great Lakes was less than the threshold for effects (Salamova et al., 2014). However, still had elicited toxicity effects (Jantunen et al., 2013) because rivers, streams and creeks draining into the Great Lakes averaged over 600 ng L⁻¹. These results indicate that current concentrations of TBOEP in the environment might have significant effects on aquatic biota but further information regarding chronic toxicity of TBOEP is required for confirmation.

Exposure of zebrafish embryos to TBOEP affected development and expression of genes along the HPT axis. Several genes which are involved in thyroid hormone synthesis pathways (*thra*, *thrβ*, *tg*, and *tshr*) were significantly up-regulated following exposure to the least concentration of 2 µg TBOEP L⁻¹, while only the *nis* gene, responsible for iodine transport into the body and thyroid hormone production (Noguchi et al., 2010) was down-regulated at 200 µg TBOEP L⁻¹. This demonstrated that down-regulation of the sodium/iodide symporter (*nis*) would likely result in decreased sodium and iodide availability and result in lesser production of thyroid hormone. This result is consistent with significant effects of exposure to 200 µg TBOEP L⁻¹ on development of larvae and is indicative of disruption along the thyroid axis. Recent results have shown that certain OPFRs such as tris (1, 3-dichloro-2-propyl) phosphate (TDCPP), triethyl phosphate (TEP), and TBOEP can disrupt the thyroid system in zebrafish and chicken embryos (Wang et al., 2013a; Egloff et al., 2014). Waterborne exposure of embryos/larvae to TDCPP significantly decreased and increased whole-body T4 and T3 concentrations, respectively, at concentrations as little as 50 µg L⁻¹. Furthermore, TDCPP up-regulated genes related to thyroid hormone metabolism (*dio1* and *ugt1ab*), synthesis of thyroid hormone (*tshβ*, *nis* and *tg*) and thyroid development (*hhex*, *nkx2.1* and *pax8*) at concentrations greater than 300 µg L⁻¹ (Wang et al., 2013b). However, chicken embryos injected with up to 45,400 ng TBOEP g⁻¹ did not exhibit altered concentrations of T4 (free T4) and thyroid gland T4 (total T4) in blood plasma (Crump et al., 2014). Exposures to 8 ng TEP L⁻¹ significantly decreased concentrations of free T4 in blood plasma and significantly reduced transthyretin (*ttr*), which in chicken embryos regulates transport of circulating THs to target tissues (Crump et al., 2014). Based on the results obtained in this study as well as the effects reported in the literature, it was hypothesized that inhibition of synthesis of T3 and T4 could be one of the main factors leading to pathologies. However, further studies are required to confirm the specific effects exposures to TBOEP induce on the thyroid hormone system.

Effects of OPFRs on the HPA axis have rarely been studied in comparison with the HPG or HPT axes. The HPA axis and its end product, cortisol, play major roles, not only in the context of regulating the body's peripheral functions such as metabolism and immunity, but also have profound effects on the brain (Rosmond, 2003). In the study, the results of which are presented here, small concentrations of TBOEP caused down-regulation of expression of *pomc* in the pituitary and *cyp11a* and *cyp17* in the adrenal gland or liver, while significant up-regulation of the expression of *mr* and *gr* also occurred. These results suggest that exposure to TBOEP, via a mechanism of positive feedback, could inhibit release of adrenocorticotrophic hormone (ACTH) by the pituitary, or synthesis of cytochrome (e.g., *cyp11a* and *cyp17*) and steroidogenesis (e.g., cortisol) in the adrenal gland or liver (Drouin et al., 1987). Modifications to expressions of these genes could affect signaling from the brain and alter normal release of hormones. Small amounts of cortisol could elicit transcription-level effects on *mr* and *ar* as part of a mechanism of negative feedback (Ware et al., 2003). These results suggest that TBOEP could cause delay of development and malfor-

mations via alterations to mechanisms of regulation (feedback) in the HPA axis.

Exposure to TBOEP affected several genes along the HPG axis and might have stimulated synthesis of 11-ketotestosterone (11KT) and E2. A complicated interaction of binding steroids, such as follistatin and gonadal steroidogenesis, such as activin and inhibin, mediates feedback regulation in the hypothalamus and pituitary, which controls concentrations of hormones and stimulates release of gonadotropins (Ingraham et al., 1994). Expression of several genes including, *er1*, *er2b* and *ar*, which are critical to regulatory feedback mechanisms of the HPG axis, were significantly altered following exposure to TBOEP. Changes in expression of these genes suggest increased synthesis of the sex hormones, 11KT and E2. Furthermore, expression of gonadotropin releasing hormone receptors, *gnrhr1*, *gnrhr2*, and *gnrhr4*, and *17βhsd* were significantly up-regulated following exposure to 2 µg L⁻¹ or 20 µg L⁻¹ TBOEP. GnRHs, are involved in propagation of signals from the hypothalamus to the pituitary, and up-regulation of their expression might cause increases in concentrations of sex steroid hormones, while up-regulation of *17βhsd*, which is involved in steroidogenesis and production of testosterone, would likely have similar effects. These results are consistent with a report of adult zebrafish exposed to TDCPP for 21 days (Liu et al., 2013), which indicated that OPFRs could disturb sex steroid hormone production along the HPG axis, eventually resulting in disruption of reproductive performance in fish.

Relatively small, environmentally relevant concentrations of TBOEP (2 µg L⁻¹) induced expression of several genes, whereas higher concentrations of 20 or 200 µg L⁻¹ generally reduced transcription of other genes, mostly those involved in steroidogenesis. Furthermore, during acute exposures of zebrafish to TBOEP varying degrees of retardation of growth and malformations that are likely associated with the observed alterations in molecular pathways reported here, were observed. However, further studies are needed to confirm the endocrine disrupting capacity at the protein or histological level based on ecologically relevant doses via waterborne or *in ovo* exposures.

5. Conclusions

Results of the current study demonstrated that exposure to TBOEP could cause lethality, reduced growth, malformations, and edema, in early life stages of zebrafish in a time- and concentration-dependent manner. Molecular effects along the HPT, HPA and HPG endocrine axes were consistent with adverse phenotypic effects observed at the organismal level. Some of the observed pathologies were likely mediated through alteration of steroidogenic pathways by TBOEP. These results might be useful for future environmental risk assessments of OPFRs in aquatic vertebrates as several of the molecular effects were observed following exposure to 2 µg L⁻¹ TBOEP⁻¹, which is in the range of environmental relevance. This study is of great importance as it raises concerns regarding the potential environmental risks of an increasingly used, frequently detected, and highly bioaccumulated OPFR to early development of aquatic organisms.

Acknowledgments

This work has been co-financially supported by National Natural Science Foundation (No. 21377053 and 20977047) and Major National Science and Technology Projects (No. 2012ZX07506-001 and 2012ZX07501-003-02) of China. Professor John P. Giesy was supported by the Program of 2012 "Great Level Foreign Experts" (#GDW20123200120) funded by the State Administration of Foreign Experts Affairs, P.R. China to Nanjing University, and the Einstein Professor Program of the Chinese Academy of Sciences. He

was also supported by the Canada Research Chair Program and a Visiting Distinguished Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution at City University of Hong Kong. Professor Markus Hecker was supported by the Canada Research Chair Program. Conceived and designed the experiments: Liu H. Performed the experiments: Ma Z. and Miao Y. Analyzed the data: Tang S. and Su G. Wrote the paper: Ma Z., Xie Y., Saunders D., Hecker M., Yu H., and Giesy J., Secured funding: Liu H.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2015.10.049>.

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Supporting Information

Effects of tris (2-butoxyethyl) phosphate (TBOEP) on endocrine axes during development of early life stages of zebrafish (*Danio rerio*)

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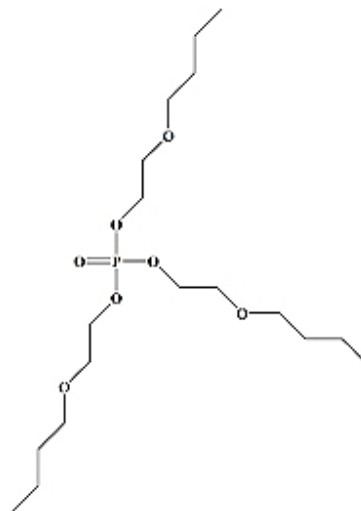
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Key words: Organophosphate flame retardant, HPT axis, HPA axis, HPG axis, qRT-PCR, Endocrine disruptor

Table S1. Structure and physico-chemical parameters of tris (2-butoxyethyl) phosphate (TBOEP).



TBOEP

Acronym	Compound name	CAS No.	Formulae	Log K_{ow}	Vapor pressure at 25°C (Pa)	Boiling point (°C)	Melting point (°C)	Density (g mL ⁻¹ at 25°C)	Water solubility at 25°C (g L ⁻¹)
TBOEP	Tris(2-butoxyethyl) Phosphate	78-51-3	C ₁₈ H ₃₉ O ₇ P	3.75	1.6×10 ⁻⁴	215-228	-70	1.006	0.027

Table S2. Endpoints measured in embryos/larvae following different durations of exposure to TBOEP.

Endpoints	12 hpf	24 hpf	36 hpf	48 hpf	54 hpf	60 hpf	72 hpf	96 hpf	120 hpf
Embryos/larvae coagulation	+	+	+	+	+	+	+	+	+
Edema				+	+	+	+	+	+
Hatchability				+	+	+	+		
Larvae length							+	+	+
Spine crooked							+	+	+

‘+’ indicates morphological changes that were selected as endpoints at different times during embryo/larval development.

Table S3. Primer sequences and accession numbers of target genes across the HPT, HPA and HPG axes in zebrafish.

Function	Gene name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Accession number	
Reference	<i>18s rRNA</i>	ttgttggtggttggctggt	ggatgctcaacaggggttcat	NM_200713	
HPT	<i>tshb</i>	gcagatcctcacttcacctacc	gcacaggttggagcatctca	AY135147	
	<i>tshr</i>	gctccttgatgtgtccgaat	cgggcagtcaggttacaat	NM_001145763	
	<i>tg</i>	ccagccgaaagatagagttg	atgctgccgtggaatagga	XM_001335283	
	<i>dio1</i>	gttcaaacagcttgcaaggact	agcaagcctctctccaagt	BC076008	
	<i>dio2</i>	gcataggcagtcgctcattt	tgtggtctctcatccaacca	NM_212789	
	<i>ttr</i>	cgggtggagtttgacacttt	gctcagaaggagagccagta	BC081488	
	<i>thra</i>	ctatgaacagcacatccgacaagag	cacaccacacacggctcatc	NM_131396	
	<i>thrb</i>	tgggagatgatacgggttgt	ataggtgccgatccaatgtc	NM_131340	
	<i>nis</i>	ggtggcatgaaggctgtaat	gcctgattggctccatacat	NM_001089391	
	<i>trh</i>	cacacagatggaggagcaga	agcagcatcaggtagcgttt	NM_001012365	
	<i>trhr1</i>	ctggtggtggtcaactcctt	gctttccaccgttgatgttt	NM_001114688	
	HPA	<i>crh</i>	ttcgggaagtaaccacaagc	ctgcactctattcgccttcc	NM_001007379
		<i>crhbp</i>	ttttcatcggcgaacctac	acgattggtcgagacactcc	NM_001003459
<i>crhr2</i>		aaagatgctggtttgggaag	cccagtaaaggcagaagcac	XM_681362	
<i>pomc</i>		aggctgactatccgcaagaa	caacctctccccctaaagc	AY158003	
<i>gr</i>		ttctacgttgctgacgatgc	ccggtgttctctgtttgat	EF567112	

	<i>mr</i>	attgggcctagtgcaaaatg	tctctgtttggetcggcttt	EF567113
	<i>mc2r</i>	ctccgttctcccttcatctg	gcagatccttgaagctgagg	NM_180971
	<i>hmgra</i>	gagccatcgactctctcctg	gaacacgactgctagcacca	BC155135
	<i>hmgrb</i>	ccaactgctccctgatcaat	tttaagaatcgcgaggcact	NM_001014292
	<i>star</i>	ctgagaatggaccacctgt	gcaataaacgtcagcaagca	NM_131663
	<i>cyp11a</i>	tcccgaaccagagcaatac	gctcaaactgctcctgacc	NM_152953
	<i>3βhsd</i>	agagaccggagaaaagagc	gggtggagtgaatctcagga	AY279108
HPG	<i>cyp19b</i>	ggcagtctctggaggatgac	cagtggtctcgaattctcca	AY780257
	<i>gnrh2</i>	gctcagcactggtctca	ctcttttgaaatcacgaa	AY657018
	<i>gnrh3</i>	tggagtggaaaggaagggtg	ttcagcatccacctattca	NM_182887
	<i>gnrhr1</i>	gtggcttcattgtggtgtg	ccagagatttcggagagcag	NM_0011144980
	<i>gnrhr2</i>	cttggcctgtggtactggtt	gacgtcctgatggaggatcat	NM_001144979
	<i>gnrhr4</i>	aaacacacgctttcgtcttt	aggttgcacacagctgacac	NM_OO1098193
	<i>fshβ</i>	acagcacaccagaaggctct	agctccccagtctgttgtgt	AY424303
	<i>lhβ</i>	gagacggtatcggtggaata	aacagtcgggcagggttaatg	AY714132
	<i>era</i>	tgagcaacaaggaatggag	gtgggtgtagatggagggttt	NM_152959
	<i>erβ</i>	tgattagctgggcgaaga	tatccagccagcagcatt	AJ414566
	<i>ar</i>	atctgtgcgctagcaggaat	caactgcgagtggaaagtca	NM_001083123
	<i>fshr</i>	cgtctcttttgcactgga	gtggcaattccaccttct	NM_001001812

<i>lhr</i>	aaaaggacgagtcgctgaaa	gctcttctgggaacatctgc	AY424302
<i>hmgra</i>	gagccatcgactctctctg	gaacacgactgctagcacca	BC155135
<i>hmgrb</i>	ccaactgctccctgatcaat	ttaagaatcgcgagcact	NM_001014292
<i>star</i>	ctgagaatggaccacctgt	gcaataaacgtcagcaagca	NM_131663
<i>cyp11a</i>	tcccgaaccagagcaatac	gctcaaactgctctgacc	NM_152953
<i>3βhsd</i>	agagaccggagaaaagagc	gggtggagtgaatctcagga	AY279108
<i>cyp17</i>	gacagtcctccgcacatct	gcatgatggtggtgttca	AY281362
<i>17βhsd</i>	gtctgatgggtcctctggaa	ttcacaagcgcctctatt	AY306005
<i>cyp19a</i>	ctgaaagggtcaggacaa	tggtcgatggtgtctgatg	AF226620

Table S4. Expression of genes in the hypothalamus-pituitary-thyroidal (HPT) axis. Transcript responses are expressed as fold change compared to corresponding controls. * $p < 0.05$, ** $p < 0.01$ indicates significant differences between exposure groups and the control.

Gene	TBOEP			Gene	TBOEP		
	Con ($\mu\text{g L}^{-1}$)	Mean	SD		Con ($\mu\text{g L}^{-1}$)	Mean	SD
<i>tshb</i>	0	1	0.09	<i>tshr</i>	0	1	0.31
	2	1.16	0.2		2	2.24**	0.45
	20	-1.37	0.1		20	1.97*	0.36
	200	-1.03	0.21		200	1.54	0.03
<i>tg</i>	0	1	0.23	<i>dio1</i>	0	1	0.25
	2	1.97*	0.29		2	1.16	0.33
	20	1.22	0.14		20	-1.22	0.19
	200	1.21	0.17		200	-1.39	0.16
<i>dio2</i>	0	1	0.26	<i>ttr</i>	0	1	0.33
	2	1.22	0.21		2	1.07	0.25
	20	-1.56	0.03		20	1.05	0.19
	200	-1.28	0.22		200	1.01	0.4
<i>thra</i>	0	1	0.17	<i>thrβ</i>	0	1	0.28
	2	1.65*	0.19		2	1.80*	0.24
	20	1.29	0.21		20	1.62	0.28
	200	1.21	0.22		200	1.36	0.32
<i>nis</i>	0	1	0.39	<i>trh</i>	0	1	0.09
	2	1.22	0.26		2	1.49	0.38
	20	-1.39	0.47		20	-1.10	0.2
	200	-4.17*	0.04		200	-1.28	0.33
<i>trhr1</i>	0	1	0.36				
	2	1.41	0.2				
	20	1.13	0.17				
	200	1.01	0.29				

Table S5. Expression of genes in the hypothalamus-pituitary-adrenal (HPA) axis. Transcript responses are expressed as fold change compared to corresponding controls. * $p < 0.05$, ** $p < 0.01$ indicates significant differences between exposure groups and the control.

Gene	TBOEP			Gene	TBOEP		
	Con ($\mu\text{g L}^{-1}$)	Mean	SD		Con ($\mu\text{g L}^{-1}$)	Mean	SD
<i>crh</i>	0	1	0.24	<i>crhbp</i>	0	1	0.21
	2	1.25	0.14		2	1.47	0.3
	20	1.02	0.13		20	1.36	0.24
	200	-1.12	0.14		200	1.14	0.14
<i>crhr2</i>	0	1	0.26	<i>pomc</i>	0	1	0.11
	2	2.18*	0.4		2	-1.49	0.12
	20	1.76	0.45		20	-1.72*	0.15
	200	1.35	0.44		200	-2.08**	0.16
<i>gr</i>	0	1	0.1	<i>mr</i>	0	1	0.29
	2	2.14*	0.2		2	1.78*	0.17
	20	1.86	0.51		20	1.32	0.28
	200	1.71	0.59		200	1.1	0.27
<i>mc2r</i>	0	1	0.24	<i>hmgra</i>	0	1	0.3
	2	1.16	0.25		2	1.44	0.19
	20	-1.59	0.2		20	-1.03	0.18
	200	-1.15	0.23		200	-1.35	0.25
<i>hmgrb</i>	0	1	0.25	<i>star</i>	0	1	0.6
	2	1.51	0.11		2	-1.18	0.09
	20	1.11	0.2		20	-1.43	0.35
	200	-1.01	0.25		200	-1.61	0.28
<i>cyp11a</i>	0	1	0.3	<i>3βhsd</i>	0	1	0.27
	2	-1.75*	0.07		2	1.15	0.28
	20	-1.59	0.1		20	-1.20	0.12
	200	-1.89*	0.09		200	-1.33	0.18
<i>cyp17</i>	0	1	0.33				
	2	-1.19	0.26				
	20	-2.13*	0.08				
	200	-1.64	0.12				

Table S6. Expression of genes in the hypothalamus-pituitary-gonadal (HPG) axis. Transcript responses are expressed as fold change compared to corresponding controls. * $p < 0.05$, ** $p < 0.01$ indicates significant differences between exposure groups and the control.

<i>Gene</i>	TBOEP			<i>Gene</i>	TBOEP		
	Con ($\mu\text{g L}^{-1}$)	Mean	SD		Con ($\mu\text{g L}^{-1}$)	Mean	SD
<i>cyp19b</i>	0	1	0.13	<i>gnrh2</i>	0	1	0.11
	2	1.4	0.11		2	1.2	0.55
	20	1.21	0.51		20	1.11	0.29
	200	1.19	0.55		200	1.13	0.19
<i>gnrh3</i>	0	1	0.22	<i>gnrhr1</i>	0	1	0.2
	2	1.09	0.41		2	1.73*	0.29
	20	-1.23	0.24		20	-1.05	0.22
	200	-1.03	0.2		200	-1.27	0.07
<i>gnrhr2</i>	0	1	0.12	<i>gnrhr4</i>	0	1	0.21
	2	1.52*	0.17		2	1.94*	0.26
	20	-1.52	0.06		20	1.21	0.26
	200	-1.25	0.24		200	1.13	0.32
<i>fshβ</i>	0	1	0.25	<i>lhβ</i>	0	1	0.93
	2	-1.09	0.21		2	-1.20	0.31
	20	-2.94*	0.04		20	-1.47	0.65
	200	-1.14	0.08		200	-1.85	0.12
<i>er1</i>	0	1	0.18	<i>er2b</i>	0	1	0.24
	2	1.72	0.48		2	1.87*	0.26
	20	1.16	0.46		20	1.61	0.41
	200	1.13	0.33		200	1.34	0.21
<i>ar</i>	0	1	0.39	<i>fshr</i>	0	1	0.2
	2	2.04**	0.14		2	1.06	0.32
	20	1.35	0.24		20	-1.45	0.26
	200	1.31	0.31		200	-1.11	0.15
<i>lhr</i>	0	1	0.25	<i>hmgra</i>	0	1	0.3
	2	1.25	0.4		2	1.44	0.19
	20	-1.15	0.04		20	-1.03	0.18
	200	-1.37	0.22		200	-1.35	0.25
<i>hmgrb</i>	0	1	0.25	<i>star</i>	0	1	0.6
	2	1.51	0.11		2	-1.18	0.09
	20	1.11	0.2		20	-1.43	0.35
	200	-1.01	0.25		200	-1.61	0.28
<i>cyp11a</i>	0	1	0.3	<i>3βhsd</i>	0	1	0.27
	2	-1.75*	0.07		2	1.15	0.28
	20	-1.59	0.1		20	-1.22	0.12
	200	-1.89*	0.09		200	-1.33	0.18

<i>cyp17</i>	0	1	0.33	<i>17βhsd</i>	0	1	0.28
	2	-1.19	0.26		2	2.88*	0.13
	20	-2.13*	0.08		20	2.72*	0.45
	200	-1.64	0.12		200	1.77	0.38
<i>cyp19a</i>	0	1	0.29				
	2	1.38	0.27				
	20	-1.04	0.13				
	200	-1.09	0.23				

Figure S1. Two-Step linear regression analysis. The first regression process was to determine the LC_{10} and LC_{50} values at each exposure time of toxicity tests (A). The second regression to extrapolate predicted no observed effect concentration (PNOEC) values were involved with plotting $LC_{50}/5$ as the dependent variable and versus reciprocal of time as independent variable (B).

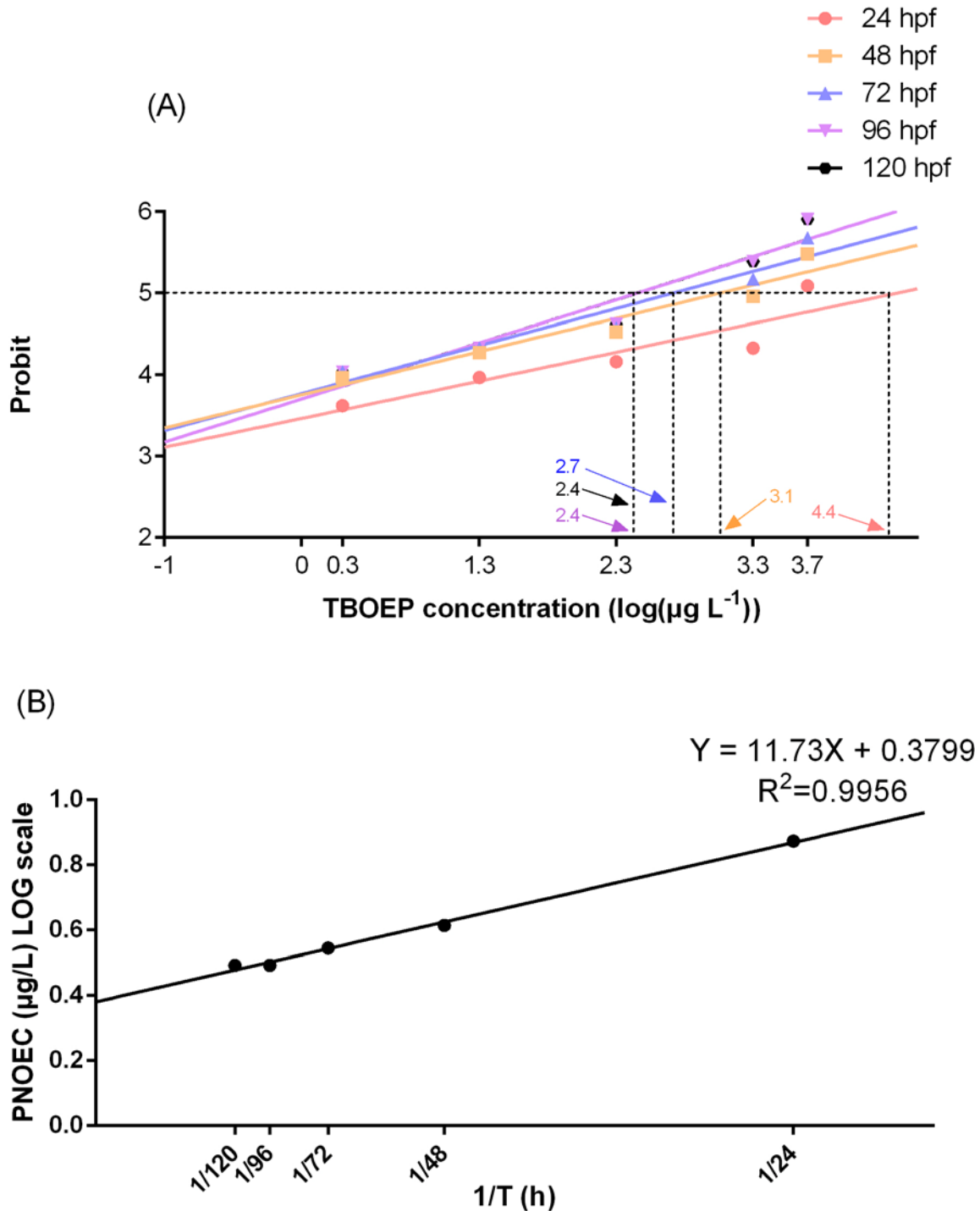


Figure S2. Expression of genes along the HPT, HPA, and HPG axes determined by qRT-PCR at 120 hpf in larvae exposed to 2, 20 or 200 $\mu\text{g TBOEP L}^{-1}$.

