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Running title: Endocrine disruption by OPFR metabolites in zebrafish

ABSTRACT

Organophosphate triesters are used as flame retardants and plasticizers to replace polybrominated diphenyl ethers. Accumulating evidence has revealed that these compounds elicit a variety of toxicity, including endocrine disruption. The present study examined estrogenicity and growth inhibition caused by organophosphate triesters and their metabolites using zebrafish embryos. Exposure to triphenyl phosphate (TPHP) and 4-hydroxylphenyl diphenyl phosphate (HO-*p*-TPHP) elicited upregulation of *cytochrome P450 19A1b* (*CYP19A1b*) expression, with a greater induction seen for the metabolite, indicating metabolic activation. Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), as well as 3-hydroxylphenyl diphenyl phosphate (HO-*m*-TPHP) and diphenyl phosphate (DPHP), did not elicit significant changes in the *CYP19A1b* expression. Reduction in body length was induced by TPHP and to a lesser extent by its hydroxylated metabolites. Altered expression of genes involved in the synthesis and action of thyroid hormones, including *iodothyronine deiodinases 1 and 2*, *thyroid hormone receptor alpha* and *transthyretin*, were commonly observed for TPHP and its two metabolites (HO-*m*-TPHP and HO-*p*-TPHP). Reduction in the body length was also seen in embryos exposed to TDCIPP, but not BDCIPP. The transcriptional effect of TDCIPP was largely different from that of TPHP, with decreased expression of *growth hormone* and *prolactin* observed only in TDCIPP-exposed embryos. Considering the concentration-response relationships for the growth retardation and gene expression changes, together with existing evidence from literature, it is likely that prolactin is in part involved in the growth inhibition caused by TDCIPP. The present study showed similarities and differences in the endocrine disruptive effects of organophosphate triesters and their metabolites.

Key words: Organophosphate triesters; Metabolites; Endocrine disruption; Growth inhibition; Flame retardants; Zebrafish

INTRODUCTION

Organophosphorus flame retardants and plasticizers (OPFRs), which consist mostly of organophosphate triester forms, are used as additives in many products, such as electronics, furniture, and textile (Hou et al., 2016; Reemtsma et al., 2008). The production and consumption of OPFRs have been increased worldwide. Globally, the amount of consumption of OPFRs was 500,000 tons in 2011, while it was estimated to be 680,000 tons in 2015 (Hou et al., 2016). Due to their increased production and usage, OPFRs are detected in a wide array of environmental matrices, including indoor and outdoor dust, air, surface soil, drinking water, and aquatic species in many countries (Choo et al., 2018; He et al., 2017; Lee et al., 2016; Li et al., 2014; McGoldrick et al., 2014; Möller et al., 2011; Stapleton et al., 2009; Tajima et al., 2014).

Increasing levels of OPFRs in indoor and outdoor environment might have led to human exposure to those compounds and subsequent detection of their metabolites in urine, as OPFRs are, in general, metabolized mainly in liver and excreted in urine in laboratory rodents (Ahmed et al., 1993; Hou et al., 2016; Minegishi et al., 1988; Suzuki et al., 1984; Van den Eede et al., 2013; Wang et al., 2020). A variety of OPFR metabolites, such as diester forms in urine have been used as biomarkers for assessment of human exposure to OPFRs (Hou et al., 2016). OPFR metabolites, like bis(2-chloroethyl) phosphate (BCEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), and diphenyl phosphate (DPHP), were found in human urine in the United States, with mean concentrations being 0.76, 0.46 and 1.1 ng/mL, respectively (Dodson et al., 2014). In urine samples collected from Australia, DPHP concentration ranged from <0.30 to 727 ng/mL, while BDCIPP concentration was in the range of <0.15 – 8.90 ng/mL, respectively, being higher in children than in adults (Van den Eede et al., 2014), possibly due to physiological and behavioral properties of children involving a different breathing zone and higher ventilation and metabolic rates than adults, in combination with an increased hand-to-mouth behavior of children (Butt et al., 2014; Van den Eede et al., 2014).

There have been a variety of studies testing effects of parent OPFRs, which revealed that the endocrine system is one of the major targets of this class of chemicals in vertebrate species. Significant decrease of testosterone concentration and the expression level of genes involved in testosterone synthesis in the testes of male ICR mice was observed after treatment with triphenyl phosphate (TPHP) and tris(2-chloroethyl)

phosphate (TCEP) (Chen et al., 2015). Endocrine disrupting effects of OPFRs have also been reported in fish. Short term waterborne exposure of adult zebrafish to tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) and TPHP caused disruption of thyroid hormone signaling in sex-dependent manner (Liu et al., 2019). Parental exposure to TDCIPP also caused increase of triiodothyronine (T3) concentration, as well as decrease of thyroxine (T4) concentration in larvae of their offspring (Ren et al., 2019). It has been reported that long term exposure to TDCIPP led to reduction in body length and mass of female zebrafish, accompanied by decreased expression levels of several genes related to the GH/IGF axis (Zhu et al., 2017). Expression level of some genes related to GH/IGF axis in zebrafish larvae were also downregulated by parental exposure to TDCIPP (Ren et al., 2019).

Several lines of evidence have shown that TPHP and its hydroxylated metabolites with triester structure, 3-hydroxylphenyl diphenyl phosphate (HO-*m*-TPHP) and 4-hydroxylphenyl diphenyl phosphate (HO-*p*-TPHP), have agonistic and antagonistic potencies toward human estrogen receptor (ER) subtypes, with those two metabolites being more estrogenic than their parent compound (Kojima et al., 2016, 2013). Diphenyl phosphate (DPHP) was shown to affect cardiac development of zebrafish, although its potency was significantly lower than TPHP (Mitchell et al., 2019). Our previous study demonstrated that TPHP, HO-*m*-TPHP and HO-*p*-TPHP caused pericardial edema and reduction of blood flow in trunk vessels of zebrafish embryos (Lee et al., 2020). A similar circulatory failure was observed for embryos exposed to TDCIPP, but not to its metabolite BDCIPP. Yet, toxicological effects of OPFR metabolites have just begun to be understood.

The main objective of the present study is to investigate endocrine disruptive potency of OPFR metabolites using zebrafish embryos. First, we investigated estrogenic potency of HO-*p*-TPHP, HO-*m*-TPHP, DPHP and BDCIPP in comparison with their parent compounds TPHP and TDCIPP, by quantifying mRNA expression level of an ER target gene identified in this species, *cytochrome P450 19A1b* (*CYP19A1b*) (Jarque et al., 2019; Lassiter and Linney, 2007; Mouriec et al., 2009). Second, we measured body length of zebrafish embryos exposed to the same set of chemicals, along with transcript levels of genes involved in growth.

MATERIALS AND METHODS

Chemicals

Dimethyl sulfoxide (DMSO) and TDCIPP were obtained from Wako Pure Chemical Industries (Osaka Japan) and TPHP was from Sigma-Aldrich (Bornem, Belgium). HO-*m*-TPHP, HO-*p*-TPHP, DPHP and BDCIPP were kindly gifted by Dr. Vladimir Belov (Max Plank Institute, Göttingen, Germany). MS and NMR techniques were used to ensure that purity of all prepared standards was above 98%. Stock and working solutions of OPFRs and their metabolites were prepared as indicated in our previous study (Lee et al., 2020). Carboxymethyl cellulose (CMC) was purchased from Sigma-Aldrich.

Zebrafish breeding

Male and female zebrafish (AB strain), which was maintained in a flow-through and multi-rack configurations as previously described (Kubota et al., 2019), were mated with 4:3 pairs, respectively. Eggs collected were placed in lab dishes (15 cm diameter) containing breeding water, and embryos were reared for 72 h in an incubator at 28.5 ℃ under a 14 h light/10 h dark diurnal cycle. Malfunctional or dead embryos were removed and water was refreshed every 24 h.

Chemical exposure

Chemical exposure was conducted similar to our previous research (Lee et al., 2020). Briefly, for body length assessment, embryos at 72 hours post fertilization (hpf) (n=10) were exposed for 24 h to either DMSO alone (0.1% DMSO, v/v) or different concentrations (i.e., 1, 3, 10, or 30 μM) of TPHP, HO-*m*-TPHP, HO-*p*-TPHP, DPHP, TDCIPP, and BDCIPP, by adding 4 μL of each chemical in 4 mL of breeding water using the 4 cm diameter petri dishes. For gene expression analysis, embryos at 72 hpf (n=20) were exposed for 24 h to the same set and concentrations of chemicals as used for the body length assessment by adding 8 μL of each chemical in 8 mL of breeding water using the 5.5 cm petri dish. After 24 h of exposure, embryos were rinsed with fresh breeding water three times, transferred to MagNA Lyser Green Beads (Roche, Mannheim, Germany), flash-frozen in liquid nitrogen, and stored at -80 ℃.

Body length assessment

Embryos at 96 hpf were immobilized with 3% CMC and observed under the inverted microscope CKX53 (Olympus, Tokyo, Japan) equipped with DP-73 (Olympus) digital camera. Body length of each embryo was calculated using the cellSens® (Olympus) software. Two separate exposure experiments were performed for body length assessment to obtain data from 20 embryos per group.

Gene expression analysis

Total RNA was isolated using QIAzol Lysis Reagent (Qiagen, MD) and NucleoSpin® (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The RNA concentration of each sample was determined using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). Total RNA (1 μg per sample) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Primer sequences for *CYP19A1b, growth hormone* (*gh*), *insulin-like growth factor 1* (*igf-1*), *prolactin* (*prl*), *thyroid peroxidase* (*tpo*), *thyroid hormone receptor alpha* (*trα*), *transthyretin* (*ttr*), *thyroid-stimulating hormone β* (*tshβ*), *iodothyronine deiodinase 1* (*dio1*), *iodothyronine deiodinase 2* (*dio2*) and *eukaryotic translation elongation factor 1α1, like 1* (*eef1a1l1*) are listed in supplemental Table S1 (Goldstone et al., 2010; Hoshijima and Hirose, 2007; Jarque et al., 2019; Mouriec et al., 2009; Yu et al., 2017, 2010). Quantitative real time PCR (qPCR) was conducted using the FastStart Essential DNA Green Master (Roche) in a LightCycler® 96 Instrument (Roche). qPCR cycle conditions consisted of 1 cycle of 95 ℃ for 10 min, 55 cycles of 95 ℃ for 10 sec, 60 ℃ for 10 sec and 72 ℃ for 10 sec, and 1 cycle of 95 ℃ for 10 sec, 65 ℃ for 1 min, and 97 ℃ for 1 sec. To ensure the amplification of a single product, a melt curve analysis was performed at the end of each PCR run. The comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) proposed by Livak and Schmittgen, 2001 was applied to comparing relative expression levels of target genes due to chemical exposure.

Statistics

R version 3. 5. 1 (R Core Team, 2018) and 4. 0. 4 (R Core Team, 2021) with nine packages (Magrittr (Bache and Wickham, 2014), dplyr (Wickham et al., 2021, 2019), ggplot2 (Wickham, 2016), tidyverse(Wickham, 2017), ggpubr (Kassambara, 2020), GGally (Schloerke et al., 2020), multcomp (Hothorn et al., 2008), nparcomp (Konietschke et al., 2015), and tibble (Muller and Wickham, 2021)) were applied to statistical analyses and graphical representation. For body length assessment, we conducted statistical analysis using data which were obtained from two separate experiments (n=20) to obtain more reliable data. Dunnett's multiple comparison test was used to determine statistically significant differences in body length and in relative gene expression levels between the vehicle control group (DMSO) and exposed groups.

RESULTS

Estrogenic effect

Exposure of embryos to HO-*m*-TPHP, HO-*p*-TPHP, and TDCIPP at 30 μM elicited significant increases in mortality (i.e., <50% of lethality) in the present study. One of the duplicate groups exposed to TPHP 30 μM and HO-*m*-TPHP 10 μM also showed >50% lethality. All other chemicals showed no significant mortality even at the highest tested concentration (30 μM).

Embryos exposed to TPHP showed significant increases in *CYP19A1b* mRNA expression level at 10 μM (2.4-fold) and 30 μM (2.1-fold) (Fig. 1A). Similarly, HO-*p*-TPHP showed a significant elevation of *CYP19A1b* transcript level at the highest concentration (10 μM), showing a 3.1-fold increase (Fig. 1C), which is greater than the fold change seen in the same concentration of TPHP. No significant upregulation of the *CYP19A1b* expression level was observed for embryos exposed to HO-*m*-TPHP or DPHP (Fig. 1B and D). Similarly, neither TDCIPP nor BDCIPP elicited significant changes in mRNA expression of *CYP19A1b* at any of the tested concentrations (Fig. 1E and F).

Growth inhibitory effect

Concentration-response relationships for effects of OPFRs and their metabolites on growth of zebrafish embryos were evaluated. Embryos exposed to TPHP and TDCIPP had a concentration-dependent decrease of body length (Figs. 2 and S1), showing significant changes at 3, 10 and 30 μM for TPHP and 10 and 30 μM for TDCIPP. A slight, but not significant, decrease of body length was observed for HO-*m*-TPHP and HO-*p*-TPHP, showing at 10 μM the lowest median value. Unlike these chemicals, other tested metabolites, including DPHP and BDCIPP, showed no clear differences in median values of body length as compared to the vehicle control (Fig. 2).

Since growth inhibition was observed for TPHP and TDCIPP, and slightly for HO-*m*-TPHP and HO-*p*-TPHP, we further examined expression levels of genes involved in growth, including *gh*, *igf-1*, *prl*, *tpo*, *trα*, *ttr*, *tshβ*, *dio1* and *dio2* (Tables 1, 2 and Figs. S2-S10). Transcript level of *gh* was significantly decreased by TDCIPP in a concentration-dependent manner and by HO-*m*-TPHP at all tested concentrations. None of the other tested chemicals exhibited any significant changes in the *gh* expression levels. Expression level of *igf-1* was significantly suppressed by the highest concentration of TPHP, DPHP, TDCIPP, and BDCIPP, and by the lowest concentration of HO-*m*-TPHP. HO-*p*-TPHP showed no significant changes in the *igf-1* expression level.

Concentration-dependent decrease of the *prl* expression level was observed for embryos exposed to TDCIPP, showing significant suppression at 10 μM (0.30-fold). No significant alterations in the *prl* level were observed for other tested chemicals.

Regarding genes involved in synthesis and action of thyroid hormones, the expression level of *tpo* showed no significant changes by any of tested chemicals, with the exception of BDCIPP at 30 μM. A significant decrease of the *trα* transcript level at higher concentrations was seen for embryos exposed to TPHP, TDCIPP, and HO-*m*-TPHP. The fold change values for decreased expression of *trα* were 0.64- and 0.59-fold for 10 and 30 μM of TPHP, 0.70-fold for TDCIPP, and 0.74-fold for HO-*m*-TPHP, respectively. For *ttr*, TPHP, HO-*m*-TPHP, HO-*p*-TPHP and TDCIPP had a similar pattern of expression, showing decreasing trend at higher concentrations. Significant downregulation of *tshβ* was elicited by TPHP at 30 μM (0.38-fold), TDCIPP at 10 μM (0.69-fold), BDCIPP at 30 μM (0.78-fold), and HO-*m*-TPHP at 1 μM (0.69-

205 fold). The expression level of *dio1* was significantly downregulated in embryos exposed to 10 and 30 μM
206 TPHP and 10 μM HO-*m*-TPHP, showing 0.53-fold, 0.26-fold and 0.43-fold changes, respectively. A similar
207 trend of reduction in the *dio1* level was seen in the highest concentration of TDCIPP (10 μM). A significant
208 increase in *dio2* level was found in TPHP at 10 μM and HO-*p*-TPHP at 10 μM, having 1.4 and 1.7-fold
209 changes, respectively. HO-*m*-TPHP also exhibited a slightly increasing pattern at 10 μM, although no
210 significant change was recorded. To the contrary, a significant decrease of *dio2* was found in BDCIPP at 3,
211 10 and 30 μM with fold changes of 0.72, 0.73 and 0.79, respectively.
212
213 **DISCUSSION**
214 The present study showed that not only OPFRs, but also several OPFR metabolites may induce endocrine
215 disrupting effects in zebrafish embryos, including estrogenicity and growth inhibition. Estrogen-like effects
216 were observed in embryos exposed to TPHP and HO-*p*-TPHP in the present study. Our results showed
217 metabolic activation of TPHP towards zebrafish ER subtypes, when *CYP19A1b* was used as a marker gene of
218 the estrogenic effect. A similar metabolic activation was shown in an *in vitro* reporter gene transactivation
219 assay where Chinese hamster ovary cells transfected with human ERα and ERβ were exposed to TPHP and
220 HO-*p*-TPHP (Kojima et al., 2016). The same study revealed that estrogenic potency of HO-*m*-TPHP towards
221 human ER subtypes was weaker than that of HO-*p*-TPHP, but was slightly higher than that of TPHP. Thus, the
222 order of estrogenicity suggested in zebrafish embryos (i.e., HO-*p*-TPHP > TPHP > HO-*m*-TPHP) is somewhat
223 different from the order of estrogenicity in human ERs shown by Kojima and colleagues (i.e., HO-*p*-TPHP >
224 HO-*m*-TPHP > TPHP). When looking at ligand binding domains (LBDs) of ER subtypes among vertebrates,
225 the amino acid sequence of human ERα is 89% identical to rat and mouse ERα, and human ERβ is 90% and
226 88% identical to rat and mouse ERβ, respectively (Harris et al., 2002). On the other hand, LBDs of zebrafish
227 and human ER subtypes are moderately conserved, showing 64% identity between hERα and zfERα, 69%
228 identity between hERβ and zfERβ1, and 68% identity between hERβ and zfERβ2 (Asnake et al., 2019).
229 Regardless of the shared LBD sequences, however, a species-specific activation of ERα was observed for
230 TDCIPP, with the rat subtype being activated (Zhang et al., 2014), but not human subtype (Kojima et al., 2016)

231 or zebrafish embryos (present study). Taken together, species differences in the ligand specificity of ER
232 subtypes may partially involve the differing response to OPFRs and their metabolites. Alternatively,
233 differences in the evaluation methods of the estrogenic potency could be attributable to the apparent species
234 differences in the estrogenicity between zebrafish and mammals. The present study quantified transcript levels
235 of *CYP19A1b*, as this gene is generally accepted as a biomarker for estrogenic potency in zebrafish embryos
236 (Cano-Nicolau et al., 2016; Jarque et al., 2019; Lassiter and Linney, 2007; Mouriec et al., 2009). On the other
237 hand, the mammalian studies measured the ER transactivation potency using the well-established reporter gene
238 assay system (Kojima et al., 2016; Zhang et al., 2014).

239 Different substitution patterns of hydroxyl group on the aromatic ring might be linked to the differing
240 estrogenic potency between HO-*m*-TPHP and HO-*p*-TPHP. There have been reports showing the importance
241 of substitution position of hydroxyl group to the estrogenicity. For instance, mono-hydroxylated
242 polychlorinated biphenyls (PCBs) with *para*-hydroxyl form were the most estrogenic, followed by *meta*- and
243 *ortho*-hydroxyl forms (Arulmozhiraja et al., 2005; Kitamura et al., 2005). An optimal estrogenic activity was
244 obtained for alkylphenols when the alkyl group was located at the *para* position of the phenyl rings relative to
245 the hydroxyl group (Routledge and Sumpter, 1997). Thus, our results on the greater estrogenic potency of HO-
246 *p*-TPHP than HO-*m*-TPHP *in vivo* could also be attributed to the relative *para* position between hydroxyl group
247 and the main structure on the phenyl ring, which is also in line with the results obtained from the cell-based
248 reporter gene assay (Kojima et al., 2016). It is also to be noted that HO-*m*-TPHP was more embryo toxic than
249 HO-*p*-TPHP (Lee et al., 2020), which might lead to the secondary effects attenuating estrogenic potencies of
250 HO-*m*-TPHP.

251 None of the other tested chemicals, including DPHP, TDCIPP or BDCIPP showed an inducible effect on
252 *CYP19A1b* expression, indicating no or weak estrogenic potency for these compounds. *In silico* docking
253 simulation suggested that OPFRs with three phenyl rings have better interaction with hERα-LBD than OPFRs
254 without that structure (Zhang et al., 2014), which supports our data showing estrogenic potency of TPHP
255 greater than DPHP, TDCIPP and BDCIPP.

256 Concentration-dependent effects on growth retardation were exerted by TPHP and TDCIPP, and to a

lesser extent by HO-*m*-TPHP and HO-*p*-TPHP. The results obtained for TPHP and TDCIPP in the present study are generally in consistent with previous studies (Isales et al., 2015; McGee et al., 2013). The novelty of the current study is that triester metabolites of TPHP, i.e., HO-*m*-TPHP and HO-*p*-TPHP slightly elicited a growth inhibitory effect even by a relatively short time exposure (24 h). Thus, the order of growth inhibitory potency for TPHP and its metabolites in zebrafish embryos is regarded as TPHP > HO-*m*-TPHP ≈ HO-*p*-TPHP > DPHP. This is somewhat different from the order of estrogenic potency mentioned above and developmental circulatory failure caused by these compounds (Lee et al., 2020), indicating that mechanisms involved are different among these three toxicity endpoints.

In order to predict possible mechanisms involved in the growth inhibition, we measured transcript levels of genes involved in the growth, including *gh*, *igf-1*, *prl*, *tpo*, *trα*, *ttr*, *tshβ*, *dio1* and *dio2*, and focused on the association of gene expression changes with reduction in the body length, and the existing evidence available from the literature.

Transcript levels of *gh* and *prl* had a clear concentration-dependent decrease in TDCIPP-exposed embryos, showing a strong correlation between these two genes in the transcript data (Spearman's rho of 0.86, see Fig. S11), possibly due to shared regulation. In the present study, TPHP exposure at 3 μM elicited significant decrease of body length without significant alterations in *gh* expression level, indicating that *gh* may not be a main regulator of growth inhibition caused by TPHP. Previous studies revealed that prolactin plays an essential role in growth and embryogenesis in zebrafish (Zhu et al., 2007). Knockdown of *prl* gene with a morpholino antisense oligonucleotide (MO) resulted in shorter body length, which was rescued by treatment with *prl* mRNA containing 5 nucleotides mismatch sequences against the Prl-MO (Zhu et al., 2007). The same study also showed that *gh* knockdown elicited no detectable morphological changes within 10 dpf (Zhu et al., 2007). These results indicate that prolactin has more important roles at least in early growth and altered expression of *prl* may cause tangible morphological changes. Taken together, it is plausible that the growth inhibition induced by TDCIPP may be associated with the decreased expression of *prl* in the present study.

Unlike TDCIPP, there was no significant decrease of expression levels of *prl* or *gh* in embryos exposed

283 to TPHP. Thus, it may be ruled out that *prl* or *gh* genes are responsible for the growth inhibition caused by
284 TPHP. Alternatively, genes involved in the biosynthesis and action of thyroid hormones, including *trα*, *ttr*,
285 *tshβ*, *dio1* and *dio2* were altered following exposure to TPHP. A similar altered expression of *trα*, *ttr*, *dio1*
286 and *dio2* was observed for embryos exposed to HO-*m*-TPHP and/or HO-*p*-TPHP.

287 In teleost, maternally-originated THs, which mostly consist of T4, are located in the egg yolk sac, and
288 iodothyronine deiodinases (Dio) that can activate T4 to T3 may play an important role in T3-dependent
289 development (Chang et al., 2012; Orozco and Valverde-R, 2005; Power et al., 2001; Walpita et al., 2010). In
290 zebrafish, knockdown of Dio1 caused no substantial developmental defects, whereas Dio2 knockdown
291 induced delayed development, suggesting that Dio2 is mainly involved in the activation of thyroid hormone
292 in zebrafish embryos (Walpita et al., 2010). In addition, double knockdown of Dio1 and Dio2 elicited more
293 severe developmental delay and malformations, indicating further that Dio1 functions under depleted thyroid
294 status where Dio2-induced production of T3 is suppressed. In the current study, the lowest concentration (3
295 μM) of TPHP induced significant decrease of body length without major changes in *dio1* or *dio2*, implying
296 that factors other than TH activation pathway may be mainly involved in the growth inhibition in embryos
297 exposed to TPHP. Nevertheless, the higher concentrations (10 and/or 30 μM) of TPHP resulted in more
298 severe growth retardation with significant decrease of *dio1* or increase of *dio2*. A similar pattern of altered
299 expression of *dio1* and/or *dio2* was observed for HO-*m*-TPHP and HO-*p*-TPHP. Thus, *dio1* and *dio2* genes
300 may be in part related to the increased severity of growth impairment posed by higher concentrations of
301 TPHP and its triester metabolites.

302 TRα is a key receptor not only for the action of THs, but also possibly for early development in
303 zebrafish (Liu et al., 2000; Ren et al., 2019). It has been shown that neither TPHP nor TDCIPP transactivates
304 hTRα or hTRβ (Kojima et al., 2013), which may indicate that the decreased expression of *trα* observed in
305 embryos exposed to TPHP and its metabolites might be independent of direct activation or inactivation of
306 TRs. Regardless of the mechanism involved, a decreased level of *trα* seen in the present study might be
307 partly associated with the growth inhibition.

308 TTR in fish is an important carrier protein that supplies THs to many target tissues (Kawakami et al.,

2006; Power et al., 2000; Yu et al., 2010). In the current study, significant reduction in *ttr* was shown in higher exposure concentrations of TPHP and its triester metabolites. Decreasing levels of TTR could lead to an increase in unbound T4 and subsequent decrease of circulating THs, as suggested in zebrafish and American kestrels (Fernie et al., 2005; Liu et al., 2019; Yu et al., 2010). This indicates that decreasing expression of *ttr* observed in embryos exposed to these chemicals triggers a reduction in TH levels, possibly leading to the growth inhibition.

In conclusion, estrogen-like and growth inhibitory effects of TPHP and its hydroxylated triester metabolites were observed in zebrafish embryos. Metabolic activation of TPHP was suggested for estrogenic effect, with HO-*p*-TPHP being greater potency than TPHP. Expression levels of genes related to the synthesis and action of THs were commonly altered in embryos exposed to TPHP and its hydroxylated triester metabolites, indicating shared mechanism of gene expression changes among the parent compound and its metabolites. The possibility that disruption of TH homeostasis occurs due to altered and unbalanced expression of TH related genes and subsequently results in growth inhibition in embryos exposed to TPHP and its hydroxylated triester metabolites warrants further investigation. Growth inhibitory effects were also shown in zebrafish embryos exposed to TDCIPP, but not its metabolite BDCIPP, whose mechanism is possibly different from that of TPHP and its metabolites, which may in part involve the decreased level of prolactin.

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Conflict of interest—The authors declare that there is no conflict of interest.

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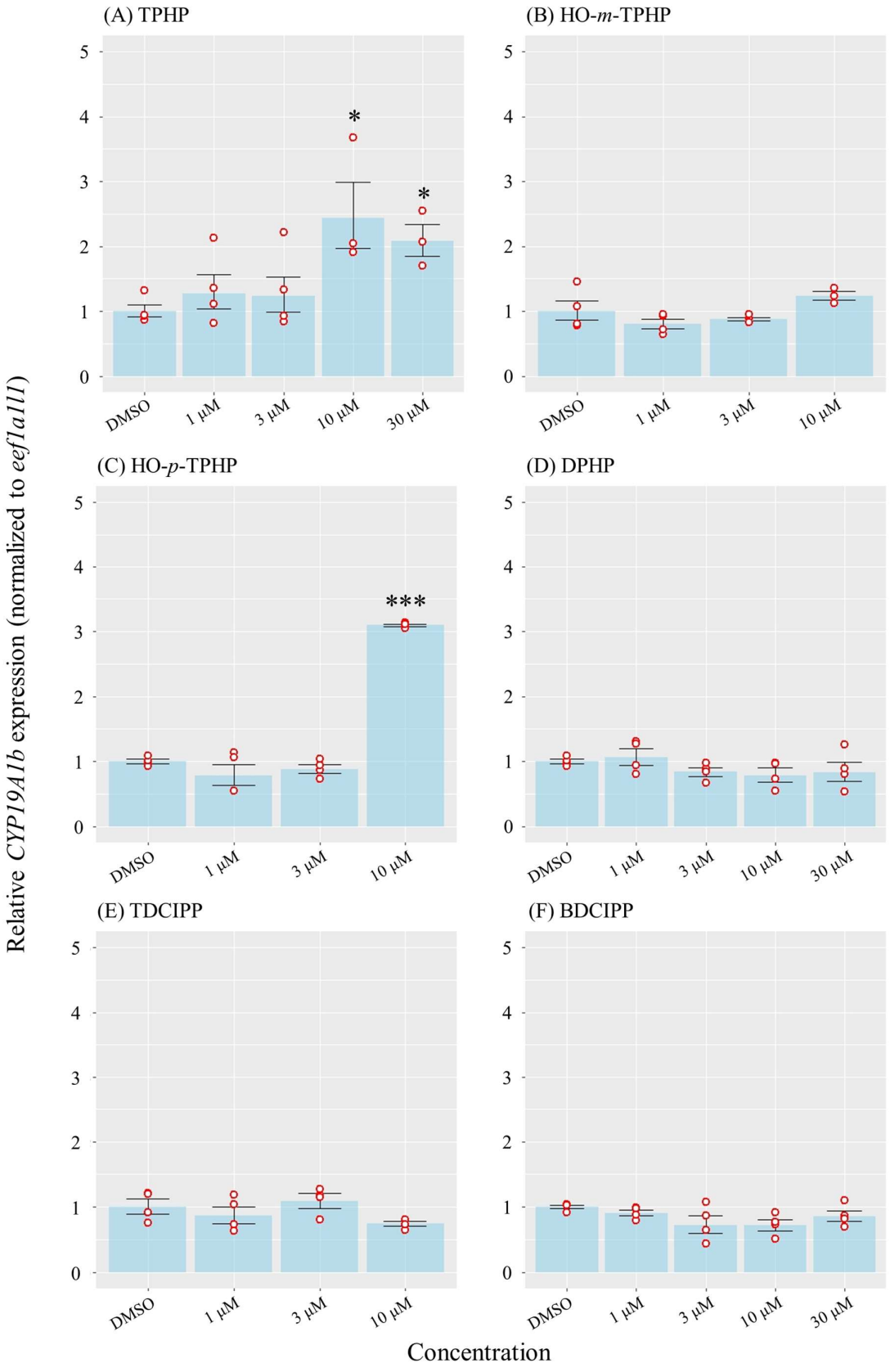
FIGURE LEGENDS

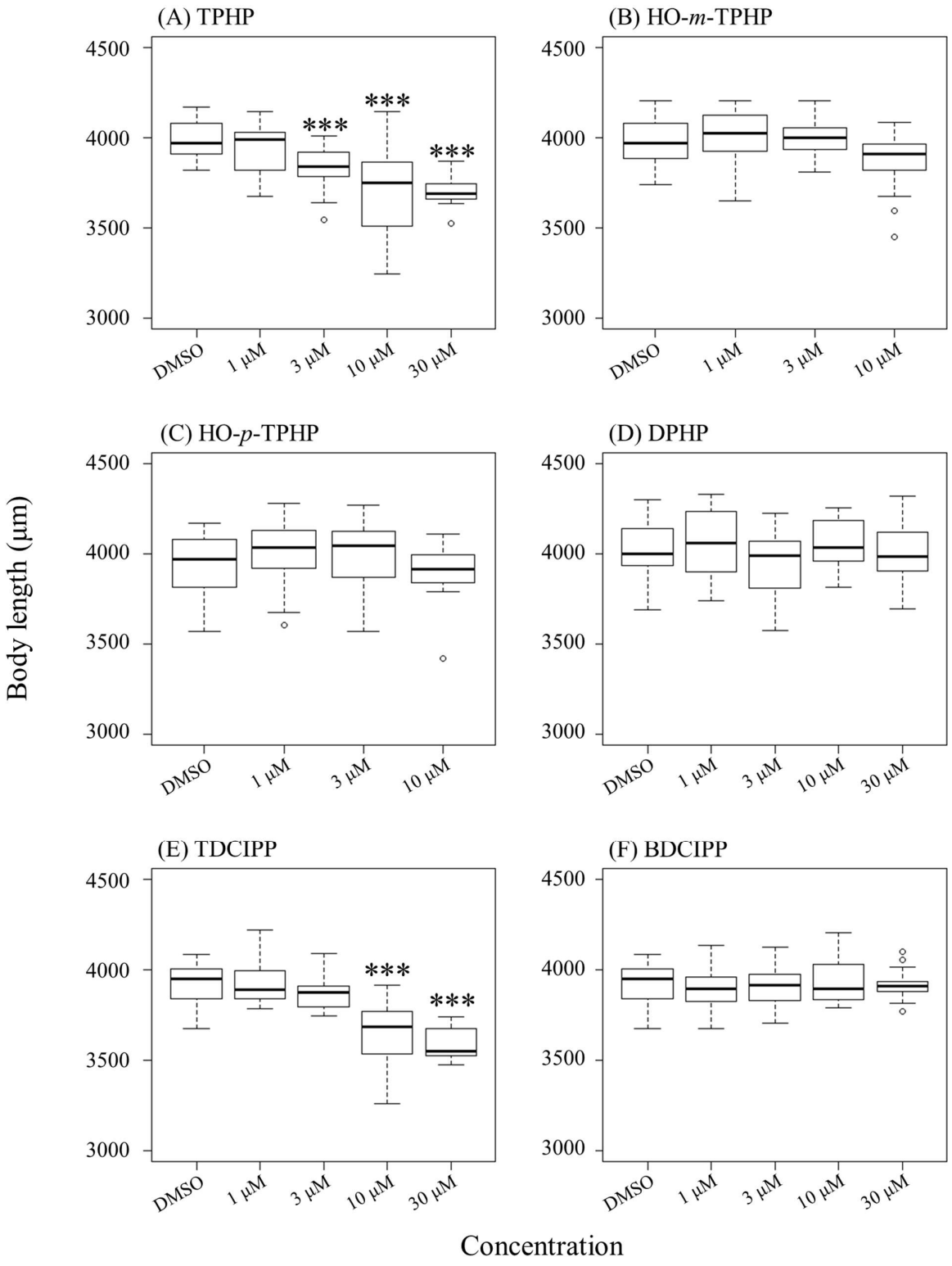
Figure 1. Effects of TPHP, TDCIPP and their metabolites on *CYP19A1b* mRNA expression levels in developing zebrafish. Zebrafish embryos (n = 20 per dish) at 72 hpf exposed to TPHP (1–30 μM) (A), HO-*m*-TPHP (1–10 μM) (B), HO-*p*-TPHP (1–10 μM) (C), DPHP (1–30 μM) (D), TDCIPP (1–10 μM) (E), or BDCIPP (1–30 μM) (F) for 24 h were used for the analysis. Error bars of these graphs show SEM of four biological replicates, except 10 and 30 μM TPHP and 10 μM HO-*m*-TPHP that have three replicates due to greater lethality of exposed embryos. Statistically significant differences between control group (DMSO) and OPFRs- or their metabolites-exposed groups were determined by one way ANOVA followed by Dunnett's post hoc test. Significant differences are shown by asterisk (*$p < 0.05$, ***$p < 0.001$).

Figure 2. Effects of TPHP, TDCIPP and their metabolites on growth of zebrafish embryos. Zebrafish embryos (n = 10 per dish) at 72 hpf exposed to TPHP (1–30 μM) (A), HO-*m*-TPHP (1–10 μM) (B), HO-*p*-TPHP (1–10 μM) (C), DPHP (1–30 μM) (D), TDCIPP (1–30 μM) (E), or BDCIPP (1–10 μM) (F) for 24 h were used for the analysis. The whiskers show data range, while the box extends from the 25th to 75th percentile. Two separate experiments were performed for each concentration group of all tested chemicals, being n = 20 per group, except 30 μM TPHP (n = 14), 10 μM HO-*m*-TPHP (n = 17) and 30 μM TDCIPP (n = 8) due to their lethality. Statistically significant differences between control group (DMSO) and OPFRs- or their metabolites-exposed groups were determined by one way ANOVA followed by Dunnett's post hoc test. Significant differences are shown by asterisk (***$p < 0.001$).


(A) TPHP
(B) HO-m-TPHP
(C) HO-p-TPHP
(D) DPHP
(E) TDCIPP
(F) BDCIPP
Relative CYP19A1b expression (normalized to eef1a1l1)
Concentration
DMSO
1 μM
3 μM
10 μM
30 μM
*
***

531

532 **Fig. 1**


(A) TPHP
(B) HO-m-TPHP
(C) HO-p-TPHP
(D) DPHP
(E) TDCIPP
(F) BDCIPP
Body length (μm)
4500
4000
3500
3000
***
DMSO
1 μM
3 μM
10 μM
30 μM
Concentration

533

534 **Fig. 2**