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Parental transfer of tris(1,3-dichloro-2-propyl) phosphate and transgenerational inhibition of growth of zebrafish exposed to environmentally relevant concentrations☆

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ABSTRACT

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is a re-emerging environmental contaminant that has been frequently detected at sub-ppb (<μg/L) concentrations in natural waters. The objective of this study was to evaluate effects of TDCIPP on growth in initial generation (F_0) zebrafish after chronic exposure to environmentally relevant concentrations, and to examine possible parental transfer of TDCIPP and transgenerational effects on growth of first generation (F_1) larvae. When zebrafish (1-month old) were exposed to 580 or 7500 ng TDCIPP/L for 240 days, bioconcentration resulted in significantly less growth as measured by body length, body mass, brain-somatic index (BSI) and hepatic-somatic index (HSI) in F_0 females but not F_0 males. These effects were possibly due to down-regulation of expression of genes along the growth hormone/insulin-like growth factor (GH/IGF) axis. Furthermore, residues of TDCIPP were detected in F_1 eggs after exposure of parents, which resulted in less survival, body length and heart rate in F_1 individuals. Down-regulation of genes in the GH/IGF axis (e.g., *gh*, *igf1*) might be responsible for transgenerational toxicity. This study provides the first known evidence that exposure of zebrafish to environmentally relevant concentrations of TDCIPP during development can inhibit growth of offspring, which were not exposed directly to TDCIPP.

1. Introduction

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is one of the primary organophosphate triesters used as flame retardants, which have been used extensively for decades in manufacturing of polymers, resins, latexes, products for infants and polyurethane foams (Dishaw et al., 2011; Stapleton et al., 2011, 2012). TDCIPP has been frequently detected in indoor and outdoor air, natural waters, sediments and aquatic species of fish, and is considered to be a re-emerging environmental pollutant (Sundkvist et al., 2010; van der Veen and de Boer, 2012). Concentrations of TDCIPP in natural waters have generally been reported at sub-per-billion (sub-ppb; <μg/L) concentrations. For example, in the Songhua River of China, concentrations of TDCIPP in water samples ranged from 2.5 to 40 ng TDCIPP/L (Cao et al., 2012; Wang et al., 2011). In seawater samples collected near the cities of Qingdao, Xiamen and Lianyungang, China, concentrations ranged from 24 to 377 ng TDCIPP/L (Hu et al., 2014).

Toxicological information has suggested that exposure to relatively great concentrations of TDCIPP can cause disruption of

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endocrine function (Crump et al., 2012; Liu et al., 2012, 2013a; Kojima et al., 2013; Wang et al., 2013; Zhang et al., 2014), neural toxicity (Dishaw et al., 2011, 2014; Ta et al., 2014; Wang et al., 2015c), developmental toxicity (Farhat et al., 2013, 2014; Fu et al., 2013; Li et al., 2015b; McGee et al., 2012; Wang et al., 2015a), and reproductive toxicity (Liu et al., 2013b; Wang et al., 2015b; Li et al., 2015a), and among these effects developmental changes might be the primary adverse effects. For example, exposure of zebrafish embryos to a large concentration of TDCIPP (1290 μg/L) inhibited rearrangement of cells at 4 h post-fertilization (hpf) and caused delay of epiboly at 5.7 and 8.5 hpf in zebrafish embryos and decreased masses of larvae (Fu et al., 2013). Exposure during early development to relatively small concentrations of TDCIPP (20 or 100 μg/L) resulted in significantly lesser body mass and body length of initial generation (F_0) zebrafish (Wang et al., 2015c). Furthermore, exposure of F_0 fish to TDCIPP (20 or 100 μg/L) resulted in transfer of TDCIPP to F_1 embryos and lesser body mass in F_1 larvae (Wang et al., 2015a).

Recently, studies of *Daphnia magna* (Li et al., 2015a, 2015b) and zebrafish (Zhu et al., 2015) demonstrated that exposure to environmentally relevant concentrations of TDCIPP causes significant growth inhibition. Specifically, treatment with 65 or 550 ng TDCIPP/L for 28 days significantly down-regulated expression of genes involved in synthesis of proteins, and expression of genes in the metabolism and endocytosis pathways, and decreased length of F_0 and first generation (F_1) *Daphnia magna* (Li et al., 2015a). While in zebrafish, exposure to 600 ng TDCIPP/L for 120 days resulted in bioconcentration of TDCIPP in tissues and lesser body length and body mass of females, and down-regulation of genes involved in production of hormones along the growth hormone/insulin-like growth factor (GH/IGF) axis was considered to be a possible mechanism of toxicity (Zhu et al., 2015). Therefore, results of the two studies demonstrated that changes in development might be critical toxic effects due to exposure to TDCIPP and suggested hazard to aquatic species.

Chronic exposure of larvae to environmentally relevant concentrations of TDCIPP due to transfer from the females during production of eggs might cause adverse effects on developing F_1 larvae. Although effects of transfer of TDCIPP from females to eggs were reported in a previous study of zebrafish, concentrations used in that study were greater than those reported in natural waters (Wang et al., 2015a). Whether exposure to environmentally relevant concentrations of TDCIPP can cause transgenerational toxicity remained unknown. To evaluate transgenerational toxicity and provide information required for assessment of hazard or risk of TDCIPP, zebrafish were exposed to environmentally relevant concentrations for 240-days. Bioaccumulation and maternal transfer of TDCIPP were evaluated, and effects on development of F_0 adult fish and F_1 larvae were examined. To elucidate possible mechanisms of development toxicity, gene expression patterns in GH/IGF axis were examined in both generations.

2. Materials and methods

2.1. Chemicals and reagents

TDCIPP was purchased from Sigma (St. Louis, MO, USA; purity: 95.7%), and was dissolved in dimethyl sulfoxide (DMSO). TDCIPP used as an analytical standard was from Tokyo Chemical Industry America (Portland, OR, USA; purity: 95%). Internal standards, d_{15}-TDCIPP and bis (1,3-dichloro-2-propyl) phosphate (BDCIPP) were purchased from Dr. Vladimir Below via Letcher Group-Organic Contaminants Research Laboratory (OCRL), NWRC (Ottawa, Canada), and purities of these two standards were >97%. MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TRIzol reagent and PrimeScript Reverse Transcription (RT) Reagent kits and SYBR Green kits were purchased from TaKaRa (TaKaRa, Dalian, China). All the other reagents used in this study were of analytical grade.

2.2. Maintenance and exposure of zebrafish to TDCIPP

Zebrafish (AB strain) were maintained according to a previously described method (Yu et al., 2011). One-month old zebrafish (fifteen fish in each of three replicated tanks for each concentration) were acclimated for 1 week in 15-L glass tanks then exposed to 0, 500 or 5000 ng/L TDCIPP for 240 days. The least concentration (500 ng/L), to which zebrafish were exposed, was comparable to that reported in natural waters along the coast of China near the city of Lianyungang (377 ng/L) (Hu et al., 2014). Exposure solutions were prepared with carbon-filtered water, and replaced daily with freshly prepared solutions containing corresponding concentrations of TDCIPP. Samples of exposure solutions were collected twice at the last day of the exposure, before and after renewal of water. Concentrations of TDCIPP and its metabolite (BDCIPP) were quantified. Both control and treated groups received 0.001% DMSO. During the exposure period, survival was recorded.

On the last day of the 240-day exposure, five females and five males from each tank were paired in clean water (without TDCIPP), and eggs were immediately collected for quantification of TDCIPP. Embryos were transferred to glass beakers containing clean water (without TDCIPP) to assess transgenerational toxicity. Hatching, survival, heart rate and growth were determined for F_1 larvae at 3-day post-fertilization (dpf) or 5-dpf. Thirty 5-dpf F_1 larvae were sampled randomly and frozen immediately in liquid nitrogen, and stored at −80 °C for the subsequent assay of gene expression. After that, fish were euthanized with 0.03% MS-222, and body length and body mass of female and male fish were recorded. Brains and livers were sampled and massed for brain-somatic index (BSI) and hepatic-somatic index (HSI) calculation, respectively. Additionally, brains and livers of females were also collected for quantitative real-time polymerase chain reactions (qRT-PCR). Since no significant effects of TDCIPP on growth in males were observed, expressions of genes in brains and livers of males were not investigated.

2.3. Quantification of TDCIPP and BDCIPP in exposure solutions, F_0 zebrafish and F_1 eggs

Concentrations of TDCIPP and BDCIPP in exposure solutions were directly measured by use of a Waters ACQUITY UPLC® I-Class system (UHPLC) coupled to Waters® Xevo™ TQ-S mass spectrometer (TQ-S/MS) (Milford, MA, USA) using electrospray ionization (ESI(+)) in the multiple reaction monitoring (MRM) mode. For more detailed information on instrumental parameters, please refer to previous publications (Su et al., 2014 and Su et al., 2015). During the analysis, decamethonium hydroxide was used as a dicationic derivatization reagent which was mixed with mobile phase post-LC separation at a constant rate of 10 μL/min with a "T" connector. TDCIPP and BDCIPP were quantified by use of transitions of m/z 430.9 > 99 and m/z 577.2 > 243.3, respectively. A 6-point calibration curve was run with each batch of samples to ensure instrumental response linearity. For the quantification of TDCIPP and BDCIPP in the exposure solutions, no background contamination was detectable, and thus the method limits of quantification were defined as a concentration that can generate instrumental response that is 10-fold greater than the signal-to-noise ratio. The method limits of quantification (MLOQs) of TDCIPP and BDCIPP were 0.01 and 0.015 ng/mL water, respectively.

Based on previous publications (Zhu et al., 2015), it was assumed

that BDCIPP was not likely bioconcentrated into F_0 zebrafish or transferred to F_1 eggs. Thus, concentrations of TDCIPP were measured only in F_0 zebrafish and F_1 eggs. A detailed protocol can be found elsewhere (Chu and Letcher, 2015), and is also provided in the Supporting Information. An ultrasonic extraction method was used for analysis of biotic samples. In brief, biotic samples were spiked with 10 ng of the internal standard, d_{15}-TDCIPP, and extracted at room temperature with 4 mL of 50/50 (v/v) DCM/HEX solvent in an ultrasonic-cleaner (1.9 L, 35 kHz, 140 W from VWR, Mississauga, Canada) for 10 min. Extraction was repeated twice and extracts combined. Further clean-up was conducted with a 300 mg aliquant of PSA bonded silica. Collected samples were analyzed by use of the same UPLC-TQ-S/MS instrument, but equipped with an atmospheric pressure chemical ionization (APCI+) source. During quantification of TDCIPP in tissues, there was unavoidable background contamination with an average of 0.31 ± 0.14 ng TDCIPP/sample. Thus, the MLOQ for TDCIPP for fish tissues was 0.42 ng/sample, which was three times the standard derivation of measurements in all solvent control fish samples. Based on its internal standard, d_{15}-TDCIPP, the mean recovery of TDCIPP for all biotic samples was 89 ± 12%.

2.4. Quantitative real-time PCR reactions

Liver, brain and F_1 larvae were preserved in TRIzol reagent (Takara, Dalian, Liaoning, China) for isolation of RNA. Real-time PCR reactions were performed according to previously published methods (Yu et al., 2014). For adult zebrafish, six fish from three replicate tanks (two fish per tank) were included for each concentration and tissue of each replicate was from a single fish. For F_1 larvae, three replicates (30 larvae for each replicate) were included for each concentration. Briefly, isolation of total RNA was performed using TRIzol reagent according to the manufacturer's instructions. Concentrations of total RNA were determined at 260 nm using the Epoch™ Microplate Spectrophotometer (Bio Tek Instruments, Inc, Vermont, USA), and purity of RNA in each sample was verified by determining the A260/A280 ratio and confirmed by use of agarose-formaldehyde gel electrophoresis with ethidium bromide staining. Synthesis of first-strand complementary DNA was performed using PrimeScript Reverse Transcription Reagent Kits (Takara, Dalian, Liaoning, China). The quantitative real-time polymerase chain reaction was done using SYBR Green PCR kits (Takara, Dalian, Liaoning, China) on an ABI Step One Plus RT-PCR (Applied Biosystem, Foster City, CA) system. Melting curve was employed to check out purity and specificity of PCR productions. Sequences of primers for selected genes were obtained using the online Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Table 1). The thermal cycle was set at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min, and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Expression of housekeeping gene ribosomal protein L8 (*rpl8*) did not change after various concentrations of TDCIPP exposure and was used as an internal control. The relative expressions of genes were determined by the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences used in the present study.

Gene	Sequence of the primer (5′-3′)	Accession number
rpl8	Forward: ttgttggtgttgttgctggt Reverse: ggatgctcaacagggttcat	NM_200713
gh	Forward: tcgttctgcaactctgactcc Reverse: ccgatggtcaggctgtttga	NM_001020492.2
ghra	Forward: ggccgaaaattccttactgtt Reverse: gctggcgttgctgattgt	NM_001083578.1
ghrb	Forward: gctgcgctctgttgataatgt Reverse: ggcggagggaggtggat	NM_001111081.1
igf1	Forward: caacgacacacaggtcttcccagg Reverse: tcggctgtccaacggtttctctt	NM_131825.2
igf1ra	Forward: gcccgtggagaagtctgtgg Reverse: gtgtgcgaaagtgttcctggtt	NM_152968.1
igf1rb	Forward: atcctcccggccttactgtt Reverse: cctgtcattgtttcggttcttgt	NM_152969.1

2.5. Statistical analyses

Normality and homogeneity of variance were verified for all data, by use of the Kolmogorov–Smirnov test or Levene's test, respectively. All data are reported as means ± standard deviation of the mean (SD). The differences of the solvent control and exposure groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS 13.0 (SPSS, Chicago, IL). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Measured concentrations of TDCIPP in exposure solutions

Measured concentrations of TDCIPP in nominal exposure to 500 (lesser) or 5000 (greater) TDCIPP ng/L exposure groups were 540 ± 23 and 7200 ± 67 ng/L before water renewal, and 630 ± 0.5 and 7700 ± 200 ng/L after water renewal, respectively. Mean concentrations of TDCIPP for samples taken before and after water renewing were 580 ± 22 and 7500 ± 140 ng/L. No TDCIPP was detected in solvent controls, and the BDCIPP metabolite was not detected in any exposure group.

3.2. Bioconcentration of TDCIPP by F_0 adult fish

In F_0 adult males or females, concentrations of TDCIPP exhibited a concentration-dependent relationship as a function of increasing exposure concentrations from 580 ± 22 to 7500 ± 140 ng/L. In females, concentrations of TDCIPP were 38 ± 1.1 and 396 ± 93 ng/g wet mass (wm), respectively (Fig. 1A). In males, concentrations of TDCIPP were 26 ± 3.8 and 317 ± 27 ng/g wm, respectively (Fig. 1A). Based on these data, bioconcentration factors (BCFs, defined as the ratio of concentrations in fish divided by that in solution) are 66 and 53 in female fish and 45 and 42 in male fish in 580 ± 22 and 7500 ± 140 ng/L treatment groups, respectively.

3.3. Parental transfer of TDCIPP

Like F_0 adult fish, a concentration-dependent profile was also observed for concentrations of TDCIPP in F_1 eggs. Concentrations of TDCIPP were 2.8 ± 1.6 ng/g wm in F_1 eggs from group exposed to the lesser concentration, and increased to 11 ± 1.2 ng/g wm in eggs collected from females exposed to the greater concentration (Fig. 1B).

3.4. TDCIPP caused female-biased inhibition of growth in F_0 fish

No mortality was observed in the solvent control- and TDCIPP-exposed groups. In females, exposure to the greater concentration of TDCIPP resulted in significantly lesser body mass and body length, whereas exposure to the lesser concentration did not cause such effects (Fig. 2A and B). BSI values were significantly smaller in fish exposed to the greater concentration than that in fish exposed to the solvent controls, while the lesser concentration did not change BSI (Fig. 2C). HSI values were significantly less in both exposure groups compared with the solvent control (Fig. 2D). In males, body mass, length of individuals, BSI and HSI values were not affected by exposure to either concentration of TDCIPP (Fig. 2A–D).

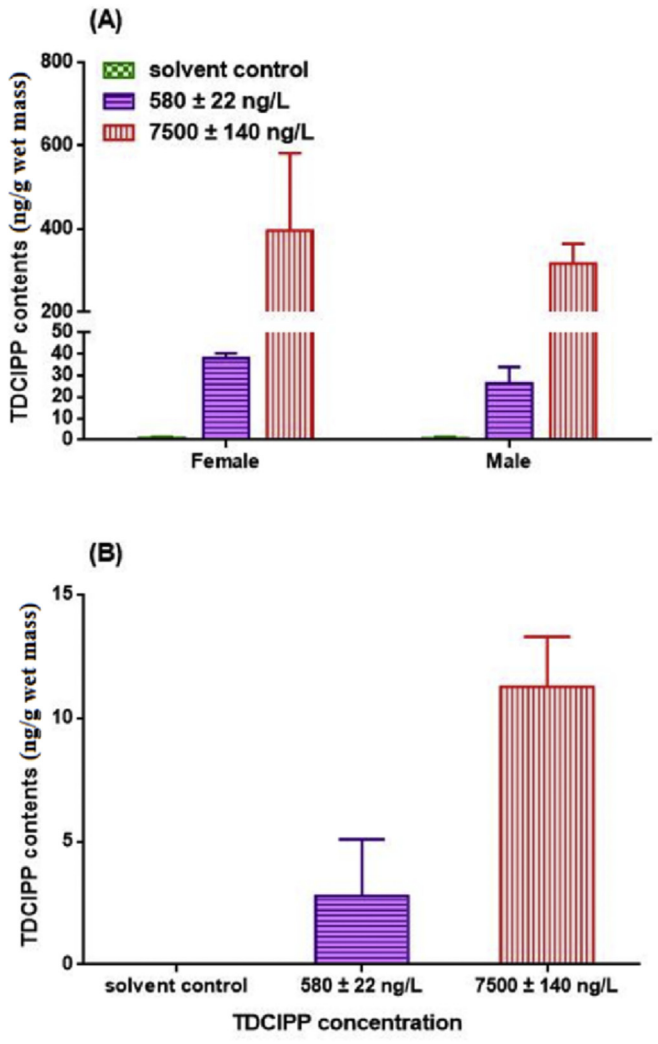

Fig. 1. Mean concentration of TDCIPP in (A) F_0 adult zebrafish after exposure to solvent control, 580 ± 22 or 7500 ± 140 ng/L TDCIPP for 240 days; (B) F_1 eggs (0 hpf). For adult fish, the values represent means ± SD of three individual fish, which were from three replicate tanks, respectively. For eggs, TDCIPP were measured in 100 eggs, with three replicate samples.

3.5. TDCIPP altered expressions of genes involved in GH/IGF axis in F_0 females

In this study, expression of genes involved in GH/IGF axis, including *gh* in brain, and *ghra*, *ghrb*, *igf1*, *igf1ra* and *igf1rb* in liver in females were examined. In brain, exposure to either of the lesser or greater concentration of TDCIPP, significantly down-regulated expression of *gh* ($P = 0.026$ and $P = 0.011$, respectively) (Fig. 3A). In liver, expressions of *ghra* and *ghrb* were significantly down-regulated in both the lesser and greater concentrations of TDCIPP (Fig. 3B). Exposure to TDCIPP also caused significant down-regulation of *igf1* and *igf1rs*. Expression of *igf1* was down-regulated by exposure to either concentration of TDCIPP ($P = 0.003$ and $P = 0.0004$, respectively) (Fig. 3B); exposure to both the lesser and greater concentrations of TDCIPP resulted in significant down-regulation of transcription of Igf1-receptor a (*igf1ra*) ($P = 0.001$ and $P = 0.002$, respectively); mRNA abundance of Igf1-receptor b (*igf1rb*) was significantly down-regulated only in F_0 adults exposed to the greater concentration of TDCIPP ($P = 0.001$) (Fig. 3B).

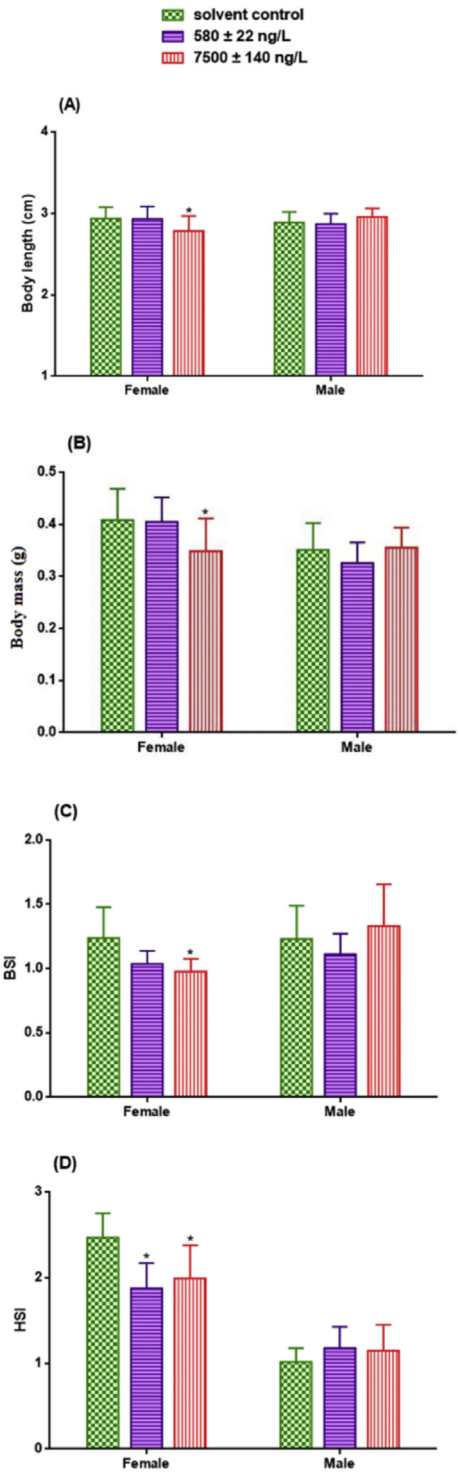

Fig. 2. Body length (A), body mass (B), brain-somatic index (BSI) (C) and hepatic-somatic index (HSI) (D) in F_0 adult zebrafish exposed to solvent control, 580 ± 22 or 7500 ± 140 ng/L TDCIPP for 240 days. Values represent mean ± SD, and 9 fish from 3 replicate tanks (three fish from each tank) were included in each concentration. Significant difference from the solvent control group is indicated by $^*P < 0.05$.

3.6. Parental TDCIPP exposure caused growth inhibition and decreased survival rate in F_1 larvae

In F_1 larvae derived from adults exposed to TDCIPP, there were no significant differences in hatching at 3 dpf, while a significant

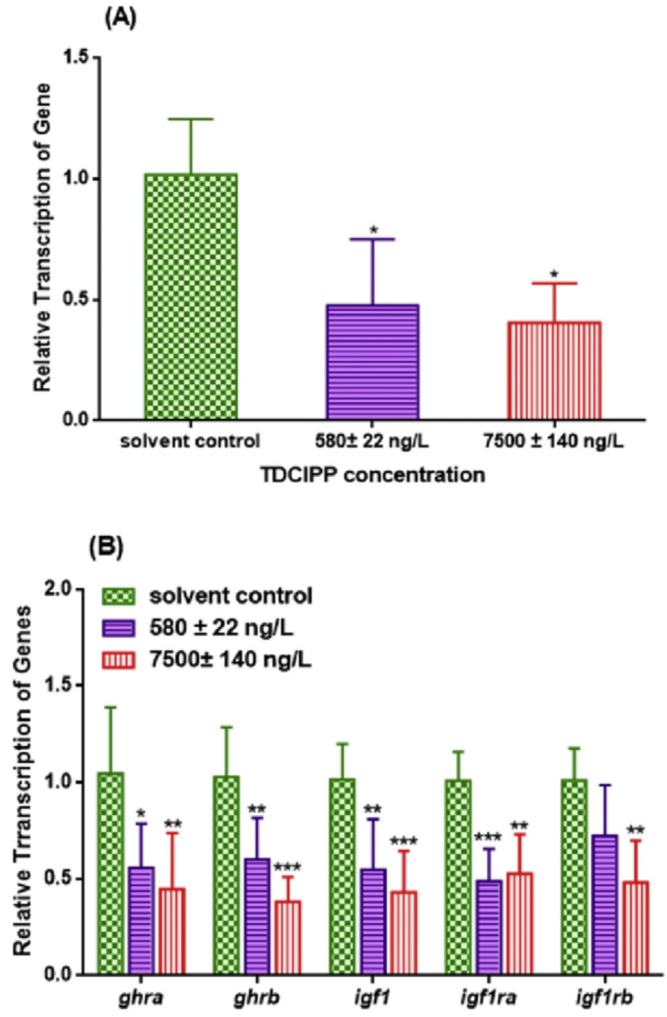

Fig. 3. Relative gene transcription of *gh* in the brain (A) and *ghra*, *ghrb*, *igf1*, *igf1ra* and *igf1rb* in the liver (B) in the female adult zebrafish exposed to solvent control, 580 ± 22 or 7500 ± 140 ng/L TDCIPP for 240 days. Values represent mean ± SD of three replicates (2 fish per replicate). Significant difference from the solvent control group is indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

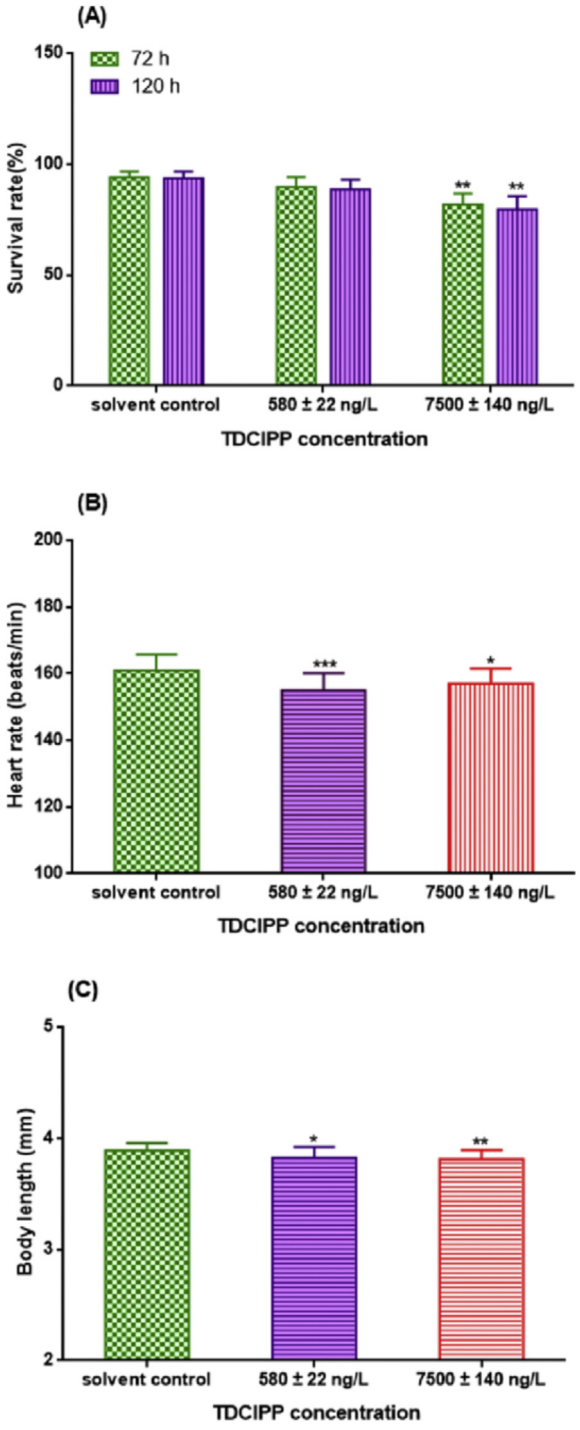

Fig. 4. Survival rate (A), heart rate (B) and body length (C) in F_1 larvae derived from parental exposure of solvent control, 580 ± 22 or 7500 ± 140 ng/L TDCIPP for 240 days. Results are given as mean values of three replicates of 30 larvae for each exposure condition at 3 or 5 dpf. Values represent mean ± SD. Significant difference from the solvent control group is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

decrease in survival was observed at 3 dpf, and 5dpf in fish derived from females exposed to the greater concentration of TDCIPP (13.0%, $P = 0.005$ and 15.0%, $P = 0.004$, respectively) (Fig. 4A). The heart rates for 3 dpf larvae derived from F_0 exposed to the lesser and greater concentrations of TDCIPP were significantly less than that of the solvent controls (3.7%, $P = 0.0003$ and 2.4%, $P = 0.019$, respectively) (Fig. 4B). Significantly lesser body length was observed at 5 dpf in F_1 larvae derived from parents exposed to the lesser or greater concentration of TDCIPP (1.6%, $P = 0.019$ and 2.0%, $P = 0.004$, respectively) (Fig. 4C). This indicated that exposure of parents to TDCIPP causes inhibition of growth of their offspring.

3.7. Parental TDCIPP exposure altered expressions of genes involved in GH/IGF axis in F_1 larvae

Expression of mRNA for the genes *gh*, *ghra*, *ghrb*, *igf1*, *igf1ra* and *igf1rb* involved in the GH/IGF axis were assessed in F_1 larvae derived from eggs of females exposed to TDCIPP (Fig. 5). At 5 dpf, *gh* expression was significantly ($P = 0.033$) downregulated in larvae of females exposed to the greater concentration of TDCIPP compared with expression of mRNA for this gene in the solvent control. There was also a significant ($P = 0.049$) down-regulation of *igf1* in larvae from eggs of females exposed to the greater concentration of TDCIPP. Expressions of *ghra*, *ghrb*, *igf1ra* and *igf1rb* were unchanged.

4. Discussion

TDCIPP is a hydrophobic chemical (log $K_{ow} = 3.65$; data source: www.chemspider.com), and has potential to be accumulated in

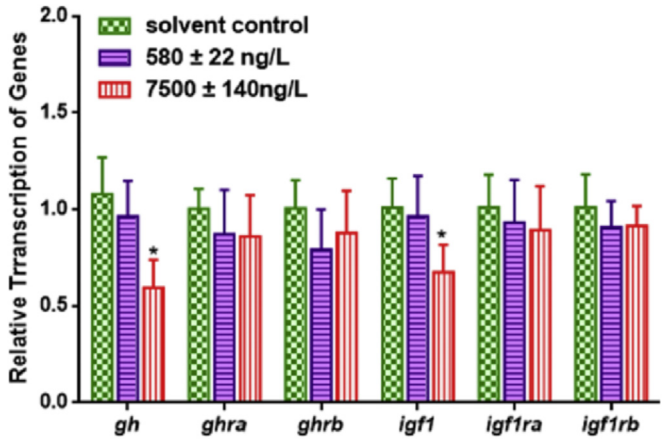

Fig. 5. Gene transcriptions in the F_1 larvae at 5-dpf derived from parental exposure of solvent control, 580 ± 22 or 7500 ± 140 ng/L TDCIPP for 240 days. Values represent mean ± SD. The data was obtained from 30 larvae for each group, and there were three replicates. Significant difference from the solvent control group is indicated by $^{*}P < 0.05$.

tissues. Indeed, greater concentrations of TDCIPP were observed in F_0 zebrafish and F_1 eggs after maternal exposure at environmentally relevant concentrations of TDCIPP. Body burdens of TDCIPP in adult fish were also observed in previous studies where it was reported that exposure to TDCIPP resulted in measureable bioconcentration in zebrafish (Wang et al., 2015a, 2015c; Zhu et al., 2015). Furthermore, BCFs for females and males were slightly different, which might be due to differences in uptake, metabolism, distribution, or elimination, as well as gender differences in nutrition (Burger et al., 2007; Greaves and Letcher, 2014). Because TDCIPP is hydrophobic (log Kow = 3.65), it is possible that more TDCIPP deposited in females were driven by relatively greater lipid contents of females. Female zebrafish contained significantly greater concentrations of TDCIPP than did males, so it was not a factor for females depurating TCDIPP into eggs. Although the character of maternal transfer was reported in a previous study after exposure to relatively great concentrations of TDCIPP (Wang et al., 2015a), the results reported here, demonstrate, for the first time that maternal exposure to environmentally relevant concentrations of TDCIPP resulted in measurable bioaccumulation by F_1 eggs, thus suggesting possible environmental risk for fish.

The negative effect of environmentally relevant concentrations of TDCIPP on growth of female zebrafish is consistent with results of a previous study, where exposure of zebrafish embryos/larvae to various concentrations of TDCIPP (50, 100, 300 or 600 μg/L) resulted in lesser in body mass of 6-dpf larvae (Wang et al., 2013). Exposure of zebrafish to relatively small concentrations of TDCIPP (4, 20, or 100 μg/L) for six months resulted in significantly less growth of both males and females (Wang et al., 2015c). Concentrations of TDCIPP, to which fish were exposed in this study (Wang et al., 2015c) were greater than those observed in aquatic environments. Recently, it was demonstrated that exposure of zebrafish to environmentally relevant concentrations of TDCIPP for 120 days caused a female-biased growth inhibition in zebrafish, including lesser body mass and body length of female zebrafish (Zhu et al., 2015). In order to assess whether such exposure could cause persistent effects on growth, a 240-day exposure was conducted. The results of that study demonstrated that similar to the previous study, TDCIPP caused a female-biased growth inhibition, but the effective concentration was greater than that reported previously (Zhu et al., 2015). The effective concentration might vary due to (1) a long-term adaptive response, and/or (2) life-stage-dependent sensitivity of zebrafish to the chemical (Maack and Segner, 2004).

Inhibition of growth of females caused by TDCIPP might be due to down-regulated expression of genes involved in the GH/IGF axis. In teleosts, Gh and Igfs are key mediators of somatic growth (Shepherd et al., 2007). In the present study, exposure to environmentally relevant concentrations of TDCIPP caused a significant down-regulation of *gh* in brain, which was consistent with previous findings (Zhu et al., 2015). The actions of Gh are initiated by binding to the Gh receptor (Ghr) in fish (Reinecke et al., 2005; Shepherd et al., 2007), and Ghra and Ghrb are two specific receptors for Gh in zebrafish (Di Prinzio et al., 2010). Levels of both *ghra* and *ghrb* mRNA declined with a dose-dependent effect in liver of female zebrafish exposed to TDCIPP. Additionally, in teleosts, Gh is the primary positive regulator of Igf-1 (Fazeli and Klibanski, 2014; Moriyama et al., 2000), and conversely, Igf-1 mediate many of the growth-promoting actions of (Li et al., 2010; Schmid et al., 2000). The results presented here further support previous findings that expression of the *igf1* gene was significantly down-regulated in female zebrafish exposed to TDCIPP and down-regulation was consistent with a decrease in transcription of *gh*. Igf-1 evokes biological responses through a widely distributed Igf-1 receptor (Igf1r) (Wood et al., 2005). In zebrafish, two functional receptors (*igf1ra* and *igf1rb*), formed by genome duplication, are critical for development (Ayaso et al., 2002; Maures et al., 2002). Specifically, in zebrafish Igf1rb is thought to play a key role in neuromuscular development (Wood et al., 2005). In this study it was found that both *igf1ra* and *igf1rb* were significantly down-regulated in liver of females. Although the mechanism remains unknown, expression of *igf1rs* was correlated with expression of *igf-1* in liver after exposure to TDCIPP. Since GH/IGF-1 signaling is essential for early growth and metabolism (Wood et al., 2005), down-regulation of *gh*, *ghra*, *ghrb*, *igf1, igr1ra* and *igf1rb* in fish exposed to TDCIPP might be the cause of lesser growth observed. These results led to the hypothesis that the GH/IGF signaling pathway is a key target for effects of TDCIPP in females.

Parental exposure to environmentally relevant concentrations of TDCIPP inhibited growth and decreased survival of F_1 larvae. A recent study reported that parental exposure to TDCIPP in zebrafish resulted in lesser body mass and reduced survival of F_1 larvae (Wang et al., 2015a). However, concentrations used in that study were greater than those detected in natural waters, and thus could not provide reliable risk assessment for TDCIPP. Also, Wang et al. (2015a) studied the effects of TDCIPP on neurodevelopment in F_1 larvae. In that study, the possible mechanism of inhibition of growth remains unclear and needs to be elucidated. The results presented here, and to our knowledge for the first time, indicated that exposure of F_0 zebrafish to environmentally relevant concentrations of TDCIPP could inhibit growth and result in lesser survival of offspring. These adverse transgenerational effects in F_1 larvae are ecologically significant because they might adversely affect growth and survival of aquatic organisms exposed to environmental realistic concentrations of TDCIPP.

Down-regulation of genes involved in the GH/IGF axis might be responsible for the observed inhibition of growth of F_1 larvae. It has been widely accepted that Gh and Igf-1 play important roles in growth and development in fish (Berryman et al., 2008). In this study, a significant down-regulation of *gh* and *igf1* was observed in the F_1 larvae, which may be responsible for the decreased body length. Growth inhibition associated with down-regulation of growth genes was also observed in the F_1 juveniles from microcystin-LR treated adult zebrafish (Liu et al., 2014). Expressions of *ghra, ghrb, igf1ra* and *igf1rb* were not changed after parental exposure to TDCIPP, which might be due to relatively small concentrations of residues of TDCIPP.

Results of this study confirmed previous results that TDCIPP

exposure caused a female-biased growth inhibition, and down-regulations of genes involved in the GH/IGF axis were considered as possible toxic mechanisms (Zhu et al., 2015). Additionally, our results indicated that maternal exposure to environmentally relevant concentrations of TDCIPP could cause transfer of the chemical to offspring in zebrafish, and resulted in transgenerational toxicity. Down-regulation of genes involved in the GH/IGF axis might be responsible for the observed toxic effect. Recently, it is accepted that the adverse outcome pathway (AOP) is effective at providing a detailed description of a toxicant's adverse effects on an organism and has the potential to be utilized to evaluate the hazards posed by a toxicant (Ankley et al., 2010; Zhou, 2015). In this study, the growth inhibition is a specific adverse outcome of TDCIPP, and can be linked to the molecular initiating event, the downregulation of genes involve in GH/IGF axis. This typical AOP might be valuable for risk assessments of TDCIPP. Collectively, the data suggested that effects on growth and development as well as reproduction with transgenerational toxicity of environmental concentrations of TDCIPP could currently be having significant adverse effects at some locations in the environment, and these data might be useful for risk prediction of TDCIPP.

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Appendix A. Supplementary data

Quantification of TDCIPP and BDCIPP in Exposure Solutions, F_0 Zebrafish and F_1 Eggs.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.09.039.

References

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29 (3), 730–741.

Ayaso, E., Nolan, C.M., Byrnes, L., 2002. Zebrafish insulin-like growth factor-I receptor: molecular cloning and developmental expression. Mol. Cell. Endocrinol. 191 (2), 137–148.

Berryman, D.E., Christiansen, J.S., Johannsson, G., Thorner, M.O., Kopchick, J.J., 2008. Role of the GH/IGF-1 axis in lifespan and healthspan: lessons from animal models. Growth Horm. IGF Res. 18 (6), 455–471.

Burger, J., Fossi, C., McClellan-Green, P., Orlando, E.F., 2007. Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals. Environ. Res. 104 (1), 135–152.

Cao, S., Zeng, X., Song, H., Li, H., Yu, Z., Sheng, G., Fu, J., 2012. Levels and distributions of organophosphate flame retardants and plasticizers in sediment from Taihu Lake, China. Environ. Toxicol. Chem. 31 (7), 1478–1484.

Chu, S., Letcher, R.J., 2015. Determination of organophosphate flame retardants and plasticizers in lipid-rich matrices using dispersive solid-phase extraction as a sample cleanup step and ultra-high performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry. Anal. Chim. Acta 885, 183–190.

Crump, D., Chiu, S., Kennedy, S.W., 2012. Effects of tris(1,3-dichloro-2-propyl) phosphate and tris(1-chloropropyl) phosphate on cytotoxicity and mRNA expression in primary cultures of avian hepatocytes and neuronal cells. Toxicol. Sci. 126 (1), 140–148.

Di Prinzio, C.M., Botta, P.E., Barriga, E.H., Rios, E.A., Reyes, A.E., Arranz, S.E., 2010. Growth hormone receptors in zebrafish (*Danio rerio*): adult and embryonic expression patterns. Gene Expr. Patterns 10 (4–5), 214–225.

Dishaw, L.V., Hunter, D.L., Padnos, B., Padilla, S., Stapleton, H.M., 2014. Developmental exposure to organophosphate flame retardants elicits overt toxicity and alters behavior in early life stage zebrafish (*Danio rerio*). Toxicol. Sci. 142 (2), 445–454.

Dishaw, L.V., Powers, C.M., Ryde, I.T., Roberts, S.C., Seidler, F.J., Slotkin, T.A., Stapleton, H.M., 2011. Is the PentaBDE replacement, tris (1,3-dichloro-2-propyl) phosphate (TDCPP), a developmental neurotoxicant? Studies in PC12 cells. Toxicol. Appl. Pharmacol. 256 (3), 281–289.

Farhat, A., Buick, J.K., Williams, A., Yauk, C.L., O'Brien, J.M., Crump, D., Williams, K.L., Chiu, S., Kennedy, S.W., 2014. Tris(1,3-dichloro-2-propyl) phosphate perturbs the expression of genes involved in immune response and lipid and steroid metabolism in chicken embryos. Toxicol. Appl. Pharmacol. 275 (2), 104–112.

Farhat, A., Crump, D., Chiu, S., Williams, K.L., Letcher, R.J., Gauthier, L.T., Kennedy, S.W., 2013. In Ovo effects of two organophosphate flame retardants—TCPP and TDCPP—on pipping success, development, mRNA expression, and thyroid hormone levels in chicken embryos. Toxicol. Sci. 134 (1), 92–102.

Fazeli, P.K., Klibanski, A., 2014. Determinants of GH resistance in malnutrition. J. Endocrinol. 220 (3), R57–R65.

Fu, J., Han, J., Zhou, B., Gong, Z., Santos, E.M., Huo, X., Zheng, W., Liu, H., Yu, H., Liu, C., 2013. Toxicogenomic responses of zebrafish embryos/larvae to tris(1,3-dichloro-2-propyl) phosphate (TDCPP) reveal possible molecular mechanisms of developmental toxicity. Environ. Sci. Technol. 47 (18), 10574–10582.

Greaves, A.K., Letcher, R.J., 2014. Comparative body compartment composition and in ovo transfer of organophosphate flame retardants in North American Great Lakes herring gulls. Environ. Sci. Technol. 48 (14), 7942–7950.

Hu, M., Li, J., Zhang, B., Cui, Q., Wei, S., Yu, H., 2014. Regional distribution of halogenated organophosphate flame retardants in seawater samples from three coastal cities in China. Mar. Pollut. Bull. 86 (1–2), 569–574.

Kojima, H., Takeuchi, S., Itoh, T., Iida, M., Kobayashi, S., Yoshida, T., 2013. In vitro endocrine disruption potential of organophosphate flame retardants via human nuclear receptors. Toxicology 314 (1), 76–83.

Li, H., Su, G., Zou, M., Yu, L., Letcher, R.J., Yu, H., Giesy, J.P., Zhou, B., Liu, C., 2015a. Effects of tris(1,3-dichloro-2-propyl) phosphate on growth, reproduction, and gene transcription of daphnia magna at environmentally relevant concentrations. Environ. Sci. Technol. 49 (21), 12975–12983.

Li, J., Giesy, J.P., Yu, L., Li, G., Liu, C., 2015b. Effects of tris(1,3-dichloro-2-propyl) phosphate (TDCPP) in Tetrahymena Thermophila: targeting the Ribosome. Sci. Rep. 5, 10562.

Li, M., Yin, Y., Hua, H., Sun, X., Luo, T., Wang, J., Jiang, Y., 2010. The reciprocal regulation of gamma-synuclein and IGF-I receptor expression creates a circuit that modulates IGF-I signaling. J. Biol. Chem. 285 (40), 30480–30488.

Liu, C., Wang, Q., Liang, K., Liu, J., Zhou, B., Zhang, X., Liu, H., Giesy, J.P., Yu, H., 2013a. Effects of tris(1,3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. Aquat. Toxicol. 128–129, 147–157.

Liu, W., Qiao, Q., Chen, Y., Wu, K., Zhang, X., 2014. Microcystin-LR exposure to adult zebrafish (Danio rerio) leads to growth inhibition and immune dysfunction in F1 offspring, a parental transmission effect of toxicity. Aquat. Toxicol. 155, 360–367.

Liu, X., Ji, K., Choi, K., 2012. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. Aquat. Toxicol. 114–115, 173–181.

Liu, X., Ji, K., Jo, A., Moon, H.B., Choi, K., 2013b. Effects of TDCPP or TPP on gene transcriptions and hormones of HPG axis, and their consequences on reproduction in adult zebrafish (Danio rerio). Aquat. Toxicol. 134–135, 104–111.

Maack, G., Segner, H., 2004. Life-stage-dependent sensitivity of zebrafish (Danio rerio) to estrogen exposure. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 139 (1–3), 47–55.

Maures, T., Chan, S.J., Xu, B., Sun, H., Ding, J., Duan, C., 2002. Structural, biochemical, and expression analysis of two distinct insulin-like growth factor I receptors and their ligands in zebrafish. Endocrinology 143 (5), 1858–1871.

McGee, S.P., Cooper, E.M., Stapleton, H.M., Volz, D.C., 2012. Early zebrafish embryogenesis is susceptible to developmental TDCPP exposure. Environ. Health Perspect. 120 (11), 1585–1591.

Moriyama, S., Ayson, F.G., Kawauchi, H., 2000. Growth regulation by insulin-like growth factor-I in fish. Biosci. Biotechnol. Biochem. 64 (8), 1553–1562.

Reinecke, M., Bjornsson, B.T., Dickhoff, W.W., McCormick, S.D., Navarro, I., Power, D.M., Gutierrez, J., 2005. Growth hormone and insulin-like growth factors in fish: where we are and where to go. Gen. Comp. Endocrinol. 142 (1–2), 20–24.

Schmid, A.C., Reinecke, M., Kloas, W., 2000. Primary cultured hepatocytes of the bony fish, Oreochromis mossambicus, the tilapia: a valid tool for physiological studies on IGF-I expression in liver. J. Endocrinol. 166 (2), 265–273.

Shepherd, B.S., Johnson, J.K., Silverstein, J.T., Parhar, I.S., Vijayan, M.M., McGuire, A., Weber, G.M., 2007. Endocrine and orexigenic actions of growth hormone secretagogues in rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146 (3), 390–399.

Stapleton, H.M., Klosterhaus, S., Keller, A., Ferguson, P.L., van Bergen, S., Cooper, E., Webster, T.F., Blum, A., 2011. Identification of flame retardants in polyurethane foam collected from baby products. Environ. Sci. Technol. 45 (12), 5323–5331.

Stapleton, H.M., Sharma, S., Getzinger, G., Ferguson, P.L., Gabriel, M., Webster, T.F., Blum, A., 2012. Novel and high volume use flame retardants in US couches reflective of the 2005 PentaBDE phase out. Environ. Sci. Technol. 46 (24), 13432–13439.

Su, G., Greaves, A.K., Gauthier, L., Letcher, R.J., 2014. Liquid chromatography-electrospray–tandem mass spectrometry method for determination of organophosphate diesters in biotic samples including Great Lakes herring gull plasma. J. Chromatogr. A. 1374, 85–92.

Su, G., Letcher, R.J., Yu, H., 2015. Determination of organophosphate diesters in urine samples by a high-sensitivity method based on ultra high pressure liquid chromatography-triple quadrupole-mass spectrometry. J. Chromgatogr. A. 1425, 154–160.

Sundkvist, A.M., Olofsson, U., Haglund, P., 2010. Organophosphorus flame retardants and plasticizers in marine and fresh water biota and in human milk. J. Environ. Monit. 12 (4), 943–951.

Ta, N., Li, C., Fang, Y., Liu, H., Lin, B., Jin, H., Tian, L., Zhang, H., Zhang, W., Xi, Z., 2014. Toxicity of TDCPP and TCEP on PC12 cell: changes in CAMKII, GAP43, tubulin and NF-H gene and protein levels. Toxicol. Lett. 227 (3), 164–171.

van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88 (10), 1119–1153.

Wang, Q., Lai, N.L., Wang, X., Guo, Y., Lam, P.K., Lam, J.C., Zhou, B., 2015a. Bioconcentration and transfer of the organophorous flame retardant 1,3-dichloro-2-propyl phosphate causes thyroid endocrine disruption and developmental neurotoxicity in zebrafish larvae. Environ. Sci. Technol. 49 (8), 5123–5132.

Wang, Q., Lam, J.C., Han, J., Wang, X., Guo, Y., Lam, P.K., Zhou, B., 2015b. Developmental exposure to the organophosphorus flame retardant tris(1,3-dichloro-2-propyl) phosphate: estrogenic activity, endocrine disruption and reproductive effects on zebrafish. Aquat. Toxicol. 160, 163–171.

Wang, Q., Lam, J.C., Man, Y.C., Lai, N.L., Kwok, K.Y., Guo, Y., Lam, P.K., Zhou, B., 2015c. Bioconcentration, metabolism and neurotoxicity of the organophorous flame retardant 1,3-dichloro 2-propyl phosphate (TDCPP) to zebrafish. Aquat. Toxicol. 158, 108–115.

Wang, Q., Liang, K., Liu, J., Yang, L., Guo, Y., Liu, C., Zhou, B., 2013. Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic-pituitary-thyroid axis. Aquat. Toxicol. 126, 207–213.

Wang, X.W., Liu, J.F., Yin, Y.G., 2011. Development of an ultra-high-performance liquid chromatography-tandem mass spectrometry method for high throughput determination of organophosphorus flame retardants in environmental water. J. Chromatogr. A 1218 (38), 6705–6711.

Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. Int. Rev. Cytol. 243, 215–285.

Yu, L., Lam, J.C., Guo, Y., Wu, R.S., Lam, P.K., Zhou, B., 2011. Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in zebrafish. Environ. Sci. Technol. 45 (24), 10652–10659.

Yu, L.Q., Zhao, G.F., Feng, M., Wen, W., Li, K., Zhang, P.W., Peng, X., Huo, W.J., Zhou, H.D., 2014. Chronic exposure to pentachlorophenol alters thyroid hormones and thyroid hormone pathway mRNAs in zebrafish. Environ. Toxicol. Chem. 33 (1), 170–176.

Zhang, Q., Lu, M., Dong, X., Wang, C., Zhang, C., Liu, W., Zhao, M., 2014. Potential estrogenic effects of phosphorus-containing flame retardants. Environ. Sci. Technol. 48 (12), 6995–7001.

Zhou, B., 2015. Adverse outcome pathway: framework, application, and challenges in chemical risk assessment. J. Environ. Sci. 35, 191–193 (China).

Zhu, Y., Ma, X., Su, G., Yu, L., Letcher, R.J., Hou, J., Yu, H., Giesy, J.P., Liu, C., 2015. Environmentally relevant concentrations of the flame retardant tris(1,3-dichloro-2-propyl) phosphate inhibit growth of female zebrafish and decrease fecundity. Environ. Sci. Technol. 49 (24), 14579–14587.

Supplementary data

Parental transfer of Tris(1,3-dichloro-2-propyl) Phosphate and Transgenerational Inhibition of Growth of Zebrafish Exposed to Environmentally Relevant Concentrations

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Detailed Protocol on Quantification of TDCIPP in F_0 Zebrafish and F_1 Eggs

Biotic samples were analyzed following a previous protocol (Chu et al., 2015). The collected samples were homogenized and transferred into a glass disposable culture tube (16×125 mm), which were further added with 10 ng of the internal standard, d_{15}-TDCIPP. The sample was mixed by use of a vortex mixer, and added with an aliquot of 4 mL of 50/50 (v/v) DCM/HEX extraction solvent. After 0.2 g sodium chloride (NaCl) and 1.2 g anhydrous magnesium sulfate ($MgSO_4$) were added into the tube, the tube was mixed with vortex mixer for 1 min. After that, the mixed sample was placed into an ultrasonic-cleaner (1.9 L, 35 kHz, 140 W from VWR, Mississauga, Canada), and ultrasonicated for 10 min at room temperature (20 ^oC). Then, the tube was centrifugated at 3500 rpm for 10 min to separate the upper solvent layer from other solid remains, and the upper layer was transferred to a disposable plain conical centrifuge tube. The ultrasonic extraction process was conducted for another two more times and all collected extracts were combined. After extraction, the combined extracts were evaporated with a steam of nitrogen to dryness, and re-dissolved with 1 mL MeOH by vortex mixing and ultrasonically extracted from 10 min. After centrifugation, the supernatant MeOH phase was transferred into another disposable plain conical centrifuge tube, and a 300 mg aliquant of PSA bonded silica was added into the sample solution for a further cleaning-up. The MeOH-silica mixture was mixed well by vortex mixing for 1 min, and then centrifuged. The supernatant was carefully transferred into a LC vial, and ready for further instrument analysis.

Reference:

Chu, S., Letcher, R. J., 2015. Determination of organophosphate flame retardants and plasticizers in lipid-rich matrices using dispersive solid-phase extraction as a sample cleanup step and ultra-high performance liquid chromatography with atmospheric pressure chemical ionization mass spectrometry. Anal. Chim. Acta. 885, 183-190