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Time-dependent inhibitory effects of Tris(1, 3-dichloro-2-propyl) phosphate on growth and transcription of genes involved in the GH/IGF axis, but not the HPT axis, in female zebrafish☆

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ABSTRACT

Growth curves were used to determine sensitive exposure windows for evaluation of developmental toxicity of chemicals to zebrafish. Dose- and time-dependent effects on body mass, body length and expression of genes involved in the growth hormone/insulin-like growth factor (GH/IGF) axis and the hypothalamic-pituitary-thyroid (HPT) axis were examined after exposure to environmentally relevant concentrations of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP). Based on growth curves, zebrafish grew most rapidly between 60 and 90 days post fertilization (dpf). Exposure to environmentally relevant concentrations of TDCIPP significantly decreased body mass and body length and down-regulated expression of several genes involved in the GH/IGF axis of female zebrafish, but no such effects were observed in male zebrafish. Exposure to TDCIPP did not change concentrations of thyroid hormones or expression of genes along the HPT axis in female and male zebrafish. These results suggest that growth stages of zebrafish between 60 and 90 dpf might be most appropriate for evaluation of developmental toxicity of chemicals, and down-regulation of genes involved in the GH/IGF axis, but not the HPT axis, might be responsible for the observed growth inhibition in females exposed to TDCIPP.

1. Introduction

Because of persistence, potential for bioaccumulation and biomagnification and toxic potency, some brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), are restricted or are being phased out of use (van der Veen and de Boer, 2012). As a result, production and usage of alternative flame retardants (FRs) are increasing. Organophosphate esters used as flame retardants (OPFRs) are one class of alternative FRs that have been produced in large volumes since the 1970s, and are added to foams, plastics, textiles, varnishes, waxes, floor polishes and electronic equipment (Reemtsma et al., 2008). Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is one major and environmentally relevant OPFR. According to USEPA's 2012 Chemical Data Reporting, in 2010 and 2011, 4500–22,700 tons/year of TDCIPP were manufactured or imported into the USA (Schreder and La Guardia, 2014). Because it is not chemically bonded into materials, TDCIPP can be easily released into aquatic environments (Kai, 2007). TDCIPP is routinely detected in natural waters (Shi et al., 2015), snow and rainfall (Regnery and Püttmann, 2009) as well as influents and

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effluents from wastewater treatment plants (Meyer and Bester, 2004; Rodil et al., 2005). For example, TDCIPP was observed at a mean concentration of 46.3 (<LOD–855) ng/L in urban surface water in Beijing, China (Shi et al., 2015). Concentrations of 24–377 ng TDCIPP/L were measured in seawaters near the cities of Qingdao and Xiamen, China (Hu et al., 2014). Concentrations as great as 3250 ng TDCIPP/L have been observed in effluents from a wastewater treatment plant in the State of Washington, USA (Schreder and La Guardia, 2014). Furthermore, TDCIPP has been detected in various aquatic wildlife (Hallanger et al., 2015; Mcgoldrick et al., 2014). For example, concentrations of TDCIPP as great as 251 ng/g lipid mass (lm) were measured in grass carp (*Cyprinus idellus*) and catfish (*Clarius fuscus*) from the Pearl River, China (Ma et al., 2013). These data suggest that TDCIPP might pose hazards or risks to exposed wildlife.

Although multiple toxic effects have been reported in various organisms exposed to relatively high concentrations of TDCIPP (Dishaw et al., 2014; Farhat et al., 2013, 2014; Fu et al., 2013; Kojima et al., 2013; Liu et al., 2013, 2016; Volz et al., 2016; Wang et al., 2015a), results of recent studies suggest that inhibition of growth is a primary apical response of both zebrafish and the protozoan *Tetrahymena thermophila* (Li et al., 2015, 2016; Yu et al., 2017; Zhu et al., 2015). For example, exposure of zebrafish to relatively small concentrations of TDCIPP (4, 20, or 100 μg/L) for 6 months resulted in significantly smaller and shorter bodies (Wang et al., 2015a). Exposure of zebrafish embryos/larvae to various concentrations of TDCIPP (50, 100, 300 or 600 μg/L) resulted in lesser body mass (Wang et al., 2013). Long-term exposure to environmentally relevant concentrations of TDCIPP resulted in significantly lesser growth of female zebrafish, relative to unexposed individuals (Yu et al., 2017; Zhu et al., 2015).

In vertebrates, growth is a multi-factorial characteristic resulting from complex genetic and molecular interactions in which growth hormone (GH) plays a major role. In teleost fish, as in other vertebrates, growth is regulated by the growth hormone/insulin-like growth factor (GH/IGF) axis (de Azevedo Figueiredo et al., 2007). Besides GH, thyroid hormones (THs), triiodothyronine (T3) and thyroxine (T4) also play major roles in regulation of growth of vertebrates (Crane et al., 2004). In fish, the thyroid endocrine system is controlled primarily by the hypothalamic–pituitary–thyroid (HPT) axis, which is responsible for maintaining thyroid hormone homeostasis (Blanton and Specker, 2007). Results of previous studies have demonstrated that exposures to TDCIPP inhibit growth and change expression of genes involved in the GH/IGF axis in zebrafish exposed to environmentally relevant concentrations (Yu et al., 2017; Zhu et al., 2015), and expression of genes involved in the HPT axis of zebrafish exposed to relatively high concentrations (Wang et al., 2013). However, it was unknown whether changes in expression of genes involved in the HPT axis due to exposure to TDCIPP are also responsible for inhibition of growth after exposure to environmentally relevant concentrations. Meanwhile, previous studies have focused on a single time point, such as 116 h (Liu et al., 2013), 142 h (Wang et al., 2013), 3 months (Wang et al., 2015a), 4 months (Zhu et al., 2015) or 8 months (Yu et al., 2017), ignoring the fact that toxicity is a time-varying process. This could have led to a bias in assessments of hazard or risk of TDCIPP to aquatic organisms (Baas et al., 2009). Therefore, the objectives of this study were to: (1) establish growth curves of zebrafish and then determine the most appropriate exposure stage and (2) evaluate time-dependent effects of environmentally relevant concentrations of TDCIPP on growth and expression of genes involved in the GH/IGF and HPT axes of zebrafish, and (3) determine which axis is the more likely primary target of TDCIPP that results in inhibition of growth.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents were purchased from the following sources: TDCIPP from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan); TRIzol reagent and reverse transcription and SYBR Green kits from Takara (Dalian, Liaoning, China); Thyroid hormone detection kits from Cloud-Clone Company (Houston, TX, USA); MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used in this study were of analytical grade.

2.2. Zebrafish maintenance, growth curves and TDCIPP exposure protocols

Zebrafish were maintained in flow-through tanks according to a previously published method (Zhu et al., 2015). Growth curves of zebrafish were established to determine the most appropriate exposure stage. Briefly, zebrafish embryos were collected as described previously (Zhu et al., 2015), and then were cultured in 10-cm glass Petri dishes until larvae could swim (almost 4–5 days post fertilization (dpf)). After that, larvae were transformed into 25-L glass tanks, where each tank contained 50 fish and were fed twice a day with the egg yolk of milled fresh hens. From 10 to 15 dpf, zebrafish larvae were co-fed with *Artemia* nauplii (Tianjin Fengnian Aquaculture Co., Ltd. Tianjin, China) and the egg yolk of milled fresh hens, and unconsumed food in tanks was cleaned every day. From 15 to 180 dpf, *Artemia* nauplii was the only dietary source. During all the culture stages, zebrafish were maintained at 28 °C under a light regime of 12 h light/12 h dark. Body mass and body length of 11 time points (3, 7, 15, 30, 45, 60, 90, 105, 120, 150 and 180 dpf) were recorded in order to establish zebrafish growth curves.

Stock solutions of TDCIPP were prepared in dimethyl sulfoxide (DMSO). Seven-day old zebrafish were acclimated in 25-L glass tanks for 1 week and then exposed to 0, 50, 500 or 5000 ng TDCIPP/L until 120 dpf. Fifty fish were exposed in each of 3 replicate tanks for each concentration. Exposure solutions were replaced daily with fresh carbon-filtered water containing corresponding concentrations of TDCIPP. Both control and treated groups received 0.005% DMSO. This experiment was ran twice in order to guarantee sufficient samples. During the exposure period, fish sampling was performed at 30, 60, 90 and 120 dpf according to the obtained growth curves of zebrafish. At 30 dpf, fish were euthanized with MS-222, thirty fish were randomly selected from 3 replicate tanks (ten fish per tank), and body mass (g) and body length (mm) were recorded. Then, six fish were randomly selected from 3 replicate tanks (two fish per tank), and the whole body of fish was collected for use in real-time PCR reactions. At 60, 90 and 120 dpf, fish were euthanized, thirty fish of each sex were randomly selected from 3 replicate tanks (ten fish each tank), and body mass (g) and body length (mm) were recorded. Then, two fish of each sex per tank were randomly selected, and a total of six fish were dissected. Brain and liver samples were collected for real-time PCR reactions.

2.3. Quantity assurance & quality control (QA&QC) on concentrations of TDCIPP in the exposure system

The QA & QC for quantification of TDCIPP during exposure to zebrafish was performed according to previously reported methods. TDCIPP was quantified in both exposure solutions and tissues of fish by use of a method based on analysis using 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(+)) and operated in multiple reaction

monitoring (MRM) mode. For detailed information on analytical methods or data QA & QC, refer to previous reports (Chu and Letcher, 2015; Su et al., 2014; Yu et al., 2017; Zhu et al., 2015). In brief, QA & QC data suggested that measured concentrations of TDCIPP in exposure system were generally comparable to nominal concentrations (i.e. 540 ± 23 versus 500 ng/L) (Yu et al., 2017), which suggested that TDCIPP in this exposure system nominal concentrations (0, 50, 500 or 5000 ng) accurately represented measured concentrations of. QA & QC data also demonstrated that no significant differences were observed between concentrations of TDCIPP before and after water renewal at any of three exposure concentrations, indicating that significant depletion of TDCIPP did not occur in the zebrafish exposure system.

2.4. Quantitative real-time PCR reactions

Quantitative real-time PCR (qRT-PCR) reactions were performed as previously described and met requirements for minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin et al., 2009). In brief, total RNA was extracted using TRIzol reagent (Takara, Dalian, Liaoning, China) according to the manufacturer's protocol. Concentrations of RNA were measured using the NanoDrop 2000 (Thermo Fisher Scientific, USA) and RNA purity was verified by determining the A260/A280 ratio and confirmed by use of agarose-formaldehyde gel electrophoresis with ethidium bromide staining. First-strand cDNA synthesis was performed using Prime ScriptTM RT reagent kits (Takara, Dalian, Liaoning, China). qRT-PCR was done using SYBR Green kits (Takara, Dalian, Liaoning, China). Sequences of primers were designed using the online primer designing tool in NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Expression of the housekeeping gene ribosomal protein L8 (*rpl8*) did not change after exposure to various concentrations of TDCIPP, and was thus selected for use as an internal control for variations among amplifications. Thermal cycling was done at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative expressions of genes were determined by the $2^{-\Delta\Delta C_T}$ method. Each concentration included six fish.

Table 1
Sequences of primers for the genes tested.

Target gene	Accession no.	Primer sequences (from 5′ to 3′)
ghrh	NM_001080092.1	F: TGGAAGACATGCTGATGCCA R: TCCACATCTTGCTTGTAGGTGT
gh	NM_001020492.2	F: TCGTTCTGCAACTCTGACTCC R: CCGATGGTCAGGCTGTTTGA
igf1	NM_131825.2	F: CAACGACACACAGGTCTTCCCAGG R: TCGGCTGTCCAACGGTTTCTCTT
igf2a	NM_131433	F: CGCCTGCCATGGATGATTAC R: TCAGTGAGCGCATCGTTGTT
igf2b	NM_001001815	F: AACCTGCCAAGTCAGAGAGGG R: GGACCTCCTGTTTTAATGCGG
igf1ra	NM_152968.1	F: GCCCGTGGAGAAGTCTGTGG R: GTGTGCGAAAGTGTTCCTGGTT
igf1rb	NM_152969.1	F: ATCCTCCCGGCCTTACTGTT R: CCTGTCATTGTTTCGGTTCTTGT
igf2r	NM_001039627.2	F: TCACGGACAGCTCCATTTCC R: TTGCTGGAGGAGCCGATTTT
crh	NM_001007379	F: TTCGGGAAGTAACCACAAGC R: CTGCACTCTATTCGCCTTCC
trα	NM_131396	F: CTATGAACAGCACATCCGACAAGAG R: CACACCACACACGGCTCATC
trβ	NM_131340	F: TGGGAGATGATACGGGTTGT R: ATAGGTGCCGATCCAATGTC
ttr	BC081488	F: CGGGTGGAGTTTGACACTTT R: GCTCAGAAGGAGAGCCAGTA
sult1 st5	NM_001199903	F: GAAAGAGGACCCTGCTCGTG R: TTTGCCATGGGGTTTTCTCG
rpl8	NM_200713	F: TTGTTGGTGTTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT

2.5. Quantification of thyroid hormones

Total concentrations of T4 and T3 were measured by use of commercial enzyme-linked immunosorbent assay (ELISA) test kits purchased from Cloud-Clone Corp. (Houston, USA). In brief, blood was collected from the caudal vein of each fish at 90 and 120 dpf and plasma was obtained by centrifugation at 3000*g* for 5 min at 4 °C. Blood plasma from five fish of the same sex was pooled to form a sample (about 30 μL). Concentrations of hormones in plasma were measured using ELISA kits according to the manufacturer's protocol. Three replicates were used in each assay. Detection limits for T4 and T3 were 1.42 ng/mL and 51.7 pg/mL, respectively. In this assay, Intra-assay and inter-assay variations were below 10% and 12%, respectively.

2.6. Statistical analyses

Normality and homogeneity of data were evaluated by Kolmogorov-Smirnow and Levene's tests, respectively. After confirmation of normality and homogeneity of variance, the differences of the control and TDCIPP exposure groups were evaluated by one-way analysis of variance (ANOVA) (SPSS 19.0, Chicago, IL, USA). Multiple comparisons were performed using Tukey's test. All data were reported as means ± standard error of the mean (SE). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Growth curve

Body mass and body length were both used to generate growth curves of zebrafish, which were fitted by a four-parametric logistic model (GraphPad Prism 6.0, GraphPad Software, Inc. CA, USA) (Fig. 1) (Equations (1)–(4)).

Body mass:

$$\text{Female}: \ Y = -0.01507 + \frac{0.67677}{1 + 10^{(\,80.64-X)*\,0.02511}};\ R^2 = 0.9959 \quad (1)$$

$$\text{Male}: \ Y = -0.01364 + \frac{0.45524}{1 + 10^{(71.39-X)*\,0.02739}};\ R^2 = 0.9947 \quad (2)$$

Body length:

$$\text{Female}: \ Y = 1.915 + \frac{33.005}{1 + 10^{(\,50.7-X)*\,0.02357}};\ R^2 = 0.9954 \quad (3)$$

$$\text{Male}: \ Y = 2.15 + \frac{31.08}{1 + 10^{(\,49.24-X)*\,0.02461}};\ R^2 = 0.9950 \quad (4)$$

Zebrafish exhibited the greatest rate of growth between 60 and 90 dpf. Furthermore, the initial stage of growth was approximately exponential, and then as the fish neared sexual maturity, growth slowed. Therefore, four sampling time points, namely 30, 60, 90 and 120 dpf, were selected in this study to evaluate effects of TDCIPP on the growth of individuals.

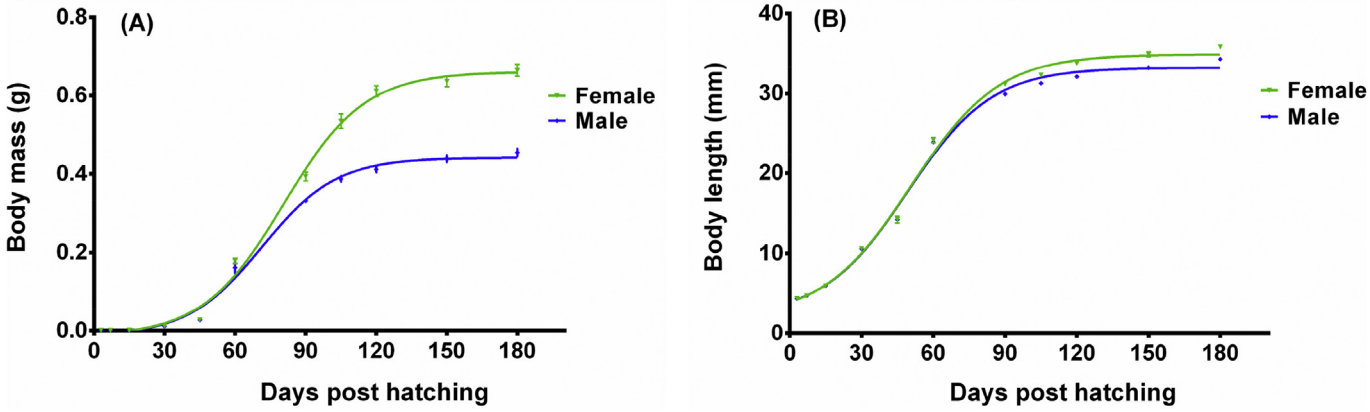

Fig. 1. Growth of zebrafish as a function of time in days post-hatch, expressed as body mass and body length. Each value is the mean ± SE of 20–30 animals.

3.2. TDCIPP caused a female-biased inhibition of growth

No statistically significant mortality was observed in any of the concentrations during the exposure period. Clear time- and dose-dependent inhibitions of growth were observed in female zebrafish after exposure to various concentrations of TDCIPP. No statistically significant effects on body mass or body length were observed at 30 (Fig. 2) or 60 dpf (Fig. 3) after exposure of TDCIPP. However, after 90 and 120 dpf, TDCIPP caused a significant inhibition of growth of females (Fig. 3). At 90 dpf, exposure to 5000 ng TDCIPP/L resulted in significantly shorter individual females, with smaller body mass, while no significant effect was observed in zebrafish exposed to the other concentrations (50 or 500 ng/L) (Fig. 3). At 120 dpf, body mass and body length of female zebrafish were significantly less in the groups exposed to 500 or 5000 ng TDCIPP/L, relative to those of controls, whereas treatment with the least concentration (50 ng TDCIPP/L) did not cause such effects (Fig. 3). There were no changes observed in body mass or body length of males at any dose of TDCIPP at any time point examined (Figs. 2 and 3).

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

Expressions of several genes involved in the GH/IGF axis were altered at 90 and 120 dpf in females exposed to TDCIPP, while no significant effects on expression of genes involved in GH/IGF axis were observed at 30 or 60 dpf (Tables 2–5). At 90 dpf, in brain of females, exposure to 50, 500 or 5000 ng TDCIPP/L significantly down-regulated expression of growth hormone (*gh*) by 1.82-, 1.85- and 3.45-fold, respectively. In liver, expressions of insulin-like growth factor 1 (*igf1*) were down-regulated by 1.61-, 2.00- and 2.38-fold, respectively, and expressions of insulin-like growth factor 2a (*igf2a*) were down-regulated by 1.75-, 2.38- and 2.78-fold in individuals exposed to 50, 500 or 5000 ng TDCIPP/L, respectively. For insulin-like growth factor 2b (*igf2b*), expression was down-regulated only in fish exposed to 5000 ng TDCIPP/L, with a 2.94-fold down-regulation. At 120 dpf, in brain of females, exposure to 50, 500 or 5000 ng TDCIPP/L significantly down-regulated expression of *gh* by 2.44-, 2.94- and 6.25-fold, respectively. In liver, expressions of *igf1* were down-regulated by 1.72-, 2.13- and 2.86-fold in individuals exposed to 50, 500 or 5000 ng TDCIPP/L, respectively, and expression of *igf2b* was down-regulated only in fish exposed to 5000 ng TDCIPP/L, with a 3.33-fold down-regulation. No significant effects on expressions of genes involved in the GH/IGF axis were observed in males exposed to 50, 500 or 5000 ng TDCIPP/L (Tables 2–5).

3.4. TDCIPP did not change expressions of genes involved in the HPT axis or concentrations of thyroid hormones

No statistically significant effects on transcriptions of genes

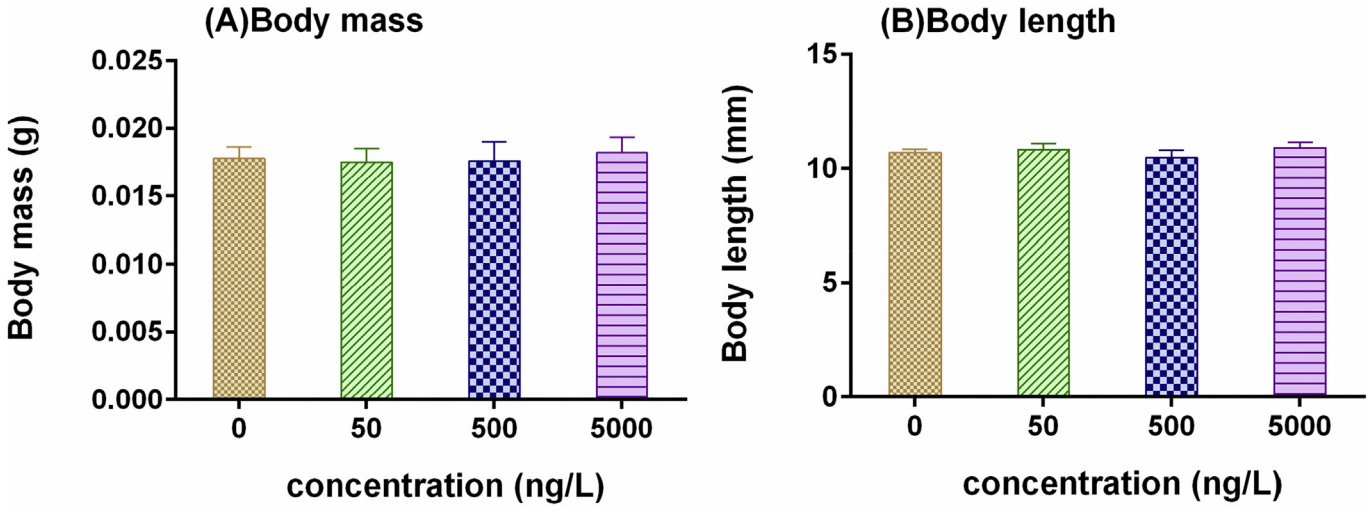

Fig. 2. Effects on body mass (A) and body length (B) of zebrafish exposed to various concentrations of TDCIPP at 30 dpf. Values are the mean ± SE (n = 30).

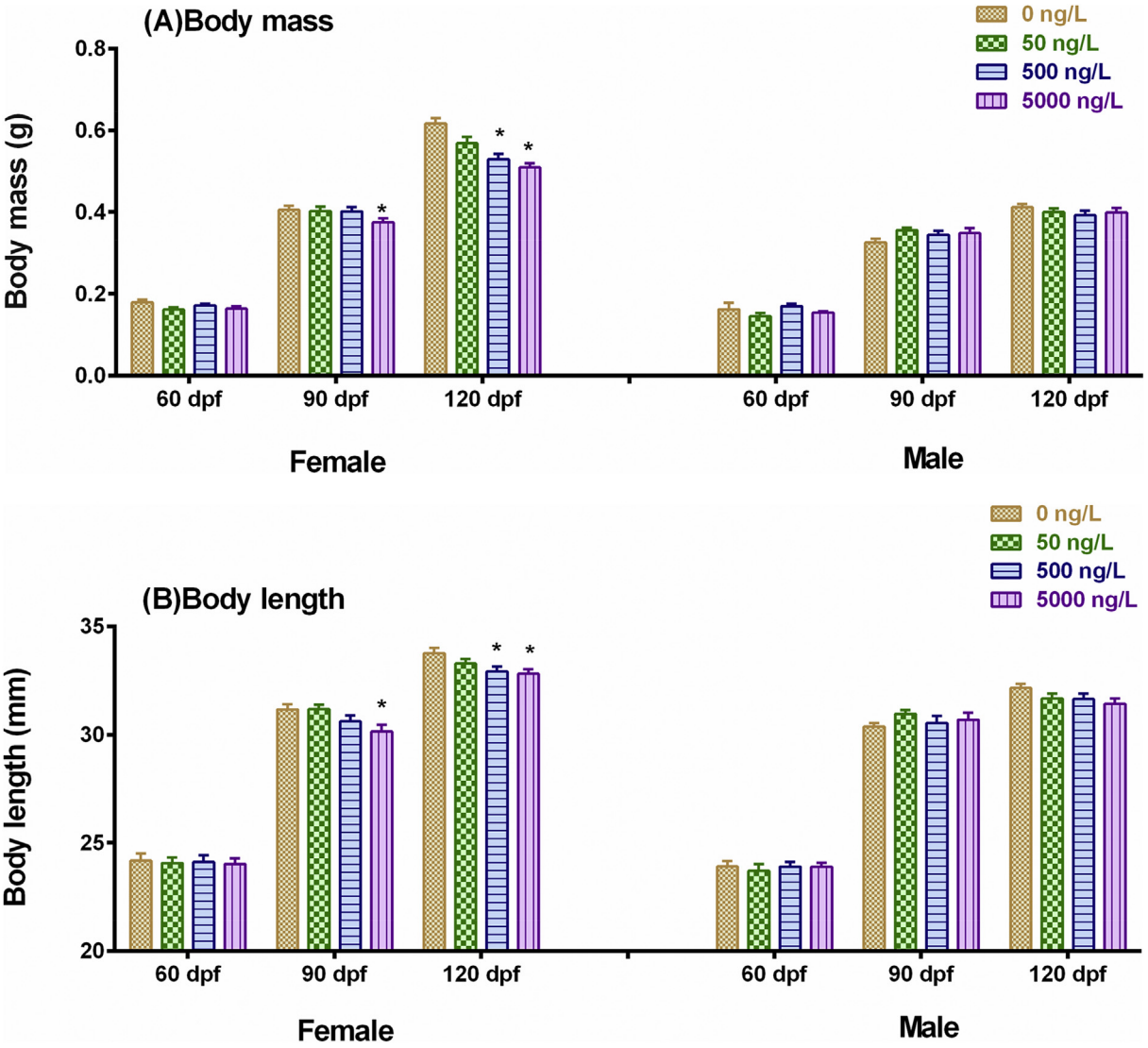

Fig. 3. Effects on body mass (A) and body length (B) of zebrafish exposed to various concentrations of TDCIPP at 60, 90 and 120 days post fertilization (dpf). Values are the mean ± SE (n = 30). *$P < 0.05$.

Table 2
Effects of various concentrations of TDCIPP on the relative expressions of genes involved in the GH/IGF and HPT axes of zebrafish at 30 days post fertilization.

TDCIPP (ng/L)	0	50	500	5000
GH/IGF axis				
gh	1.02 ± 0.12	1.22 ± 0.12	1.07 ± 0.12	0.79 ± 0.20
ghrh	1.04 ± 0.14	1.21 ± 0.29	0.72 ± 0.45	1.23 ± 0.28
igf1	1.02 ± 0.10	0.89 ± 0.10	0.98 ± 0.17	0.92 ± 0.09
igf2a	1.03 ± 0.11	1.01 ± 0.09	1.17 ± 0.15	1.15 ± 0.23
igf2b	1.02 ± 0.09	0.84 ± 0.06	1.00 ± 0.13	1.09 ± 0.12
igf1ra	1.02 ± 0.09	1.04 ± 0.07	1.08 ± 0.08	1.11 ± 0.08
igf1rb	1.02 ± 0.10	0.89 ± 0.05	1.10 ± 0.08	1.06 ± 0.10
igf2r	1.04 ± 0.13	1.15 ± 0.08	1.24 ± 0.19	1.26 ± 0.11
HPT axis				
crh	1.05 ± 0.15	1.09 ± 0.14	0.76 ± 0.09	0.84 ± 0.12
trα	1.04 ± 0.14	1.05 ± 0.06	1.16 ± 0.13	1.22 ± 0.09
trβ	1.02 ± 0.11	1.04 ± 0.07	1.19 ± 0.14	0.99 ± 0.06
ttr	1.02 ± 0.08	1.35 ± 0.07	1.01 ± 0.11	1.05 ± 0.12
sult1 st5	1.01 ± 0.06	1.12 ± 0.07	1.12 ± 0.05	1.15 ± 0.08

Values are calculated using $2^{-\triangle\triangle C_T}$ method and represent mean ± SE (n = 6).

involved in the HPT axis, including corticotropin releasing hormone (*crh*), transthyretin (*ttr*), thyroid hormone receptor alpha (*trα*), thyroid hormone receptor beta (*trβ*) and sulfotransferase family 1 cytosolic sulfotransferase 5 (*sult1 st5*) were observed in males or females exposed to any concentration of TDCIPP (Tables 2–5). Furthermore, exposure to TDCIPP did not significantly affect concentrations of T3 and T4 in plasma of females and males (Table 6).

4. Discussion

As a small fish model, zebrafish were widely used in the studies of environmental toxicology and several exposure protocols were established to evaluate developmental toxicity of chemicals (Nishimura et al., 2016; Song et al., 2014; Zhang et al., 2015). However, to the best of our knowledge, no studies have been performed to produce growth curves of zebrafish for determining the most appropriate exposure stage for evaluation of developmental toxicity of chemicals. In this study, for the first time, growth curves of zebrafish from 0 to 180 dpf were produced, and the results demonstrated that there was an exponential growth stage for zebrafish between 30 and 90 dpf. These results suggested that this zebrafish growth stage might be a sensitive window for evaluation of developmental toxicity of chemicals. This would provide the

Table 3
Effects of various concentrations of TDCIPP on relative expressions of genes involved in the GH/IGF and HPT axes of female and male zebrafish at 60 days post fertilization.

	Females				Males			
TDCIPP (ng/L)	0	50	500	5000	0	50	500	5000
GH/IGF axis								
Brain								
gh	1.03 ± 0.11	1.15 ± 0.16	1.03 ± 0.15	0.89 ± 0.13	1.08 ± 0.19	0.97 ± 0.14	1.07 ± 0.16	0.89 ± 0.16
ghrh	1.01 ± 0.05	1.02 ± 0.14	1.20 ± 0.19	1.08 ± 0.09	1.03 ± 0.11	1.05 ± 0.09	0.95 ± 0.09	1.03 ± 0.05
Liver								
igf1	1.02 ± 0.08	0.89 ± 0.13	0.82 ± 0.08	0.85 ± 0.07	1.02 ± 0.09	0.84 ± 0.11	0.85 ± 0.12	0.97 ± 0.16
igf2a	1.07 ± 0.20	0.97 ± 0.10	1.07 ± 0.16	1.08 ± 0.14	1.04 ± 0.17	1.15 ± 0.29	1.08 ± 0.29	1.30 ± 0.06
igf2b	1.05 ± 0.12	0.99 ± 0.32	1.36 ± 0.21	1.33 ± 0.14	1.10 ± 0.27	0.72 ± 0.08	1.13 ± 0.25	0.81 ± 0.18
igf1ra	1.05 ± 0.14	1.20 ± 0.28	0.76 ± 0.11	1.14 ± 0.16	1.05 ± 0.16	1.15 ± 0.22	0.85 ± 0.18	0.75 ± 0.10
igf1rb	1.02 ± 0.10	1.09 ± 0.24	1.10 ± 0.28	1.18 ± 0.15	1.04 ± 0.16	0.70 ± 0.14	0.74 ± 0.16	1.00 ± 0.27
igf2r	1.09 ± 0.19	1.02 ± 0.29	1.17 ± 0.13	1.01 ± 0.09	1.00 ± 0.04	1.06 ± 0.10	1.02 ± 0.26	1.04 ± 0.33
HPT axis								
Brain								
crh	1.04 ± 0.13	0.80 ± 0.11	0.98 ± 0.19	0.84 ± 0.27	1.02 ± 0.10	1.00 ± 0.15	1.14 ± 0.15	1.18 ± 0.16
Liver								
trα	1.03 ± 0.10	1.01 ± 0.17	1.25 ± 0.12	1.22 ± 0.11	1.02 ± 0.10	0.74 ± 0.09	1.17 ± 0.33	0.73 ± 0.05
trβ	1.00 ± 0.04	0.90 ± 0.08	1.04 ± 0.09	0.97 ± 0.09	1.05 ± 0.15	1.02 ± 0.14	1.06 ± 0.21	0.80 ± 0.14
ttr	1.04 ± 0.12	0.85 ± 0.14	0.89 ± 0.15	1.24 ± 0.24	1.02 ± 0.15	0.88 ± 0.09	1.30 ± 0.29	0.87 ± 0.19
sult1 st5	1.06 ± 0.14	0.90 ± 0.12	0.92 ± 0.12	0.81 ± 0.07	1.02 ± 0.10	1.30 ± 0.18	0.74 ± 0.23	1.18 ± 0.12

Values are calculated using $2^{-\triangle\triangle C_T}$ method and are the mean ± SE (n = 6).

Table 4
Effects of various concentrations of TDCIPP on relative expressions of genes involved in the GH/IGF and HPT axes of female and male zebrafish at 90 days post fertilization.

	Females				Males			
TDCIPP (ng/L)	0	50	500	5000	0	50	500	5000
GH/IGF axis								
Brain								
gh	1.03 ± 0.15	0.55 ± 0.10*	0.54 ± 0.04*	0.29 ± 0.05*	1.04 ± 0.12	0.84 ± 0.11	0.98 ± 0.20	0.93 ± 0.12
ghrh	1.04 ± 0.18	0.96 ± 0.11	1.01 ± 0.03	0.87 ± 0.04	1.12 ± 0.21	0.98 ± 0.08	0.94 ± 0.13	0.73 ± 0.10
Liver								
igf1	1.01 ± 0.06	0.62 ± 0.06*	0.50 ± 0.10*	0.42 ± 0.08*	1.09 ± 0.22	1.02 ± 0.17	0.88 ± 0.10	0.98 ± 0.16
igf2a	1.03 ± 0.14	0.57 ± 0.07*	0.42 ± 0.06*	0.36 ± 0.04*	1.07 ± 0.16	1.12 ± 0.25	1.16 ± 0.25	0.80 ± 0.17
igf2b	1.07 ± 0.22	0.78 ± 0.12	0.54 ± 0.31	0.34 ± 0.05*	1.08 ± 0.26	1.06 ± 0.21	0.96 ± 0.25	0.81 ± 0.10
igf1ra	1.01 ± 0.08	1.37 ± 0.10	1.42 ± 0.11	1.29 ± 0.13	1.07 ± 0.18	1.36 ± 0.20	1.32 ± 0.11	1.17 ± 0.23
igf1rb	1.01 ± 0.10	1.18 ± 0.26	1.70 ± 0.41	1.18 ± 0.08	1.07 ± 0.23	0.79 ± 0.15	0.84 ± 0.10	0.90 ± 0.32
igf2r	1.03 ± 0.10	1.33 ± 0.14	1.15 ± 0.15	0.89 ± 0.08	1.02 ± 0.09	1.04 ± 0.20	1.12 ± 0.09	0.94 ± 0.06
HPT axis								
Brain								
crh	1.02 ± 0.11	0.86 ± 0.05	0.88 ± 0.04	0.88 ± 0.11	1.07 ± 0.17	0.97 ± 0.38	0.76 ± 0.09	0.79 ± 0.12
Liver								
trα	1.01 ± 0.08	0.98 ± 0.11	0.90 ± 0.08	0.75 ± 0.08	1.01 ± 0.07	0.97 ± 0.11	0.97 ± 0.16	0.56 ± 0.14
trβ	1.01 ± 0.07	1.27 ± 0.11	1.19 ± 0.13	0.98 ± 0.13	1.02 ± 0.08	1.23 ± 0.17	1.17 ± 0.09	1.18 ± 0.09
ttr	1.03 ± 0.12	1.24 ± 0.13	0.79 ± 0.14	0.99 ± 0.08	1.02 ± 0.09	1.02 ± 0.23	0.76 ± 0.09	0.83 ± 0.08
sult1 st5	1.13 ± 0.25	0.78 ± 0.13	0.83 ± 0.21	0.71 ± 0.11	1.03 ± 0.10	0.88 ± 0.23	0.74 ± 0.24	0.88 ± 0.13

Values are calculated using $2^{-\triangle\triangle C_T}$ method and are the mean ± SE (n = 6). *$P < 0.05$.

greatest power for discrimination among exposures to precisely derive thresholds for effects by use of the point of departure. Results presented here are consistent with those of previous studies where zebrafish generally took 3 months to reach sexual maturity and allocate most of the ingested energy for non-reproductive growth purposes (Gómez-Requeni et al., 2010).

Exposure to environmentally relevant concentrations of TDCIPP caused a dose- and time-dependent growth inhibition in a female-biased manner. In this study, three environmentally relevant concentrations (50, 500 and 5000 ng/L) and 4 sampling time points were used to evaluate dose- and time-dependent effect of TDCIPP on individual growth of zebrafish. Previously, it had been reported that measured concentrations in exposure solutions were comparative with nominal concentrations when zebrafish were exposed to 50, 500 or 5000 ng TDCIPP/L, and thus TDCIPP concentrations in exposure solutions were not repeatedly measured in the present study (Zhu et al., 2015). In this study, a clear time- and dose-dependent inhibition of growth by TDCIPP was observed. Inhibition of the growth of individuals by TDCIPP has been reported in chicken (Farhat et al., 2013, 2014) and zebrafish embryos (Fu et al., 2013) exposed to relatively high concentrations and in *Daphnia magna* (Li et al., 2015) and *T. thermophile* (Li et al., 2016) exposed to environmentally relevant concentrations. Furthermore, Zhu et al. (2015) and Yu et al. (2017) reported that exposure to environmentally relevant concentrations of TDCIPP significantly decreased body length and body mass of female zebrafish, but no such effects were observed in males. These results are consistent with the findings of the study, results of which are presented here, although the underlying mechanisms of the gender-related differences in response to TDCIPP remains unknown. Gender-dependent effects were also observed in fish exposed to other chemicals. For example, long-term exposure to small concentrations of PBDEs resulted in greater accumulation into male zebrafish (Yu et al., 2011). Fish exposed to paper mill effluent exhibited significant differences in effects between genders, including: decreased growth, masculinized embryo sex ratio, decreased embryo or oocyte production, and development of a “phallus-like” morphology and reproductive behavior in females; hypermasculinized phallus morphology, increased testis mass, and demasculinized reproductive behavior in males (Burger et al., 2007). It has

Table 5
Effects of various concentrations of TDCIPP on relative expressions of genes involved in the GH/IGF and HPT axes of female and male zebrafish at 120 days post fertilization.

	Females				Males			
TDCIPP (ng/L)	0	50	500	5000	0	50	500	5000
GH/IGF axis								
Brain								
gh	1.10 ± 0.22	0.41 ± 0.08*	0.34 ± 0.18*	0.16 ± 0.05*	1.12 ± 0.30	1.38 ± 0.74	1.07 ± 0.56	1.27 ± 0.24
ghrh	1.02 ± 0.12	1.01 ± 0.36	1.03 ± 0.14	0.69 ± 0.14	1.05 ± 0.16	0.65 ± 0.14	0.64 ± 0.06	0.77 ± 0.09
Liver								
igf1	1.05 ± 0.16	0.58 ± 0.04*	0.47 ± 0.10*	0.35 ± 0.09*	1.04 ± 0.13	0.84 ± 0.15	0.70 ± 0.11	1.05 ± 0.24
igf2a	1.19 ± 0.34	1.25 ± 0.24	1.56 ± 0.35	0.90 ± 0.17	1.03 ± 0.11	1.26 ± 0.24	0.75 ± 0.07	1.21 ± 0.15
igf2b	1.04 ± 0.16	0.74 ± 0.25	0.52 ± 0.06	0.30 ± 0.06*	1.03 ± 0.10	1.00 ± 0.15	0.73 ± 0.04	0.79 ± 0.09
igf1ra	1.04 ± 0.14	1.22 ± 0.12	1.48 ± 0.12	1.40 ± 0.13	1.03 ± 0.12	1.04 ± 0.12	0.90 ± 0.05	0.98 ± 0.15
igf1rb	1.10 ± 0.27	1.79 ± 0.41	1.46 ± 0.29	1.58 ± 0.26	1.01 ± 0.07	0.96 ± 0.09	0.76 ± 0.09	0.75 ± 0.11
igf2r	1.01 ± 0.06	1.23 ± 0.10	1.33 ± 0.10	1.24 ± 0.06	1.06 ± 0.16	1.13 ± 0.11	0.82 ± 0.06	0.97 ± 0.14
HPT axis								
Brain								
crh	1.26 ± 0.49	0.85 ± 0.17	0.96 ± 0.10	1.20 ± 0.16	1.06 ± 0.17	1.04 ± 0.20	0.72 ± 0.23	0.77 ± 0.18
Liver								
trα	1.02 ± 0.08	0.78 ± 0.16	0.75 ± 0.10	0.70 ± 0.06	1.03 ± 0.13	0.79 ± 0.04	0.76 ± 0.08	0.63 ± 0.05
trβ	1.00 ± 0.04	1.01 ± 0.09	1.04 ± 0.07	0.84 ± 0.03	1.03 ± 0.10	0.90 ± 0.11	0.78 ± 0.04	0.68 ± 0.08
ttr	1.16 ± 0.30	1.68 ± 0.15	1.46 ± 0.21	1.15 ± 0.16	1.06 ± 0.15	1.30 ± 0.18	1.07 ± 0.22	1.53 ± 0.31
sult1 st5	1.13 ± 0.26	1.11 ± 0.31	1.59 ± 0.19	1.39 ± 0.14	1.06 ± 0.17	0.91 ± 0.09	0.73 ± 0.07	0.69 ± 0.10

Values are calculated using $2^{-\triangle\triangle C_T}$ method and are the mean ± SE (n = 6). *$P < 0.05$.

Table 6
Total concentrations of T4 and T3 in blood plasma of zebrafish after exposure to various concentrations of TDCIPP at 90 and 120 days post fertilization (dpf). Values represent the mean ± SE of three replicates, and each replicate included five fish.

	TH levels (ng/mL)	Sex	TDCIPP (ng/L)			
			0	50	500	5000
90 dpf	T3	female	1.51 ± 0.06	1.38 ± 0.08	1.32 ± 0.16	1.15 ± 0.13
		male	1.48 ± 0.07	1.37 ± 0.18	1.43 ± 0.17	1.33 ± 0.14
	T4	female	13.07 ± 1.77	14.19 ± 1.69	13.63 ± 0.75	12.54 ± 1.18
		male	12.16 ± 0.47	11.78 ± 0.38	12.43 ± 1.22	13.38 ± 0.62
120 dpf	T3	female	1.61 ± 0.08	1.44 ± 0.08	1.37 ± 0.20	1.24 ± 0.12
		male	1.50 ± 0.08	1.30 ± 0.07	1.27 ± 0.07	1.41 ± 0.11
	T4	female	14.33 ± 1.27	12.15 ± 1.88	13.10 ± 0.70	13.47 ± 1.72
		male	12.77 ± 1.22	14.40 ± 0.88	11.94 ± 2.17	11.91 ± 1.45

been reported that this phenomenon might be correlated with differences in uptake, distribution and metabolism, as well as nutritional requirements of the genders (Burger et al., 2007). In the present study, differences in effects on growth between the genders, appeared first at 90 dpf. Based on growth curves, between 60 and 90 dpf, the growth rate of female was greater than that of male's, which might result in more bioaccumulation of TDCIPP. This hypothesis was consistent with previous findings in zebrafish exposed to nominal concentrations of 50, 500 or 5000 ng TDCIPP/L (Zhu et al., 2015). Therefore, for this study, it was hypothesized that different rates of growth between males and females would lead to different bioaccumulation of TDCIPP, which might be one of the main reasons for observed gender-dependent effects of TDCIPP.

Down-regulation of expression of genes involved in the GH/IGF axis, but not the HPT axis, might be responsible for the observed growth inhibition of female zebrafish exposed to TDCIPP. In this study, a time- and dose-dependent study was conducted to evaluate effects of TDCIPP on expression of genes involved in the GH/IGF and HPT axes. In fish, growth is controlled in part by the GH/IGF axis. The hypothalamus produces antagonistic polypeptides, growth hormone-releasing hormone (GHRH) and growth hormone-inhibiting hormone, which stimulates the pituitary gland to produce GH (Moriyama et al., 2000). GH binds to its receptors in the target organs, mainly in the liver, and stimulates synthesis and release of IGFs. IGFs exerts on cells through binding to IGF receptors (Maures et al., 2002; Reinecke et al., 2005). The liver appears to be the primary site of production of IGFs (igf1, igf2a and igf2b) and their receptors (igf1ra, igf1rb and igf2r). Both IGF-1 and IGF-2 stimulate cellular responses mainly through IGF-1R. In contrast, the IGF-2R is primarily responsible for clearing IGF-2 (Duan, 1997; Le Roith et al., 2001). GH and IGFs are key mediators of somatic growth (Shepherd et al., 2007). GH participates in almost all major physiological processes in fishes, including carbohydrate, lipid and protein metabolism, soft tissue and skeletal growth, reproduction and immune function, as well as regulation of ionic and osmotic balances (Reinecke et al., 2005). The liver appears to be the primary site of production of IGF-1 (Duan, 1997). IGF-1 plays a central role in mediating somatic growth of fishes and concentrations of IGF-1 in blood plasma are positively correlated with growth in a variety of fishes (Wood et al., 2005). It has been reported that IGF-2 and IGF-1 potently activate DNA synthesis and proliferation of embryonic cells of zebrafish (Pozios et al., 2001). In addition, recombinant tilapia IGF-2 can stimulate proliferation in both teleost and mammalian cell lines (Hu et al., 2004). In the present study, *gh* in brain and *igf1*, *igf2b* in liver were significantly down-regulated in female zebrafish, which was accompanied by significantly lesser body mass and body length, which indicated that the GH/IGF signaling pathway might be a key target for effects of TDCIPP on growth of females. Besides GH and IGFs, Thyroid hormones also play an important role in maintenance of a normal physiological homeostasis of fishes. THs assist in control of activities of a variety of tissues and biological functions, such as somatic growth, development and metabolism, as well as post-hatching metamorphosis (Blanton and Specker, 2007). In the present study, exposure to environmentally relevant concentrations of TDCIPP affected neither concentrations of T3 or T4 in blood plasma nor expressions of genes

involved in the HPT axis throughout exposures. Therefore, the results of this study suggest that inhibitory effects of environmentally relevant concentrations of TDCIPP on growth might be caused by down-regulation of genes involved in the GH/IGF axis, but not the HPT axis in female zebrafish, although results of previous study has demonstrated that exposure of zebrafish to relatively high concentrations (4, 20, or 100 μg/L) of TDCIPP for 3 months significantly altered concentrations of T3 and T4 in blood plasma of female zebrafish (Wang et al., 2015b). Furthermore, changes in concentrations of thyroid hormones and expressions of genes involved in the HPT axis were also observed in rats exposed to 250 mg TDCIPP/kg/d, via oral gavage for 21 days (Zhao et al., 2016) and zebrafish embryos/larvae after acute exposure to 100 μg TDCIPP/L (Wang et al., 2013). Possible reasons for the discrepancy between results obtained in this study compared with previous studies (Wang et al., 2013; Zhao et al., 2016) might be that exposure concentrations used in previous studies were greater than those in this study. Therefore, results of the present study suggested that down-regulation of expressions of genes involved in the GH/IGF axis, but not the HPT axis, might be responsible for observed inhibition of growth of female zebrafish exposed to environmentally relevant concentrations of TDCIPP.

Exposure to environmentally relevant concentrations of TDCIPP caused a time-dependent change in the expression of genes of GH/IGF axis in females. Evaluation of time-dependent effects is equally important compared with the evaluation of dose-dependent effects when assessing environmental risks of chemicals. However, in most studies, dose-dependent effects of chemicals were examined (Li et al., 2015; Liu et al., 2016; Wang et al., 2015b; Yu et al., 2017), and time-dependent effects were usually ignored. In this study, time-dependent effects on expressions of genes involved in the GH/IGF axis of female zebrafish exposed to TDCIPP were examined. Treatment with TDCIPP significantly down-regulated expressions of *gh*, *igf1* and *igf2b* in a time-dependent manner, which was comparative with results of a previous study (Zhu et al., 2015). It should also be noted that expression of *igf2a* was down-regulated at 90 dpf in females, but it was unchanged at 120 dpf. Differences in responses of *igf2a* between 90 and 120 dpf might be due to life-stage-dependent sensitivity of zebrafish to the chemical (Maack and Segner, 2004) and the reversibility of the chemical binding at the biological target site as well as the ability to recover (Jager et al., 2011). Those results indicated that the earliest and most sensitive responses to exposure to TDCIPP on growth of zebrafish was 60–90 dpf, which was the period of most rapid growth. Therefore, 90 dpf is the optimum sampling point for toxicological studies of TDCIPP developmental toxicity.

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