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Environmentally Relevant Concentrations of the Flame Retardant Tris(1,3-dichloro-2-propyl) Phosphate Inhibit Growth of Female Zebrafish and Decrease Fecundity

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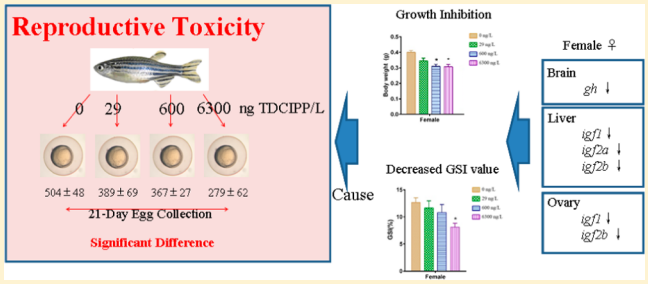
Supporting Information

ABSTRACT: Bioconcentrations of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) in brain, gonad, and liver as well as effects on fecundity and development of zebrafish (*Danio rerio*) were determined. Zebrafish (1-month old) were exposed to environmentally relevant concentrations of 29 ± 2.1, 600 ± 21, or 6300 ± 130 ng TDCIPP/L. After 120 days of exposure, TDCIPP accumulated in the brain, gonad, and liver with bioconcentration factors of 460, 38, and 87 in females and 26, 55, and 110 in males, respectively. TDCIPP accumulated to a greater extent in brains of females than those of males. Exposure to 6300 ± 130 ng TDCIPP/L resulted in significantly ($P < 0.05$) fewer eggs being produced, but the histology of the gonad, plasma concentrations of estradiol and 11-ketotestosterone, and expression of genes involved in hypothalamic−pituitary−gonadal−liver axis were not significantly ($P > 0.05$) different between individuals exposed to TDCIPP and the unexposed control fish. Exposure to TDCIPP resulted in shorter body length, lighter body mass, and lower gonadal−somatic index in females. These effects were possibly due to down-regulation of expression of genes along the growth hormone/insulin-like growth factor (GH/IGF) axis. Correlations between the production of eggs and developmental parameters or expression of genes along the GH/IGF axis further suggested that environmentally relevant concentrations of TDCIPP could have adverse effects on reproduction, possibly due to the inhibition of the growth of females.



INTRODUCTION

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is a synthetic organophosphate flame retardant (FR) and plasticizer that has been used for decades in various products, such as plastics, foams, textiles, varnishes, electronics equipment, and furniture.[1] It is estimated that the annual production of TDCIPP in the United States in the years of 1998, 2002, and 2006 ranged from 4500 to 22 700 tonnes.[1] Recently, because the use of brominated FRs are generally being voluntarily reduced and phased out or banned, the consumption of organophosphate FRs such as TDCIPP has been increasing.[1,2]

TDCIPP is an additive FR that is not chemically bonded to polymeric products, whereas reactive FRs are chemically bonded into products. Therefore, TDCIPP is more likely to be released into the environment, as evidenced by its frequent detection in aquatic environmental compartments.[1,3−14] For example, 2.5−40 ng TDCIPP/L was reported in waters of the Songhua River, China.[5,6] In seawater along the coast of China near the cities of Qingdao and Xiamen, concentrations of TDCIPP ranged from 24 to 377 ng/L.[7] In effluents from sewage treatment plants in Sweden, concentrations as great as 3

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μg TDCIPP/L have been reported.[13] TDCIPP has also been detected in various aquatic species of fish. Yellow perch (*Perca fluviatilis*) from Djupasjön Lake in Sweden contained concentrations as great as 140 ng of TDCIPP/g of lipid weight (lw).[8] Catfish (*Clarius fuscus*) and grass carp (*Cyprinus idellus*) from the Pearl River, China, were reported to have concentrations of TDCIPP as great as 251 ng TDCIPP/g lw.[15] These reported concentrations of TDCIPP in aquatic organisms were at least 10−100-fold greater than concentrations in water collected from the same areas, which indicates the substantial bioconcentration/bioaccumulation potential of TDCIPP in aquatic systems.

Limited toxicological information suggests that exposure to TDCIPP has the potential to disrupt the endocrine system[16−21] and neurological,[22−25] developmental, and reproductive functions,[26−33] depending on the species tested. For example, a high concentration of TDCIPP was shown to delay remethylation in zygotes and inhibit embryonic epiboly in embryos of zebrafish, as well as decreased lengths of head and bill, masses, and size of gallbladder in chicken embryos.[29] Acute exposure to TDCIPP altered expression of steroidogenic genes and production of testosterone (T) and 17β-estradiol (E2) in exposed H295R cells and zebrafish, and fewer eggs were produced by zebrafish.[32] Exposure of zebrafish to relatively small concentrations (4, 20, or 100 μg TDCIPP/L) for 6 months significantly decreased body mass, body length, and condition factor (K); affected transcription of genes involved in the hypothalamic−pituitary−gonadal−liver (HPGL) axis; increased concentrations of T and E2 in blood plasma; and resulted in fewer eggs being produced.[17] Collectively, these studies suggested that exposure to TDCIPP could cause adverse effects on development and reproduction of zebrafish.[17,29,32] However, concentrations to which fish were exposed in these studies were greater than environmentally relevant concentrations ever reported (≤377 ng/L), and thus could not provide reliable thresholds for effects that could be used in assessments of risk. Recently, using *Daphnia magna* as a model, it was found that environmentally relevant concentrations of TDCIPP significantly decreased fecundity as well as the length of F_0 and F_1 generations, and development of F_1 individuals was found to be a more sensitive end point than was fecundity of F_0 individuals.[34] Alterations of expression of genes related to the synthesis of proteins and metabolism in endocytosis pathways might be responsible for TDCIPP-induced effects on reproduction and development of *D. magna*.[34] These data for *D. magna* suggested a need for further studies to investigate the potential toxicological effects due to exposure of other aquatic organisms to environmentally relevant concentrations of TDCIPP, such as fish.

In the present study, bioaccumulation of TDCIPP was evaluated, and effects on development and reproduction were examined, after chronic, water-borne exposures of zebrafish to environmentally relevant concentrations of TDCIPP.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals and reagents were purchased from the following sources: TDCIPP for zebrafish aquatic exposure was from Sigma-Aldrich (St. Louis, MO), TRIzol reagent and reverse transcription and SYBR Green kits were from Takara (Dalian, Liaoning, China), hormone detection kits were from Cayman Chemical Co. (Ann Arbor, MI), MS-222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt) was from Sigma-Aldrich, TDCIPP for use as an analytical standard was from Tokyo Chemical Industry America (Portland, OR), and TDCIPP-d_{15} was purchased from Dr. Vladimir Below via Organic Contaminants Research Laboratory (OCRL), NWRC (Ottawa, Canada). All the reagents used in this study were of analytical grade.

Zebrafish Maintenance and TDCIPP Exposure Protocols. Stock solutions of TDCIPP were prepared in dimethyl sulfoxide (DMSO). Zebrafish were maintained in flow-through tanks, according to previously published methods.[35] One-month-old zebrafish were acclimated in 15-L glass tanks for 1 week and then exposed to 0, 50, 500, or 5000 ng TDCIPP/L for 4 months. Twenty fish were exposed in each of three replicate tanks for each concentration. During semistatic exposure, solutions were replaced daily with fresh carbon-filtered water containing corresponding concentrations of TDCIPP. Exposure solutions were sampled at the last day of exposure, and concentrations of TDCIPP and bis(1,3-dichloro-2-propyl) phosphate (BDCIPP) were quantified. Both control and treated groups received 0.001% DMSO since previous study demonstrated that no significant effects on development and reproduction were observed in zebrafish when DMSO concentrations were ≤0.01%.[36]

To examine the effects of TDCIPP on reproduction, fish were spawned in groups during the last 21 days of exposure. For each concentration, groups containing four females and four males from each replicate (n = 3) were grouped and spawned after lights on in the morning, and the number of eggs spawned was recorded daily. Fecundity was reported as cumulative eggs per female in the last 21 days of exposure.

After exposure, fish were euthanized with MS-222, and body mass (g) and snout-to-vent length (mm) were recorded. Blood was collected for quantification of 11-ketotestosterone (11-KT) (for males) and E2 (for females), and gonads were sampled and weighed for determination of the gonadal−somatic index (GSI) and then fixed in Bouin's solution for subsequent imbedding and histological examination. Brain, gonad, and liver samples were collected for quantification of TDCIPP or BDCIPP and real-time PCR reactions.

Quantification of TDCIPP and BDCIPP in Exposure Solutions and Fish Tissues. Both TDCIPP and BDCIPP were quantified in water and zebrafish samples. Detailed protocols used for identification and quantification of TDCIPP in water or tissues have been published previously[11,37,38] and are also provided in the Supporting Information (SI). An internal calibration method was used for quantification of TDCIPP and BDCIPP in zebrafish tissue samples.

During quantification of TDCIPP and BDCIPP in water, 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. In tissue samples, there was unavoidable background contamination, with an average of 0.29 ± 0.12 ng TDCIPP/sample, consistently observed in all control fish samples. Thus, the MLOQ for TDCIPP was background-corrected for fish tissues and was calculated to be 0.36 ng/sample, which was three times the standard derivation of measurements in all control fish samples. On the basis of its internal standard, TDCIPP-d_{15}, the mean recovery of TDCIPP for all samples of tissues was 99 ± 12%. Like TDCIPP, unavoidable background contamination (0.2 ± 0.05 ng/sample) was also observed for BDCIPP in analyzed blank

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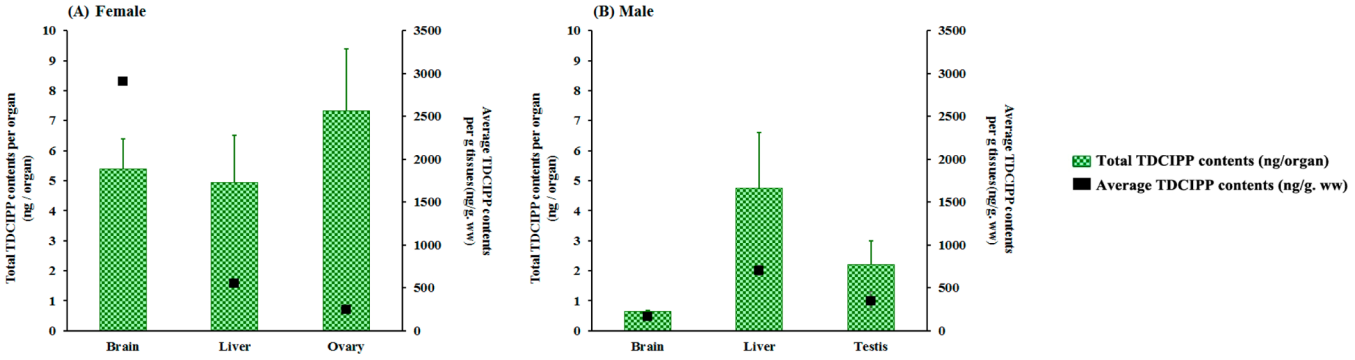

Figure 1. Total and average concentrations of TDCIPP in brain, gonad, and liver of female (A) and male (B) zebrafish after exposure to 6300 ± 130 ng/L for 120 days. Values represent mean ± SE (n = 3).

samples. The MLOQ of BDCIPP in fish tissues was 0.19 ng BDCIPP/sample, and its mean recovery for all analyzed samples was 85 ± 17%.

Histological Examination. Histological examination of gonads was performed according to previously published methods.[39] First, gonadal tissues were dehydrated in 70% ethanol for 15 min, 80% ethanol for 15 min, 90% ethanol for 15 min, 95% ethanol for 30 min, and 100% ethanol for 40 min. Second, tissues were hyalinized in a mixture of xylene and ethanol (v/v, 1:1) for 8 min and 100% xylene for 8 min. Third, tissues were immersed in paraffix wax for 60 min at 58 °C and then were embedded. Lastly, tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Stages of oogenesis and spermatogenesis were identified and quantified using previously reported methods.[40] Six fish for each sex and treatment group were included in the assay, and a total of three sections were collected from each fish.

Quantification of Hormone Concentrations. Quantification of concentrations of 11-KT and E2 in blood plasma of males and females, respectively, was performed following previous methods using commercial kits from Cayman Chemical Co.[35,39] In brief, blood from each fish was centrifuged at 5000g for 5 min at 4 °C, and then plasma from two fish was collected and pooled to form a sample. Plasma from each pooled sample was diluted with ultrapure water and extracted with ethyl ether, and then evaporation was performed. EIA buffer provided in the kits was used to redissolve residues, and concentrations of hormones were measured according to the manufacturer's instructions. Limits of quantification for 11-KT and E2 were 1.3 and 19 pg/mL, respectively. Three biological replicates were used in this assay. To compare effects of TDCIPP on expression of genes and production of hormones, concentrations of 11-KT and E2 in plasma were expressed as fold-change relative to control.

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.[35,41] In brief, TRIzol reagent was used for the isolation of total RNA. Purity and total concentrations of RNA were measured using the Epoch microplate spectrophotometer (Bio Tek Instruments, Inc.). Reverse transcription and qRT-PCR were performed using Prime ScriptTM RT reagent kits and SYBR Green kits, respectively, from Takara (Dalian, China). Sequences of primers were designed by use of Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (see Table S1, SI). Expression of the housekeeping gene *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 gene expression to DMSO control was calculated using the $2^{-\Delta\Delta C_T}$ method, and for down-regulated gene, fold change was calculated using the formula "$-1/2^{-\Delta\Delta C_T}$ value" according to the previous protocol.[42] In this study, we selected differentially expressed genes based on fold change >1.5 and P value <0.05. There were three replicated tanks for each concentration, and two fish from each tank were used; thus, in total six fish were analyzed in each treatment.

Statistical Analyses. All statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL). The normality and homogeneity of data were evaluated by Kolmogorov–Smirnow and Levene's tests, respectively. If necessary, data were log-transformed to approximate normality. One-way analysis of variance (ANOVA) and Tukey's multiple range test were used to determine significant differences between the control and TDCIPP treatment groups. Spearman correlation analyses with Bonferroni correction were used to examine the relationship between the number of eggs produced and developmental parameters or gene expression. Significant differences between treatments and corresponding control were identified by P-value < 0.05.

RESULTS

Concentrations of TDCIPP/BDCIPP in Exposure Solutions. Nominal concentrations of TDCIPP in exposure solutions were 50, 500, and 5000 ng TDCIPP/L, while measured concentrations of TDCIPP in these same three solutions were 29 ± 2.1, 600 ± 21, and 6300 ± 130 ng/L, respectively. No TDCIPP was detected in control solutions. BDCIPP was detectable in the solution containing the greatest concentration of TDCIPP, but accurate quantification was not possible due to the extremely low instrumental response (<MLOQ). BDCIPP was not detected in controls or TDCIPP standard; thus, the background BDCIPP contribution from the TDCIPP dose is not of concern.

Concentrations of TDCIPP/BDCIPP in Fishes. Concentrations of TDCIPP in brain, ovary, and liver of female zebrafish were 2900 ± 890, 2400 ± 33, and 550 ± 26 ng/g wet mass (ww), respectively, and the total mass of TDCIPP in the same organs were 5.4 ± 1.0, 7.3 ± 2.1, and 4.9 ± 1.6 ng, respectively (Figure 1A). In male zebrafish, concentrations of TDCIPP in brain, testis, and liver were 170 ± 15, 350 ± 11, and 700 ± 57

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ng/g ww, respectively, and the TDCIPP burdens in the same organs were 0.63 ± 0.06, 2.2 ± 0.8, and 4.8 ± 1.9 ng, respectively (Figure 1B). A statistically significant difference in accumulation of TDCIPP between females and males was observed in brain, but no significant effects were observed in liver and gonad. Concentrations of BDCIPP determined in homogenates of zebrafish collected from the group exposed to the greatest concentrations of BDCIPP were consistently less than its MLOQ.

Exposure to TDCIPP Decreased Cumulative Production of Eggs. TDCIPP caused a dose-dependent reduction in cumulative production of eggs after commencement of the 21 d of exposure (Figure 2). Exposure to 29 ± 2.1 or 600 ± 21 ng

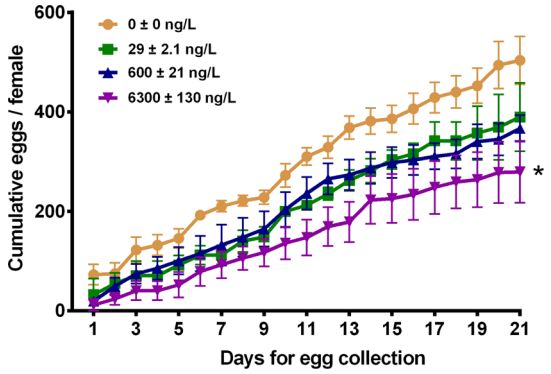

Figure 2. Cumulative fecundity in zebrafish exposed to various concentrations of TDCIPP. Data represent the mean cumulative number of eggs per female collected from three replicate tanks, each containing four pairs of fish. The asterisk indicates a value significantly different ($P < 0.05$) from that of the control group.

TDCIPP/L resulted in slightly fewer eggs produced than by the controls, but the effect was not statistically significant. Exposure to 6300 ± 130 ng TDCIPP/L resulted in significantly (44.6%) fewer eggs being produced relative to the unexposed controls (Figure 2).

TDCIPP Caused Female-Biased Growth Inhibition. Body mass and fork length (snout-to-vent) of the body and GSI were measured to evaluate effects of TDCIPP on growth and development of gonads. TDCIPP caused inhibition of growth of females (Figure 3). In females, exposure to 600 ± 21 or 6300 ± 130 ng TDCIPP/L resulted in significantly shorter individuals with smaller mass of bodies, whereas treatment with the least concentration (29 ± 2.1 ng TDCIPP/L) did not cause such effects (Figure 3A,B). GSI values were significantly less than that of controls only for fish exposed to the greatest concentration (6300 ± 130 ng/L), while no significant effect was observed in zebrafish exposed to the other concentrations (29 ± 2.1 or 600 ± 21 ng TDCIPP/L) (Figure 3C). In males, mass, length of individuals, and GSI values were not affected by exposure to any concentration of TDCIPP (Figure 3A–C).

TDCIPP Did Not Change Gonad Histology and Concentrations of 11-KT and E2. Relative proportions of oocytes and sperm were not affected by exposure to TDCIPP. Percentages of primary, cortical alveolar, early vitellogenic, and late/mature oocytes in control females were 70 ± 5.3, 7.4 ± 2.7, 15 ± 3.6, and 6.4 ± 1.0%, respectively (Figure S1A, SI). Exposure to each of the three concentrations tested did not affect percentages of these oocytes in ovaries (Figure S1A, SI). Percentages of spermatogonia, spermatocytes, and spermatids in males of the control group were 12 ± 0.6, 51 ± 2.6, and 38 ±

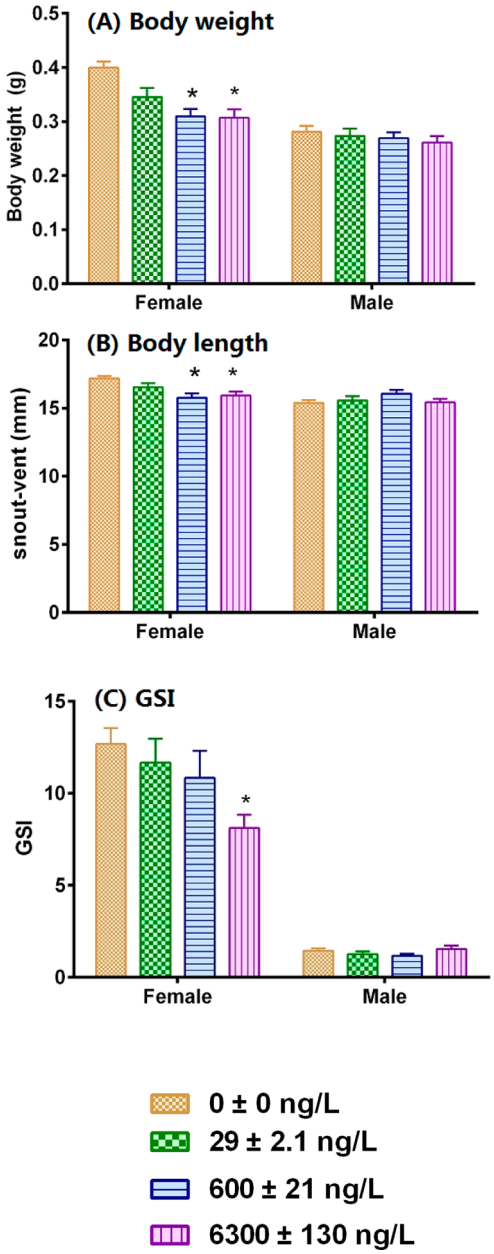

Figure 3. Effects on body weight (A), body length (B), and gonadal–somatic index (GSI) (C) in zebrafish exposed to various concentrations of TDCIPP for 120 days. Value represent mean ± SE ($n = 15$). *$P < 0.05$.

2.8%, respectively (Figure S1B, SI). Treatment with each of the three concentrations tested did not affect percentages of spermatogonia, spermatocytes, or spermatids in the testis (Figure S1B, SI).

The mean concentration of E2 in blood plasma of females was 1100 pg/mL, and concentration of 11-KT in plasma of males was 1300 pg/L in the control group (Figure S2, SI). There was not significant change in the concentrations of the two hormones in any of the treatments (Figure S2, SI).

Expressions of Genes Involved in HPGL Axis Were Not Altered after TDCIPP Exposure. Expression of genes involved in the HPGL axis, including *fshβ* and *lhβ* in brain; *cyp19a*, *activin-βa2*, and *3βhsd* in gonad; and *vtg1* in liver of females and males, were measured after exposure to TDCIPP. No statistically significant effects on transcription of these genes

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Table 1. Effects of Different Concentrations of TDCIPP (ng/L) on the Relative Expression of Genes Involved in the GH/IGF Axis of Female and Male Zebrafish[a]

	Females				Males			
	0 ± 0	29 ± 2.1	600 ± 21	6300 ± 130	0 ± 0	29 ± 2.1	600 ± 21	6300 ± 130
				Brain				
ghrh	0.99 ± 0.42	0.94 ± 0.32	1.03 ± 0.32	1.58 ± 0.09	1.60 ± 0.58	1.37 ± 0.57	1.51 ± 0.35	0.55 ± 0.21
gh	1.11 ± 0.28	0.46 ± 0.11[b]	0.32 ± 0.08[b]	0.23 ± 0.10[b]	1.04 ± 0.16	0.42 ± 0.04[b]	0.37 ± 0.07[b]	0.32 ± 0.06[b]
				Liver				
igf1	1.03 ± 0.11	0.13 ± 0.04[b]	0.15 ± 0.03[b]	0.26 ± 0.04[b]	1.01 ± 0.07	0.35 ± 0.05[b]	0.24 ± 0.03[b]	0.49 ± 0.06[b]
igf2a	1.02 ± 0.12	0.45 ± 0.09[b]	0.36 ± 0.09[b]	0.23 ± 0.05[b]	1.04 ± 0.15	0.84 ± 0.28	0.71 ± 0.18	1.67 ± 0.37
igf2b	1.01 ± 0.09	0.25 ± 0.05[b]	0.29 ± 0.05[b]	0.35 ± 0.04[b]	1.03 ± 0.16	0.81 ± 0.21	0.51 ± 0.13	1.03 ± 0.17
				Gonad				
igf1	1.06 ± 0.22	0.64 ± 0.07[b]	0.50 ± 0.16[b]	0.38 ± 0.08[b]	1.08 ± 0.19	1.82 ± 0.32	1.67 ± 0.26	1.58 ± 0.39
igf2a	1.01 ± 0.09	0.66 ± 0.09	0.73 ± 0.10	0.75 ± 0.08	1.02 ± 0.10	1.08 ± 0.15	1.12 ± 0.21	1.27 ± 0.42
igf2b	1.03 ± 0.12	0.73 ± 0.07	0.68 ± 0.08	0.45 ± 0.06[b]	1.01 ± 0.08	1.04 ± 0.12	1.20 ± 0.25	1.15 ± 0.22

[a]Values are calculated using the $2^{-\Delta\Delta C_T}$ method and represent the mean ± SE ($n = 6$). [b]$P < 0.05$.

Table 2. Spearman Rank Correlation Coefficients and Probabilities (see footnotes) between Production of Eggs and Developmental Parameters and Expressions of Genes in Ovary of Female Zebrafish

	developmental parameters			genes		
	GSI	snout−vent	body weight	*Igf1*	*Igf2a*	*Igf2b*
cumulative production of eggs	0.834[b]	0.648[b]	0.862[b]	0.752[b]	0.721[b]	0.500[a]

[a]Correlation is significant at the 0.05 level (two-tailed). [b]Correlation is significant at the 0.01 level (two-tailed).

were observed in males and females exposed to any of the treatments (Table S2, SI).

TDCIPP Altered Expressions of Genes Involved in GH/IGF Axis in Female-Biased Manner. Expression of genes involved in the GH/IGF axis, including *ghrh* and *gh* in brain and *igf1*, *igf2a*, and *igf2b* in gonad and liver, were examined in females and males after exposure to TDCIPP (Table 1). In males, only expressions of *gh* in brain and *igf1* in liver were significantly down-regulated after exposure to low (29 ± 2.1 ng/L), medium (600 ± 21 ng/L), or high (6300 ± 130 ng/L) concentrations of TDCIPP, while transcriptions of the other genes were not significantly changed compared with the solvent control. However, expressions of most of the genes examined were altered in females exposed to TDCIPP. In the brain of females, exposure to low, medium, or high TDCIPP concentrations significantly down-regulated expression of *gh* by 2.2-, 3.1-, and 4.3-fold, respectively, while expressions of *ghrh* were not affected. In ovary, expressions of *igf1* and *igf2b* were significantly down-regulated, while expression of *igf2a* was not altered. Expressions of *igf1* were down-regulated by 1.6-, 2.0-, and 2.6-fold in individuals exposed to low, medium, or high TDCIPP concentrations, respectively. Expression of *igf2b* was down-regulated only in fish exposed to high TDCIPP concentration, with a 2.2-fold down-regulation. In liver, expressions of *igf1*, *igf2a*, and *igf2b* were strongly down-regulated in all the exposure groups. Expressions of *igf1* were down-regulated by 7.7-, 6.7-, and 3.8-fold in fish exposed to low, medium, or high TDCIPP concentrations, respectively. Expressions of *igf2a* were down-regulated by 2.2-, 2.8-, and 4.3-fold relative to controls in fish exposed to low, medium, or high TDCIPP concentrations, respectively. Expressions of *igf2b* were down-regulated by 4.0-, 3.4-, and 2.9-fold in individuals exposed to low, medium, or high TDCIPP concentrations, respectively.

Correlations between the Number of Eggs Produced and Developmental Parameters and Expression of Genes in Ovary. Correlations between the number of eggs produced and GSI, body length, body mass, or expression of genes in ovary were examined (Table 2). The number of eggs produced was significantly correlated with GSI, body length, and body mass. The number of eggs produced was also significantly correlated with expressions of *igf1*, *igf2a*, and *igf2b* in ovary of fish exposed to various concentrations of TDCIPP, while no significant correlations were observed between the number of eggs produced and expressions of genes in the HPGL axis (data not shown).

DISCUSSION

On the basis of results of previous in vitro or in vivo studies, BDCIPP is generally regarded as a primary metabolite of TDCIPP.[43−45] In the present study, concentrations of BDCIPP in zebrafish or their exposure solutions were consistently less than the MLOQs. These results are in a good agreement with previously published results,[43] in which concentrations of BDCIPP in whole zebrafish embryos were generally 100-fold less than those of its parent TDCIPP. The fact that BDCIPP is barely detectable in both zebrafish and exposure solutions suggests (1) the low depletion rate of TDCIPP in the zebrafish exposure system and/or (2) little bioconcentration of BDCIPP in tissues of zebrafish. In fact, while TDCIPP or triphenyl phosphate (TPHP, another OPFR) was incubated with hepatocytes of embryos of chicken,[45−47] the formed diester metabolites [i.e., BDCIPP or diphenyl phosphate (DPHP)] are less prone to accumulate into cells than parent TDCIPP or TPHP. Mean BCFs (TDCIPP concentration ratios between tissue and water) for brain, gonad, and liver were 460, 38, and 87 in females, respectively, and 26, 55, and 110 in males, respectively, which suggests bioconcentration of TDCIPP into all three investigated organs of zebrafish. Observed BCFs of TDCIPP among the three organs might be explained by various factors, including lipid content, metabolic capacity, or even differences in nutritional needs among the organs.[48,49] The brain tissue of female zebrafish contained significantly greater

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concentrations of TDCIPP than did brains of males. This might explain greater effects of TDCIPP observed in females rather than males, since gh is an important upstream regulator of the GH/IGF axis and its transcription in brain was more strongly down-regulated in females than that in males after exposure to TDCIPP.

Exposure to environmentally relevant concentrations of TDCIPP resulted in lesser production of eggs than in unexposed controls, but it did not affect histology of the gonads, plasma concentrations of 11-KT and E2, or expression of genes involved in the HPGL axis. The reproductive system is controlled primarily by the HPGL axis, and this system is responsible for regulating hormone dynamics by coordinating hormone synthesis, secretion, transport, and metabolism.[50] Results of previous studies have demonstrated that exposure of zebrafish to relatively great concentrations (0.04, 0.2, or 1 mg/L) of TDCIPP for 21 days significantly decreased cumulative production of eggs and caused alterations in expression of genes involved in the HPGL axis. Concentrations of plasma testosterone and E2 were also altered.[33] Similarly, results of another study demonstrated that chronic exposure of zebrafish to relatively small concentrations of TDCIPP (4, 20, or 100 μg/L) resulted in significant accumulation of TDCIPP and BDCIPP in gonads, altered expression of genes involved in the HPGL axis, altered histology of ovary, increased plasma concentrations of testosterone and E2 in females, and decreased production of eggs.[17] Results of those two studies suggest that TDCIPP can affect the HPGL axis and thus decrease the fecundity of zebrafish. However, effects of environmentally relevant concentrations of TDCIPP on fecundity and HPGL axis remained unknown. To support reliable risk assessment, experiments studying chronic and environmentally relevant concentrations of exposure were performed. Unexpectedly, exposure of adult zebrafish to 6300 ± 130 ng TDCIPP/L resulted in a lesser cumulative production of eggs, relative to that of controls. However, other biological parameters studied, such as gonad histology, plasma concentrations of 11-KT and E2, and expression of genes of the HPGL axis, were not affected. Results of the study presented here suggest that other metabolisms might be involved in the reproductive toxicity caused by exposure to environmentally relevant concentrations of TDCIPP, and these mechanisms (possibilities) are discussed below.

TDCIPP caused inhibition of growth of female zebrafish relative to that of controls, possibly by altering expression of genes involved in the GH/IGF axis. Inhibition of growth by TDCIPP has been observed in chicken, zebrafish, and a unicellular flagellate (*Tetrahymena thermophile*).[17,27,30,51] However, concentrations used in these studies were greater than those observed in aquatic environments. Recently, using *D. magna* as a model, it was found that environmentally relevant concentrations of TDCIPP significantly decreased body lengths of F_0 and F_1 generations,[34] which suggested the need for further studies to evaluate the risk of exposure for other aquatic organisms, such as fish, to environmentally relevant TDCIPP concentrations. In this study, to fill this gap, a chronic exposure of zebrafish was performed. Environmentally relevant concentrations of TDCIPP caused a female-biased growth inhibition, which was consistent with observations in a previous study, although exposure concentrations used were greater than environmental concentrations.[17] Furthermore, expressions of genes involved in the GH/IGF axis were examined in this study to explore possible mechanisms of actions of TDCIPP. In fish, growth is mainly controlled by the GH/IGF axis, where factors such as gh and igf are included in the axis and cooperate with each other to regulate fish organ development.[52,53] In this study, for the first time, we found that TDCIPP caused a female-biased down-regulation of GH/IGF genes in brain, gonad, and liver, which may be responsible for the observed female-biased inhibitory effects on GSI, body length, and body weight. Transcriptional responses of several GH/IGF genes (e.g., *igf1*, *igf2b*, etc.) to TDCIPP did not follow a concentration−response relationship, and the reasons might be that exposure to relatively great concentrations of TDCIPP (e.g., 600 ± 21 and 6300 ± 130 ng/L) caused possible feedback responses that in turn partly up-regulated the expressions of the several GH/IGF genes.

Inhibition of growth caused by exposure to TDCIPP might be responsible for the decreased production of eggs. Besides the HPGL axis and steroid hormones, in fish reproduction is also regulated by hormones of the GH/IGF axis.[52] For example, some factors, such as igfs, can affect gonad development and modulate reproduction of fishes.[53] In this study, the results of which are presented here, a lower GSI was observed in female zebrafish after exposure to TDCIPP, which suggested disruption of ovary development. GSI is usually used as a parameter in toxicological studies, and studies have demonstrated that in fishes, GSI is correlated with the number of eggs produced.[54] Therefore, it was postulated that TDCIPP-induced reproductive toxicity might be caused by inhibition of development. To test the hypothesis, correlations between production of eggs and developmental parameters were examined. The relatively large correlations between production of eggs and GSI and expression of genes involved in the GH/IGF axis confirmed that hypothesis. Recently, the concept of adverse outcome pathway (AOP) has been rapidly accepted worldwide in risk assessments of chemicals.[55−59] An AOP is a linear pathway, which includes a molecular initiating event (MIE), key events (KE), and an adverse outcome (AO).[56] These factors (e.g., MIE, KE, and AO) are causally linked together; therefore, a pathway-based analysis of chemical effects would allow an application of nontraditional approaches for understanding the risks of chemical exposure.[56] In this study, exposure to TDCIPP did not significantly change expression of genes included in the HPG axis, production of steroid hormones, and histology of gonads in zebrafish, but fecundity was significantly reduced. Furthermore, inhibition of growth caused by TDCIPP exposure might be responsible for the decreased production of eggs, and down-regulations of genes included in the GH/IGF axis were considered as possible toxic mechanisms. Collectively, the data suggest that inhibition of individual growth by down-regulating expression of genes included in the GH/IGF axis could cause reduced fecundity due to chemical exposure, and this information might be valuable for risk assessments of chemicals according to the AOP concept and support a paradigm shift in environmental toxicology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b03849.

Protocols for analysis of TDCIPP and BDCIPP in exposure solutions and in zebrafish organs, sequences of primers for selected genes (Table S1), effects of TDCIPP

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on expression of genes involved in the HPGL axis (Table S2), histological examination of gonad development after TDCIPP exposure (Figure S1), and effects on plasma E2 in females and 11-KT in males after TDCIPP exposure (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) van der Veen, I.; de Boer, J. Phosphorus flame retardants: Properties, production, environmental occurrence, toxicity and analysis. *Chemosphere* **2012**, *88* (10), 1119−1153.

(2) Ceresana. *Market Study: Flame Retardants*, 3rd ed.; Ceresana: Constance, Germany, 2015; available at http://www.ceresana.com/en/market-studies/additives/flame-retardants/ceresana-market-study-flame-retardants.html.

(3) Andresen, J. A.; Grundmann, A.; Bester, K. Organophosphorus flame retardants and plasticisers in surface waters. *Sci. Total Environ.* **2004**, *332* (1−3), 155−166.

(4) Bacaloni, A.; Cavaliere, C.; Foglia, P.; Nazzari, M.; Samperi, R.; Laganà, A. Liquid chromatography/tandem mass spectrometry determination of organophosphorus flame retardants and plasticizers in drinking and surface waters. *Rapid Commun. Mass Spectrom.* **2007**, *21* (7), 1123−1130.

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

(6) Wang, X.; Liu, J.; Yin, Y. 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* **2011**, *1218* (38), 6705−6711.

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

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

(9) Carignan, C. C.; McClean, M. D.; Cooper, E. M.; Watkins, D. J.; Fraser, A. J.; Heiger-Bernays, W.; Stapleton, H. M.; Webster, T. F. Predictors of tris(1,3-dichloro-2-propyl) phosphate metabolite in the urine of office workers. *Environ. Int.* **2013**, *55* (4), 56−61.

(10) Meeker, J. D.; Cooper, E. M.; Stapleton, H. M.; Hauser, R. Urinary Metabolites of Organophosphate Flame Retardants: Temporal Variability and Correlations with House Dust Concentrations. *Environ. Health Persp.* **2013**, *121* (5), 580−585.

(11) Chen, D.; Letcher, R. J.; Chu, S. Determination of non-halogenated, chlorinated and brominated organophosphate flame retardants in herring gull eggs based on liquid chromatography−tandem quadrupole mass spectrometry. *J. Chromatogr. A* **2012**, *1220* (2), 169−174.

(12) Hartmann, P. C.; Bürgi, D.; Giger, W. Organophosphate flame retardants and plasticizers in indoor air. *Chemosphere* **2004**, *57* (8), 781−787.

(13) Marklund, A.; Andersson, B.; Haglund, P. Organophosphorus flame retardants and plasticizers in air from various indoor environments. *J. Environ. Monit.* **2005**, *7* (8), 814.

(14) Stackelberg, P. E.; Furlong, E. T.; Meyer, M. T.; Zaugg, S. D.; Henderson, A. K.; Reissman, D. B. Response to Comment on "Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *Sci. Total Environ.* **2006**, *354* (1), 93−97.

(15) Ma, Y.; Cui, K.; Zeng, F.; Wen, J.; Liu, H.; Zhu, F.; Ouyang, G.; Luan, T.; Zeng, Z. Microwave-assisted extraction combined with gel permeation chromatography and silica gel cleanup followed by gas chromatography−mass spectrometry for the determination of organophosphorus flame retardants and plasticizers in biological samples. *Anal. Chim. Acta* **2013**, *786* (13), 47−53.

(16) Zhang, Q.; Lu, M.; Dong, X.; Wang, C.; Zhang, C.; Liu, W.; Zhao, M. Potential Estrogenic Effects of Phosphorus-Containing Flame Retardants. *Environ. Sci. Technol.* **2014**, *48* (12), 6995−7001.

(17) Wang, Q.; Lam, J. C. W.; Han, J.; Wang, X.; Guo, Y.; Lam, P. K. S.; Zhou, B. 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.* **2015**, *160*, 163−171.

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

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

(20) Crump, D.; Chiu, S.; Kennedy, S. W. 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.* **2012**, *126* (1), 140−148.

(21) Farhat, A.; Crump, D.; Porter, E.; Chiu, S.; Letcher, R. J.; Su, G.; Kennedy, S. W. Time-dependent effects of the flame retardant tris(1,3-dichloro-2-propyl) phosphate (TDCPP) on mRNA expression, *in vitro* and *in ovo*, reveal optimal sampling times for rapidly metabolized compounds. *Environ. Toxicol. Chem.* **2014**, *33* (12), 2842−2849.

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

(23) Dishaw, L. V.; Hunter, D. L.; Padnos, B.; Padilla, S.; Stapleton, H. M. Developmental Exposure to Organophosphate Flame

DOI: 10.1021/acs.est.5b03849
Environ. Sci. Technol. 2015, 49, 14579−14587

Retardants Elicits Overt Toxicity and Alters Behavior in Early Life Stage Zebrafish (*Danio rerio*). *Toxicol. Sci.* **2014**, *142* (2), 445−454.
(24) Ta, N.; Li, C.; Fang, Y.; Liu, H.; Lin, B.; Jin, H.; Tian, L.; Zhang, H.; Zhang, W.; Xi, Z. Toxicity of TDCPP and TCEP on PC12 cell: Changes in CAMKII, GAP43, tubulin and NF-H gene and protein levels. *Toxicol. Lett.* **2014**, *227* (3), 164−171.
(25) Wang, Q.; Lam, J. C.; Man, Y.; Lai, N. L.; Kwok, K. Y.; Guo, Y. Y.; Lam, P. K.; Zhou, B. Bioconcentration, metabolism and neurotoxicity of the organophorous flame retardant 1,3-dichloro 2-propyl phosphate (TDCPP) to zebrafish. *Aquat. Toxicol.* **2015**, *158*, 108−115.
(26) Wang, Q.; Lai, N. L.; Wang, X.; Guo, Y.; Lam, P. K.; Lam, J. C.; Zhou, B. 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.* **2015**, *49* (8), 5123−5132.
(27) Farhat, A.; Crump, D.; Chiu, S.; Williams, K. L.; Letcher, R. J.; Gauthier, L. T.; Kennedy, S. W. *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.* **2013**, *134* (1), 92−102.
(28) Farhat, A.; Buick, J. K.; Williams, A.; Yauk, C. L.; O'Brien, J. M.; Crump, D.; Williams, K. L.; Chiu, S.; Kennedy, S. W. 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.* **2014**, *275* (2), 104−112.
(29) McGee, S. P.; Cooper, E. M.; Stapleton, H. M.; Volz, D. C. Early Zebrafish Embryogenesis Is Susceptible to Developmental TDCPP Exposure. *Environ. Health Persp.* **2012**, *120* (11), 1585−1591.
(30) Fu, J.; Han, J.; Zhou, B.; Gong, Z.; Santos, E. M.; Huo, X.; Zheng, W.; Liu, H.; Yu, H.; Liu, C. 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.* **2013**, *47* (18), 10574−10582.
(31) Wang, Q.; Liang, K.; Liu, J.; Yang, L.; Guo, Y.; Liu, C.; Zhou, B. 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.* **2013**, *126*, 207−213.
(32) Liu, X.; Ji, K.; Choi, K. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish. *Aquat. Toxicol.* **2012**, *114−115*, 173−181.
(33) Liu, X.; Ji, K.; Jo, A.; Moon, H.; Choi, K. 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.* **2013**, *134−135*, 104−111.
(34) Li, H.; Su, G.; Zou, M.; Yu, L.; Letcher, R. J.; Yu, H.; Giesy, J. P.; Zhou, B.; Liu, C. Effects of tris(1,3-dichloro-2-propyl) phosphate on growth, reproduction, and gene transcription of *Daphnia magna* at environmentally relevant concentration. *Environ. Sci. Technol.* **2015**, *49*, 12975.
(35) Dang, Y.; Giesy, J. P.; Wang, J.; Liu, C. Dose-dependent compensation responses of the hypothalamic-pituitary-gonadal-liver axis of zebrafish exposed to the fungicide prochloraz. *Aquat. Toxicol.* **2015**, *160*, 69−75.
(36) Han, X. B.; Yuen, K. W. Y.; Wu, R. S. S. Polybrominated diphenyl ethers affect the reproduction and development, and alter the sex ratio of zebrafish (*Danio rerio*). *Environ. Pollut.* **2013**, *182*, 120−126.
(37) Su, G.; Greaves, A. K.; Gauthier, L.; Letcher, R. J. Liquid chromatography-electrospray−tandem mass spectrometry method for determination of organophosphate diesters in biotic samples including Great Lakes herring gull plasma. *J. Chromatogr. A* **2014**, *1374*, 85−92.
(38) Chu, S.; Letcher, R. J. 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* **2015**, *885*, 183−190.
(39) Liu, C.; Deng, J.; Yu, L.; Ramesh, M.; Zhou, B. Endocrine disruption and reproductive impairment in zebrafish by exposure to 8:2 fluorotelomer alcohol. *Aquat. Toxicol.* **2010**, *96* (1), 70−76.
(40) Yu, L.; Liu, C.; Chen, Q.; Zhou, B. Endocrine disruption and reproduction impairment in zebrafish after long-term exposure to DE-71. *Environ. Toxicol. Chem.* **2014**, *33* (6), 1354−1362.
(41) Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L.; Vandesompele, J.; Wittwer, C. T. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **2009**, *55* (4), 611−622.
(42) Schmittgen, T. D.; Livak, K. J. Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* **2008**, *3* (6), 1101−1108.
(43) McGee, S. P.; Cooper, E. M.; Stapleton, H. M.; Volz, D. C. Early Zebrafish Embryogenesis Is Susceptible to Developmental TDCPP Exposure. *Environ. Health Persp.* **2012**, *120* (11), 1585−1591.
(44) Van den Eede, N.; Maho, W.; Erratico, C.; Neels, H.; Covaci, A. First insights in the metabolism of phosphate flame retardants and plasticizers using human liver fractions. *Toxicol. Lett.* **2013**, *223* (1), 9−15.
(45) Farhat, A.; Crump, D.; Porter, E.; Chiu, S.; Letcher, R. J.; Su, G.; Kennedy, S. W. Time-dependent effects of the flame retardant tris(1,3-dichloro-2-propyl) phosphate (TDCPP) on mRNA expression, *in vitro* and *in ovo*, reveal optimal sampling times for rapidly metabolized compounds. *Environ. Toxicol. Chem.* **2014**, *33* (12), 2842−2849.
(46) Su, G.; Letcher, R. J.; Crump, D.; Gooden, D. M.; Stapleton, H. M. *In Vitro* Metabolism of the Flame Retardant Triphenyl Phosphate in Chicken Embryonic Hepatocytes and the Importance of the Hydroxylation Pathway. *Environ. Sci. Technol. Lett.* **2015**, *2* (4), 100−104.
(47) Su, G.; Crump, D.; Letcher, R. J.; Kennedy, S. W. Rapid *in Vitro* Metabolism of the Flame Retardant Triphenyl Phosphate and Effects on Cytotoxicity and mRNA Expression in Chicken Embryonic Hepatocytes. *Environ. Sci. Technol.* **2014**, *48* (22), 13511−13519.
(48) Greaves, A. K.; Letcher, R. J. Comparative Body Compartment Composition and *In Ovo* Transfer of Organophosphate Flame Retardants in North American Great Lakes Herring Gulls. *Environ. Sci. Technol.* **2014**, *48* (14), 7942−7950.
(49) Burger, J.; Fossi, C.; McClellan-Green, P.; Orlando, E. F. Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals. *Environ. Res.* **2007**, *104* (1), 135−152.
(50) Cyr, D. G.; Eales, J. G. Interrelationships between thyroidal and reproductive endocrine systems in fish. *Rev. Fish Biol. Fisher.* **1996**, *6* (2), 165−200.
(51) Li, J.; Giesy, J. P.; Yu, L.; Li, G.; Liu, C. Effects of Tris(1,3-dichloro-2-propyl) Phosphate (TDCPP) in *Tetrahymena Thermophila*: Targeting the Ribosome. *Sci. Rep.* **2015**, *5* (10562), 10562.
(52) Reinecke, M. Insulin-like Growth Factors and Fish Reproduction. *Biol. Reprod.* **2010**, *82* (4), 656−661.
(53) Reinecke, M.; Björnsson, B. T.; Dickhoff, W. W.; McCormick, S. D.; Navarro, I.; Power, D. M.; Gutiérrez, J. Growth hormone and insulin-like growth factors in fish: Where we are and where to go. *Gen. Comp. Endocrinol.* **2005**, *142* (1−2), 20−24.
(54) Hutchinson, T. H.; Ankley, G. T.; Segner, H.; Tyler, C. R. Screening and testing for endocrine disruption in fish-biomarkers as "signposts," not "traffic lights," in risk assessment. *Environ. Health Persp.* **2006**, *114*, 106−114.
(55) NRC. Toxicity testing in the 21st centruy. In *A Vision and a Strategy*; The National Academies Press: Washington DC, 2007.
(56) Garcia-Reyero, N. Are adverse outcome pathways here to stay? *Environ. Sci. Technol.* **2015**, *49*, 3−9.
(57) 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. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* **2010**, *29* (3), 730−741.

DOI: 10.1021/acs.est.5b03849
Environ. Sci. Technol. 2015, 49, 14579−14587

(58) Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M.; Ottinger, M. A.; Vergauwen, L.; Whelan, M. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol. Sci.* **2014**, *142* (2), 312−320.
(59) Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M.; Ottinger, M. A.; Vergauwen, L.; Whelan, M. Adverse outcome pathway (AOP) development II: best practices. *Toxicol. Sci.* **2014**, *142* (2), 321−330.

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Supporting Information

Environmentally Relevant Concentrations of the Flame Retardant Tris(1,3-dichloro-2-propyl) Phosphate (TDCIPP) Inhibits Growth of Female Zebrafish and Decreases Fecundity

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Supporting Text 1: Protocol of analysis of TDCIPP and BDCIPP in exposure solutions

Triplicates of beakers were conducted for each concentration and concentrations of TDCIPP and BDCIPP were measured. Concentrations of TDCIPP in exposure solutions were quantified on the last day of exposure. Detailed protocols for identification and quantification of these two residues can be found elsewhere [1, 2]. In brief, analyses were conducted using a Waters ACQUITY UPLC® I-Class system (UHPLC) coupled to Waters® Xevos™ TQ-S mass spectrometer (TQ-S/MS) (Milford, MA, USA) using electrospray ionization (ESI(+)) in multiple reaction monitoring (MRM) mode. Due to minimal ionization in the ESI source which resulted in a poor limit of detection for BDCIPP, 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. LC separation was carried out on a Cortecs™ UHPLC C18 column (2.1 mm × 50 mm, 1.6 μm particle size) (Waters, Mississauga, ON, Canada). Mobile phases for LC were water (A) and methanol (B), and both contained 2 mM of ammonium acetate. The flow rate of the mobile phase was 0.5 mL/min and the gradient was as follows: 0 min, 5% B; 0−5 min, 95% B (linear); hold for 1 min; 6−6.1 min, 5% B (linear) and hold for 4.9 min. The capillary voltage was 0.5 KV. The source and desolvation temperatures were 150 and 600 °C, respectively. The desolvation and cone gas flow rates were 800 and 150 L/h, respectively. TDCIPP and BDCIPP were quantified by use of transitions of 430.9 > 99 and 577.2 > 243.3, respectively. A 6-point calibration curve was run with each batch of samples to ensure linearity of the instrument's response. Method limits of quantification were 0.01 ng/mL and 0.015 ng/mL water for TDCIPP and BDCIPP, respectively.

Supporting Text 2: Protocol for quantification of TDCIPP in zebrafish organs

Detailed methods have been published previosuly [3]. In brief, organs collected from zebrafish were homogenized and transferred into a glass disposable culture tube (16×125 mm). A volume of 4 mL of 50/50 (v/v) DCM/HEX extraction solvent was added to the tube. After vortexing, 10 ng of the internal standard, d_{15}-TDCIPP, was spiked into the sample and mixed. After that, 0.2 g sodium chloride (NaCl) and 1.2 g anhydrous magnesium sulfate (MgSO4) were added into the sample. The sample tube was mixed with vortex for 1 min. The sample was then ultrasonicated in an ultrasonic-cleaner (1.9 L, 35 kHz, 140 W from VWR, Mississauga, Canada) for 10 min at room temperature. After centrifugation the extract was transferred into a disposable plain conical centrifuge tube. The ultrasonic extraction process (with DCM/HEX extraction solvent) was repeated two more times and the extracts were combined.

After solvents in the extract were evaporated with a steam of nitrogen to dryness, 1 mL MeOH was added into the sample. The sample was well mixed by vortex and ultrasonically extracted from 10 min. After centrifugation the supernatant (MeOH phase) was transferred into another disposable plain conical centrifuge tube. A 300 mg aliquant of PSA bonded silica was added into the sample solution.

The sample was mixed well by vortex mixing for 1 min, and then centrifuged. The supernatant was carefully transferred into a LC vial. The sample was ready for UHPLC-MS/MS analysis.

Supporting Text 3: Protocol of analysis of BDCIPP in zebrafish homogenates

After spiking with 10 ng of deuterated d_{10}-BDCIPP, samples were subjected to accelerated solvent extraction (ASE; Dionex ASE 200, Sunnyvale, CA, USA) with 50:50 acetone(ACE) /HEX with 1% acetic acid at 100 ^{o}C and 1500 psi for 3 cycles. The extract was reduced to a volume of approximately 1 ml under a gentle flow of nitrogen. This concentrated fraction was then further cleaned-up on a 1 g ISOLUTE® aminopropyl silica gel SPE column (Biotage, Charlotte, NC, USA) packed into a 6 mL Superclean^{TM} glass cartridge (Sigma-Aldrich). The SPE column was pre-washed with 10 mL of 5:95 water/methanol (MeOH) containing 0.1 M ammonium acetate, and then 3 mL each of MeOH and ACE, to clean and condition the silica gel absorbent. After loading the concentrated fraction onto the column with 3 mL ACE, the column was further rinsed with 6 mL ACE, 3 mL MeOH, 4 mL 5:95 water/MeOH containing 0.005 M ammonium acetate, and finally 1.5 mL 5:95 water/MeOH containing 0.1 M ammonium acetate. All of these eluates were discarded, and the target OP diesters were eluted in a final 4 mL of 5:95 water/MeOH containing 0.1 M ammonium acetate. After evaporation of MeOH using a constant flow of nitrogen the remaining water was diluted with 4 mL ACE and 1.5 g of sodium sulfate was added to remove moisture and ammonium acetate. The acetone portion of the solution was transferred into a new glass tube and evaporated to dryness under a flow of nitrogen. The residue was re-dissolved with 1000 μL of MeOH, and filtered through a centrifugal filter (0.2 μm Nylon membrane, 500 μL; VWR, Mississauga, ON, Canada). The resulting 1000 μL of filtrate was transferred to a vial for quantification by LC-MS/MS.

References:

1. Chen, D.; Letcher, R. J.; Chu, S. Determination of non-halogenated, chlorinated and brominated organophosphate flame retardants in herring gull eggs based on liquid chromatography–tandem quadrupole mass spectrometry. *J. Chromatogr. A* **2012,** *1220* (2), 169-174.

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

3. Chu, S.; Letcher, R. J. 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* **2015,** *885*, 183-190.

Table S1. 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
fshβ	NM_205624.1	F: ACAGCACACCCAGAAGGTCT
		R: AGCTCCCCAGTCTGTTGTGT
lhβ	NM_205622.2	F: GAGACGGTATCGGTGGAAAA
		R: AACAGTCGGGCAGGTTAATG
vtg1	NM_001044897.2	F: CTGCGTGAAGTTGTCATGCT
		R: GACCAGCATTGCCCATAACT
cyp19a	AF226620.1	F: CCGTTCTTATGGCAGGTGAT
		R: TTGTGTGGTCGATGGTGTCT
3βhsd	AY279108.1	F: TGCCAGTCTTCATCTACACCAG
		R: TTCCCAGAGGCTCTTCTTCGTG
Activin-βA2	AJ238980	F: GCTGCTCATAACTCCAACTGTT
		R: TCCAACAACCAGTCCTGTTGGA
rpl8	NM_200713	F: TTGTTGGTGTTGTTGCTGGT
		R: GGATGCTCAACAGGGTTCAT

Table S2 Effects of different concentrations of TDCIPP on the relative expression of genes involved in HPGL axis of female and male zebrafish.

	Females				Males			
TDCIPP (ng/L)	0 ± 0	29 ± 2.1	600 ± 21	6300 ± 130	0 ± 0	29 ± 2.1	600 ± 21	6300 ± 130
Brain								
fshβ	1.07 ± 0.17	1.36 ± 0.38	1.48 ± 0.62	1.29 ± 0.36	1.19 ± 0.28	1.20 ± 0.23	0.75 ± 0.12	0.94 ± 0.19
lhβ	1.45 ± 0.67	1.74 ± 0.38	1.21 ± 0.19	1.79 ± 0.23	1.20 ± 0.43	1.18 ± 0.48	0.31 ± 0.08	0.36 ± 0.10
Liver								
vtg1	1.12 ± 0.21	0.60 ± 0.36	0.76 ± 0.23	1.78 ± 0.22	1.08 ± 0.26	0.52 ± 0.16	1.69 ± 0.28	0.97 ± 0.29
Gonad								
cyp19a	1.10 ± 0.21	0.77 ± 0.24	1.48 ± 0.45	1.56 ± 0.32	1.02 ± 0.10	1.66 ± 0.40	1.46 ± 0.45	1.82 ± 0.50
Activin-βA2	1.08 ± 0.18	0.82 ± 0.13	0.67 ± 0.10	0.99 ± 0.15	1.06 ± 0.18	1.30 ± 0.32	1.68 ± 0.27	1.67 ± 0.07
3β-hsd	1.05 ± 0.14	1.05 ± 0.07	1.15 ± 0.15	1.00 ± 0.15	1.02 ± 0.10	1.19 ± 0.26	1.34 ± 0.22	1.18 ± 0.16

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

Figure S1

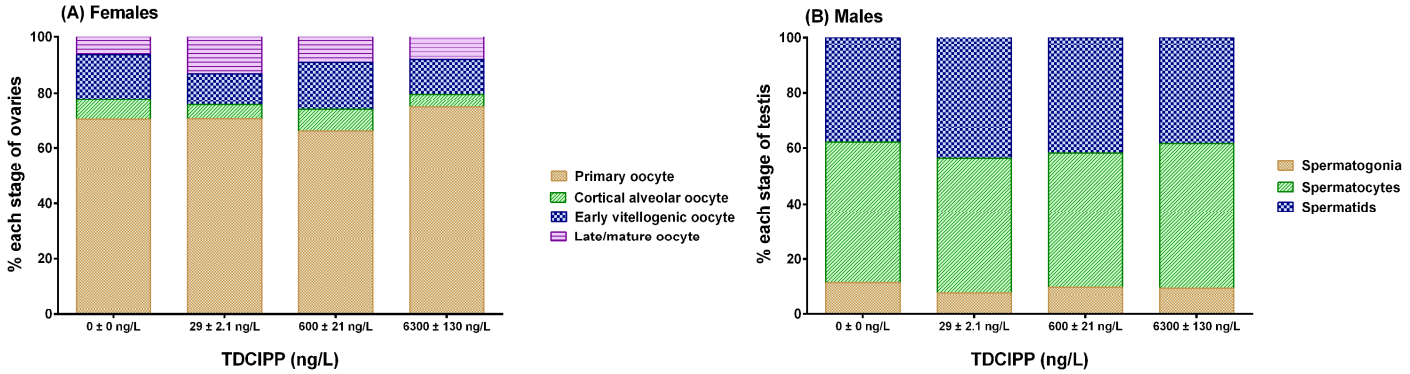

Figure S1: Histological examination of gonad development in female (A) and male (B) zebrafish after exposure to various concentrations of TDCIPP for 120 days. Values represent the mean ± SE of six individual fish from 3 replicate tanks.

Figure S2

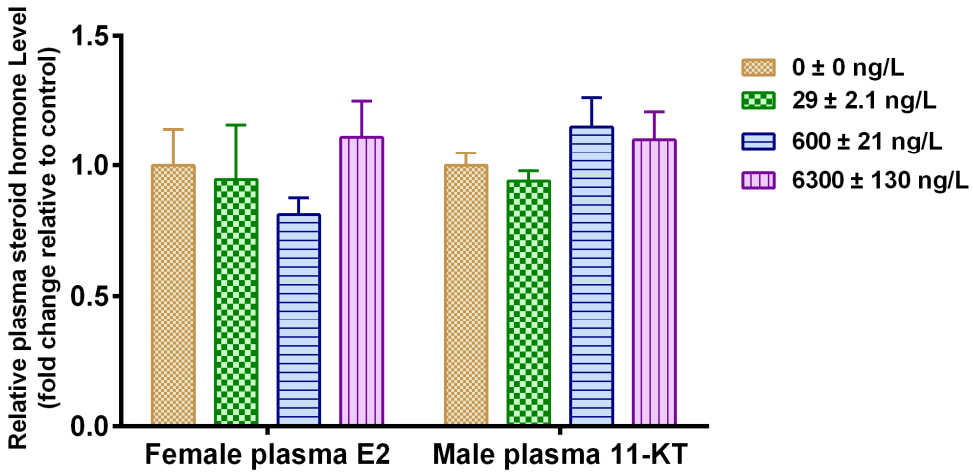

Figure S2: Effects on plasma estradiol (E2) in females and 11-ketotestosterone (11-KT) in males after exposure to various concentrations of TDCIPP for 120 days. Value represent mean ± SE (n=3).