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Endocrine Disruption Potentials and Related Mechanisms of Several Organophosphate Flame Retardants

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Abstract

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Organophosphate flame retardants (OPFRs) have been widely used as alternatives to polybrominated diphenyl ethers (PBDE) to prevent fire. Several OPFRs have been frequently detected in environment and biota, however knowledge on their potential toxicological effects are limited. Recently, these compounds are reported to be associated with altered hormones like sex steroid, thyroid hormone and prolactin, and decreased semen quality in adult men. In the present study, various cell lines and a zebrafish (*Danio rerio*) model were employed to investigate the endocrine disruption potency and underlying mechanisms of several OPFRs. The results of this study will provide essential toxicity information for ecological risk management about this re-emerging group of environmental contaminants. For this purpose, four separate sets of experiments were conducted.

Firstly, two human cell lines (H295R and MVLN) and zebrafish were employed to screen the endocrine disrupting potentials of six OPFRs, i.e., tris-(2-chlorethyl) phosphate (TCEP), tris-2-chloroisopropyl phosphate (TCPP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris-(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPP), and tricresylphosphate (TCP). By all six OPFRs, both 17β-estradiol (E2) and testosterone (T) concentrations increased in H295R cells. In addition, transcription of four major steroidogenic genes (*CYP11A1*, *CYP11B2*, *CYP19A* and *3βHSD2*) was up-regulated and that of two sulfotranferase genes (*SULT1E1* and *SULT2A1*) was

down-regulated. In MVLN cells, no OPFRs acted as estrogen receptor (ER) agonists, while TDCPP, TPP, and TCP acted as ER antagonists inhibiting binding of E2. Following 14 d exposure to adult zebrafish, TDCPP, TPP, or TCP significantly increased plasma T and E2 concentrations. Among males, both T and 11-KT decreased and E2 increased. In general, transcription of *CYP17* and *CYP19a* genes in gonad was significantly up-regulated in both sexes, while *VTG1* gene was down-and up-regulated in female and male fish, respectively.

In second experiment, a 21 d reproduction test was conducted using adult zebrafish to understand the effects of two major OPFRs on reproduction performances. The study OPFRs included TDCPP and TPP, which are most widely used and also exhibited clear evidence of hormone alterations previously *in vitro* cell assay. In this experiment, paired adult zebrafish were exposed to various concentrations of TDCPP and TPP (0, 0.04, 0.2, and 1.0 mg/L) for 21 d, and the effects on gene transcription, proteins, and several reproduction related parameters were evaluated. Fecundities were significantly decreased by both OPFRs, and these reproduction changes were accompanied with significant increases of plasma E2, E2/T and E2/11-KT, and decreases of T and 11-KT in male and female fish. Significant increase of plasma vitellogenin (VTG) level was also observed. Altered transcription of genes along HPG axis was sex-dependent, and was supportive of the protein levels observations. The results of the second experiment demonstrated that both TDCPP and TPP could perturb the function of HPG axis, and then influence the sex hormone balance as well as reproduction performance.

In third experiment, the effects on fish thyroid hormones (THs) were investigated after 14 d exposure. Plasma concentrations of THs, and transcription of related genes involved in thyroid synthesis, transport, binding, or regulation were examined in brain, thyroid, and liver of zebrafish. The concentrations of peripheral triiodothyronine (T3) and thyroxine (T4) decreased significantly in male fish following the exposure to both OPFRs, but the trend was opposite in female fish. The transcription of genes along hypothalamus-pituitary-thyroid (HPT) axis by the exposure was sex-

dependent: In male fish, transcriptions of *CRH* and *TSH* in brain were significantly greater as a compensation of hypothyroidism, but *TG*, or *Dio2* were decreased in thyroid or liver. In contrast, transcriptions of *CRH* and *TSH* genes were significantly down-regulated in females, but increases in transcription of other genes did not support the compensatory efforts of the brain.

In the fourth experiment, a longer term exposure from the fertilization of the eggs was conducted to understand the effects from very early stage of the life, and possibly to assess cross-talks among the HPG, HPT and hypothalamic-pituitary-adrenal (HPA) axes. During this test, zebrafish embryos were exposed to 0.005, 0.05 and 0.5 mg/L TDCPP or TPP for 120 d, and the growth parameters, hormone levels, as well as the transcriptional profiles along the HPG, HPA, and HPT axes were examined. Following the exposure, conditional factor (CF), gonad somatic index (GSI), and liver somatic index (LSI) were significantly decreased in exposed fish. In female fish, exposure to TDCPP or TPP led to increase of cortisol, follicle stimulating hormone (FSH), luteinizing hormone (LH), T4, T3, and E2, and lesser concentration of 11-KT. In males, exposure to these two chemicals resulted in decrease of cortisol, FSH, LH, T4, T3, T, and 11-KT. Sex dependent changes were also evident: Transcription of genes along HPG, HPA and HPT axis were mostly up-regulated in female, while down-regulated in males.

The observations of a series of experiments showed that (1) all six selected OPFRs could disturb the synthesis, metabolism or activation of sex hormone, and that (2) TDCPP and TPP influenced balances of sex hormones and THs, and could cause reproductive impairments in zebrafish. In addition, (3) Life cycle exposure to TDCPP or TPP caused developmental retardation, and disturbed hormone balance at concentrations that could occur in the environment. Ecological implications of these observations deserve further investigation.

Key words: organophosphate flame retardants, endocrine disruption, hypothalamus-pituitary-gonad, hypothalamus-pituitary-thyroid, hypothalamus-pituitary-adrenal, sex steroid, thyroid hormone

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Abbreviation

AR: Androgen receptor

CRH: corticotrophin-releasing hormone

CRHBP: Corticotrophin-releasing hormone binding protein

CRHR2: Corticotrophin-releasing hormone receptor

C*t*: Threshold cycle

CYP11A: Cytochrome P450 side-chain cleavage

CYP11B: Cytochrome P450 11B

CYP17: Cytochrome P450 17

CYP19A: Cytochrome P450 19A

CYP19B: Cytochrome P450 19B

Dio: deiodinase

DMEM/F12: Dulbecco's modified eagle's medium and Ham's F-12 Nutrient mixture

DMSO: Dimethyl sulfoxide

E2: 17β-estradiol

ER: Estrogen receptor

FSH: Follicle stimulating hormone

FSHβ: Follicle stimulating hormone β

FSHR: Follicle stimulating hormone receptor

GnRH: Gonadotropin-releasing hormone

GnRHR: Gonadotropin-releasing hormone receptor

GR: Glucocorticoid receptor

Hhex: Hematopoietically-expressed homeobox protein

HSD: Hydroxysteroid dehydrogenase

HMGR: Hydroxymethylglutaryl CoA reductase

IQR: Interquartile range

LH: luteinizing hormone

LHβ: Luteinizing hormone β

MC2R: Melanocortin 2 receptor

MR: Mineralocorticoid receptor

NIS: Sodium iodide symporter

OPEs: organophosphate esters

OPFR: Organophosphate flame retardant

Pax8: paired box gene 8

RLU: Relative Light Unit

StAR: Steroidogenic acute regulatory protein

STPs: sewage treatment plants

SULT: Sulfotransferase

T: Testosterone

11-KT: 11 ketotestosterone

TBEP: Tris(2-butoxyethyl) phosphate

TCP: Tricresylphosphate

TCEP: Tris(2-chloroethyl) phosphate

TCPP: Tris(chloropropyl) phosphate

TDCPP: tris-(1,3-dichloro-2-propyl) phosphate

TPP:Triphenyl phosphate

T3: triiodothyronine

T4: thyroxine

TG: thyroglobulin

TPO: thyroperoxidase

TR: thyroid hormone receptor

TRH: thyrotropin releasing hormone

TRHR: Thyrotropin-releasing hormone receptor

TSH: thyroid-stimulating hormone

TTR: transthyretin

TR: Thyroid hormone receptor

Chapter 1. Introduction

1.1 organophosphate flame retardants

Flame retardants are chemicals that used to resist or inhibit the spread of fire in manly products, in which included construction materials, furniture, plastics, electronics equipment, textiles, and other materials. Until recently, polybrominated diphenyl ethers (PBDEs) accounted for a large proportion of flame retardants used in polyurethane foam and electronic applications [Consumer Product Safety Commission (CPSC) 2006]. However, because of the persistence, bioaccumulation and adverse health effects, components of the Penta and Octa-BDE commercial mixtures have been added to the Persistent Organic Pollutants list of the Stockholm Convention (Ashton et al., 2009) and have been banned or voluntarily phased out . Even use of Deca-BDE will discontinue by the end of 2012 or 2013 (Hess et al., 2009; Chemtura et al., 2009; Albemarle et al., 2009). Since the ban on PBDEs, the use of alternate flame retardants, such as organophosphate flame retardants (OPFRs) has been on the rise (Stapleton et al., 2010).

OPFRs can be divided in three main groups, inorganic, organic and halogen containing FRs. The six OPFRs selected in this study is limited to OPFRs mentioned in the literature as potential substitutes for PBDEs, and also have been widely detected in environment and biota samples recently, which include three chlorinated OPFRs, like tris (chloropropyl) phosphate (TCPP), tris (1,3-dichloro-2-propyl) phosphate (TDCPP) and tris (2-chloroethyl) phosphate (TCEP), three non-chlorinated OPFRs, like triphenyl phosphate (TPP), tricresyl phosphate (TCP) and tris-(2-butoxyethyl) phosphate (TBEP). Even though ubiquitous of OPFRs have been reported, the toxicity information of OPFRs is lacked, especially aquatic toxicity of OPFRs. In this study, the endocrine disruption potentials and underlying mechanisms of OPFRs was investigated.

1.2 Properties and production of OPFRs

There are great variations in physiological properties of OPFRs. For example, water solubility range from 0.36 (TCP) to $7.0X10^{3}$ (TCEP) (Table 1.1). This is consistent with previous study, which showed the solubility of OPFRs versus the molecular mass. The solubility decreases by increasing molecular mass. In case their hydrolysis half life is equal, the OPFRs with lower masses are therefore more likely to be found in the aquatic environment than those with higher molecular masses. Besides, all six OPFRs s have a positive log Kow value, which means they are more lipophilic than hydrophilic, especially TDCPP, TCP and TPP. There is also a variety in vapor pressures and bioconcentration factors (BCF).The vapor pressure at 25℃ ranges from $1.1X10^{-4}$ mm Hg for TCEP to $7.4X10^{-8}$ mm Hg for TDCPP, and the BCF ranges from 1.37 for TCEP to $8.56X10^{3}$ for TCP. According to previous studies, the BCF generally increase with increasing molecular mass, except for chlorine containing compounds. The non-halogen OPFRs with higher molecular masses are therefore more like to be found in nature.

Since the ban of PBDE, the usages of OPFRs, especially chlorinated OPFRs, are increasing. In Table 1.2 an overview of the studies OPFRs is given with their production or usage volumes. Out of this table some observations can be made. The total consumption of FRs in Europe in 2005 was 85, 000 tones, OPFRs were responsible for 20%, of which 11% were chlorinated and 9% were non-chlorinated OPFRs, and showed 46, 000 tones and 39, 000 tones respectively. In 2007 the use of chlorinated OPFRs had increased to 51, 000 tones. The use of TCPP has continued to grow since the mid-1960s, might be explained by the fact that TCPP is often used as replacement of TCEP (WHO, 1998).

Table 1.1. physico-chemical properties of six OPFRs.

Abbreviation	Molar mass (g/mol)	Boiling Point (℃)	Melting Point (℃)	Solubility in water (mg/L)	Vapor pressure (mm Hg) at 25℃	Log K_{ow}	Log K_{oc}	Bioconcentration factor (BCF)
TCPP	327.57	342	-40	$1.6X10^{3}$	$1.9X10^{-6}$	2.59	2.21	8.51
TCEP	285.49	351	-55	$7.0X10^{3}$	$1.1X10^{-4}$	1.44	2.48	1.37
TDCPP	430.91	457	88	1.50	$7.4X10^{-8}$	3.8	2.35	13.5
TCP	368.37	439	77	0.36	$1.8X10^{-7}$	5.11	4.35	$8.56X10^{3}$
TPP	326.3	370	49	1.9	$1.2X10^{-6}$	4.59	3.72	113
TBEP	398.48	200-230	-70	1.1-1.3	$2.8X10^{-7}$	3.65	4.38	$1.08X10^{3}$

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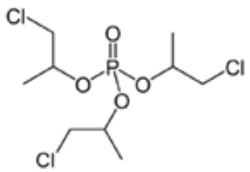
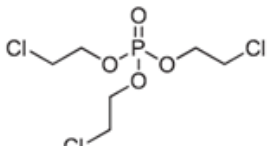


TCPP

Tris (chloropropyl) phosphate

Formula: C9H18C1304P

CAS No: 13674-84-5

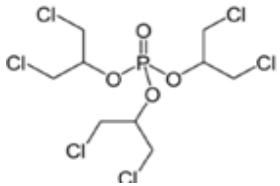


TCEP

Tris (2-chloroethyl) phosphate

Formula: C6H12C1304P

CAS No: 115-96-8

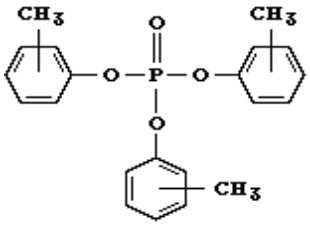


TDCPP

Tris (1,3-dichloro-2-propyl) phosphate

Formula: C9H15C1604P

CAS No: 13674-87-8

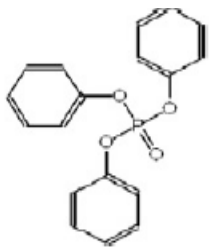


TCP

Tricresyl phosphate

Formula: C21H2104P

CAS No: 1330-78-5

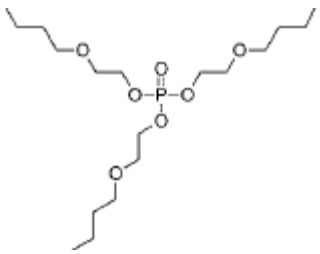


TPP

Triphenyl phosphate

Formula: C18H1204P

CAS No: 115-86-8



TBEP

Tris-(2-butoxyethyl) phosphate

Formula: C18H3907P

CAS No: 78-51-3

Table 1.2. Production volume of six OPFRs.

OPFRs	Location	Year	Production/usage volume (tons) per year
TCPP	Worldwide	1997	40 000
	Europe	1995	22 950
	UK	1995	2750
	Norway	2008	42.7
	Denmark	2008	177
	Finland	2008	16 429
	Sweden	2008	132
TCEP	Europe	1995	2040
	UK	1995	400
	Norway	2003	1286
	Norway	2004	798.5
	Finland	2004	1598
	United States	2006	227-454
	Norway	2008	261.3
	Denmark	2008	0.1
	Finland	2008	198
	Sweden	2008	0
TDCPP	Worldwide	1997	8000
	United States	1998	454-4500
	United States	1998-2006	4500-22 700
	Europe	2000	<10000
	Denmark	2000	132.8
	Denmark	2001-2006	134.1
TPP	United States	1998-2002	4500-22 700
	Europe	2000	20 000-30 000
	Norway	2004	55
	Norway	2005	6.7
	Sweden	2005	1592
	Norway	2008	18.4
	Denmark	2004-2008	2.3-16.7
	Finland	2004-2008	9.8-57.1
	Sweden	2003-2008	46-88
TCP	United States	1998-2006	454-4500
	Norway	2008	0.8
	Denmark	2008	0.6
	Finland	2008	3.6
	Sweden	2008	5.0
TBEP	Worldwide	2002	5000-6000
	United States	2002	<1000
	Norway	2003-2004	1.3-1.5
	Norway	2005	0.6

WHO (1997), UNEP (2002), US-EPA (2006), EU (2008), Green et al (2008) and SPIN (2011).

1.3 Occurrence of OPFRs in aqueous

OPFRs are additives and not chemically bonded to the final products, therefore are easy to release to the environment. Occurrences of OPFRs have been widely detected in environment or biota; studies showed that OPFRs are ubiquitous contaminants in the environment, which included aqueous environment.

OPFRs have been widely detected in aqueous system, included wastewater, surface water, ground water, or even in drinking water (Table1.3). Meyer and Bester (Meyer et al 2004) investigated six OPFRs in two waste water treatment plants (WWTPs) in Germany, showed around or over 100 ng/L for six OPFRs, chlorinated OPFRs were hardly removed, together with moderate elimination of TPP, TCEP, TCPP and TDCP showed no significant removal. Same with that, in a Swedish study (Marklund et al., 2005), TCPP and TBEP were the most abundant OPFRs. Remarkably high influent concentration of TBEP was recorded in another German WWTP (Rodil et al 2005). Recently, a study of 16 WWTPs in Austria confirmed the prominent of TCPP. It is believed that WWTPs are major source of OPFRs in surface water. In rural areas, ditches have become contaminated by TCP due to leaching from large amounts of plastic films used for greenhouses. Emission into groundwater may occur via landfill leachate and release into the marine environment from dump sites. Table1.4 included most relevant studies of OPFRs in surface, ground and drinking water. However, the compounds found and the levels detected at certain locations in rivers and lakes strongly depend upon the local situation of emissions and dilution. Typical concentrations in surface water cannot therefore be given, and the OPFRs encountered at a certain site may differ. However, some aliphatic and some chlorinated triesters appear to be ubiquitous. A long-term study covering various rivers in Japan in the period 1976–90 has been published (Fukushima et al 1992), showing regional as well as temporal trends. A more recent study was performed for seven triesters along the River Ruhr in a densely populated region of Germany (Andresen et al., 2004). Most OPFRs occurred at concentrations of 10–200 ng/L, with the highest levels being found downstream of WWTP discharges. Similar

concentrations of TCEP, TCPP, TBP and TBEP were recently reported for Austrian rivers (Elena et al., 2007). The occurrences of OPFRs in drinking suggest that water-treatment processes were inappropriate for the removal of OPFRs in these cases.

OPFRs and its metabolite were also detected in aqueous organisms (Table 1.5). Green et al., (2008) analyzed mussel and cod liver from Norway, and found TPP, TCP or TCPP below the limit of detection (LOD), while TCEP or TDCPP showed <5μg/kg in cod liver and <10-30 μg/kg in mussel. Evenset et al., (2009) reported 1.4-2.9 μg/kg of TCPP in fish muscle and 5.5-8.9 μg/kg in fish liver from Norway. In same study, TCEP was found 1.5-5.0 μg/kg in muscle tissue, and 13-26 μg/kg in fish liver tissue, TDCPP was <0.3-6.7 μg/kg in muscle and <0.3-6.7 in liver. TPP was 0.3-3.2 μg/kg in muscle and 5.7-13 μg/kg in fish tissue. Leonards et al., (2011) reported TCPP, TCEP, TDCPP, TCP, TPP and TBEP in beach crab, cod liver, trout and in bird blood and bird eggs from Norway, the TCPP was dominant, followed by TCEP, TDCPP, TPP and TCP. Sundkvist et al., (2010) showed TPP and TCPP dominated in the biota with levels ranging from 170-770ng/g for TCPP in perch and 21-180ng/g for TPP. In milk samples, TCPP was the most frequently occurring OPFRs. But study of Campone et al., (2010) showed that all 14 detected OPFRs including TCPP, TCEP, TDCPP, TCP, TPP and TBEP were below the LOD.

Like PBDE, OPFRs were also persistent in aqueous. Aryl-phosphates, included TCP and TPP, in leachate rapidly decreased to less than the detection limit within 20 days under aerobic condition (Kawagoshi et al., 2002), suggesting high biodegradability. TBEP decreased slowly in all samples but that of sterilized distilled water. Slightly degradation of TCEP and TDCP were observed, but TCPP could remain in the leachate over 80 days.

Table1.3. Occurrence of six OPFRs in wastewater (μg/L) (range or mean value)

Country	Type of matrix	Concentration	Reference
Sweden	Influents, effluents, and sludge from 11 Swedish sewage treatment plants (STPs).	TCPP: 1.1-4.0; TCEP: 0.09-34; TBEP: 3.1-35; TDCP: 0.18-3.0; TPP: 0.041-3.0;	Marklund et al., 2005
Austria	Effluents of 16 WWTPs	TCPP: 0.27-1.4; TCEP: <LOD-1.6; TBEP: 0.053-5.4; TDCP: 0.019-1.4; TPP: <LOD-0.17; TCP: <LOD-0.055	Elena et al., 2007
Germany	Influent, effluent and sludge of one WWTP	TCPP: 0.043-1.0;	Bester et al., 2005
Germany	Influent and effluent of two WWTPs	TCPP: 0.94-6.6; TCEP: 0.25-0.64; TBEP: 0.79-8.0; TDCP: 0.18-0.31; TPP: 0.031-0.29;	Meyer et al., 2004
Germany	Influent and effluent of one WWTP	TCPP: 3.1; TCEP: 0.33; TDCP: 0.21; TBEP: 12; TPP <LOD;	Rodil et al., 2005
Germany	Influent and effluent of one WWTP	<LOD(DBEP); ; 0.18(DPP);	Quintana et al., 2007
Spain	Influent and effluent of one STP	Unit (ng/L) TCPP: 0.31-0.91; TCEP: <LOD-0.7; TBEP: 0.11-33.73; TPP: <LOD-0.47;	Rodriguez et al., 2006

Table1.4. Occurrence of six OPFRs in surface, ground or drinking water (ng/L) (range or mean value)

Country	Type of matrix	Concentration	Reference
Germany	Six rivers	TCEP: Nd-1,236; TBEP: 121-952;	Fries et al., 2001
Germany	Oder river	TCEP: 352; TBEP: 2,955;	Fries et al., 2003
Germany	River Ruhr	TCPP: 85-126; TCEP: 23-61; TBEP: <LOD-53; TDCP: 17-32;	Regnery et al., 2010a
Germany	nine lentic surface waters	TCPP: <LOD-5,791; TCEP: <LOD-390; TBEP: <LOD-1,616; TDCP: <LOD-532;	Regnery et al., 2010b
Japan	Yamato Osaka river	TCPP: 1-9.8; TCEP: 0.082-2.3; TBEP: 1.4-230; TDCP: 0.069-18; TPP: 0.11-2.6; TCP: 0.086-7.5;	Schou et al., 1981
Austria	Four rivers	TCPP: 270-1400; TCEP: <LOD-1600; TBEP: 13-5400; TDCP: 23-1400; TPP: <LOD-170; TCP: <KLOD-55;	Elena et al., 2007
Germany	Drinking water	TCPP: 50-150; TCEP: 10-130; TBEP: <3-180; TDCP: 10-40; TPP: <0.3-7.2;	Andressen et al., 2006
USA	Drinking water	TCEP: 500; TBEP: 500; TDCP: 500; TPP: 500;	Stackelberg et al., 2004
Italy	Drinking water	TCPP: 54-117; TCEP: <LOD-7; TBEP: 87-323; TDCP: <LOD; TPP: 11-165; TCP: <LOD	Bacaloni et al., 2007

Table1.5. Occurrence of six OPFRs and metabolites in aquatic organisms

Compounds	Type of matrix	Reference	Sample
TPP, TCEP, TCPP, TDCP TCP, TBEP	Salmon (fresh &smoked) and cod samples from supermarkets in Stockholm (Sweden).	Campone et al., 2010	Fish tissue
TPP, TCEP, TCPP, TDCP TCP, TBEP	Crab, cod, trout (Norway)	Leonards et al., 2011	Marine organisms
TDCPP, TCPP TCEP, TPP	Fish muscle, liver (Norway)	Evenset et al., 2009	Fish tissue
TCEP, TCPP, TBEP, TPP, TCP	Mussel liver, cod liver (Norway)	Green et al., 2008	Fish tissue
BPP, TCP, TDCP,TCEP, TPP, TCPP	Freshwater organisms (mussels, salmon, herring, eelpout, perch) (Swed)	Sundkvist et al., 2010	Marine organisms

1.4. Endocrine toxicity of OPFRs

OPFRs are of concern because they leach or diffuse out of the products over the course of their lifetime with exposure to humans through ingestion, inhalation of dust particles and dermal absorption (WHO 1990; Hughes et al., 2001).

Unlike PBDE, the endocrine disrupting toxicities of OPFRs have not been investigated systematically. According to U.S Environmental Protection Agency, endocrine disrupting chemical (EDC) is external compound that interferes with the endocrine system, which included disturb or mimics natural hormones in the body that are responsible for the maintenance, reproduction, development, and/or behavior of an organism (1997). EDC can adversely affect reproductive biology and the thyroid system directly through hormone receptors or indirectly through their synthesis, metabolism or transport. In 2010, Meeker and Stapleton found concentration of TDCPP or TPP in house dust associated with hormone balance in male human being, suggested the possible endocrine disrupting potential of OPFRs.

According to previous studies, TCEP is possible reproductive toxicant (Tilson et al., 1990; Umezu et al., 1998). TDCPP induced histopathological abnormalities in testis or seminal vesicle in male rat, but no effects on male reproductive performance in rabbits were observed, and the possible effect on female reproduction has not been investigated. Reproduction toxicity was not shown in TPP when exposed to cat up to 1 g/kg or to Sprague-drawly rats for 4 month. Toxicity study of TCP showed reproduction damage and neurotoxicity to male fisher 344 rats (Environment health criteria 110). Carlton et al., (1987) also showed reproduction effect of TCP. While, reproductive toxicity of TCPP or TBEP have not been studied. The underlying mechanisms of these reproduction toxicities were not known. *In vitro* study showed TBEP could bind beta-adrenergic receptor and interact with beta-adrenergic transport proteins (Sager et al 1989). Triaryl phosphate, include TCP or TPP are constitutively active receptor (CAR) and pregnane X receptor (PXR) activators that might influence steroid-dependent biological pathways and cause reproductive effects.

Since the main sink of such substances are surface waters, and OPFRs have already widely detected in water or aquatic organisms, thus, the aquatic vertebrates, such as fish are most endangered. Previous aquatic toxicity of OPFRs was more focused on calculating PNEC, NEC or HQ values by acute or chronic toxicity with survival as endpoints. The aquatic toxicity information of OPFRs in very limited, especially the development and reproduction toxicity as well as underlying mechanisms. Therefore, this study was set up to fill this toxicity gap.

1.5. Research objective and study design

In this study, development and reproduction toxicity, as well as underlying mechanisms of OPFRs were investigated. Zebrafish (*Danio rerio*) was used as *in vivo* model. As we know, zebrafish has many advantages as a model organism for investigating endocrine disruption, such as the available genomic resources and the long experience in toxicity testing. It is easily possible to establish molecular endpoints for EDC effects assessment.

Since the endocrine system of fish is generally organized like most vertebrates, with hierarchic structures for several endocrine feedback mechanisms. Hypothalamus-pituitary-endocrine gland axes exist for adrenal cortex, gonad and thyroid system. The hypothalamus-pituitary-gonad (HPG) axis plays the major role for sexual differentiation and regulation of reproduction. Thyroid hormones (THs), which were regulated by hypothalamus-pituitary-thyroid (HPT) axis, regulate growth, morphological development, and play an important role in the perinatal development of the central nervous system. Hypothalamus-pituitary-adrenal (HPA) axis in fish was thought to be involved in regulation of stress response, and involved in metamorphosis, interfering to some extent with the HPT or HPG axis. These systems are responsible for regulating hormone dynamics by coordinating their synthesis, secretion, transport, and metabolism. Each endocrine axis interacts with the other endocrine axes to integrate bodily function. EDC-induced changes along one endocrine axis are likely to lead to changes in the other endocrine axes. Therefore, in the present study, the effects of OPFRs on

whole endocrine system that regulate development or reproduction, which include HPG, HPT and HPA axes was investigated.

The outline for the whole dissertation project is demonstrated in Figure 1.1. In chapter 2, effect of OPFRs on synthesis, metabolism and activation of sex hormone was investigated by *in vitro* cell screening, and *in vivo* ,zebrafish. In chapter 3, a 21 d reproduction test was set up, effects of TDCPP or TPP on reproduction performance of zebrafish, and system that regulate the reproduction, called HPG axis was investigated. In chapter 4, effects of TDCPP or TPP on thyroid hormone levels and system of HPT axis was studies after 14 d short term exposure of TDCPP or TPP to adult zebrafish. In chapter 5, after life cycle exposure of zebrafish to TDCPP or TPP was conducted, development toxicity and cross talk between HPG, HPT and HPA axes was investigated. This study was expected to provide toxicological information in ecological risk assessment of OPFR.

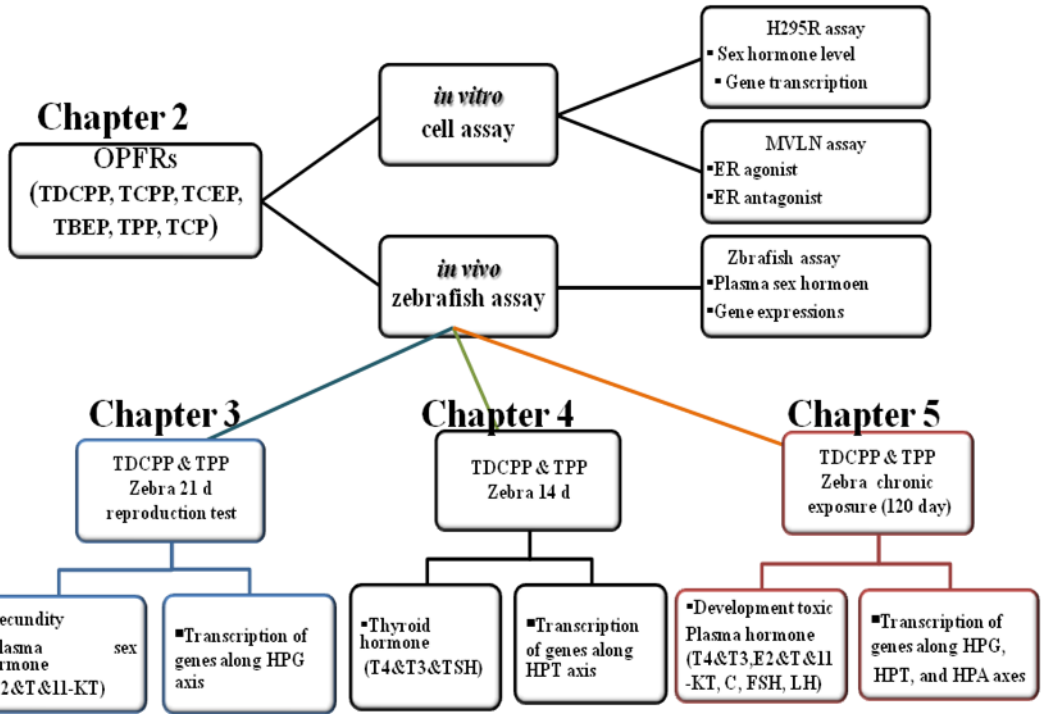


Figure 1.1 Study diagram to investigate endocrine disrupting potential and underlying mechanisms of OPFRs.

Chapter 2. Endocrine disruption potentials of organophosphate flame retardants and related mechanisms in H295R and MVLN cell lines and in zebrafish

2.1 Introduction

Because of potential adverse health effects of polybrominated diphenyl ethers (PBDEs), many industrial countries have banned the use of these compounds as flame retardants. As a consequence, alternative chemicals have been increasingly used and organophosphate flame retardants (OPFRs) are one of such examples. OPFRs have been produced and used in high volumes. The use of OPFRs in Europe amounted to 85,000 tons in 2005, and 91,000 tons in 2006 (http://www.cefic-efra.com), and their usage is expected to rapidly increase in the future. OPFRs are anthropogenic chemicals that are utilized also as plasticizers in various consumer products and building materials (Nagase et al., 2003; Stapleton et al., 2009), or even in baby products (Stapleton et al., 2011).

OPFRs are widely distributed owing to their extensive use, and volatility, and are considered to be persistent in water and air (Reemtsma et al., 2008). Since OPFRs are not covalently bound to host materials, these compounds could diffuse out into surroundings relatively easily by volatilization, leaching and/or abrasion (Marklund et al., 2003; Reemtsma et al., 2008). Several OPFRs such as tris-(2-chlorethyl) phosphate (TCEP), tris-2-chloroisopropyl phosphate (TCPP), tris(1,3-dichloro-2-propyl) phosphate (TDCPP), tris-(2-butoxyethyl) phosphate (TBEP), triphenyl phosphate (TPP), and tricresylphosphate (TCP) have been frequently detected in the environment including indoor dust, air, soil and sediment worldwide (Garcia-Lopez et al., 2008; Stapleton et al., 2009; Takigami et al., 2009). Water is a preferred

medium of distribution for OPFRs(Verbruggen et al., 2005), and therefore this group of chemicals have been detected in surface waters, effluents of wastewater treatment plants, urban precipitation, and storm-water runoff with concentrations ranging from mid to high ng/l. TCPP is among the most dominant compounds detected in water environment (Bacaloni et al., 2008; Fries and Püttmann, 2003; Martínez-Carballo et al., 2007; Marklund et al., 2005; Regnery and Püttmann, 2010a; 2010b). OPFRs have also been detected in biota including aquatic organisms and in human breast milk with TCPP and TPP being the most dominant OPFRs in Sweden. In perch, TCPP and TPP were detected at levels ranging between 170 and 770, and 21 and 180 ng/g, respectively (Sundkvist et al., 2010).

The potential adverse effects of OPFRs to the ecosystem and human health have been suggested in several studies (European Commission 2006, 2007; Reemtsma et al., 2008; Ren et al., 2008). Exposure to OPFRs has been associated with adverse reproductive, neurologic, and other systemic effects. Alteration in thyroid function and change in relative liver weight were also reported in laboratory animals (National Research Council (NRC) 2000; U.S. EPA 2005). Recently, Meeker and Stapleton (2010) suggested that TDCPP and TPP might be associated with disrupted hormone levels and decreased semen quality among adult human males. Each interquartile range (IQR) increase of TPP in house dust was associated with a 19% decrease in sperm concentrations and a 10% increase in prolactin level of the adult males. In addition, each IQR increase in dust TDCPP was associated with a 17% increase in prolactin and a 3% decline in free thyroxine (T4). Although the contribution of house dust as an exposure pathway for OPFRs is not elucidated, the observed association underlines a need for investigating mechanisms underlying the endocrine disruption of the OPFRs in male humans. However, little is known about the toxicological consequence of exposure to OPFRs among aquatic organisms.

In the present study, I investigated the effects of major OPFRs on endocrine disruption using both in vitro and in vivo models. Two cell lines, i.e., H295R and MVLN cells and a fish (*Danio rerio*) were used to elucidate both

mechanisms of endocrine disruption. H295R cells, a human adrenocortical carcinoma cell, have frequently been used to evaluate effects of chemicals on steroidogenesis by measuring transcription of several key steroidogenic genes and concentrations of sex hormones (Hecker et al., 2006). The affinity of chemicals toward estrogen receptor alpha (ERα) was investigated using MVLN cell line. In addition, zebrafish was employed to assess consequences of endocrine disruption and related mechanisms.

2.2 Materials and Methods

2.2.1 Chemicals and cell cultures

Six OPFRs that were investigated in the present study include TCEP (CAS No. 115-96-8. Purity: 97%), TCPP (CAS No. 13674-84-5), TDCPP (CAS No. 13674-87-8. Purity: 95%), TBEP (CAS No. 78-51-3. Purity: 94%), TPP (CAS No. 115-86-6), and TCP (CAS No. 1330-78-5. Purity: 90%). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Chem Service (West Chester, PA, USA). Dimethyl sulfoxide (DMSO) and methanol were used as solvent for H295R and MVLN cell assay, respectively. In both cells, final concentrations of solvent in the exposure media were less than 0.1% (v/v).

H295R cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma–Aldrich) supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA, USA), 2.5% Nu-Serum (BD Biosciences), and 1.2 g/l Na_2CO_3 (Sigma–Aldrich). Medium was renewed every 4 d, and cell subculture was performed every 7 d or when cell confluence was over 80%. Culture medium for MVLN was hormone-free DMEM/F12 nutrient mixture, 1 mM sodium pyruvate, and 1 mg/l of insulin (Sigma-Aldrich) supplemented with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT, USA).

2.2.2 H295R and MVLN cell assay

Concentrations of sex hormones were determined and transcriptions of several key genes involved in steroidogenesis were quantified using H295R cell bioassays. H295R cell line was obtained from American Type Culture Collection (Manassas, VA, USA), and was cultured following Hilscherova et al. (2004). H295R cells were seeded and incubated in 24-well plates for 24 h, before the medium were changed and the cells were dosed with various concentration of each OPFR (0.001, 0.01, 0.1, 1, 10, or 100 mg/l) plus a control. Each treatment included three replicates included three replicates, and exposure duration was 48 h. Nominal concentrations were used throughout the study. To avoid influence of cytotoxicity on H295R cells, cell viability was checked with an MTT bioassay, and only the non-cytotoxic doses (>80% of cell viability compared to that of control) were used for evaluation of effects on hormone production (Hecker et al., 2006) and steroidogenic gene transcription (Hilscherova et al., 2004). In brief, H295R cells were exposed to chemicals for 48 h, and culture medium and remaining cells were used for hormone measurements and gene transcription analysis, respectively.

Binding affinity to estrogen receptor was measured using MVLN cell line following Snyder et al. (2001) and Preuss et al. (2006). A 250 μl culture medium (12.5×104 cells/ml) was added per well and was incubated for 24 h in a standard 96-well plate. After 72 h of incubation with 2.5 μl of a given OPFR, luciferase activity was measured using Steady^{Glo}-Lucifearse Assay System (Promega Corp., Madison, WI, USA). The relative potency of OPFRs was determined following Coors et al. (2003). The maximum response of 17β-estradiol (E2) was set to 100% and the relative light unit (RLU) for each sample well was calculated as a percentage of the maximum induction of luciferase activity (% E2 max Luc). All the tests were conducted in three replicates. For each plate, a blank and a solvent control were prepared. In order to evaluate estrogen receptor antagonism, 100 nM E2 was incubated with a given OPFR in methanol and a binding affinity of E2 was measured.

2.2.3 Zebrafish culture and exposure

Adult wild-type zebrafish (~4 m old) were obtained from a commercial vendor and were acclimated for >10 d in the laboratory aquaria filled with aerated dechlorinated water. Fish culture water was monitored routinely for chemistry: pH 7.2-7.8; total hardness 160-170 mg/L, dissolved oxygen >7 mg/l, and temperature 24 ± 2oC. Based on results of cytotoxicity tests and bioassays with H295R and MVLN cell lines, three OPFRs that showed more evident responses among the test chemicals were chosen, and zebrafish exposure was conducted for these three OPFRs. For each treatment, three replicates (3 fish for each replicate) were employed for each sex. Concentrations of sex hormones (E2, T and 11-KT) in blood plasma, and transcription of related genes, i.e., CYP17 and CYP19A in gonad, and VTG 1 in liver, were measured. Blood samples collected from the two fish of the same sex were pooled to increase the sample volume. The blood sample was centrifuged at 5000g for 5 min at 4^{o}C, and then the supernatant was collected and stored at -80oC before analysis. Male and female fish were exposed and measured separately. OPFRs that were evaluated (their target concentrations) included TDCPP (0, 0.04, 0.2 and 1 mg/l), TPP (0, 0.04, 0.2 and 1 mg/l), and TCP (0, 0.008, 0.4 and 0.2 mg/l). During the exposure, no significant mortality was observed. Fish were fed with *Artemia* nauplii (<24 h after hatching) twice a day, and exposure medium was renewed every other day. At day 14, all surviving fish were anesthetized with ice-cold water, and blood was collected using a glass capillary tube from caudal vein. The blood sample of fish was then placed in a 1.5 ml microcentrifuge tube for hormone analysis. Liver and gonad were collected and preserved in RNA Later® for subsequent RNA extraction.

2.2.4 Measurement of sex hormones and gene transcription in H295R cell and zebrafish

In both H295R medium and zebrafish plasma, sex steroid hormones were measured by competitive enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Cayman Chemical; Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]). In zebrafish, plasma sex hormones were measured in male and female fish separately. 11-ketotestosterone (11-KT) was also measured in zebrafish by ELISA with commercially available kits (Cayman Chemical, 11-ketotestosterone [Cat # 582751]). The intra- and inter-assay coefficients of variation (CV) were <30% (Detection limits: 19 pg/ml for E2, 6 pg/ml for T and 1.3 pg/ml for 11-KT).

Expression of mRNA was quantified by real-time polymerase chain reaction (PCR) (For details, see supplement). For H295R cells, total RNA was isolated from each well using a total RNA isolation kit (Agilent Technologies, Ontario, CA, USA). For fish, total RNA of liver and gonad were extracted by RNeasy mini-kit (QIAGEN). For both H295R cell and the fish, the complementary DNAs were synthesized from the purified RNA samples using iScript™ cDNA Synthesis kit (BioRad, Hercules, CA, USA). The ABI 7300 real-time polymerase chain reaction (PCR) system (Applied Biosystems) was used to perform quantitative real-time PCR. PCR reaction mixtures (20 μl) contained 10 μl TaqMan gene expression master mix, 1 μl TaqMan gene expression assay, 5 μl RNase-free water, and 4 μl cDNA template. Thermal cycling was 50℃ for 2 min, 95℃ for 10 min, 40 cycles at 95℃ for 15 s, and 60℃ for 1 min. Primer sequences of the steroidogenic genes and sulfotransferase (SULT) genes in H295R cells are shown in Table 2.1. Primer sequences of the related genes of fish are shown in Table 2.2.

The amount of PCR product was quantified by using the *Ct* number. For each selected gene, quantification was conducted in three replicate samples (n=3) which were repeated twice. The expression level of each target mRNA was normalized to that of a reference gene (*β-actin* gene) using ΔΔ Ct method (Livak and Schmittgen, 2001).

Table 2.1. Primers that were evaluated for gene expression in H295R cells by quantitative polymerase chain reaction (qPCR).

	ion No	ID	equence (5'- -3')[a]
β-actin	NM_001101	Hs99999903_m1	
CYP11A1	NM_001099773	Hs00167984_m1	
CYP11B2	NM_000498	Hs01597732_m1	
CYP17A1	NM_000102	Hs01124136_m1	
CYP19A1	NM_031226	Hs00903413_m1	
CYP21A2	NG_007941	Forward primer	AGAAATTCGGGCCCATCTACAG
		Reverse primer	CTCAATGGTCCTCTTGGAGTTCAG
		TaqMan probe	FAM-CACCACCACATCTTGC-NFQ
HSD3β2	NM_001166120	Hs00605123_m1	
17βHSD1	NM_000413	Forward primer	GTTCTGCTGCTGCCCTTTG
		Reverse primer	CTGGGCCGCACTCGAT
		TaqMan probe	FAM-CAGGCTCAAGTGGACCC-NFQ
17βHSD4	NM_000414	Hs00264973_m1	
SULT1A1	NM_001055	Forward primer	TGGGACCTGGGACAGCT
		Reverse primer	CCAGGATCCGTAGGACACTTC
		TaqMan Probe	FAM-TCCGACCATGAACTTC-NFQ
SULT2A1	NM_003167	Hs00193690_m1	
SULT2B1	NM_004605	Hs00234219_m1	
SULT1E1	NM_005420	Hs00190268_m1	

Table 2.2. Primers that were evaluated for gene expression in zebrafish by qPCR.

	ion	ID	equence (5’- -3’)
β-actin	NM_131031	Forward primer	CCATCGGCAATGAGCGTTTC
		Reverse primer	CAAGATTCCATACCCAGGAAGGA
		TaqMan probe	FAM-CCCGAGGCTCTCTTC-NFQ
VTG1	NM_001044897	Forward primer	GGAGGAATCCATGAAGCTCTTCTAAA
		Reverse primer	TGCTCTCAGTGTACGCTTAATCTTT
		TaqMan probe	FAM-ACGGTCAACACTTTCATCTG-NFQ
CYP17	NM_212806	Forward primer	GGACTCCAGTGTTGGTGAATACA
		Reverse primer	GGGTTCTTCCATTCCTTCTCATCAT
		TaqMan probe	FAM-CCCGCGTCCCTTTC-NFQ
CYP19A	NM_131154	Forward primer	GGCTGCACAAGAAGCACAA
		Reverse primer	CAGAGCTGTGATGGCATCCT
		TaqMan probe	FAM-CTCCTGAGCATCTCTC-NFQ

2.2.5 Statistical analysis

Data were checked for distribution of normality and homogeneity of variances by use of Shapiro-Wilk's and Levene's test, respectively. One-way analysis of variance (ANOVA) with Dunnett's test or Kruskal-Wallis test as well as linear regression analysis were performed using SPSS 16.0 for Windows® (SPSS, Chicago, IL, USA) to test for differences among all treatments, or dose-response relationship. Differences with $p < 0.05$ were considered significant.

2.3 Results

2.3.1 Cytotoxicity of OPFRs in H295R and MVLN cells

Based on MTT assay, TDCPP, TPP, and TCP had greatest cytotoxicity in H295R cells (i.e., showing <80% cell viability at 10 mg/l and greater), followed by TCEP and TBEP (<80% cell viability at 100 mg/l and greater). TCPP did not show cytotoxicity even at 100 mg/l (data not shown). Only non-cytotoxic doses were used for the H295R assay. The MTT assay was also conducted using MVLN cells, and only non-cytotoxic doses were used for the MVLN luciferase assay.

2.3.2 Hormone synthesis and related gene transcription in H295R cell

All six OPFRs significantly increased E2 and T production by H295R cells. The ratio between E2 and T (E2/T ratio) increased as well. However the potency varied by chemical (Figure 2.1A, 2.1B). TDCPP showed a statistically significant increase in both E2 and T at concentrations as low as 0.01 mg/l. Statistically significant increase of both sex hormones was noted at 0.1 mg/l for TCEP and TCP, at 1 mg/l for TBEP and TPP, and at 100 mg/l for TCPP. Significant increase of E2/T ratio was observed at 0.01 mg/l for TCP and TDCPP, at 0.1 mg/l for TCEP and TPP, at 1 mg/l for TBEP, and at 100 mg/l for TCPP (Figure 2.1C). Exposure to forskolin increased E2 and T concentrations, as well as E2/T ratio (Figure 2.2).

All six OPFRs influenced transcription of important steroidogenic genes such as *CYP11A1* and *CYP11B2* in dose dependent manner (Figure 2.3A, 2.3B). Transcription of *CYP19A1* was significantly up-regulated when exposed to TCEP, TDCPP, TPP, or TCP (Figure 2.3C). Transcription of *HSD3β2* was up-regulated after exposure to TCEP, TCPP, TDCPP, or TCP (Figure 2.3D). While transcription of *CYP17A1* was down-regulated by TCPP treatment, transcription of *CYP21A1*, *17βHSD1* and *17βHSD4* were not interfered by any test OPFRs (Figure 2.4). For forskolin that was used as a positive control chemical, all eight steroidogenic mRNAs were up-regulated

(Figure 2.5).

The transcription of *SULT1E1* gene was down-regulated when exposed to 1 mg/l TDCPP, TPP, or TCP, and to 10 mg/l for TCEP, TCPP or TBEP dose-dependently (Figure 2.6A). SULT2A1 gene transcription was also down-regulated when exposed to 0.1 mg/l for TDCPP, 1 mg/l for TPP or TCP, or 10mg/L for TCEP, TCPP or TBEP (Figure 2.6B). The transcription of *SULT1A1* and *SULT2B1* genes was not interfered by any OPFRs that were studied (Figure 2.7). Forskolin down-regulated transcription of *SULT1E1*, *SULT2A1*, and *SULT2B1* genes, but not *SULT1A1* gene (Figure 2.8).

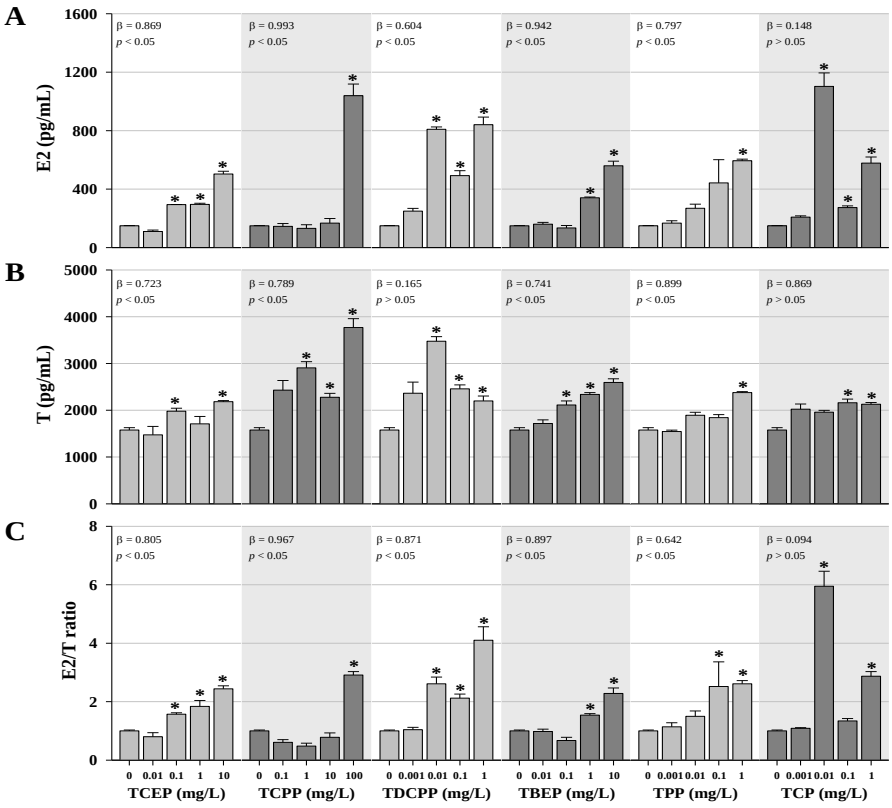


Figure 2.1. Effects on steroid hormone 17β-estradiol (E2) concentration (A), testosterone (T) concentration (B) and E2/T ratio (C) in H295R cell line after exposure to TCEP (0.01-10 mg/L), TCPP (0.1-100 mg/L), and TDCPP (0.001-1 mg/L), TBEP (0.01-10 mg/L), TPP (0.001-1 mg/L), and TCP (0.001-1 mg/L) for 48 h. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO). The β and p value were determined based on linear regression analysis.

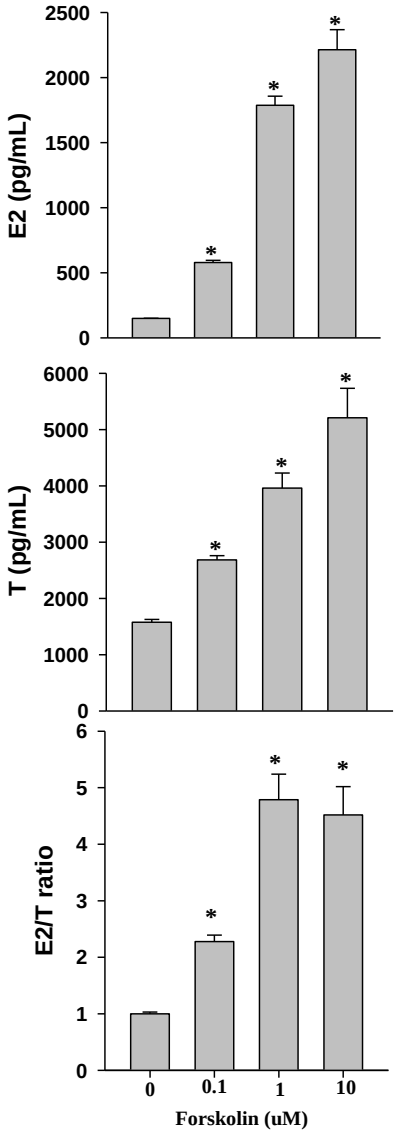


Figure 2.2. Changes on steroid hormone 17β-estradiol (E2) concentration (A), testosterone (T) concentration (B), and E2/T ratio (C) in H295R cell line after exposure to forskolin (0.1-10 μM) for 48 h. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO).

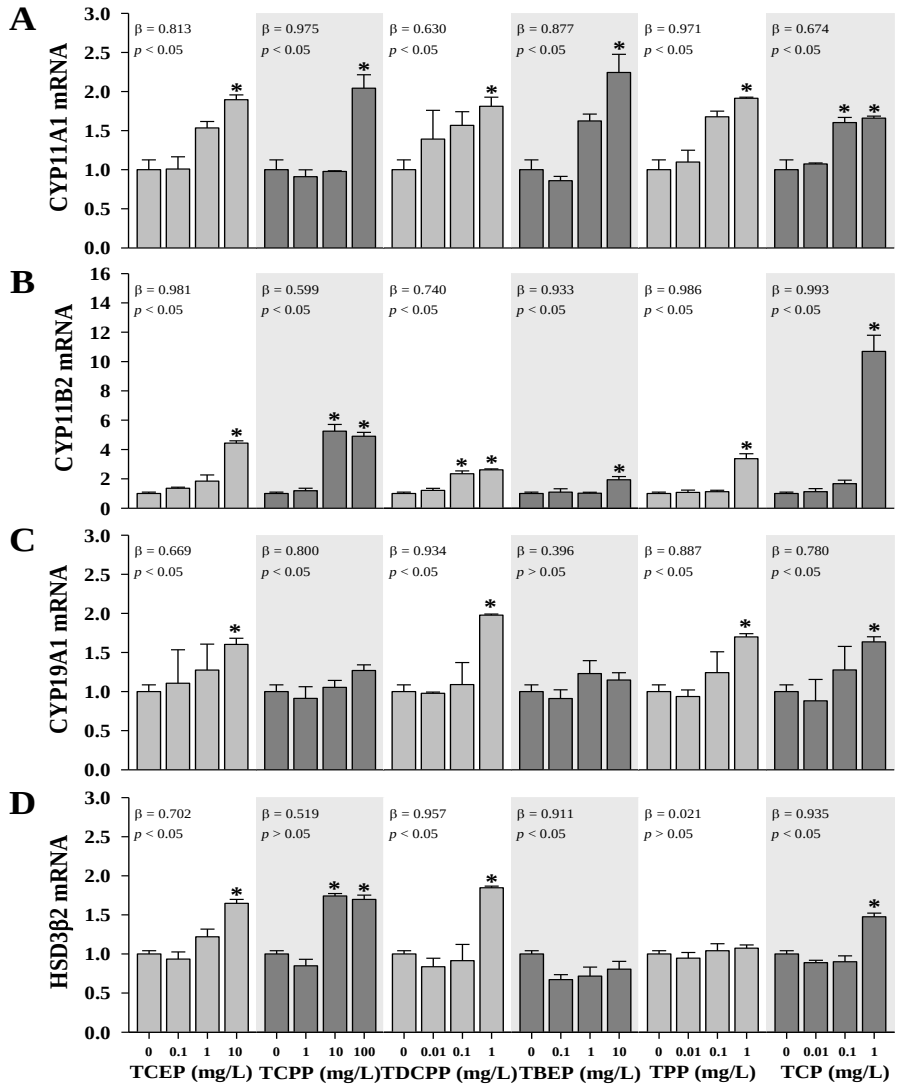


Figure 2.3. Effects on *CYP11A1* (A), *CYP11B2* (B), *CYP19A1* (C), and *HSD3β2* (D) gene transcription in H295R cell line after exposure to TCEP (0.1-10 mg/L), TCPP (1-100 mg/L), TDCPP (0.01-1 mg/L), TBEP (0.1-10 mg/L), TPP (0.01-1 mg/L), and TCP (0.01-1 mg/L) for 48 h. The results are shown as mean ± SD of three replicate. Asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO). The β and p value were determined based on linear regression.

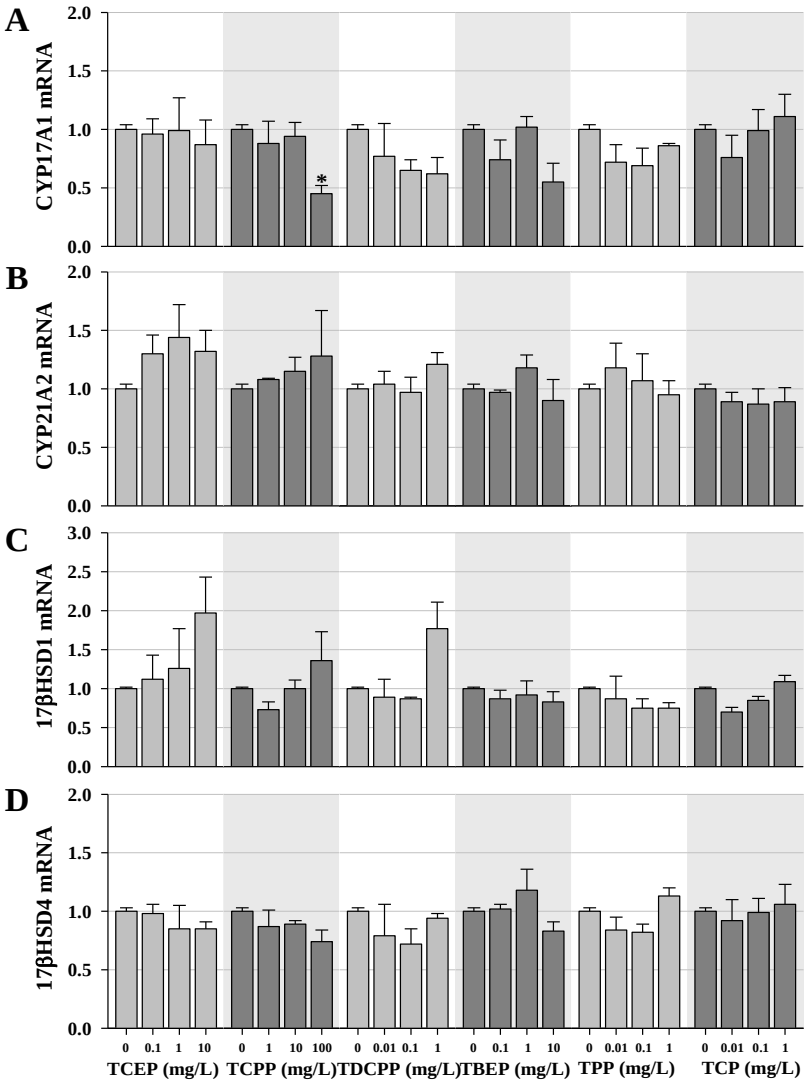


Figure 2.4. Effects on *CYP17A1* (A), *CYP21A2* (B), *17βHSD1* (C), and *17βHSD4* (D) gene transcription in H295R cell line after exposure to TCEP (0.1-10 mg/L), TCPP (1-100 mg/L), TDCPP (0.01-1 mg/L), TBEP (0.1-10 mg/L), TPP (0.01-1 mg/L), and TCP (0.01-1 mg/L) for 48 h.

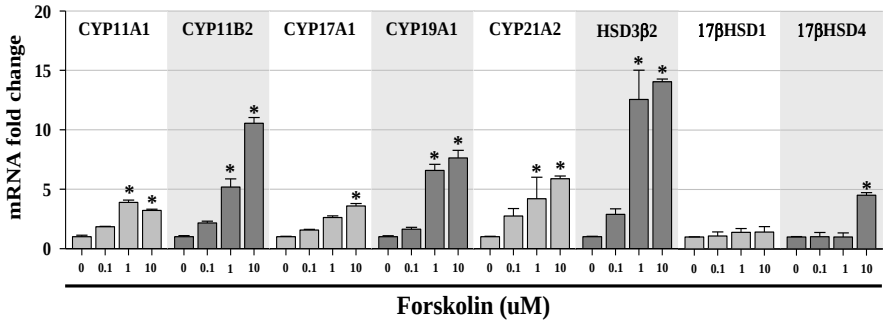


Figure 2.5. Expression of eight steroidogenic gene transcription in H295R cell line after exposed to forskolin (0.1-10 μM) for 48 h.

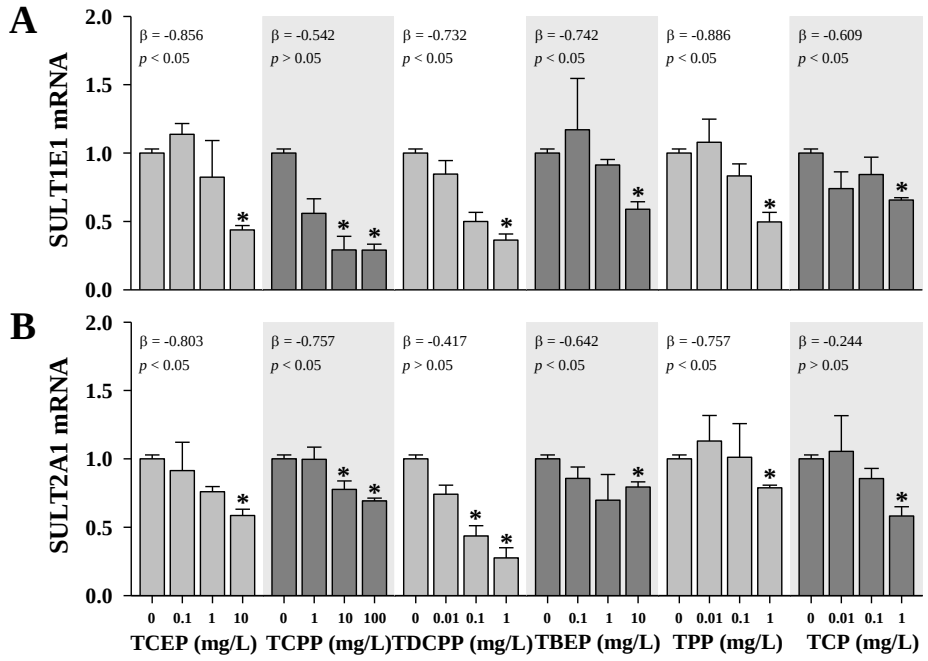


Figure 2.6. Effects on *SULT1E1* (A) and *SULT2A1* (B) gene transcription in H295R cell line after exposure to TCEP (0.1-10 mg/L), TCPP (1-100 mg/L), TDCPP (0.01-1 mg/L), TBEP (0.1-10 mg/L), TPP (0.01-1 mg/L), and TCP (0.01-1 mg/L) for 48 h. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO). Each treatment group has conducted linear regression analysis, β and p value was provided.

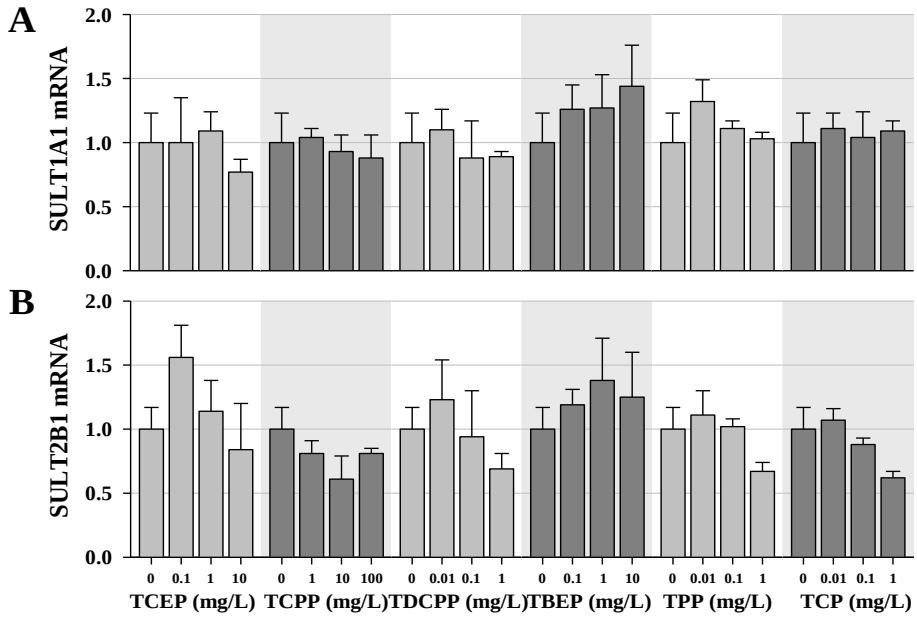


Figure 2.7. Effects on SULT1A1 (A) and SULT2B1 (B) gene transcription in H295R cell line after exposure to TCEP (0.1-10 mg/L), TCP (0.01-1 mg/L), TBEP (0.1-10 mg/L), TPP (0.01-1 mg/L), TCPP (1-100 mg/L), and TDCPP (0.01-1 mg/L) for 48 h.

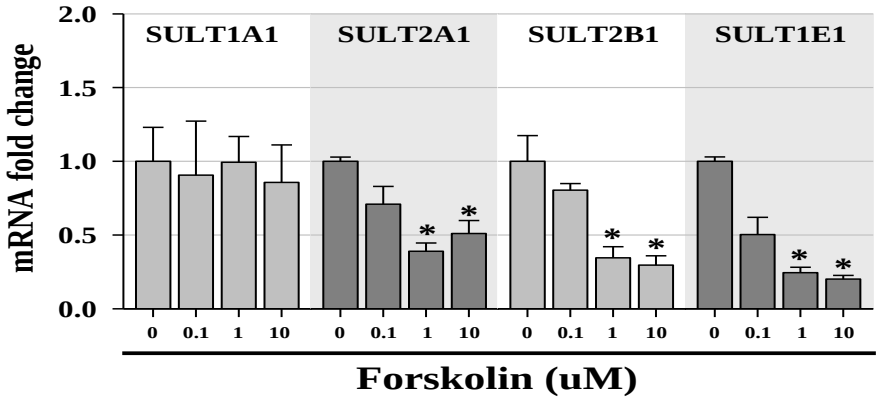


Figure 2.8. Expression of four sulfotransferase (SULT) gene transcription in H295R cell line after exposure to forskolin (0.1-10 uM) for 48 h.

2.3.3 MVLN luciferase assay

Binding affinity to E2 receptor was not detected for TCEP, TDCPP, TBEP, TPP or TCP in MVLN cells. However, exposure to TDCPP, TPP or TCP significantly reduced the binding affinity of E2 to estrogen receptor in a concentration-dependent manner. Statistically significant reduction in the binding affinity was observed at concentrations as low as 10, 0.001, and 0.01 mg/l for TDCPP, TPP, and TCP, respectively (Figure 2.9).

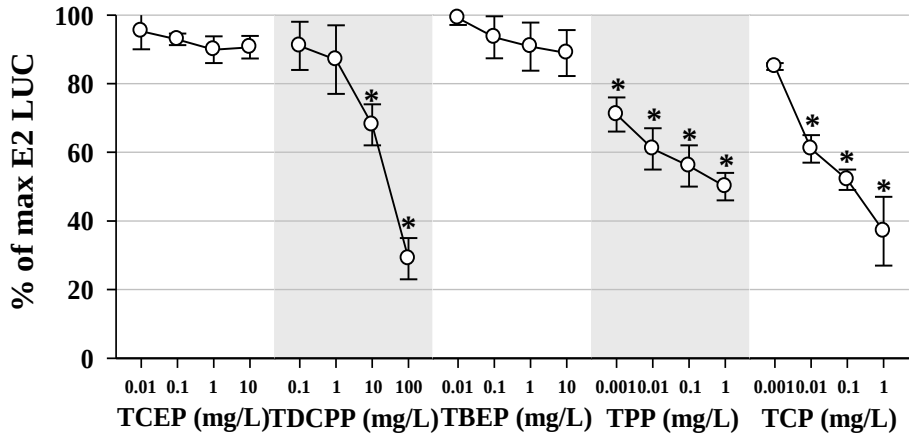


Figure 2.9. Effects of TCEP, TDCPP, TBEP, TPP, and TCP in co-incubation with 100 nM 17β-estradiol (E2) in the MVLN cell luciferase assay. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from control (100 nM E2).

2.3.4 Effect on plasma sex hormones and related gene transcription in zebrafish

Plasma E2 concentration significantly increased in male fish after exposure to 1 mg/l for TDCPP or TPP, or to 0.2 mg/l for TCP (Figure 2.10A), while T and 11-KT concentrations generally decreased (Figure 2.10B and C). Both E2/T and E2/11-KT ratios significantly increased at l mg/l for TDCPP or TPP, or 0.2 mg/l for TCP in male fish (Figure 2.10D and E). In female fish, the plasma E2 concentrations significantly increased after exposure to 1 mg/l TDCPP and TPP, while T concentrations also significantly increased after exposure to 1 mg/l TDCPP, or 0.2 mg/l TCP (Figure 2.10A and B). Plasma 11-KT, however, did not show significant changes (Figure 2.10C). Among the female zebrafish, E2/T ratio significantly decreased at l mg/l for TDCPP, or 0.2 mg/l for TCP (Figure 2.10D), while E2/11-KT increased after exposure to l mg/l TDCPP or TPP, or 0.2 mg/l TCP (Figure 2.10E).

Changes in transcription of a couple of steroidogenic genes and vitellogenin gene were observed. In male fish, exposure to 1 mg/l of TDCPP and TPP significantly increased *CYP17* and *CYP19A* gene transcription. *CYP19A* gene transcription was up-regulated by 0.2 mg/l TCP, but such up-regulation was not evident for *CYP17* gene (Figure 2.11A and B). Transcription of *VTG1* gene increased when exposed to as low as 1 mg/l TDCPP, 0.04 mg/l TPP, or 0.2 mg/l TCP among male fish (Figure 2.11C). In female fish, exposure to 1 mg/l of TDCPP or TPP significantly increased the transcription of *CYP17* and *CYP19A*. *CYP17* gene transcription was significantly up-regulated by 0.04 and 0.2 mg/l TCP, but such up-regulation was not clear for *CYP19A*. Among female fish, expression of *VTG 1* decreased when exposed to as low as 0.2 1 mg/l TDCPP, 1 mg/l TPP or 0.04 0.2 mg/l TCP (Figure 2.11A, B and C).

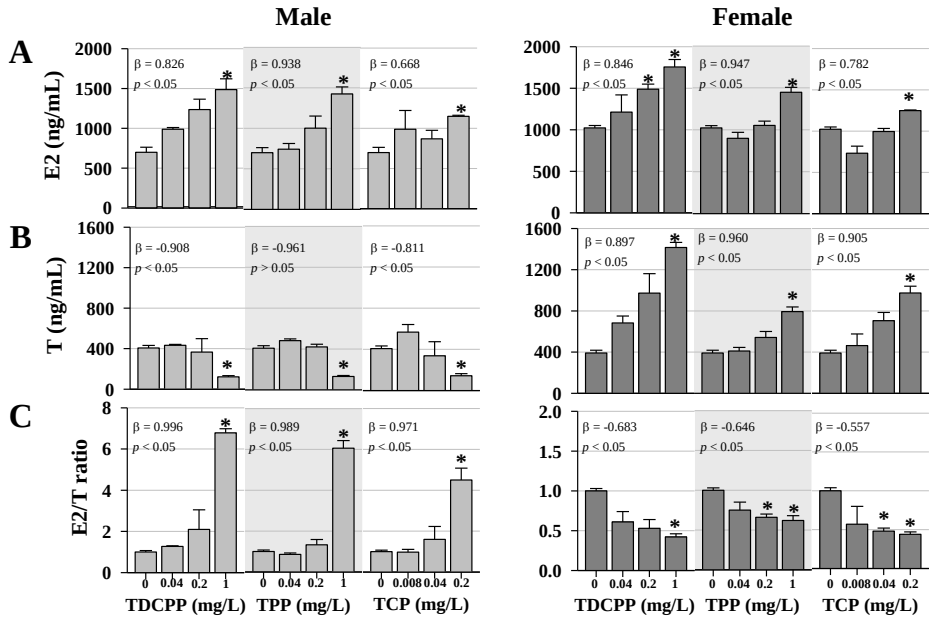


Figure 2.10. Effects on plasma 17β-estradiol (E2) concentration (A), testosterone (T) concentration (B), 11-ketotestosterone (11-KT) concentration (C), E2/T ratio (D), and E2/11-KT ratio (E) in male and female zebrafish exposed in 0.04, 0.2, or 1 mg/L of TDCPP, 0.04, 0.2, or 1 mg/L of TPP, and 0.008, 0.04, or 0.2 mg/L of TCP for 14 d. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.01% DMSO). The β and p values were determined based on linear regression analysis.

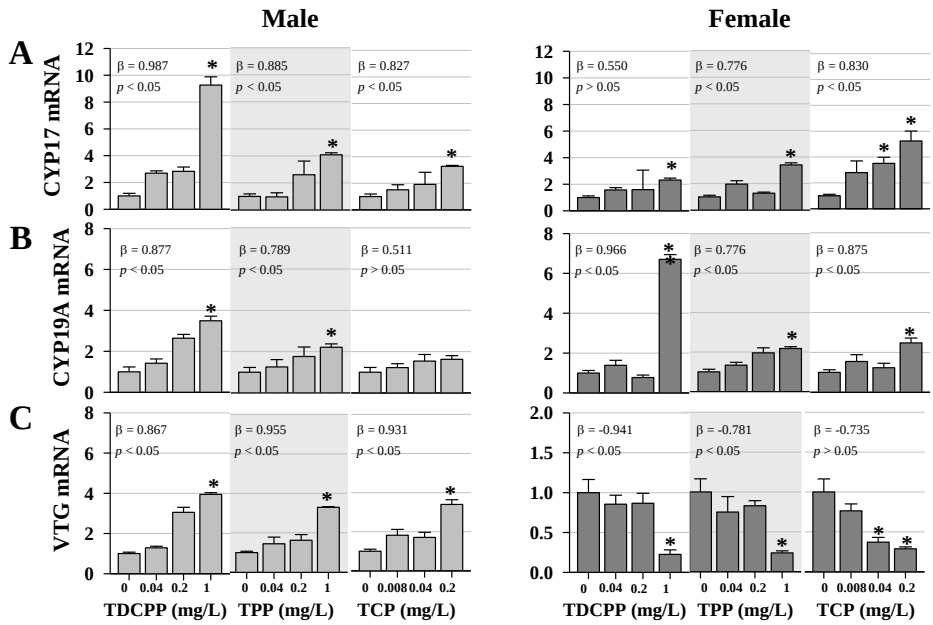


Figure 2.11. Effects on *CYP17* (A), *CYP19A* (B), and *VTG 1* (C) gene transcription in male and female zebrafish gonad and liver exposed in 0.04, 0.2, or 1 mg/L of TDCPP, 0.04, 0.2, or 1 mg/L of TPP, and 0.008, 0.04, or 0.2 mg/L of TCP for 14 d. The results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.01% DMSO). The β and p values were determined based on linear regression analysis.

2.4 Discussion

Both *in vitro* and *in vivo* observations suggest evidences of endocrine disruption by OPFRs. Results of in vitro assays showed that all six OPFRs have the potential to disrupt steroidogenic pathway and increase E2/T ratio. More than one mechanism is involved in this effect. These compounds could influence the synthesis of E2 and T by altering transcription of major steroidogenic genes such as *CYP11A1*, *HSD3β2* and *CYP19A1*. CYP11B2 which regulates the synthesis of aldosterone was most sensitively influenced by the exposure to all six OPFRs. HSD3β2 is a steroid-metabolizing enzyme that is essential for adrenal production of mineralocorticoids and glucocorticoids. The up-regulation of *HSD3β2* suggests the possibility of OPFRs to influence cortisol production. Transcriptions of *CYP19A1*, which catalyzes the conversion of T to E2, increased by exposure to TCEP, TDCPP, TPP, or TCP. T synthesis increased by exposure to OPFRs, however, the increase in E2 synthesis generally outweighed that of T and hence E2/T ratio increased by the exposure. Up-regulation of *CYP19A1* and increased E2/T ratio suggest that exposure to OPFRs could result in an estrogenic responses by enhancing production of and conversion to endogenous E2.

Secondly, OPFRs could inhibit transcription of several *SULT* genes such as *SULT1E1* and *SULT2A1* in H295R cell line, suggesting that OPFRs increase E2 concentration through inhibition of E2 metabolism. SULT enzymes may inactivate E2 by sulfonation (Hanet et al., 2008) and hence transcription of SULT genes may be used to determine effects of chemicals on endocrine system. Prusakiewicz et al. (2007) showed that parabens might increase estrogenicity as a result of inhibition of estrogen sulfotransferase activity. Four major SULT enzymes (SULT1A1, SULT1E1, SULT2A1, and SULT2B1) of which gene transcriptions were measured in the present study are well distributed in human beings, and are responsible for conjugation of sulfonate to E2 or its precursors (Gamage et al., 2006). SULT1A1 is phenolsulfotransferase, which accepts estrogens as substrates at relatively

high concentration (Harris et al., 2000). SULT2A1 is responsible for the sulfonation of deoxycorticosterone and dehydroepiandrosterone as well as pregnenolone and 17-hydroxypregnenolone in human adrenal glands (Dujaili et al., 2010). *SULT1E1* is a major isoform for the inactivation of E2 that regulates the activity of E2 at physiological concentrations (Zhang et al., 1998). The *SULT2B1* isoforms are highly selective for the sulfation of 3β-hydroxysteroids including DHEA and prognenolone, which are found in several human tissues including adrenal cortex (Dongning et al., 2004). Along with up-regulation of steroidogenic genes, down-regulation of transcription of *SULT1E1* and *SULT2A1* may be linked to increased endogenous E2 concentrations by OPFRs. In contrast, estrogen receptor antagonism was suggested by MVLN assay for TDCPP, TPP, and TCP, while no OPFRs showed binding affinities to estrogen receptor of MVLN cell. This observation is comparable to a couple of studies that suggested androgenicity of TPP in rat uterine cytosol (Fang et al., 2000, 2003).

A few OPFRs were reported to exhibit endocrine disruption potential in both in vitro and in vivo studies, although many of them are remained less studied. TPP was reported to activate the enzymes involved in steroid hormone metabolism in vitro (Honkakoski et al., 2004). TCEP is also reported to be a reproductive toxicant (Matthews et al., 1993) which could lead to a decrease in fertility in both rats and mice (Beth-Hubner, 1999). However adverse effects of TCPP and TBEP on endocrine disruption have rarely been reported to date (World Health Organization, 2000). In the present study, we demonstrated that both TCPP and TBEP might also lead to endocrine disruption by altering steroidogenic processes in cell lines. Further investigation on implications of such endocrine disruption is needed with aquatic organisms.

In zebrafish, exposure to TDCPP, TPP or TCP led to disruption of sex hormone balances (Figure 2.5). While effects of the OPFRs on E2 hormone production were generally similar, the effects on T and 11-KT were different by sex (Figure 2.5A-2.5C). Based on E2/T ratio, exposure to OPFRs resulted in estrogenicity among males and generally androgenicity among females

(Figure 2.5D). However the E2/11-KT ratio showed generally the same direction in both sexes, i.e., higher E2/11-KT ratio, although the extent of increase was much greater among the male fish (Figure 2.5E). Decrease of 11-KT concentrations among male fish may affect the spermatogenesis and potentially fertility of the fish. Since 11-KT plays an important role in the spermatogonial proliferation, decreased 11-KT level may arrest meiosis and germ cell maturation (Schulz et al., 2010). The ratio of sex hormones could be used as a sensitive biomarker of abnormal sex hormones in fishes (Orlando et al., 2004). Alteration of sex hormone balance in zebrafish was in part accounted for by the changes in transcriptions of *CYP17* and *CYP19A* in the gonad (Figure 1.6). Unlike TDCPP and TPP, alterations of *CYP17* and *CYP19A* gene transcription by exposure to TCP were different by sex, suggesting different mechanisms of steroiogenic alteration of this compound in fish (Figure 2.6A, 2.6B). The transcription of *VTG 1* gene, a frequently used biomarker of endocrine disruption, decreased in female fish by exposure to TDCPP, TPP or TCP, but increased in male fish. VTG is essential for oocyte maturation and successful reproduction of female fish, is synthesized in the liver of female fish in response to estrogens (Liu et al., 2009). While *VTG* gene transcription may not be as biologically relevant as the VTG protein, up-regulation of *VTG 1* mRNA coupled with increased E2/T or E2/11-KT ratios strongly suggest potential estrogenic activities of TDCPP, TPP or TCP especially in male fish. In contrast, down-regulation of *VTG 1* mRNA was coincided with decreased E2/T ratio but not with E2/11-KT ratio in female fish, which deserves further study.

The mechanisms underlying the sex dependent responses in zebrafish are not clear. Physiological differences by sex including differences in gonadotropins (FSH, LH, or prolactin) and thyroid hormones may explain the observed differences by sex. Sex dependent responses in hormone balance were also reported in zebrafish after exposure to 8:2 fluorotelomer alcohols (Liu et al., 2010). While exposure to 8:2 fluorotelomer alcohols significantly increased the concentrations of both E2 and T in plasma among female zebrafish, the exposure resulted in marked decrease of T and increase of

plasma E2 concentrations among the male fish. These observations might be due to the differences by sex in regulations of transcription of FSHβ and LHβ genes in the pituitary.

Effects on male reproduction have been reported for several OPFRs. Exposure to high level of TDCPP (80 mg/Kg-d oral dose) significantly increased interstitial-cell tumors and histopathological abnormalities in testis or seminal vesicle of male rat (NRC 2000). Besides, butylated TPP and TCP have been associated with endocrine effects, which included reduced male fertility (Latendresse et al., 1994). Meeker and Stapleton (2010) suggested that the exposure to TDCPP or TPP might be associated with reduced sperm concentrations among male human adults. Considering the decrease of T and increase of E2 concentrations in plasma are often associated with reduced semen production and density in male fish (Liu et al., 2010), our observation of alterations in sex hormone balances and transcriptions of steroidogenic genes in zebrafish suggests potential reproduction related effects of OPFRs on aquatic organisms. The concentrations that were determined to be effective in endocrine disruption in the present study were several orders of magnitude greater than those reported in the water environment such as lake water, rain water, effluents of wastewater treatment plants, or storm-water runoff (Bacaloni et al., 2008; Fries and Püttmann, 2003; Martinez-Carballo et al., 2007; Regnery and Püttmann, 2010a; 2010b). Thus, the direct effects of OPFRs on endocrine systems may not be expected at their current levels of occurrence.

Chpater 3. Effects of TDCPP or TPP on reproduction and hormones and gene transcriptions of HPG axis in adult zebrafish (*Danio rerio*)

3.1 Introduction

Among the OPFRs, tris(1,3-dichloro-2-propyl)phosphate (TDCPP) and triphenyl phosphate (TPP) have been widely used in polyurethane foams which are commonly found in sofas, chairs, and car upholstery (Marklund et al., 2003; Reemtsma et al., 2008; Stapleton et al., 2009). These chemicals have been also detected in effluents of sewage treatment plants (STPs), indicating the inputs from households, industrial sites, and stormwater (Mihajlovic et al., 2011; Regnery and Puttmann, 2010). High concentrations of TDCPP up to several μg/Lwere detected in water at waste disposal sites (Kawagoshi et al., 1999). TDCPP was also detected in effluents of a sewage treatment plant (STP) at 3 μg/L (Marklund et al., 2005) and in perch caught from the ambient water near STPs at 36-140 ng/g lipid weight (Sundkvist et al., 2010). TPP is more degradable than TDCPP, however the concentrations of TPP detected in marine organisms showed the similar range of detection in perch, i.e., between 21 and 180 ng/g (Sundkvist et al., 2010). Metabolites of TDCPP and TPP have also been reported in human urine (Cooper et al., 2011). As to aquatic toxicity, for triaryl and chlorinated phosphates, TPP and TDCPP seem to have the highest toxicity to aquatic organisms, with 96 h LC_{50} values of 0.36 and 1.1 mg /L, respectively, for rainbow trout (Environmental criteria 209). TCPP and TCEP are even less toxic to fathead minnow (51 mg/L) and goldfish (90 mg/L), respectively (Environmental criteria 209). Further, low to moderate acute oral toxicities have been reported for chlorinated OPFRs. Therefore, further toxicity and underlying of TDCPP or TPP was investigated.

While information on endocrine disruption capacity of OPFRs is generally limited (CPSC 2006; NRC 2000; U.S. EPA 2005), but available reports suggest that some OPFRs may cause adverse effects on endocrine system and even on reproduction. TPP could bind with androgen receptor (Fang et al., 2003) and activate enzymes involved in steroid hormone metabolism *in vitro* (Honkakoski et al., 2004). Besides, butylated TPP could reduce male fertility and alter female reproductive cycles in rats (Latendresse et al., 1994). In rats, TDCPP could induce a tumor in testicular interstitial cells and increase abnormalities in the testis, epididymis and seminal vesicle (NRC 2000). Among humans, potential association between OPFRs and hormonal changes was suggested. TDCPP and TPP in house dust levels were correlated with the altered thyroid hormone levels, sex steroid hormone and prolactin levels, as well as decreased semen quality in men (Meeker and Stapleton, 2010). We previously reported that TDCPP and TPP could disrupt sex hormone balance and transcriptions of steroidogenic genes in H295R cell, a human adrenal cell. Both compounds could also disturb sex hormone balances in zebrafish after 14 d exposure (Liu et al., 2012).

Steroid hormones are important regulators of reproductive processes; they would play direct roles on gametogenesis and reproductive maturations. Endocrine disruptors can either interact with receptors or alter expression of the enzymes that involved in synthesis and metabolism of steroid hormones, and therefore may eventually impair reproduction (Hilscherova et al., 2004). In fish, reproduction is closely regulated by the hypothalamus-pituitary-gonadal axis (HPG) (Sofikitis et al., 2008). Alteration of HPG axis regulation is therefore often associated with adverse consequences on endocrine system and reproduction (Sofikitis et al., 2008; Zhang et al., 2008). Steroidogenesis in gonadal tissues is also important in reproduction processes (Sofikitis et al., 2008). Steroid hormone have direct effect on gametogenesis and reproductive maturation, endocrine disruptor can directly disturb steroid hormone synthesis or metabolism, and then, impair reproduction (Ma et al., 2012),

Zebrafish (*Danio rerio*) is an attractive model organism for evaluating

reproductive toxicity and endocrine-disrupting effects of xenobiotics, because of its small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Segner, 2009). In the present study, I employed zebrafish and measured effects of TPP and TDCPP on reproduction-related parameters and the transcription of relevant genes of the HPG axis in the fish, after a 21 d exposure.

3.2 Materials and methods

3.2.1 Preparation of testing materials

TDCPP (CAS No. 13674-87-8, purity 95%) and TPP (CAS No. 115-86-6,) were purchased from Chem Service (West Chester, PA, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Dimethyl sulfoxide (DMSO) was used as solvent.

3.2.2 Fish maintenance and exposure design

Wild-type adult male and female zebrafish (4 – 5 m old) were obtained from a local supplier and acclimated for > 1 month in a temperature-controlled room at 27℃ ± 1℃ in the Environmental Toxicology Laboratory at Seoul National University (Seoul, Korea). The fish were maintained under a photoperiod of 14:10 h light:dark and fed with *Artemia nauplii* (< 24 h after hatching) *ad libitum* twice daily. The fish were cultured and maintained in filtered tap water after aeration for > 24 h. Water quality parameters, including pH, conductivity, temperature, and dissolved oxygen, were measured regularly.

Adult zebrafish were exposed to 0, 0.04, 0.2, or 1.0 mg/L of TDCPP or TPP for 21 d. Four replicates with three pairs of fish per replicate were used in each treatment or as a control (n = 12 pairs per treatment). A 15-L glass tank was used as a test vehicle and the test medium was renewed every other day. As a solvent, 0.005% (v/v) DMSO was used in both treatments and controls. Among the exposed fish, six pairs per treatment were examined to assess reproduction. Spawned eggs were collected and enumerated 2 h after the light was turned on every morning. The frequency of spawning and the number of eggs per spawning event were recorded throughout the entire test period.

After 21 d of exposure, all fish were anesthetized, and body weights and lengths were measured. Gonads and livers were removed and weighed, and gonadosomatic index (GSI = 100 × [gonad weight (g)/body weight (g)]), hepatosomatic index (LSI = 100 × [gonad weight (g)/body weight (g)]), and

condition factor (CF = 100 × [body weight (g)/total length^3 (cm)]) values were calculated.

Samples of 4 – 10 L of blood per fish was collected from the caudal vein and stored in heparinized microcapillary tubes. Blood was sampled from five fish per treatment group or the controls. Collected blood samples were centrifuged at 5000 × *g* for 20 min at 4℃ and the supernatants were separated and stored at –80℃ until analysis. The brain, gonads, and liver were dissected out from each fish, weighed, and preserved in 250 μL of RNA Later reagent (Qiagen, Korea Ltd., Seoul, Korea) at –80℃ until further analysis.

3.2.3. Chemical analysis

The actual concentrations of TPP or TCDPP were measured from the exposed media using a gas chromatograph interfaced with a mass spectrometer (GC/MS). Water samples from each treatment group were taken directly from the test beakers before (0 h) and after 48 h of exposure (48 h), and were stored at –80℃ until chemical analysis. The following procedures were used for extraction and measurement. Water samples (20 mL) were spiked with surrogate standard (tri-*n*-butyl-d_{27} phosphate; CDN Isotope, Pointe-Claire, QB, Canada) and extracted in 5 mL of DCM for 30 min with mechanical shaking. The samples were extracted twice with 5 mL of DCM. Anhydrous sodium sulfate was added to the DCM extracts to remove any residual water. The extracts were concentrated to approximately 1 mL under a stream of nitrogen gas, and were added to 5 mL of *n*-hexane for solvent exchange. A GC/MS (Agilent 7890A/5975C MSD; Agilent Technologies, Wilmington, DE) was used for identification and quantification. The MS was operated under positive electron impact mode and ions were monitored using the selected ion monitoring (SIM) mode. The capillary column used to separated TDCPP and TPP was a DB5-MS (30 m length, 0.25 mm inner diameter, 0.25 m film thickness; J&W Scientific, Palo Alto, CA). The recovery rate of spiked tri-*n*-butyl-d_{27} phosphate was 106%. Solvents that were injected before and after the injection of standards showed negligible contamination or carryover. Procedural blanks were processed with each set

of 9 water samples to check for laboratory contamination. Blanks did not contain quantifiable amounts of TDCPP or TPP. Limits of detection (LOD) for TDCPP (0.06 ng/mL) and TPP (0.12 ng/mL) were calculated as 3 times the signal to noise ratio.

3.2.4 Sex hormone and vitellogenin measurement

Plasma sex steroid hormones were measured in both male and female fish by competitive enzyme-linked immunosorbent assay (ELISA) using commercially available kits (testosterone [Cat # 582701], 17β-estradiol [Cat # 582251]; Cayman Chemical, Ann Arbor, MI). 11-Ketotestosterone (11-KT) was measured by ELISA using a kit (11-ketotestosterone [Cat # 582751; Cayman Chemical]). Intra- and inter-assay coefficients of variation (CV) were < 30% (detection limits: 19 pg/mL for estradiol (E2), 6 pg/mL for testosterone (T), and 1.3 pg/mL for 11-KT). Plasma vitellogenin (VTG) levels were measured in both male and female fish using a zebrafish ELISA kit (Biosense Laboratory, Bergen, Norway) in accordance with the manufacturer's instructions. All samples and standards were run in triplicate. The coefficients of variation for these assays were < 10%.

3.2.5 RNA isolation and qRT-PCR

Total RNA was extracted from brains and gonads using RNeasy minikits (Qiagen). cDNAs were synthesized from purified RNA samples using iScript™ cDNA Synthesis kits (BioRad, Hercules, CA). Quantitative real-time PCR was performed with SYBR Green PCR kits (Toyobo, Tokyo, Japan) using an ABI 7300 system (PerkinElmer Applied Biosystems, Foster City, CA). PCR mixtures (20 L) contained 1.8 L (0.9 μM) of forward and reverse primers, 3 L of cDNA sample, and 10 L of SYBR Green™ PCR Master Mix (Applied Biosystems). Primer sequences for 22 selected genes, in addition to a housekeeping gene (*β-actin*), are listed in Supplementary Table S1. The PCR profile consisted of an initial denaturation step at 50℃ for 2 min, 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. The amount of PCR product obtained was quantified using the

threshold cycle (Ct) number, which corresponds to the cycle at which an increase in the signal associated with exponential growth in the PCR product was detected. For each selected gene, real-time PCR was performed for three replicate samples that were repeated twice. The expression level of each target mRNA was normalized to that of the housekeeping gene using the *Ct* method.

3.2.6 *Ex vivo* brain exposure

Ex vivo whole-brain cultures that included the hypothalamus and pituitary were produced using the previously described method (Tomizawa et al., 2001). TDCPP and TPP were dissolved in DMSO and diluted with DMEM/F12 medium (Sigma). Then, 1 mL of culture medium containing TDCPP (0.04, 0.2, or 1 mg/L) or TPP (0.04, 0.2, or 1 mg/L) was added to each well of a 6-well microplate (Falcon, Franklin Lakes, NJ). Intact brain tissue that was freshly excised from a male or female zebrafish was then placed in the well for 48 h. After exposure, the tissue was collected and gene transcription levels were determined. Six samples were used for each treatment and the control.

3.2.7 Statistical analyses

Statistical analyses were performed using SPSS® (version 18.0; SPSS Inc., Chicago, IL). Levels of gene transcription in tissues were expressed as fold changes relative to the solvent control. The Shapiro–Wilk test and Levene's test were used to evaluate the normality of distributions and homogeneity of variances, respectively. Differences between groups were tested using Dunnett's one-way analysis of variance (ANOVA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

3.3 Results

3.1. Concentrations of TDCPP or TPP in exposure media

The measured concentrations of TDCPP were consistent over the renewal interval, i.e., 48 h (Table 3.1). However, measured concentrations of TPP after 48 h exposure were less than that of the fresh water sample, suggesting greater degradability of TPP and possible underestimation the toxicity. For simplicity, the nominal concentrations were used for the presentation of the results in this paper.

3.3.1 Effects on K, GSI, HSI and reproduction

No mortality was observed in any of the experimental concentrations during the exposure period. No significant differences were seen in CF, GSI, or HSI in both exposed male and female fish (Table 3.2). Although statistical significance was not observed, GSI in the male fish decreased at 1.0 mg/L TDCPP. HSI of male fish decreased after exposure to TDCPP and TPP, but the opposite trend was observed among the female fish.

The egg reproduction per female was significantly reduced by the exposure (Fig. 3.1). The number of eggs per spawning and the number of spawning events also decreased among the fish exposed to TDCPP or TPP.

Table 3.1. Nominal and measured concentrations of TDCPP or TPP in exposure waters during 14 d exposure.

	Nominal	Measured			Nominal	Measured	
		0 h	48 h			0 h	48 h
TDCPP	0.00	0.00	0.00	TPP	0.00	0.00	0.00
	0.04	0.04	0.03		0.04	0.03	0.00
	0.2	0.15	0.13		0.2	0.14	0.00
	1.0	0.75	0.62		1.0	0.89	0.38

Unit: mg/L.

Table 3.2. Condition factor (K), gonadosomatic index (GSI) and hepatosomatic index (HSI) for adult male and female zebrafish after 21 days exposure to TDCPP or TPP (n=5).

Chemical	Conc.	K		GSI		HSI	
	(mg/L)	Male	Female	Male	Female	Male	Female
TDCPP	0	0.94±0.05	1.18±0.06	1.00±0.24	11.98±0.77	2.31±0.11	4.16±0.27
	0.04	0.90±0.12	1.16±0.28	1.16±0.15	13.09±1.27	2.10±0.08	4.30±0.36
	0.2	0.77±0.21	1.14±0.26	1.03±0.18	12.09±1.14	1.99±0.56	4.66±0.94
	1.0	0.80±0.13	1.19±0.05	058±0.23	14.55±0.74	1.69±0.23	4.64±0.74
TPP	0	0.92±0.09	0.99±0.08	1.28±0.08	10.98±1.10	2.66±0.06	4.18±0.35
	0.04	1.25±0.20	1.15±0.13	1.50±0.72	13.43±1.26	2.57±0.33	4.29±1.20
	0.2	1.10±0.15	1.53±0.37	1.26±0.53	12.37±3.34	2.91±0.41	4.11±0.28
	1.0	1.25±0.13	1.20±0.24	1.36±0.26	12.66±0.57	2.11±0.14	4.63±0.61

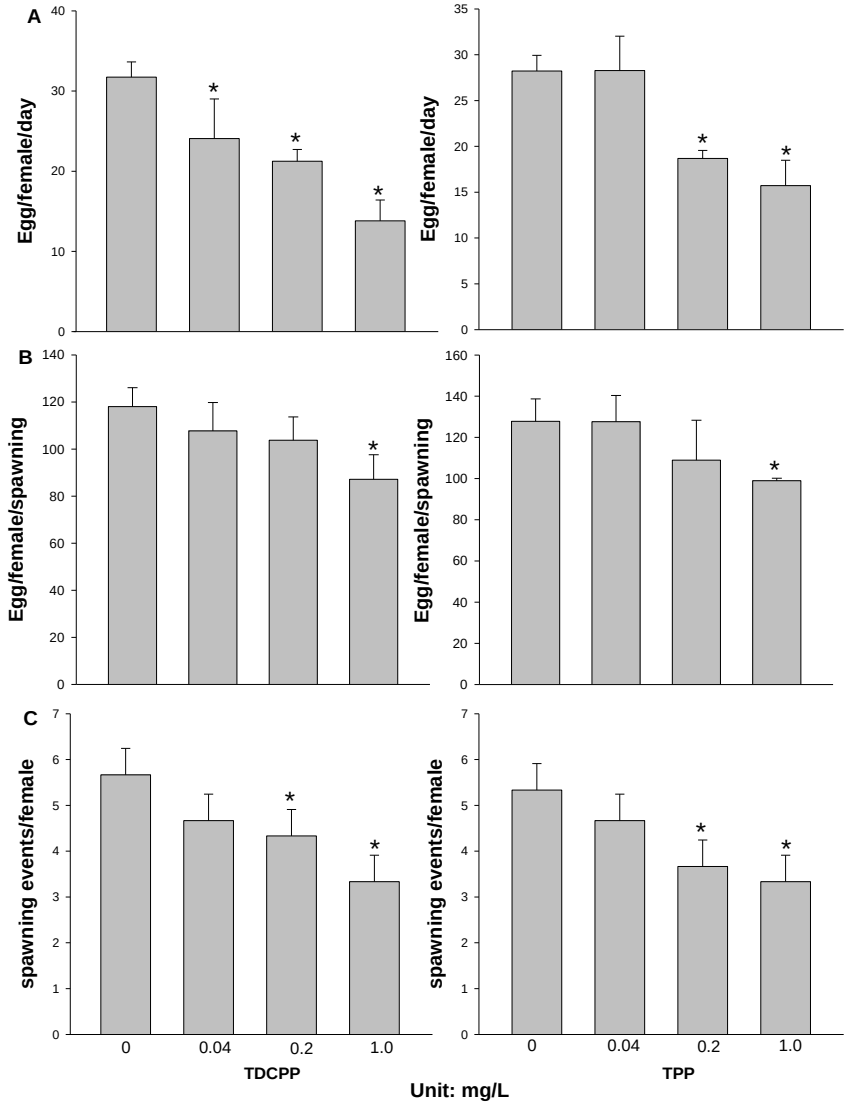


Figure 3.1. Effects of TDCPP or TPP on reproductive parameters of zebrafish breeding pairs including (A) eggs per female per day, (B) egg per female per spawning, and (C) the number of spawning event per female. Data are expressed as mean ± SD of six replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.005% DMSO). The p value was determined based on ANOVA analysis.

3.3.2 Effects on sex hormone and vitellogenin levels

Plasma E2 concentrations significantly increased in female fish after the exposure to 1.0 mg/L TPP or TDCPP (Fig. 3.2A), while T and 11-KT concentrations significantly decreased (Fig. 3.2B and C) leading to greater estrogenicity at as low as 0.2 mg/L TDCPP or TPP (Fig. 3.2D and E). In male fish, the plasma E2 concentrations significantly increased at 0.2 mg/L TPP or 1.0 mg/L TDCPP (Fig. 3.2A), while T and 11-KT concentration significantly decreased by TPP (Fig. 3.2B and C), although the trends were not monotonous. Both E2/T and E2/11-KT ratios significantly increased at 0.04 mg/L TDCPP or TPP and greater (Fig. 3.2D and E).

Plasma vitellogenin (VTG) concentrations increased by both compounds regardless of the fish sex (Fig. 3.3). In male fish, significant increase of plasma VTG was observed at as low as 0.04 mg/L TDCPP or 1 mg/L TPP. In female fish, significantly increased VTG was observed at 0.2 mg/L TDCPP, and 0.2 and 1.0 mg/L TPP.

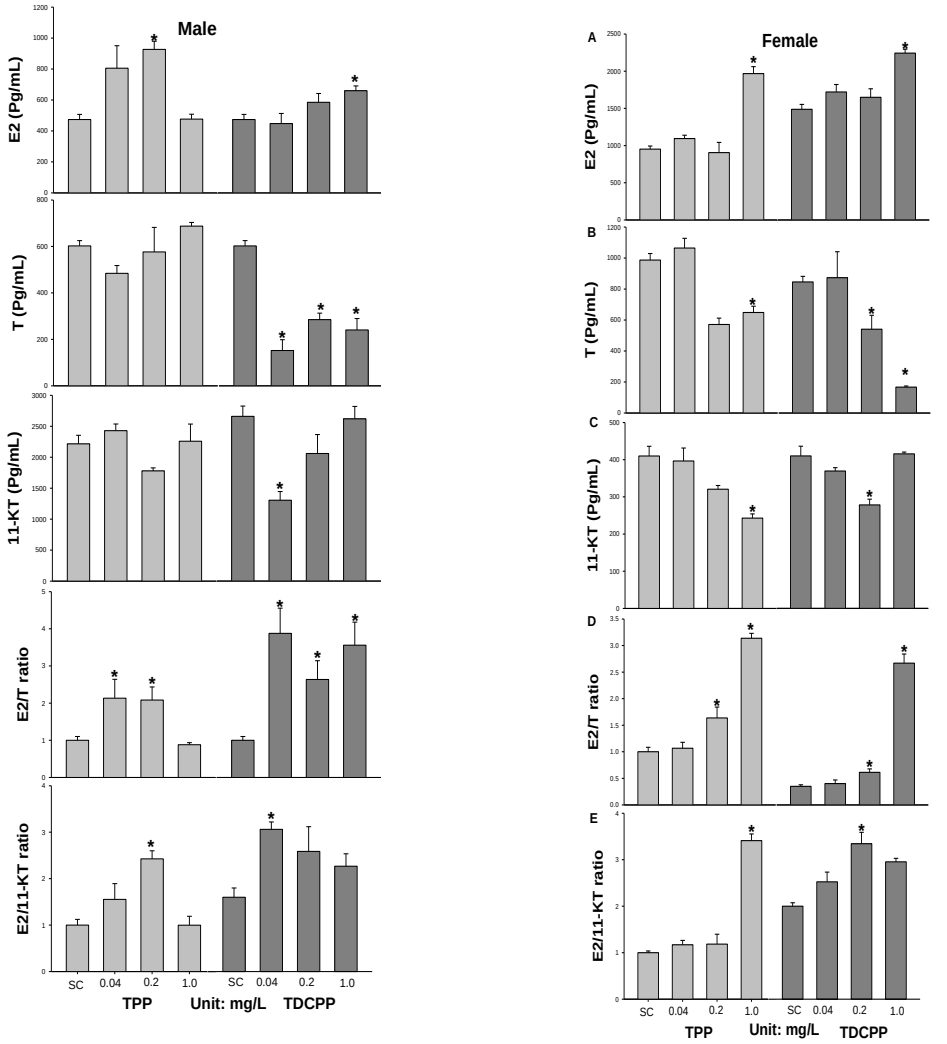


Figure 3.2. Effects of TDCPP or TPP on plasma level of (A) E2, (B) T, (C) 11-KT, (D) E2/T ratio and (D) E2/11-KT ratio, after 21 day exposure to TDCPP or TPP. Data are expressed as mean ± SD of five replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.005% DMSO). The p value was determined based on ANOVA analysis.

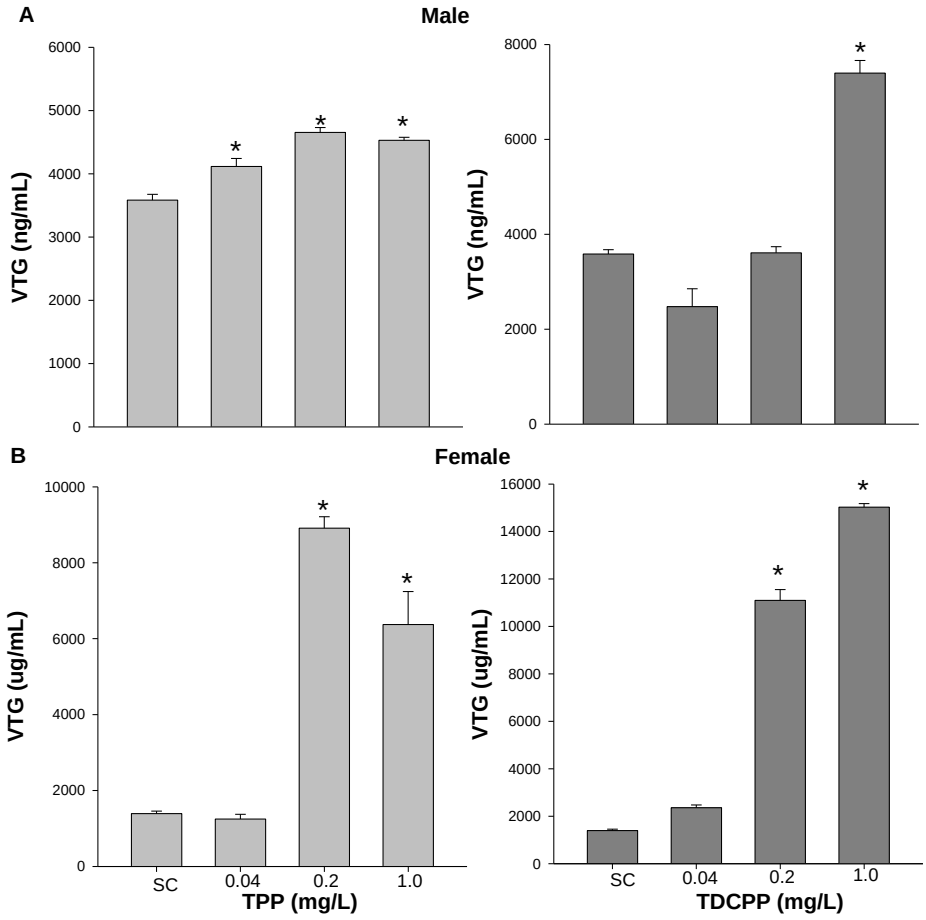


Figure 3.3. Effects of TDCPP or TPP on plasma VTG level in (A) males and (B) females after 21 day exposure to TDCPP or TPP. Data are expressed as mean ± SD of five replicates. Asterisk indicates significant difference from solvent control (SC, treated with 0.005% DMSO). The p value was determined based on ANOVA analysis.

3.3.3 Transcriptional response along the HPG axis

The transcriptions of the selected genes of the HPG axis were influenced by the exposure to TDCPP or TP (Figs. 3.4 and 3.5, and more detailed information refer to Table 3.3 and 3.4). Sex-specific transcriptional patterns were observed in each organ.

3.3.3.1 Brain

In males, *GnRH2* was significantly up-regulated in both TDCPP and TPP treatment groups (Fig. 3.4), however the direction of change of *GnRH3* gene transcription was the opposite (Fig. 3.5). Among the four GnRH receptors that were measured, only *GnRHR3* was significantly up-regulated in male and down-regulated in female fish, by both chemicals. Transcription of other pituitary receptor genes was also affected but the trend was not consistent by sex and the compound. Regardless of sex, *FSHβ* was significantly up-regulated, but transcription of *LHβ* decreased in male but increased in female fish by both compounds. Except for *AR* that was down-regulated, *CYP19B*, *ERα*, *ER2β1* were generally up-regulated by exposure to both compounds.

3.3.3.2 Gonad

Exposure to TDCPP or TPP led to significant transcriptional changes in both testis and ovaries of fish (Figs. 3.4 and 3.5). After the exposure to both compounds, LHR gene was significantly down-regulated in testis, but up-regulated in ovary. FSHR gene was also significantly up-regulated in ovary by both compounds.

In male fish, among steroidogenic genes, transcriptions of *CYP11A*, *CYP17*, *CYP19A*, *3βHSD* or *17βHSD* were significantly up-regulated in TDCPP treatment group. By TPP, transcription of *HMGRA*, *StAR* or *17βHSD* were significantly down-regulated, while those of *CYP11A*, *CYP17* and *CYP19A* were significantly up-regulated in testis.

In female fish, major steroidogenic genes, including *HMGRA*, *StAR*, *CYP17* or *CYP19A* were significantly up-regulated by exposure to TDCPP or TPP, while transcription of *HMGRB* was significantly up-regulated by TDCPP but down-regulated by TPP. The transcription of *17βHSD* showed the opposite trend.

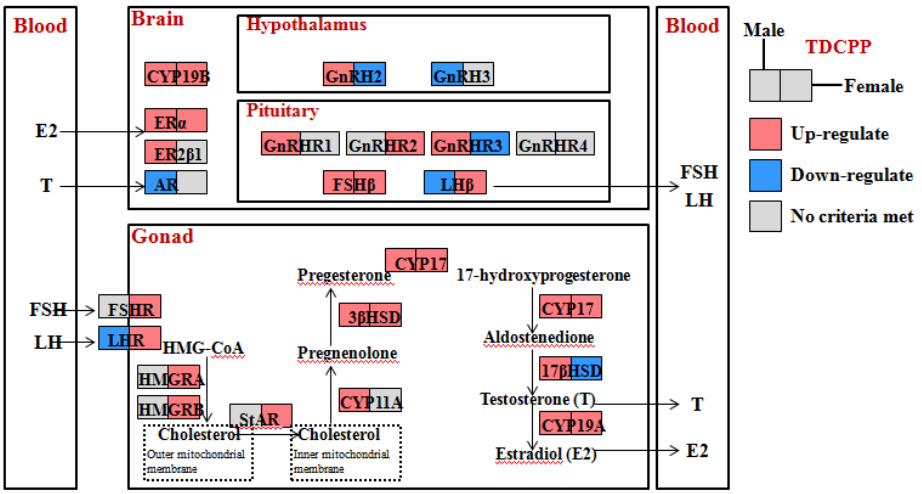


Figure 3.4. Striped view of sex-dependent response profile in adult zebrafish after exposure to TDCPP. Gene expression data from zebrafish treated by 0.04, 0.2 and 1.0 mg TDCPP/L are shown as striped color sets on the selected endocrine pathways along zebrafish HPG axis. The legend listed in the upper right corner of the graph describes the order of sex and the three colors designating different change trend. Red: significantly up-regulate; Blue: significantly down-regulate; Gray: no statistically significant change.

Table 3.3. Changes of gene transcription in hypothalamus-pituitary-gonad axis in male and female zebrafish by the exposure to TDCPP.

Gene	Male			Female		
	0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
Brain						
GnRH2	0.89±0.15	0.79±0.15	1.92±0.06*	1.02±0.04	0.70±0.06*	0.56±0.09*
GnRH3	0.75±0.02	0.68±0.18	0.45±0.14*	0.96±0.38	0.83±0.11	0.87±0.23
GnRHR1	0.89±0.13	0.77±0.14	1.79±0.07*	1.12±0.35	1.18±0.13	0.76±0.08
GnRHR2	0.92±0.35	0.68±0.13	1.11±0.26	1.36±0.14	1.54±0.07*	2.01±0.06*
GnRHR3	1.36±0.14	1.55±0.08	2.01±0.06*	1.06±0.31	0.86±0.01	0.69±0.02*
GnRHR4	1.13±0.53	0.86±0.10	1.61±0.53	0.90±0.13	0.77±0.43	1.17±0.46
LHβ	1.03±0.29	0.80±0.07	0.72±0.20*	1.34±0.16	3.26±0.56	7.01±0.73*
FSHβ	1.49±0.59	1.86±0.16*	1.37±0.11*	1.76±0.38	1.59±0.11*	3.02±0.29*
CYP19B	0.81±0.18	0.95±0.08	1.80±0.26*	1.20±0.11	0.94±0.03	1.57±0.03*
AR	0.79±0.04	1.25±0.19	0.58±0.01*	0.99±0.12	0.83±0.16	1.20±0.10
ERα	1.11±0.25	1.42±0.25	2.25±0.07*	1.59±0.31*	0.79±0.15	2.10±0.24*
ER2β1	0.96±0.27	1.11±0.50	1.60±0.11*	1.15±0.08	0.95±0.18	1.38±0.21
Gonad						
LHR	0.59±0.03*	0.59±0.13*	0.29±0.07*	1.10±0.16	3.60±1.55	11.77±1.48*
FSHR	0.85±0.17	1.10±0.15	1.26±0.06	1.06±0.38	1.04±0.41	2.68±0.16*
HMGRA	1.04±0.05	1.05±0.23	0.81±0.05	0.96±0.06	4.94±1.80	8.93±0.36*
HMGRB	0.82±0.17	1.00±0.12	0.83±0.07	1.61±0.31	3.30±0.44*	2.85±0.64*
StAR	0.88±0.07	0.80±0.17	0.86±0.10	0.75±0.21	5.47±0.09*	2.13±0.26*
CYP11A	1.21±0.05	1.62±0.24*	1.78±0.23*	1.39±0.24	1.58±0.50	1.14±0.35
CYP17A	1.29±0.19	1.50±0.33	1.37±0.04*	3.09±0.95	3.23±0.21*	2.14±0.26*
CYP19A	0.98±0.13	1.67±0.33*	1.93±0.08*	1.46±0.45	4.61±0.60*	2.58±0.27*
3βHSD	0.92±0.08	0.99±0.05	1.38±0.14*	1.98±0.33	3.90±0.52*	2.49±0.71*
17βHSD	1.30±0.22	1.57±0.25	1.79±0.13*	1.07±0.02	0.55±0.20*	0.37±0.12*

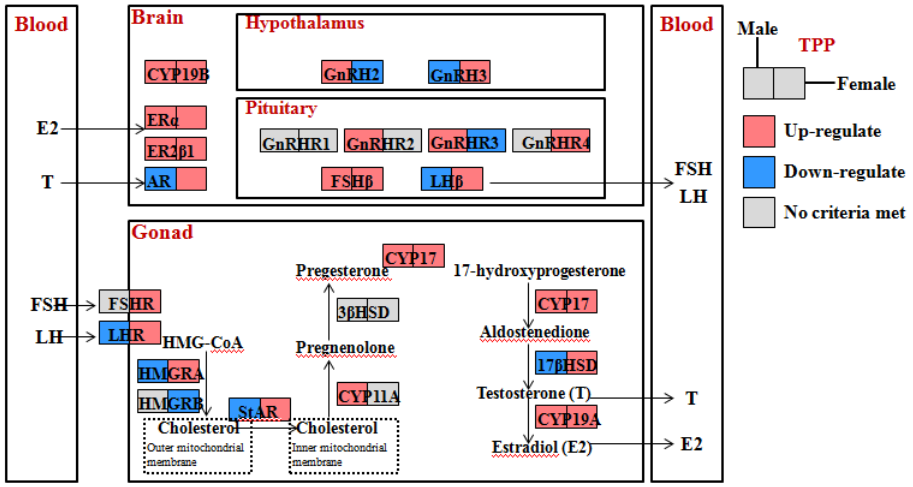


Figure 3.5. Striped view of sex-dependent response profile in adult zebrafish after exposure to TPP. Gene expression data from zebrafish treated by 0.04, 0.2 and 1.0 mg TPP/L are shown as striped color sets on the selected endocrine pathways along zebrafish HPG axis. The legend listed in the upper right corner of the graph describes the order of sex and the three colors designating different change trend. Red: significantly up-regulate; Blue: significantly down-regulate; Gray: no statistically significant change.

Table 3.4. Changes of gene transcription in hypothalamus-pituitary-gonad axis in male and female zebrafish by the exposure to TPP.

Gene	Male			Female		
	0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
Brain						
GnRH2	1.60±0.17*	1.63±0.53	1.79±0.07*	1.36±0.26	1.10±0.19	0.51±0.02*
GnRH3	0.37±0.02*	0.60±0.12*	0.65±0.02*	1.30±0.43	2.37±0.11*	1.79±0.26*
GnRHR1	0.90±0.03	1.19±0.09	1.11±0.21	1.31±0.39	1.31±0.13	1.11±0.13
GnRHR2	1.41±0.12*	1.54±0.20*	1.96±0.20*	1.51±0.28	1.34±0.12	1.35±0.46
GnRHR3	1.10±0.28	1.16±0.11	2.23±0.22*	0.71±0.16	0.69±0.03*	0.59±0.17*
GnRHR4	1.11±0.54	1.40±0.17	1.16±0.03	1.38±0.26	1.35±0.40	1.75±0.07*
LHβ	0.21±0.01*	0.36±0.09*	0.76±0.07*	0.70±0.16	3.00±0.17*	2.98±0.41*
FSHβ	2.62±1.00	2.85±0.96	4.69±0.32*	1.09±0.24	2.97±0.24*	1.85±0.06*
CYP19B	0.72±0.07	2.35±0.49	2.90±0.30*	1.24±0.16	1.60±0.08*	2.11±0.04*
AR	0.72±0.20	1.09±0.15	0.70±0.06*	1.33±0.12	1.55±0.33*	1.74±0.04*
ERα	0.79±0.14	1.12±0.17	1.77±0.18*	1.02±0.31	0.95±0.05	2.32±0.40*
ER2β1	1.17±0.36	1.59±0.21*	2.94±0.15*	1.28±0.27	1.44±0.22	2.12±0.22*
Gonad						
LHR	0.26±0.12*	0.26±0.02*	0.47±0.10*	2.03±0.43	4.53±1.33	16.41±4.31*
FSHR	1.01±0.14	1.00±0.31	1.15±0.13	1.06±0.38	1.04±0.41	2.68±0.16*
HMGRA	0.78±0.06	0.85±0.17	0.47±0.10*	0.93±0.19	4.13±1.21	2.31±0.26*
HMGRB	0.91±0.11	0.99±0.31	0.82±0.01	1.27±0.22	0.63±0.14	0.56±0.04*
StAR	0.89±0.10	0.91±0.02	0.34±0.14*	1.39±0.10	2.41±0.24*	8.50±1.04*
CYP11A	1.22±0.18	3.33±0.63*	3.55±0.14*	1.20±0.54	0.84±0.06	0.71±0.05
CYP17A	0.90±0.28	1.13±0.02	2.78±0.13*	1.26±0.19	0.96±0.08	1.59±0.05*
CYP19A	1.29±0.22	1.57±0.25	1.79±0.13*	7.52±2.95*	6.39±0.65*	13.14±2.53*
3βHSD	0.73±0.10	0.93±0.16	0.95±0.12	1.16±0.09	0.72±0.09	1.15±0.11
17βHSD	1.07±0.02	0.55±0.20*	0.37±0.12*	2.86±0.15*	2.55±0.12*	2.02±0.19*

3.3.4 Transcriptional response after *ex vivo* exposure of brain

In *ex vivo* exposure of the male fish brain, all measured genes, including *CYP19B, GnRH2, GnRH3, GnRHR1, GnRHR2, GnRHR4, FSHβ, LHβ, ERα* or *AR* were significantly up-regulated by TDCPP or TPP (Tables 3.5 and 3.6). In female *ex vivo* brain, gene transcription of *CYP19B*, *GnRH2* and *ERα*were significantly up-regulated, while transcription of GnRHR3, *FSHβ*, *LHβ* and *AR* were significantly down-regulated by exposure to TDCPP or TPP.

Table 3.5. Sex and dose-dependent transcriptional responses in adult zebrafish *ex vivo* brain exposed to 0, 0.04, 0.2, and 1.0 mg/L TDCPP.

Sex	Gene	Concentration		
		0.04 mg/L	0.2 mg/L	1 mg/L
Female	*CYP19B*	0.98 ± 0.03	1.02 ± 0.39	3.77 ± 0.09*
	GnRH2	1.33 ± 0.56	1.68 ± 0.17	3.33 ± 0.32*
	GnRH3	0.80 ± 0.31	0.73 ± 0.17	0.55 ± 0.07*
	GnRHR1	1.06 ± 0.38	0.69 ± 0.19	2.65 ± 0.41*
	GnRHR2	0.90 ± 0.52	0.67 ± 0.08	0.70 ± 0.26
	GnRHR4	1.06 ± 0.08	0.44 ± 0.04*	0.54 ± 0.80*
	FSHβ	0.88 ± 0.10	0.49 ± 0.05*	1.07 ± 0.10
	LHβ	0.76 ± 0.37	0.43 ± 0.03*	1.41 ± 0.11
	ERα	1.59 ± 0.74	1.04 ± 0.33	1.88 ± 0.00*
	AR	0.90 ± 0.07	0.53 ± 0.15*	1.29 ± 0.28
Male	*CYP19B*	1.06 ± 0.09	2.42 ± 0.13*	1.33 ± 0.04*
	GnRH2	1.33 ± 0.19	2.93 ± 0.08*	2.01 ± 0.11*
	GnRH3	0.96 ± 0.56	2.89 ± 0.31*	2.90 ± 0.07*
	GnRHR1	1.47 ± 0.10	3.03 ± 0.25*	1.79 ± 0.10*
	GnRHR2	0.76 ± 0.23	2.98 ± 0.23*	1.54 ± 0.05*
	GnRHR4	0.53 ± 0.27	1.77 ± 0.02*	0.91 ± 0.04
	FSHβ	0.69 ± 0.41	2.46 ± 0.09*	2.17 ± 0.14*
	LHβ	0.70 ± 0.34	1.98 ± 0.07*	1.17 ± 0.13
	ERα	0.75 ± 0.27	3.11 ± 0.29*	1.84 ± 0.10*
	AR	0.78 ± 0.15	2.58 ± 0.10*	1.33 ± 0.03*

Values represent the mean ± SE of five replicate samples. Gene expressions were expressed as fold change relative to solvent control. * indicates significant difference between exposure groups and the corresponding control group ($P<0.05$).

Table 3.6. Sex and dose-dependent transcriptional responses in adult zebrafish *ex vivo* brain exposed to 0, 0.04, 0.2, and 1.0 mg/L TPP.

Sex	Gene	Concentration		
		0.04 mg/L	0.2 mg/L	1 mg/L
Female	*CYP19B*	0.93 ± 0.20	0.94 ± 0.03	1.61 ± 0.08*
	GnRH2	0.70 ± 0.16	0.94 ± 0.15	1.92 ± 0.08*
	GnRH3	0.83 ± 0.17	0.50 ± 0.25	0.54 ± 0.08*
	GnRHR1	1.18 ± 0.15	0.75 ± 0.19	0.85 ± 0.19
	GnRHR2	0.80 ± 0.20	0.73 ± 0.10	0.78 ± 0.31
	GnRHR4	0.90 ± 0.21	0.37 ± 0.05*	0.51 ± 0.21
	FSHβ	0.61 ± 0.26	0.33 ± 0.07*	0.59 ± 0.28
	LHβ	0.68 ± 0.30	0.53 ± 0.00*	0.45 ± 0.09*
	ERα	1.28 ± 0.50	1.82 ± 0.50	1.73 ± 0.04*
	AR	1.17 ± 0.19	0.40 ± 0.07*	0.80 ± 0.11
Male	*CYP19B*	1.17 ± 0.13	2.04 ± 0.13*	1.59 ± 0.09*
	GnRH2	1.95 ± 0.04*	3.25 ± 0.42*	3.55 ± 0.53*
	GnRH3	1.56 ± 0.22	2.54 ± 0.04*	2.80 ± 0.14*
	GnRHR1	0.97 ± 0.38	2.07 ± 0.12*	1.55 ± 0.16
	GnRHR2	1.38 ± 0.07	2.65 ± 0.16*	1.33 ± 0.13
	GnRHR4	1.38 ± 0.18	1.63 ± 0.13	0.96 ± 0.06
	FSHβ	1.50 ± 0.20	1.67 ± 0.05*	1.34 ± 0.01
	LHβ	1.81 ± 0.62	2.21 ± 0.91*	1.15 ± 0.01
	ERα	1.48 ± 0.05	1.29 ± 0.31	1.52 ± 0.11
	AR	0.92 ± 0.03	1.80 ± 0.10*	1.15 ± 0.05

Values represent the mean ± SE of five replicate samples. Gene expressions were expressed as fold change relative to solvent control. * indicates significant difference between exposure groups and the corresponding control group ($P<0.05$).

3.4. Discussion

Impaired reproduction was observed in adult zebrafish after 21 d of exposure to TDCPP or TPP. This impairment corresponded well with increases in plasma E2 and VTG levels in both male and female fish, and altered transcription levels of regulatory and steroidogenic genes of the HPG axis. Alterations in HPG axis regulation and gonadal steroidogenesis are often associated with adverse effects on reproduction (Sofikitis et al., 2008). Steroidogenesis is important for spermatogenesis and oocyte maturation, and is controlled by LH and FSH in the pituitary or GnRH in the hypothalamus; therefore, all of these compartments play a significant role in regulating reproduction. Therefore, chemicals that disturb steroidogenesis or the HPG axis can have adverse effects on reproduction (Zhang et al., 2008).

Following 14 d of exposure, similar observations of increased estrogenicity, e.g., increases in E2 concentrations and E2/11-KT ratios, were reported in adult zebrafish (Liu et al., 2012a). In the present study, plasma VTG levels also increased in both male and female zebrafish after exposure to TDCPP and TPP, consistent with the observed changes in sex hormones. In fish, VTG, which induces oocyte maturation, is synthesized in the liver in response to estradiol stimulation (Nilsen et al., 2004). Synthesis of VTG in the liver is also facilitated by exogenous estrogenic chemicals that bind to specific ERs. Hence, the increased plasma VTG levels in male and female fish exposed to the two OPFRs could either be the consequence of increased E2 synthesis in the plasma or may suggest that both of the OPFRs act as xenoestrogens. Increases in the E2/11-KT ratio (Fig. 2E) support the hypothesis of increased estrogenicity through altered steroidogenesis. It is unlikely that both OPFRs act as xenoestrogens and bind to ERs. TDCPP and TPP inhibit the binding of E2 to ER in MVLN cells (Liu et al., 2012a).

Sex steroid hormones play crucial roles in sex differentiation, sexual maturation, and various behaviors that are associated with reproduction (Devlin and Nagahama, 2002). Any chemicals that can act on the HPG axis

can have adverse effects on reproduction (Brain et al., 2007). In the present study, we found that both TDCPP and TPP caused significant decreases in fecundity during a 21-d exposure period. Exposure to TDCPP and TPP also increased estradiol levels (E2), but decreased testosterone levels (T) in both female and male fish. The transcription levels of many genes that are related to the HPG axis were also influenced. The steroidogenesis pathway in the gonad is considered to be the major source of sex steroid hormones. Increased levels of both *CYP19A* and *CYP19B* in the testis and brain can be attributed to increased conversion of T to E2. Responses in steroidogenesis-related gene transcription to TDCPP or TPP exposure correspond well with changes in sex hormones. Gene transcription varied somewhat by sex, but the underlying mechanism remains to be determined.

The results showed clear sex-dependent responses in the brain to TDCPP or TPP exposure. The transcription level of the *GnRH2* gene increased in males but decreased in females following exposure to either compound. Transcription of *GnRH3* decreased in males but increased in females following TPP exposure. Transcription levels of *GnRHR3* and *LH* showed similar sex-dependent trends. GnRHs and gonadotropins are important regulators of sex steroids; therefore, they can be affected by sex hormones through negative feedback mechanisms. GnRHs act as neuromodulators and regulate reproductive behaviors (Okuzawa et al., 2003). Therefore, any alterations in the balances of GnRHs and GnRHRs can lead to interruptions in the sex hormone balance in fish.

The present observations also provide evidence that pituitary gonadotropins are affected by exposure to OPFRs in fish. In the present study, upregulation of *FSHβ* and *LHβ* in the pituitary and *FSHR* and *LHR* in the ovaries appeared to lead to the stimulation of E2 synthesis in female fish. Meanwhile, upregulation of *FSHβ* and downregulation of *LHβ* gene expression with a decrease in the transcription of *LHR* in the testis may lead to the stimulation of E2 and inhibition of T synthesis. Similar observations were reported in male goldfish after exposure to monocrotophos (Tian et al., 2010). Exposure resulted in significantly higher plasma E2 levels but lower T concentrations in

goldfish, with an accompanying increase in FSH and a decrease in LH, with respect to both protein and gene transcription. In mammals, FSH is involved in the synthesis of E2 and VTG, while the main role of LH is to enable Leydig cells to generate androgens. In amphibians, these functions appear to be the same, i.e., plasma FSH levels correspond to E2 levels, while LH is correlated with androgen levels (Polzonetti-Magni et al., 1998). Although the precise functions of FSH and LH in teleosts are still largely unknown, several lines of evidence suggest that they are involved in steroidogenesis in ways that are similar to their roles in mammals (Swanson et al., 2003).

The differences in transcriptional responses between the *in vivo* and *ex vivo* brain (e.g., in the *GnRH2* or *GnRH3* genes) indicate that feedback from other organs is important for hormone regulation throughout the HPG axis. In addition to sex hormones, thyroid hormones may also regulate FSH or LH in the pituitary gland. Zhang et al. (2008) suggested that lower concentrations of T4 and T3 caused by exposure to propylthiouracil would result in increased production of FSH and LH, which would in turn promote steroidogenesis. Clinical studies have also demonstrated that boys with hypothyroidism exhibit elevated concentrations of FSH and LH (Meikle, 2004). Based on previous observations, TDCPP and TPP would reduce plasma thyroid hormones in adult male zebrafish but would result in increases in their levels in female fish (Liu et al., 2012b).

These results showed that short-term (21 d) exposure to TDCPP and TPP in adult zebrafish impaired reproductive capacity, increased plasma E2 and VTG levels in both male and female fish, and influenced the transcription of genes of the HPG axis in a sex-dependent manner. With increasing use of these compounds, the consequences of exposure in aquatic ecosystems deserve further investigation.

Chpater 4. Effects of TDCPP or TPP on thyroid hormone and related gene transcriptions in hypothalamus-pituitary-thyroid (HPT) of zebrafish (*Danio rerio*)

4.1 Introduction

Acute toxicity of OPFRs towards aquatic organisms varies by compound and species. TPP and TDCPP appeared to be relatively toxic with 96 h median lethal concentrations ranging between 0.26 and 1.1 mg/L for rainbow trout (Sundkvist et al., 2010). Probably due to the chemical structure which is similar to those of organophosphate insecticides (Bacaloni et al., 2007), these chemicals are suspected for endocrine disruption and reproduction related problems. High dose exposure to TDCPP caused a significant increase in testicular interstitial cell tumors and histopathologic abnormalities of testis in rats (NRC 2000). TDCPP and TPP were reported for sex hormone disruption in a fish and a human adrenal cell line (Liu et al., 2012). In addition, potential effects of OPFRs on thyroid hormones (THs) are suspected among humans. Meeker and Stapleton (2010) reported that increased concentrations of TDCPP or TPP in the house dust were associated with a reduced concentration of thyroxine (T4) in the serum of adult men. In cultured avian hepatocytes, administration to TDCPP and TCPP influenced the genes that were associated with the thyroid hormone pathway, among others (Crump et al., 2012).

The importance of THs balance is well recognized in the development and growth of many organisms. Therefore the chemicals that may influence the function of thyroid should deserve attention and warrant appropriate management. Several chemicals are reported to have impacts on thyroid gland and its function. Some chemicals may influence directly on TH synthesis, transport, and binding, and others may affect the thyroid system by

influencing feedback mechanisms (Kloas and Lutz, 2006). Several environmental chemicals, including PBDE (DE-47, DE-71), polychlorinated biphenyls (PCB), and chlorinated paraffins are reported to disturb TH balances through affecting their transport or elimination (Hallgren and Darnerud, 2002; Yu et al., 2010; Yu et al., 2011). In addition, PFOS or a fungicide triadimefon were reported to alter transcription of the genes along the hypothalamus-pituitary-thyroid gland (HPT) axis, and therefore influence the synthesis, regulation and activation of THs in fish (Shi et al., 2009; Liu et al., 2011b). For OPFRs, rats dosed with high levels of TDCPP showed altered thyroid weights, but its effects on THs levels or underlying mechanisms are not known. However, thyroid disrupting effects of OPFRs in fish and related mechanisms have not been reported yet, to our knowledge.

Neuro-endocrine cells are important regulators of thyroid hormones. For example, transcriptions of CRH or TSHβ in hypothalamus or pituitary gland play important roles in regulations of thyroid hormones. Chemicals that act as agonists/antagonists or modulators of the synthesis and/or metabolism of neuropeptides, neurotransmitters, or neurohormones, may alter diverse physiological, behavioral, or hormonal processes, and therefore affect an animal's capacity to reproduce, develop and grow, or deal with stress and other challenges (Waye and Trudeau 2011). However, only a small proportion of the published researches directly examined the effects of endocrine disruptors on neuro-endocrine processes (Majdoubi, 2011). Teleost fish is suggested as a good model for investigation of neuro-endocrine effects of endocrine disruptors (Page et al., 2011). *Ex vivo* brain culture can be used to understand the direct responses of brain against the exposure to potential endocrine disruptors (Liu et al., 2011a). Tomizawa et al (2001) showed that whole brain of zebrafish (including hypothalamus and pituitary gland) could be cultured *ex vivo* for 7 d, and be used for neurobiological studies. Liu et al (2011a) also successfully used whole brain culture of zebrafish to study neuro-endocrine effects of prochloraz or propylthiouracil (48 h exposure).

In the present study, I used zebrafish to evaluate the effects of TDCPP and TPP on thyroid system and to identify potential mechanisms of TH disruption.

In addition, *ex vivo* brain exposure was conducted to understand the direct effects of these compounds on hypothalamus and pituitary gland. Only a few studies have been conducted on the effects of these OPFRs on thyroid status, and detailed mechanisms of such effects are yet to be elucidated. The results of this study will provide mechanistic understanding of the effects of TDCPP and TPP on thyroid of fish, and help better grasp their potential consequences in aquatic environment.

4.2 Materials and methods

4.2.1 Preparation of testing materials

Same with chapter 3.

4.2.2 Fish maintenance and exposure

Fish maintenance was similar with chapter 3.

Five male and female zebrafish per group were exposed to TDCPP and TPP (0, 0.04, 0.2, and 1.0 mg/L) in 3 L beakers filled with approximately 2.6 L of test medium or control. The exposure duration was 14 d, during which the fish were fed with *Artemia* nauplii *ad libitum* twice daily. Exposure medium was renewed at least three times per week. Mortality was recorded daily until the test termination, and dead organisms were removed as soon as noted. After 14 d of exposure, all surviving fish were euthanized and employed for further analysis.

4.2.3 Thyroid hormone measurement

After exposure, the tail of each zebrafish was transected, and blood was collected in a heparinized glass capillary tube. Bloods from five fish were pooled to make >15 μL in a 1.5 mL microcentrifuge tube, and centrifuged at 5,000g for 15 min, and 330 μL enzyme-linked immunosorbent assay (ELISA) buffer was added for hormone analysis. Triiodothyronine (T3), T4 and thyroid-stimulating hormone (TSH) levels were measured using ELISA kit (Uscnlife, Wuhan, China), following the manufacturer's instruction. The supernatant was employed for measurement of total triiodothyronine (T3), tT4 and thyroid-stimulating hormone (TSH) levels using ELISA kits (Cat No. E90453Ge for general T3, E90452Ge for general T4, and E90463Mu for mouse TSH, Uscnlife, Wuhan, China), following the manufacturer's instruction. Since different volume of blood samples were employed, the concentrations of THs were calculated using different dilution factors. The intra-assay variations are 4.0% for tT4, 4.1% for tT3 and 4.3% for TSH, and

the inter-assay variations for tT4, tT3 and TSH are 7.8%, 7.9% and 7.8%, respectively. The reported detection limits for tT4, tT3 and TSH are 1.2 ng/ml, 0.1 ng/ml and 0.1 mIU/ml, respectively.

4.2.4 RNA isolation and qRT-PCR

Whole brain, thyroid, and liver were collected from each fish and preserved in 250 μL RNAlater reagent at -80^{o}C until analysis. For separation of thyroid gland, whole pharyngeal tissues were sampled. In zebrafish, thyroid follicles are loosely dispersed along the ventral midline of the pharyngeal mesenchyme. The expression levels of *β-actin*, *thyrotropin-releasing hormone* (*TRH*), *corticotrophin-releasing hormone (CRH)*, *TSHβ*, *thyroid hormone receptor* (*TR*) *alpha*, and *TRβ* mRNAs in brain with includes hypothalamus and pituitary gland, *TRα*, *TRβ*, *thyroglobulin* (*TG*), *thyroperoxidase* (*TPO*), *deiodinase* (*Dio*) *1*, *Dio2*, *paired box gene 8* (*pax8*) mRNAs in thyroid, and *TRα*, *TRβ*, *Dio1*, *Dio2*, and *transthyretin* (*TTR*) mRNAs in liver were measured. The primers and the TaqMan probes (Applied Biosystems, Foster City, CA, USA) are shown in Table 4.1. Total RNAs were extracted from the brain, thyroid gland, and liver using RNeasy mini-kit (QIAGEN, Valencia, CA, USA). Complementary DNAs were synthesized from the purified RNA samples using iScript^{TM} cDNA Synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was performed using ABI 7300 real-time PCR system (Applied Biosystems). PCR reaction mixtures contained 10 μL TaqMan Gene Expression Master Mix, 1 μL TaqMan gene expression assay (Applied Biosystems), 5 μL RNase-free water, and 4 μL complementary DNA templates. The PCR reaction comprised an initial denaturation step at 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, followed by 40 cycles at 95 ^{o}C for 15 s, and 60 ^{o}C for 1 min.

For each gene, real-time PCR reactions were performed in triplicates, which were repeated separately twice (n=6). The expression level of each target mRNA was normalized to that of a reference gene (*β-actin* gene) using the delta delta *C*t method (Livak and Schmittgen, 2001). Up-regulation or down-regulation was expressed as a "fold difference" compared to normalized DMSO control values. All data were statistically analyzed as "fold difference" between exposed and DMSO control.

4.2.5 *Ex vivo* brain exposure

In order to identify the direct responses of brain against the exposure to TDCPP or TPP, the *ex vivo* brain exposure was performed in 6-well microplates (Falcon, Franklin Lakes, NJ, USA). The methods described elsewhere (Liu et al., 2011; Tomizawa et al., 2001) were followed. For this exposure, whole brain was freshly separated from the fish, and was used for a treatment. TDCPP and TPP dissolved in DMSO were diluted with Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (DMEM/F12) medium (Sigma-Aldrich Korea, Gyeonggi, Korea). The 1 mL of culture medium containing TDCPP (0.04, 0.2, and 1 mg/L) and TPP (0.04, 0.2, and 1 mg/L) were added to each well. Transcription of the selected genes including *TRH*, *CRH*, *TSHβ*, *TRα* and *TRβ* were determined after 48 h of exposure. Each treatment or control had six replicates. Both control and treatment group received 0.01% DMSO as solvent.

4.2.6 Statistical analyses

Statistical analysis was similar with chapter 3.

4.3 Results

4.3.1 Survival of fish and concentration of thyroid hormones in blood

No lethality was observed in zebrafish at the experimental concentrations of both chemicals. In male fish, T3 and T4 concentrations decreased and TSH concentration increased significantly at as low as 0.2 mg/L TDCPP or 1 mg/L TPP. In female fish, the direction was the opposite. T4 levels increased and TSH decreased significantly at 1 mg/L of both chemicals (Figure 4.1). Plasma TH concentrations were higher in males than in females. The concentrations of T4 and T3 were 20.56±2.12 and 1.44±0.3 ng/mL among male fish, and 17.49±1.6 and 0.85±0.3 ng/mL in female fish, respectively. However, plasma TSH level in male fish (7.91±0.23 mIU/L) was lower than in female fish (9.45±0.09 mIU/L). This sex difference was independent of OPFRs exposure.

Table 4.1. qPCR primers used for gene expression assays

Gene	Accession NO	Assay ID	Sequence (5'-3')
β-actin	NM_131031	Forward primer	CCATCGGCAATGAGCGTTTC
		Reverse primer	CAAGATTCCATACCCAGGAAGGA
		TaqMan probe	FAM-CCCGAGGCTCTCTTC-NFQ
TRH	NM_001012365	Forward primer	GCTCTCTCCGTCGGTCTGTT
		Reverse primer	GCGAGATCCGTGCTGATGA
		TaqMan probe	FAM-CATGTTCTGTGAGCT-NFQ
TSHβ	NM_181494	Dr03150633_m1	
CRH	NM_001007379	Forward primer	GCGCAGGTCGGAGGAG
		Reverse primer	CTCGTAGCAGATGAAAGGTCAGATC
		TaqMan probe	FAM-CCGCCGATTTCCCT-NFQ
TG	XM_689200	Dr03986645_m1	
TPO	EU267076	Forward primer	ACAGTATCAACAACTACCTGCATGT
		Reverse primer	GCTGACCCAGACACCTCTCTA
		TaqMan probe	FAM-CCCTTCCTGCTCCCC-NFQ
TTR	NM_001005598	Forward primer	GCTGATGTGGTGTTTGAAGCT
		Reverse primer	CAGAAGGAGAGCCAGTGTGTAATG
		TaqMan probe	FAM-ATGCAGAGGGACATCGT-NFQ
TRα	NM_131396	Dr03131486_m1	
TRβ	NM_131340	Dr03138250_m1	
DIO1	NM_001007283	Forward primer	GAGACCGCTGATCCTCAACTTC
		Reverse primer	GCGCTCAGACGGGTCAT
		TaqMan probe	FAM-CTCCTGACCGCCCTTC-NFQ
DIO2	NM_212789	Dr03088603_m1	

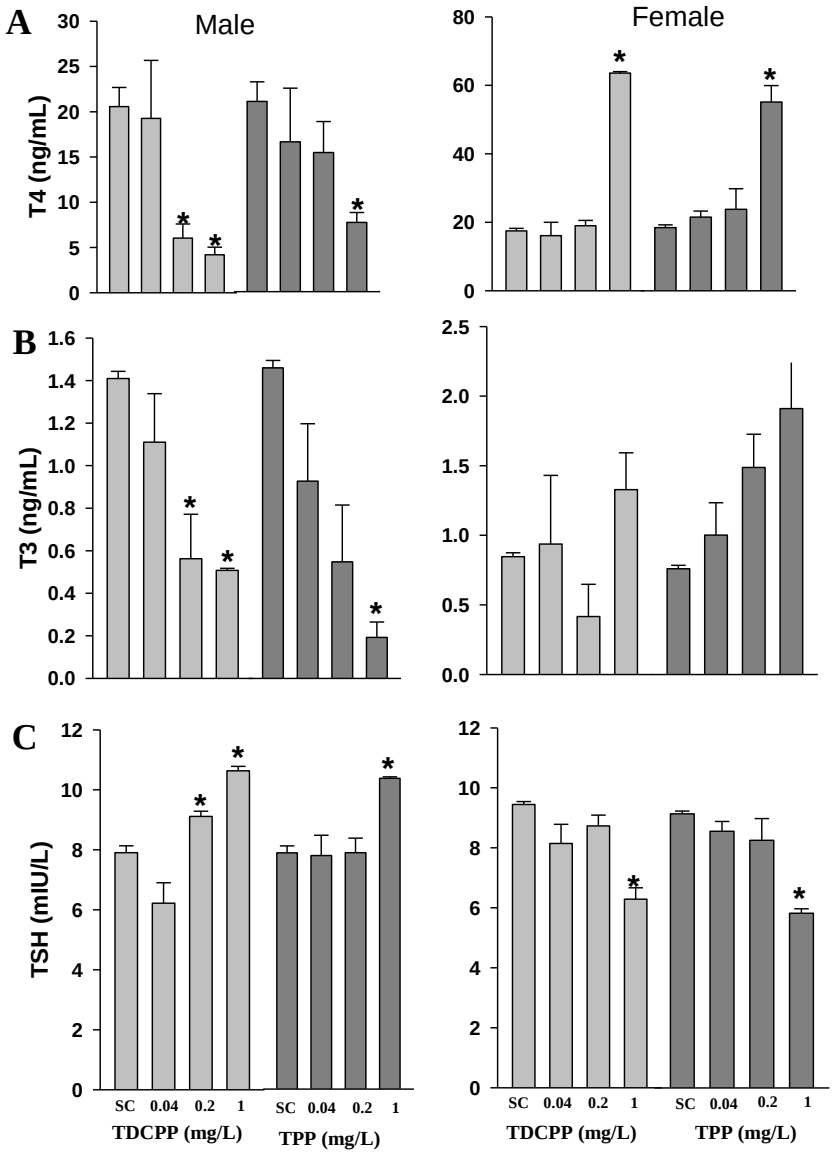


Figure 4.1. Effects on plasma (A) thyroxine (T4) concentration, (B) triiodothyronine (T3), and (C) thyroid-stimulating hormone (TSH) concentrations in male and female zebrafish exposed to 0.04, 0.2, or 1 mg/L of TDCPP, or 0.04, 0.2, or 1 mg/L of TPP for 14 d. Results are shown as mean ± SD of three replicates. Asterisk indicates significant difference from the solvent control.

4.3.2 Effects on transcription of related genes after *in vivo* exposure

Changes in transcriptional profiles in zebrafish exposed to TDCPP and TPP were chemical- and sex-dependent (Figure 4.2). In male fish, exposure to TDCPP significantly up-regulated mRNA level of *TRH*, corticotrophin-releasing hormone (*CRH*) and *TSHβ* gene in brain (Figure 4.2, also see Table 4.2). Transcription of *TRα* gene was significantly down-regulated, but *TRβ* was up-regulated in brain. In thyroid, transcriptions of *TG* and *TRα* genes were significantly down-regulated, and transcription of *Dio2* was up-regulated. In liver, transcriptions of *TRα*, *TRβ*, *Dio1* and *Dio2* were significantly down-regulated. *TTR* mRNA level in liver also showed a trend toward decreased abundance following exposure but statistical significance was not detected. In female fish, exposure to TDCPP led to significant down-regulation of *CRH* and *TSHβ*, and up-regulation of *TRβ* transcription in brain. In thyroid, mRNA level of *TG*, *TPO* and *Dio2* significantly increased, while *TRβ* and *Dio1* were down-regulated by the exposure. In liver, exposure to TDCPP led to decreased relative transcription of *TTR*, *TRα* and *Dio1* gene and increased relative transcription of *TRβ* and *Dio2* gene.

Exposure to TPP led to significant up-regulation of *TRH*, *CRH*, *TSHβ* and *TRβ* in brain of male fish. In male thyroid, transcription of *Pax8* and *TG* gene significantly decreased, while *TPO*, *TRα* and *TRβ* showed decreasing trend but without statistical significance. In liver, exposure to TPP also significantly down-regulated transcriptions of *TRα*, *TRβ*, *Dio1* and *Dio2* gene (Figure 4.3, also see Table 4.2). In female fish, transcriptions of *CRH* and *TSHβ* were significantly down-regulated while those of *TRH*, *TRα* and *TRβ* were significantly up-regulated in brain. In thyroid, significant increase in transcription of *TG*, *TRα* and *DIO2* gene were observed. In liver, *TRα* and *Dio2* were significantly up-regulated, while expression of *TRβ* and *Dio1* were down-regulated after exposure to TPP.

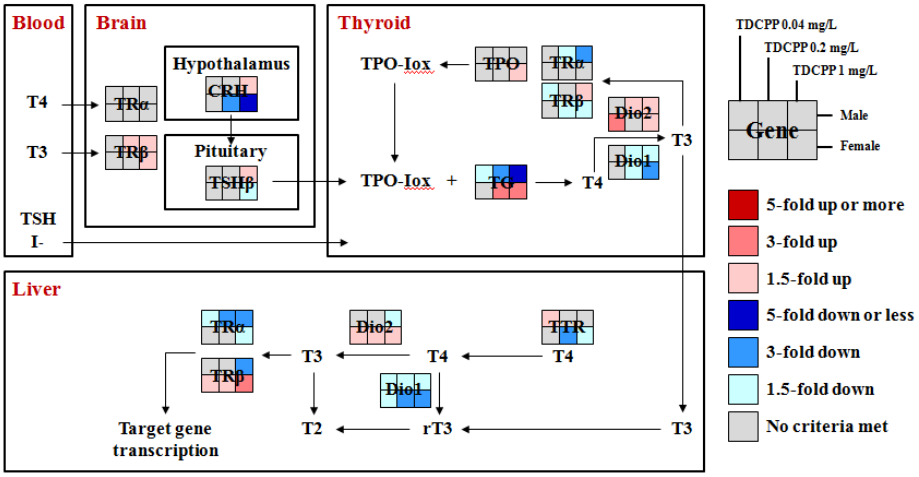


Figure 4.2. Dose-dependent effects and proposed pathways of action by TDCPP on hypothalamus-pituitary-thyroid (HPT) axis of male or female fish.

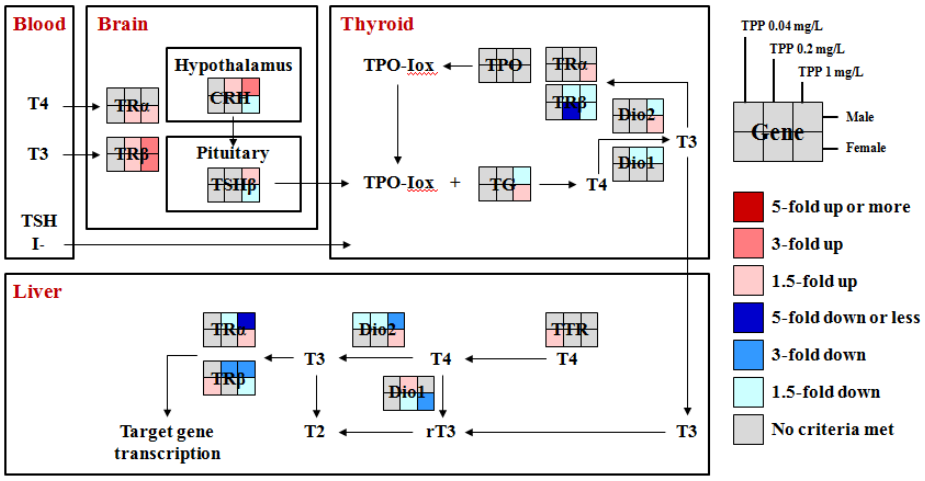


Figure 4.3. Dose-dependent effects and proposed pathways of action by TPP on hypothalamus pituitary-thyroid (HPT) axis of male or female fish.

Table 4.2. Changes of gene transcription in brain, thyroid, and liver, in male or female zebrafish by the exposure to TDCPP or TPP

Tissue	Gene	Male			Female		
		TDCPP			TDCPP		
		0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
Brain	*CRH*	1.23±0.04	1.59±0.05	2.09±0.05*	0.96±0.10	0.33±0.17*	0.19±0.01*
	TRH	1.11±0.03	1.56±0.05*	1.51±0.03*	0.86±0.02	0.97±0.11	1.29±0.06
	TSH	1.16±0.12	1.38±0.11	1.53±0.11*	1.23±0.08	1.04±0.24	0.46±0.04*
	TRα	1.13±0.05	1.31±0.14	0.73±0.04*	0.72±0.09	0.65±0.19	0.89±0.32
	TRβ	1.23±0.04	1.59±0.05*	2.09±0.05*	1.16±0.17	1.36±0.19	1.65±0.11*
Thyroid	*TG*	0.56±0.03*	0.25±0.02*	0.15±0.03*	0.88±0.19	3.23±0.63	3.61±0.16*
	TPO	1.36±0.34	1.28±0.50	1.19±0.07	1.43±0.24	0.90±0.34	2.84±0.11*
	TRα	1.21±0.41	0.46±0.03	0.32±0.07*	1.31±0.52	1.20±0.50	0.88±0.15
	TRβ	0.49±0.07	0.96±0.02	2.21±1.04	0.84±0.28	0.42±0.06*	0.45±0.03*
	Dio2	0.88±0.05	1.91±0.16*	1.80±0.06*	3.02±0.71	1.17±0.60	2.69±0.55*
	Dio1	0.84±0.10	0.63±0.04	0.60±0.04	0.85±0.17	0.36±0.08*	0.29±0.04*
Liver	*TTR*	1.53±0.39	1.00±0.24	0.70±0.08	1.05±0.09	0.23±0.01*	0.52±0.21
	TRα	0.56±0.10	0.22±0.17*	0.25±0.09*	1.16±0.31	0.91±0.26	0.60±0.02*
	TRβ	1.02±0.07	1.03±0.38	0.20±0.03*	1.94±0.16	2.52±0.67	3.64±0.54*
	Dio2	0.88±0.09	1.17±0.39	0.50±0.10*	2.56±0.86	2.86±0.46	1.67±0.04*

	Dio1	0.44±0.06*	0.33±0.03*	0.34±0.01*	0.61±0.06*	0.22±0.04*	0.22±0.06*
			TPP			TPP	
		0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
Brain	*CRH*	1.02±0.03	1.84±0.03*	4.69±0.37*	0.48±0.13	0.64±0.12	0.44±0.02*
	TRH	0.96±0.02	2.79±0.21*	3.40±0.07*	0.90±0.06	2.76±0.14*	2.32±0.09*
	TSHβ	0.86±0.02	0.94±0.09	2.46±0.42*	1.15±0.09	0.82±0.09	0.42±0.06*
	TRα	0.93±0.08	0.80±0.32	1.26±0.06	1.29±0.60	1.88±0.02*	1.93±0.19*
	TRβ	1.02±0.03	1.84±0.03*	4.90±0.02*	1.04±0.09	1.93±0.14*	4.09±0.11*
Thyroid	*TG*	1.02±0.19	0.73±0.07	0.65±0.24	0.90±0.04	1.47±0.55	1.56±0.21
	TPO	0.95±0.33	1.20±0.30	0.84±0.21	1.29±0.09	1.20±0.47	1.08±0.33
	TRα	1.03±0.06	1.04±0.16	0.79±0.04	1.45±0.13	1.48±0.13	2.14±0.10*
	TRβ	1.29±0.24	0.38±0.17	0.44±0.24	0.83±0.22	0.17±0.10*	0.35±0.28
	Dio2	1.05±0.14	0.79±0.10	0.51±0.01*	1.27±0.30	0.97±0.14	2.03±0.15*
	Dio1	0.64±0.18	0.44±0.03	0.47±0.04	1.14±0.48	0.79±0.05	1.14±0.37
Liver	*TTR*	1.05±0.05	1.22±0.15	1.08±0.23	2.39±0.34	1.26±0.09	0.78±0.12
	TRα	1.03±0.14	0.42±0.01*	0.10±0.01*	0.96±0.25	0.86±0.42	1.88±0.05*
	TRβ	1.30±0.52	0.28±0.02*	0.21±0.03*	1.54±0.22	0.78±0.13	0.41±0.07*
	Dio2	0.64±0.08	0.35±0.12*	0.28±0.02*	0.92±0.34	1.36±0.04	2.50±0.27
	Dio1	0.93±0.03	1.59±0.71	0.69±0.04	0.92±0.13	0.63±0.13*	0.31±0.03*

4.3.3 Effects on transcription of related genes after *ex vivo* exposure of brain

In *ex vivo* exposure of the male fish brain, *TRH* and TR*β* were significantly down-regulated, while *CRH*, *TSHβ* and TR*α* were significantly up-regulated by TDCPP or TPP (Table 4.3). In female *ex vivo* brain, *TRH* and *CRH* were significantly up-regulated, while *TSHβ* and TR*α* were significantly down-regulated by the exposure.

Table 4.3. Changes of gene transcription in male or female *ex vivo* brain by the exposure to TDCPP or TPP

Genes	Male			Female		
	TDCPP			TDCPP		
	0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
CRH	1.64±0.12	1.00±0.01	2.49±0.17*	1.38±0.35	1.08±0.15	1.73±0.06*
TRH	0.70±0.06	1.03±0.06	0.72±0.02	0.64±0.08	0.85±0.18	1.88±0.45*
TSHβ	0.86±0.23	2.04±0.47*	1.18±0.43	1.08±0.06	0.44±0.05*	0.46±0.03*
TRα	1.03±0.05	2.01±0.09*	1.31±0.03*	0.95±0.02	0.40±0.07*	0.49±0.21
TRβ	0.91±0.03	1.29±0.23	0.44±0.02*	1.09±0.27	0.91±0.08	1.59±0.07*
	TPP			TPP		
	0.04 mg/L	0.2 mg/L	1.0 mg/L	0.04 mg/L	0.2 mg/L	1.0 mg/L
CRH	0.58±0.03	1.00±0.03	1.93±0.17*	0.51±0.11	0.82±0.21	2.01±0.10*
TRH	1.38±0.20	0.21±0.04*	0.27±0.05*	0.86±0.14	0.87±0.04	4.47±0.27*
TSHβ	1.03±0.23	1.23±0.31	2.39±0.44*	0.81±0.32	0.45±0.04*	0.70±0.06
TRα	0.75±0.11	2.67±0.08*	1.17±0.27	0.58±0.07	0.54±0.16*	0.91±0.08
TRβ	0.82±0.11	0.65±0.12	0.57±0.02*	1.09±0.37	1.08±0.06	2.26±0.16*

4.4. Discussion

Thyroid function is dependent on various factors which include iodine uptake, TH synthesis, transport, tissue-specific TH deiodination, and binding of TH to thyroid hormone receptors (TRs) (Yen and Chin, 1994). Therefore thyroid disrupting chemicals may exert the impacts either by directly influencing the synthesis of THs via iodide uptake, thyroperoxidase (TPO) activity, and thyroglobulin (TG) synthesis. These chemicals may also indirectly interfere with the functions of hypothalamus-pituitary-thyroid axis. For example, the disturbance of circulating THs may trigger the alteration in TSH expression and may influence synthesis of transthyretin (TTR) and deiodinase (Dio) which will subsequently affect TH bioavailability.

By the exposure to the test OPFRs, blood TH levels showed clear sex-dependent alternations. THs significantly decreased in male fish, but increased in female fish (Figure 4.1). Therefore, increase of TSH in male, and decrease in female may be interpreted as compensation against hypo- and hyperthyroidism in male and female fish, respectively. A few studies strongly suggested some OPFRs could disrupt the thyroid, however detailed mechanisms of action were not clear. Rats dosed with high levels of TDCPP resulted in increased thyroid/body weight ratio compared to the controls (NRC 2000). Among adult human males, serum free T4 levels showed inverse association with TDCPP concentrations in the indoor house dust (Meeker and Stapleton 2010), which is in line with our observation in male fish. While direct extrapolation is not possible from fish to humans, our observation may provide possible mechanistic links between THs and OPFRs, which could be further elucidated in the future.

The thyroid regulating responses of the brain, e.g., signals like *CRH* or *TSH*, were different by sex. OPFR exposure resulted in increase of *TSHβ* in male but decrease of *TSHβ* in female fish in both *in vivo* and *ex vivo* brain. TSH is important regulator of THs stimulating synthesis of THs. *TSHβ* transcription in brain therefore can be interpreted as a compensation for the changes in

plasma THs. *TRH*, however, did neither show differences by sex, nor correspond well with transcription of *TSHβ* (Table S2). In mammals, TRH is reported to stimulate the secretion of TSH and regulates TH synthesis. In non-mammalian vertebrates like amphibians and fish, however, CRH appears to be a more important stimulator of TSH secretion (De Groef et al., 2006) and might therefore function as a common regulator of the thyroid homeostasis.

It is interesting that increasing *CRH* transcription is coincided with increasing *TSHβ* in male brain but with decreasing *TSHβ* in female brain *ex vivo* (Table 2). This *ex vivo* observation in female fish brain is different from the *in vivo* observation which showed the decreases of both *CRH* and *TSHβ* transcriptions (Figures 3 and 4). Such decreases of *CRH* and *TSHβ* transcriptions in female brain clearly show negative feedback against increased plasma THs in female fish. Similar observations have been reported elsewhere (Chen et al., 2012; Liu et al., 2011b; Yu et al., 2010). The reason that *TSHβ* was not responsive to increase of CRH in *ex vivo* brain test is not clear. Other regulatory mechanisms of *TSHβ* might be present in brain, but further investigation is needed to confirm this hypothesis. TSH levels in plasma are consistent with *TSHβ* transcription in brain. But the reason why thyroid gland is not responsive to this signal is also obscure. TSH can increase the synthesis of THs, but genes related to thyroid development, and conversion of THs may not be toward the compensation of hypothyroidism in male fish.

In thyroid gland, the transcriptions of *TG* gene corresponded well with the changes of THs in plasma. Down-regulation of *TG* gene in male fish, but up-regulation in female fish by exposure to both OPFRs might partly contribute to the reduction of T4 in male and induction of T4 in female fish, respectively. TG and TPO are involved in TH synthesis. TPO catalyzes the iodination of tyrosyl residues in TG, and their coupling into iodothyronines (Dunn and Dunn, 2001) is highly conserved among vertebrates. In the present study, exposure to TDCPP or TPP resulted in significant up-regulation of *TG* gene in female, but down-regulation in male fish. The transcription of TPO did not show any significant changes but showed generally similar trends. The

relationship between TPO activity and TG iodination is not well understood, but based on our observation, TG was more responsive to exposure to OPFRs. Similar observation was reported for DE-71 elsewhere (Yu et al., 2010). Our observations are contrary to Yu et al. (2010) or Chen et al. (2012), which reported that increased gene transcription of *TG* to compensate for the reduced T4.

TTR is an important transport protein for TH that regulates the supply of the hormone to various target tissues (Kawakami et al., 2006). Many environmental contaminants that exhibit structural resemblance to THs influence the levels of circulating THs by competing for their binding sites on transport proteins, and by interfering with TH homeostasis by binding TTR, in vertebrates (Morgado et al., 2009). Reduction in TTR would subsequently cause more unbound T4 which may be more susceptible to hepatic catabolism, resulting in decrease of circulating TH concentration. Unlike PBDEs which significantly down-regulate transcription of *TTR* gene (Yu et al., 2010; Chen et al., 2012), in present study, only TDCPP significantly down-regulated transcription of *TTR* gene in female fish, but not in male fish. Exposure to TPP did not significantly influence *TTR* gene transcription in both male and female fish. Crump et al. (2012) showed that TDCPP have no significant effect of transcription of *TTR* in primary cultures of avian hepatocytes. Reduced T4 levels in male fish could not be explained by the change in *TTR* transcription.

Deiodinase (Dio) enzymes are important regulators of circulating and peripheral TH levels (Orozco and Valverde, 2005). Dio2 converts T4 into T3, and plays important role in plasma TH homeostasis. Previous studies have shown that Dio activity in fish is sensitive to environmental chemicals, and its relative mRNA expression has been recommended as a sensitive biomarker of thyroid disruption in fish (Li et al., 2009). Our observation of increased *Dio2* transcription in female, but generally decreased *Dio2* in male fish, explains the differences of TH levels by sex. Decrease in Dio2 in male fish may result in limited conversion from T4 to more active T3. Significant decreases of THs in concentration dependent manner can be explained partly by the reduced

Dio2 transcription. Dio1, which is responsible for iodine recovery and TH degradation, is generally decreased by the exposure to both OPFRs in both sex fish. Dio1 is generally considered to play minimal role in TH homeostasis in fish (Liu et al., 2011b). Our data which showed unanimous reduction in *Dio1* transcription but different directions of TH change in male and female fish also support this idea.

The exposure of fish to TDCPP or TPP affected the transcription of *TRs* gene in tissue specific manner. However sex-dependent pattern of *TRs* transcription was not detected. THs act by binding to specific TRs and play important roles in the embryogenesis and larval development in the early stages of the fish life cycle (Liu and Chan, 2002). Therefore abnormal *TRα* and *TRβ* might result in the failure of THs to bind and activate the appropriate post-receptor response cascades (Wu and Koenig, 2000).

To our knowledge, this is the first report that shows sex-dependent responses of thyroid gland to OPFRs in fish. Sex-dependent changes of THs related gene transcriptoin were reported in rats after exposure to thimerosal, and this sex-dependent difference was attributed to different sensitivity by sex (Khan et al., 2012; Sulkowski et al., 2012). The reason for sex-dependent responses to OPFRs is not clear. The fact that OPFRs could disturb sex hormones like E2 of zebrafish in sex-dependent manner (Liu et al., 2012) may explain the difference of response in THs. In fish, CRH, which stimulates TSH release, is suggested as one of the primary targets for E2. Besides, it is also possible that the sex-dependent THs levels were explained by different transcription of *TG* or *Dio2* by sex, but exact mechanisms for such different responses are not known.

It should be noted that adult fish were used in the present study. Adult zebrafish may not be sensitive enough to indicate potential impacts of these chemicals on the fish. Embryo-larval stage fish are generally favored as a reliable model for screening of thyroid disrupting chemicals (Yu et al., 2010), because early developmental stages tend to be more susceptible and the effects on this period may determine the status of later stages in life. However, using the larvae limits the amount of bio-specimen, e.g., for hormone

measurements, therefore understanding tissue-specific responses against chemical exposure is not easy (Johnson and Lema, 2011). Another limitation of the present study is the difficulties of isolation of organs like thyroid and pituitary glands in zebrafish. For example, thyroid glands are scattered throughout the pharyngeal region of the adult zebrafish (Porassi et al., 2009), and it is virtually impossible to isolate thyroid follicles without any inadvertent inclusion of non-thyroidal tissues. Likewise, the responses of pituitary glands were measured in the whole brain sample which includes other tissues as well. Because of the influence of non-target tissues, therefore quantitative comparison of the gene transcription responses measured at these organs may not be appropriate.

In conclusion, the present study showed that in zebrafish both TDCPP and TPP could modulate the transcription of several genes in the HPT axis and led to changes in TH levels in sex-dependent manner. The compensatory efforts of brain regarding TH disruption in blood, e.g., changes in *CRH* or *TSHβ* gene transcription were not supported by gene transcriptions in thyroid, e.g., *TG*. Sex-dependent effects on regulation of genes for thyroid development and TH synthesis are evident but warrant further investigation on detailed mechanisms. THs are crucial for the development, growth, and differentiation of fish, hence, consequences of longer term exposure of developing embryos in development and neuroendocrine system deserves further study.

Chapter 5. Effects of long-term exposure to TDCPP or TPP in zebrafish (*Danio rerio*) Alternations of hormone balance and gene transcriptions along HPG, HPA, and HPT axes

5.1 Introduction

In vertebrates, hypothalamic-pituitary-gonad (HPG), hypothalamic-pituitary-thyroid (HPT), and hypothalamic-pituitary-adrenal (HPA) axes are important regulators of the reproduction, thyroid and adrenal endocrine systems, respectively. Each axis is responsible for regulating the synthesis, secretion, transport, and metabolism of different hormones (Cyr and Eales, 1996; Mcgonnell and Fowkes, 2006; Zoeller, et al., 2007). In fish, reproduction process is mainly regulated by HPG axis and gonadal steroidogenic pathway (Nagahama and Yamashita, 2008; Sofikitis et al., 2008). Thyroid hormones (THs) play an important role in the development, growth and metabolism of vertebrates. Thyroid function is dependent on iodine uptake, synthesis, transport, tissue-specific deiodination, and binding to thyroid hormone nuclear receptor (TRs), of which processes are mainly regulated by HPT axis (Yen and Chin, 1994). The function of HPA axis is important in response to stressor exposures in teleosts (Iwama et al., 2006), while the adrenal gland has been rather neglected in regulatory endocrine disruption screening or test schemes (Harvey et al., 2007).

HPG, HPT, and HPA axes interact with one another rather than function independently (Milla et al., 2009; Senthilkumaran, 2007). Previous studies showed that exposure to E2 would increase plasma cortisol concentration in juvenile Atlantic Salmon (Lerne et al., 2007).Thyroid hormones have been associated with the modulation of the expression of some genes along HPG axis in teleost (Swapna and Senthilkumaran, 2007). It is likely that chemical-induced changes along one endocrine axis would lead to changes in other endocrine axes. For example, fadrozole, an aromatase inhibitor, would

decrease plasma 17β-estradiol (E2) concentration of fathead minnow (*Pimephales promelas*) or Japanese medaka (*Oryzias latipes*), and also modulate thyroid hormones in a frog (*Silurana tropicalis*) (Ankley et al., 2002; Zhang et al., 2008; Langlois et al., 2010). Exposure to propylthiouracil resulted in lesser concentration of T4 and T3 and greater concentration of E2 in plasma of zebrafish (Liu et al., 2011). Besides, exposure to polychlorinated biphenyl (PCB) 126 decreased plasma thyroxine (T4) and triiodothyronine (T3), but increased plasma concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), and E2 in rats (Desaulniers et al., 1999). However, detailed mechanisms of cross-talk among the axes have rarely been elucidated.

Tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and triphenyl phosphate (TPP) have been widely used as additive organophosphate flame retardants (OPFRs) in polyurethane foams, which are commonly found in sofas, chairs, car upholstery, and related products (Marklund et al., 2003; Reemtsma et al., 2008; Stapleton et al., 2009). Both compounds have been widely detected in air and water environment (Marklund et al., 2005; Reemtsma et al., 2008; Stapleton et al., 2011). While toxicological information on TDCPP and TPP is limited (National Research Council, 2000; U.S. Environmental Protection Agency, 2005), available reports strongly suggest endocrine disrupting potentials both *in vitro* and *in vivo*. TPP shows moderate binding affinity to androgen receptor (Fang et al., 2003) and can activate enzymes involved in metabolism of steroid hormones *in vitro* (Honkakoski et al., 2004). Besides, butylated TPP reduced male fertility and altered female reproductive cycles in rats (Latendresse et al., 1994). Exposure of rats to TDCPP resulted in a significant induction of testicular interstitial cell tumors and increased abnormalities in the testis, epididymis and seminal vesicle (NRC 2000). Concentrations of TDCPP or TPP in house dust showed positive association with increased prolactin and decreased thyroxine (T4) and testosterone (T) concentrations in man (Meeker and Stapleton, 2010).The house dust concentrations of TDCPP and TPP were also associated with decreased semen quality suggesting a link with infertility among the human adult males.

We previously observed that TDCPP and TPP could influence the synthesis, metabolism and activation of E2 in human adrenal H295R cells (Liu et al., 2012a). Both compounds could also damage the reproduction performance and alter the transcription of genes along HPG axis in zebrafish after 21 d exposure, and could change thyroid hormone levels and related gene expression along HPT axis in adult zebrafish on sex-dependent pattern after 14 d of exposure (Liu et al., 2013 in review; Liu et al., 2013 in review). However, the exposure duration was rather short up to 21 d, and the endocrine disrupting effects were investigated separately. The aim of the present study is to investigate the effects of long-term exposure to TDCPP or TPP, on the development of zebrafish and a series of events along the different endocrine regulating axes in zebrafish.

5.2 Materials and Methods

5.2.1 Testing chemicals

Same with Chapter 3.2.1

5.2.2 Zebrafish chronic exposure

5.2.2.1. Embryos phase

Adult zebrafish were placed in a spawning aquarium (40 L) and allowed to mate under a photoperiod of 16:8 h light:dark. The embryos were collected at 8 h post-fertilization (hpf) and examined under a steromicroscope. Embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Approximately 100 normal embryos were randomly selected and distributed into glass beakers containing 300 mL of test solutions. Tested concentrations were determined by preliminary range finding tests: the concentrations for TDCPP included 0, 0.005, 0.05, and 0.5 mg/L, and those of TPP were 0, 0.005, 0.05, and 0.5 mg/L. None of the test concentrations caused any distinct deformation or mortality during the exposure duration. Four replicates were employed for each treatment.

5.2.2.2 Larval-juvenile-adult phase

Newly hatched fries were transferred into 3 L glass beakers until 5-30 dpf, and then moved to 15 L tanks until the end of the experiment at 120 dpf. Four replicates were employed for each treatment. After 5 d post-fertilization (dpf), larvae were fed with (paramecia or *Artemia* nauplii) twice per day. During the exposure, approximately half of the exposure solution was renewed every two days. Mortality was recorded daily, and dead organisms were removed as soon as noted. Ten larvae and ten juvenile fish were randomly selected from each group at 14 dpf and 40 dpf, respectively, and were measured for body length (mm).

All the surviving fishes were sacrificed at 120 dpf, and the blood samples were collected from caudal vein using a heparinized capillary tube. Blood sample (20-40 μL) was centrifuged at 5,000 g for 20 min at 4^{o}C, and the supernatants were collected and frozen at -80℃ until analysis. The weights of whole body, length, gonads and livers were measured, and condition factor (K; 100 x [body weight (g)/total length^{3} (cm)]), gonadosomatic index (GSI; 100 x [gonad weight (g)/ body weight (g)]) and hepatosomatic index (HIS; 100 x [liver weight (g)/ body weight (g)]) were calculated. Brain, adrenal (anterior kidney), gonad, and thyroid were collected and preserved in RNAlater® reagent (QIAGEN, Korea Ltd., Seoul, Korea) at -80 ^{o}C for subsequent RNA isolation.

5.2.3 Hormone measurement

Hormones including testosterone (T; Cat # 582701), 17β-estradiol (E2; Cat # 582251), 11-keto testosterone (11-KT; Cat # 582751), follicle stimulating hormone (FSH; Cat # 500710), luteinizing hormone (LH; Cat # 500720) and cortisol (Cat # 500360) were measured by competitive enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Cayman Chemical, Company, Ann Arbor, MI, USA). The intra- and inter-assay coefficients of variation (CV) were <30% (detection limits: 6 pg/mL for T, 19 pg/mL for E2, 1.3 pg/mL for 11-KT, 5 mIU/mL for FSH and LH, 7.8 pg/mL for cortisol). Triiodothyronine (T3; Cat # E0453f) and thyroxine (T4; Cat # E0452f) levels were measured using ELISA kit (Uscnlife, Wuhan, China), following the manufacturer's instruction. ELISAs for T3 and T4 were validated for the use of zebrafish samples by demonstrating parallelism between a series of diluted and spiked samples in relation to the standard curve. Absorbance of each sample was measured at 415 nm by use of a microplate reader (Tecan Infinite 200, Tecan, Männedorf, Switzerland).

5.2.4 Quantitative real-time PCR or mRNA array

Extraction of total RNA, determination of purity of the RNA, synthesis of first-strand cDNA and quantitative real-time PCR were performed by use of previously described methods (Liu et al., 2012). In brief, total RNA were extracted from the brain, adrenal, gonad and thyroid samples using RNeasy mini-kit (QIAGEN). The complementary DNAs were synthesized from the purified RNA samples using iScript^{TM} cDNA Synthesis kit (BioRad, Hercules, CA, USA). The ABI 7300 Real-Time PCR system (PerkinElmer Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. The PCR reaction comprised an initial denaturation step at 50^{o}C for 2 min, 95^{o}C for 10 min, followed by 40 cycles at 95^{o}C for 15 s, and 60^{o}C for 1 min. Transcriptions of 44 genes plus one housekeeping gene (*β-actin*) were measured. Primer information for the measured genes are shown in Table 5.1. The amount of PCR product obtained was quantified by using the threshold cycle (*Ct*) number, which corresponds to the cycle in which an increase in the signal associated with the exponential growth of PCR product is detected. For each selected gene, real-time PCR reactions were performed for three replicate samples and repeated twice. The expression level of each target mRNA was normalized to that of a reference gene (*β-actin*) using the delta delta *Ct* method.

5.2.5 Statistical analysis

Same with Chapter 3.2.7

Table 5.1. qPCR primers used for gene expression assays

Gene name	Accession No.	Description (Tm℃)	Sequence (5'-3')
β-actin	NM_131031	Forward (59.5)	TGCTGTTTTCCCCTCCATTG
		Reverse (58.3)	TCCCATGCCAACCATCACT
GnRH2	AY657018	Forward (59.0)	CTGAGACCGCAGGGAAGAAA
		Reverse (59.6)	TCACGAATGAGGGCATCCA
GnRH3	NM_182887	Forward (59.3)	TTGCCAGCACTGGTCATACG
		Reverse (58.3)	TCCATTTCACCAACGCTTCTT
GnRHR1	NM_001144980	Forward (58.9)	ACCCGAATCCTCGTGGAAA
		Reverse (59.9)	TCCACCCTTGCCCTTACCA
GnRHR2	NM_001144979	Forward (58.8)	CAACCTGGCCGTGCTTTACT
		Reverse (58.7)	GGACGTGGGAGCGTTTTCT
GnRHR3	NM_001177450	Forward (56.9)	GAGGCGCAGCGGAACA
		Reverse (60.6)	AAGACCCCTGTCGTTCCAATC
GnRHR4	NM_001098193	Forward (58.4)	CACCAACAACAAGCGCAAGT
		Reverse (58.5)	GGCAACGGTGAGGTTCATG
FSHβ	NM_205624	Forward (58.4)	GCTGTCGACTCACCAACATCTC
		Reverse (58.3)	GTGACGCAGCTCCCACATT
LHβ	NM_205622	Forward (58.3)	GGCTGCTCAGAGCTTGGTTT
		Reverse (58.1)	TCCACCGATACCGTCTCATTTA
CYP19B	AF183908	Forward (58.3)	GTCGTTACTTCCAGCCATTCG
		Reverse (59.1)	GCAATGTGCTTCCCAACACA
ERα	NM_152959	Forward (58.7)	CAGACTGCGCAAGTGTTATGAAG
		Reverse (59.4)	CGCCCTCCGCGATCTT
ER2β	NM_174862	Forward (58.3)	TTCACCCCTGACCTCAAGCT
		Reverse (58.6)	TCCATGATGCCTTCAACACAA
AR	NM_001083123	Forward (58.1)	TCTGGGTTGGAGGTCCTACAA
		Reverse (59.4)	GGTCTGGAGCGAAGTACAGCAT
FSHR	NM_001001812	Forward (59.1)	CGTAATCCCGCTTTTGTTCCT
		Reverse (58.9)	CCATGCGCTTGGCGATA
LHR	AY424302	Forward (58.5)	GGCCATCGCCGGAAA
		Reverse (59.8)	GGTTAATTTGCAGCGGCTAGTG
HMGRA	BC155135	Forward (58.7)	GAATCCACGGCCTCTTCGT
		Reverse (58.4)	GGGTTACGGTAGCCACAATGA
HMGRB	NM_001014292	Forward (59.9)	TGGCCGGACCGCTTCTA
		Reverse (58.4)	GTTGTTGCCATAGGAACATGGA
StAR	NM_131663	Reverse (58.2)	GGTCTGAGGAAGAATGCAATGAT

		Reverse (58.8)	CCAGGTCCGGAGAGCTTGT
CYP11A	NM_152953	Forward (58.0)	GGCAGAGCACCGCAAAA
		Reverse (59.3)	CCATCGTCCAGGGATCTTATTG
3βHSD	AY279108	Forward (58.7)	AGGCACGCAGGAGCACTACT
		Reverse (58.0)	CCAATCGTCTTTCAGCTGGTAA
CYP17	AY281362	Forward (58.4)	TCTTTGACCCAGGACGCTTT
		Reverse (59.9)	CCGACGGGCAGCACAA
17βHSD	AY306005	Forward (59.7)	TGCATCTCGCATCAAATCCA
		Reverse (58.0)	GTCCAAGTTCCGCATAGTAGCA
CYP19A	AF226620	Forward (58.5)	GCTGACGGATGCTCAAGGA
		Reverse (58.8)	CCACGATGCACCGCAGTA
CRH	NM_001007379	Forward (59.8)	GCGCTGCAGCTCCAGTTAAC
		Reverse (58.1)	GGCCGATGTTTCCAACTTTC
CRHBP	NM_001003459	Forward (58)	TGGAGAGCTCAGCCTTGGA
		Reverse (59)	AGCGCAGCCAAGAATGGA
CRHR2	XM_681362	Forward (58.3)	TTACCAAGGGCCTGTGATTCTAG
		Reverse (58.4)	GCGCACAATGTTGAAAAGAAAC
POMC	AY158003	Forward (58.2)	CGCAGACCCATCAAGGTGTA
		Reverse (58.2)	CGTTTCGGCGGATTCCT
GR	EF567112	Forward (59.7)	GCCTGTCGTTTCCGCAAAT
		Reverse (58.2)	TTGCTTTTACGTGCCTCTAGGTT
MR	EF567113	Forward (58.3)	TCGGCTATTGTTGGCGTTAAT
		Reverse (58.3)	TGCGCTCCAATCTGGTAATG
MC2R	NM_180971	Forward (58.8)	AAACCGAATCGCGTCTATGC
		Reverse (59.8)	CAGGCCGCTTTTCCTGTGT
CYP11B	DQ650710	Forward (59.8)	TGGGCCACACATCGAGAGA
		Reverse (58.9)	CGTCCCGTTCTTGAGGAAGA
TRH	NM_001012365	Forward (58.1)	GCTCTCTCCGTCGGTCTGTT
		Reverse (59.5)	GCGAGATCCGTGCTGATGA
TRHR1	NM_001114688	Forward (58.2)	CAGTGCCATCAACCCTCTGA
		Reverse (58.6)	GGCAGCGCGGAACTTCT
TSHβ	AY135147	Forward (59.7)	CCATCTGCATGGGCTTCTGT
		Reverse (59.9)	CGAGGACCCACCAACTCCTT
TRα	NM_131396	Forward (58.4)	GCCGCTTCCTGCACATG
		Reverse (58.3)	AGCGGCGGGAACAGTTC
TRβ	NM_131340	Forward (59.2)	TGGCATGGCTACAGACTTGGT
		Reverse (58.3)	TCAGCTTCCGCTTGGCTAA
TSHR	NM_001145763	Forward (58.5)	GCGAGAAGGGAGAGGAGGTT

		Reverse (58.9)	TCCTCGCAAGGGTTGAACTC
Pax8	XM_001339857	Forward (59.8)	AAGACCCCTGTCGTTCCAATC
		Reverse (60.3)	AAACTCGTACTGCAGGGATCCA
Hhex	NM_130934	Forward	TGCTTGTCATGCCAGTGGAT
		Reverse	GACATCTCTTCCACCAGGATCTTC
TPO	EU267076	Forward (58.8)	GTTCGGTCTGCCAGGACACT
		Reverse (59.9)	TCCAAGCGCTTCAGCAGAGT
TG	XM_689200	Forward (58.1)	GTCTCTTGAGTGTTCGAATGACAAG
		Reverse (58.6)	AAAGGCGGGCCATTAAGG
NIS	NM_001089391	Forward (59.3)	AATCAAGCCACAGGCCTGAA
		Reverse (58.3)	AATGTGCAGATGAGCCCAGTT
DEIO1	BC076008	Forward (58.2)	AACTTGGAGGAGAGGCTTGCT
		Reverse (58.6)	AGGGCATGGAGGGTCTTCTT
DEIO2	NM_212789	Forward (59.5)	CGCGAAATGGGCTTGCT
		Reverse (58.1)	CCAGGCAAAATCTGCAAAGTTA

5.3 Results

5.3.1. Concentrations of TDCPP or TPP in exposure media

The concentrations of TDCPP and TPP in the exposure media were measured and presented in Table 5.2. The measured concentrations of TDCPP were in a range between 87% and 100% of the nominal concentrations, and were generally consistent over the 48 h of the water renewal interval. For TPP, the actual concentrations at 0 h were similar to nominal concentrations with a range between 71% and 92%, but the concentrations measured at 48 h showed notable decrease. For simplicity, the nominal concentrations of TDCPP and TPP were used for presentation of the results throughout this paper.

Table 5.2. Nominal and measured concentrations of TDCPP or TPP in exposure waters during 120 d exposure.

	Nominal	Measured			Nominal	Measured	
		0 h	48 h			0 h	48 h
TDCPP	0.000	0.000	0.000	TPP	0.000	0.000	0.000
	0.005	0.005	0.005		0.005	0.004	0.000
	0.050	0.050	0.050		0.050	0.046	0.000
	0.500	0.436	0.405		0.500	0.355	0.180

5.3.2. Developmental toxicity of TDCPP and TPP

The K of larval and juvenile fish was not affected even at the highest experimental concentrations of TDCPP and TPP, respectively (Table 5.3). However, in adult fish the changes were significant. K, GSI and HSI in adult female fish were significantly reduced at 0.5 mg/L TDCPP. Similarly decreasing patterns were observed in the adult fish following the exposure to TPP.

Table 5.3. Growth endpoints determined in zebrafish exposed to TDCPP and TPP.

Chemicals	Concentration	Larvae	Juvenile	Adult (Male)			Adult (Female)		
	(mg/L)	K	K	K	GSI	HSI	K	GSI	HSI
TDCPP	SC	1.32±0.26	0.58±0.07	1.51±0.15	1.67±0.68	1.54±0.24	1.79±0.07	16.57±2.15	3.53±0.28
	0.005	1.17±0.11	0.64±0.06	1.46±0.13	1.51±0.36	1.15±0.58	1.74±0.12	14.41±1.43	2.94±0.83
	0.05	1.22±0.17	0.62±0.04	1.50±0.14	1.36±0.17	1.50±0.37	1.83±0.15	15.65±3.83	3.28±0.45
	0.5	1.39±0.34	0.66±0.08	1.29±0.18	1.52±0.27	1.37±0.25	1.50±0.06*	9.53±0.98*	3.16±0.17*
TPP	0.005	1.26±0.24	0.61±0.04	1.47±0.15	1.56±0.34	1.51±0.69	1.70±0.15	13.41±3.26	3.39±0.60
	0.05	1.26±0.43	0.59±0.07	1.57±0.10	1.32±0.25	1.67±0.46	1.71±0.16	14.49±3.86	2.99±0.74
	0.5	1.35±0.27	0.67±0.05	1.31±0.15	1.18±0.48	1.08±0.36	1.81±0.17	12.8±3.27	3.03±0.63

5.3.2. Changes in hormones by exposure to TDCPP

For most of the hormones that were measured in the present study, statistically significant changes were observed after the exposure to TDCPP. In females, the concentrations of plasma cortisol, FSH and LH were significantly increased at all the experimental concentrations from 0.005 mg/L TDCPP. Cortisol levels were also increased at as low as 0.05 mg/L TDCPP (Figure 5.1). The E2 levels and E2/T ratios were significantly increased at ≥ 0.005 mg/L TDCPP. E2/11-KT ratios were also significantly increased at 0.05 mg/L TDCPP (Figure 5.2). The concentrations of T4 and T3 were significantly increased after exposure to TDCPP among the female fish (Figure 5.3). Among male fish, however, the pattern of hormonal change was different. The concentrations of cortisol, FSH, LH, T, T4, and T3 were significantly decreased, and E2/T and E2/11-KT ratios were increased following the exposure (Figures 5.1, 5.2, and 5.3).

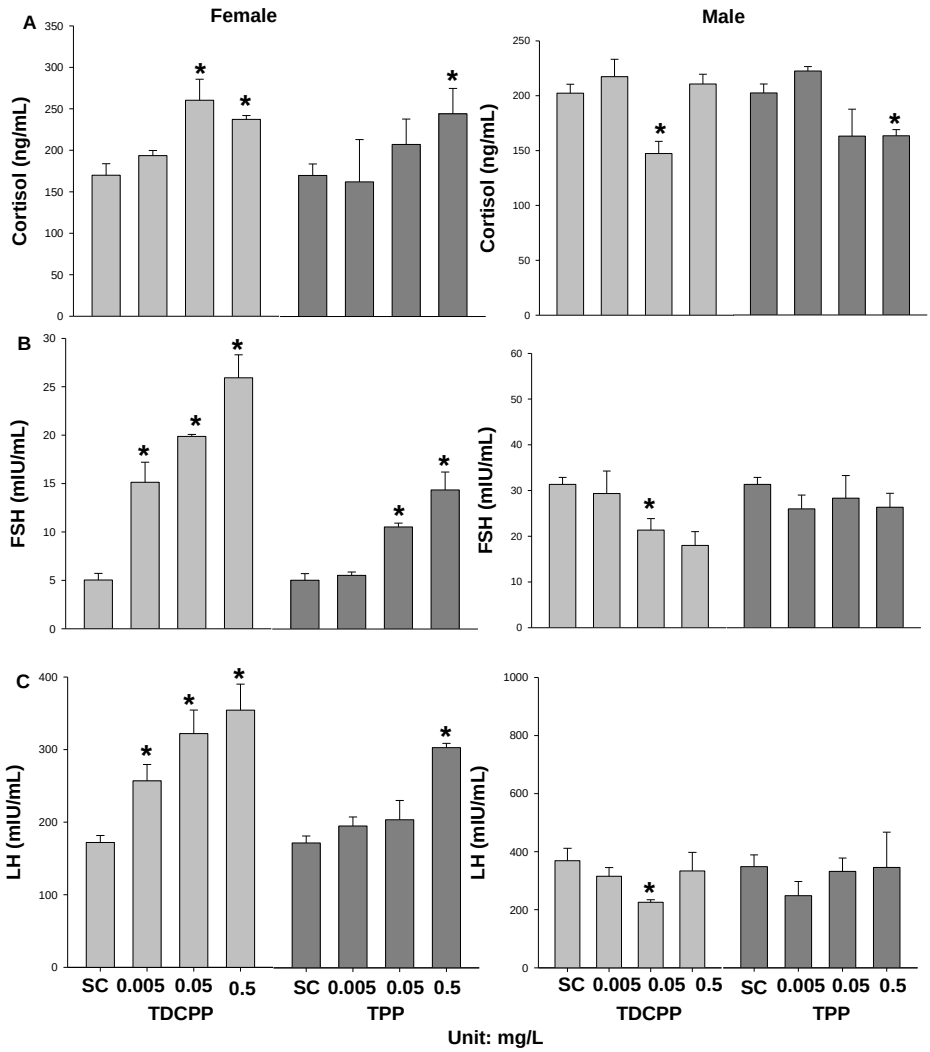


Figure 5.1. Effects of 120 d exposure to TDCPP or TPP on plasma level of cortisol (A), follicle stimulating hormone (FSH) (B), and luteinizing hormone (LH) in female and male zebrafish. Data are expressed as mean ± SD of six replicates. Asterisk indicates significant difference (p<0.05) from the solvent control (SC, treated with 0.005% DMSO).

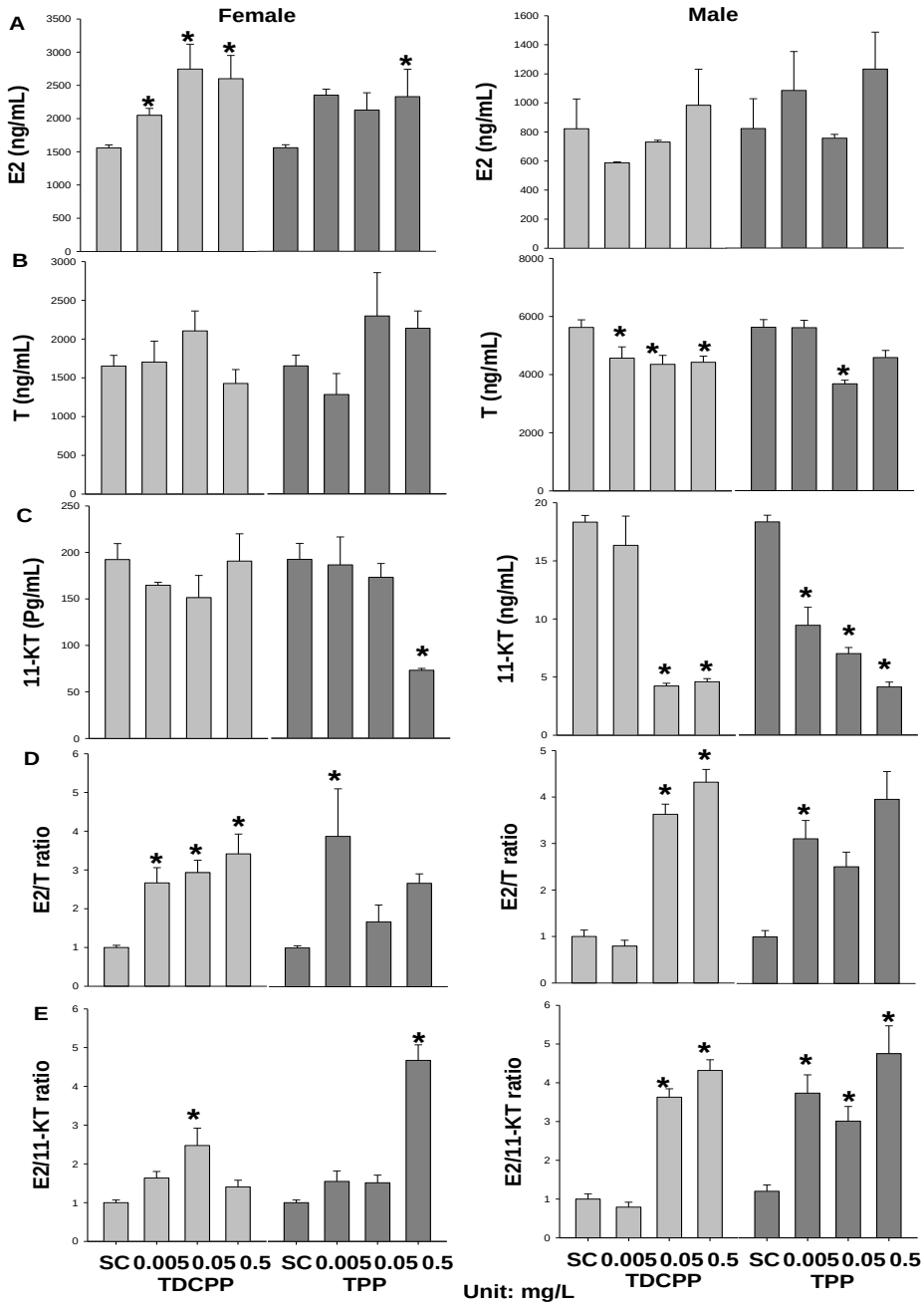


Figure 5.2. Effects of 120 d exposure to TDCPP or TPP on plasma E2 (A), T (B), 11-KT (C) levels and E2/T ratio (D), E2/11-KT ratio (E) in female and male zebrafish. Data are expressed as mean ± SD of six replicates. Asterisk indicates significant difference (p<0.05) from the solvent control (SC, treated with 0.005% DMSO).

5.3.3 Changes in hormones by exposure to TPP

The concentrations of plasma cortisol were significantly increased among the female fish after the exposure to TPP. Concentrations of FSH in blood plasma were significantly greater in female fish following the exposure, whereas opposite trend was observed for males (Figure 5.1). After the exposure, E2 levels, E2/T ratio, and E2/11-KT ratio were significantly increased, while 11-KT levels were significantly decreased among the female fish (Figure 5.2). In males, concentrations of T and 11-KT were significantly decreased, whereas E2/T ratio and E2/11-KT ratio were significantly increased. Concentrations of T4 and T3 were significantly increased in both female and male fish after exposure to 0.05 mg/L and 0.5 mg/L TPP, respectively (Figure 5.3).

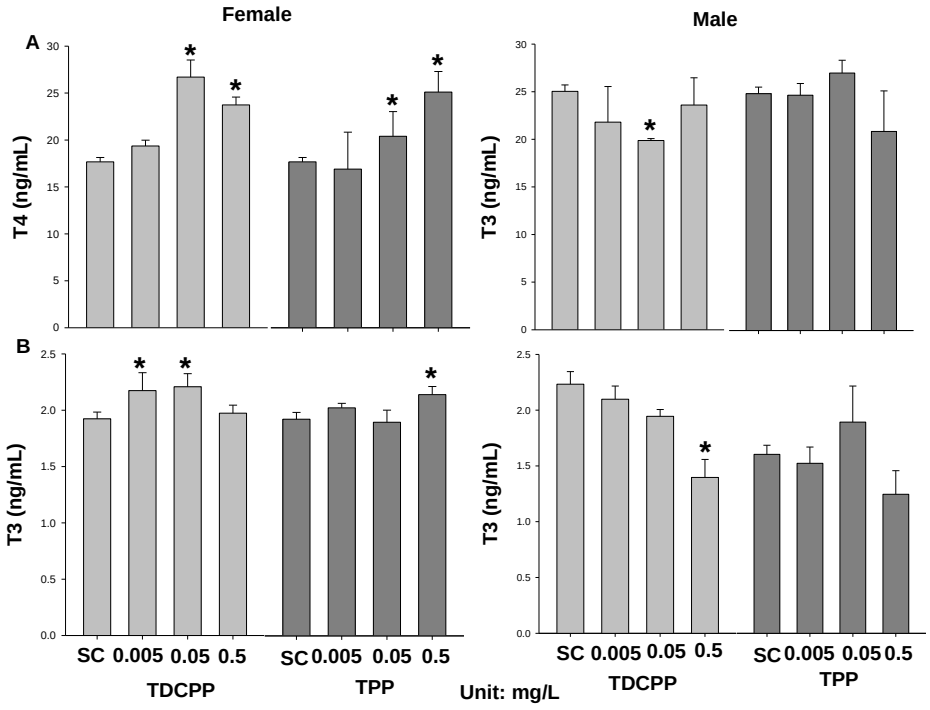


Figure 5.3. Effects of 120 d exposure to TDCPP or TPP on plasma thyroxine (T4) and triiodothyronine (T3) level in female (A) and male fish (B). Data are expressed as mean ± SD of five replicates. Asterisk indicates significant difference (p<0.05) from the solvent control (SC, treated with 0.005% DMSO).

5.3.4. Changes in gene transcription by exposure to TDCPP

Changes in transcriptional profiles of major genes of HPG, HPA, and HPT axes in zebrafish following exposure to TDCPP were dose- and sex-dependent (Figure 5.4 and Table 5.4). In females, transcriptions of *GnRH3*, *GnRHR2*, *GnRHR3*, *ERα*, *FSHβ*, and *LHβ* genes were significantly up-regulated in brain, while no significant changes were observed for *GnRH2*, *GnRHR1*, GnRHR4, *CYP19B*, *ER2β1*, and *AR*. In gonad, significant up-regulation of *FSHR*, *LHR*, *HMGRA*, *StAR*, *CYP11A*, and *CYP17* were observed after exposure to TDCPP. Along the HPA axis, transcriptions of *POMC* and *MR* genes in brain, and *CYP11A*, *CYP17*, *17βHSD* and *CYP11B* genes in adrenal gland were significantly up-regulated. Along the HPT axis, transcriptions of *TRHR2* in brain, and *TSHR*, *TPO*, *Pax8* and *DIO2* in thyroid gland were significantly up-regulated. In males, transcriptions of *GnRH3*, *FSHβ*, *LHβ*, *StAR*, *HMGRA*, and *TRHR2* genes of HPG or HPT axes were significantly down-regulated, while those of *POMC* and *CYP11B* genes of HPA axis was significantly up-regulated.

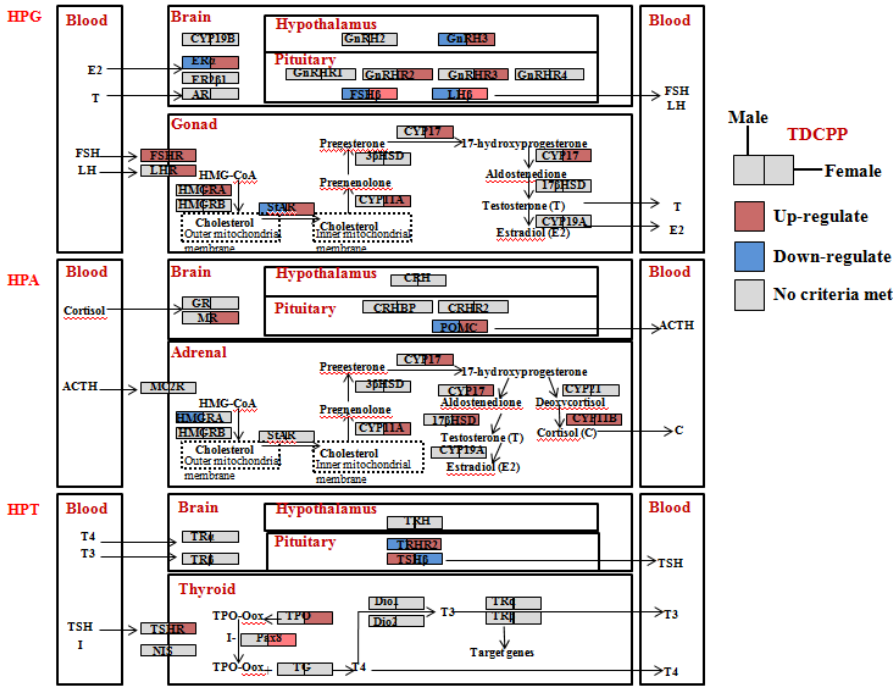


Figure 5.4. Stripped view of sex-dependent response profile in adult zebrafish after 120 d exposure to TDCPP. Gene transcription data from zebrafish treated by 0.005, 0.05, or 0.5 mg TDCPP/L are shown as striped color sets on the selected endocrine pathways along HPG, HPA, or HPT axis of zebrafish. The legend listed in the upper right corner of the graph describes the order of sex and the three colors depict different change thresholds. Red: statistically significant up-regulate; Blue: statistically significantly down-regulate; Gray: no statistically significant change.

5.3.5. Changes in gene transcription by exposure to TPP

In females, transcriptions of *GnRH3*, *ERα*, *FSHβ*, *LHβ*, *FSHR*, *LHR*, *StAR*, and *CYP19A* genes of HPG axis, and *POMC*, *MR*, *MC2R*, *CYP11A*, *CYP17*, *17βHSD* and *CYP11B* genes of HPA axis were significantly up-regulated (Figure 5.5 and Table 5.5). Among the genes of HPT axis, transcriptions of *NIS*, *Pax8*, and *TPO* genes were significantly up-regulated. In males, transcriptions of *GnRH3*, *StAR*, and *CYP17* genes of HPG axis were significantly down-regulated. Except for significant up-regulation of *CYP11B* and down-regulation of TRH, no genes of HPA and HPT axes were significantly altered.

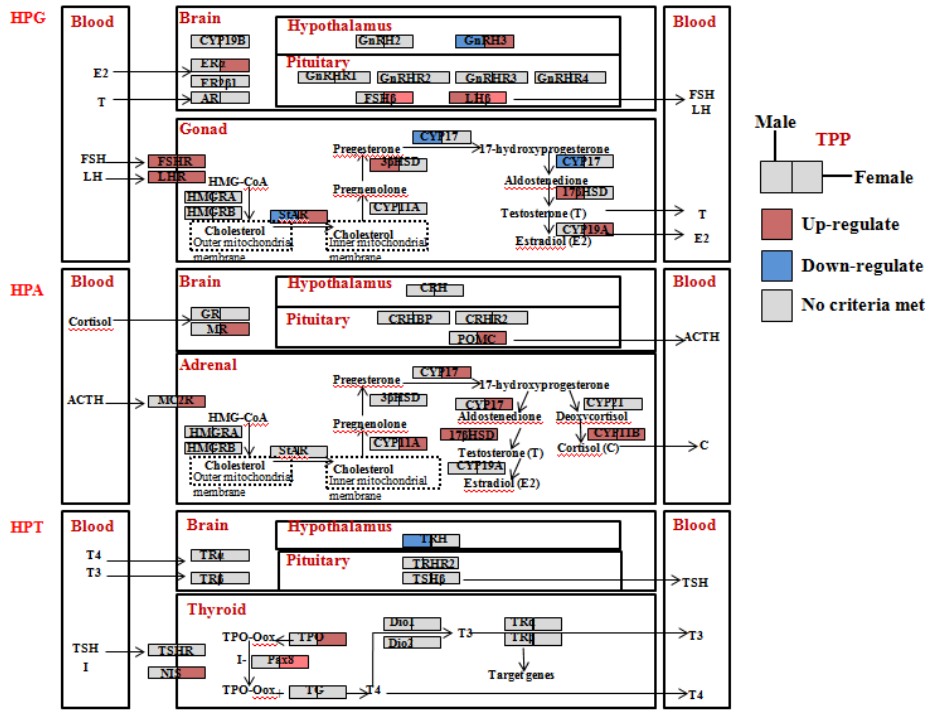


Figure 5.5. Stripped view of sex-dependent response profile in adult zebrafish after 120 d exposure to TPP. Gene expression data from zebrafish treated by 0.005, 0.05 or 0.5 mg TPP/L are shown as striped color sets on the selected endocrine pathways along HPG, HPA, or HPT axis of zebrafish. The legend listed in the upper right corner of the graph describes the order of sex and the three colors depict different change thresholds. Red: significantly up-regulate; Blue: significantly down-regulate; Gray: no statistically significant change.

Table 5.4. Changes in gene transcription of HPG, HPA and HPT axes in male and female zebrafish by the 120 d exposure to TDCPP

Axes	Tissue	Gene	Female				Male			
			0 mg/L	0.005 mg/L	0.05 mg/L	0.5 mg/L	0 mg/L	0.005 mg/L	0.05 mg/L	0.5 mg/L
HPG	Brain	*GnRH2*	1.0±0.30	0.79±0.25	1.43±0.41	0.85±0.14	1.0±0.30	0.73±0.32	1.03±0.37	1.05±0.17
		GnRH3	1.0±0.08	0.97±0.18	2.57±0.59*	1.90±0.08*	1.0±0.23	0.79±0.06	0.63±0.07*	0.67±0.10
		GnRHR1	1.0±0.29	0.78±0.24	1.28±0.19	1.13±0.07	1.0±0.13	0.97±0.35	0.69±0.13	0.70±0.10
		GnRHR2	1.0±0.15	1.97±0.65	2.25±0.45	1.49±0.08*	1.0±0.17	2.20±1.32	1.13±0.09	1.42±0.17
		GnRHR3	1.0±0.11	2.39±0.17*	2.45±0.54	2.01±0.49*	1.0±0.14	1.22±0.44	0.67±0.17	0.93±0.22
		GnRHR4	1.0±0.11	1.45±0.62	1.54±0.35	1.12±0.16	1.0±0.07	1.17±0.20	0.90±0.08	1.05±0.07
		CYP19B	1.0±0.14	0.98±0.31	1.23±0.29	1.28±0.31	1.0±0.03	1.42±0.49	0.89±0.03	0.97±0.04
		ERα	1.0±0.24	1.73±0.99	1.61±0.77	3.52±0.84*	1.0±0.23	1.00±0.63	0.58±0.19	0.71±0.33
		ER2β1	1.0±0.33	1.20±0.18	0.94±0.06	1.43±0.38	1.0±0.17	1.35±0.24	1.15±0.44	0.90±0.08
		AR	1.0±0.05	1.40±0.18	1.46±0.31	1.36±0.13	1.0±0.09	1.36±0.12	1.15±0.02	1.09±0.11
		FSHβ	1.0±0.06	1.15±0.40	1.73±0.13*	3.45±0.34*	1.0±0.19	0.94±0.13	0.50±0.08*	1.07±0.07
		LHβ	1.0±0.09	1.18±0.08	0.96±0.16	4.25±0.57*	1.0±0.16	1.20±0.59	0.41±0.13*	0.46±0.06*
	Gonad	*FSHR*	1.0±0.05	0.99±0.19	0.82±0.11	2.31±0.19*	1.0±0.08	1.47±0.31	1.68±0.18*	1.93±0.16*
		LHR	1.0±0.16	0.98±0.08	1.86±0.28	2.64±0.33*	1±0.10	1.30±0.23	2.18±0.78	1.84±0.22
		HMGRA	1.0±0.31	1.02±0.16	1.82±0.69	3.20±0.30*	1.0±0.19	1.15±0.03	1.05±0.15	1.01±0.31
		HMGRB	1.0±0.16	0.98±0.18	1.09±0.53	1.64±0.30	1.0±0.05	1.58±0.08	1.34±0.85	1.38±0.32

		StAR	1.0±0.04	0.93±0.21	1.19±0.06	1.69±0.13*	1.0±0.04	1.10±0.61	0.81±0.20	0.66±0.01*
		CYP11A	1.0±0.27	0.82±0.05	1.52±0.15*	1.73±0.22*	1.0±0.19	1.53±0.08	0.87±0.08	1.42±0.38
		CYP17	1.0±0.21	0.96±0.16	1.27±0.14	1.75±0.37*	1.0±0.58	0.99±0.50	1.21±0.29	1.19±0.11
		CYP19A	1.0±0.13	0.72±0.14	1.18±0.20	1.46±0.05	1.0±0.09	1.35±0.33	0.97±0.47	1.02±0.40
		3βHSD	1.0±0.07	0.99±0.30	2.21±0.83	1.59±0.16	1.0±0.07	1.74±0.50	1.71±0.46	2.24±0.60
		17βHSD	1.0±0.15	0.77±0.38	1.04±0.05	1.49±0.35	1.0±0.07	0.82±0.12	1.38±0.42	1.88±0.30
HPA	Brain	*CRH*	1.0±0.10	0.91±0.36	1.28±0.48	0.85±0.23	1.0±0.22	0.73±0.08	0.86±0.18	0.96±0.10
		CRHBP	1.0±0.10	0.80±0.32	1.22±0.22	0.90±0.03	1.0±0.08	0.82±0.10	0.80±0.13	0.94±0.14
		CRHR2	1.0±0.10	0.79±0.22	1.23±0.28	0.82±0.30	1.0±0.25	1.13±0.32	0.86±0.02	0.92±0.03
		POMC	1.0±0.15	1.08±0.07	1.32±0.41	5.26±0.57*	1.0±0.15	1.15±0.23	1.50±0.01	1.62±0.07*
		GR	1.0±0.21	0.97±0.35	1.28±0.21	1.08±0.08	1.0±0.21	1.07±0.19	0.81±0.11	0.81±0.12
		MR	1.0±0.19	1.18±0.18	1.02±0.12	6.45±1.08*	1.0±0.14	1.11±0.08	1.06±0.20	1.03±0.19
	Adrenal	*MC2R*	1.0±0.20	0.90±0.20	0.96±0.35	1.22±0.09	1.0±0.10	0.98±0.11	1.59±0.22	0.96±0.10
		HMGRA	1.0±0.09	0.99±0.11	1.13±0.20	1.16±0.10	1.0±0.10	1.03±0.38	0.80±0.30	0.55±0.07*
		HMGRB	1.0±0.09	0.97±0.21	0.97±0.35	0.90±0.17	1.0±0.06	0.96±0.04	1.02±0.10	0.89±0.09
		StAR	1.0±0.11	0.99±0.23	1.31±0.12	1.13±0.44	1.0±0.15	0.92±0.16	1.40±0.31	2.22±0.61
		CYP11A	1.0±0.07	0.98±0.26	1.20±0.10	1.61±0.11*	1.0±0.11	1.20±0.58	1.18±0.11	0.93±0.16
		CYP17	1.0±0.07	2.47±0.92	1.43±0.24	4.11±0.37*	1.0±0.22	1.18±0.16	1.05±0.13	0.95±0.35
		CYP19A	1.0±0.24	0.89±0.19	0.86±0.14	0.93±0.17	1.0±0.17	1.22±0.23	1.02±0.35	0.88±0.33

		3βHSD	0.90±0.05	0.94±0.25	0.90±0.24	1.45±0.25	1.0±0.22	1.35±0.26	0.95±0.19	1.11±0.02
		17βHSD	1.0±0.29	0.94±0.51	1.32±0.58	3.04±0.72*	1.0±0.19	0.94±0.48	0.83±0.05	0.90±0.28
		CYP11B	1.0±0.15	1.02±0.20	1.72±0.48	2.11±0.37*	1.0±0.07	0.85±0.16	2.31±0.21*	2.26±0.07*
HPT	Brain	*TRH*	1.0±0.01	0.80±0.25	1.22±0.34	0.82±0.19	1.0±0.25	1.06±0.18	1.03±0.21	0.99±0.11
		TRHR2	1.0±0.19	1.14±0.10	1.30±0.22	2.43±0.19*	1.0±0.18	0.65±0.15	0.19±0.09*	0.58±0.15*
		TSHβ	1.0±0.06	1.17±0.17	1.02±0.31	0.49±0.13*	1.0±0.17	1.00±0.24	1.46±0.06	2.25±0.07*
	Thyroid	*TSHR*	1.0±0.12	1.42±0.67	1.13±0.18	2.49±0.38*	1.0±0.32	1.29±0.31	2.09±0.58	1.55±0.41
		NIS	1.0±0.17	1.34±0.22	0.94±0.25	1.06±0.10	1.0±0.04	1.21±0.07	1.32±0.34	1.29±0.35
		TG	1.0±0.24	0.98±0.09	0.64±0.20	0.99±0.10	1.0±0.27	1.22±0.15	1.01±0.41	0.92±0.27
		TPO	1.0±0.06	1.50±0.72	1.30±0.29	2.62±0.57*	1.0±0.11	0.85±0.20	0.98±0.44	1.01±0.10
		Pax8	1.0±0.15	0.89±0.22	1.25±0.40	2.19±0.13*	1.0±0.19	1.37±0.04	2.89±1.32	1.74±0.27
		Hhex	1.0±0.27	1.53±0.09	1.77±0.62	2.26±0.42	1.0±0.26	1.79±0.67	1.50±0.16	1.77±0.67
		DIO1	1.0±0.05	0.77±0.24	0.73±0.17	1.05±0.14	1.0±0.05	1.31±0.21	1.20±0.25	1.13±0.44
		DIO2	1.0±0.08	0.96±0.17	1.09±0.43	2.38±0.22*	1.0±0.34	1.25±0.49	1.00±0.35	1.06±0.16
		TRα	1.0±0.16	1.14±0.20	0.78±0.50	1.29±0.24	1.0±0.20	1.22±0.23	1.25±0.07	1.34±0.14
		TRβ	1.0±0.27	0.86±0.28	0.90±0.27	1.60±0.33	1.0±0.13	1.25±0.26	1.79±0.46	1.66±0.46

Table 5.5. Changes in gene transcription of HPG, HPA and HPT axes in male and female zebrafish by the 120 d exposure to TPP

Axes	Tissue	Gene	Female				Male			
			0 mg/L	0.005 mg/L	0.05 mg/L	0.5 mg/L	0 mg/L	0.005 mg/L	0.05 mg/L	0.5 mg/L
HPG	Brain	*GnRH2*	1.0±0.30	0.97±0.08	0.86±0.06	0.99±0.12	1.0±0.30	0.99±0.26	1.42±0.42	1.64±0.29
		GnRH3	1.0±0.08	1.56±0.34	1.02±0.13	1.84±0.03*	1.0±0.23	1.29±0.17	0.88±0.23	0.60±0.02*
		GnRHR1	1.0±0.29	0.98±0.39	0.83±0.25	0.99±0.06	1.0±0.13	0.89±0.16	1.15±0.05	0.77±0.19
		GnRHR2	1.0±0.15	1.16±0.14	1.21±0.11	1.04±0.21	1.0±0.17	2.20±1.32	1.13±0.09	1.42±0.17
		GnRHR3	1.0±0.11	1.10±0.59	1.42±0.59	1.22±0.41	1.0±0.14	1.60±0.38	0.94±0.12	1.57±0.26
		GnRHR4	1.0±0.11	1.13±0.06	0.99±0.23	0.89±0.14	1.0±0.07	1.22±0.27	1.04±0.09	1.07±0.32
		CYP19B	1.0±0.14	1.25±0.11	1.03±0.46	1.20±0.04	1.0±0.03	1.47±0.28	1.08±0.31	1.23±0.17
		ERα	1.0±0.24	0.87±0.13	0.86±0.36	1.60±0.22*	1.0±0.23	0.93±0.22	0.76±0.15	1.10±0.29
		ER2β1	1.0±0.33	0.91±0.09	0.75±0.27	0.96±0.31	1.0±0.17	1.09±0.23	1.06±0.60	1.58±0.42
		AR	1.0±0.05	1.0±0.13	1.02±0.11	0.94±0.07	1.0±0.09	1.24±0.12	1.24±0.12	1.38±0.28
		FSHβ	1.0±0.06	1.82±0.25*	2.12±0.69	2.48±0.29*	1.0±0.19	0.81±0.19	1.08±0.46	1.28±0.14
		LHβ	1.0±0.09	2.63±0.52	1.35±0.07	3.60±0.68*	1.0±0.16	0.53±0.13	1.31±0.19	2.93±0.48*
	Gonad	*FSHR*	1.0±0.05	1.33±0.58	1.67±0.58	1.90±0.46	1.0±0.08	1.67±0.38*	1.03±0.21	2.12±0.25*
		LHR	1.0±0.16	1.09±0.29	2.11±0.53	5.83±0.21*	1±0.10	0.88±0.27	0.95±0.14	2.87±0.03*
		HMGRA	1.0±0.31	1.19±0.09	2.73±0.44	2.13±0.78	1.0±0.19	1.08±0.06	0.92±0.20	1.20±0.12
		HMGRB	1.0±0.16	1.27±0.23	1.54±0.61	2.27±0.72	1.0±0.05	1.07±0.20	1.01±0.32	1.28±0.20

		StAR	1.0±0.04	1.15±0.37	2.38±0.09*	2.21±0.14*	1.0±0.04	1.01±0.11	0.86±0.47	0.84±0.02*
		CYP11A	1.0±0.27	0.89±0.10	1.05±0.14	1.26±0.30	1.0±0.19	1.13±0.32	1.02±0.07	1.30±0.06
		CYP17	1.0±0.21	1.33±0.15	1.33±0.44	1.13±0.13	1.0±0.58	0.79±0.16	0.61±0.35	0.57±0.09*
		CYP19A	1.0±0.13	1.25±0.24	1.06±0.38	1.59±0.06*	1.0±0.09	0.80±0.03	0.83±0.17	0.91±0.05
		3βHSD	1.0±0.07	1.50±0.32	1.38±0.17	0.97±0.25	1.0±0.07	1.10±0.19	1.12±0.46	1.42±0.00*
		17βHSD	1.0±0.15	1.18±0.22	1.23±0.26	0.84±0.15	1.0±0.07	1.76±0.46	2.21±0.16*	1.12±0.27
HPA	Brain	*CRH*	1.0±0.10	1.16±0.27	1.14±0.11	0.91±0.17	1.0±0.22	1.35±0.17	1.31±0.19	1.05±0.29
		CRHBP	1.0±0.10	1.15±0.26	0.99±0.20	0.92±0.25	1.0±0.08	1.07±0.05	1.09±0.16	1.05±0.24
		CRHR2	1.0±0.10	1.18±0.24	1.06±0.13	0.93±0.20	1.0±0.25	0.93±0.07	1.08±0.07	1.00±0.27
		POMC	1.0±0.15	0.91±0.16	0.96±0.30	1.85±0.15*	1.0±0.15	1.26±0.13	1.06±0.31	1.64±0.08*
		GR	1.0±0.21	0.97±0.13	0.95±0.13	1.02±0.09	1.0±0.21	1.05±0.09	1.18±0.21	1.09±0.16
		MR	1.0±0.19	1.02±0.08	0.86±0.08	2.34±0.20*	1.0±0.14	1.11±0.27	1.08±0.11	1.15±0.05
	Adrenal	*MC2R*	1.0±0.20	0.91±0.20	0.83±0.07	1.93±0.11*	1.0±0.10	1.31±0.43	1.37±0.46	0.77±0.14
		HMGRA	1.0±0.09	0.95±0.04	0.83±0.28	1.49±0.49	1.0±0.10	1.17±0.18	1.16±0.35	1.31±0.46
		HMGRB	1.0±0.09	1.21±0.45	1.27±0.30	1.17±0.10	1.0±0.06	1.33±0.26	1.36±0.17	1.27±0.45
		StAR	1.0±0.11	1.03±0.17	1.46±0.31	1.30±0.09	1.0±0.15	1.11±0.06	1.33±0.24	4.21±1.03
		CYP11A	1.0±0.07	0.98±0.33	1.48±0.11	2.03±0.05*	1.0±0.11	0.99±0.12	1.35±0.19	0.95±0.08
		CYP17	1.0±0.07	0.97±0.10	1.63±0.21	2.38±0.44*	1.0±0.22	1.31±0.37	0.73±0.21	0.78±0.22
		CYP19A	1.0±0.24	0.86±0.18	0.67±0.27	0.69±0.14	1.0±0.17	1.50±0.34	1.16±0.26	1.79±0.30

		3βHSD	0.90±0.05	0.85±0.22	1.36±0.16	1.17±0.12	1.0±0.22	1.49±0.27	0.49±0.10	1.26±0.08
		17βHSD	1.0±0.29	1.07±0.38	1.41±0.44	1.90±0.14*	1.0±0.19	0.76±0.08	0.99±0.36	2.19±0.07*
		CYP11B	1.0±0.15	1.71±0.83	2.25±0.71	2.64±0.30*	1.0±0.07	0.96±0.11	0.96±0.16	3.12±0.24*
HPT	Brain	*TRH*	1.0±0.01	1.22±0.07	1.00±0.31	0.85±0.20	1.0±0.25	1.27±0.30	1.01±0.06	0.60±0.07*
		TRHR2	1.0±0.19	0.94±0.26	0.99±0.15	2.37±0.31	1.0±0.18	0.55±0.18	0.77±0.08	0.71±0.11
		TSHβ	1.0±0.06	0.98±0.61	1.05±0.15	1.00±0.15	1.0±0.17	1.14±0.33	0.59±0.14	1.29±0.47
	Thyroid	*TSHR*	1.0±0.12	1.05±0.59	0.97±0.18	0.91±0.04	1.0±0.32	1.41±0.18	1.97±0.55	1.70±0.39
		NIS	1.0±0.17	1.23±0.28	1.14±0.45	1.36±0.15	1.0±0.04	1.15±0.01	1.71±0.08*	1.19±0.16
		TG	1.0±0.24	1.04±0.40	1.27±0.47	0.94±0.35	1.0±0.27	1.22±0.15	1.02±0.41	0.92±0.27
		TPO	1.0±0.06	1.51±0.68	1.44±0.48	1.85±0.10*	1.0±0.11	0.83±0.18	1.24±0.24	1.06±0.66
		Pax8	1.0±0.15	0.89±0.22	1.25±0.40	2.19±0.13*	1.0±0.19	1.35±0.25	1.51±0.37	2.17±0.70
		Hhex	1.0±0.27	1.40±0.31	1.84±0.51	2.25±0.62	1.0±0.26	1.04±0.38	1.21±0.17	1.56±0.74
		DIO1	1.0±0.05	0.87±0.11	0.65±0.23	0.81±0.37	1.0±0.05	1.35±0.11	0.88±0.24	1.22±0.12
		DIO2	1.0±0.08	0.82±0.19	1.26±0.36	1.01±0.24	1.0±0.34	0.99±0.43	1.66±0.24	1.21±0.14
		TRα	1.0±0.16	0.73±0.18	0.90±0.26	1.08±0.40	1.0±0.20	1.30±0.54	1.56±0.58	1.60±0.54
		TRβ	1.0±0.27	0.99±0.04	1.34±0.37	1.72±0.35	1.0±0.13	0.89±0.35	1.32±0.35	1.04±0.13

5.4 Discussion

The observation of disruptions of hormonal balance corresponds generally well with the reports of a previous study (Liu et al., 2012): The concentrations of E2 and T were increased in females, while E2 increased but T decreased in males, 11-KT was decreased both in males and females. The difference was that E2/T ratio in this study was increased both in males and females. Even though the change pattern of T or E2/T ratio was different, the change pattern of E2, 11-KT and E2/11-KT ratio in female fish, and T, 11-KT and E2/11-KT in male fish was the same. Liu et al. (2012) demonstrated that TDCPP and TPP could up-regulate several steroidogenic genes, e.g., including *CYP11A1*, *CYP11B2*, *CYP19A*, or *3βHSD* in H295R cells, or up-regulate *CYP17* and *CYP19A* in the gonad of adult zebrafish. In the present study, in female fish, exposure to TDCPP and TPP led to increase of GnRH/gonadaotropin and activation of steroidogenesis, which might be responsible for the increased plasma E2 level. While, in male fish, exposure to both TDCPP and TPP showed inhibition of GnRH/gonadotropin and steroidogenesis, which might be responsible for the decreased plasma T and 11-KT levels.

For THs, similar to Liu et al. (2012), exposure to TDCPP significantly increased T4 and T3 levels in female fish, while decreased T4 and T3 in male fish. TPP significantly increased concentration of T4 in both female and male fish, but have no significant effects on T3 (see Chapter 4 for data). Significant alteration of THs in zebrafish suggests thyroid disruption capacity of the OPFRs. In fish, THs are secreted from the thyroid gland and play major roles in the development and growth of fish. Exposure to polybrominated diphenyl ethers (PBDEs) could significantly alter T4 and T3 levels and also recude body weight of zebrafish larvae (Yu et al., 2010, 2011). In this study, we observed that the CF, GSI and LSI of zebrafish were reduced by exposure to TDCPP or TPP, which suggest adverse effects of OPFRs on the growth of fish.

Changes of plasma cortisol suggest the activation or inhibition of HPA axis in fish. In teleosts, cortisol is a major corticosteroid secreted by the interrenal

tissue, or adrenal cortex in tetrapods, that plays an important role in the physiological adjustments essential to cope with general stress. Proopiomelanocortin (POMC) is one of the major components of HPA axis, it is the common precursor protein of several biologically active peptides, which include pituitary adrenocorticotropin hormone (ACTH), which promotes cortisol production in adrenal cells (Hontela., 2005). The up-regulation of *POMC*, *CYP11A* and *CYP11B* in females or down-regulation of *POMC* in males were accompanied with the increased cortisol in female and decreased in male fish have also been reported by Palermo et al. (2012). *GR* an *MR* are ligand activated transcription factors that play an essential role of translating the cortisol signal. Cortisol binding to *MR* may provide key signaling for zebrafish development (Alsop and Vijayan, 2009). Therefore up-regulation of *MR* in female fish after exposure to TDCPP and TPP would be related to developmental toxicity of these chemicals.

CRH has been demonstrated to have similar activity as TRH in nonmammalian vertebrates including fish. *CRH* is a more potent factor than *TRH* for stimulating TSH release. Along the HPT axis that regulate the production of THs, *CRH* and *TSHβ*, similar with a previous study, the down- or up-regulation of *CRH* and *TSHβ* were modulated by THs via negative feedback (Chiamolera and Wondisford, 2009). The expression of sodium/iodide symporter (*NIS*), *TG* and *TPO* genes are known to be involved in THs synthesis, and the transcription of NIS gene was regulated by *Pax8*,and stimulated by TSH (Zoeller et al., 2007). Pax8 protein is essential for the late differentiation of the follicular cells (Wendl et al., 2002). The up-regulation of *Pax8* and *TPO* observed in the present study suggests that TDCPP and TPP would induce gene expression and promote the growth of thyroid primordium, which might be responsible for the up- or down-regulation of THs levels. Transcriptions of TRH or TRHR, as well as two thyroid receptor (*TR*: *TRα* and *TRβ*), and two deiodinase (*Dio1* and *Dio2*) were not significantly changed, suggesting that these genes are less sensitive to OFPRs stress and may not be reliable endpoints for adverse effects of OPFRs exposure.

Interactions between HPA and HPG axes were observed elsewhere (Liu et al., 2011; Lerner et al., 2007; Poursaeid et al., 2012). Lerner et al (2007) reported that the exposure to E2 resulted in an increase of plasma cortisol levels in juvenile Atlantic salmon. Poursaeid et al. (2012) also showed that a long-term stimulation of cortisol would alter sex steroid levels and gonadal development in cultured great sturgeon *Huso huso*. Liu et al (2011) suggest that the inhibition of E2 by prochloraz in zebrafish would be responsible for the decreased plasma cortisol levels. It was demonstrated that several putative transcription regulation elements including two ER and several activation protein 1 (AP-1) and glucorticoid receptor (GR) regulation elements, were found in the proximal promoter of zebrafish CRH (Yao and Denver, 2007). Conversely, cortisol may also influence sex steroids suggesting their reciprocal interaction along the reproductive cycle. In Arctic charr, cortisol affected the vitellogenin production controlled by estrogen (Berg et al., 2004), and also inhibited the production of estrogen in rainbow trout (Pankhurst and Van Der Kraak, 2000). There are also reports about the effects of cortisol on GnRH and gonadotropins production. Huang et al. (1999) showed that cortisol treatments induced elevation of pituitary LH in rainbow trout. Even though cortisol mainly displayed direct deleterious effects on female gametogenesis, cortisol can also exhibit a spectrum of direct positive activities during oocyte maturation or ovulation (Milla et al., 2009). In female fish, androgens are reported to involve in the regulations of final stages of synchronization and secretion of GnRHs and gonadotropins (Nagahama et al., 1994). Many studies indicate that cortisol inhibits male reproductive physiology over the whole reproductive cycle. Cortisol would directly inhibit androgen or inhibit gonadotropins (Consten et al., 2001, Lister et al., 2008). Cortisol can also influence male reproduction: Under stress or cortisol treatment, a delay in the testicular development has been reported along with a few positive effects on spermatogenesis (Ozaki et al., 2006). In the present study, in the female fish, TDCPP and TPP exposure increased plasma cortisol levels, which in turn, might activate GnRHs and gonadotropin, and then activate the steroidogenesis and increased sex hormone. While, in the male fish, decreased plasma cortisol

was accompany with the inhibition of GnRH/gonadotropin and steroidogenes, which would responsible for the decreased levels of T or 11-KT.

Cortisol has been also reported to influence thyroid activity and thyroid hormones in fish (Walpita et al., 2007). Cortisol treatment led to decrease of plasma T3 levels in Coho salmon or in rainbow trout (Redding et al., 1984; Brown et al., 1991), but also to increase of T4 to T3 conversion in brook charr (Vijayan et al., 1988). Suchiang et al. (2012) suggested that the effects of cortisol on the thyroid hormones are species- and phase/season dependent. Besides, cortisol and T4 exhibit synergistic interactions during larval development in fish (De Jesus et al., 1990). T4 also promotes the secretion of cortisol by the interregnal tissue (Young and Lin, 1988). Their plasma levels follow similar patterns during physiological events or chronic exposure to contaminants (Redding et al., 1991; Hontela et al., 1995). Consistent with that, in the present study, after chronic exposure to TDCPP or TPP, the similar increase of plasma cortisol and THs in females, and decrease of cortisol and THs in males were observed. The decreased plasma cortisol in males suggested the cortisol impairment, which would reduce physiological competence of fish and might responsible for decreased THs. Sex-dependent change patterns in HPG and HPT axes might be associated with cortisol, however underlying mechanisms of cortisol and endocrine systems, and sex-dependent responses to stress caused by TDCPP or TPP warrant further studies.

Chapter 6. Conclusions

6.1 Conclusion

Our observations from H295R and MVLN cells show that six major OPFRs, i.e., TCPP, TCEP, TDCPP, TPP, TBEP and TCP, have endocrine disruption potency through multiple mechanisms including steroidogenesis, and metabolism and activation of E2. TDCPP and TPP, which showed greater potentials of endocrine disruption were chosen for in vivo studies employing fish. Following a 21 d exposure, both TDCPP and TPP disturbed balance of the sex hormones, and impaired reproduction performance of adult zebrafish. Exposure to TDCPP or TPP also modulated the balance of THs and altered transcription of the gens of HPG and HPT axes, in sex-dependent manner. Besides, developmental retardation and hormonal disturbance were also found after longer term (120 d) exposure to TDCPP or TPP from fertilized eggs. Following the long-term exposure, increased plasma levels of cortisol, FSH, LH, T4, T3, and E2, and decreased levels of 11-KT were observed in females, while the opposite trends were generally observed among male fish. Gene transcriptions along HPG, HPT and HPA axes support the observations on hormone level changes.

6.2 Implication of this study

Endocrine disrupting toxicity of OPFRs and possible underlying mechanisms were identified in zebrafish. Zebrafish has been suggested as one of the relevant models for human endocrine systems since crucial principles and components of endocrine systems are shared (Segner et al., 2003). For this reason, zebrafish has been widely used to investigate endocrine disrupting mechanisms that are also relevant to human endocrinology. Since the function of endocrine systems is similar between fish and humans, potential implications in humans and other mammalian models are of concern (Lohr and Hammerschmidt, 2011).

Potentials of ecological risk of OPFRs that have been identified in the

present study deserve consideration. To date, only limited chronic toxicity data have been available, e.g., those from *Daphnia* and algae (EU risk assessment report 2008). Based on the present study, a 21-day *Daphnia* reproduction (NOEC of 40 μg/L) was determined to be the most sensitive endpoint by TPP exposure. An assessment factor of 100 was employed and a PNEC value of 0.4 μg/L was obtained. For TDCPP, the 21-day *Daphnia* reproduction NOEC value was below 40 μg/L, and PNEC of TDCPP was estimated to be below 0.4 μg/L. Besides, a 120 d exposure to 5 μg/L TDCPP or TPP showed hormonal disturbance in fish which may lead to impairments of reproduction, which suggest potential ecological consequences of OPFRs. Further studies using chronic fish exposure are needed to refine current PNECs for TDCPP and TPP.

6.3 Future works

Several studies were identified as future research topics. Studies on cross-talk among the various regulators of development and reproductive activities are required to understand the consequences of OPFR exposure on the population level. The underlying mechanisms for sex-dependent changes in hormones and gene transcriptions related to endocrine systems also deserve further study. Besides, long-term fish toxicity tests employing ecologically relevant endpoints would be needed to better understand ecological risks associated with the test OPFRs.

Health implications of OPFRs among humans should also be investigated. Several studies have reported that the concentrations of OPFRs in house dust or air are similar to or often greater than those of PBDEs (Reemtsma et al., 2008; Stapleton et al., 2009). Considering results of observational studies on human population (Meeker and Stapleton, 2010), more studies are required to understand health consequences of exposure to OPFRs.

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