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Effects of triclosan on gonadal differentiation and development in the frog *Pelophylax nigromaculatus*

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ABSTRACT

Previous studies have reported that triclosan (TCS) could possess an androgenic activity. We aimed to investigate the effects of TCS on gonadal differentiation and development in the frog *Pelophylax nigromaculatus*, a sensitive amphibian species to androgenic chemicals. *P. nigromaculatus* tadpoles at stage 24 were exposed to TCS (3, 30, and 300 nmol/L) to stage 46 in a semi-static exposure system. At the end of exposure, gonadal morphology and histology, sex ratio and gonadal expression of sex-biased genes were examined in *P. nigromaculatus*. In each TCS treatment group, we found several individuals whose gonads exhibited morphological and/or histological abnormalities. Gonadal histological abnormalities were characterized by few oocytes and many somatic cells. Although the percentage of the individuals with abnormal gonads was low (7.8%) among all animals treated with TCS, statistical test revealed the sex ratios in the 3 and 300 nmol/L TCS treatment groups were significantly different from the solvent control. In the 30 nmol/L TCS treatment group, abnormal gonads were also observed, although the sex ratio was not changed compared with the solvent control, which was possibly due to the smaller sample size in this group. In all the TCS treatment groups, the sex ratios were not obviously male-biased, but the expression levels of some sex-biased genes were significantly altered by TCS. Altogether, our results suggest that TCS, even at environmentally relevant concentrations, could disrupt gonadal differentiation and development in *P. nigromaculatus*, but we are not sure whether the disrupting effects were associated with masculinization or feminization.

Introduction

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether, TCS) is a broad-spectrum bacteriostatic agent widely used in personal care products, household items, and medical devices for over 40 years (Yueh and Tukey, 2016). It was allowed to be added into personal care products at a limited percentage of 0.3% in most countries. Consequently, TCS has become a prevalent anthropogenic contaminant in the aquatic environment worldwide (Wang and Tian, 2015). According to a review, TCS has been detected in surface water worldwide with the values ranging 0.2–1023 ng/L (Bedoux et al., 2012) and in waste water with the concentrations among 1.4–40,000 ng/L (Scientific Committee on Consumer Safety, 2010). Subsequently, TCS was detectable in organisms like algae, fish and snail (Dhillon et al., 2015). As a result of its wide exposure *via* consumer products

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and polluted food and water, TCS was frequently detectable in human body fluids, such as breast milk, serum and urine (Bedoux et al., 2012; Witorsch, 2014). Recently, a regulation released by U.S. Food Drug Administration (FDA, 2016) would like to prohibit the sales of consumers containing TCS and other 18 antimicrobials, but the existed TCS in the environment still possesses a threat to organisms.

Many studies have reported the adverse effects of TCS on growth (Fort et al., 2011), lipid metabolism (Ho et al., 2016), behavior (Fritsch et al., 2013; Schultz et al., 2012), hepatotoxicity (Haggard et al., 2016) and embryonic development (Macedo et al., 2017) in organisms. According to the report by WHO-UNEP (2012), TCS was listed as one of potential endocrine disrupting chemicals (EDCs). One of the most important endocrine disrupting effects of TCS is reproductive disruption, which has attracted more attention in recent decades. Studies reported that TCS could possess estrogenic effects and anti-estrogenic effects both *in vitro* and *in vivo* models (Ahn et al., 2008; Gee et al., 2008; Ishibashi et al., 2004; Jung et al., 2012; Raut and Angus, 2010; Stoker et al., 2010). However, Foran et al. (2000) reported that exposure of medaka to 100 μg/L TCS resulted in an augmentation of fin length, a sexually dimorphic characteristic, exhibiting a weak masculinizing effect. A luciferase assay in MDA-kb2 cells showed that 1 μmol/L TCS alone could cause 4-fold induction of androgen receptor-responsive gene expression relative to the solvent control group and enhanced the 5-dihydrotestosterone-induced activities up to 180% in the co-exposure assay (Christen et al., 2010). These *in vivo* and *in vitro* studies collectively indicated that TCS could have a potential androgenic activity. Given that TCS is ubiquitous in the environment and organisms, it is likely to possess a threat to species that are sensitive to androgenic chemicals, such as fish and amphibians.

The frog *Pelophylax nigromaculatus* is a widely distributed species in East Asia, especially in China. Our previous studies have demonstrated that gonadal differentiation of *P. nigromaculatus* is sensitive to androgenic chemicals (Li et al., 2015; Xu et al., 2015). Shirane (1986) reported that 50 μg/L testosterone can induce female-to-male reversal in *P. nigromaculatus*. Our previous studies found that environmentally relevant concentrations of 17β-trenbolone and 5α-dihydrotestosterone (DHT) could result in complete female to male reversal of almost all individuals in *P. nigromaculatus* (Li et al., 2015; Xu et al., 2015), showing high sensitivity of gonadal differentiation to androgenic chemicals in this species.

Shortly, we aimed to investigate whether TCS has masculinizing effects on gonadal differentiation and development in the frog *P. nigromaculatus*. DHT was set as the positive control to test the potential masculinizing effects of TCS. Besides gonadal morphology and histology, sex ratio and gonadal expression of sex-biased genes were evaluated in *P. nigromaculatus* following treatment with 3–300 nmol/L TCS. Considering inconsistent findings that TCS affected amphibian metamorphic development in previous studies (Veldhoen et al., 2006; Fort et al., 2010, 2011, 2017), we also assessed effects of TCS on *P. nigromaculatus* metamorphic development and thyroid histology, whose abnormalities in some cases are associated with changes in metamorphic development.

1. Materials and methods

1.1. Animals and rearing conditions

P. nigromaculatus was raised as described particularly in our previous study (Li et al., 2015; Lou et al., 2014). In brief, adult frogs were raised in glass tanks with sand and dechlorinated water, and fed with mealworm (*Terzebrio molitor*) daily. The water quality for raising *P. nigromaculatus* was as follows: chlorine concentration < 5 μg/L, pH 6.5–7.0, dissolved oxygen concentration > 5 mg/L, and water hardness ($CaCO_3$) approximately 150 mg/L. Water temperature was maintained at (24 ± 1)°C. Fluorescent lighting provided a photoperiod of 12 hr light/12 hr dark (600 to 1000 lx on the water surface for tadpoles, 100 to 300 lx for adult frogs).

A pair of adult frogs were induced to breed by injecting 25 μg luteinizing hormone releasing hormone (LHRH, Ningbo Second Hormone Factory, China) and human chorionic gonadotropin (HCG, Yantai North Pharmaceutical Co. Ltd., China; 400 IU for female, 200 IU for male) dissolved in 0.6% NaCl, accompanied by simulating rainfall. Fertilized eggs were incubated in dechlorinated tap water and developed to Gosner stage 24 tadpoles on the 5th day post fertilization (Gosner, 1960). The tadpoles were fed with commercial diets (Totoro Supplies, Hong Kong, China) twice daily.

1.2. Exposure of TCS to *P. nigromaculatus*

Triclosan (TCS, >98.0%; CAS# 3380-34-5; Tokyo Chemical Industry, Japan) was dissolved in dimethyl sulfoxide (DMSO, >99.5%; CAS#67-68-5; Sigma-Aldrich, USA) to prepare 0.6, 6 and 60 mmol/L TCS stock solutions, which were sub-packaged and stored at −20°C. The volume of 50 μL stock solution was added into 10 L dechlorinated tap water to prepare exposure solutions of 3, 30 and 300 nmol/L TCS respectively. All groups including the solvent control group contained 0.0005% DMSO. Given that low dose of DMSO had no effects on aquatic organisms in previous studies (Oka et al., 2008; Pereira et al., 2011), no blank control was conducted in this experiment. The positive control group was treated with 1 nmol/L 5α-dihydrotestosterone (DHT, >99.5%; CAS# 521-18-6; Dr. Ehrenstorfer, Germany), referring to our previous study (Xu et al., 2015). Tadpoles at stage 24 were randomly assigned to each tank with 10 L water, ensuring the density was less than 1.5 tadpoles/L. Each treatment group consisted of three replicate tanks with 14 tadpoles per tank, while the solvent control had four replicate tanks. The experimental water was totally changed every other day in a semi-static exposure system. When tadpoles reached stage 42, they were transferred to amphibious environment for metamorphosis. When the *P. nigromaculatus* reached stage 46, they were dissected immediately for latter analysis.

1.3. TCS analysis in experimental water

In the middle of exposure, when the tadpoles reached stages 35/36, experimental water was sampled for TCS analysis following the methods described in previous studies (Li et al., 2015; Shen et al., 2012; Zhao et al., 2010). On day 0, day 1 and day 2 in a water renewal cycle, water samples of 1 L,

0.1 L, 0.01 L were collected in glass bottles respectively from 3, 30, 300 nmol/L TCS treatment groups (no supplement after sampling) and stored in the dark at 4°C before analysis. Supelclean™ENVI™-18SPE (SUPELCO, USA; 500 mg/6 mL) column was used to isolate and preconcentrate TCS from water samples. After the columns were conditioned by 5 mL ethyl acetate, 5 mL methanol and 10 mL ultrapure water sequentially, water samples passed through the columns at a flow rate of 5–10 mL/min. Finally, TCS was eluted from the columns by 3 mL ethyl acetate three times, and the samples were dried and redissolved in 500 μL acetonitrile.

TCS concentrations were determined by LC–MS/MS (EVOQ Elite&EVOQ Qube, Bruker, USA) with ACQUITY UPLC™ BEH C18 column (Waters, USA; 50 mm × 2.1 mm, 1.7 μm). As for the LC conditions, water (A) and acetonitrile (B) were used as the mobile phase and the flow rate was 0.2 mL/min. The gradient was as follows: 0 min (20% B), 3 min (90% B), 8 min (90% B), 12 min (100% B). Multiple reaction monitoring (MRM) mode was used for quantitative analysis of TCS. The qualitative ion and the quantitative ion for TCS were 286.7/35 and 289/35.2, respectively. External standard method was applied to quantify the TCS concentrations in water samples. The standard curve of serially-diluted TCS was analyzed and the R^2 was 0.9995 (5 concentrations ranging from 0.675 to 10.8 μmol/L, Fig. S1). Laboratory blanks were also analyzed along with the samples. The recoveries ranged from 70% to 98%.

1.4. Sample collection and sex identification

After all individuals finished metamorphosis in *P. nigromaculatus*, the survival rates and the time from stage 24 to 46 of froglets in each tank were recorded. After anaesthetization in 100 mg/L tricaine methane sulfonate (MS-222, 98%; CAS# 886-86-2; Sigma-Aldrich, USA), froglets were examined for body weight and length. All gonad-kidney complexes were photographed using a microscope (SZ660, Chongqing Optec Instrument Co., Ltd., China) connected SLR camera (EOS700D, Canon, China) at the same magnification. Sex identification of *P. nigromaculatus* was based on morphological observation described in detail in our previous studies (Li et al., 2015; Xu et al., 2015). A typical testis is smooth and pear-shaped, and has no visible metamere. In contrast, a typical ovary is composed of 7 to 8 ovary lobes separated by metameres and obviously longer and wider than a testis. The individuals with typical ovaries or testes were identified as females or males, respectively. The individuals whose gonads cannot be identified as neither typical testes nor ovaries were regarded to be sexually ambiguous. Sexual identification based on gross gonadal morphology was assessed blindly.

Gonad length was measured using the image analysis software OPTPro2012_Chs (Chongqing Optec Instrument Co., Ltd., China). The gonad-kidney complexes of the twenty firstly completing metamorphosis froglets were sampled for sex-related gene expression analysis, and the thyroids of these twenty froglets were immersed in Bouin's fixative (75% (V/V) picric acid, 25% formaldehyde and 5% acetic acid, the reagents here were obtained from Beijing Chemical Reagent Co., Ltd., China). In addition, the gonad-kidney complexes of the remaining froglets were also immersed in Bouin's fixative for histological analysis. All animal experiments in this study were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission, 1988).

1.5. Histological examination of gonad

After fixation, the gonad-kidney complexes and thyroids were dehydrated in a graded series of alcohol solutions (70%–100%), embedded in paraffin, and transversely sectioned at 6 μm thickness. Hematoxylin and eosin (Jiuzhou Bailin biotechnology Co., Ltd., China) staining was performed and the gonad and thyroid histology were observed under Axioskop standard microscope (Axio Scope A1, Carl Zeiss, Germany).

1.6. RNA extraction and reverse transcription

Total RNA was isolated from the gonad-kidney complexes using RNA prep pure animal tissue total RNA extraction kit (TIANGEN Biotech Co., Ltd., Beijing, China) following the manufacturer's instructions. The concentrations of total RNA were measured using spectrophotometer (NanoDrop2000, Thermo Scientific, USA), and the quality of RNA was tested by agarose gel electrophoresis. RNA (1 μg) was reverse transcribed into cDNA using FastQuant RT kit (TIANGEN Biotech Co., Ltd., Beijing, China). The cDNA was stored at −80°C for further expression analysis for sex-biased and thyroid related genes.

1.7. Gene expression analysis by qPCR

Previous studies have demonstrated CYP19, SOX3, TBPL2 and Emi2 display ovary-biased expression in stage 46 *P. nigromaculatus*, whereas CYP17, Srd5α2, and SOX9 exhibited testis-biased expression (Xu et al., 2015). Additionally, anti-Mullerian hormone gene (AMH) was employed as it was reported to be involved in testicular differentiation process and exhibited testis-biased expression in anuran species (Jansson et al., 2016; Kodama et al., 2015). In the present study, thus, we used CYP19, SOX3, TBPL2 and Emi2 as ovarian molecular markers and CYP17, Srd5α2, SOX9 and AMH as testicular molecular markers to evaluate the effects of TCS on gonadal differentiation and development in *P. nigromaculatus* at molecular level.

Quantitative polymerase chain reaction (qPCR) was used to analyze the expression levels of these genes. Ribosomal protein L8 (rpl8) was used as a reference gene to normalize mRNA expression of sex-biased genes according to our previous studies (Li et al., 2015; Lou et al., 2014). Primers were synthesized by Sangon Biotech Co., Ltd. (Beijing, China), and the detailed information are shown in Table 1. PCR efficiency (E, %) was calculated according to the equation: $E = (10^{-1/\text{slope}} - 1) \times 100\%$, based on amplification of a standardized dilution series of the template cDNA (triplicates). The quantification of target gene expression was based on a comparative CT (cycle threshold) method. The fold change of each gene was analyzed by the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001). Quantitative data are shown as mean ± standard error of the mean (SEM).

1.8. Statistical analysis

Data of growth time, body weight and length, gonad length were analyzed using one-way analysis of variance (ANOVA).

Table 1 – Primer pairs (in the 5′ to 3′ direction) used for qPCR assay and related information.

Gene	Primer sequence	Annealing temperature (°C)	PCR efficiency (%)	Amplify length (bp)	GeneBank ID
Rpl8	Forward: GCTGTCGACTTCGCAGAAAGGCA Reverse: ACCTGTAAGGGTCACGGAAGGCA	60	100%	115	XM_018556352.1
AMH	Forward: AGTCTGTCCTTGACCGACCTC Reverse: GCCATTCTCATGTGCTACCATC	60	100%	80	XM_018573247.1
CYP19	Forward: GTGACATGCCAAACCTTAAAGT Reverse: CATCATCCTCCATAGCCTTGC	62	108%	100	GBET01000003
SOX3	Forward: CCAGCCTACAACCAGCAGACCT Reverse: GTGTGGGAAGTGATGGCAGGTG	62	107%	104	AB295441.1
TBPL2	Forward: TGGATGCTTCTGGCCTTTCACT Reverse: TGGGACGAAGGCTGGAACCA	62	102%	81	GBET01000019
Emi2	Forward: TGTGGGCTGCCAAACCAATTC Reverse: AGATCCTGCACGGTGGTCTG	60	97%	129	GBET01000009
CYP17	Forward: GGTGTGCTCCTTGTGCTTCA Reverse: CCACTGTGTCCACTATGCCTT	60	110%	102	GBET01000007
Srd5α	Forward: CCAGCCAAATACGCCTGGTTCA Reverse: TACATCCCAGGGTGTCCCATCG	62	100%	97	GBET01000016
SOX9	Forward: TACAGCGACCAGCAGCAACAG Reverse: TGAGCCTTGGTGTTCGGTGTAG	62	97%	129	GBET01000015

Rpl8: ribosomal protein L8; AMH: anti-Mullerian hormone; CYP19: cytochrome P450 aromatase 19; SOX3: sex-determining region Y-box 3; TBPL2: TATA box-binding protein-like 2; Emi2: early mitotic inhibitor 2; CYP17: cytochrome P450 aromatase 17 A; Srd5α: steroid 5a–reductase type 2; SOX9: sex-determining region Y-box 9.

The Chi-square test was used for significant differences in sex ratios compared with the solvent control. The sex-differences in gene expression levels in each group were analyzed using independent-samples T Test. The differences among experimental groups were analyzed using one-way ANOVA followed by Dunnett (equal variances assumed) and Dunnett's T3 (equal variances not assumed). With no obvious difference in samples from replicate tanks, the data of three/four replicate tanks in each treatment group was combined. Significance was set at $p < 0.05$.

2. Results

2.1. Actual TCS concentrations in experimental water

The actual concentrations of TCS in the experiment water on day 0, day 1, day 2 are shown in Table 2. No pollution was detected in the solvent control group. On day 0, the actual concentrations of TCS in the 3, 30, 300 nmol/L treatment groups were 3.18, 31.75, 308.20 nmol/L, respectively, approaching the nominal concentrations. Decreases in TCS concentrations were observed in all the treatment groups on day 1 and day 2. On day 1, the TCS concentrations in the 3, 30, 300 nmol/L treatment groups reduced to 0.89, 9.58, 127.66 nmol/L, respectively, accounting for about 28%–42% of the initial concentrations. On day 2, however, only 0.42, 2.26, 39.48 nmol/L TCS remained, accounting for 7%–13% of the initial concentrations.

Table 2 – Actual triclosan (TCS) concentrations (nmol/L) in the experimental water.

	Day 0	Day 1	Day 2
Solvent control	ND	ND	ND
TCS-3 nmol/L	3.18 ± 0.24	0.89 ± 0.02	0.42 ± 0.01
TCS-30 nmol/L	31.75 ± 2.49	9.58 ± 0.96	2.26 ± 0.24
TCS-300 nmol/L	308.20 ± 10.19	127.66 ± 10.54	39.48 ± 8.15

ND: not detected. Data are shown as mean ± standard deviation ($n = 3$). TCS: triclosan.

2.2. Survival and metamorphic development

The survival rate (94.6%) in the solvent control group was higher than the survival rate (90%) required in standardized testing protocols. Survival rates were 95.2%, 85.7%, 95.2% respectively in the 3, 30 and 300 nmol/L TCS treatment groups. Most of the dead tadpoles were drowned because we failed to immediately transfer them to amphibious environment when they reached stage 42. The survival rate in the 30 nmol/L TCS treatment group was slightly low but with no significance compared with the solvent control group. No significant difference was observed in body weight and body length between the solvent control group and TCS treatment groups (Table 3).

The average time for firstly 80% individuals to reach stage 46 in the solvent control and DHT groups were 49 and 45 days, with 45, 48 and 46 days in the 3, 30 and 300 nmol/L TCS treatment groups, respectively (Fig. 1). Compared with the solvent control, the time to reach stage 46 in the 3 and 300 nmol/L TCS treatment groups was significantly shorter, whereas no significant difference was found in 30 nmol/L TCS group. Further study on thyroid histology showed no differences between treatments and the solvent control (Fig. 2).

2.3. Gonadal morphology and histology

Based on gonadal morphology, all the gonads in the solvent control group were regarded as typical ovaries or testes (Fig. 3a, b). With regard to histology, the typical ovary was suffused with

Table 3 – Survival (rate), body weight (g) and body length (cm) of *Pelophylax nigromaculatus* in treatment groups compared with the solvent control (SC).

Endpoint	SC	DHT	TCS (nmol/L)		
			3	30	300
Survival number (rate)	53 (94.6%)	42 (100%)	40 (95.2%)	36 (85.7%)	40 (95.2%)
Body weight	0.57 ± 0.12	0.56 ± 0.13	0.56 ± 0.09	0.60 ± 0.12	0.56 ± 0.13
Body length	1.89 ± 0.14	1.94 ± 0.15	1.87 ± 0.18	1.89 ± 0.14	1.91 ± 0.13

DHT: 5α-dihydrotestosterone.
Data are shown as mean ± standard deviation.

oocytes (Fig. 3a1), whereas the typical testis was filled with numerous spermatogonium and somatic cells (Fig. 3b1). In the DHT treatment group, all gonads were identified as typical testes, with normal testicular histology. In the 3 nmol/L TCS treatment group, all the gonads were identified astypical ovaries or testes based on gonadal morphology, but four morphological ovaries (Fig. 3c) displayed abnormal histological structures. As shown in Fig. 3c1, the histological abnormality in the gonad was characterized by fewer oocytes and more somatic cells. In the 30 nmol/L TCS treatment group, one sexually ambiguous individual whose gonadal length was between typical testes and ovaries was found, but its gonads displayed normal testicular structure (data no shown). In contrast, one morphologically typical ovary in this group exhibited abnormal histological structure, like Fig. 3c1. In the 300 nmol/L TCS treatment group, two individuals were identified to be sexually ambiguous in term of gonadal morphology (Fig. 3d), with abnormal gonadal histology (Fig. 3d1). Additionally, another two individuals whose gonads looked like typical ovaries exhibited histological abnormality, like Fig. 3d1.

The numbers of individuals with ovaries, testes and sexually ambiguous gonads based on gonadal morphology in each group were labeled in Fig. 4, where the average gonad lengths were also shown. The average gonad lengths of the males and females in the solvent control group were 0.08 cm and 0.22 cm, respectively (Fig. 4). A significant increase in ovarian length was observed in the 30 nmol/L TCS treatment group compared with the control ovaries. The gonad lengths in other treatment groups had no obvious difference from the solvent control (Fig. 4).

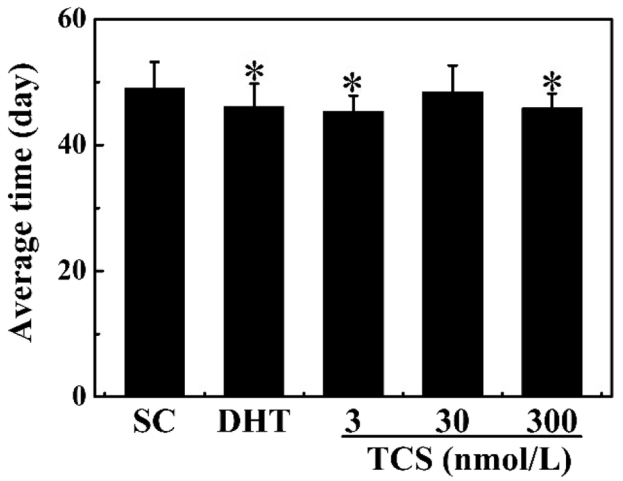

Fig. 1 – The average time (day) for firstly 80% individuals to reach stage 46 in the treatment groups compared with the solvent control (SC). Data are shown as mean ± standard deviation. * indicates a significant difference between TCS treatment and the solvent control ($p < 0.05$).

2.4. Sex ratios

The sex ratios were calculated based on gonadal morphology and histology (Fig. 5). The sex of gonads sampled for gene expression analysis were identified based on morphology only. The solvent control group contained 43.4% males ($n = 23$) and 56.6% females ($n = 30$). Exposure to 1 nmol/L DHT resulted in female-to-male sex reversal of all *P. nigromaculatus*. In the 3, 30, 300 nmol/L TCS treatment groups, the female percentages were 40%, 52.8%, 30%, with 50%, 44.4%, 60% males, respectively. The percentages of abnormal individuals in the 3, 30, 300 nmol/L TCS treatment groups were 10% (4/40), 2.8% (1/36) and 10% (4/40), respectively. Significant differences in the sex ratios were observed between the 3, 300 nmol/L TCS treatment groups and solvent control group, with the lack of difference between 30 nmol/L TCS treatment group and the solvent control. There were totally 9 abnormal individuals among 116 TCS-treated *P. nigromaculatus*, namely 7.8% individuals exhibited gonadal abnormalities in term of gross morphology and/or histology.

2.5. Expression of sex-biased genes

We analyzed the expressions of sex-biased genes in gonads of *P. nigromaculatus* following the treatment of TCS. As shown in Fig. 6, CYP19, TBPL2, Emi2 and SOX3 displayed high expression in ovaries than in testes in the solvent control, while AMH, Srd5α, CYP17 and SOX9 had higher expression in testes than in ovaries. DHT significantly elevated AMH and SOX9 expression, and also resulted in weak up-regulation of Srd5α and CYP17 regardless of the lack of statistical significance. DHT had no influence on expression of the ovary-biased genes.

In the male *P. nigromaculatus*, 3–300 nmol/L TCS displayed DHT-like stimulatory effects on AMH and Srd5α expression, with similar up-regulation of CYP17 expression following exposure to 3–30 nmol/L TCS. In the female *P. nigromaculatus*, all concentrations of TCS elevated Srd5α expression, while 30 nmol/L TCS promoted AMH expression. No change in SOX9 expression was observed in both males and females from treatment groups.

As for 4 ovary-biased genes, 30 nmol/L TCS exhibited stimulatory effects on TBPL2 and Emi2 in both males and females. Also, both 3 and 30 nmol/L TCS resulted in increases in CYP19 expression in males.

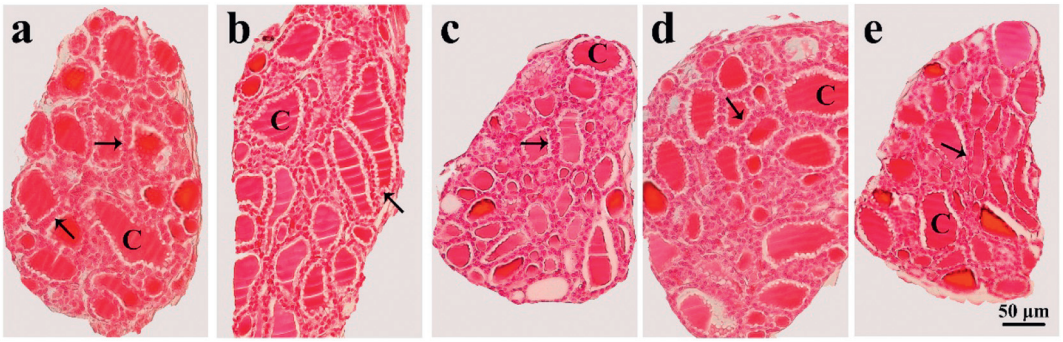

Fig. 2 – Thyroid histology in the *Pelophylax nigromaculatus* in all groups. (a) the solvent control; (b) 5α-dihydrotestosterone; (c) 3 nmol/L triclosan (TCS); (d) 30 nmol/L TCS; (e) 300 nmol/L TCS. C: colloid. The black arrows indicate the follicular epithelial cells.

3. Discussion

Considering the previous reports suggesting androgenic activities of TCS (Foran et al., 2000; Christen et al., 2010), we intended to investigate whether TCS has masculinizing effects on gonadal differentiation and development in *P. nigromaculatus*, a sensitive amphibian species to androgenic chemicals. As expected, DHT (1 nmol/L, 290 ng/L), serving as a positive control, induced complete female to male reversal of all the individuals, at gonadal morphological, histological or molecular levels. In the TCS treatment groups, nine individuals (7.8%) with abnormal morphological and/or histological gonads were found, but no gonadal abnormality was observed in the solvent control. In particular, the histological abnormalities were characterized by too few oocytes and too many somatic cells (Fig. 3c1, d1). These results show that TCS has significant influences on gonadal differentiation and development in *P. nigromaculatus*, despite a low abnormal percentage (7.8%). To our knowledge, the morphological and histological abnormalities in gonads following treatment with TCS have never been reported previously. In a recent study, we found similar histological abnormalities in the intersex gonads induced by tetrabromoethyl- cyclohexane (TBECH) in *P. nigromaculatus*. In the present study, however, we are not sure that the histological abnormalities in the gonads following TCS treatment are intersexes because we cannot determine whether too many somatic cells are ovary-specific or testis-specific, and whether the gonad is a genetic male or female. In term of sex ratios, there were significant differences in the 3 and 300 nmol/L treatment groups compared with the solvent control, with the lack of significant difference in the 30 nmol/L treatment group. However, the occurrence of gonadal abnormal individual (2.8%) in the 30 nmol/L treatment group show that exposure to 30 nmol/L TCS was able to induce gonadal abnormality. Maybe, the lack of significant difference in the sex ratio between the 30 nmol/L treatment group and the solvent control could be due to the small sample size in this group. Altogether, we conclude that TCS in the concentration range of 3–300 nmol/L disrupted gonadal differentiation and development in *P. nigromaculatus*, but the disrupting effects cannot be attributed to masculinization or feminization. Foran et al. (2000) found that exposure of medaka to 100 μg/L TCS resulted in augmentation of fin length, a sexually dimorphic

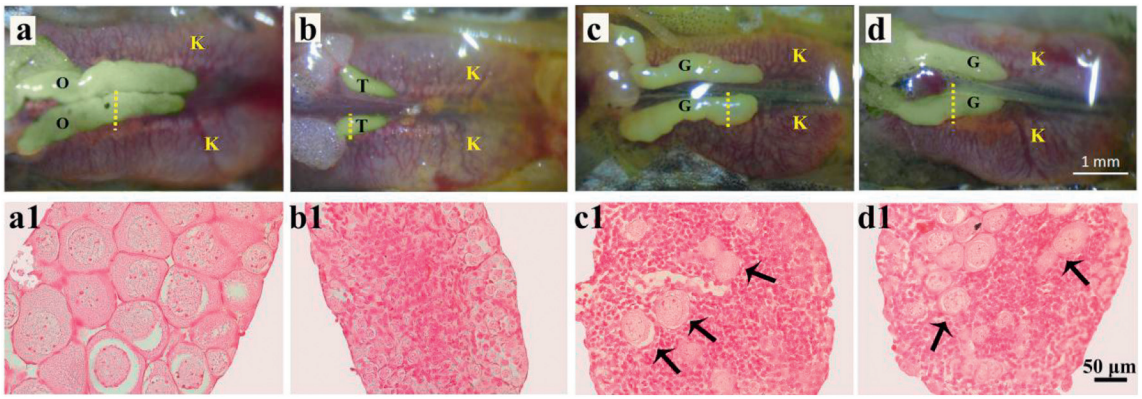

Fig. 3 – Gonadal morphology (a-d) and histology (a1-d1) of *Pelophylax nigromaculatus*. The section position is labeled with dashed line in the morphological photograph. (a) Solvent control ovary; (b) solvent controltestis; (c) triclosan (TCS) treated ovary; (d) TCS-induced sexually ambiguous gonad. In the histology results (a1-d1), ovary is filled with oocytes (a1), whereas testis contains numerous spermatogonium and some somatic cells (b1). Abnormal gonads in TCS treatment groups display with many somatic cells and a few oocytes (c1, d1, the oocytes are shown with black arrow). O: ovary; T: testis; G: TCS treated gonad; K: kidney.

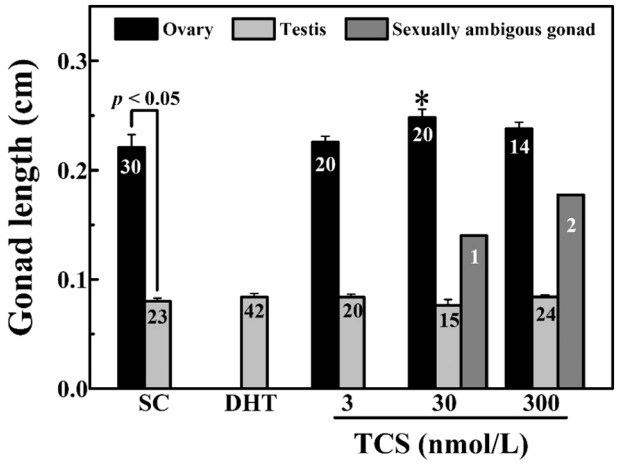

Fig. 4 – Gonad length of *Pelophylax nigromaculatus* in treatment groups compared with the solvent control (SC). Sex was identified based on gross gonadal morphology. Ligatures indicate $p < 0.05$. The numbers of tadpoles were labeled in the columns. Data are shown as mean ± standard error of the mean. * indicates a significant difference from the solvent control ovaries ($p < 0.05$).

characteristic, and concluded a weak masculinizing effect of TCS. However, the authors reported no changes in sex ratio following exposure to TCS by examining sexually dimorphic characteristics. Their results are inconsistent with our finding that 3and 300 nmol/L TCS altered sex ratio in *P. nigromaculatus* based on gonadal morphological and histological examination.

In the present study, TCS stimulated the expression of AMH, Srd5α and CYP17 compared with the solvent control. These results suggest TCS seemed to exhibit masculinizing effects on *P. nigromaculatus* though mimicking the action of DHT. However, the promotion of ovary-biased genes TBPL2, Emi2 and CYP19 by TCS are surprising, which appears to be associated with estrogenic effects. Thus, it is interesting to

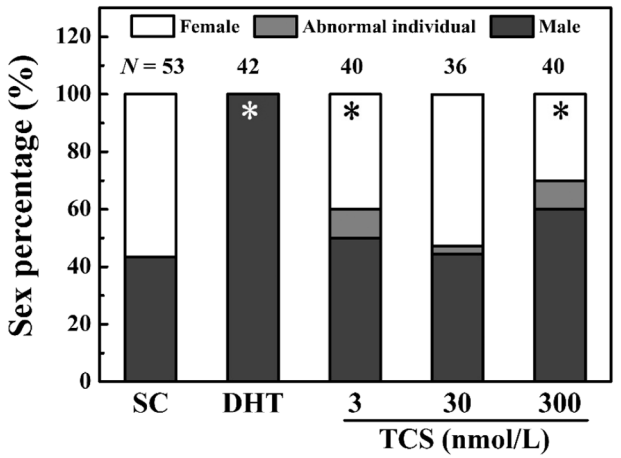

Fig. 5 – Sex percentages in treatment groups compared with the solvent control (SC). DHT, 5α-dihydrotestosterone. The sex ratios were measured according to the gross morphology and histology of the gonads. * indicates a significant difference from the solvent control group ($p < 0.05$).

suppose that the effects of TCS on *P. nigromaculatus* may involve androgenic and estrogenic activities, as previous studies reported both androgenic and estrogenic properties of TCS *in vivo* and *in vitro* (Yueh and Tukey, 2016). As described by Veldhoen et al. (2006), halogenated biphenolic compounds were likely to affect multiple targets because of the presence of receptor crosstalk in biological systems. However, the exact reason of these disrupting effects is currently unknown and further studies are required to investigate the mechanisms of endocrine disruption of TCS. In addition, it is noteworthy that the effects of TCS on sex-biased gene expression seemed to display nonlinear concentration-response relationships. For example, 3 and 30 nmol/L TCS dramatically promoted CYP17 expression in the males, but 300 nmol/L TCS had a weak effect with a lack of significant difference from the solvent control. Moreover, 30 nmol/L TCS resulted in up-regulation of TBPL2 and Emi2 in females and males, whereas 3and 300 nmol/L TCS had no effects. Nonlinear concentration-response seem to be not a rare phenomenon, but it is difficult to explain with several possible mechanisms involved (Vandenberg et al., 2012).

In the present study, both 3 and 300 nmol/L TCS were found to accelerate *P. nigromaculatus* development from stage 24 to 46 and resulted in early metamorphosis. In the 30 nmol/L TCS treatment group, no significant effect on *P. nigromaculatus* development was observed. In a recent study, we observed that 3, 30 and 300 nmol/L TCS resulted in early metamorphosis in *Xenopus laevis*. Therefore, we speculated that no observed effect of 30 nmol/L TCS was mainly because the tadpoles to early reach stage 42 died before stage 46 and were not counted for the time to reach stage 46. In other words, 30 nmol/L TCS as well as other two concentrations is possibly able to promote metamorphic development in *P. nigromaculatus*. A previous study (Fort et al., 2011) reported that 32 day-exposure to TCS from stage 47 had no effect on developmental stages, and only resulted in increases in body weight and body length of *X. laevis*, showing that TCS could not affect metamorphosis and only promote the growth. Veldhoen et al. (2006) also reported that exposure to TCS for 18 days had no effect on hindlimb growth of premetamorphic *Rana catesbeiana*, although it promoted T3-induced metamorphosis. In our study, *P. nigromaculatus* tadpoles were exposed to TCS from the earliest premetamorphic stage until complete metamorphosis, and the exposure duration is longer than those in the two previous studies, which is one possible reason for different findings between our study and previous reports. In accordance with previous studies (Fort et al., 2010, 2011), we found no changes in thyroid histology in *P. nigromaculatus* following exposure to TCS. The effects of TCS on metamorphic development in *P. nigromaculatus* we observed might be the result of nonthyroidal mechanisms.

In a previous study concerning exposure of tadpoles using a semi-static system, TCS concentrations declined to 50%–80% of the initial concentrations after 48 hr (Veldhoen et al., 2006). Following the study, we chose a semi-static exposure system with the exposure water changed every other day. In our study, the actual concentrations of TCS in the experiment water decreased to 7%–13% (0.42, 2.26, 39.48 nmol/L) of the initial concentration on day 2. The discrepancy of the residual TCS concentrations may result from the different exposure conditions between the two studies. As described previously, the concentrations of TCS in aquatic environment ranged

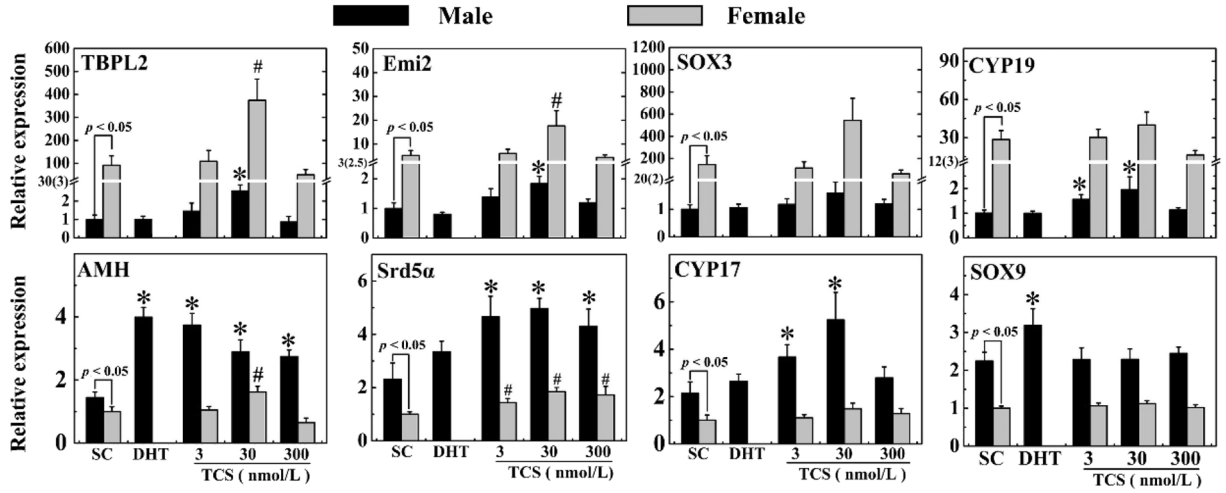

Fig. 6 – Gonadal expression of sex-biased genes in *Pelophylax nigromaculatus* following treatments compared with the solvent control (SC). For females, *n* = 12, 10, 13 and 11 in SC, 3, 30 and 300 nmol/L TCS groups respectively. For males, *n* = 10, 20, 10, 7 and 8 in SC, DHT, 3, 30 and 300 nmol/L TCS groups respectively. Ligatures indicate $p < 0.05$; Data are shown as mean ± standard error of the mean. * indicates a significant difference from the solvent control males ($p < 0.05$); # indicates a significant difference from the solvent control females ($p < 0.05$).

among 1.4–40,000 ng/L, namely 0.005–138.16 nmol/L. In our study, the actual concentrations of TCS ranged from 0.42 to 308 nmol/L, which were within the range of environmentally relevant concentrations. Thus, the disrupting effects of TCS on gonadal differentiation and development we observed in this study should receive more attention in further studies.

4. Conclusions

In conclusion, we found that TCS could disrupt gonadal differentiation and development in *P. nigromaculatus* based on the observations at gonadal morphological, histological and molecular levels as well as sex ratios. As the exposure concentrations are environmentally relevant in this study, the disrupting effects of TCS on gonadal differentiation and development of amphibians should receive more attention in further studies.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.05.040.

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