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Endocrine disrupting effects of tebuconazole on different life stages of zebrafish (*Danio rerio*)☆

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ABSTRACT

Tebuconazole is a widely used fungicide that has been detected in water ecosystems, of which the concentrations may affect the endocrine function of aquatic organisms. At present study, tissue-specific bioaccumulation of tebuconazole was found in ovary of adult zebrafish, indicating a potential risk of endocrine disruption. In order to evaluate the potential endocrine disrupting effects, three life stages (2 hpf (hours post-fertilization) –60 dpf (days post-fertilization), Stage I; 60–120 dpf, Stage II; 180–208 dpf, Stage III) of zebrafish (*Danio rerio*) were chronically exposed to tebuconazole at the concentrations ranging from 0.05 mg/L to 1.84 mg/L. Result showed that exposed to tebuconazole could lead to a male-biased sex differentiation in juvenile zebrafish and significant decrease of the percentage of germ cells in sexually-mature zebrafish. Egg production was significantly inhibited by 57.8% and 19.2% after Stage II- and Stage III-exposures, respectively. The contents of 17β-estradiol in gonad decreased by 63.5% when exposed to 0.20 mg/L tebuconazole at Stage II and by 49.5% after exposed to 0.18 mg/L tebuconazole at Stage III, respectively. For all stages exposure, reductions in 17β-estradiol/testosterone ratio were observed, indicating an imbalance in steroids synthesis. Additionally, tebuconazole reduced the expression of *cyp19a*, which was consistent with the decrease of E2 level. In overall, the present findings indicated that, playing as an anti-estrogen-like chemical, tebuconazole inhibited the expression of Cyp19, thereby impairing steroid hormones biosynthesis, leading to a diminished fecundity of zebrafish.

1. Introduction

Tebuconazole is one of the most frequently used broad-spectrum triazole fungicides in grains, vegetables and fruits (Zhang et al., 2015). Due to its widespread application, tebuconazole can undergo runoff to surface water with the concentrations ranging from 0.6 to 200 μg/L (Richardson and Kimura, 2007; Rabiet et al., 2010; Zhang et al., 2015). Previous study demonstrated that tebuconazole was persistent in soil, with a degradation half-life (time required to reach 50% degradation) even reaching 600 days (Cui et al., 2018). In Italy, evidence showed that the metabolites of tebuconazole were detected in agricultural workers with the levels ranging from 3 to 473 μg/L (Mercadante et al., 2014). These clearly suggested that tebuconazole might pose risks to humans and ecosystem at environmentally realistic concentrations.

Ecological toxicity of tebuconazole has been assessed over last few years. For instance, exposed to 1.19 mg/L tebuconazole reduced growth, induced oxidative stress, and promoted metabolism of

Abbreviations: AOP, adverse outcome pathway; BCA, bicinchoninic acid; BCF, bioconcentration factor; BW, body weight; BW_e, body weight at the end of exposure; BW_i, body weight at the initial time of exposure; CM, corrected mortality; DMSO, dimethyl sulfoxide; dpf, days post-fertilization; DT_{50}, time required to reach 50% disappearance; DT_{95}, time required to reach 95% disappearance; ECM, embryo-culture medium; EP, egg production; epf, eggs per female; ESR, estrogen receptor; FC, fold of change; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, gender ratio; GSI, gonadal somatic index; HSI, hepatic somatic index; hpf, hours post-fertilization; HPG, hypothalamic-pituitary-gonadal; HRP, horse radish peroxidase; PVDF, polyvinylidene fluoride; rpm, runs per minute; SR, survival rate; TMS, tricaine methanesulfonate; VC, vehicle control; WBL, whole body length; WBL_e, whole body length at the end of exposure; WBL_i, whole body length at the initial time of exposure.

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glycogen in fish (Toni et al., 2011). Increased levels of cholesterol and triglycerides as well as vitellogenin were observed in male zebrafish after 14 days of tebuconazole exposure (Sancho et al., 2010). Previous studies have determined thyroid disruption that tebuconazole significantly increased levels of triiodothyronine (T3) by 27.8% and transcriptions of *tshβ* and *trβ* in zebrafish by 2.18-fold and 2.61-fold respectively after exposure for 120 dpf (Yu et al., 2013). Similar thyroid toxicity was observed in amphibia. Metamorphosis of *Hyla intermedia* (Italia tree frog) was decreased by 58.0% and 73.9% after exposed to tebuconazole for 78 days at concentrations of 5 and 50 μg/L, respectively (Bernabò et al., 2016). Additionally, effects on reproduction following tebuconazole exposure had been found in organisms. For example, in *D. magna*, the reproduction rates decreased by 44% and 68% after exposed to 0.71 mg/L tebuconazole for 14 and 21 days, respectively (Sancho et al., 2015). An *in vitro* study showed that tebuconazole suppressed functions of human placental trophoblast cell via protease systems and hormones (Liu et al., 2015). 17β-estradiol (**E2**) levels were suppressed in adult male *Xenopus laevis* after 27 days exposures of 0.1, 1 and 100 μg/L tebuconazole (Poulsen et al., 2015). Previous research reported that tebuconazole and other azole compounds might cause endocrine disruption by inhibiting steroids biosynthesis (Nellemann, 2010; Villeneuve et al., 2014). These studies indicated that tebuconazole had potential risks to reproduction of different species. In general, reproductive and endocrine impairments caused by tebuconazole have been reported in some invertebrates and vertebrates. However, few study has been done on the chronic effects of tebuconazole on gonadal development in fish.

In the present study, using an aquatic model species, *Danio rerio,* we aimed to validate the hypothesis that tebuconazole acted through down-regulation of steroids (specifically E2) in aquatic organisms and evaluate the linkage of its chronic exposure with the diminished reproduction of aquatic organisms. Since fish are often exposed to environmental contaminants in their lifetime (involving several life stages), to address these issues, the present study characterized the effects caused by tebuconazole on zebrafish in three life stages: gonad forming and differentiating stage, gonad undergoing to maturation stage and gonad maturated stage. To confirm the adverse outcome pathway (AOP) of tebuconazole-mediated reproductive toxicity in zebrafish, steroid hormones including E2 and testosterone (T) were measured following tebuconazole exposure. To further explore the molecular mechanisms potentially involved in tebuconazole toxicity, genes expressions of estrogen receptor (*esr*), androgen receptor (*ar*), cytochrome P450 aromatase 17 (*cyp17*), cytochrome P450 side-chain cleavage (*cyp11a*), 17β-hydroxysteroid dehydrogenase (*hsd17b*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), steroidogenic acute regulatory protein (*star*), and protein expression of gonadal aromatase Cyp19a (the protein of Cytochrome P450 Family 19 Subfamily A) were evaluated.

2. Materials and methods

2.1. Chemicals and materials

Tebuconazole (CAS 107 534-96-3) standard (purity>98%) was purchased from Shanghai Yuanji Chemical Co., Ltd. (Shanghai, China). Tebuconazole stock solution (20 000 mg/L) was prepared by dissolving 5 g standard material in 250 mL dimethyl sulfoxide (DMSO) and stored at −20 °C for no more than three months. A series of given volumes of stock solution were diluted by embryo-culture medium (ECM, containing 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$ and 4.2 mM $NaHCO_3$) or charcoal filtered tap water (DMSO as co-solvent, containing 0.01%, v/v) to form a set concentrations of exposure solutions. Tricaine methanesulfonate (TMS) and DMSO were obtained from Sigma (St. Louis, MO, USA). Lysis buffer, bicinchoninic acid (BCA) protein kit, reference protein GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and HRP (horse radish peroxidase)-conjugated secondary antibody (goat anti-rabbit IgG antibody) were all purchased from Servicebio (Wuhan, China). Methanol and acetonitrile were obtained from Merck (Darmstadt, Germany).

2.2. Zebrafish maintenance

The cultivation of zebrafish (AB strain, purchase from Institute of Hydrobiology, Chinese Academy of Sciences) and artificial insemination of eggs were mainly performed according to the previous studies (Wang et al., 2015a; Ma et al., 2016). Embryo, larvae and adult fish were maintained as described in Supporting Information (S1.1). All procedures were conducted in accordance with The Guide for the Care and Use of Laboratory Animals (Council, 2010).

2.3. Bioaccumulation and depuration

Adult zebrafish of 4-month-age were exposed to 0.18 mg/L ($\frac{1}{100}$ LC_{50} (96 h) for adult zebrafish) (Yu et al., 2013) tebuconazole solution (containing 0.01% (v/v) DMSO) for 28 days (accumulation phase), and the exposure solution was renewed at an interval of 3 days. Subsequently, the fish were transferred to tebuconazole-free water and cultured for 21 days with daily renewal of water (depuration phase). The test was conducted with 70 fish in a barrel-type glass tank (Ø40 (i. d.) × 40 cm) which filled with 35 L solution (nearly 1 g bw/L). Zebrafish in tebuconazole-free charcoal filtered tap water (containing 0.01% DMSO, v/v) was set as vehicle control (VC). Each treatment was conducted in triplicates. During accumulation phase, three fish per time were randomly sampled from each tank at 1st, 2nd, 3rd, 5th,7th, 10th, 14th, 21st and 28th d after exposure for whole-body-quantification of tebuconazole, and ten fish (at 28th d after exposure) were sampled and dissected to separate the brain, gonad, liver and torso muscle for tissue-specific bioaccumulation assessment. During depuration phase, three fish per time were sampled from each tank at 1st, 2nd, 3rd, 5th, 7th, 10th, 14th and 21st d after depuration for whole-body-quantification of tebuconazole to evaluate the depuration effect. During the whole 49-d period, the test system was maintained at 26 ± 1 °C. The quantification of tebuconazole in exposure solutions and fish tissues were described in Supporting Information (S1.2). Pseudo first-order (Ofomaja et al., 2010) and first-order kinetic models were used to fit the bioaccumulation and depuration kinetics of tebuconazole in zebrafish, respectively. The pseudo first-order kinetic model was expressed via Eq. (1), while the first-order kinetic model was expressed via Eq. (2):

$$\ln(Q_e\text{-}Q_t) = \ln(Q_e)\text{-}k_1\ t \quad (1)$$

$$Q_t = Q_0\ e^{-k\ t} \quad (2)$$

Where Q_e and Q_t mean the accumulation concentrations of tebuconazole in zebrafish (mg/kg) at equilibrium and at exposure or depuration time t (d), respectively. Q_0 means the initial concentration of tebuconazole in zebrafish (mg/kg) at the beginning of depuration (or, in other words, at the end of exposure). The model parameters k_1 and k mean the pseudo first-order uptake rate constant (d^{-1}) and first-order depuration rate constant (d^{-1}), respectively.

2.4. Zebrafish chronic exposure

The chronic exposures were performed in three life stages of zebrafish. Stage I exposures were performed from 2 hpf (hours post-fertilization) to 60 dpf (days post-fertilization). This duration was selected because fish were undergoing gonadal sex differentiation. Stage II exposures occurred from 60 to 120 dpf. This duration was selected because gonadal sex differentiation of fish was finished and gradually undergoing to maturation. Stage III exposures were conducted from 180 to 208 dpf. This duration was selected because fish had already reached sexual maturity.

Stage I Exposure. Three hundred randomly selected embryos (2 hpf) were placed in a glass culture dish containing 60 mL ECM with a given concentration of tebuconazole. Nominal exposure concentrations of tebuconazole were set as 0.05, 0.20 and 0.50 mg/L (represent $\frac{1}{10}$ ingLC$_{10}$, $\frac{1}{4}$ LC$_{10}$ and LC$_{10}$ of embryo lethality, respectively) (Yu et al., 2013), and tebuconazole-free solution (containing DMSO, 0.01%, v/v) was set as VC. Each treatment was conducted in triplicates. The exposure solution was renewed at an interval of 72 h during exposure (no renewal of exposure solution during 2 hpf to 5 dpf), and dead embryos or larvae were removed after daily observations. Additionally, beginning from 6 dpf, the exposure solutions were prepared by charcoal filtered tap water but not ECM, and the fish from each repeat were termly transferred to bigger glass tanks and cultured in different volumes of exposure solution (0.5 L, 2 L and 5 L exposure solution when zebrafish were in ages of 6–20, 21–40 and 40–60 dpf, respectively). After exposure, the fish were sampled for sex appraisal by histological examination.

Stage II Exposure. The protocol was the same as Stage I exposures except that 40 zebrafish (60 dpf, unknown gender) with uniform whole body length (WBL, 20 ± 2 mm) and body weight (BW, 50 ± 5 mg) were selected and placed in a glass tank filled with 5 L exposure solution (different life stage using different volume of exposure solution, i.e., 5 L, 20 L and 35 L exposure solution when zebrafish were in ages of 60–80, 81–100 and 101–120 dpf, respectively). The nominal exposure concentrations of tebuconazole were also set as 0.05, 0.20 and 0.50 mg/L. A self-made device displayed in Fig. S1 was used for the measurement of body length of zebrafish at the beginning of exposures.

Stage III Exposure. The protocol was the same as Stage II exposures except that 30 females and 30 males of 180 dpf old zebrafish (uniform WBL and BW of (39 ± 2) mm, (550 ± 55) mg for female and (37 ± 2) mm, (400 ± 40) mg for male, respectively) were selected and exposed in 35 L exposure solution lasting for 28 d at concentrations of 0.18, 0.92 and 1.84 mg/L tebuconazole (representing $\frac{1}{100}$ LC$_{50}$, $\frac{1}{20}$ LC$_{50}$ and $\frac{1}{10}$ LC$_{50}$ of adult lethality, respectively) (Yu et al., 2013). Each treatment was conducted in triplicates and the exposure solution was renewed at an interval of 48 h.

At the end of each exposure stage, the collected fish were firstly anesthetized by TMS (10 mg/L) and measured for BW and WBL. Subsequently, sex appraisal was conducted by histological examination for stage I and gender ratio (GR, male/female) was calculated. While for stage II and stage III, males and females (identified by shape) in each tank were individually collected and fish with same gender were randomly separated into two subgroups. For each gender, fish (10 fish) from one subgroup were used for blood collection (drawn from caudal vein) (Wang et al., 2015a) and hormone measurement, and then the fish was dissected to separate the gonad and the liver for the calculation of the gonadal somatic index (GSI) and hepatic somatic index (HSI), respectively. The separated gonads were then collected for mRNA analysis. Fish (10 fish) from another subgroup were directly dissected to separate the ovary or testis for histological examination. The histological examination of gonad was described in Supporting Information (S1.3).

2.5. Quantification of steroid hormones in tissues and plasma

Steroid hormones of E2 and T in plasma or whole body of zebrafish were quantified by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, MI, USA) according to manufacturer's instructions, with the detection limits of 19 pg/mL and 6 pg/mL for E2 and T, respectively. Each sample was analyzed in 3-well replicates in a same plate with a coefficient of variation less than 20%.

2.6. Quantitative Real-Time PCR assay

The total RNA was extracted using TRIzol reagent as described by Li et al. (2016). RNA extraction and gene expression analysis were conducted as described in the Supporting Information (S1.4). Quantitative Real-Time PCR (qRT-PCR) was performed to examine the selected genes (*cyp11a,cyp17a, cyp19a, esr1,esr2b, star, hsd3b1, hsd17b1* and *hsd17b3*) in HPG (hypothalamic-pituitary-gonadal) axis and the primer sequences (Table S3) of those genes were performed as previously described (Wang et al., 2015b; Ma et al., 2016).

2.7. Cyp19a western blot analysis

Cyp19a in gonad of fish was selected to analysis of its protein expression level at the end of Stage II exposure. A western blot was performed by using 3 ovaries or 3 testes from each replicate. After 0.3 mL ice cold Lysis buffer was added, selected tissues were homogenized with IKA T10 (Staufen im Breisgau, Germany) at 8000 rpm (runs per minute) for 1 min. Then the content of total protein for each tissue lysate was determined by BCA protein kit. Each protein sample was electrophoresed in a 12.5% (m/m) SDS-polyacrylamide gel, using a Mini-Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA, USA). After that, the proteins were transferred to PVDF (polyvinylidene fluoride) membrane (Darmstadt, Germany) and blocked with 5% (m/v) fat-free milk for 1 h at room temperature. The membrane was washed and further incubated at room temperature for 3 h with primary rabbit anti-Cyp19a antibody (1:1000, v/v) (Abcam, USA) or reference protein (GAPDH, 1:10 000, v/v), and the blots were developed with HRP-conjugated secondary antibody (1:3000, v/v) and visualized by enhanced chemiluminescence. The relative optical density of protein imprinting band was analyzed using Image Lab 5.2 Software (Bio-Rad, USA). A quantitative measure of protein expression was obtained by densitometry, with the results normalized to GAPDH expression.

2.8. Statistical analysis

Statistical analysis was performed using SPSS® version 20.0 (SPSS, USA). The data were initially verified for normality and homogeneity of variance using the Kolmogorov-Smirnov test and Levene's test. Data were expressed as means ± standard deviation (SD). Statistical differences in BW, WBL, gene expression and steroids concentrations were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test to the *p*-values. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Accumulation and depuration of tebuconazole in zebrafish

After 28 days of exposure, the average concentration of tebuconazole measured in the exposure medium was 0.177 ± 0.013 mg/L with a fluctuation of 0.153–0.197 mg/L (Fig. S2). The maximum

cumulative mortality of zebrafish (vehicle control groups) was 8.6%, which conformed to the requirements of OECD (Organization for Economic Cooperation and Development) Guideline 203 (OECD, 1992). In fish, the content of tebuconazole gradually increased, and reached a steady-state on 21st d after exposure with the equilibrium concentration of 3.859 ± 0.301 mg/kg (Fig. 1A). The measured bioconcentration factor (BCF, the ratio of equilibrium concentration of tebuconazole in fish to exposure concentration of tebuconazole in water) was approximately 21.8, indicating moderate accumulation (China, 2014). Meanwhile, the contents of tebuconazole in liver, gonad, brain and muscle on 28th d after exposure were 5.711 ± 0.212, 3.459 ± 0.323, 0.374 ± 0.041 and 2.503 ± 0.094 mg/kg, respectively (Fig. 1B). The cumulative curve of tebuconazole in zebrafish fitted the pseudo-first-order dynamic model ($R^2 = 0.984$) with calculated Q_e values of 3.794 mg/kg and k_1 of 0.2127 d^{-1}, respectively (Fig. 1C).

In the depuration phase, the maximum cumulative mortality of remaining zebrafish was 6.7% (including control groups and depuration groups). The content of tebuconazole in zebrafish slowly decreased from 3.859 ± 0.301 mg/kg (at the end of exposure) to 1.997 ± 0.168 mg/kg (on 21st day after depuration) (Fig. 1A). The depuration curve fitted the first-order dynamic model ($Q_t = 3.908\ e^{-0.0301t}$, $R^2 = 0.9752$). The DT_{50} and DT_{95} (time required to reach 50% and 95% disappearance of tebuconazole in fish, respectively) were 23.0 d and 100.4 d, respectively. The depuration rate constant (k) was 0.0301 d^{-1} (Fig. 1D).

3.2. Developmental and reproductive toxicity of tebuconazole to zebrafish

The survival rates (SRs) of fish were higher than 80% whatever in Stage II exposures or in Stage III exposures, while it was slightly lower in Stage I exposures (ranging from 68.0% to 74.9%). However, the corrected mortalities of fish in all groups were lower than 10%. Additionally, at a same exposure stage, there was no significant difference in SR or corrected mortality (CM) among treatments. Moreover, result showed that exposed to tebuconazole at three life stages of zebrafish all resulted in developmental and reproductive toxicities. Data were detailedly listed in Table 1.

Overall, tebuconazole had a negative effect on BW of zebrafish. Compared with VC, significant decrease of BW_e (body weight at the end of exposure) at Stage I exposures was only found in 0.05 mg/L treatment (reducing by 32.6%, $p = 0.036$). For Stage II exposures, the BW of fish for all groups increased 5.27- to 8.31-fold from the beginning to the end of exposures. However, general decreases of BW_e were found in all tebuconazole treatments, and significance decrease occurred in 0.20 mg/L treatment whatever for female (reducing by 26.0%, $p = 0.001$) or male (reducing by 23.3%, $p = 0.004$). Similar variations of BW_e were both found in male and female fish in Stage III exposures. After treated with 0.18, 0.92 and 1.84 mg/L of tebuconazole, BW_e were 419.5 ± 20.8 mg ($p > 0.05$), 386.0 ± 16.3 mg ($p = 0.018$), 392.5 ± 19.2 mg ($p = 0.043$) for males and 55.6 ± 40.3 mg ($p > 0.05$), 466.9 ± 30.2 mg ($p = 0.037$), and 461.9 ± 28.4 mg ($p = 0.032$) for females respectively. While in VC, BW_e for male and female fish were 434.1 ± 13.6 mg and 570.0 ± 44.9 mg, respectively. Additionally, compared with BW_i (body weight at the initial time of exposure), slight variations of BW which could be ignored were found in VC and 0.18 mg/L groups (BW_e/BW_i, 0.98- to1.05-fold, $p > 0.05$), while significant decreases of BW of female fish in 0.92 and 1.84 mg/L groups (0.87-fold ($p = 0.041$) and 0.83-fold ($p = 0.018$), respectively) and male fish in 1.84 mg/L group (0.90-fold ($p = 0.045$)) were observed.

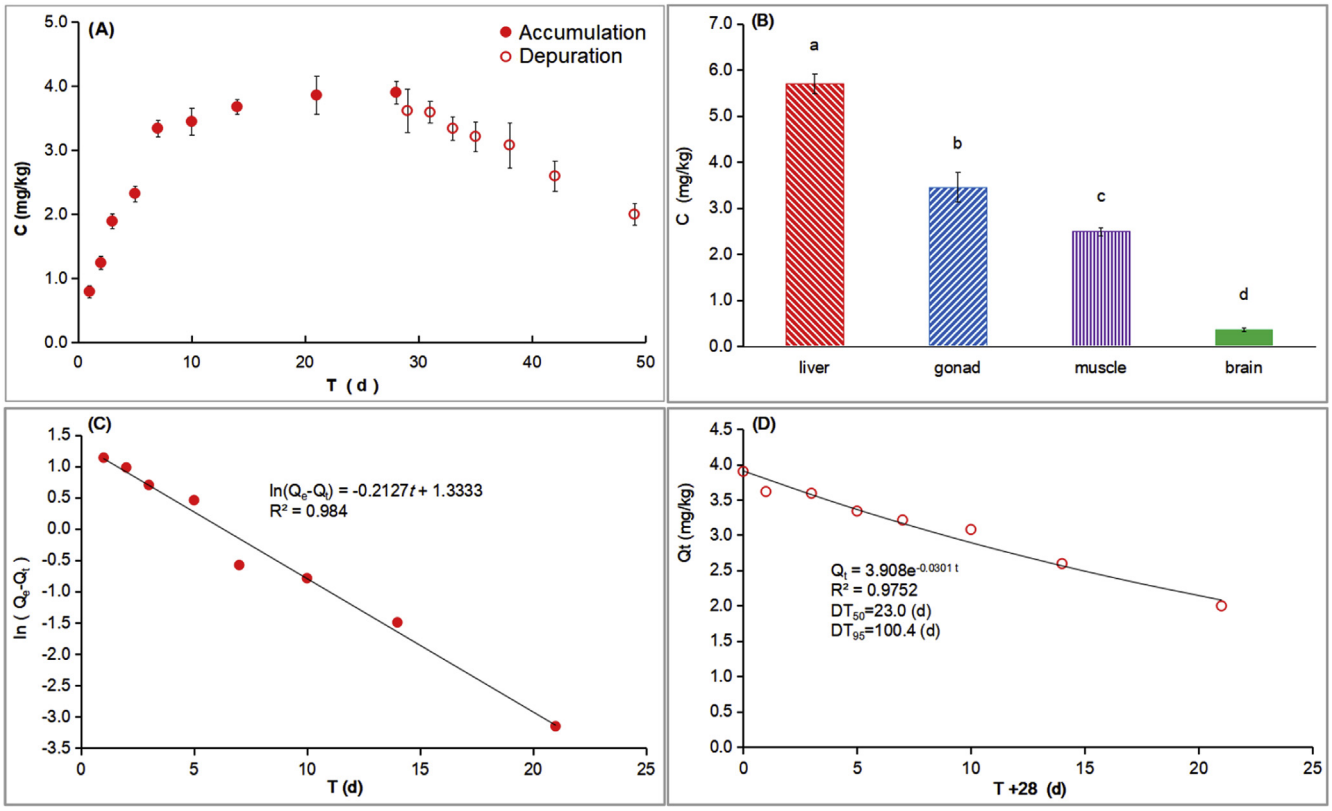

Fig. 1. Accumulation/depuration dynamics and tissue-depend distribution of tebuconazole in zebrafish. (A) Accumulation-depuration curve of tebuconazole in zebrafish; (B) Tissue-depend distribution of tebuconazole in zebrafish; (C) The pseudo-first-order dynamic curve for accumulation of tebuconazole in zebrafish; (D) The first-order dynamic curve for depuration of tebuconazole in zebrafish. The letters a,b,c,d represented significant difference among tissues ($p < 0.05$).

Table 1
Developmental and reproductive toxicities of tebuconazole to zebrafish.

Exposure stage	Toxicity index	Treatment [a]			
		VC	*L*	*M*	*H*
Stage I	SR [b] (%)	74.7 ± 3.2	74.9 ± 3.8	71.9 ± 2.2	68.0 ± 9.3
	CM [c] (%)	/	−0.3	3.7	8.9
	BWe [d] (mg) female	49.4 ± 7.0	53.2 ± 5.5	42.8 ± 6.3	33.3 ± 4.4*
	BWe (mg) male	45.2 ± 2.3	44.5 ± 2.6	40.1 ± 3.1	31.2 ± 4.9*
	WBLe [e] (mm) female	18.8 ± 0.1	19.3 ± 0.5	17.6 ± 0.7	16.7 ± 0.6*
	WBLe (mm) male	18.0 ± 0.4	16.9 ± 0.4	16.8 ± 0.7	15.7 ± 1.3
Stage II	BWi [f] (mg)	50.9 ± 4.0	48.6 ± 2.7	52.4 ± 2.9	50.5 ± 3.9
	WBLi [g] (mm)	19.3 ± 0.3	18.6 ± 0.5	18.9 ± 0.4	18.4 ± 0.6
	SR (%)	86.2 ± 6.9	84.4 ± 4.2	87.8 ± 7.5	87.5 ± 5.8
	CM (%)	/	2.12	−1.84	−1.50
	BWe (mg) female (Fold of change, BWe/BWi)	421.8 ± 14.8 (8.29)	403.8 ± 25.3 (8.31)	312.3 ± 29.0** (5.96)	406.1 ± 24.4 (8.04)
	BWe (mg) male (Fold of change, BWe/BWi)	360.0 ± 37.3 (7.07)	329.2 ± 60.4 (6.77)	276.2 ± 19.1** (5.27)	335.9 ± 39.1 (6.65)
	WBLe (mm) female	39.4 ± 2.1	35.9 ± 0.5*	32.4 ± 0.1**	36.3 ± 7.9
	WBLe (mm) male	38.0 ± 1.2	35.0 ± 2.6	32.4 ± 1.2***	35.3 ± 1.9*
	HSI[h] (%) female	1.92 ± 0.09	1.31 ± 0.16**	1.63 ± 0.38	1.47 ± 0.72
	HSI (%) male	0.90 ± 0.11	0.73 ± 0.31	1.09 ± 0.27	0.95 ± 0.37
	GSI[i] (%) female	12.15 ± 0.53	10.93 ± 1.73	10.92 ± 3.21	10.49 ± 0.32**
	GSI (%) male	1.90 ± 0.17	1.29 ± 0.57	0.97 ± 0.38***	1.62 ± 0.21*
	EP [j] (eggs per female)	49.3 ± 5.5	41.6 ± 1.1	20.5 ± 5.2**	20.8 ± 2.1**
Stage III	BWi (mg) female	543.3 ± 53.0	545.9 ± 41.1	534.7 ± 25.7	554.3 ± 22.2
	BWi (mg) male	421.9 ± 15.2	428.5 ± 36.2	418.2 ± 21.5	434.6 ± 9.2
	WBLi (mm) female	39.6 ± 1.3	39.2 ± 1.1	39.5 ± 1.3	40.0 ± 1.7
	WBLi (mm) male	37.9 ± 1.5	36.7 ± 1.6	36.9 ± 1.1	37.1 ± 1.8
	SR (%) female + male	95.6 ± 2.5	94.4 ± 2.5	92.9 ± 1.9	88.9 ± 4.2
	CM (%) female + male	/	1.2	2.9	7.0
	BWe (mg) female (Fold of change, BWe/BWi)	570.0 ± 44.9 (1.05)	555.6 ± 40.3 (1.02)	466.9 ± 30.2*# (0.87)	461.9 ± 28.4*# (0.83)
	BWe (mg) male (Fold of change, BWe/BWi)	434.1 ± 13.6 (1.03)	419.5 ± 20.8 (0.98)	386.0 ± 16.3* (0.92)	392.5 ± 19.2*# (0.90)
	WBLe (mm) female	40.7 ± 1.5	39.5 ± 0.7	38.3 ± 1.3	38.8 ± 1.2
	WBLe (mm) male	39.0 ± 1.3	37.5 ± 1.5	37.3 ± 1.0	37.8 ± 2.4
	HSI (%) female	2.25 ± 0.18	2.32 ± 0.26	1.85 ± 0.13*	1.73 ± 0.09*
	HSI (%) male	1.85 ± 0.14	1.62 ± 0.15	1.45 ± 0.12*	1.38 ± 0.21*
	GSI (%) female	11.83 ± 0.55	11.31 ± 1.36	10.31 ± 0.62*	9.87 ± 0.74*
	GSI (%) male	1.64 ± 0.15	1.64 ± 0.18	1.36 ± 0.16	1.32 ± 0.11*
	EP (eggs per female)	92.7 ± 4.7	86 ± 4.4	74.3 ± 6.5**	71.6 ± 7.9*

*$p < 0.05$ and **$p < 0.01$ indicates significant differences between exposure groups and the corresponding vehicle control group.
[a] ***VC*** means vehicle control. ***L*** means 0.05 mg/L treatment for Stages I and II exposure, and 0.18 mg/L treatment for Stage III exposure. ***M*** means 0.20 mg/L treatment for Stages I and II exposure, and 0.90 mg/L treatment for Stage III exposure. ***H*** means 0.50 mg/L treatment for Stages I and II exposure, and 1.84 mg/L treatment for Stage III exposure.
[b] SR means survival rate.
[c] CM means corrected mortality.
[d] BWe means body weight of fish at the end of exposure.
[e] WBLe means whole body length of fish at the end of exposure.
[f] BWi means body weight of fish at the initial time of exposure.
[g] WBLi means whole body length of fish at the initial time of exposure.
[h] HSI = liver weight/body weight × 100; [i] GSI = gonad weight/body weight × 100.
[j] EP means egg production. All the data are expressed as the mean ± SD.

Negative effects of tebuconazole exposure on WBL of immature zebrafish were also observed. For Stage I, significant decrease of WBL_e (whole body length at the end of exposure) occurred in 0.50 mg/L treatment. While for Stage II, there was no significant difference of WBL_i (whole body length at the initial time of exposure) among different treatments. However, compared with VC, significant decreases of WBL_e of female fish were found in all three exposure groups and male fish in 0.20 and 0.50 mg/L groups. There was no significant change in WBL_e for both male and female fish among treatments after Stage III exposures.

The effects of tebuconazole on HSI and GSI of zebrafish were only performed at the end of stage II and stage III exposures because the liver and gonad of zebrafish at the end of stage I exposure were too small to be separated. Generally weak inhibition of tebuconazole on HSI for stage II exposure fish was found, and significant decrease of which was only occurred in 0.05 mg/L group for female fish (1.31 ± 0.16, $p = 0.001$). Comparatively, strong inhibitory effect of tebuconazole on HSI was found after Stage III exposures, and significant decreases were found in 0.92 and 1.84 mg/L groups both for male and female fish. Significant reductions in GSI were observed for female in groups of 0.50 mg/L (Stage II, 10.49 ± 0.32, $p = 0.004$), 0.92 mg/L (Stage III, 10.31 ± 0.62, $p = 0.035$) and 1.84 mg/L (Stage III, 9.87 ± 0.74, $p = 0.034$) and male in groups of 0.20 mg/L (Stage II, 0.97 ± 0.38, $p < 0.001$) and 0.5 mg/L (Stage II, 1.62 ± 0.21, $p = 0.034$) and 1.84 mg/L (Stage III, 1.32 ± 0.11, $p = 0.044$).

Tebuconazole could greatly inhibit the fecundity of zebrafish. Compared with VC (49.3 ± 5.5 eggs per female (epf) for Stage II and 92.7 ± 4.7 epf for Stage III), exposed to tebuconazole, egg production (EP) significantly reduced by 58.4% (20.5 ± 5.2 epf, $p = 0.019$) and 57.8% (20.8 ± 2.1 epf, $p = 0.023$) in 0.20 and 0.50 mg/L groups of Stage II, 19.8% (74.3 ± 6.5 epf, $p = 0.007$) and 22.8% (71.6 ± 7.9 epf, $p = 0.024$) in 0.92 and 1.84 mg/L groups of Stage III, respectively.

3.3. Histological examination

Gonadal histological examination of zebrafish was carried out to observe the pathological change and the development of germ cell after Stage II and Stage III exposures. Due to too small of gonad of zebrafish at 60 dpf to be separated, histological examination was

conducted on the mid-section of zebrafish body after stage I exposure to identify the gender and then calculate GR. Result showed that obvious pathological change was occurred neither in ovary nor in testis of zebrafish for all treatments (Fig. S3, S4 and S5). However, tebuconazole obviously disrupted the sex differentiation of zebrafish in Stage I. After exposed to 0.05, 0.20 and 0.50 mg/L tebuconazole, GR were 1.18 ± 0.23 ($p = 0.853$), 1.61 ± 0.16 ($p = 0.038$) and 4.04 ± 0.88 ($p = 0.025$), respectively. While in VC group, GR was 1.15 ± 0.20. Data showed that GR gradually increased accompanying with the rising of exposure concentration, indicating that tebuconazole plays as an androgen-like or antiestrogen-like chemical (Fig. 2A).

For stage II, no significant difference was found in GR after exposure (data not shown) due to the sex differentiation of the zebrafish used had already finished at the beginning of exposure (Santos et al., 2017). But ovarian and testicular histology both showed significant differences in developing degree of germ cells, i.e., exposed to tebuconazole caused variation in the proportion of primary oocytes (PO), cortical alveolar oocytes (CAO), early vitellogenic oocytes (EVO) and late/mature oocytes (LMO) in female and spermatozoa (Sz), spermatocytes (Sc) and spermatogonia (Sg) in male, respectively. In ovary, compared with VC, the percentages of LMO significantly decreased by 57.4% ($p < 0.001$), 44.1% ($p = 0.007$) and 32.7% ($p = 0.048$) after exposure to tebuconazole at 0.05, 0.20 and 0.50 mg/L, respectively. Meanwhile, in all three exposure groups the proportions of CAO increased by 148.4% ($p = 0.004$), 246.8% ($p < 0.001$), 140.3% ($p = 0.023$), respectively (Fig. 2B). In testis, compared with VC, the percentages of Sc in 0.05 mg/L and 0.50 mg/L exposure groups significantly reduced by 53.8% ($p < 0.001$) and 38.4% ($p = 0.014$), respectively. Moreover, the

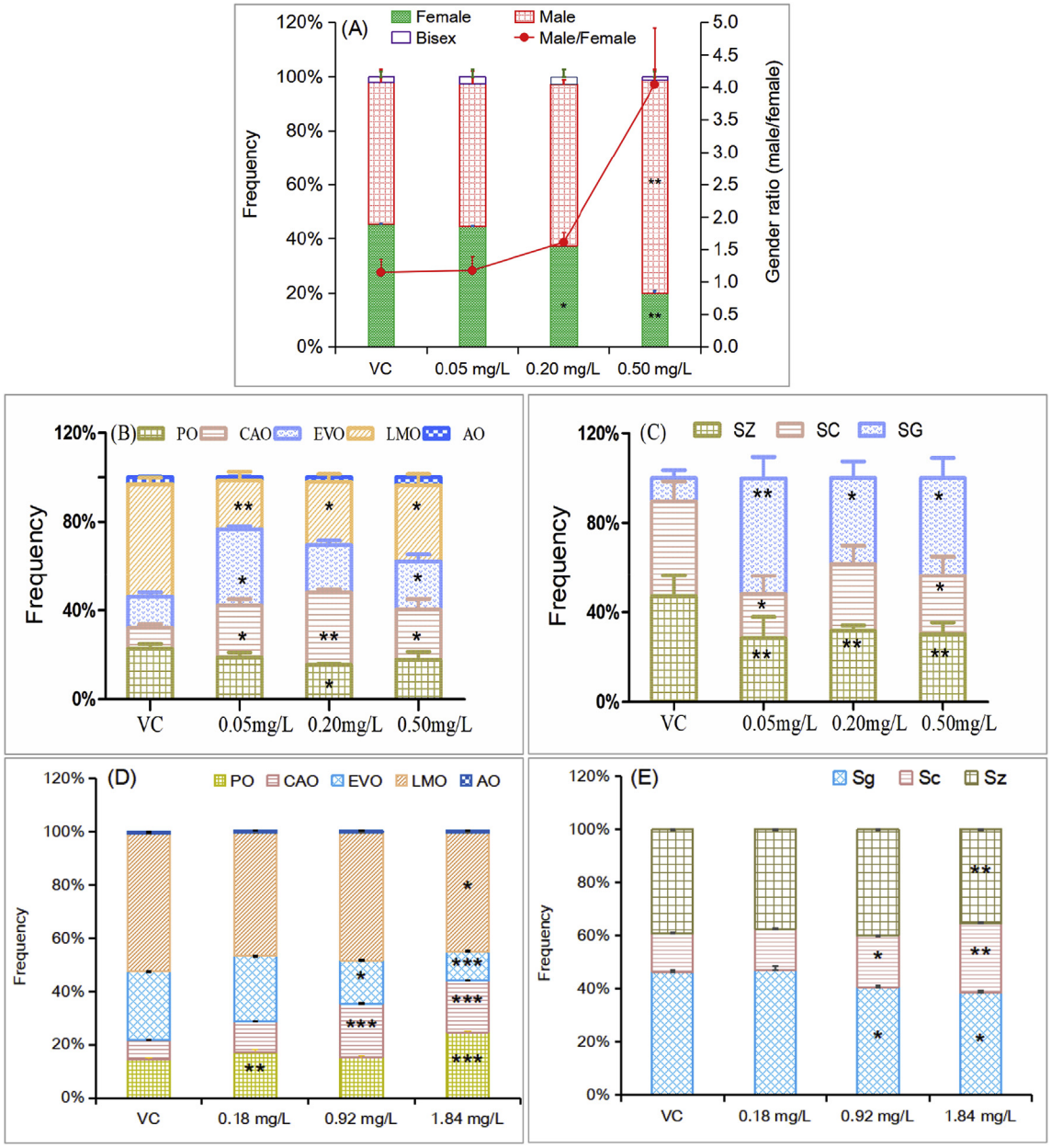

Fig. 2. Effects of tebuconazole on the development of germ cells of zebrafish after (A) stage I, (B) stage II -female, (C) stage II-male, (D) stage III-female and (E) stage III-male exposures. PO, primary oocytes; CAO, cortical alveolar oocytes; EVO, early vitellogenic oocytes; LMO, late/mature oocytes; AO, Atreticoocyte; Sz, spermatozoa; Sc, spermatocytes; Sg, spermatogonia.

percentages of Sz significantly reduced by 39.6% ($p < 0.001$), 32.8% ($p < 0.001$) and 36.1% ($p < 0.001$) in 0.05, 0.20 and 0.50 mg/L tebuconazole treatments, respectively. In contrast, the percentages of Sg increased by 498.2% ($p < 0.001$), 368.4% ($p = 0.029$) and 419.5% ($p = 0.001$), respectively (Fig. 2C).

Similar results were found in ovary of female fish at the end of stage-III exposure**.** Compared with VC, the percentages of LMO decreased by 10.8% ($p = 0.093$), 7.3% ($p = 0.068$) and 13.9% ($p = 0.011$), while CAO conversely increased by 57.5% ($p = 0.101$), 176.7% ($p < 0.00.001$) and 167.1% ($p < 0.001$) after exposed to tebuconazole at 0.18, 0.92 and 1.84 mg/L, respectively (Fig. 2D). In testis of stage-III exposed male fish, compared with VC, there was no significant variation in portions of Sg, Sc and Sz in low concentration (0.18 mg/L) of tebuconazole treatment. But significant variations of those were found in high concentration (1.84 mg/L) of tebuconazole treatment, in which Sc significantly increased by 81.2% ($p < 0.001$, from 14.6% (VC) to 26.4%), Sg and Sz significantly reduced by 17.0% ($p = 0.014$, from 46.3% (VC) to 38.4%) and 10.1% ($p = 0.003$, from 39.1% (VC) to35.2%) respectively (Fig. 2E).

3.4. Effects of tebuconazole on sex hormones of zebrafish

The effects of different concentrations of tebuconazole on the expression of sex hormones (T and E2) in plasma of zebrafish were conducted after exposure and the folds of change (FCs) for T, E2 or E2/T ratio (all compared with VC) were calculated (Fig. 3). For female fish (Fig. 3A), the FCs of T and E2 in plasma displayed opposite variation tendencies at the end of stage I exposures. Generally, FC of T increased (1.14 ($p > 0.05$), 1.56 ($p < 0.05$) and 1.92 ($p < 0.05$) in 0.05, 0.20 and 0.50 mg/L groups, respectively) while those of E2 decreased (0.88 ($p > 0.05$), 0.64 ($p < 0.05$) and 0.51 ($p < 0.05$) in 0.05, 0.20 and 0.50 mg/L groups, respectively), giving a positive and a negative dose-response relationship with tebuconazole, respectively. Similar results were obtained for FCs of T and E2 at the end of stage II exposures, but no dose-response relationship was found (1.09 ($p > 0.05$), 1.04 ($p > 0.05$) and 1.39 ($p < 0.05$) for FC of T and 0.77 ($p > 0.05$), 0.24 ($p < 0.05$) and 0.36 ($p < 0.05$) for FC of E2 in 0.05, 0.20 and 0.50 mg/L groups, respectively). No significant alternation was observed for FC of T in Stage III groups (0.78 ($p > 0.05$), 1.03 ($p > 0.05$) and 1.09 ($p > 0.05$) in 0.05, 0.20 and 0.50 mg/L groups, respectively), but those of E2 reduced significantly in all three exposure treatments (0.67 ($p < 0.05$), 0.69 ($p < 0.05$) and 0.44 ($p < 0.05$) in 0.05, 0.20 and 0.50 mg/L groups, respectively). For male fish (Fig. 3B), the variations of T and E2 were very closed to those of females. Additionally, the ratio of E2/T generally went down accompanying with the increase of tebuconazole in all three exposure stages, whatever in females or in males. It indicated that tebuconazole could inhibit the synthesis of E2 and promote the content of T, leading to the disorders of sex hormones in zebrafish.

3.5. Influence of tebuconazole on HPG axis-related genes expression of zebrafish

The expression of genes associated with HPG-axis were assessed in zebrafish exposed to tebuconazole at all stages (Fig. 4).

After stage I exposures, in female fish (Fig. 4A), the expression of *cyp19a* was diminished in 0.20 (0.58-fold, $p = 0.027$) and 0.50 mg/L (0.59-fold, $p = 0.005$) groups. Decreases in mRNA expression of *esr1* (0.40-fold, $p = 0.002$; 0.63-fold, $p = 0.023$; 0.43-fold, $p = 0.003$) and *hsd17b1* (0.30-fold, $p = 0.002$; 0.39-fold, $p = 0.010$; 0.29-fold, $p = 0.001$) were observed in 0.05, 0.20 and 0.50 mg/L tebuconazole treatments in females. Transcripts of *esr2b* were significantly reduced in 0.05 (0.34-fold, $p = 0.001$) and 0.50 mg/L (0.65-fold, $p = 0.012$) tebuconazole treatments of female fish. In contrast, Transcripts of *hsd3b1* (1.67-fold, $p = 0.007$; 1.80-fold, $p = 0.033$) and *cyp11a* (1.79-fold, $p = 0.027$; 1.53-fold, $p = 0.016$) in females were significantly increased in 0.20 and 0.50 mg/L tebuconazole treatments. In male zebrafish (Fig. 4B), after stage I exposures, the mRNA expression of *esr1* and *esr2b* were diminished in all tebuconazole treatments. The FCs were 0.36-fold ($p = 0.002$), 0.36-fold ($p = 0.003$) and 0.43-fold ($p = 0.014$) for *esr1,* and 0.28-fold ($p = 0.002$), 0.35-fold ($p = 0.003$) and 0.32-fold ($p = 0.009$) for *esr2b* in 0.05, 0.20 and 0.50 mg/L tebuconazole treatments, respectively. The transcripts of *cyp19a*, *hsd17b1* and *hsd17b3* were decreased while *hsd3b1* was contrarily increased in 0.50 mg/L tebuconazole treatment in male fish. The FCs were 0.68-fold ($p = 0.003$), 0.61-fold ($p = 0.002$), 0.46-fold ($p = 0.001$) and 1.97-fold ($p = 0.010$) for *cyp19a, hsd17b1*, *hsd17b3* and *hsd3b1,* respectively.

After stage II exposures, in female fish (Fig. 4C), transcripts of *esr1* were significantly down-regulated in 0.20 and 0.50 mg/L tebuconazole treatments (0.71-fold ($p = 0.023$) and 0.77-fold ($p = 0.019$), respectively). Transcript of *esr2b* was significantly reduced in 0.50 mg/L tebuconazole treatment (0.70-fold, $p = 0.042$). In 0.20 and 0.50 mg/L tebuconazole treatments, the transcripts of *cyp19a* were significantly decreased but *cyp11a, star* and *hsd3b1* were significantly up-regulated, with the FCs of 0.71-fold ($p = 0.018$) and 0.55-fold ($p = 0.036$) for *cyp19a*, 1.50-fold ($p = 0.026$) and 1.75-fold ($p = 0.013$) for *cyp11a*, 2.28-fold

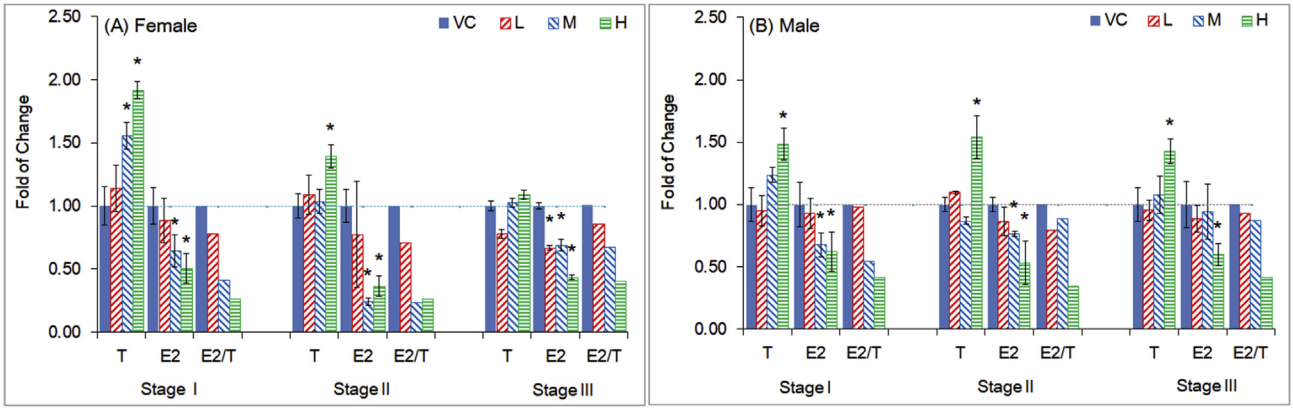

Fig. 3. Influence of tebuconazole on the expression of sex hormones T and E2 in (A) female and (B) male zebrafish exposed to different life stages. VC meant vehicle control; L, M and H meant low, medium and high concentrations of tebuconazole, representing 0.05, 0.20 and 0.5 mg/L for Stage I and II exposures, and 0.18, 0.92 and 1.84 mg/L for Stage III exposure, respectively.

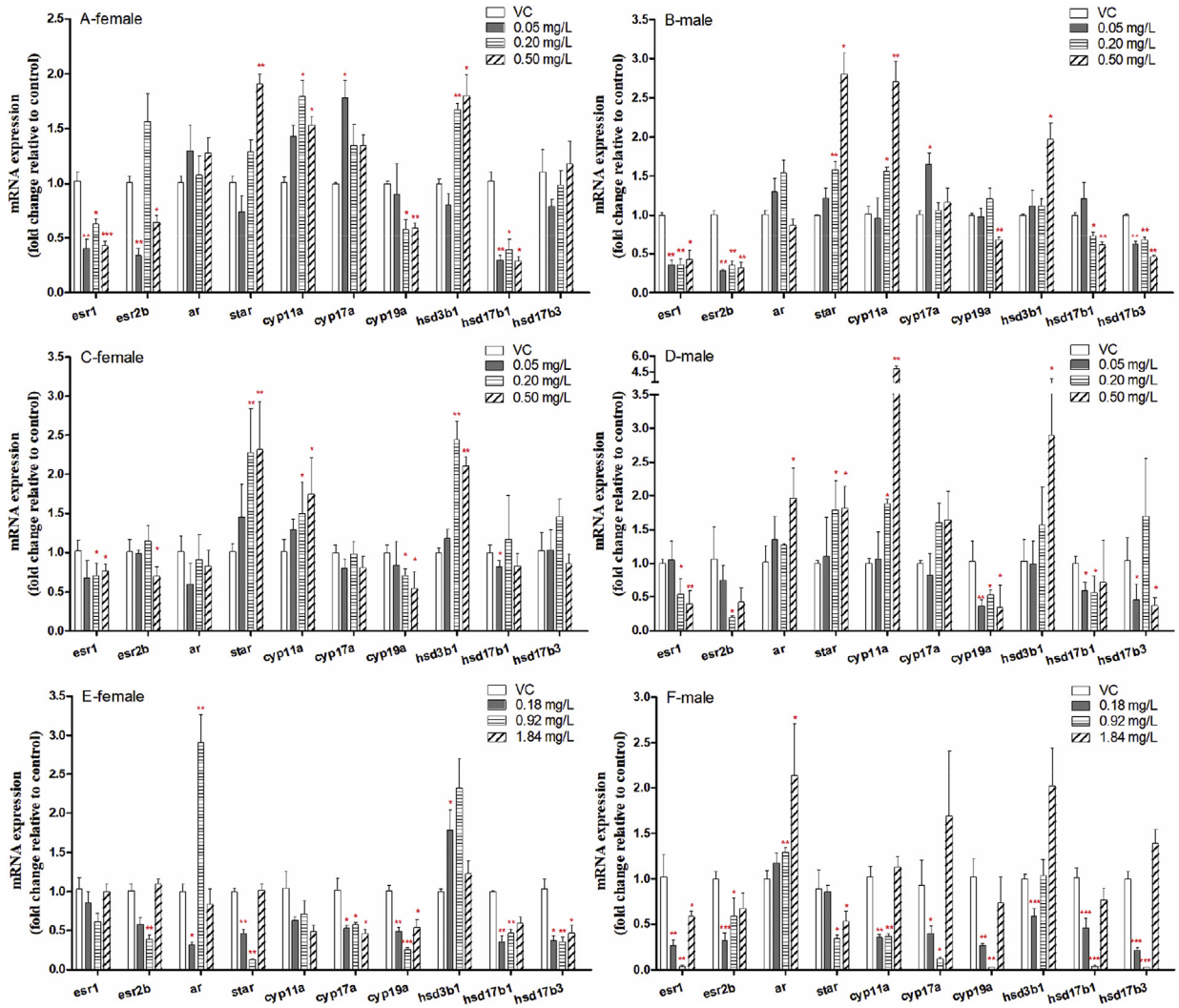

Fig. 4. Influence of tebuconazole on HPG-axis related gene expression in female and male fish after three stage exposures. (A) Stage-I, female fish, (B) Stage-I, male fish, (C) Stage-II, female fish, (D) Stage-II, male fish, (E) Stage-III, female fish and (F) Stage-III, male fish. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, indicating significant differences between exposure groups and the corresponding control group.

($p = 0.006$) and 2.32-fold ($p = 0.008$) for *star*, and 2.44-fold ($p = 0.006$) and 2.12-fold ($p < 0.001$) for *hsd3b1*, respectively. Decrease of *hsd17b1* mRNA expression (0.82-fold, $p = 0.026$) was observed in 0.05 mg/L tebuconazole treatment. In male zebrafish (Fig. 4D), transcripts of *esr1* were significantly down-regulated in 0.20 and 0.50 mg/L tebuconazole treatments (0.54-fold ($p = 0.030$) and 0.40-fold ($p = 0.007$), respectively). The mRNA expressions of *cyp19a* were significantly decreased in all tebuconazole exposure groups. General decreases of *hsd17b1* mRNA expression were found in all tebuconazole treatments, but significances only occurred in 0.05 and 0.20 mg/L treatments. The mRNA expression of *ar* (1.97-fold, $p = 0.020$), *star* (1.82-fold, $p = 0.011$), *cyp11a* (4.80-fold, $p = 0.003$) and *hsd3b1* (2.90-fold, $p = 0.023$) were significantly increased in 0.50 mg/L tebuconazole treatment.

At stage III, gonadal transcripts of *esr2b* and *hsd17b1* as well as *star* in females were significantly decreased in 0.18 mg/L (0.58-fold ($p = 0.022$), 0.36-fold ($p = 0.009$) and 0.46-fold ($p = 0.009$), respectively) and 0.92 mg/L (0.39-fold ($p = 0.002$), 0.47-fold ($p = 0.007$) and 0.13-fold ($p = 0.003$), respectively) tebuconazole treatments (Fig. 4E). Significant diminished transcripts of *cyp17a* and *cyp19a* in females occurred in 0.18, 0.92 and 1.84 mg/L treatments, with the FCs of 0.53-fold ($p = 0.025$), 0.58-fold ($p = 0.013$) and 0.64-fold ($p = 0.023$) for *cyp17a*, and 0.49-fold ($p = 0.002$), 0.26-fold ($p < 0.001$) and 0.54-fold ($p = 0.012$) for *cyp19a*, respectively. Transcripts of *ar* increased in 0.92 mg/L treatment (2.9-fold, $p = 0.021$), but reduced in 0.18 mg/L group (0.32-fold, $p = 0.007$). Transcription of *hsd3b1* in females was enhanced 1.78-fold ($p = 0.032$) in 0.18 mg/L tebuconazole group. In male fissSeven transcripts of *esr1*, *esr2b*, *cyp11a*, *cyp17a*, *cyp19a*, *hsd17b1* and *hsd17b3* were significantly down-regulated in both 0.18 mg/L group (0.27-fold ($p = 0.001$), 0.32-fold ($p < 0.001$), 0.36-fold ($p = 0.006$), 0.40-fold ($p = 0.034$), 0.27-fold ($p = 0.008$), 0.46-fold ($p < 0.001$) and 0.22-fold ($p < 0.001$), respectively) and 0.92 mg/L group (0.04-fold ($p = 0.005$), 0.59-fold ($p = 0.027$), 0.37-fold ($p = 0.007$), 0.12-fold ($p = 0.013$), 0.03-fold ($p = 0.003$), 0.04-fold ($p < 0.001$) and 0.03-fold ($p < 0.001$), respectively). In contrast, mRNA expression of *ar* was increased in 0.20 and 0.50 mg/L groups (1.29-fold ($p = 0.007$) and 2.13-fold ($p = 0.043$), respectively).

3.6. Effect of tebuconazole on expression of Cyp19a protein in gonad

The expression of Cyp19a protein in gonad of zebrafish at stage-II was consistent with that of its mRNA (Fig. 5). Result illustrated

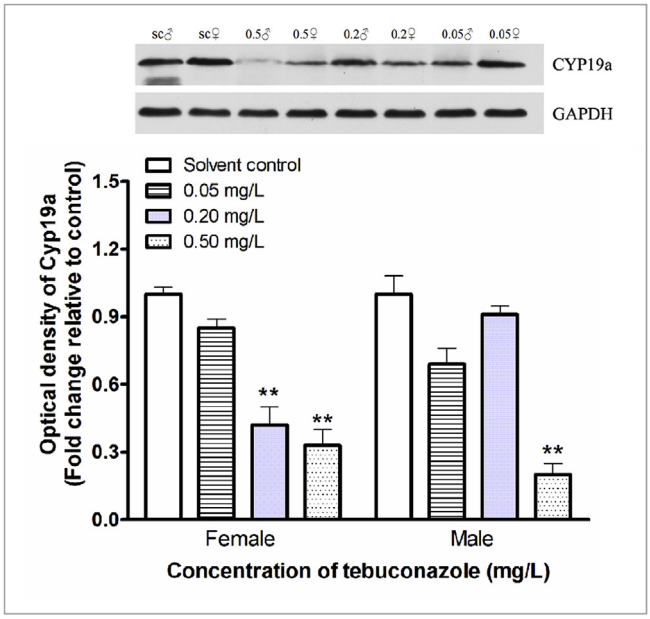

Fig. 5. Western blot analysis of the expression of protein Cyp19a in male and female zebrafish of Stage II after exposed to tebuconazole. The representative western blot of Cyp19a expression at stage I, GAPDH was used as an internal reference. The relative quantification of protein expression of Cyp19a after tebuconazole exposure. Symbols of '♀' and '♂' represents female fish and male fish, respectively. VC means vehicle control.

that compared with VC, the contents of protein Cyp19a in females were reduced by 15.2% ($p = 0.085$), 57.8% ($p = 0.006$) and 66.7% ($p = 0.002$) after treated with 0.05, 0.20 and 0.50 mg/L tebuconazole, respectively. Meanwhile, the expressions of protein Cyp19a in males were also down-regulated in overall but significant down-regulation of which was only found in 0.50 mg/L treatment (reducing by 80.1%, $p = 0.002$). Generally, it suggested that exposed to tebuconazole inhibited the expression of protein Cyp19a in both female and male zebrafish.

4. Discussion

Tebuconazole is a triazole fungicide that has been increasingly used in agricultural settings, leading to exposure in humans and wildlife (Toni et al., 2011; Mercadante et al., 2014; Bernabò et al., 2016). Limited studies have been performed so far to evaluate the molecular effects of tebuconazole in male and female species (Slotkin et al., 2008; Mu et al., 2015). In the present study, three developmental stages of zebrafish were used to assess the reproductive and endocrine effects of tebuconazole.

Tissue-specific bioaccumulation of adult zebrafish displayed that tebuconazole was significantly higher in gonads than in other tissues (except liver). Previous studies have suggested that accumulation of xenobiotics in gonads might be associated with endocrine disorders or reproductive dysfunction (Wang et al., 2015a). Consistent with accumulated values, GSI was significantly reduced after exposed to tebuconazole at adolescence and adult life stages. GSI is widely used as a biomarker of gonad condition in aquatic wildlife (Wang et al., 2015b). As a response to the decreased GSIs in males and females, in the present study, there were significant reductions in fecundity after adolescaria and adult stages exposures. Therefore, tebuconazole might inhibit gonadal growth of zebrafish. Simultaneously, bioaccumulation test also showed the highest enrichment of tebuconazole in liver of zebrafish. In view of general decreases of HSI both in males and females in the present study, tebuconazole might have hepatotoxicity to zebrafish.

Histological results indicated that exposed to tebuconazole in adolescence and adult life stages (sexually differentiated life stages, i.e., Stages II and III at present study) disrupted the gonadal development of zebrafish. Tebuconazole might directly affect the percentage of LMO–the largest oocytes in ovary, and might lead to change in GSI value (Zhao et al., 2015). However, a previous study reported that the decrease of GSI in zebrafish ovaries was mainly due to the significant decrease of late vitellogenic oocytes (Zucchi et al., 2014). In the present study, the alterations of GSI in 0.50 mg/L of Stage II and 1.84 mg/L of Stages III exposure groups conformed with the findings reported by Zhao et al. (2015). Growth and maintenance of gonads are regulated by steroid hormones, and significant correlations have been established between lower steroid levels and reduction of GSI (Liu et al., 2011; Wang et al., 2015b). Consistent with the reduction of GSI, E2 levels were diminished in the present study. In addition, various factors (e.g. hormones and genes) are differentially expressed throughout follicle development and play important roles in regulating oocytes development (Tokarz et al., 2013; Clelland and Peng, 2009). The present findings demonstrated that tebuconazole significantly altered the balance between estrogens and androgens in both female and male zebrafish. Decreases in E2/T ratio might further lead to a delay in ovarian development and a consequent decline in fecundity, supporting the anti-estrogenic effect of tebuconazole on zebrafish. For testes, exposed to tebuconazole reduced the portions of **Sz** in both adolescence and adult fish, which might also contribute to the injury in fecundity. Combined with the result of GSI reduction in males in the present study, tebuconazole did not promote but delayed the maturation of sperm cells in spite of the fact that increased hormone of T was found in exposed fish. It suggested that tebuconazole might not be an androgen function-like chemical and the reasons remained unknown.

Additionally, histological results also indicated that exposed to tebuconazole in juvenile stage (sexually differentiating stage, i.e., Stage I at present study) altered the gonadal differentiation of zebrafish, tending to result in a male sex ratio bias. As known, zebrafish is considered as a class of undifferentiated gonochoristic species (Hoar and Randall, 1969). In recent years, evidences have been found that some synthetic chemicals in environment could affect the sexual differentiation of zebrafish (Chen et al., 2016; Hua et al., 2016; Lee et al., 2017; Santos et al., 2017; Delomas and Dabrowski, 2018; Shi et al., 2018; Yang et al., 2018), playing roles as environmental endocrine disruptors. Delomas and Dabrowski (2018) reported that down-regulation of E2 in juvenile zebrafish induced a male-biased development. Svensson et al. (2016) described that anti-estrogen drugs (levonorgestrel and progesterone) could affect sexual development in juvenile fish, leading to a male phenotype. Additionally, it was reported that 100% mutant zebrafish developed to males when gonadal aromatase gene *cyp19a1a* (a key gene which regulates the synthesis of E2) was knocked out (Lau et al., 2016). Contrarily, promoted E2 and gene *cyp19a* in juvenile fish during the sex differentiation period after exposed to perfluorooctanesulphonic acid (Chen et al., 2016) or monocrotophos (Gao, 2011) produced a female-biased sex ratio. Therefore, considering the overall down-regulations of hormone E2, aromatase Cyp19a, and genes of *cyp19a, esr1* and *ers2b,* as well as male-biased development in the present study, it was deduced that tebuconazole inhibiting E2 signal pathway might be the key reason for the disruption of tebuconazole on sexual differentiation of juvenile zebrafish. However, the process of zebrafish gonadal differentiation is heretofore still poorly understood due to the scarcity in available information (Santos et al., 2017). The molecular mechanism of tebuconazole disrupting on sex differentiation of zebrafish remained further investigation.

Tebuconazole had a profound impact on the expression of genes

involved in steroid hormone biosynthesis in zebrafish. Steroid hormones regulate neurogenesis, sex determination, reproductive processes and behavior of fish (Tokarz et al., 2013). Steroidogenesis requires the catalytic reactions of a number of enzymes including cytochrome P450s (CYPs) and hydroxysteroid dehydrogenases (HSDs) (Zhao et al., 2014). The mRNA expression of cytochrome P450 19 (*cyp19*), 3β-hydroxysteroid dehydrogenase (*hsd3b*), and 11β-hydroxysteroid dehydrogenase (*hsd11b*) were significantly changed in both male and female zebrafish by tebuconazole exposure, which was consistent with the reduction of estrogen formation. Although reductions in the expression of enzymes that regulate the synthesis of steroid hormones were consistent with the declines of steroid hormones in female fish after exposed to tebuconazole, it was unclear whether tebuconazole could affect the upstream regulation pathways of these enzymes. In addition, estrogen receptors (ESRs) transcripts were also reduced. ESRs including ESR1, ESR2a and ESR2b are nuclear receptors, which play important roles in sexual development by mediating estrogen (Tohyama et al., 2015). The transcriptional activity of *esr* is regulated in a ligand-dependent manner directly through binding of estrogens or anti-estrogens (Smith and O Malley, 2004; Berno et al., 2008). Consistent with the down-regulated estrogen, transcripts of *esr1* and *ers2b* significantly reduced. The present findings demonstrated that tebuconazole significantly changed transcripts of genes along the HPG-axis and ultimately reduced E2.

Aromatase (Cyp19a) plays a critical role in converting androgens into estrogens (Lou et al., 2013; Luthra, 2015). Previous studies have demonstrated that the reduced expression of *cyp19a* resulted in down-regulation of E2, probably leading to accumulation of androstenedione or T (Kinoshita and Chen, 2003). In the present study, dose-dependent reductions were observed both in transcripts of *cyp19a* and protein levels of Cyp19a in gonad of zebrafish. Results of the present study were consistent with previous studies on the reduction of E2 concentration in male frog by tebuconazole (Poulsen et al., 2015). However, it was unclear how the expression of aromatase is diminished. As reported, azole pesticides have competitive inhibitory effects on a number of cytochrome P450 enzymes (Zhang et al., 2008; Liu et al., 2011). Previous studies suggested that azole pesticides might be more likely to act as upstream enzymes inhibitors of steroidogenesis enzymes than as inhibitors of aromatases which are the terminal enzymes of E2 biosynthesis (Villeneuve et al., 2007; Zhang et al., 2008). This hypothesis was confirmed in our study by the transcriptional responses observed in gonad of female zebrafish. For instance, a significant increase in *hsd3b* transcript might be regarded as a compensatory response to the inhibitory effect on steroidogenesis enzymes. In addition, to confirm the potential inhibitory effect on aromatase activity, down-regulated expression of *cyp19a* was measured in female fish after exposed to tebuconazole. Multiple regulatory elements have been observed in Cyp19a promoter in zebrafish (Zhao et al., 2017). Wang et al. (2015c) demonstrated that diminished binding of the androgen-androgen receptor complex to the androgen responsive element in Cyp19a1 promoter resulted in suppression of aromatase expression. Similarly, down-regulated estrogen would be expected to decrease the binding to the estrogen responsive elements in Cyp19a promoter. However, further studies were needed to confirm this mechanism.

5. Conclusion

In summary, our comprehensive analysis demonstrated that exposed to tebuconazole at environmental dose could produce anti-estrogen-like endocrine disrupting effects on zebrafish. Chronically exposed to tebuconazole could lead to a male-biased sex differentiation in juvenile zebrafish, and delay the development of germ cells in adolescence and adult zebrafish, thereby further cause adverse effects on reproduction. These anti-estrogen-like endocrine disrupting effects might mainly attribute to the reason that exposed to tebuconazole inhibited the expression of aromatase Cyp19a thereby down-regulated steroid hormone E2. However, the underlying molecular mechanisms of tebuconazole disrupting on sex differentiation and fecundity remained to be further studied.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.03.067.

Conflicts of interest

The authors declare no competing financial interest.

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