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Disruption of sex-hormone levels and steroidogenic-related gene expression on *Mongolia Racerunner* (*Eremias argus*) after exposure to triadimefon and its enantiomers

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HIGHLIGHTS

- Triadimefon and its enantiomers can disturb the synthesis of sex steroid hormones.
- The biotoxicity of the two enantiomers is different and (S)-TF show higher biotoxicity than (R)- TF.
- The plasma testosterone level was elevated, while the estradiol level was reduced.
- The expression of steroidogenic-related genes was changed.

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ABSTRACT

Triadimefon (TF) is a widely used chiral fungicide with one chiral centre and two enantiomers (TF_1 and TF_2). However, little is reported about the ecological toxicity of reptiles on an enantioselective level. TF is a potential endocrine disruptor that may interfere with sex steroid hormones, such as testosterone (T) and 17beta-estradiol (E_2). In our study, the lizards *Mongolia Racerunner* (*Eremias argus*) were orally exposed to TF and its enantiomers for 21 days. Plasma sex steroid hormones and steroidogenic-related genes, including 17-beta-hydroxysteroid (*hsd17β*), cytochrome P450 enzymes (*cyp19* and *cyp17*), and steroid hormone receptors (*erα* and *Ar*) were evaluated. After exposure, the plasma testosterone level in the 100 mg/kg^{bw} group was elevated, while the oestradiol level was reduced. This phenomenon may be caused by the transformation of *cyp19*, which may inhibit the conversion of testosterone to oestradiol and affect sexual behaviour. In addition, the two enantiomers have different effects on hormone levels, which testified to the previously reported biotoxic dissimilarity between TF_1 and TF_2 in organisms. Furthermore, the *cyp19* mRNA level in liver and gonad of the TF_2 and TF group (100 mg/kg^{bw}) were significantly down-regulated, while the *cyp17* and *hsd17β* mRNA levels were up-regulated. The expression of *erα* and *Ar* mRNA levels were up-regulated in males but not in females, which may indicate that TF has sex differences on these two genes. As seen from the above results, TF and its enantiomers may have endocrine-disrupting effects on lizards (*E. argus*) by acting sensitively on sex steroid hormones and steroidogenic-related genes.

1. Introduction

Conazoles are a type of broad-spectrum fungicide that contain a 1, 2, 4-triazole ring or an imidazole moiety. Triazoles are a kind of conazoles and they are widely used in agriculture to inhibit fungal growth on trees, grasses, fruits and vegetables with low resistance risk and high biological activity (Crowell et al., 2010; Wu et al., 2001; Schwinn, 1984). Triazoles are potentially ergosterol biosynthesis inhibitors, which may influence the sterol 14-alpha-demethylase (CYP 51) in biosynthesis of ergosterol, an essential component of fungal cell walls (Zarn et al., 2003). Because of the broad use of triazoles, they may transport from cultivated soils in significant amounts, which may post threats to land ecological environment.

Although most triazoles are sold as racemates, they are chiral

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compounds with at least one or two chiral centres (Liu et al., 2014). The enantiomers of triazoles have nearly the same physicochemical properties in an achiral environment. However, they may show significant differences in a chiral environment, especially in biological properties, because of the effect of enzymes, organic matter or other chiral macromolecules (Qi et al., 2016; Zhang et al., 2016). One enantiomer of triadimenol strongly shows the highest toxicity and is up to 100-fold more active than the other three enantiomers (Burden et al., 1987).

Triadimenfon (TF), with one chiral centre and two enantiomers, is one of the registered systemic fungicides (Liu et al., 2011). In recent years, more and more studies have discovered that excessive triadimefon use can cause a series of health problems, such as neurotoxicity in rodents (Moser and Macphail, 1989) or teratogenic effects on rat embryos (Menegola et al., 2000). In addition, an increasing number of investigations also proved that triadimefon is a type of cytochrome p450 enzyme inhibitor (e.g., *cyp11b*, *cyp17* and *cyp19a*). Triadimefon could block the synthesis of 17β-estradiol (E2) from testosterone (Brande-Lavridsen et al., 2008; Mason et al., 1987), which may have reproductive toxicity to animals and even human beings (Vinggaard et al., 1999, 2000; Chu et al., 2016). Furthermore, all of the above studies only focus on the racemization of TF. There is lack of ecotoxicology data on triadimefon and its enantiomers (Chu et al., 2016). No clear research has pointed out whether the enantiomers of TF have different effects on the sexual gland system.

The sexual gland system plays a significant role in the whole life of vertebrates by determining the next generation of organisms. Sex steroid hormone is synthesized in the gonad and acts on promoting the maturity of the gonad, developing secondary sex characteristics and maintaining sexual function (Kime, 1993). Testosterone (T) and 17beta-estradiol (E_2) are the most important sex steroid hormones in vertebrates. Testosterone is one of the primary hormones produced in testis that is vital for spermatogenesis (Guan et al., 2012). E_2 has been reported to exist in ovaries, but males also need E_2 to regulate spermatogonial proliferation and sertoli cell physiology (Miura et al., 1991). As is known to all, the steroid hormones are synthesized from cholesterol through a series of reactions involving many types of enzymes, such as the 17-beta-hydroxysteroid (*hsd17β*) and cytochrome P450 enzymes (*cyp19*, *cyp17*). The enzymes can control the synthesis of sex steroid hormones and the rate testosterone converts to oestrogen, thereby influencing reproductive behaviour (Costa et al., 2015). T and E_2 generally act via the activation of androgen receptor (*Ar*) and oestrogen receptor (*Er*), which influence target gene expression and/or modulate intracellular signalling cascades.

In recent years, reptiles appear to be decreasing on a worldwide scale largely because of the unsustainable use of pesticides (McIntyre and Whiting, 2012). During the farming season (May to Sept.), which is also the reproduction season of lizards, many types of pesticides sprayed on crops may lead to excessive pesticide residue. Reptiles, especially lizards, prey on many types of insects and lived close to the cropland that is easily polluted by the insecticides. Thus, they are considered as a useful bioindicator organism (Bishop and Gendron, 1998). As for all organisms, it is necessary to investigate the research of lizards in order to protect them from endangerment. *Eremias argus* (*E. argus.*) is a species of lizard that is widely distributed north of the Yangtze River, including the North China Plain and Northeast China Region, the main agricultural areas of China. This species is listed as endangered breed under the Protection of Wild Fauna and Flora Act of the Korean Ministry of Environment (Kim et al., 2012; Wang et al., 2014).

In this study, *E. argus* was chosen as the test organism, and the effects of TF and its enantiomers on the endocrine system were studied, including histological analysis, sex steroid hormone level and steroidogenic-related gene transcriptional profiles. In addition, the biotoxicity differences of the two enantiomers were evaluated.

2. Materials and methods

2.1. Chemicals

Analytical standards of TF (purity>99%) were kindly provided by the college of science, China Agricultural University (Beijing, China). The stock solution of TF was prepared in acetonitrile (analytical grade, Beijing chemical reagent Co. Ltd, China) and stored in darkness at 4 °C. As to the dosage, TF and its enantiomers were dissolved in acetonitrile and then dispersed in corn oil (acetonitrile: corn oil was 1:9, V/V). The lactescence was stored in darkness at 4 °C and balanced to room temperature (28 °C) and then mixed evenly before dosing.

2.2. The separation of triadimefon enantiomers

The (R)-triadimefon (TF_1) and (S)-triadimefon (TF_2) were separated on an Agilent 1260 high-performance liquid chromatograph (HPLC) system with a chiral column (CHIRALPAK IC), and the UV detection wavelength was 225 nm. The mobile phase was a mixture of *n*-hexane and isopropyl alcohol (90:10, V/V) at a flow rate of 1.5 mL/min, and the injection volume was 20 μL. According to our previous study (Wang et al., 2014), the absolute configurations of TF enantiomers were determined and the purity of the enantiomers was >96%.

2.3. Test lizard and culture conditions

The juvenile E. argus were collected in Abag Banner, Inner Mongolia (China), which has no history of chemical application, and maintained in a laboratory since July 2009. The lizards were roomed in aquariums that were 5 × 1.2 × 0.4 m in size with approximately 15 cm mollisol on the bottom and contained water dishes and cardboard that provided a refuge and basking location. The temperature of the domestication room ranged from 26 °C to 30 °C with a light-dark cycle of 14:10 h, and the humidity was from 25% to 30%. All of the lizards received enough UV exposure from UV lamps in order to maintain daily life. The lizards were fed 1–2 live meal-worms (1.5 cm in length) once a day. The excreta of the lizards were cleaned every two days.

2.4. Exposure experiment

Healthy, sexually mature (approximately 2 years old) lizards that were similar in body weight and length were used in the exposure experiment. These lizards were grouped randomly by sex ratio (1:1) and housed in 30 × 30 × 20 cm glass cage before dosing. The test conditions were the same as the domestic conditions. The test lizards (n = 270) were divided into seven groups (each group n = 54), one control group and six test groups. The test lizards were dosed a different concentration of TF and its enantiomers every week (1, 7 and 14 days) according to weight. Two dose concentrations (10, 100 mg/kg^{bw}) were selected according to previous studies (Wang et al., 2014; Garrison et al., 2011).

Three male and female lizards in each group were euthanized and dissected at 7, 14, 21 days, and three replicate were prepared at each time point. The bodies and excised livers were weighted after dissection. The blood was collected from the neck and centrifuged immediately, and then the serum was kept frozen at −20 °C for subsequent use. The livers, gonads, brain and kidney were also collected and kept in an RNA sample store solution (purchased from

TIANGEN Biotech, Beijing).

2.5. Histopathological analysis

Approximately 4–5 μm of excised liver of the lizards was cut down and drenched in paraformaldehyde solution. The tissues were made into pathology slices and stained with haematoxylin and eosin and then analysed using light microscopy.

2.6. Sex hormone (E_2, T) levels in plasma

Commercialized ELISA kit products purchased from Elabscience Biotechnology Co., Ltd, were used to measure the testosterone (T) and oestradiol (E_2) level in serum. Each experimental sample (approximately 50 μl) was analysed by demonstrating parallelism between a series of diluted and spiked serum samples in relation to the standard curve. The inter-assay coefficients variation of both T and E_2 is less than 10%. Cross reactivity between T and E_2 antibodies was less than 0.5%. The sensitivity of the assay was 9.38 pg/mL for E_2 and 0.19 ng/mL for T.

2.7. RNA extraction and cloning of cDNA

The total RNA was isolated from the livers and gonads of male and female lizards using 1 mL of Trizol reagent (TIANGEN Biotech, Beijing China). Then, RNA samples were dissolved in ribonuclease-free water and stored at −80 °C until the process of reverse-transcriptase polymerase chain reaction.

Complementary DNA was synthesized with mixtures containing 11 μl of total RNA, 2 μl of 10 μM oligo (dT) $_{15}$, and 2.5 mM of deoxyribonucleoside triphosphate. The mixture was heated to 70 °C for 5 min and then immediately ice-bathed for 5 min. After cooling, 200 U of reverse transcriptase (TIANGEN Biotech) and 40 units of RNAsin (RNAase inhibitor; TIANGEN Biotech) were added to the mixture. The total mixtures were incubated for 50 min at 42 °C and heated to 95 °C for 5 min to inactivate the reverse transcription.

2.8. Real-time PCR and primer design

Quantitative real-time PCR with SYBR Green detection was performed using a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 20 μl according to protocols established by the manufacturer, 500 nM forward primer and 500 nM reverse primer. The PCR conditions included initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C (30 s), 54 °C (40 s) and 72 °C (40 s). The primers were all designed according to the conserved region of the known sequences of other lizard species and the specificity of the primers was tested with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Relative expression was calculated using *β-actin* (housekeeping gene) and the sequences of gene-specific primers are listed in Table S1. During the experiment, all samples were tested in triplicate and the average expression derived from the triplicate was used for later analysis. The results were analysed according to delta-delta Ct method.

2.9. Tissue distribution of erα, Ar, cyp17, cyp19 and hsd17β

To analyse the distribution of *erα*, *Ar*, *cyp17*, *cyp19* and *hsd17β* in different tissues, 12 normal lizards (6 females and 6 males) were used. Quantitative real-time PCR was conducted with liver, gonad, brain and kidney tissues of sexually maturity lizards, with the *β-actin* gene used as an endogenous control.

2.10. Statistical analysis

All experimental data were presented as the mean ± SD and statistically analysed with SPSS 21.0 software. A *t*-test was used to check the variance and all data met the assumptions of equal variance. Significant differences between the control and treated groups were evaluated using one-way ANOVA at 95% confidence limits, $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. Body weight, liver index and clinical signs

During the exposure time, the changes in body weight and liver index of females and males were recorded at 7 d, 14 d and 21 d (Table 1). As shown, there was no significant difference in body weight or liver index between the control group and the test groups after 21 days of exposure. Additionally, during the experiment, the daily food consumption of the lizards was unchanged, and there were no treatment-related clinical signs.

3.2. Liver histopathology

After 21 days of exposure, liver histopathological changes at 100 mg/kg^{bw} were detected (Fig. 1). In the control group (Fig. 1A and D), normal hepatic cells have well-preserved cytoplasm, prominent nuclei and nucleoli, visible central veins and thin sinusoids. However, the hepatic cells in the two-enantiomer groups have fewer nuclei, loosened cytoplasm and a disordered arrangement of cellula, which may imply the damage of hepatic cells.

3.3. Plasma sex hormone levels

Exposure to TF and its enantiomers (10 mg/kg^{bw} and 100 mg/kg^{bw}) has different effects on the level of oestradiol (E_2) and testosterone (T) (Fig. 2). The plasma sex hormone level was not affected by TF_1 exposure compared to the control group. In the TF_2 group, the T level was dramatically up-regulated in males ($p < 0.01$). The E_2 concentration of the TF_2 group was significantly down-regulated compared to the control group ($p < 0.05$). A similar situation happened to females in the TF group ($p < 0.05$) on E_2 level. However, there was an interesting phenomenon that the males had a higher E_2 level than females in the TF_2 and TF group. There were no significant changes in the E_2 and T level at 10 mg/kg^{bw}.

3.4. Tissue distribution of the steroidogenic-related genes in female and male lizard

Steroidogenic-related genes (*erα*, *Ar*, *cyp17*, *cyp19* and *hsd17β*) exhibit a wide distribution in the tissue of the lizards (Table 2). Rt-PCR was carried out to examine the expression of the five steroidogenic-related genes in the brain, liver, kidney, ovary and testis of sexually mature *Eremias argus*. Differential expression was seen from the five genes compared to the internal control transcript (*β-actin*). For all the tissues, the *erα* mRNA was highly expressed in female liver and ovary. However, the *Ar* mRNA was strongly expressed in male liver and testis. The other three steroidogenic-related genes also could be examined in the tissues, especially in the liver, ovary and testis. In addition, the expressions of the five genes in the brain are similar and moderate, which is in contrast to the kidney.

Table 1
Body weight and liver index of lizards after 21 days exposure.

Conc. (mg/kg)	Compound	Body weight (g)			Liver index (%)		
		7D	14D	21D	7D	14D	21D
	CK	3.06 ± 0.57	2.94 ± 0.12	3.03 ± 0.48	2.89 ± 0.12	2.85 ± 0.20	2.78 ± 0.23
10	TF1	2.99 ± 0.37	2.88 ± 0.36	2.96 ± 0.64	2.76 ± 0.18	2.94 ± 0.31	2.68 ± 0.24
	TF2	2.69 ± 0.82*	2.99 ± 0.23	2.92 ± 0.42	2.98 ± 0.21	2.86 ± 0.23	2.75 ± 0.14
	TF	3.07 ± 0.72	2.76 ± 0.31	3.14 ± 0.43	2.88 ± 0.15	2.81 ± 0.30	2.81 ± 0.20
100	TF1	2.89 ± 0.68	2.94 ± 0.24	2.97 ± 0.09	3.08 ± 0.51	2.99 ± 0.04	2.71 ± 0.24
	TF2	2.94 ± 0.51	2.81 ± 0.67	2.81 ± 0.72	2.94 ± 0.15	2.84 ± 0.21	2.69 ± 0.72
	TF	2.84 ± 0.40	3.05 ± 0.45	3.05 ± 0.38	2.89 ± 0.10	2.87 ± 0.15	2.74 ± 0.31

Asterisks indicate significant difference from the control ($p < 0.05$).

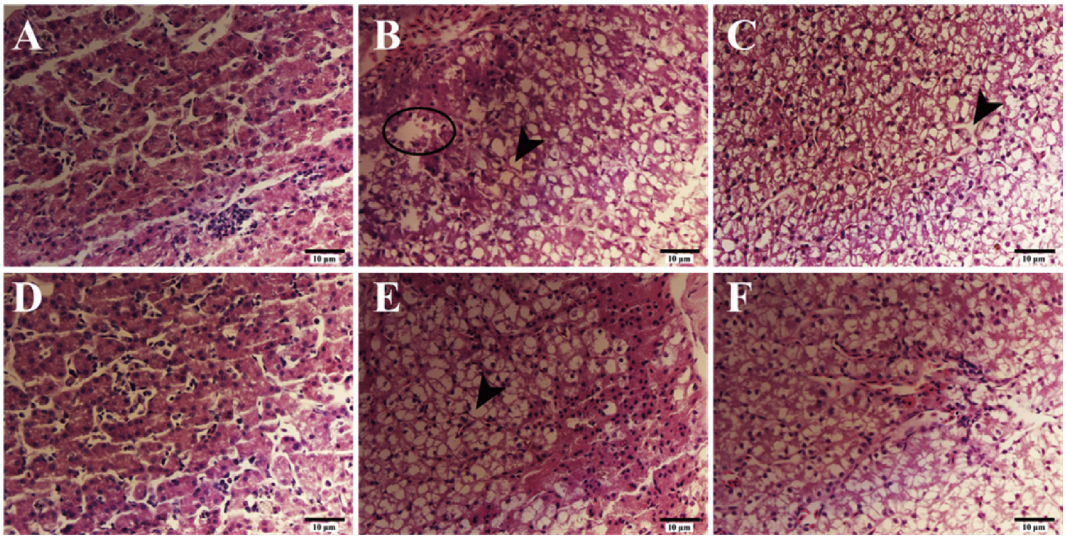

Fig. 1. Liver sections of male and female lizards. (A) normal hepatic tissue from the female control; (B) hepatic tissue from 100 mg/kg^{bw} TF_1 treated group in females; (C) hepatic tissue from 100 mg/kg^{bw} TF_2 treated group in females; (D) normal hepatic tissue from the male control; (E) hepatic tissue from 100 mg/kg^{bw} TF_1 treated group in males; (F) hepatic tissue from 100 mg/kg^{bw} TF_2 treated group in males.

3.5. Quantitation of erα, Ar, cyp17, cyp19 and hsd17β mRNA by real-time PCR

The expression profiles of steroidogenic-related genes in liver after exposure to triadimefon and its enantiomers, such as *erα*, *Ar*, *cyp17*, *cyp19* and *hsd17β* are shown in Fig. 3 (100 mg/kg^{bw}) and Fig. 4 (10 mg/kg^{bw}). The expression of *cyp19* mRNA level was decreased in the TF_2 and TF group (100 mg/kg^{bw}) compared with that in the control group ($p < 0.05$). However, the expression of *cyp19* in the TF_1 group had no significant change. After a 21-day exposure, up-regulation of *cyp17* and *hsd17β* was easily observed in the livers (100 mg/kg^{bw}) of the TF_2 ($p < 0.01$) and TF group ($p < 0.05$). The expression of *Ar* was increased in 100 mg/kg^{bw} ($p < 0.05$). In the lower concentration group (10 mg/kg^{bw}), down-regulation of *cyp19* and up-regulation of *hsd17β* were observed, which is in accordance with the 100 mg/kg^{bw} group. However, the other steroidogenic-related genes had no significant changes in the liver, with values remaining at 10 mg/kg^{bw}.

In gonads, the expression of *cyp19* is down-regulated ($p < 0.05$) which is similar to that of the liver (Fig. 5). As to *hsd17β* and *cyp17*, clear excess expression could be seen, while it is more sensitive in males than in females (100 mg/kg^{bw}, $p < 0.01$). The *erα* and *Ar* mRNA expression were increased ($p < 0.05$), especially in males ($p < 0.01$), compared to the control group, in contrast with that of the liver. Furthermore, the females seem not as sensitive as the males in *erα* and *Ar* expression.

4. Discussion

4.1. Histopathology effect of TF on liver tissues

The liver is a very critical organ for detoxifying processes and oxidative stress, with an abundance of metabolic detoxification enzymes, such as the cytochrome P450 (CYP) enzymes (Sozen et al., 2015). Many xenobiotic pollutants may be degraded in the liver. Nevertheless, if the exposed quantity of TF is beyond the capacity the liver can sustain, it may lead to inactivation of CYP enzymes and may cause hepatocellular damage. With increases in the exposing quantity and time, the damage is severe. In our study, the TF_1 and TF_2 test groups show different degrees of hepatocellular damage (Fig. 1), and this may harm normal physiological activities, such as development and reproduction. Similar reports proved that fluconazole exposure to rats may also cause the same damage in liver (Sun et al., 2006). Thus, it can be seen that short-term exposure to TF and its enantiomers may cause hepatocellular damage and influence the normal physiological function of liver.

4.2. Effects of TF and its enantiomers on sex hormone levels

In reptiles as well as other vertebrates, the regulation of sexual reproduction depends on normal ontogenesis of gonads and a complex network of signalling pathways via hypothalamus-pituitary-gonads axis (Chakraborty et al., 2011). The main sex

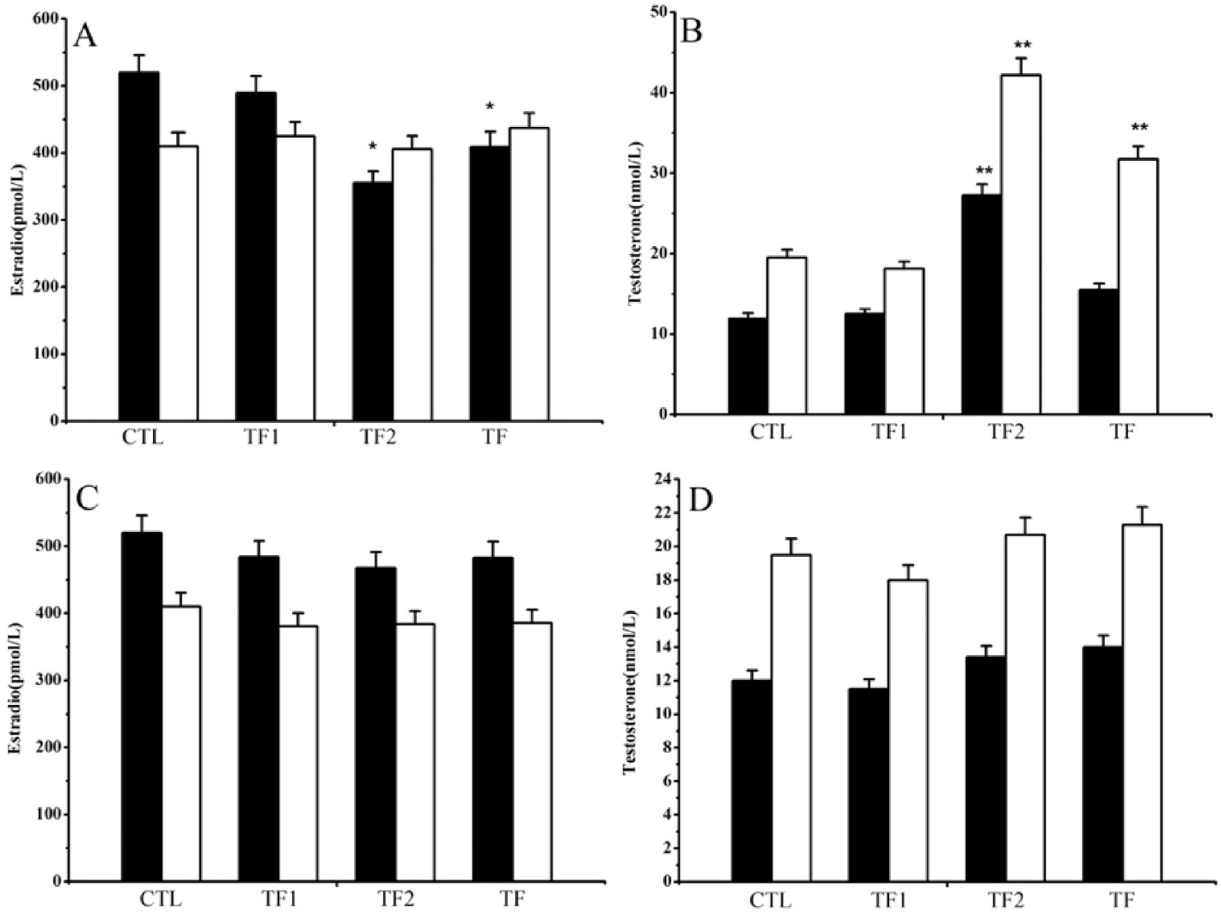

Fig. 2. Plasma concentration of estradiol (E_2) and testosterone (T) after TF and its enantiomers (100 mk/kg^{bw}) exposure for 21days in male (□) and female (■). (A)estradiol concentration (pmol/L) of 100 mk/kg^{bw}; (B) testosterone concentration (nmol/L) of 100 mk/kg^{bw}; (C) estradiol concentration (pmol/L) of 10 mk/kg^{bw}; (D) testosterone concentration (nmol/L) of 10 mk/kg^{bw}. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β-actin. Data are expressed as mean ± S.E., $^{*}p < 0.05$; $^{**}p < 0.01$, relative to control.

Table 2
RT-PCR analysis of steroidogenic-related gene in various of tissues of adult lizard.

Gene	Liver		Brain		Ovary	Testis	Kidney	
	Females	Males	Females	Males	Female	Males	Females	Males
erα	2.30 ± 0.16	1.75 ± 0.38	1.83 ± 0.48	1.26 ± 0.30	2.35 ± 0.07	1.55 ± 0.25	0.93 ± 0.20	1.13 ± 0.27
Ar	1.70 ± 0.40	2.69 ± 0.15	1.67 ± 0.35	1.63 ± 0.15	0.99 ± 0.13	2.72 ± 0.25	0.83 ± 0.13	1.42 ± 0.05
Cyp17a	2.07 ± 0.34	1.76 ± 0.13	1.65 ± 0.11	1.67 ± 0.16	2.53 ± 0.09	1.72 ± 0.04	1.06 ± 0.21	0.85 ± 0.18*
Cyp19a	1.98 ± 0.21	2.11 ± 0.37	1.49 ± 0.64	1.53 ± 0.16	2.66 ± 0.27	2.52 ± 0.13	1.08 ± 0.34	1.39 ± 0.10
Hsd17β	1.54 ± 0.21	2.13 ± 0.42	1.44 ± 0.56	2.08 ± 0.19	2.84 ± 0.81	2.53 ± 0.05	1.23 ± 0.22	0.79 ± 0.08*

Asterisks indicate significant difference from the control ($p < 0.05$).

steroid hormones, such as T and E_2, are in dynamic balance and the alteration of this balance could affect sexual behaviour including sexual maturation, reproductive success and the appearance of secondary sexual characteristics (Tokarz et al., 2015; Li et al., 2015). Lizards have more opportunity to be in contact with xenobiotic chemicals because their environment is easily polluted by contaminants, and this may lead to endocrine disorders (Jones and Swain, 1996; Jones, 2011). Changes in sex hormone levels and related gene expressions may be effective bio-markers to evaluate the damage of xenobiotic chemicals.

In our study, the E_2 level in TF_2 (100 mg/kg^{bw}) females was decreased (Fig. 2), which is consistent with the finding that propiconazole and fadrozole exposure to female *Fathead Minnows* may also restrain the synthesis of E_2 (Villeneuve et al., 2009a,b; Skolness et al., 2013). However, the T level was significantly increased in the TF_2 and TF male lizards, and this phenomenon also appeared in the study of Ankley et al. (2002) and Goetz et al. (2007) (Ankley et al., 2002; Goetz et al., 2007). Previous reports have noted that conazoles often share a common biochemical characteristic of toxic action, for they can disrupt the synthesis of sex steroid hormones. Liao et al. (2014) found that exposure of medaka fish to letrozole at an early life stage altered phenotypic sex development and reproduction in adults and skewed the sex ratio (Liao et al., 2014). Propoconazole at 500 and 1000 μg/L can reduce the plasma E_2 concentration and egg production of female *Fathead Minnow* (Skolness et al., 2013). Based on this, we may suggest that TF and TF_2 may disturb the endocrine system of lizards by restraining the sex steroid hormone levels, and this may

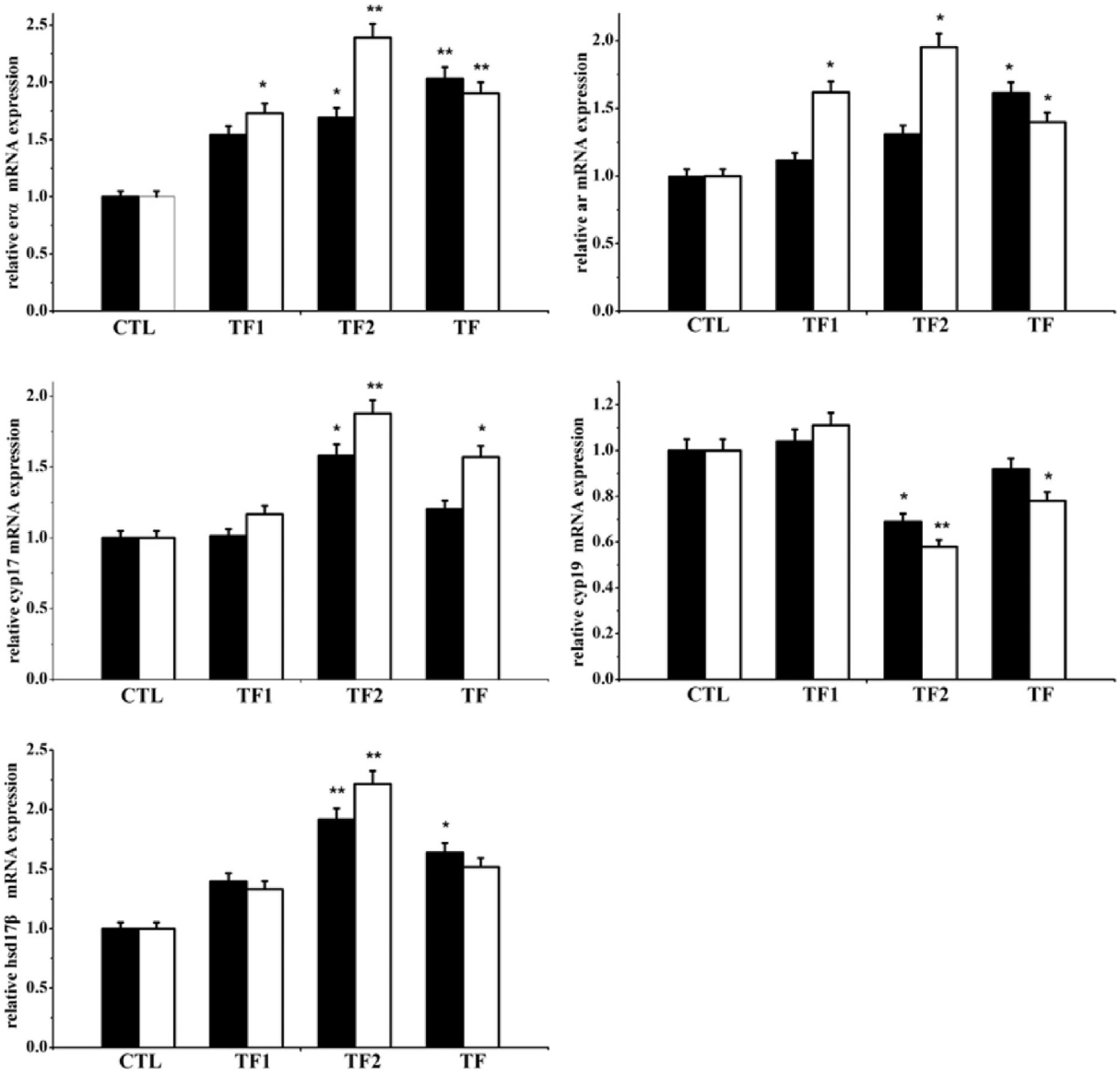

Fig. 3. Relative expression levels of steroidogenic-related genes in liver (100 mg/kg^{bw}) of males (□) and females (■) after 21 days exposure. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β-actin. Data are expressed as mean ± S.E., *p < 0.05; **p < 0.01, relative to control.

impact on the reproductive behaviour of the lizards (Parsley et al., 2014).

In contrast to the TF_2 group, the lizards in the TF_1 group are not affected according to the sex steroid hormone level (Fig. 2), which verifies that the triazoles have different biological activity and toxicity when inside organisms (Chen and Liu, 2008). According to previous studies, many chiral pesticides can be metabolized enantioselectively in a variety of environmental media (Garrison, 2006; Jarman et al., 2005), becoming depleted in one enantiomer while enriched in the other. Furthermore, the metabolites of triazoles are often chiral. It is well known that triadimefon degrades relatively fast and its metabolite triadimenol (TN) degrades slowly. Triadimenol has two chiral centers and four chiral enantiomer forms. The four enantiomers show different toxicity effects, and the (1S, 2R)-TN isomer metabolized from TF_2 is 1000 times more active than the other three (Deas et al., 1986; Burden et al., 1987). As a result, we may conjecture that the two enantiomers of TF may have different toxicities because of the stereoselective metabolism to TN.

There was an interesting phenomenon that the E_2 levels of males in the TF_2 and TF group are higher than those of females (Fig. 2). A similar phenomenon also appeared in fluoride exposure (Li et al., 2016). This reversal phenomenon may come from the expression change of *cyp19* after exposure because *cyp19* is a terminal enzyme that could convert testosterone into oestradiol (He et al., 2012). The study of Ankley et al. (2002) also demonstrated that T levels were increased in male fathead minnow (*Pimephales promelas*) following an 8 or 21 days exposure to fadrozole, which may also make T concentrations increased (Ankley et al., 2002). Many reports have noted that the triazoles are potent aromatase (*cyp19*) inhibitors that block the synthesis of oestradiol to testosterone (Demers, 1994). As the data above show, we may conclude that TF exposure may do harm to the aromatase (*cyp19*) and prevent the conversion between T and E_2.

4.3. Effects of TF and its enantiomers on expressions of steroidogenic-related genes

The excessive use of TF has caused increased risk to soil, reptiles

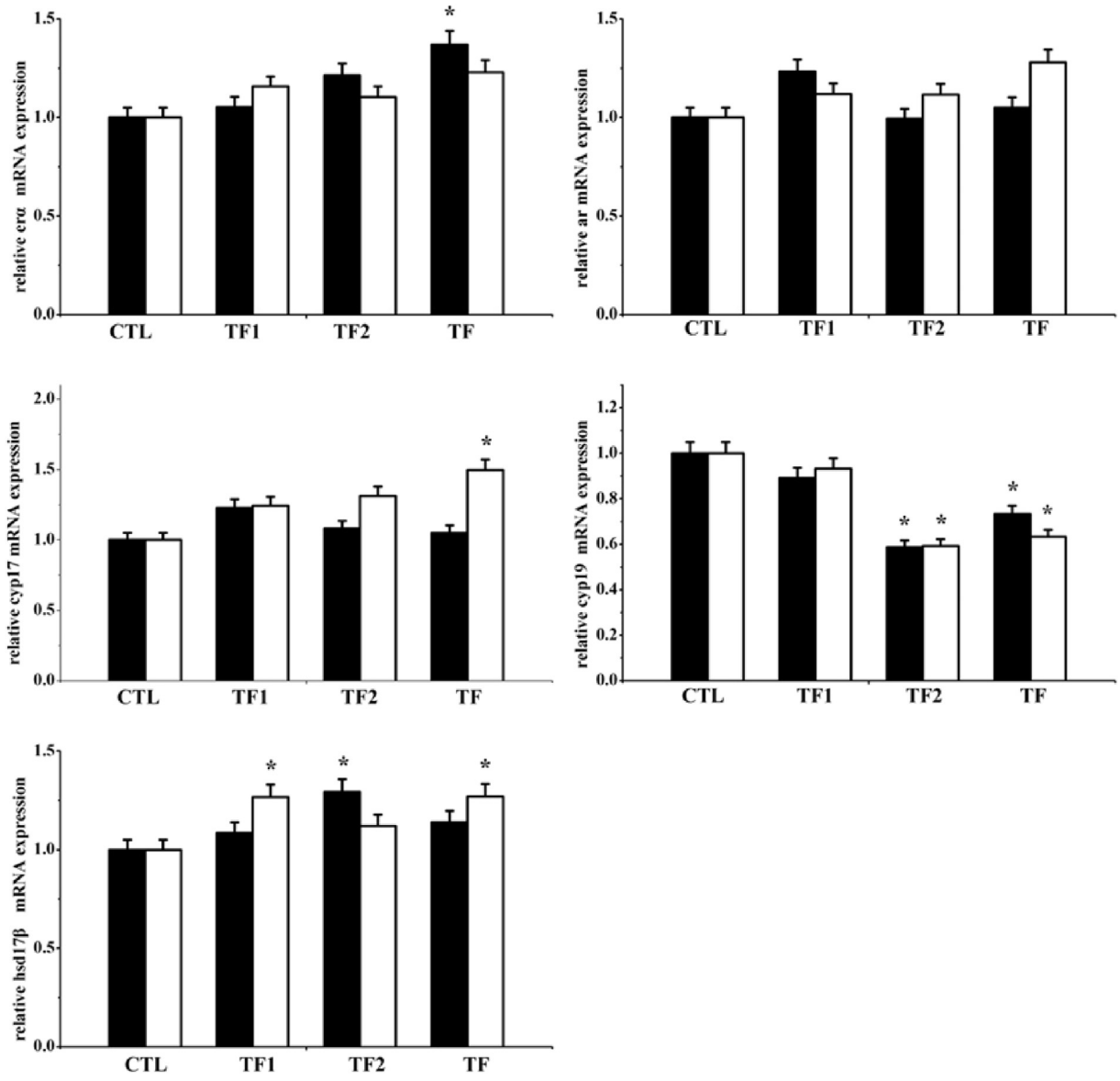

Fig. 4. Relative expression levels of steroidogenic-related genes in liver (10 mg/kg^{bw}) of males (□) and females (■) after 21 days exposure. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β-actin. Data are expressed as mean ± S.E., *$p < 0.05$; **$p < 0.01$, relative to control.

and other vertebrates. Exposure of triadimenfon to rare minnow decreased body length, body weight, heart rate and also caused notable changes in enzyme activities and mRNA expression (Zhu et al., 2014). Some studies have reported triazoles are designed to inhibit cytochrome P450 (CYP), especially *cyp19*, potentially disrupting the synthesis of sex hormones in vertebrates.

In our study, the expression of *cyp19* mRNA levels in the liver and gonad were decreased in the TF_2 and TF groups (100 mg/kg^{bw}) compared with that in the control group (Figs. 3 and 5), which also has been observed in letrozole exposure in medaka (Sun et al., 2011). A similar result also appeared in the study by Liao et al. (2014). (Liao et al., 2014). Skolness et al. (2013) determined that propiconazole could alter the expression of *cyp19*, *cyp17* and *cyp11a* in adult fathead minnows (Skolness et al., 2013), leading to a decrease in egg production and an increase in relative testis weight. The author notes that the induction of *cyp19* is like a compensatory response to aromatase inhibition in multiple vertebrates (Zhang et al., 2008; Villeneuve et al., 2009a,b). *Cyp19* is the terminal enzyme during the biosynthesis of oestrogen, and it also participates in the conversion of testosterone into oestrogens (Simpson et al., 1994). Once the transcription of *cyp19* is damaged, it may affect the synthesis of sex steroid hormones and the conversion between testosterone and oestrogen and may even lead to disorder of the endocrine system. Because gene transcriptional responses represent the primary interaction site between chemicals and organisms, they provide essential clues for understanding how chemical exposure can affect organisms (Moens et al., 2007). Thus, TF and its enantiomers could change the expression of *cyp19* and break the balance of sex hormone levels.

Cyp17 is the key enzyme in androstenedione synthesis, the direct precursor of testosterone, and *hsd17β* is also responsible for the synthesis of T. In the present research, the expression of *cyp17* in the TF group is up-regulated at 100 mg/kg^{bw} in male livers (Fig. 3), while no markable change was observed in females. This is in accordance with ketoconazole (another type of triazole pesticides) exposure to *Pimephales promelas*, the mRNA transcripts of two steroidogenic enzymes (including *cyp17*) were elevated after 21 days of exposure. Changes in both transcript levels and the

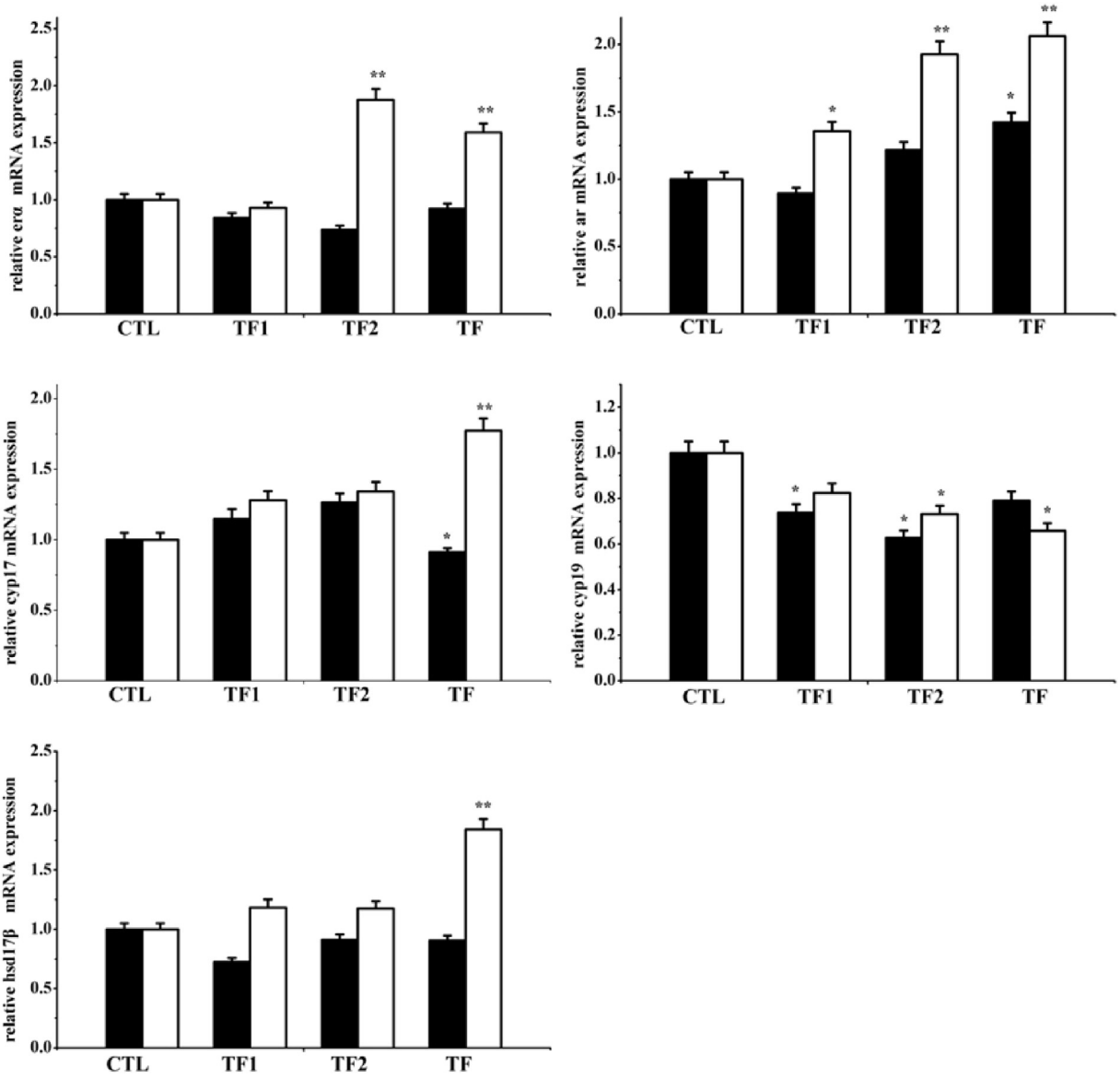

Fig. 5. Relative expression levels of steroidogenic-related genes in gonad (100 mg/kg^{bw}) of males (□) and females (■) after 21 days exposure. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β-actin. Data are expressed as mean ± S.E., *p < 0.05; **p < 0.01, relative to control.

proliferation of liver tissue represent potential adaptive or compensatory responses to impaired steroidogenic capacity (Ankley et al., 2007). For the increase of *cyp17* and *hsd17β* in lizards may accelerate the transformable rate from androstenedione to testosterone and lead to an abundance of T, which can explain the high concentration of T in the TF_2 and TF group (Fig. 2) mentioned above.

From the present study, we know that TF may damage not only the transcripts of *cyp19* but also those of *cyp17*. For example, there is evidence that ketoconazole interacts with CYPs other than *cyp19* (e.g., *cyp11* and *cyp17*), which are upstream of T production. Exposure of triadimenfon and myclobutanil to rat livers at their maximum tolerant dose level may also induce the up-regulation of *cyp1a* and *cyp3a* families of cytochromes (Sun et al., 2007). From the variation of *cyp17* and *cyp19* after exposure (Figs. 3 and 5), we may demonstrate that TF and its enantiomers can inhibit one or more expression of cytochrome P450 enzymes and disturb the concentration of steroid hormone and, as a result, interfere with reproductive behaviour. The expression of *cyp17* and *cyp19* in the TF_1 group (liver and gonad) had no significant change, which is also different from the TF_2 group, and may provide more evidence about the biotoxicity discrepancy in the enantiomers of TF.

The steroid hormone receptors (*erα* and *Ar*) mainly exist in the cytoplasm and cell nucleus, which can regulate the transcription of target genes by combining with the relative hormone gland (Li et al., 2016). *Erα* and *Ar* are the main sex hormone receptors and also play an important role in the reproduction process in vertebrates. Androgens regulate genes by binding to androgen receptors and ultimately leading to changes in gene expression. Thus, the exchange in steroid hormone level may associate with the expression of *erα* and *Ar*. This point of view was confirmed in the present study (Figs. 3 and 5), since the *erα* and *Ar* were obviously increased in males. However, no significant transformation was observed in females. Liao et al. (2014) also found that *erα* expression in medaka after letrozole exposure showed different changes in males and females (Liao et al., 2014). From the different expression of *erα* and *Ar* in female and male lizards, we may speculate that TF had sex differences on the two genes.

5. Conclusion

In summary, the present study reveals and explains that TF and its enantiomers may have an endocrine disrupting effect by disturbing sex steroid hormone levels, including those of 17beta-estradiol and testosterone. The different metabolites may lead to higher biotoxicity of TF_2 than TF_1. The relative expression of steroidogenic-related *genes* (*erα*, *Ar*, *cyp17*, *cyp19* and *hsd17β*) also affected after 21 day exposure of TF, which may lead to abnormal reproductive behaviour. The present study provides essential data for future investigations about the toxicity of TF and its enantiomers in reptiles and other organisms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.12.096.

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