



Stereoselective degradation and thyroid endocrine disruption of lambda-cyhalothrin in lizards (*Eremias argus*) following oral exposure[☆]



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ABSTRACT

The disturbance of the thyroid system and elimination of chiral pyrethroid pesticides with respect to enantioselectivity in reptiles have so far received limited attention by research. In this study, bio-accumulation, thyroid gland lesions, thyroid hormone levels, and hypothalamus-pituitary-thyroid axis-related gene expression in male *Eremias argus* were investigated after three weeks oral administration of lambda-cyhalothrin (LCT) enantiomers. In the lizard liver, the concentration of LCT was negatively correlated with the metabolite-3-phenoxybenzoic acid (PBA) level during 21 days of exposure. (+)-LCT exposure induced a higher thyroid follicular epithelium height than (–)-LCT exposure. The thyroxine levels were increased in both treated groups while only (+)-LCT exposure induced a significant change in the triiodothyronine (T3) level. In addition, the expressions of hypothalamus-pituitary-thyroid axis-related genes including thyroid hormone receptors (*trs*), deiodinases (*dios*), uridinediphosphate glucuronosyltransferase (*udp*), and sulfotransferase (*sult*) were up-regulated after exposure to the two enantiomers. (+)-LCT treatment resulted in higher expression of *trs* and (–)-LCT exposure led to greater stimulation of *dios* in the liver, which indicated PBA-induced antagonism on thyroid hormone receptors and LCT-induced disruption of thyroxine (T4) deiodination. The results suggest the (–)-LCT exposure causes higher residual level in lizard liver while induces less disruption on lizard thyroid activity than (+)-LCT.

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1. Introduction

The value of reptiles as an integral part of natural ecosystems and an indicator organism for environmental equality has been widely recognized (Adams et al., 1995). Reptiles are sensitive to contaminants and accumulate pollutants to levels equal to or greater than those of birds and mammals (De Falco et al., 2007). In recent years, the number of reptiles has dramatically declined, which might be caused by habitat loss, disease, and environmental pollution (Gibbons et al., 2000). The widespread use of pesticides, which could induce endocrine disruption, might be a fundamental

cause of the population decline of reptiles (Sun et al., 2009). However, less than 1% of studies in vertebrate ecotoxicology involve reptiles (Sparling et al., 2010).

As in other vertebrates, the thyroid system of reptiles is controlled by the hypothalamus–pituitary–thyroid (HPT) axis (Meyer et al., 2014). The HPT axis regulates the synthesis and secretion of thyroid hormones (THs). THs play a crucial role in the development, growth, and reproduction of reptiles (Jugan et al., 2010). Disruption of the reptilian thyroid system may affect these important functions as well as the thyroid hormone homeostasis, and HPT axis-related gene expression (Rivera and Lock, 2008). The mechanisms by which pesticides disrupt circulating hormone levels are complex and involve direct effects on the pathways of hormone biosynthesis, transport, and metabolism, or indirect effects on feedback mechanisms (Brasfield et al., 2008). Previous studies have shown structural or functional differences in the

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thyroid glands of reptiles after pesticides exposure (Sciarrillo et al., 2008; Bicho et al., 2013). However, few studies have investigated the potential toxic mechanisms of pesticides associated with reptilian HPT axis-related gene expression.

Reptiles are not required test subjects under the minimal requirements of federal regulations. However, a toxicity assay using lizards has been developed as an important model for ecotoxicological studies of reptiles in recent years (Amaral et al., 2012). Lizards have a wide geographic distribution, are small in size and easy to feed in the laboratory. What's more, most lizards are insectivorous (Bishop and Gendron, 1998) and thus likely have high contact with pesticides. A Chinese native lizard species—*Eremias argus* (*E. argus*) is widely distributed in the north of the Yangtze River. We collected juvenile *E. argus* from the wild in the Inner Mongolia Province and have maintained them in our laboratory for more than 4 years. The reproduction method of *E. argus* has also been established, so *E. argus* is regarded as an ideal model for reptilian risk assessment.

Pyrethroids, a class of endocrine-disrupting insecticides, contain one to three chiral centers and are now the fourth most used group of insecticides worldwide (Brander et al., 2016). The estrogenic activity and thyroid disruption of pyrethroid pesticides in mice or aquatic vertebrates have been previously investigated (Jin et al., 2015; Wang et al., 2011; Tu et al., 2016) but little is known about their effects on reptiles (Hopkins, 2000). Pyrethroids have been detected in the sediment of Sacramento-San Joaquin Delta of California (Weston and Lydy, 2010). The application rate of pyrethroid pesticides in field crops ranges from 5 to 15 g/ha, and applications are repeated at intervals of 1–2-week (Velmurugan et al., 2007). The lizards living in cropland will be exposed to these pesticides. The enantiomers of pyrethroids are known to selectively interact with biological systems (Liu et al., 2005) and may show enantioselective bioaccumulation and toxicity. Whether the enantiomers of pyrethroids are enantioselective with respect to their thyroid disruption potential in reptiles is still unclear.

To understand the role of pyrethroids in disruption of the reptilian thyroid axis, two enantiomers of the type II pyrethroid insecticide lambda-cyhalothrin (LCT) (Fig. S1) were orally administered to *E. argus* for three weeks. Thyroid histopathology, THS level and the transcription of HPT axis-related genes in the liver and brain were assessed to elucidate the potential mechanisms of thyroid disruption by different LCT enantiomers. The LCT and its metabolite-3-phenoxybenzoic acid (PBA) concentrations in the liver were also analyzed to determine the dose-response and time-relationship on lizard thyroid system. Our findings are intended to provide new insights into the enantioselective impact of pyrethroids on the thyroid axis of reptiles.

2. Materials and methods

2.1. Animals and husbandry

Immature (1–2 years old, before sexual maturity) male *E. argus* were obtained from our breeding colony in Changping district, Beijing, China. The average body weight and body length were 2.5 g and 35.6 mm respectively. The lizards were kept in a 5 × 1.2 × 0.4 m solid bottom indoor aquarium covered with 10 cm of mollisol and fallen leaves. Ultraviolet lamps were set on a 12 h:12 h light/dark cycle to provide enough light and maintain the necessary temperature. The temperature and humidity were maintained at 25–30 °C, 30–50%, respectively. The lizards were fed with live mealworms twice each day and sprayed with water several times a day. The excreta were cleaned every day.

2.2. Chemicals

LCT (racemate, 98% pure, CAS 91465-08-6) was provided by J&K Chemical Technology (Beijing, China). All solvents of acetone, acetonitrile, n-hexane, and isopropanol were of HPLC grade and purchased from Dikma (Beijing, China).

2.3. Chromatographic separation and concentration analysis

The enantiomers of LCT were separated by HPLC on a CHIRALCEL[®] cellulose TRIS (3,5-dimethylphenyl-carbamate) (OD) column (Fig. S2). A volume of 20 µL was injected for chiral separation in the normal-phase mode. N-hexane and isopropanol (95:5, v/v) were used with a flow rate of 2.5 mL min⁻¹. The signal at 236 nm was recorded. The resolved enantiomers were manually collected into separate glass vials at the HPLC outlet. All the solvent fractions of the enantiomers were evaporated to dryness and redissolved in ethanol. A stock solution was prepared by dilution in corn oil following Wang et al. described method (Wang et al., 2014).

Acetonitrile was employed to extract the lizard samples. The analysis of LCT concentration was performed on a Thermo-TSQ 8000 GC/MS/MS equipped with an electron-impact ionization (EI) source and column-TR-35MS (0.25 mmΦ × 30 mID × 0.25 mm, Thermo). The two enantiomers of LCT can be separated completely by BGB-172 chiral capillary column (20% *tert*-butyldimethylsilyl-β-cyclodextrin dissolved in 15% diphenyl-polysiloxane and 85% dimethyl-polysiloxane, GBG Analytik, Adliswil, Switzerland) on GC/MS/MS (Fig. S3). The purity for both enantiomers was determined to be ≥ 99.9%. The PBA analysis was performed on HPLC/MS/MS equipped with a TSQ Quantum Access MAX triple quadrupole MS, an Accela 600 pump/auto sampler HPLC and a C₁₈ column (2.1 mmΦ × 100 mm × 5 µm, Thermo). Blank liver and faeces samples were detected and no target chemicals were found. Detailed protocols for extraction, clean-up, identification and quantification are provided in previous studies (Chang et al., 2016a, 2016b).

2.4. Exposure experiment and sampling

Male lizards were randomly separated into three groups—a control group, a (+)-LCT exposure group, and a (–)-LCT exposure group (total n = 81, n = 27 for each group). Prior to the experiments, each group was allowed to acclimate to the experimental conditions for one week in the experimental glass cages (60 × 60 × 40 cm). Each glass cage contained 9 lizards and there were 3 cages for each group. The lizards were dosed orally with 10 mg kg⁻¹ body weight (bw) of the LCT enantiomers or corn oil once a week. The dosing was operated with a GC instrument injection syringe to deliver a volume of 10–20 µL corn oil-ethanol or corn oil-ethanol LCT enantiomer into the oral cavity of each lizard (Wang et al., 2014). There are no direct toxicity data of LCT in reptiles and birds are usually used as surrogates in risk assessment (Weir et al., 2010). The LD₅₀ value of LCT in birds is greater than 2000 mg kg⁻¹, and 0.5% of the LD₅₀ value was selected in this study. This dosage is also similar with pyrethroid bioaccumulation studies on earthworm (Chang et al., 2016b) and rabbit (Liu et al., 2011). The every week exposure is to mimic the usual usage of LCT on the farmland. Lizards from each group were euthanized 7, 14, and 21 days after exposure. Three lizards were selected randomly from each cage, and three replicates were prepared. The body weights were measured. The brain and liver were collected, weighed, and frozen at –80 °C with RNA store. A part of liver was left for concentration analysis. The blood was immediately centrifuged at 2500 × g for 10 min, and the plasma was stored at –80 °C for TH analysis. The collected faeces of the lizards from the same

treatment group were combined together every 7 days and stored at -20°C before analysis.

2.5. Thyroid gland histology

Lizard thyroids were sampled at 21 days after dosing and stored in 4% paraformaldehyde for histopathological analysis. The method generally followed that previously described by McFarland (McFarland et al., 2008). Thyroid gland was processed for paraffin wax embedding. Sections were cut, stained with hematoxylin and eosin (HE), and then examined by light microscopy (Olympus DP73).

2.6. Thyroid hormone analysis

Plasma samples were collected after 21 days of exposure. The thyroxine (T4) and triiodothyronine (T3) levels in the plasma were determined by an enzyme-linked immunosorbent assay (ELISA) kit specified for lizards (T3 kit was from Elabscience Biotechnology Co., LTD, T4 kit was from Cloud-Clone Corp.) according to the manufacturer's instructions. Duplicate aliquots (50 μL) of the diluted plasma were used in the T3 and T4 assay. Preliminary analysis demonstrated correspondence between the diluted samples and the standard curves. The assay sensitivity was 0.84 pg mL^{-1} and 3.7 ng mL^{-1} for T3 and T4, respectively.

2.7. Isolation of RNA, cDNA synthesis and real-time PCR

Total RNA was isolated from the liver and brain of the lizards using Trizol reagent (Life Technology, Beijing, China). Traces of DNA were removed by incubation with DNase-I (Ambion). The RNA was dissolved in RNase-free water and stored at -80°C .

Reverse transcription reaction mixtures contained 22 μL of total RNA, 2 μL of Oligo (dT)₁₅ primers, and 4 μL DNTP. The mixtures were heated at 70°C for 5 min and quickly chilled on ice. After cooling, 8 μL $5 \times$ buffer, 2 μL M-mlv, and 40 units RNAsin (an RNase inhibitor) were added to a total volume of 41 μL . The mixture was incubated for 50 min at 42°C and then heated to 95°C for 5 min to inactivate the reverse transcription reaction.

Genes selected in this study (thyroid hormone receptors ($\text{tr}\alpha$, $\text{tr}\beta$), deiodinases (dio1 , dio2), uridinediphosphate glucuronosyl-transferase (udp), and sulfotransferase (sult)) and their respective primers are listed in Table S1. All primers were designed by the authors using Primer-Blast from NCBI. Real-time PCR was performed in a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) using the SYBR GREEN PCR kit (Tiangen Biotech, Beijing, China). The thermal cycle parameters were: 5 min at 95°C , 40 cycles of 30 s at 95°C , 40 s at 54°C and 40 s 72°C . All of the samples were analyzed in triplicate with MxPro software. As for the quantification of PCR, three of the most used housekeeping genes were selected, specially, rpl8 (ribosomal protein L8), 18s (18 s ribosomal RNA) and β -actin. β -actin gene was selected as the most stable reference gene according to the result from geNorm analysis. A dissociation curve analysis was performed for each gene and only one peak was observed for the amplification, indicating the specific amplification of the target gene. We also sequenced the products and did the sequence alignment to further validate the specificity of the products. The gene expression data showed changes relative to the control animals for the same treatment period.

2.8. Data analysis

Hepatosomatic index was calculated as follows:

$$\text{hepatosomatic index\%} = \frac{\text{liver weight}}{\text{body weight}} \times 100$$

Statistical analysis of the data were performed using analysis of variance (ANOVA) on SPSS (version 13.0; USA). A probability of $p < 0.05$ was considered statistically significant. The data from three replicates are statistically independent. Correlation analysis was conducted with Pearson's test.

3. Results

3.1. Growth and hepatosomatic index

The body weight of lizards increased with time in the control group, indicating a normal growth. In the (+)-LCT exposed group, the body weight was significantly lower than that of the control group at the end of the exposure (one-way ANOVA, $p = 0.005$) (Table 1). In addition, after 21 days of exposure, significant decrease of the hepatosomatic index (HIS) was noted in the (+)-LCT treated groups (one-way ANOVA, $p = 0.008$). No significant differences in the body weight and HIS were observed between the control group and (–)-LCT exposure group.

3.2. The bioaccumulation, metabolism of LCT in the liver and faeces

The concentration of LCT in the liver was analyzed at 7, 14 and 21 days. In the (+)-LCT exposure group, the concentration of LCT was highest (0.084 mg kg^{-1}) at 7 days and decreased to the lowest level (0.009 mg kg^{-1}) at 14 days (Fig. 1a). In the (–)-LCT exposure group, the LCT concentration gradually increased with time (0.025–0.058 mg kg^{-1}) and was greater than that in the (+)-LCT exposure group at 14 (Multivariate ANOVA, $p < 0.001$) and 21 days (Multivariate ANOVA, $p = 0.035$).

One of LCT metabolites, PBA is regarded as an indicator to assess the metabolic rate of LCT (Chang et al., 2016b). The concentration of PBA in the (+)-LCT exposure group was lowest at 7 days and increased to the highest at 14 days (Fig. 1c). In the (–)-LCT exposure group, the concentration of PBA in the liver was highest at 7 days and decreased gradually from 7 to 21 days. The level of PBA at 21 days in (–)-LCT exposure group was significantly lower than that in (+)-LCT exposure group (Multivariate ANOVA, $p = 0.019$). The PBA concentration was negatively correlated with the LCT concentration after the (+)-LCT ($R^2 = -0.96$) or (–)-LCT ($R^2 = -0.77$) exposure (Table S2).

Our previous study demonstrated that LCT could be excreted through the lizard skin and faeces while 90% of the LCT was detected in the faeces (Chang et al., 2016a). In this study, the LCT concentration in the faeces was significantly higher in the (+)-LCT exposure group than that in the (–)-LCT exposure group at all time point (Multivariate ANOVA, $p = 0.001$). The average excretion concentration of LCT in the (+)-LCT exposed group was 3.65 mg kg^{-1} , more than 3-fold greater than that in the (–)-LCT

Table 1

Body weight and hepatosomatic index (HIS) of lizards (*E. argus*, 1–2-year old) during exposure to lambda-cyhalothrin (LCT) enantiomers.

Compound	HIS (%)			Body weight/g		
	7d	14d	21d	7d	14d	21d
Control	4.0 \pm 0.2	4.2 \pm 0.3	4.3 \pm 0.2	2.5 \pm 0.3	3.2 \pm 0.3	3.5 \pm 0.4
(+)-LCT	4.5 \pm 0.4	4.0 \pm 0.2	3.4 \pm 0.2*	2.6 \pm 0.2	2.7 \pm 0.3	2.5 \pm 0.2*
(–)-LCT	3.6 \pm 0.2	4.6 \pm 0.3	4.6 \pm 0.4	2.5 \pm 0.3	3.3 \pm 0.3	3.2 \pm 0.2

Data are expressed as mean \pm S.E. of each treatment (n = 9).

*Statistically significant difference from the control (one-way ANOVA, $p < 0.05$).

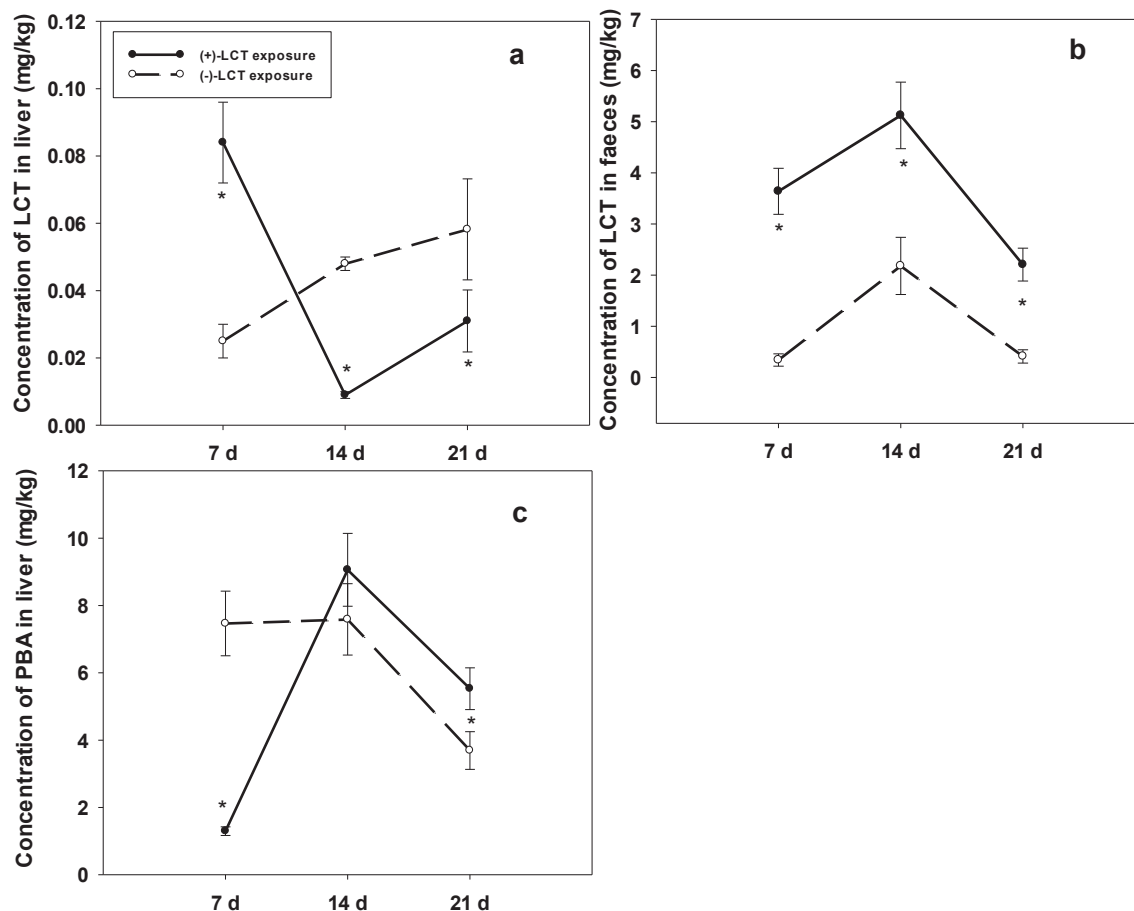


Fig. 1. The concentration of lambda-cyhalothrin (LCT) in the lizard (*E. argus*, 1–2-year old) liver (a) and faeces (b) after 7, 14 and 21 days of (+)-LCT or (-)-LCT exposure. The concentration of 3-phenoxybenzoic acid (PBA) in the lizard liver (c) after 7, 14 and 21 days of (+)-LCT or (-)-LCT exposure. The data are expressed as mean \pm S.E. of each treatment ($n = 3$). * represents significant difference between (+)-LCT exposure and (-)-LCT exposure group (Multivariate ANOVA, $p < 0.05$).

exposed group (Fig. 1b).

3.3. Histopathological changes in thyroid gland

Morphologically, the thyroid of reptile differs from that of fish (Schmidt and Braunbeck, 2011). The thyroid gland of *E. argus* was a single discrete ribbon-like structure which transversely crossed the middle of trachea. It was formed by thyroid follicles containing follicular epithelium cells and colloids. The height of follicular epithelium cell and the colloid area changed with respect to the functional state of thyroid gland.

In the control group (Fig. 2a), thyroid showed a medium-high ($0.67 \pm 1.67 \mu\text{m}$, Table S3) follicular epithelium. The follicle lumen was full of colloid. Samples treated with the (+)-LCT and (-)-LCT enantiomer showed different morphological signs of thyroid activity. The thyroid gland treated with the (+)-LCT enantiomer (Fig. 2b) showed an irregular shape of the follicles with roundish nuclei as well as a significant high follicular epithelium ($1.28 \pm 0.25 \mu\text{m}$, one-way ANOVA, $p < 0.05$). There were clear signs of reabsorption in the follicle lumen (white arrow). In the (-)-LCT enantiomer exposure group, the follicular area was enlarged and the number of follicles was decreased (Fig. 2c). However, the colloids were also decreased and no reabsorbing vacuoles were observed. The height of the epithelium was $0.89 \pm 0.19 \mu\text{m}$, but no statistical significance was observed compared with the control group.

3.4. Plasma thyroid hormone level

The thyroid follicular cells are responsible for synthesis, storage and excretion of THs (Cunha and van Ravenzwaay, 2005). In that case, TH levels may also be affected. The effects of the LCT enantiomers on the plasma T3 and T4 levels after 3 weeks of exposure were presented in Fig. 3. Both the T3 and T4 concentrations increased in the LCT enantiomer-treated groups. The T3 concentration in the (+)-LCT exposed group was 2.33-fold greater than that in control group (6.02 ng L^{-1}) (one-way ANOVA, $p = 0.002$). In contrast, no significant difference in the T3 concentration was observed between the control and the (-)-LCT treated group. The T4 content in the control group was $137.72 \text{ ng mL}^{-1}$ and was significantly up-regulated in LCT enantiomer-treatment groups (one-way ANOVA, $p = 0.018$ for the (+)-LCT exposure and $p = 0.002$ for the (-)-LCT exposure) although no statistically significant difference was observed between the two groups. In both the (+)-LCT and (-)-LCT treated groups, the ratio of T3/T4 was significantly decreased, and the (-)-enantiomer exposure group showed a relatively lower value.

3.5. Quantification of *tra*, *trb*, *dio1*, *dio2*, *udp* and *sult* mRNA by real time PCR

The alterations of THs can feedback to the HPT axis-related genes. The expression of the *tra*, *trb*, *dio1*, *dio2*, *udp*, and *sult* in the lizard livers was determined weekly (Fig. 4). The *tra*, *trb*, *dio1*,

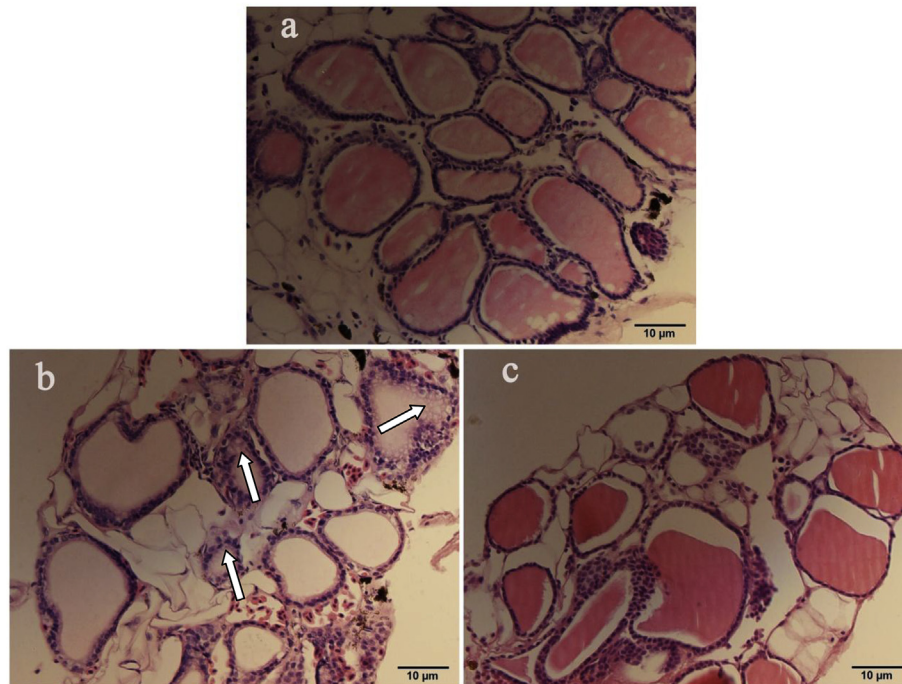


Fig. 2. A thyroid gland section of lizard (*E. argus*, 1–2-year old) stained with hematoxylin and eosin (HE). **a)** representative section from a lizard treated with corn oil (control group); note the medium-high thyroid follicular epithelium. **b)** representative section from a lizard treated with the (+)-lambda-cyhalothrin enantiomer; note the thyroid follicular epithelium was very high and colloid showed obvious vacuolization (white arrows). **c)** representative section from a lizard treated with the (-)-lambda-cyhalothrin enantiomer; the size of thyroid follicles became larger while the colloid was decreased.

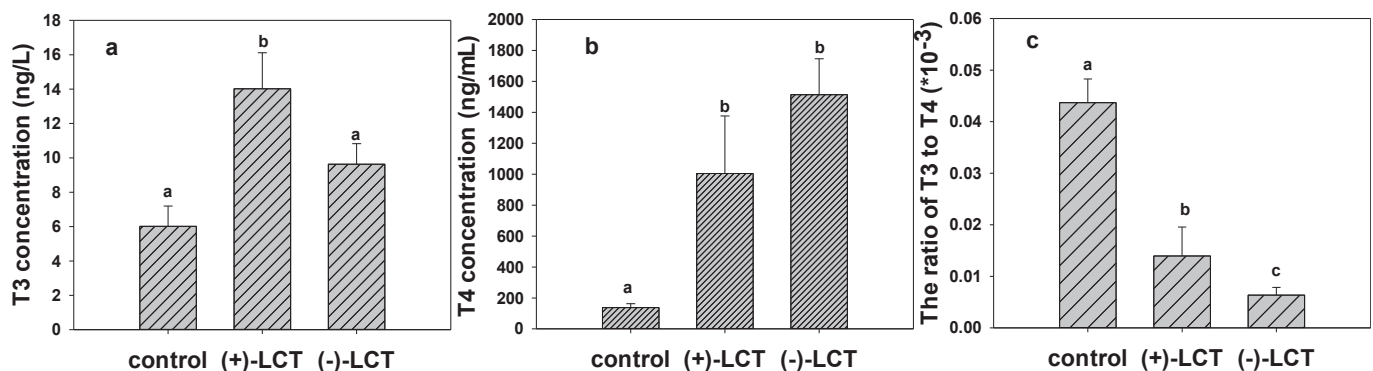


Fig. 3. **a-b)** Variations of triiodothyronine (T3) and thyroxine (T4) levels in the plasma of lizard (*E. argus*, 1–2-year old) subjected to different lambda-cyhalothrin (LCT) enantiomers after 21 days. **c)** The ratio of T3 to T4. Values are showed as mean \pm S.E (n = 9). Groups with different letters are significantly different from each other ($p < 0.05$), whereas the same letter indicates no significant difference (one-way ANOVA).

dio2, and *udp* mRNA levels were significantly higher in the treatment groups after 7 days of exposure when comparing with the control group respectively (one-way ANOVA, $p < 0.05$, the exact p values were shown in Table S4). No significant difference was seen between the (+)-LCT and (-)-LCT exposed groups in the *tra*, *dio1*, and *dio2* levels. However, the expression of the *trb* and *udp* mRNA levels in the (+)-LCT treated group were all higher than those in the (-)-LCT group (one-way ANOVA, $p < 0.05$). Compared with 7 days of exposure, the expression of *tra*, *trb*, *dio1*, *dio2*, *udp*, and *sult* genes was significantly lower after 14 days of (+)-LCT treatment and only *tra*, *trb*, and *sult* gene expressions were significantly decreased in (-)-LCT exposure group (one-way ANOVA, $p < 0.05$, the exact p values were shown in Table S4). The *tra*, *trb*, *dio1*, *dio2*, *udp* and *sult* mRNA levels indicated no significant difference between the control and (+)-LCT exposed groups at 14 days. In the (-)-LCT exposed

group, the *dio1*, *dio2*, *udp*, and *sult* mRNA levels were significantly higher than those in the control or the (+)-LCT treated group (one-way ANOVA, $p < 0.05$, the exact p values were shown in Table S4). At the end of exposure, the expressions of *tra*, *trb*, *dio2*, *udp*, and *sult* mRNA were all up-regulated in LCT enantiomer-treated groups. Furthermore, the *tra*, *trb*, *dio2*, and *sult* mRNA levels were significantly higher than those at 14 days (one-way ANOVA, $p < 0.05$, the exact p values were shown in Table S4). The expressions of *tra* (1.5-fold), *trb* (1.8-fold), and *udp* (2.4-fold) mRNA were significantly higher in the (+)-LCT exposed group while the *dio1* (1.5-fold) and *dio2* (4.0-fold) mRNA levels were significantly lower compared with the (-)-LCT treated group (one-way ANOVA, $p < 0.05$). No significant difference was observed in the *sult* mRNA level between the treated groups.

The expression of *tra*, *trb*, *dio1*, and *dio2* in lizard brain was

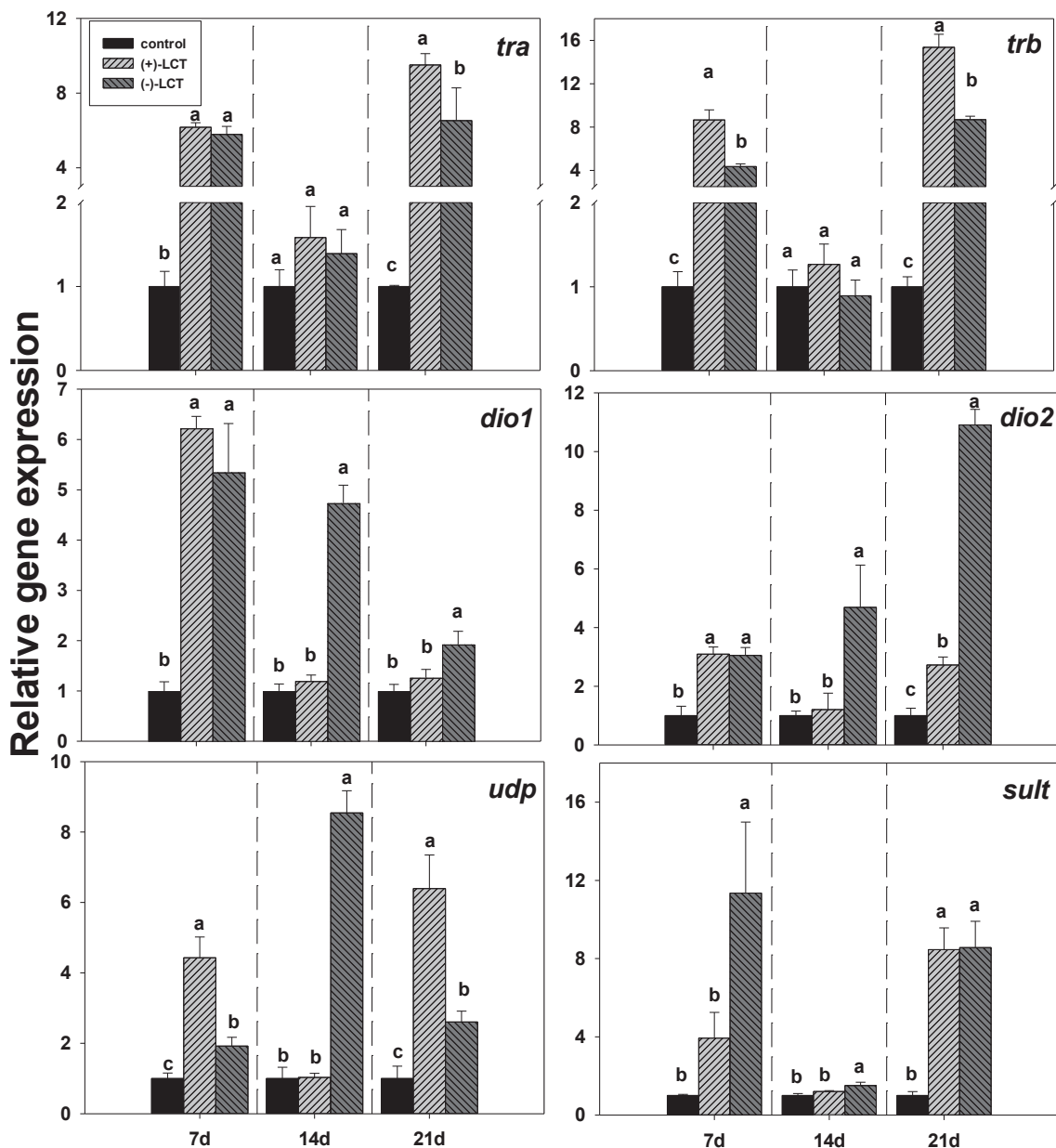


Fig. 4. Relative gene levels of thyroid hormone receptors (*tra*, *trb*), deiodinases (*dio1*, *dio2*), uridinediphosphate glucuronosyltransferase (*udp*), and sulfotransferase (*sult*) in the lizard (*E. argus*, 1–2-year old) liver after three weeks exposure of lambda-cyhalothrin (LCT). The result was evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. The results are expressed as the mean \pm S.E for each treatment ($n = 3$). Two bars labeled with totally different letters indicate a significant difference on gene expression at the same time point between the control group and treatment groups at a significant level of $p < 0.05$ (one-way ANOVA).

analyzed weekly (Fig. 5). At 7 days, the *dio2* mRNA levels were significantly increased after LCT enantiomers exposure while the expression of *dio1* gene was only significantly up-regulated after (–)-LCT treatment (one-way ANOVA, $P < 0.05$). No significant difference was observed on the *dio1* and *dio2* gene level between the (+)-LCT and (–)-LCT exposure group after 7-day exposure. However, exposure to (+)-LCT resulted in 2.8- and 1.7-fold up-regulation of *tra* and *trb* compared with the (–)-LCT exposed group (one-way ANOVA, $P < 0.05$). At 14 days of exposure, the *tra*, *trb*, and *dio1* mRNA levels were all up-regulated and significantly higher in (+)-LCT exposed group compared with (–)-LCT treated group (one-way ANOVA, $P < 0.05$). However, after 21 days of exposure, no significant variations in the *tra* and *trb* mRNA levels were seen between LCT enantiomer-treated groups. The expression of *dio1*

(1.8-fold) and *dio2* (4.1-fold) was more seriously affected by the (+) enantiomer than (–) enantiomer (one-way ANOVA, $p < 0.05$). The *tra* and *trb* mRNA levels increased with exposure time in the exposure groups and were significantly higher at 21 days than those at 7 days (one-way ANOVA, $P < 0.05$, the exact p values were shown in Table S4).

4. Discussion

This study evaluated the growth, histology, TH levels, and the changes in HPT axis-related gene expression in *E. argus* exposed to the pyrethroid pesticide – LCT to study the enantioselective effect of LCT on the lizard thyroid system and to lay a foundation for the risk assessment of LCT in reptiles.

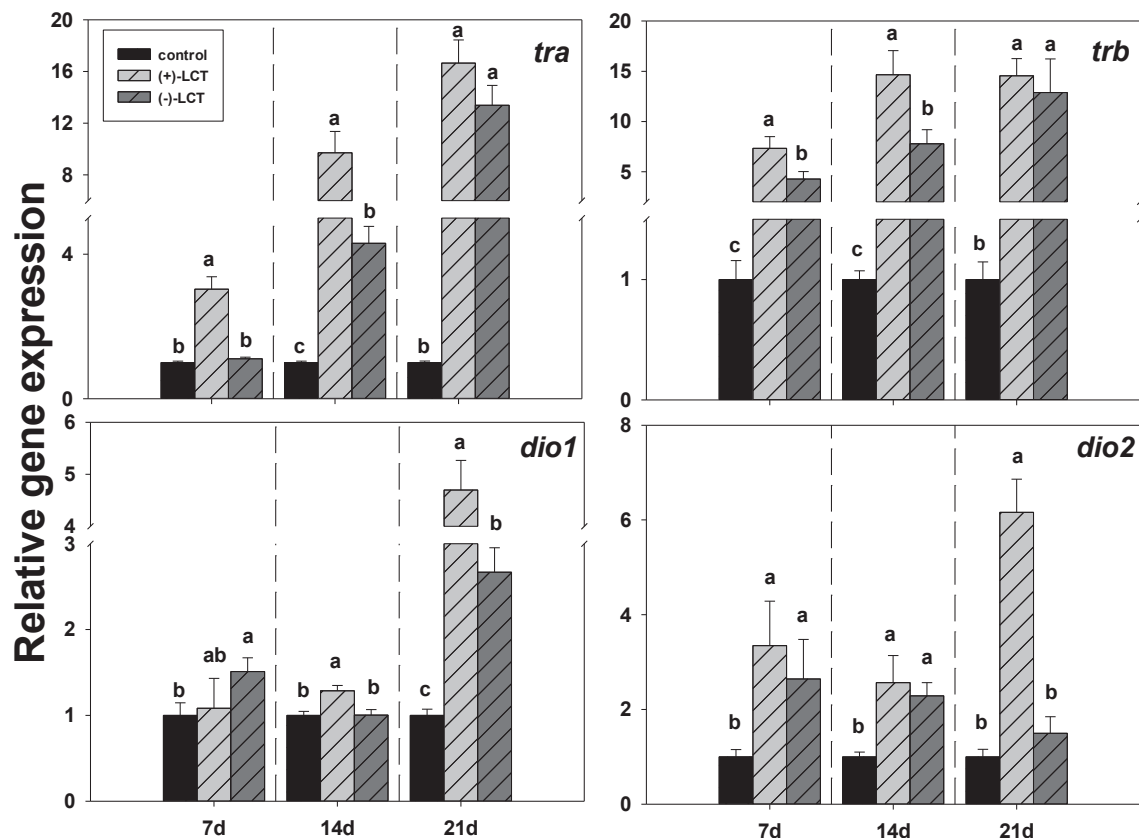


Fig. 5. Relative gene levels of thyroid hormone receptors (*tra*, *trb*), deiodinases (*dio1*, *dio2*) in the lizard (*E. argus*, 1–2-year old) brain after three weeks exposure of lambda-dyhalothrin (LCT). The result was evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. The results are expressed as the mean \pm S.E for each treatment ($n = 3$). Two bars labeled with totally different letters indicate a significant difference on gene expression at the same time point between the control group and treatment groups at a significant level of $p < 0.05$ (one-way ANOVA).

Bioaccumulation is a key factor to affect the toxicity of compounds. Our previous study has shown that the highest LCT concentration was found in the liver when comparing with the brain, gonad, plasma, heart and fat (Chang et al., 2016a). However, the maximum concentration of LCT in the hypothalamus of rat was 1.3 times higher than in the liver after a single 20 mg kg^{-1} oral dose (Anadon et al., 2006). These results indicated that the bioaccumulation of LCT in tissues was species-dependent. In the present study, the LCT concentration ranged from 0.031 to 0.058 mg kg^{-1} in the lizard liver after exposed to 10 mg kg^{-1} LCT enantiomers for three weeks. The lizard liver preferred to accumulate (-)-LCT after 14 days of treatment, which was further demonstrated by the relatively higher excretion level of LCT in the faeces after (+)-LCT exposure. Several previous studies have shown that (+)-LCT was more rapidly eliminated in a series of fish (Corcellas et al., 2015) and rabbit tissues (Liu et al., 2011) while preferred to accumulate in earthworm (Chang et al., 2016b). The metabolic breakdown of LCT enantiomers may be varied in different organisms. Another interesting finding was that the PBA concentrations were much higher than the LCT levels in the lizard liver. Furthermore, the level of PBA was higher at the (+)-LCT exposure group than that at the (-)-LCT group after 21 days of treatment. As pyrethroid metabolisms could also cause endocrine disruption, sometimes to a greater extent than the parent compounds (DeGroot and Brander, 2014), it is possible for us to predict that the different thyroid effects between the two enantiomers might be due to the differential metabolism and actions caused by the metabolism themselves.

Because THs are essential for growth, body and tissue weight are generally thought to be suitable endpoints for assessing the effects of thyroid disruption (Sciarrillo et al., 1999). In this study, oral administration of (+)-LCT affected the normal growth of the lizards because of the significantly decreased body weight. This phenomenon could be due to either a reduction in the daily feed intake or a disturbance in the level of THs (Dunlap, 1995). As the feed intake of lizards did not decrease for the 21 days of exposure and the T3 level was significantly increased, it was more possible to be caused by the disruption of THs. In addition, the decrease of HIS in the (+)-LCT exposure group at 21 days may be caused by the higher metabolic rate of (+)-LCT in the lizard liver. These results indicated that the thyroid system of lizards may be more easily affected by (+)-LCT than (-)-LCT.

According to histopathological analysis, there was a correlation between LCT enantiomers exposure and thyroid gland activity in the lizards. The increase in follicular epithelium cell height, highly vacuolated colloid and enlarged follicular area indicated the stimulation of lizard thyroid activity (Virgilio et al., 2003; Bicho et al., 2013). Meanwhile, the reduced colloids with few reabsorption vacuoles revealed poor functional activity of thyroid gland of lizards (Sciarrillo et al., 2008). In this study, (+)-LCT enantiomer exposure induced higher level in thyroid follicular epithelium height, more reabsorption vacuoles in colloids than the control exposure group, which indicated that the (+)-LCT stimulated the thyroid activity. Consistent with this result, flufenoxuron exposure also activate the thyroid gland of lizards (Chang et al., 2017). In contrast, (-)-LCT enantiomer exposure not only enlarged the

follicular area but also reduced the colloid area. According to the complicated result, it was difficult to determine the effect of (–)-LCT on the lizard thyroid activity. Through comparing the thyroid follicular epithelium height, we can predict that the thyroid activity was more seriously affected by (+) enantiomers than (–) enantiomer.

LCT enantiomers administration (10 mg kg⁻¹ once a week) also induced a massive increase in the synthesis and release of THs in the lizards. Recent studies showed 1.5–3 times up-regulation of the T3 and T4 levels in zebrafish embryos (Tu et al., 2016), whereas the THs levels were 1.5–5-fold decreased in freshwater teleost fish after LCT treatment (Dey and Saha, 2014). The various results may be associated with different species, development stages, and dosage of LCT used in the experiment. In this study, the TH levels of *E. argus* in the control group also showed differences when comparing with other kind of lizards. For example, the T4 concentration in *podarcis sicula* ranges from 0.1 to 6.7 ng mL⁻¹ according to different seasons (Sciarrillo et al., 2000) while for male western fence lizard, it is from 12.2 to 33.1 ng mL⁻¹ (Brasfield et al., 2008). The male 1–2-year old *E. argus* tested in our study showed relatively higher T4 content (137.72 ng mL⁻¹). These differences may be explained by the different physiology and ages of lizards. Another speculation was that the volume of plasma in *E. argus* (~50 µL) was less than western fence lizard which may cause higher TH levels. In the exposure groups, the two enantiomers of LCT did not show chiral discrimination regarding the influence of T4 concentration, but they were both more than 7-fold higher than in the control group. Only the (+)-LCT exposed group showed a sharp increase in the T3 content compared with the control group (Fig. 3), which was also consistent with the stimulated thyroid gland activity. It may also be because of the more seriously impact of (–)-LCT on reducing the activities of deiodinase enzymes which catalyze the conversion from T4 to T3. The (–)-LCT exposure induced lower T3 to T4 ratio compared with its counterpart, which further supported our hypothesis. The disruption of T4 deiodination has also been used to explain the changes of TH levels in birds (Wada et al., 2009) and turtle (Meyer et al., 2014) after mercury exposure. The significant elevation of T3 after (+)-LCT treatment could be detrimental because its primary function is to regulate the transcription of HPT axis-related genes and it is also involved in the timing of reproduction in lizards (Brasfield et al., 2008). It suggested that (+)-LCT might not only affect the activity of thyroid system, but also cause reproduction disruption in lizards, which need further study.

In order to understand the underlying mechanism of the TH disruption caused by LCT enantiomers, the expression levels of HPT axis-related genes were investigated in different tissues. There are few studies which incorporate alterations in mRNA levels to assess the effects of pesticides in the vertebrate endocrine system (Buchholz et al., 2006). We demonstrated that LCT enantiomers altered the steady-state mRNA levels of key THs gene targets.

THs exert their effects on development and physiology primarily through interactions with specific nuclear proteins, such as the thyroid hormone receptors. In the present study, the expression of thyroid hormone receptor genes (*trs*) was enhanced in the liver especially at 21 days (Fig. 4). This result is consistent with a previous study suggesting a significant up-regulation of *trs* after T3 administration in neuronal cells of birds (Crump et al., 2008). Accordingly, the elevations of *trα* and *trβ* gene expression in this study were attributed to the increased circulating TH levels at the end of exposure. Furthermore, the up-regulation of *trα* and *trβ* mRNA levels was higher in the liver of the (+)-LCT treated group than that in the (–)-LCT group at 21 days (Fig. 4). As LCT was found to interact with thyroid hormone receptor proteins (Tu et al., 2016), one speculation was that the binding affinity of (+)-LCT with thyroid hormone receptor proteins was higher, which was also

supported by the T3 up-regulation. Du et al. (2010) have indicated that both LCT and PBA showed antagonistic effects on thyroid hormone receptors (Du et al., 2010). Thus, another speculation was that the higher concentration of PBA in lizard liver after 21 days of (+)-LCT exposure induced the higher expression of *trα* and *trβ* genes as a compensatory mechanism. Thyroid hormone receptors can form heterodimers by making combination with retinoid X receptors (RXR) (Jahangir et al., 2016). Thus the expression of RXR genes also need further research to investigate the complicated function of LCT in lizard thyroid system. Neither *trα* nor *trβ* gene was significantly affected after LCT enantiomer treatment at 14 days, which indicated that the alteration of *trα* and *trβ* gene level of was time-dependent and pollutant concentration-dependent in the lizard liver. In the brain, the *trs* expression increased with time in the enantiomer treated groups, indicating the brain was more sensitive with long time exposure. Although the expression of *trα* and *trβ* varied between the two enantiomer treatment groups from 7 days to 14 days, no significant difference was observed in the brain at the end of exposure (Fig. 5). Thus, there may be a similar LCT and PBA residual level in the lizard brain after LCT enantiomers administration. Results from this study showed that *trα* and *trβ* gene expressions were related with different tissues and the (+) enantiomer exhibited more serious influence on *trs* in the liver after 21 days of exposure.

Deiodinase genes (*dios*) including *dio1* and *dio2* are responsible for the synthesis and metabolism of THs. The *dio1* gene mainly regulate the iodine recovery and THs degradation and *dio2* gene has a considerable influence on the synthesis active T3 from T4 in amphibian or fish (Brown, 2005; Orozco and Valverde-R, 2005). As the *dios* in fish and amphibian share properties with other vertebrate organisms, the *dios* in the reptiles might have similar functions. In the present study, the mRNA alterations of *dio1* and *dio2* gene were investigated in the liver and brain of lizards. In the liver, the expressions of *dio1* and *dio2* were up-regulated in the treated groups compared with the control group. Additionally, lizards in the (–)-LCT treated group showed significant higher alteration than (+)-LCT treatment at 14 days and 21 days, which was positively correlated with the LCT concentration in the liver (Pearson's test, R² = 0.97 for *dio1*, R² = 0.94 for *dio2*). LCT in the liver may disrupt the activity of deiodinase enzymes and then influence the *dio1* and *dio2* gene expression. The lower T3/T4 value in the exposure groups was also an indicator for the decreased deiodinases activity (Li et al., 2016). At 21 days, the T4 levels were significantly increased in the treatment groups. As *dio1* and *dio2* genes were responsible for the T4 degradation or conversion, their up-regulation may be attributed to the increased T4 content. In the brain, the (+)-LCT treated group provoked significantly elevation of *dio2* expression while the impact of (–)-LCT on that was lower than (+)-LCT exposure at 21 days (Fig. 5). This result was opposite with that in the liver and consistent with the T3 level, suggesting that the highly elevated *dio2* gene level in the brain may be the primary reason for the elevation of T3 level (Gong et al., 2016).

THs metabolism by pathways besides deiodination also involve conjugation, deamination, decarboxylation, or cleavage at the ether linkage between the aromatic rings (McNabb, 2007). In mammals, 80% T4 is degraded through deiodination and the major conjugation of T4 is regulated by *udp* gene (Engler and Burger, 1984). Previous study showed that sulfo-conjugation accounted for the largest metabolism of T3 followed by deiodination in rat (Deherder et al., 1988). However, there is no study about the THs metabolism routes in reptiles. In the current study, the expression of *udp* and *sult* genes was mainly detected in the lizard liver. The (+)-enantiomer exposure caused more than 3-fold higher *udp* gene expression and relatively lower concentration of T4 comparing with the (–)-enantiomer exposure at 21 days. The expression of *sult*

gene was similar between two enantiomers treatment. In that case, the relatively higher T3 level in the (+)-enantiomer group was mainly influenced by *dios*.

In conclusion, this is the first report on the enantioselective thyroid system disruption of LCT in lizards. Both LCT enantiomers altered the expression of HPT axis-related genes. Although (–)-LCT appeared to accumulate higher level in the liver, its toxic effects on lizard growth, T3 level and thyroid activity were relatively lower than those of (+)-LCT. It suggested that (–) enantiomer caused less disruption on lizard thyroid than (+) enantiomer. The usage of (–)-LCT in the field would be less harmful to the lizard thyroid system. As (–)-LCT showed higher toxicity in fish, it is still not appropriate to make LCT with only one enantiomer. Although this study provides fundamental data for understanding the role of pyrethroid pesticide enantiomers in disruption of reptilian thyroid system, their reproductive effects on lizards still need further research as reproduction is also important to keep the lizard populations.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.envpol.2017.09.072>.

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