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Oral and dermal diflubenzuron exposure causes a hypothalamic–pituitary–thyroid (HPT) axis disturbance in the Mongolian racerunner (*Eremias argus*)☆

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ABSTRACT

Diflubenzuron (DFB) is a potential endocrine-disrupting chemical. However, its thyroid endocrine effect on reptiles has not been reported. In this study, immature lizards (*Eremias argus*) were exposed to 20 mg kg^{-1} DFB once a week for 42 days through oral or dermal routes. Their body weight, plasma thyroid hormone levels, thyroid gland histology and the transcription of hypothalamic–pituitary–thyroid (HPT) axis-related genes in different tissues were assessed to explore the effects of DFB on the HPT axis of lizards. The body weight decreased significantly only after the dermal exposure to DFB. Triiodothyronine (T3) to thyroxine (T4) ratio in the male plasma also significantly increased after the dermal exposure. After oral exposure, the activity of thyroid gland was positively related to the thyroid hormone levels. Furthermore, the alterations in thyroid hormone levels affected the HPT axis-related gene expression, which was tissue dependent and sexually selected. The thyroid hormone receptor genes (trα and trβ) in the brain and thyroid were more sensitive to oral exposure. However, only the dermal treatment affected the *trα*, *trβ* and *type 2 deiodinase* (*dio2*) genes in the male liver. These results suggest that DFB exposure caused sex-specific changes in the thyroid function of lizards, and the dermal treatment may be an important route for the risk assessment of reptiles.

1. Introduction

Diflubenzuron (DFB) is widely used to inhibit the synthesis of chitin during insect moulting (Olsvik et al., 2013). Because chitin does not exist in vertebrates, including mammals, DFB is considered less dangerous than other pesticides. However, large quantities of DFB residuals have been detected in soil (Levot, 2011) and water (Fait et al., 2007). A previous study showed that fish could accumulate DFB at a level greater than water (Ahmed and Eid, 1991), while DFB has a relatively small effect on wild Atlantic cod (Olsvik et al., 2013). Because of its reproductive toxicity to shellfish, DFB has been considered a potential endocrine-disrupting chemical (Hester and Harrison, 1999). DFB concentration in the soil after aerial application varies between 0.30 (Nigg et al., 1986) and 2.68 mg kg^{-1} (Bull and Ivie, 1978). Although DFB preferentially accumulates in the soil, the long-term endocrine toxicity of DFB to terrestrial organisms remains less considered (Desneux et al., 2007).

Reptiles, which are one among the terrestrial organisms, are regarded as integral parts of the ecological environment and as important indicators for natural ecosystem balance. The International Union for the Conservation of Nature (IUCN) listed 28% of the evaluated reptiles as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) in 2010. Reptiles can be directly exposed to pesticides through various routes, including inhalation, food ingestion and skin penetration (Amaral et al., 2012). Thus, pesticides have been identified as one of the main factors that drive global reptile declines (Bohm et al., 2013). However, very few publications have investigated the toxic effects of pesticides on reptiles. Recently, several researchers have focused on the reptile ecotoxicology (McFarland et al., 2008; Weir et al., 2015; Amaral et al., 2012), but reptiles remain the least studied of all

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vertebrates (Rainwater et al., 2005). In addition, dermal exposure is not explicitly considered relative to the dietary pathway, even though reptiles have a greater percentage of body contact with soil and vegetation (Weir et al., 2010). Reptiles and mammals may show similar skin permeability (Weir et al., 2016). Dermal uptake has been regarded an important route of human exposure to organic pollutants (Wu et al., 2016). Researchers have also indicated that dermal contaminant exposure should be addressed because dermal-exposure modelling may be more important than other exposure routes in the reptilian risk assessment (Weir et al., 2014).

Because of the lack of regulatory requirements for toxicity assay on reptiles, there is no established model for ecological investigation. Lizards have potential as a laboratory model for evaluating the effects of numerous pesticides because of their abundant presence, richness and conspicuousness in agro-environments (Talent et al., 2002). We collected a native Chinese lizard species, *Eremias argus*, from the wild in the Inner Mongolia Province. *E. argus* has been maintained in our laboratory for more than 4 years. The reproduction method of *E. argus* was also established, and *E. argus* is considered an excellent model for reptilian risk assessment (Wang et al., 2014).

Thyroid hormones are directly involved in many physiological processes in vertebrates. In general, the biologically active triiodothyronine (T3) is derived from the outer ring deiodination of thyroxine (T4), which is mediated by deiodinase enzymes (Croteau et al., 1996). Thyroid hormones bind to the thyroid hormone receptors to exhibit their biological activity (Buchholz et al., 2006) and are eliminated by hepatic enzymes such as uridine diphosphate glucuronosyltransferase and sulfotransferase (Boas et al., 2006). Similar to that in numerous other vertebrates, thyroid hormones in lizards can regulate thermoregulation, growth, and metabolism and are also indirectly involved in a permissive capacity in other endocrine-dependent systems (Brasfield et al., 2008). The hypothalamic–pituitary–thyroid (HPT) axis of lizards may also be responsible for controlling the synthesis, release and metabolism of thyroid hormones (Blanton and Specker, 2007). However, little attention has been paid to the mechanism of the HPT axis of lizards.

Chitin-inhibiting chemicals can affect the growth of insects, and we hypothesize that it can also affect the growth of non-target animals through disturbing the HPT axis. The goal of the present study was to gain insight into the potential thyroid endocrine disruption in developmental stage lizards (*E. argus*) after the oral or dermal exposure to DFB for six weeks. Lizard body weight, plasma thyroid hormone levels, thyroid gland lesions and the expression of HPT axis-related genes in select tissues (liver, brain and thyroid gland) were measured to evaluate the effects of DFB on the thyroid system of *E. argus*. To our knowledge, no studies have yet assessed DFB exposure in this species.

2. Materials and methods

2.1. Chemicals

DFB (98% purity, Fig. A1) was purchased from J&K Chemical Technology (Beijing, China). Solvents including methanol and acetone were of analytical grade and purchased from Dikma (Beijing, China). DFB was dissolved in methanol and diluted by corn oil to prepare the stock solution.

2.2. Animals and husbandry

Immature (1–2-year-old) male and female *E. argus* were obtained from our breeding colony at Changping district, Beijing, China. Their average body lengths were 38 (male) and 35 (female). The lizards were kept in a 5 × 1.2 × 0.4 m solid-bottom indoor aquarium containing 10 cm of mollisol and fallen leaves (collected from our breeding colony, which have not received pesticides for more than 5 years). Ultraviolet lamps were set at 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 and 30–50%, respectively. The lizards were fed with live mealworms twice a day and sprayed with water 3–4 times a day. The excreta were cleaned every other day. In general, the lizards were housed together. Prior to the experiments, the lizards were allowed to acclimate in the experimental conditions for one week in experimental glass cages (60 × 60 × 40 cm).

2.3. Exposure experiment and sample collection

Pesticides such as DFB are routinely applied to crops several times per growing season. In this experiment, each lizard was administered a total of six doses of DFB or corn oil over 42 days (one dose every week). The dose interval was selected according to our toxicokinetics study of DFB in lizard tissues. After a single exposure with 20 mg kg^{-1} body weight DFB, its concentration in the lizard tissues decreased to 0.19–1.80 mg kg^{-1} at 7 days, which is approximately equal to its environmental concentration (0.30–2.70 mg kg^{-1}) in the soil after aerial application (Ramos et al., 2013). The lizards were administrated with DFB every week to ensure that the DFB concentration in the tissues was relatively higher than the environmental concentration and gain insight into the toxic effects of DFB.

2.3.1. Experiment 1

Body length is considered a better indicator of age and gender of lizards than body mass (Kim et al., 2010). Following acclimatisation, the lizards were sorted by body length and distributed evenly into exposure and control groups (n = 108, each group n = 54, sex ratio 1:1, for the male lizards, their tails are longer and thicker, have narrower abdomen and longer body length and the colour of skin is also brighter than the female lizards. When sampling, we can accurately separate the male and female lizards according to the sex organs.) The lizards in the exposure group were orally administered with 20 mg kg^{-1} body weight DFB stock solution using the injection needle (10–20 μL) once a week. The lizards in the control group were administrated with methanol diluted by corn oil alone. The body weight of lizards was recorded once a week. Lizards from both exposure and control groups were euthanized (lizards became unconscious in a sealed bottle filled with diethyl ether and suffered decollation) at 14, 28 and 42 days. Six lizards (three males and three females) were selected randomly from each group, and three replicates were prepared (sampling three times from the same group and at the same time point). The body weight of the lizards was measured. The liver, brain and thyroid gland were collected and frozen at - 80 °C with RNA Store. The blood was collected when the lizards were suffering the decollation and immediately centrifuged at 2500 × g for 10 min, and the plasma was stored at - 80 °C for thyroid hormone analysis.

2.3.2. Experiment 2

A dermal exposure experiment was also conducted. For solvent carrier exposure, the same micropipette approach used for the oral exposure was used. DFB (the stock solution was prepared with acetone solvent) was placed on the belly (anterior to the pelvic girdle and posterior to the throat) of lizards once a week. Only acetone solvent was used in the control groups. Dermal treatment lizards were held for approximately 30 s to evaporate acetone. The exposure concentration was also 20 mg kg^{-1} body weight. To save more lizards, the lizards in the dermal exposure group (n = 36, each

group n = 18) were euthanized only at 42 days to compare the toxicity in this group with that in the oral exposure group.

2.4. Thyroid hormone analysis

Plasma samples were collected after 42 days of exposure. The T3 and T4 levels in the plasma were analysed using an enzyme-linked immunosorbent assay (ELISA) kit (purchased from Elabscience Biotechnology Co., Ltd, Wuhan, China) following the manufacturer's instructions. This ELISA kit uses competitive ELISA, and the results are measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The assay sensitivity was 0.84 pg mL^{-1} for T3 and 3.7 ng mL^{-1} for T4.

2.5. Histopathology

When sampled at 42 days after dosing, the thyroid gland tissue was stored in 4% paraformaldehyde for histopathological analysis. The thyroids were processed for paraffin wax embedding. Sections were cut, stained with haematoxylin and eosin and examined by light microscopy (Olympus DP73).

2.6. Isolation of mRNA and quantitative real-time polymerase chain reaction (qRT-PCR)

The liver, brain, and thyroid gland samples were homogenized and prepared for total RNA isolation using TRIzol reagent (Life Technology, Beijing, China). The cDNA was synthesized by reverse transcription reactions according to the manufacturer's instructions. Reverse transcription reaction mixtures contained 22 μL of total RNA, 2 μL of Oligo $(dT)_{15}$ primers and 4 μL of DNTP. The mixtures were heated at 70 °C for 5 min and quickly cooled on ice. After cooling, 8 μL of 5 × 6 buffer, 2 μL of M-mlv and 40 units of 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.

The HPT axis-related genes chosen in this study and their respective primers are listed in Table 1. All the primers were designed by the authors using the National Center for Biotechnology Information (NCBI) website. Real-time PCR was performed in a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 20 μL, including the SYBR Green RealMasterMix, 1 μM forward primer and 1 μM reverse primer. The thermal cycle parameters were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, 54 °C for 40 s and 72 °C for 40 s. All the samples were analysed in triplicate, and the delta-delta Ct method was used to analyse the results. The thyroid hormone receptor (trα and *trβ*), deiodinase (*dio1* and *dio2*) and sulfotransferase (*sult*) mRNA expressions were normalized by *β-actin* mRNA expression. A dissociation curve analysis was performed for each gene to check the amplification of the untargeted fragments. Only one peak was observed for the amplification, which indicates the specific amplification of the target gene. The gene expression data showed changes relative to the control animals for the same treatment period.

2.7. Data analysis

Statistical analysis of the data was performed by the SPSS (version 13.0; USA) using one-way analysis of variance (ANOVA), repeated-measures ANOVA and multivariate ANOVA. The assumptions were checked, and a probability of $p < 0.05$ or $p < 0.01$ was considered statistically significant.

3. Results

3.1. Body weight

No mortality was observed during the experimental period in the exposure and control groups. The body weight of lizards at different time points is shown in Fig. 1. In control groups, the body weight gradually increased with time, but no significant difference was observed between different time points (one-way ANOVA, $F = 0.544$, $p = 0.767$). There was also no significant difference in the body weight between control and oral (repeated-measures ANOVA, $F = 1.473$, $p = 0.292$) or control and dermal (repeated-measures ANOVA, $F = 1.696$, $p = 0.263$) exposure groups during the 42-day treatment. However, when comparing the body weight between control and exposure groups at the same time point (multivariate ANOVA), after 35 days, the body weight of the lizards in the dermal exposure group began to decrease significantly ($F = 7.942$, $p = 0.048 < 0.05$ at 35 days and $F = 7.870$, $p = 0.049 < 0.05$ at 42 days) compared to those in the control group.

3.2. Thyroid hormone levels

In this study, the T4 and biologically active T3 levels were assessed after the oral or dermal DFB exposure for 42 days (Fig. 2). In both oral and dermal control groups, the T3 (>1.5-fold) and T4 (>2.0-fold) concentrations were higher (one-way ANOVA, for the F and *p* values, please see Table A2) in female lizards than in male ones (Fig. 2a).

In the oral exposure group, the male lizards exhibited significantly higher T3 (1.6-fold) concentrations, while the female lizards had relatively lower T3 (2.6-fold) concentrations, than those in the control groups at 42 days (one-way ANOVA, for the F and *p* values, please see Table A2). Consistent with the changes in the T3 level, the T4 concentration was significantly up-regulated (1.9-fold) in

Table 1
Primers used for PCR and the quantification of the mRNA expression by real-time PCR.

Gene	Sequence (5′-3′)	Product size (bp)	GenBank accession no.
β-actin	TCTTCCAGCCCTCATTCCT ACGGTGTTGGCATACAGGT	186	KX459399
trα	GCAGCCAATGTTCGGTGAAA CCTTGCAGCCTTCACAGGTA	135	KX494867
trβ	TGCTGATGAAAGTGACGGA TGACAGACACCCAGTAGTGCT	176	KX459400
dio1	TCCCACTCCACCCTTGTAGA CCCTCATGTCCAGTGGTTGT	120	KX452398
dio2	AGATGCCTACAAACAGGTGAAG GCTGAGCCAAAGTTGACCA	103	KX459401
sult	CAGTTGGTACCAGATTTGCAG CCTACAGCTCTTTGCAGCC	103	KX459403

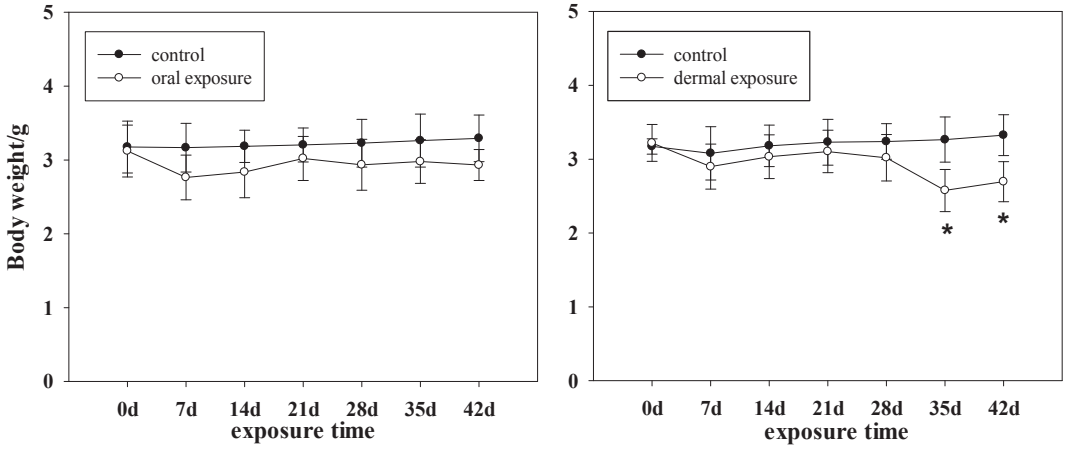

Fig. 1. Changes in the body weight over time of the lizards (*Eremias argus*) dosed with diflubenzuron (DFB) through oral and dermal administration for 42 days. The data are expressed as mean ± S.E. (n = 3). $^{*}p < 0.05$ indicates a significant difference compared to the control (repeated-measures and multivariate ANOVA).

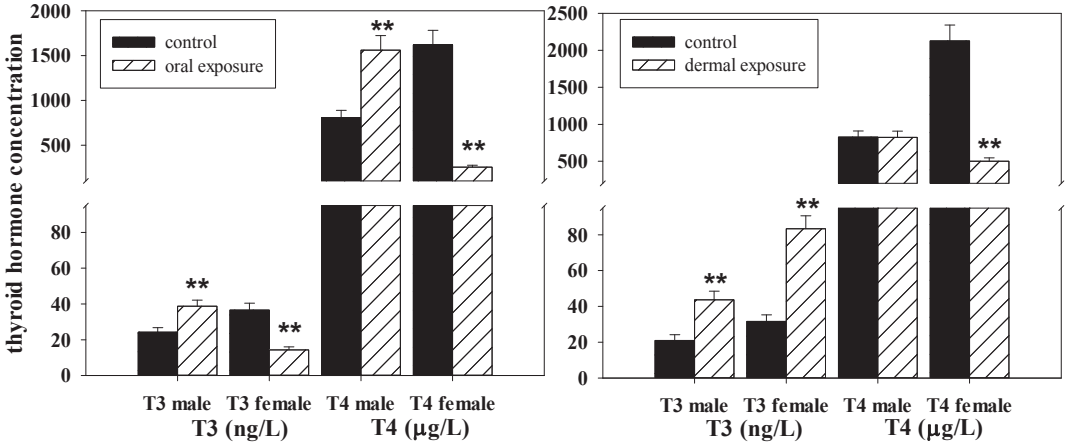

Fig. 2. Variations in triiodothyronine (T3) and thyroxine (T4) levels in the plasma of *Eremias argus* after 42 days of oral (a) and dermal (b) exposures. The data are expressed as mean ± S.E. (n = 3). $^{**}p < 0.01$ indicates an extremely significant difference compared to the control (one-way ANOVA).

the male lizards and significantly down-regulated (6.4-fold) in the female lizards (one-way ANOVA, for the F and *p* values, please see Table A2). The T3 to T4 ratio significantly increased in the female plasma alone (Fig. A2).

Dermal DFB exposure caused significant increases in both male and female plasma T3 levels, and the T4 levels decreased significantly (4.2-fold) in the female lizards alone (Fig. 2b) (one-way ANOVA, for the F and *p* values, please see Table A2). The T3 to T4 ratio significantly increased in both male and female plasmas (Fig. A2).

3.3. Thyroid histopathology

The thyroid gland lesion was analysed after 42 days of exposure. Only oral DFB exposure caused significant injury to the thyroid gland tissue (Fig. 3). The follicles with epithelium cells were fully filled with colloids. However, compared with the male lizards in the control group (Fig. 3a), those in the exposure group (Fig. 3b) showed many reabsorbed vacuoles (white arrows) in the colloids. In addition, the follicular area of thyroid gland in the exposure group was larger than that in the control group. In contrast, there was no significant difference in the follicular epithelial cell height between the control and exposure groups (Table A1).

The thyroid glands of female lizards from both the control and DFB exposure groups (Fig. 3c–d) had many reabsorbed vacuoles (white arrows). No significant difference was observed in the follicular epithelial cell height between the control and exposure groups. In the exposure group, the thyroid glands of female lizards had smaller follicular areas and more number of follicles, which suggests the suppression of the thyroid gland activity.

3.4. Quantitation of trα, trβ, dio1, dio2 and sult mRNA in the lizard tissues after oral exposure

In the oral exposure group, the expressions of *tra, trb, dio1, dio2* and *sult* genes in the lizard liver were determined every two weeks (Fig. 4a–b). At the same time point, different gene expressions in the control group were normalized to 1.00 when comparing with those in the exposure group. No significant difference was observed among the different gene expressions in the control group at the same time point (one-way ANOVA). After 14 days of exposure, in the female liver, the *trα, trβ, dio1* and *sult* mRNA levels increased significantly, while the *dio2* mRNA level decreased significantly. In the male liver, the *sult* gene expression was significantly up-regulated, and the *dio1* gene expression was significantly down-regulated. No significant difference was observed in the *trα, trβ* and *dio2* gene expressions. After 28 days of exposure, the mRNA level of *dio1* in the male and that of *dio2* in the female liver

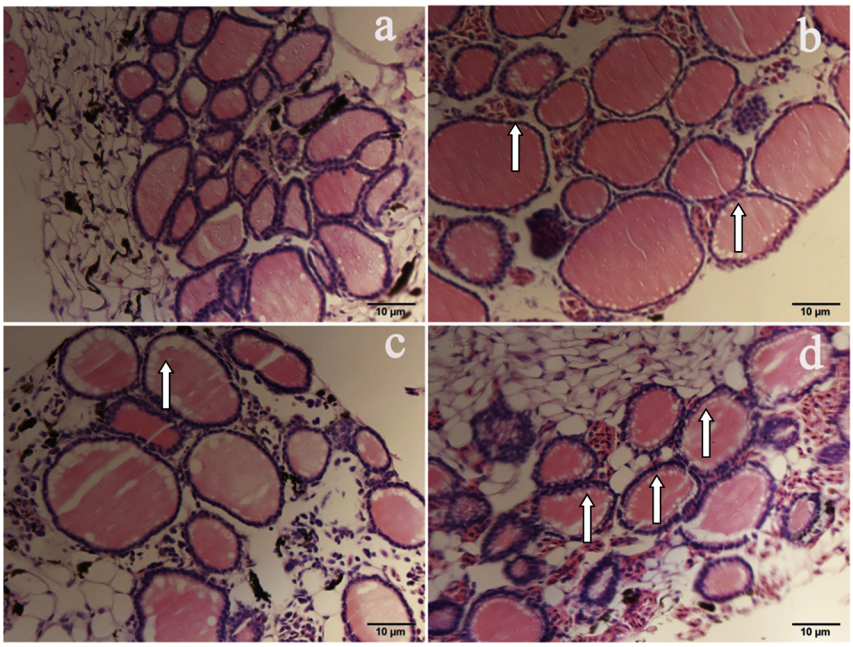

Fig. 3. Thyroid gland sections of the male and female *Eremias argus*. Representative sections of the male a) and female c) lizard from the control groups at 42 days; Representative sections of the male b) and female d) lizard from the diflubenzuron (DFB) exposure groups at 42 days.

continued to decrease. Compared with that at 14 days, the up-regulation of *dio1* and *sult* in the females showed a sharp decrease after 28 days. After 42 days of exposure, the expression of *sult* was significantly up-regulated in the male, and the *trα, trβ* and *dio1* mRNA levels were significantly increased in the female lizards. Nevertheless, the expression of *dio2* in the female and that of *dio1* in the male continued to be at low levels.

In the brain, the mRNA levels of *trα, trβ*, *dio1* and *dio2* were measured every 2 weeks (Fig. 4c–d). After exposure for 14 days, the expressions of *trα, trβ* and *dio2* mRNA levels significantly decreased in the female brain. In the male brain, the *trβ* and *dio2* gene expressions significantly increased. In the female brain, the trα and *dio2* gene expressions were significantly up-regulated, while the *trβ* gene expression returned to the control level after 28 days of exposure. In comparison, the *trα, trβ* and *dio2* gene expression continued to increase in the male brain. At the end of 6 weeks, no significant differences were observed in the *dio1* gene expression in both male and female brains compared with that in the control, while the expressions of *trα, trβ* and *dio2* gene increased to a higher level.

The *trα, trβ* and *dio1* gene expressions in the thyroid tissue were also assessed (Fig. 4e–f). After 14 days of oral exposure, in the female lizards, the trα gene expression was significantly down-regulated, while the *dio1* gene expression was up-regulated. The *trβ* and *dio1* mRNA levels decreased significantly in the male lizards. After 28 days of exposure, the *trα, trβ* and *dio1* gene expressions were significantly down-regulated in the female thyroid. In the male thyroid, only the trα and *dio1* levels significantly decreased. The results obtained after 42 days of exposure showed that the trα and *trβ* expressions were significantly down-regulated in the female thyroid and significantly increased in the male thyroid.

3.5. Quantitation of trα, trβ, dio1, dio2 and sult mRNA in the lizard tissues after dermal exposure

In this study, the disturbance in HPT axis-related gene expressions in the lizard liver, brain and thyroid gland was evaluated after 42 days of dermal exposure (20 mg kg^{-1} every week) (Fig. 5). At 42 days, the *trα, trβ*, *dio1*, *dio2* and *sult* genes were significantly up-regulated in the male lizard liver (Fig. 5a). In the female liver, only the trα and *trβ* gene expressions were significantly up-regulated, whereas the *dio2* and *sult* gene expressions were significantly down-regulated (Fig. 5b).

The *trα, trβ*, *dio1* and *dio2* gene expressions in the brain are shown in Fig. 5c–d. No significant difference was observed in the *trα, trβ and dio1* gene expressions in both male and female lizards after the DFB dermal exposure. The *dio2* mRNA expression was significantly down-regulated in the female brain, while it was significantly up-regulated in the male brain.

Although no significant thyroid lesion was observed after the DFB dermal exposure, the *dio1* gene expression was significantly down-regulated in both male and female thyroids (Fig. 5e–f). In the male thyroid, only the *trβ* mRNA level significantly decreased.

4. Discussion

DFB is widely used to inhibit the growth of insects, while its effect on the growth of non-target organisms, especially reptiles, is poorly recognized. This is the first study that examined the effects of DFB on the lizard thyroid endocrine system under laboratory conditions.

Our study found that there was no significant difference in the body weight of lizards at the control and oral exposure groups during the 42 days of exposure. In contrast, our previous study showed that oral exposure to flufenoxuron induced loss of body weight in lizards at both proliferation and resting stages (Chang et al., 2017). Flufenoxuron may cause more serious effects on the growth of lizards. The body weight was significantly decreased at 35 and 42 days after dermal DFB exposure. This suggested that dermal administration inhibited the growth of lizards after a long-term exposure. The changes in the body weight of lizards after contaminant exposure might be partially associated with the disruption of thyroid hormone homeostasis. Dermal exposure of

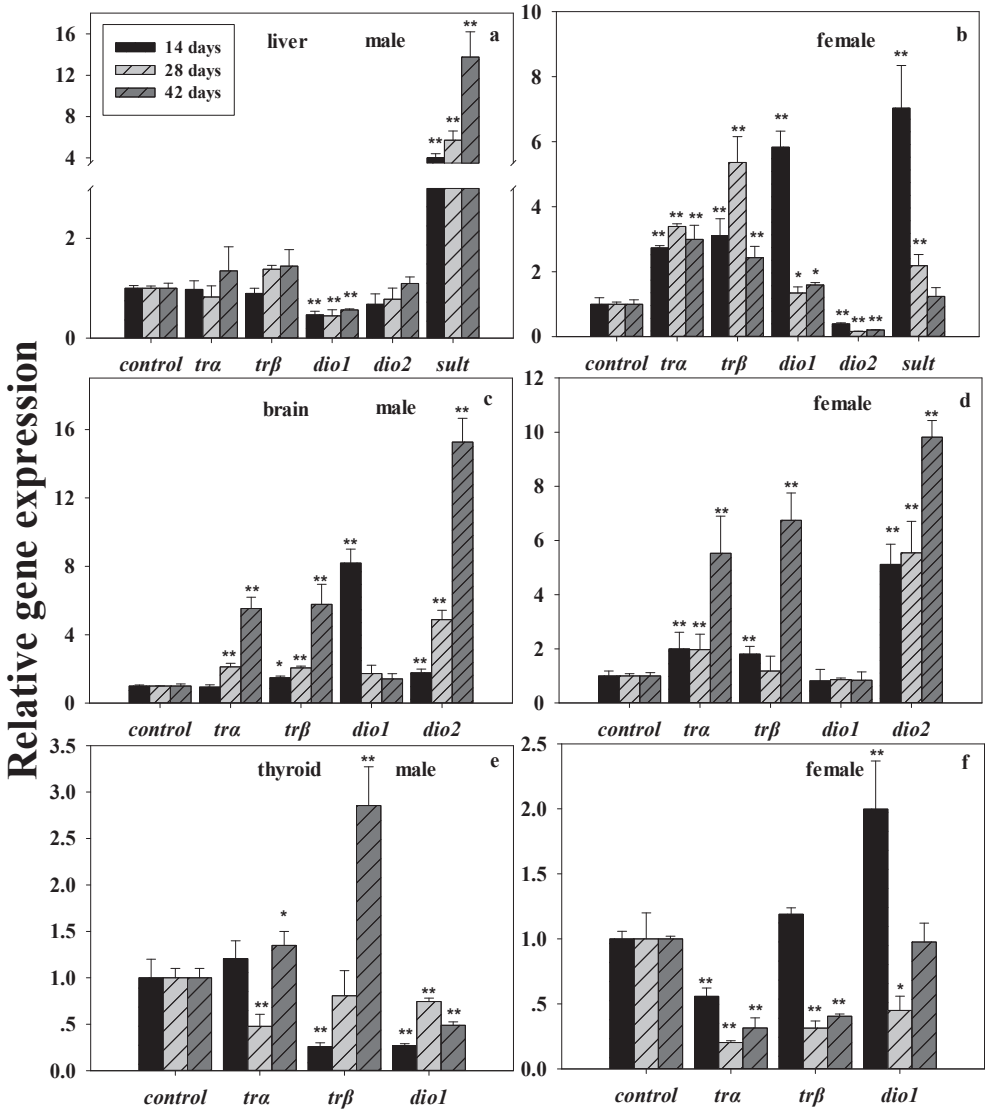

Fig. 4. mRNA expression in the hypothalamic–pituitary–thyroid (HPT) axis in the lizard liver (a–b), brain (c–d) and thyroid (e–f) after 14, 28 and 42 days of oral exposure to 20 mg kg^{-1} diflubenzuron. The data are expressed as mean ± S.E. (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference and an extremely significant difference compared to the control, respectively (one-way ANOVA).

the reptiles could be relatively more important than other routes, which is consistent with a previous study (Weir et al., 2010).

The changes in thyroid hormone levels in lizard plasma after DFB exposure further demonstrated our hypothesis. Both oral and dermal DFB exposures disturbed the thyroid hormone homeostasis in the lizards. In the oral treatment group, the T3 and T4 levels were significantly increased in the male and significantly decreased in the female lizards. In addition, the follicular area of thyroid gland was larger in the male and smaller in the female compared with the controls, respectively. These results indicate that the thyroid endocrine system was stimulated in the male lizards and suppressed in the female lizards after DFB oral exposure. Similar to our results, a recent study also reported differential regulation of thyroid hormone levels in different sex mice exposed to chlorpyrifos (Haviland et al., 2010). This phenomenon could be explained by the different physiological stages of the male or female animals before exposure. Above all, the DFB oral exposure could disturb the thyroid hormone level and thyroid gland activity in both male and female lizards. Neuman-Lee et al. (2015) also found that the exposure of non-model snake to PBDEs induced increased size and higher thyroid follicular height (Neuman-Lee et al., 2015). These results suggest that the thyroid endocrine system of reptiles is easily disturbed. In the dermal exposure group, the T3 level was significantly increased in both male and female plasmas. However, no significant difference in T4 level in the male lizards was found between the control and dermal exposure groups. Moreover, the T3/T4 ratio in the male plasma was significantly increased by the dermal DFB exposure alone. The transition of T4 to T3 is modulated by deiodinase enzymes. It was possible that the dermal DFB treatment affected the activity of deiodinases.

The HPT axis-related genes are regarded the target genes for the thyroid hormone. The *trα*, *trβ*, *dio1*, *dio2* and *sult* gene expressions in the lizard liver, brain and thyroid gland were determined for further investigation. The results in the liver showed that the trα and *trβ* gene expressions were more sensitive in the female liver than in the male liver after oral exposure to DFB at all the time points. A previous study also indicated that the sensitivity to styrene exposure of the female was higher than that of the male rats

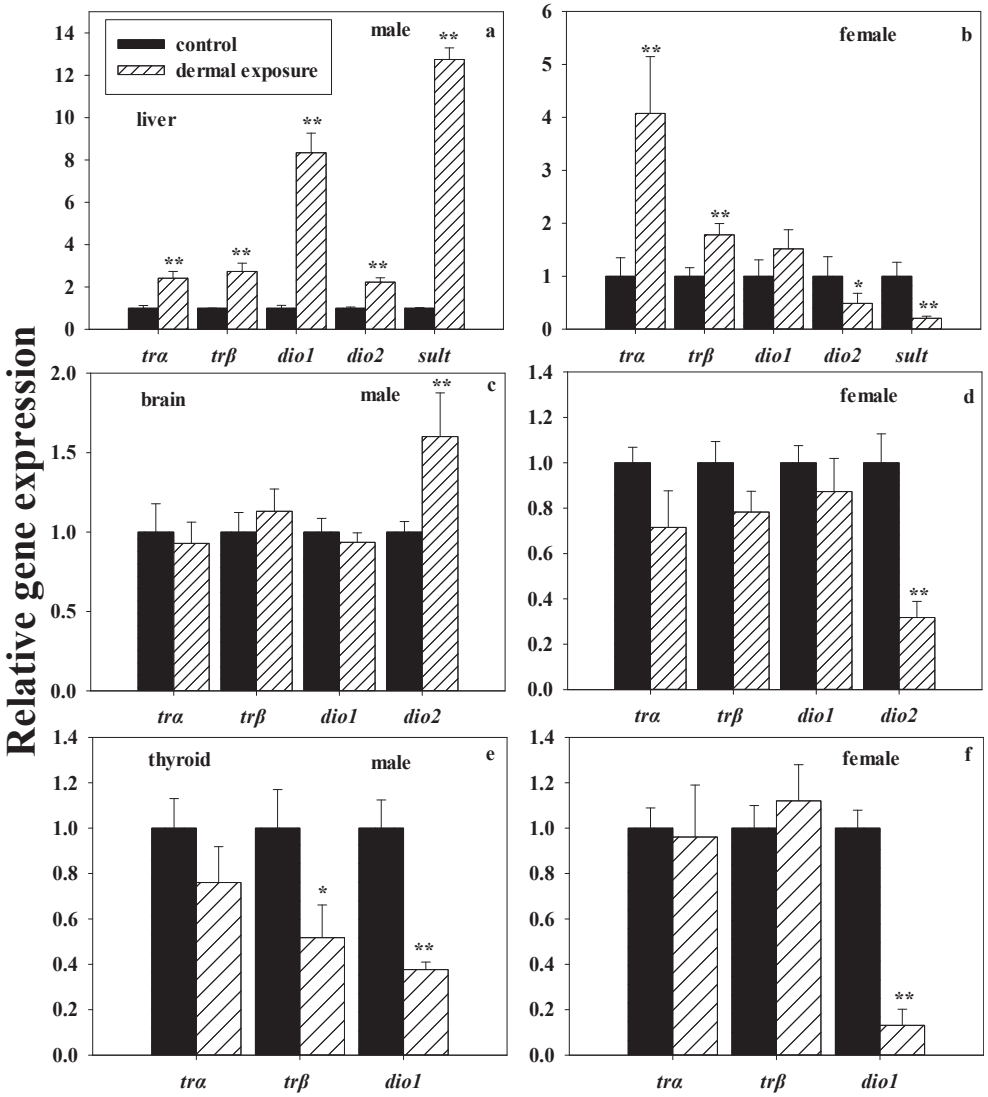

Fig. 5. mRNA expression in the hypothalamic–pituitary–thyroid (HPT) axis in the lizard liver (a–b), brain (c–d) and thyroid gland (e–f) after 42 days of dermal exposure to 20 mg kg^{-1} diflubenzuron. The data are expressed as mean ± S.E. (n = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate a significant difference and an extremely significant difference compared to the control, respectively (one-way ANOVA).

(Umemura et al., 2005). Thyroid hormones affect the development and physiology primarily through interactions with specific nuclear proteins—the thyroid hormone receptors. In this study, DFB was supposed to be a thyroid hormone congener and stimulate the thyroid hormone receptor gene expression. The T3 negative feedback effect could increase the expressions of trα and *trβ* genes in the female liver. The combined two effects resulted in the up-regulation of the thyroid hormone receptor genes in the female liver after oral exposure. The expressions of trα and *trβ* genes in the male liver was significantly increased only after the dermal exposure. The increase in trα and *trβ* gene expressions also indicates that DFB might show stronger bonding to the thyroid hormone receptors through dermal exposure than oral exposure.

The deiodinases (*dio1* and *dio2* genes) are important regulators for maintaining the T4 to T3 ratio (Opitz et al., 2006). A previous study showed that treatment of tilapia (*Oreochromis mossambicus*) with a thyroid hormone synthesis inhibitor increased the activity of *dio2* gene in the liver (Van der Geyten et al., 2001). In our study, the plasma T3 level might mainly regulate the *dio1* gene expression in the liver after oral exposure. The significantly increased *dio2* gene expression in the male liver after dermal exposure further demonstrates our speculation that the activity of deiodinase in the male was affected by the dermal exposure route. Another phenomenon is that both oral and dermal DFB exposures increased the *sult* mRNA expression in the male liver. The sulfotransferase regulates the metabolism of thyroid hormones mainly through conjugating with T3 (Deherder et al., 1988). The significant up-regulation of *sult* gene in the male liver was considered a response to the significant increase in T3 level at 42 days. In the female liver, the *sult* gene was not affected by the changes in T3 level in the oral exposure group and significantly decreased in the dermal treatment group. Thus, the *sult* mRNA is likely to be the target gene for the elevation of T3 level in the males, while the changes in T3 level in the females mainly affected the *trα*, *trβ*, *dio1* and *dio2* genes. Rooney et al. (2003) also showed that atrazine exposure suppressed the immune function in male but not in female rats (Rooney et al., 2003). The sex specificity of pesticides' toxic effect may result from the ability of pesticides to dampen the pulsatile gonadotropin-releasing hormone secretion from the hypothalamus (Cooper et al., 2000), which needs further research.

The expressions of the *trα*, *trβ* and *dio2* genes increased with oral exposure time in both the male and female brain, which is different from that in the liver. The *trα*, *trβ* and *dio2* genes in the brain may be more seriously affected by DFB rather than the thyroid hormone level changes. These results indicate that the changes in HPT axis-related gene expressions was tissue specific after oral exposure to DFB (Shi et al., 1996). In contrast, there was no significant difference in the *trα*, *trβ*, *dio1* gene expressions in both the male and female brain after DFB dermal exposure. This result suggests that the dermal exposure induced less toxic effects on the lizard brain.

After oral exposure, the trα and *trβ* gene expressions were significantly increased in the male thyroid and significantly decreased in the female. It was presumed that the thyroid hormone receptor genes in the thyroid gland responded positively to the changes in thyroid hormone levels and the thyroid gland activity after oral DFB exposure. After dermal exposure, although there was no obvious thyroid gland lesion, the expression of *dio1* gene was significantly decreased in both the male and female thyroids. Combined with the up-regulation of T3 levels, the decrease in *dio1* gene expression may be attributed to the negative feedback mechanism.

In conclusion, the pesticide DFB was evaluated under laboratory conditions in lizards to assess thyroid disruption in reptiles. Although no significant change was observed in the body weight after DFB oral exposure, the thyroid hormone levels were significantly increased in the males and significantly decreased in the females. The activity of the thyroid gland was positively correlated with the changes in thyroid hormone level. In addition, the transcription of trα and *trβ* genes in the thyroid responded positively to the changes in T3 levels and thyroid activity after oral DFB exposure. In contrast, dermal exposure to DFB led to a significant decrease in the body weight after the long-term treatment. The T3/T4 ratio was significantly increased in both the male and female plasmas, although the thyroid did not show a significant lesion. DFB, which binds to thyroid hormone receptors competitively with T3, may partially be responsible for the up-regulation of *trα*, *trβ* and *dio2* genes in the male liver and the changes in thyroid hormone levels after dermal exposure. These results indicate that oral and dermal DFB exposure exhibited different thyroid endocrine-disrupting mechanisms to lizards. Different exposure routes should be considered when evaluating the ecotoxicological effects of contaminants in reptiles.

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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.2017.08.115.

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