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Xenopus laevis tadpoles exposed to metamifop: Changes in growth, behavioral endpoints, neurotransmitters, antioxidant system and thyroid development

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ABSTRACT

Pesticides are a major cause of the reduction in the global amphibian population. In this study, the acute toxicity and chronic effects of metamifop on *Xenopus laevis* (*X. laevis*) tadpoles were investigated. The 96 h-LC_{50} value of metamifop on *X. laevis* tadpoles was 0.634 mg/L, which indicated that metamifop was highly toxic to tadpoles. In the chronic toxicity study, tadpoles were exposed to 0.063 mg/L of metamifop. After 14, 21 and 35 d of exposure, metamifop significantly inhibited the body weight and neurotransmitter synthesis of tadpoles, caused abnormal behavior and interfered with fat metabolism. According to the results of antioxidant enzymes and malondialdehyde (MDA), tadpoles exposed to 0.063 mg/L metamifop suffered severe lipid oxidative damage. Compared with the control group, the thyroid hormone (TH) levels and related gene expression in tadpoles in the treatment group were affected, reflecting the endocrine interference effect of metamifop. The data of this study can enrich our knowledge of the effects of aryloxyphenoxy propionate pesticides on amphibians and highlight the role of metamifop and other pesticides play in global decline of amphibians.

1. Introduction

The decline in the number of amphibians worldwide poses a major threat to global biodiversity. The assessment showed that about 32% of the amphibians in the world were on the verge of extinction (Hayes et al., 2010). Pollution has been identified as the second-highest risk factor, which declines amphibian populations. Pesticides are the main group responsible for pollutants (Lehman and Williams, 2010). Pesticides might enter amphibian habitats through runoff and aerial drifting. Amphibians living in habitats adjacent to agricultural fields are vulnerable to pesticides because of the permeability of their skins and sensitivity to environmental chemicals (Khan and Law, 2005). Li et al. (2016a) studied the effects of seven fungicides on *Xenopus laevis* (*X. laevis*) embryos, and the results showed that the embryos had a strong embryo teratogenic effect (Li et al., 2016a). Lanctôt et al. (2014) found that long-term exposure to glyphosate-based herbicide directly affected the metamorphosis of tadpoles of the wood frog (*Lithobates sylvaticus*) by reducing the development rate (Lanctôt et al., 2014). Therefore, it is essential to evaluate the toxicity of pesticides to amphibians.

Metamifop is a novel aryloxyphenoxy propionate herbicide developed by Hannong Chemical Co., Ltd. (Republic of Korea) used for controlling grassy weeds in paddy fields, such as barnyard grass and crabgrass (Fig. S1) (Xia et al., 2016). It inhibits the activity of acetyl-CoA carboxylase and lipid synthesis to control weeds. However, the wide application of metamifop may bring potential risks to many non-target species (Dong et al., 2017); indeed, many studies identified the LD_{50} values in different species and found that the acute oral and percutaneous. The acute oral and percutaneous LD_{50} for rats was >2000 mg/kg; the acute oral LD_{50} for bees (48 h) was 100 μg/bee; the EC_{50} for algae (72 h) was 2.03 mg/L; EC_{50} (48 h) for *Daphnia magna* was 0.288 mg/L (Lewis et al., 2016; Tomlin, 2005). Zhao et al. (2019) reported that metamifop showed high acute toxicity to zebrafish (*Danio rerio*), and it induced severe oxidative stress and apoptosis in embryos after 96 h of exposure (Zhao et al., 2019). However, studies on the toxicity of metamifop to amphibians are still limited.

Amphibian larvae are an effective model for studying the behavioral effects of pesticide exposure (Mikó et al., 2017). Measuring behavioural

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change does not require lethality as an endpoint and provides an understanding of the complex connections between an organism's internal biochemistry and its external environment (Denoël et al., 2012). Some reports have observed that aquatic pollutants can cause changes in several aspects of animal behavior, including preference or avoidance of areas with high concentrations of pollutants, feeding behavior and abnormal motility (Mikó et al., 2017). The thyroid of amphibians plays an important role in metamorphosis regulation and protein synthesis (Brown, 2000), which is responsible for the secretion of thyroid hormones (TH). During the metamorphosis of amphibians, the hypothalamic-pituitary-thyroid (HPT) axis can regulate the reconstruction of multiple tissues and organs, such as leg bud formation, tail absorption, craniofacial and digestive system reconstruction (Blanton and Specker, 2007). HPT axis is also responsible for controlling the synthesis, release and metabolism of TH. Some pesticides affected the normal growth and development of organisms by inhibiting the synthesis, secretion and transport of TH, or interfering with the binding of thyroid hormone receptors (TR). Li et al. (2016b) demonstrated that triadimefon altered HPT axis-related genes and TH levels in *X. laevis* tadpoles, which led to thyroid endocrine disorder, thus delaying the development of TH-dependent metamorphosis (Li et al., 2016b). Boone et al. (2013) analyzed the effect of carbaryl on the thyroid of green frog (*Lithobates clamitans*) tadpoles and found that carbaryl changed the mRNA abundance distribution of TH regulatory genes (*trα, trβ and dio2*) during the development (Boone et al., 2013). Therefore, pesticide residues in the aquatic environment may have endocrine-disrupting effects on the thyroid of amphibians, affecting their normal growth and development.

X. laevis is an ideal model organism in ecological risk assessment because of its high sensitivity to environmental pollutions (Kloas and Lutz, 2006). In this study, *X. laevis* tadpoles were exposed to metamifop for 96 h to determine the 96 h-LC_{50} value; moreover, the effects of metamifop on the behavioral endpoints, body weight, triglycerides (TG) and total cholesterol (T-CHO), neurotransmitters contents (glycine and γ-aminobutyric acid) and oxidative stress including total superoxide dismutase (T-SOD), catalase (CAT) and malondialdehyde (MDA) were also studied. After 14, 21, and 35 d of exposure, TH [triiodothyronine (T3) and thyroxine (T4)] contents and expressions of related genes (*tshα*, *tshβ*, tr *α*, tr *β*, *dio2* and *dio3*) in *X. laevis* tadpoles were measured. We aimed to explore the effect of metamifop on tadpoles and provide an understanding of the risk of pesticide use to amphibian populations.

2. Materials and methods

2.1. Chemical reagents and X. laevis

Metamifop (purity 96.0%; catalog number: PD20110752) and 3-aminobenzoic acid ethyl ester (MS-222) were purchased from J&K Scientific Ltd. (Beijing, China). All analytical grade reagents were purchased from Yili Fine Chemicals (Beijing, China). Metamifop stock solution was prepared with acetone (1000 mg/L). *X. laevis* tadpoles were obtained from the State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-environmental Sciences, Beijing, China.

Tadpoles were not fed before the experiment and were acclimated for 24 h under controlled laboratory conditions in charcoal-filtered tap water at 24 ± 1 ℃ and 12/12 h light/dark cycle. According to the Nieuwkoop and Faber system (Johnson, 1968), acute toxicity experiment was implemented when tadpoles were around stage 45–46.

2.2. Acute toxicity study

Tadpoles were randomly selected around stage 45–46 and placed in a 500 ml beaker containing 500 ml of filtered solution. According to the pre-experiment, tadpoles were exposed to 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mg/L of metamifop. The control specimens were exposed to the same amount of acetone than the exposed specimens. The concentration of acetone added to the control and treatment groups was 0.01% (v/v), and the level used was below recommended level by the American Society for Testing Materials (ASTM). Each treatment contained three replicate tanks with ten tadpoles per replicate. Exposure solutions were renewed every day. After 96 h of exposure to metamifop, mortality was recorded to calculate the LC_{50} value.

2.3. Chronic toxicity study

To study the chronic toxicity of metamifop to *X. laevis* tadpoles, 60 tadpoles around stages 45–46 were randomly selected and put into a glass tank containing 20-L of test solution. One-tenth of the 96 h-LC_{50} value (0.063 mg/L) was selected as the exposure concentration of the chronic toxicity test. A blank solvent control group was set up. Each treatment was performed in triplicate, and the test solutions were renewed every 3 d. Tadpoles were fed with commercial food (Totoro Supplies, Hong Kong, China) twice daily, under the previously described conditions. Behavioral endpoints of tadpoles of each treatment group were observed after 14, 21 and 35 d of exposure. The average body weight, and triglyceride (TG), total cholesterol (T-CHO), neurotransmitter contents, TH contents, gene expressions and antioxidant enzyme activities of tadpoles were measured after anesthetizing with MS-222 (100 mg/L).

2.4. Behavioral observation

Behavioral biomarkers were recorded according to the method described by Denoël et al. (2012), which are described in the Supplementary material.

2.5. Quantification of TG and T-CHO contents

Ten tadpoles from each tank were collected after 14, 21 and 35 d of exposure for the assay (N = 30). After anesthetizing with MS-222, tadpoles were homogenized with phosphate buffer (5.0 mmol/L, pH = 7.8) and centrifuged at 10,000 g for 10 min at 4 ℃. The supernatant was used for the determinations. Bovine serum albumin was used as a standard to determine the total soluble protein concentration. The contents of TG and T-CHO were assayed by test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and measured as described in the Supplementary material.

2.6. Determination of antioxidant enzyme activity and MDA

According to the manufacturer's instructions, activities of superoxide dismutase (SOD) and catalase (CAT) and MDA content were determined by test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and measured as described in the Supplementary material.

2.7. Determination of neurotransmitter contents

The contents of glycine (Gly) and γ-aminobutyric acid (GABA) in tadpoles were determined by an enzyme-linked immunoassay assay (ELISA) kit purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) according to the instructions in the Supplementary material.

2.8. Determination of TH

The contents of T3 and T4 were determined by an ELISA kit (CloudClone Corp. , Wuhan , China) according to the manufacturer's instructions, and the measurements were performed as described in the Supplementary material.

2.9. Relative expression of genes

After 14, 21 and 35 d of exposure, five tadpoles from each tank (N = 15) were selected to determine the relative expression of genes.

Total RNA of tadpoles was extracted using TRNzol Universal reagent (Tiangen Biotech Co., Ltd., Beijing, China), following the manufacturer's instructions. Total RNA concentration was calculated from the absorbance at 260 nm using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). To ensure RNA purity, the quality was verified by gel electrophoresis using 1% agarose gel, and RNA samples with OD260/OD280 between 1.7 and 2.0 were used for complementary DNA (cDNA) synthesis. cDNA was generated from 1 μg total RNA using FastKing RT kit (with gDNase) (Tiangen Biotech Co., Ltd., Wilmington, DE, USA). Gene expression analysis was conducted as described in the Supplementary material. All operations were performed according to the manufacturer's instructions.

2.10. Data analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0. LC_{50} and associated 95% confidence intervals were calculated using a probit equation. According to (Livak and Schmittgen, 2001), the relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method. For body weight, the frequency of behavioral endpoints, relative gene expression, neurotransmitters, TG, T-CHO and TH contents and antioxidant enzymes activities, statistical differences between treatment groups were compared by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) post-hoc analyses. Statistical significance was established at the $P < 0.05$ level. All data were presented as mean ± standard deviation (SD).

3. Results and discussion

3.1. Acute toxicity

As shown in Table 1, the 96 h-LC_{50} value of metamifop on *X. laevis* tadpoles was 0.634 mg/L, indicating that metamifop was highly toxic to the non-target aquatic organisms, including *X. laevis* tadpoles. A previous study displayed that the 96 h-LC_{50} values of metamifop on zebrafish embryos and larvae were 0.648 and 0.216 mg/L, respectively (Zhao et al., 2019). Zhu et al., (2015, 2017) studied the acute toxicity of other aryloxyphenoxy propionate pesticides to aquatic organisms, and the 96 h-LC_{50} values of quizalofop-p-ethyl and cyhalofop-butyl on zebrafish embryos were 0.23 and 0.57 g/ml respectively (Zhu et al., 2017, 2015). These results indicated that aryloxyphenoxypropionate pesticides, such as metamifop, quizalofop-p-ethyl and cyhalofop-butyl, were highly toxic to aquatic vertebrates at the early life stages.

Table 1
96 h-LC_{50} value of metamifop to tadpoles.

Concentrations (mg/L)	Mortality values (%)	LC_{50} (mg/L)[a]	Confidence intervals (mg/L)[b]	R^{2}[c]
0.4	13.33	0.634	0.590–0.897	0.902
0.5	20.00			
0.6	43.33			
0.7	60.00			
0.8	73.33			
0.9	83.33			

[a] LC_{50}: the lethal concentration that causes 50% mortality compared with the control group.

[b] 95% confidence intervals.

[c] Correlation coefficient.

3.2. Body weight and lipid metabolism

After 35 d of exposure, the weight of tadpoles in the treatment group decreased by 40.82% compared with the control group. TG contents at 14, 21 and 35 d after exposure were significantly lower than those of the control group by 17.27%, 46.14% and 57.20%. T-CHO contents at 14, 21 and 35 d after exposure were significantly lower by 41.48%, 50.28% and 66.41%, respectively (Fig. 1). The main fatty tissue in tadpoles is the fat body, which has the highest lipid content compared with other

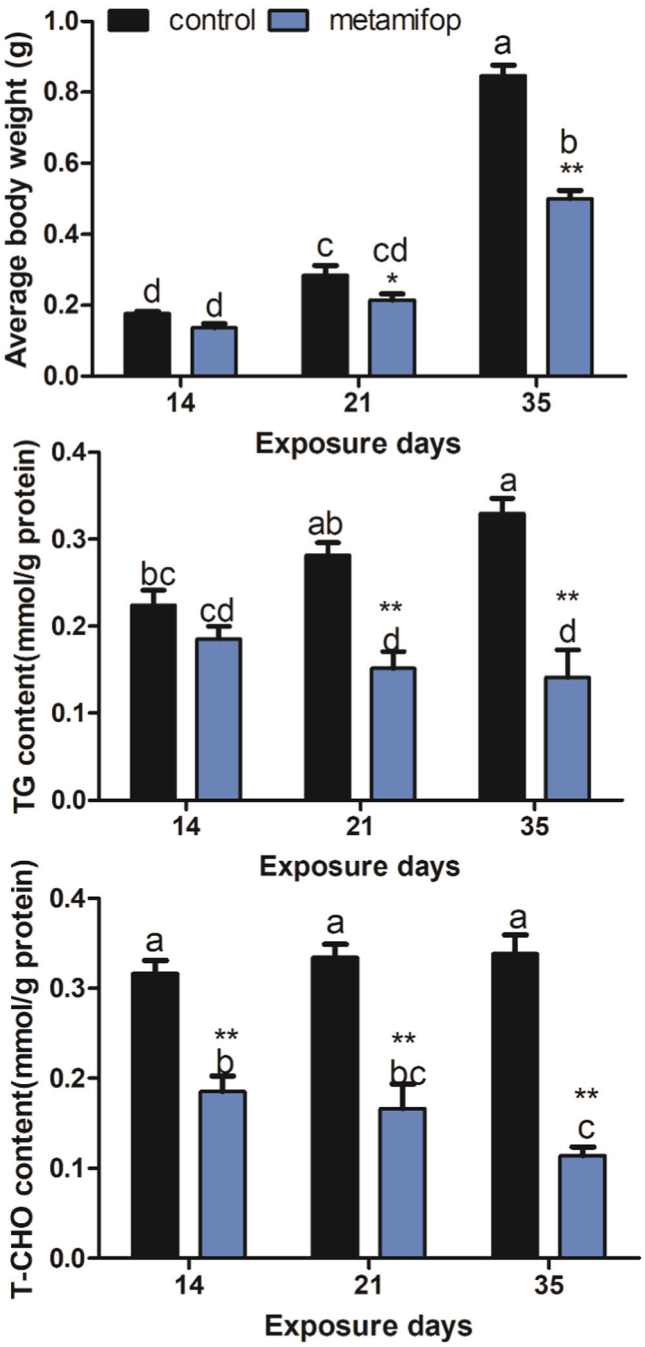

Fig. 1. Average body weight, TG and T-CHO contents in *X. laevis* tadpoles exposed to metamifop for 14,21 and 35 days (N = 3). abc: different letters represent statistically significant differences (SNK) at $P < 0.05$, whereas the same letter indicates no significant difference. * Denotes significant difference between control and treatments at $P < 0.05$, ** denotes significant difference between control and treatments at $P < 0.01$. Error bars indicate the standard deviation.

tissues (Wright et al., 2011). The amount of fat reflects body shape and physical condition. Both TG and T-CHO are the main components of lipids, and their lower levels may lead to the weight loss of tadpoles. Lipid metabolism disorder affects the energy metabolism of tadpoles during metamorphosis and adversely affect their development (Bender et al., 2018). We inferred that metamifop could inhibit lipid accumulation and lead to weight loss, which was time-dependent. In our study, the decrease in tadpole weight, TG and T-CHO could also be explained by the decrease in foraging (Table S2). Similar to our results, after 28 d of exposure to 10 mg/L triadimefon, the weight of pond frog (*Rana nigromaculata*) tadpole was significantly lower than the control group (Zhang et al., 2018). Compared with the control group, the butter frog (*Leptodactylus latrans*) tadpoles in glyphosate and 2,4-D mixture treatments showed shorter length and lower mass (Pavan et al., 2021). In this study, the decrease in tadpole weight after exposure to metamifop could be due to the disruption of thyroid metabolism (Hersikorn and Smits, 2011). However, further studies are needed to establish the underlying mechanisms.

3.3. Behavioral endpoints

Tadpoles exposed to metamifop showed more behavioral abnormalities (Table S2). Metamifop increased the percentages of tadpoles swirling. After 35 d of exposure, the swirling percentage in the treatment group (13.8 ± 4.9) increased by 84.00% compared with that of the control group (7.5 ± 2.6). After exposure to metamifop, the side-lying significantly increased. The lateral side-lying or swirling caused by the lack of correct balance posture is a biomarker for detecting the sublethal effects of neurotoxic chemicals (Denoël et al., 2012). Metamifop inhibited the feeding of tadpoles, which was not obvious at 14 d of exposure, but the inhibitory effect became more significant with the increase of exposure time. We found that the frequency of air-breathing of tadpoles increased significantly in the metamifop treatment group. A previous study reported that the stress of chemicals increased air-breathing in Perez's frog (*Pelophylax perezi*) tadpoles (Egea-Serrano et al., 2011). Metamifop significantly increased the activity of tadpoles after 35 d of exposure, and the activity of the treatment group (28.8 ± 7.5) was 285.15% of that of the control group (10.1 ± 2.9). After exposure to 0.063 mg/L metamifop, tadpoles tended to use more peripheral area, which might be due to tadpoles staying in the surrounding area of the tank to avoid the pesticide solution. Tadpoles behaved abnormally after exposure to pesticides. David et al. (2012) studied the behavioral changes of tadpoles induced by cypermethrin at sublethal concentrations, including twisting, writhing, uncoordinated swimming and avoidance (David et al., 2012). After exposure to 1.5 mg/L of malathion, tadpoles exhibited abnormal behavior. They lied inverted on their dorsal surface by quickly turning over the backs (David and Kartheek, 2015). These behavioral results confirmed the validity of these six endpoints in ecotoxicology studies. In our study, tadpoles were excited after exposure to metamifop. Compared with the control group, tadpoles in metamifop treatment group showed abnormal behavior and increased activity.

3.4. Neurotransmitters

Amino acids, such as Gly and GABA are considered to be important neurochemicals. As the main inhibitory neurotransmitters, Gly and GABA increase the permeability of chloride ions, hyperpolarize nerve and muscle cells, interfering with the transmission of neuromuscular and lead to death (Cully et al., 1994; Mellin et al., 1983).

Compared with the control group, the contents of Gly and GABA in tadpoles treated with metamifop decreased significantly (Fig. 2), and with the increasing of the exposure time, the contents of the two inhibitory neurotransmitters gradually decreased. After 35 d of exposure, the contents of Gly and GABA decreased by 54.69% and 49.74% compared with the control group, respectively. After inhibition, they

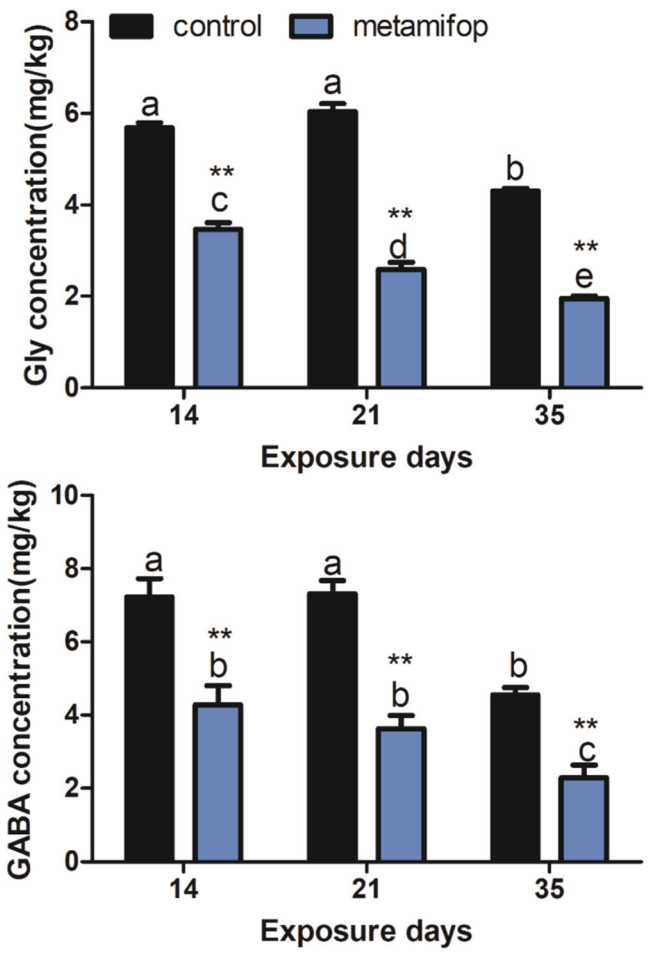

Fig. 2. Gly and GABA concentrations in *X. laevis* tadpoles exposed to metamifop for 14,21 and 35 days (N = 3). abc: different letters represent statistically significant differences (SNK) at $P < 0.05$, whereas the same letter indicates no significant difference. * Denotes significant difference between control and treatments at $P < 0.05$, ** denotes significant difference between control and treatments at $P < 0.01$. Error bars indicate the standard deviation.

could not effectively prevent the excessive excitatory discharge of main neurons, which leads to increased muscle contraction, exercise and oxygen consumption and provides a reasonable explanation for the increase of activity and air-breathing of tadpoles in the metamifop treatment group. Low environmental concentrations of pesticides can change the content of neurotransmitters, hyperpolarized nerves and muscle cells of amphibians and affect neuromuscular transmission, resulting in behavioral disorders (Novelli et al., 2012; Zortéa et al., 2017).

3.5. Levels of antioxidant enzyme

Compared with the control group, the activity of T-SOD in tadpoles increased after exposure to metamifop (Fig S2). The activity of T-SOD in the treatment group increased significantly by 20.55% and 65.69% compared with the control group after 21 and 35 d of exposure. Similar to the results of T-SOD activity, the CAT activities showed a time-dependent increase in the metamifop treatments. The CAT activities significantly increased at 14 d and reached a maximum level at 35 d, with an activity increase of 135.82%. MDA content in metamifop treatment group was higher than that in the control group and was not significant at 14 and 21 d. After 35 d of exposure, the content of MDA in tadpoles increased significantly, which was 135.82% of the control group.

Exposure to pesticides may cause oxidative stress in aquatic animals by producing reactive oxygen species (ROS) and affecting their growth and development (Pašková et al., 2011). SOD and CAT are two important antioxidant enzymes that control excess free radicals. Compared with the control group, SOD and CAT activities significantly increased after exposure to metamifop, which might protect against free radical stress induced by metamifop. MDA is an indicator of oxidative stress and lipid peroxidation level. It is a kind of membrane lipid peroxidation produced by ROS (Chaoui et al., 1997). After exposure to metamifop for 35 d, MDA content significantly increased in the treatment group compared with the control group. Under stress caused by pesticides for a long time, SOD and CAT antioxidants might not prevent oxidative damage by ROS but lead to the peroxidation of membrane lipids. Similar to our findings, metamifop induced oxidative stress in zebrafish embryos, with increased contents of ROS and MDA (Zhao et al., 2019).

ROS plays an important role in TH synthesis (Denef et al., 1996). Excessive accumulation of ROS could induce cell destruction and inflammation (Wollman and Breitman, 1970). Chlorpyrifos-ethyl had an endocrine-disrupting effect on Nile tilapia (*Oreochromis niloticus*), which may be caused by oxidative stress (Oruç, 2010). It could be inferred that changes in oxidative stress levels might also affect thyroid development and its relative gene expression level. Considering the changes in oxidative stress induced by metamifop, it is necessary to investigate the effects of metamifop on thyroid development and related gene expression levels in tadpoles.

3.6. Levels of TH

During the metamorphic development of amphibians, the HPT axis can regulate the remodeling of multiple tissues and organs, such as leg bud formation, tail absorption, craniofacial and digestive system remodeling (Miyata and Ose, 2012). The main function of HPT axis is to maintain the normal circulation level of THs (mainly T3 and T4). The system is related to each other through hormones. These important hormones include: T4 and T3 synthesized and secreted by the thyroid, thyroid stimulating hormone (TSH, encoded by *tshα* and *tshβ*) synthesized and secreted by the pituitary gland and TSH releasing hormone secreted by hypothalamus. T4 is the major product released from the thyroid gland (Fernandez et al., 2018).

As shown in Fig. 3, that metamifop had an inhibitory effect on the contents of T3 and T4. After 35 d exposure to metamifop, the contents of T3 and T4 in tadpoles significantly reduced by 61.01% and 58.64% compared with the control group. In this study, the decrease of T3 and T4 levels could explain the growth retardation and weight loss of tadpoles. Zortéa et al. (2017) reported that triadimefon changed TH levels in zebrafish (Zortéa et al., 2017). However, to date there has been only a few studies reported the effect of metamifop on the TH contents in tadpoles. Our study is the first report of metamifop inhibiting the synthesis of T3 and T4.

3.7. Relative expression of TH-dependent genes

TSH plays an essential role in regulating the secretion of THs (Ock et al., 2013). After 14 d of exposure to metamifop, the mRNA expression levels of *tshα* and *tshβ* were not significantly different from the control group, but with the prolonged exposure time, the mRNA levels of the two genes significantly increased. Compared with the control group, tadpole *tshα* and *tshβ* mRNA expression levels were significantly up-regulated by 4.09 and 4.89 fold after exposure to metamifop for 35 d, indicating that up-regulation of mRNA level may be a compensatory response to the decrease of TH concentration. The mRNA levels of TSH gene transcription in *X. laevis* were up-regulated after exposure to triadimefon for 21 d (Li et al., 2016b). Liu et al. (2011) reported the decrease of T3 level and the up-regulation of *tshβ* in zebrafish larvae after exposure to triadimefon (Liu et al., 2011).

TH regulates gene expression mainly by combining with TR. TR

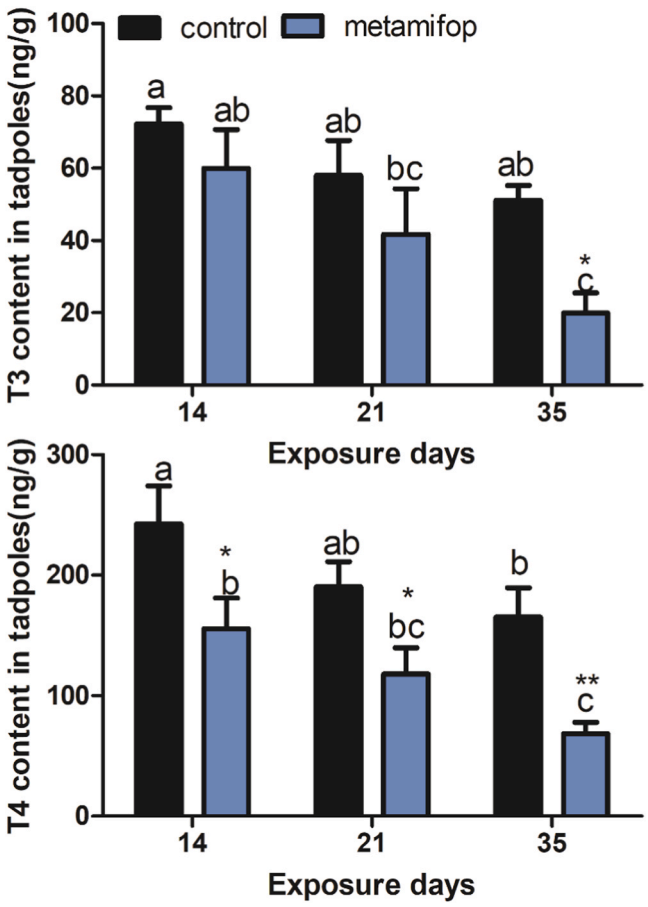

Fig. 3. T3 and T4 levels in *X. laevis* tadpoles exposed to metamifop for 14,21 and 35 days (N = 3). abc: different letters represent statistically significant differences (SNK) at $P < 0.05$, whereas the same letter indicates no significant difference. * Denotes significant difference between control and treatments at $P < 0.05$, ** denotes significant difference between control and treatments at $P < 0.01$. Error bars indicate the standard deviation.

determines the specific action site and mode of TH. There are two subtypes of TR, including TRα and TRβ (encoded by tr α and tr β). These were involved in cell proliferation, differentiation and apoptosis (An et al., 2010). The tr α and tr β mRNA expression levels were shown in Fig. 4. Metamifop significantly inhibited the mRNA levels of tr α and tr β in a time dependent manner. After 35 d of exposure, the mRNA expression levels of tr α and tr β significantly decreased by 6.5 and 12.2-fold, respectively. tr α-knockout tadpoles grew slower than wild-type (Choi et al., 2017). tr β gene mutation had similar developmental defects with hypothyroidism (Forrest et al., 1996). Consistent with our research, after 14 and 21 d of exposure to triadimefon, tr α and tr β expression levels in *X. laevis* were down-regulated (Li et al., 2016b).

TH is activated or inactivated by the deiodination reaction, which is the stepwise removal of iodine from the outer or the inner ring and requires the catalysis of deiodinases (Navarro-MartínLaia et al., 2012). Deiodinases mainly include type 2 deiodinase (DIO2; encoded by *dio2*) and type 3 deiodinase (DIO3; encoded by *dio3*). The activity of deiodinase is essential for regulating the concentration of TH in tissues and was strongly affected by the concentration of serum TH. The intracellular concentration of T3 was regulated by the activities of DIO2 and DIO3 (St. Germain et al., 2009). DIO2 uniquely activates TH by catalyzing the conversion of T4 to T3 (Xie et al., 2019). Therefore, DIO2 plays a key role in the growth and development of tadpoles. As shown in Fig. 5, the expression of *dio2* in tadpoles was inhibited after 14 d of exposure to metamifop. With the increase of growth time, the expression of *dio2* decreased significantly. After 35 d of exposure, *dio2* expression level was

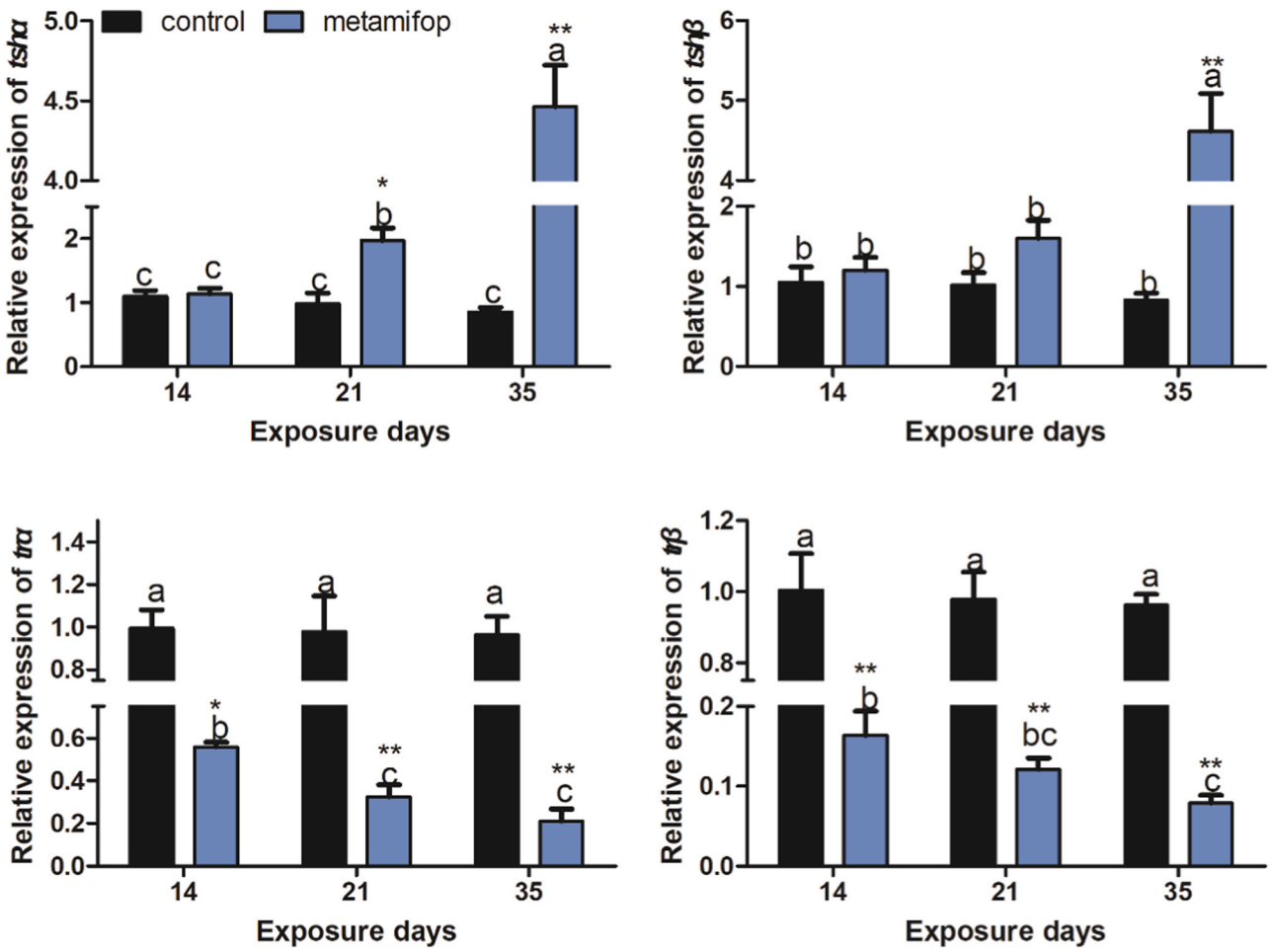

Fig. 4. The mRNA relative expression of thyroid stimulating hormone α (*tshα*), thyroid stimulating hormone β (*tshβ*), thyroid hormone receptors α (tr *α*) and thyroid hormone receptors β (tr *β*) in *X. laevis* tadpoles exposed to metamifop for 14, 21 and 35 days. abc: different letters represent statistically significant differences (SNK) at $P < 0.05$, whereas the same letter indicates no significant difference. * Denotes significant difference between control and treatments at $P < 0.05$, ** denotes significant difference between control and treatments at $P < 0.01$. Error bars indicate the standard deviation.

12.3% of the control group. The results showed that metamifop decreased the expression of *dio2* gene, reducing the transformation from T4 to T3 and causing the decrease of T3 level.

DIO3 inactivates TH by converting T4 into biologically inactive reverse T3 (rT3) and converting T3 into biologically inactive diiodothyronine (T2) (Navarro-Martín et al., 2014). Consistent with the results of *dio2*, metamifop inhibited the expression of *dio3*. With the extension of exposure time, the decrease in *dio3* expression level was increasingly significant compared with the control group. In our study, the down-regulation of *dio2* and *dio3* gene expression in metamifop treatment group was consistent with the low-level of T3 and T4. Therefore, we concluded that metamifop destroyed the HPT regulation at the gene level. That clearly showed the thyroid endocrine-disrupting effect of metamifop in *X. laevis* tadpoles, which might have potentially profound ecological effects.

4. Conclusion

The results indicated that metamifop had high acute toxicity to *X. laevis* tadpoles. After a long period of exposure, metamifop inhibited the synthesis of neurotransmitters, caused abnormal behavior and interfered with fat metabolism, which might affect the normal growth of tadpoles and inhibit body weight. Moreover, the neurotransmitter levels of tadpoles significantly changed after exposure to metamifop, and tadpoles showed abnormal behavior in a time-dependent manner, suggesting that metamifop might cause excitotoxicity to tadpoles. According to the results of antioxidant enzymes, tadpoles exposed to metamifop suffered severe lipid oxidative damage. We also found that compared with the control group, TH levels and relative expression of TH-dependent genes in tadpoles in the treatment group were affected, reflecting the endocrine interference effect of metamifop.

Pesticides are one of a major threat to amphibians, since the larval development of amphibians usually occur in the same season when the pesticides are frequently applied. Besides, amphibians play a key role in transferring energy and nutrients between terrestrial and aquatic ecosystems. Therefore, a comprehensive assessment of the toxicity of pesticides to amphibians is of high importance. Our study highlighted the acute and chronic toxicity of metamifop in amphibians and revealed the potential adverse effects of metamifop on the individual level. Moreover, the present findings enrich our current knowledge of the effect of pesticides on amphibians and highlight the role of pesticides in the global decline of amphibians. These findings provide valuable information for more restrictive regulation of pesticide levels in the aquatic environment to support the maintenance of healthy ecosystems.

CRediT authorship contribution statement

Hongjun Zhang and Jinling Diao conceived the idea of the study, Yinan Qin analyzed the data, Rui Liu and Yinan Qin interpreted the results, Rui Liu wrote the paper. All authors discussed the results and revised the manuscript.

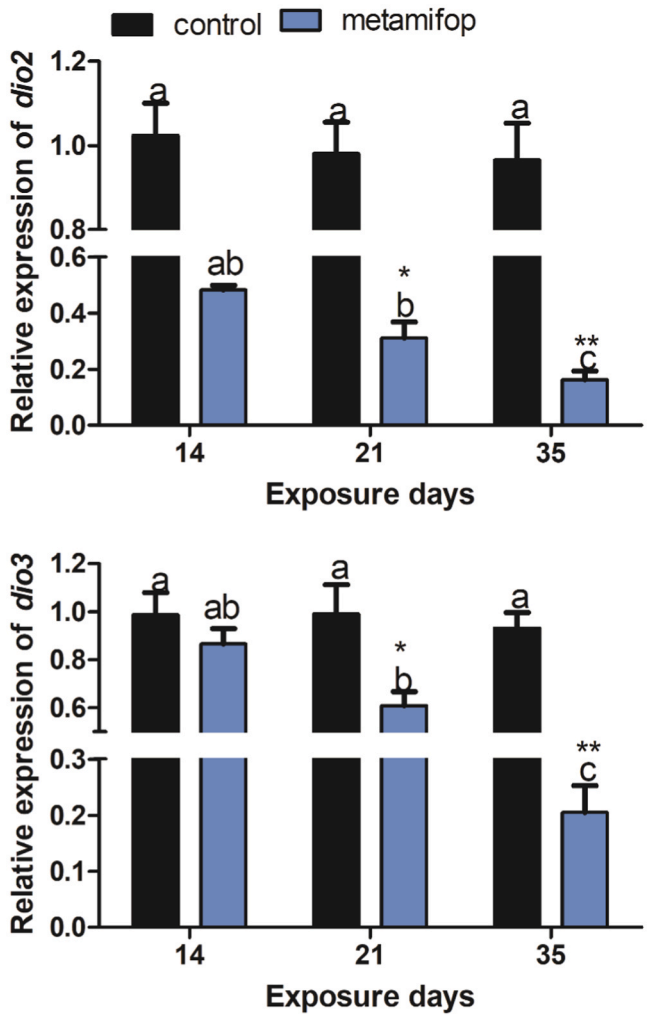

Fig. 5. The mRNA relative expression of type II deiodinase (*dio2*) and type III deiodinase (*dio3*) in *X. laevis* tadpoles exposed to metamifop for 14, 21 and 35 days. abc: different letters represent statistically significant differences (SNK) at $P < 0.05$, whereas the same letter indicates no significant difference. * Denotes significant difference between control and treatments at $P < 0.05$, ** denotes significant difference between control and treatments at $P < 0.01$. Error bars indicate the standard deviation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112417.

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