Effects of exposure to acetochlor on the expression of thyroid hormone related genes in larval and adult rare minnow (*Gobiocypris rarus*)

Wei Li, Jinmiao Zha, Zhaoli Li, Lihua Yang, Zijian Wang*

State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China

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ABSTRACT

Acetochlor is a commonly used herbicide in agricultural environments, which can accelerate T_3 -induced metamorphosis in amphibians. However, effects of acetochlor on the thyroid system in fish are still unclear at environmentally relevant concentrations. In this study, expression profiles of thyroid hormone receptor alpha (α), deiodinase ($d1$ and $d2$), malic enzyme (me), and sodium iodide symporter (nis) genes were determined in larval and adult rare minnow (*Gobiocypris rarus*) after exposure to different levels of acetochlor (20, 200, and 2000 ng/l) for 21 days, respectively. Furthermore, plasma thyroid hormones (THs) levels in adults were evaluated simultaneously. The results showed that $d1$, $d2$, me , and nis mRNA levels were significantly down-regulated in the larvae. Similar expression changes of these genes in female brains were found. However, in adult livers, these gene expressions had no significantly effects, except the α mRNA level significantly up-regulated. These results indicated that exposure to acetochlor could result in tissue-specific alternative expression of TH-related genes in adults. Moreover, the expression of $d2$ and me showed a positive correlation with plasma T_4 levels in female brains. Therefore, larval development and adult brain of rare minnow could be affected by acetochlor at environmentally relevant concentrations.

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1. Introduction

In recent years, a lot of endocrine disrupting chemicals (EDCs) have been released into the aquatic environment (Petrovic et al., 2004). Previous studies confirmed that some EDCs could cause disturbance of thyroid hormone homeostasis, hypothyroidism, and thyroid gland abnormalities in fish (Arukwe and Jenssen, 2005). As a significant portion of EDCs, several pesticides are potential endocrine disrupters, which alter the normal functioning of the endocrine system (Charlier and Plomteux, 2002). The use of pesticides on agricultural land may result in contamination of adjacent surface waters and thus pose a potential risk to a range of aquatic organisms such as fish (Guo et al., 2008). However, studies on disruption of the thyroid system in fish are limited (van der Ven et al., 2006).

Acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-acetamide) is a common herbicide all over the world (Lengyel and Foldenyi, 2003), which has been widely used in China (Ye, 2003). Previous studies have demonstrated the presence of acetochlor in surface water (Hladik et al., 2008), soil (Chao et al., 2007), and sediment (Xue et al., 2005). Measurable concentrations of acetochlor ranged from 0.05 to 2.5 $\mu\text{g/l}$ in surface water (Boyd, 2000;

Hladik et al., 2008; Kolpin et al., 1996). Acetochlor has been classified by the US EPA as a B-2 carcinogen (U.S. EPA, 1994). Furthermore, acetochlor can accelerate T_3 -dependent metamorphosis in amphibians (Cheek et al., 1999; Crump et al., 2002; Helbing et al., 2006). However, the effects of acetochlor alone on the thyroid system of fish are unclear.

The thyroid hormones (THs) triiodothyronine (T_3) and thyroxine (T_4) have a wide range of biological effects in physiological processes of vertebrates (Power et al., 2001). In fish, THs have been implicated as being important regulators in differentiation, growth, metabolism, and salinity adaptation (Crane et al., 2004; Orozco et al., 2002). The effects of exposure to EDCs may be multiple targets with the complex regulatory network of thyroid hormone metabolism and action: TH receptors (TR), mediating gene regulation in response to T_3 (Marchand et al., 2001); deiodinase enzyme (D1 and D2), outer ring deiodination of T_4 to the biologically active T_3 (Orozco and Valverde, 2005); malic enzyme (ME), having a central role providing NADPH for fatty acid biosynthesis lipid metabolism and being a well-characterised endpoint of THs action (Rosebrough et al., 2006); and the sodium iodide symporter (NIS), an integral plasma membrane glycoprotein of thyroid hormone synthesis (Dohan et al., 2003). Previous studies indicated that the gene expressions of TH receptors and deiodinases were sensitive molecular biomarkers for thyroid disruption in fish exposed to environmental contaminants (Picard-Aitken et al., 2007; Scholz and Mayer, 2008). However, little

* Corresponding author. Tel.: +86 10 62849140; fax: +86 10 62929140.
E-mail address: wangzj@rcees.ac.cn (Z. Wang).

is known about the *me* and *nis* genes as potential biomarkers in fish.

Rare minnow (*Gobiocypris rarus*) is considered to be an appropriate species for the assessment of endocrine disrupting chemicals due to its small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Zha et al., 2007). Rare minnow is an ideal model fish in the laboratory. We have been focused on changes of the hypothalamic–pituitary–gonad (HPG) axis in Chinese rare minnow by environment pollutants (Zha et al., 2008; Zhang et al., 2008). However, the potential disruption of hypothalamic–pituitary–thyroid (HPT) axis is largely unknown.

With the advances in molecular approaches, the application of toxicogenomics could be a powerful tool for evaluating the effects and discovering molecular mechanisms underlying toxic response (Nie et al., 2006). Therefore, in this study, we suppose that the expressions of *trα*, *d1*, *d2*, *me*, and *nis* genes can be potential biomarkers to study on disruption of the thyroid system. Our studies aimed at investigating the effects of environmentally relevant concentrations of acetochlor on plasma THs levels and the expression of THs related genes of larvae and adult rare minnow, and discussing the possible molecular mechanisms underlying toxic response.

2. Materials and methods

2.1. Chemicals

Acetochlor was purchased from Sigma–Aldrich Chemical Co. (USA). Stock solution of acetochlor was prepared by dilution in dimethylsulfoxide (DMSO) (Absolute grade), which was purchased from Tedia Company Inc. (Fairfield, OH, USA). To obtain the final concentration for exposure, the appropriate amount of the stock solution was added to the aquarium water via a combination of polytetrafluoroethylene (PFTE) and isoversinic tubes (Abimed, Langenfeld, Germany). A vehicle treatment containing a combination of DMSO served as a control. The ratio of vehicle to water was 1:100,000 (vol/vol).

2.2. Test fish and culture conditions

The brood stock of rare minnow was raised in a flow-through system with dechlorinated tap water (pH 7.2–7.6; hardness 44.0–61.0 mg CaCO₃/l) at a constant temperature (25 ± 1 °C) with a photoperiod of 16:8 h (light:dark) and has been used for testing chemicals in our laboratory for more than 6 years (Zha et al., 2007). Fish were fed a commercial food pellet (Trea, Germany) at a rate of 0.1% body weight per day and newly hatched brine shrimp (*Artemia nauplii*) two times daily.

2.3. Exposure and experimental design

Experiment 1: Newly hatched larvae (*n* = 120) were the offspring from the same pair of brood stock and were randomly divided into four groups. Larvae were exposed to various concentrations (control, 20, 200, and 2000 ng/l; nominal concentrations) of acetochlor for 21 days. Larvae were under flow-through conditions at 25 ± 1 °C with a photoperiod of 16:8 h (light:dark). After 3 days post-hatching, larvae were fed newly hatched brine shrimp (*A. nauplii*) two times daily. Waste and residue were removed daily while the test equipment and chambers were cleaned once a week. After 21 days exposure, larvae were sacrificed and stored at –80 °C until RNA extractions.

Experiment 2: Healthy adult rare minnows (*n* = 120), 5 months old and the offspring from the same pair of brood stock, were randomly divided into four groups. Fish were exposed under flow-through conditions to various concentrations (control, 20, 200, and

2000 ng/l; nominal concentrations) of acetochlor. Fish in each group were sacrificed after 21 days exposure. Blood was collected from each fish in a heparinized microcapillary tube. The samples were immediately centrifuged (8000 × *g*, 10 min, 4 °C), and the plasma were kept frozen at –80 °C until use. In addition, the livers and brains were excised, flash-frozen in liquid nitrogen and stored at –80 °C until analysis.

2.4. Plasma THs measurement

Plasma T₄ and T₃ were measured using commercial radioimmunoassay kits purchased from Beijing North Institute of Biotechnology, China, using sample volumes of 50 μl for T₄ and T₃. The RIAs for T₄ and T₃ were validated for use with rare minnow samples by demonstrating parallelism between a series of diluted and spiked plasma samples in relation to the standard curve. The inter-assay coefficients of variation for both T₄ and T₃ were less than 5% and intra-assay coefficients of variation less than 10%. Cross reactivity between T₄ and T₃ antibodies was less than 0.5%. Assay sensitivities were 3 and 0.25 ng/ml for T₄ and T₃, respectively.

2.5. Real-time PCR

Total RNA was isolated from whole larvae and the livers and brains of adults using Trizol reagent (Life Technology). Then RNA samples were dissolved in ribonuclease-free water and stored at –80 °C until the process of reverse-transcriptase polymerase chain reaction.

The reverse transcription reaction mixtures containing 10 μl of total RNA, 2 μl (0.05 μg/μl) of Oligo(dT)₁₅, and diethyl pyrocarbonate-treated water (a total volume of 12 μl), were heated to 70 °C for 5 min and quickly chilled on ice. After cooling, 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl₂, 2 mM deoxynucleotide triphosphate (10 mM each), 40 units of RNAasin (RNAase inhibitor; Promega), and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) were added to a total volume of 25 μl and incubated for 1 h at 37 °C. Thereafter, the reaction mixture was heated to 70 °C for 10 min to inactivate the reverse transcription.

Real-time PCR was performed in a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 25 μl, consisting of the Brilliant II SYBR Green QPCR master mix, 300 nM forward primer and 300 nM reverse primer. The forward and reverse primers used and PCR products were listed in Table 1. The thermal cycle parameters used were: 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 1 min 57 °C and 30 s at 72 °C. All the samples were analyzed in triplicate and the mean value of these triplicate measurements were used for calculations of mRNA expressions. Results were analyzed according to delta-delta Ct method. The *trα*, *d1*, *d2*, *me*, and *nis* mRNA expressions were normalized for *β-actin* mRNA expression. Dissociation curve analysis was performed for each gene to check the amplification of untargeted fragments. Only one peak was observed for each amplification, indicative for the amplification of the target gene only. Gene expression data are presented as change relative to control animals within the same treatment period.

2.6. Statistics

All statistical analyses were performed with the SPSS (version 13.0; USA). All quantitative data are expressed as the mean ± S.E. of the mean (S.E.M.). Statistical analysis of the data was performed using analysis of variance, followed by Bonferroni's Multiple Comparison Test. A probability of *p* < 0.05 was considered statistically significant.

Table 1

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

Gene	Sequence (5' → 3')	Product size (bp)	Genbank accession no.
<i>β-actin</i>	F: CAGGGCGTGATGGTGGGAT R: GGTGGCTTTGGGGTTGAG	226	DQ539421
<i>trα</i>	F: ATGGACTTGGTCTGGACGA R: ATTGGTGTGGCGATGAGCCT	173	EU725804
<i>d1</i>	F: GTTCAACAGCTTGTCAGGACTTC R: AGCAAGCCTCTCTCCAAGTT	142	EU725801
<i>d2</i>	F: GGATGAGTCGGAAGGTGAA R: CCACACTAAGCAAGCCATT	88	EU725803
<i>ttr</i>	F: TGTATCGGGTGGAGTTTGACA R: GAAGAAGAGCCAGACTGTAA	130	EU725802
<i>nis</i>	F: TGGGTGTAGTGAACGGTCCA R: CGGTTTATTGTGGCGGGAAC	71	FJ624477
<i>me</i>	F: TGTCAGGCTGTGGTGGTGA R: AGGCAACTCTCAGTCCGAT	133	EU744545

Table 2

Growth, gonadosomatic index (GSI) and hepatosomatic somatic index (HSI) of rare minnow at the end of acetochlor exposure.

Concn. (ng/l)	Body length (mm)			GSI (%)		HSI (%)	
	Larvae	Males	Females	Males	Females	Males	Females
Control	12.5 ± 0.5	46.1 ± 2.0	47.9 ± 2.2	4.1 ± 1.0	17.7 ± 2.6	1.0 ± 0.5	1.9 ± 0.6
20	11.5 ± 0.4	45.9 ± 2.0	46.4 ± 2.0	4.4 ± 0.7	15.5 ± 3.4	1.2 ± 1.0	1.9 ± 1.0
200	11.1 ± 0.5	43.6 ± 2.1 ^a	45.7 ± 3.8	3.2 ± 1.1 ^a	17.2 ± 3.4	1.4 ± 0.6	1.5 ± 0.3
2000	11.9 ± 0.5	43.0 ± 2.3 ^a	46.1 ± 1.4	3.8 ± 0.8	19.6 ± 4.5	1.3 ± 0.3	2.1 ± 0.5

Data expressed as mean ± S.E. of each treatment (larvae, *n* = 30; males or females, *n* = 15).^a Statistically significant difference from the control (*p* < 0.05).

3. Results

3.1. The exposure experiment in larvae

3.1.1. Mortality and growth

No mortality was observed in treatment and control groups during the exposure period. At the end of acetochlor exposure, there was no significant difference in growth of larvae (Table 2).

3.1.2. Quantitation of *trα*, *d1*, *d2*, *me*, and *nis* mRNA at the larval stage

The mRNA expressions of *trα*, *d1*, *d2*, *me*, and *nis* in whole larvae were determined by real-time PCR after the 21-day exposure to acetochlor (Fig. 1). The *trα* mRNA levels were significantly increased at 20 ng/l (*p* < 0.05), but significantly decreased at 200 ng/l. The *d1*, *d2*, and *nis* mRNA levels were significantly down-regulated at 200 and 2000 ng/l compared with the control (*p* < 0.05). Moreover, the expression of *me* mRNA was significantly down-regulated only at 2000 ng/l (*p* < 0.05).

3.2. The exposure experiments in adults

3.2.1. Mortality and growth

No mortality was observed in treatment and control groups during the exposure period. The body length of males treated at 200 and 2000 ng/l were significantly lower than those in controls (*p* < 0.05). In contrast, no significant change in body length was observed in females. The GSI of males was significantly affected at 200 ng/l (*p* < 0.05). However, the HSI of females and males at all treatments did not show statistically differences compared to the controls (Table 2).

3.2.2. Plasma thyroid hormone levels

At the end of exposure, plasma *T*₄ levels at 200 and 2000 ng/l were significantly lower than those at the controls (*p* < 0.05)

(Table 3). The plasma *T*₃ levels were significantly decreased at the concentration of 200 ng/l (*p* < 0.05), whereas no significant differences in plasma *T*₃ were observed among other treatment and control groups.

3.2.3. Quantitation of *trα*, *d1*, *d2*, *me*, and *nis* mRNA in livers and brains of adults

In the liver of rare minnow, only *trα* mRNA levels were significantly increased in all treatment groups for both males and females (*p* < 0.05) (Fig. 2), whereas no significant effects for expression of *d1*, *d2*, and *me* mRNA at the end of the exposure (*p* < 0.05).

In the brain of rare minnow, the *d1* mRNA could not be expressed at high levels, whereas the *nis* mRNA expressed at the highest level (data not shown). For males, the *trα* mRNA levels were significantly increased in brains at 20 ng/l, whereas significant decreases were observed at 200 and 2000 ng/l. The *d2* mRNA levels were significantly down-regulated at all treatments. No significant effects were observed in *nis* and *me* mRNA levels compared with the control. For females, the *trα* mRNA levels were significantly increased in brains at 200 ng/l, whereas a significant decrease was observed at 2000 ng/l. The expression of *d2* and *me* were significantly down-regulated at 200 and 2000 ng/l (*p* < 0.01). The expression of *nis* were significantly down-regulated at all treatments. Moreover, the

Table 3Plasma concentrations of *T*₄ and *T*₃ after acetochlor exposure in males and females.

Concn. (ng/l)	<i>T</i> ₄ (ng/ml)		<i>T</i> ₃ (ng/ml)	
	Males	Females	Males	Females
Control	80.1 ± 4.3	101.9 ± 6.3	5.2 ± 0.6	5.8 ± 0.7
20	74.8 ± 5.2	89.9 ± 6.4	4.5 ± 0.5	5.3 ± 0.5
200	37.4 ± 6.4 ^a	66.5 ± 5.8 ^a	2.3 ± 0.5 ^a	3.2 ± 0.4 ^a
2000	21.8 ± 5.3 ^a	69.8 ± 7.4 ^a	4.9 ± 0.7	4.6 ± 0.6

Data expressed as mean ± S.E. of each treatment (*n* = 5).^a Statistically significant difference from the control (*p* < 0.05).

Fig. 1. Relative expression levels of *trα*, *d1*, *d2*, *me*, and *nis* in larvae after 21 days of exposure to acetochlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. Data expressed as mean $\pm$ S.E. of each treatment ($n = 5$). * $p < 0.05$, relative to control.

expression of *d2* and *me* in brains showed a positive correlation with plasma T_4 levels in females ($r = 0.991$, $p = 0.009$; $r = 0.996$, $p = 0.007$). The changes of *trα* mRNA levels in larvae showed a significant positive correlation with those in brains of females ($r = 0.976$, $p = 0.024$).

4. Discussion

Synthetic organic pesticides have been detected frequently in ground water, surface water, aquatic biota and sediment, and atmosphere in decades (Barbash et al., 2001). As a common pre-emergent herbicide all over the world, measurable concentrations of acetochlor can accelerate T_3 -induced metamorphosis in amphibians. In this study, effects of environmentally relevant concentrations of acetochlor on the expression of *trα*, *d1*, *d2*, *me*, and *nis* genes were investigated in larvae and adults rare minnow under laboratory conditions.

Our studies found that there was no significant difference in growth of larvae, whereas the body length of males treated at 200 and 2000 ng/l were significantly lower than those in controls after 21-day exposure to acetochlor (Table 2). Previous studies observed that 10 nM (2.7 μ g/l) acetochlor accelerated T_3 -induced forelimb emergence, but acetochlor alone did not alter metamorphosis of *Rana pipiens* after 7-day exposure (Cheek et al., 1999). Furthermore, no differences in body area of *Xenopus laevis* were observed after

72 h of exposure to 10 nM acetochlor (Crump et al., 2002). These results indicate that acetochlor alone may not affect the development of early life stage of aquatic organisms at environmentally relevant concentrations.

In this study, treatment with acetochlor at 200 and 2000 ng/l significantly decreased plasma T_4 levels in adult rare minnow, as well as decreased plasma T_3 levels at 200 ng/l (Table 3). Acetochlor can increase glucuronidation of THs and decrease THs levels in rodents (Hurley, 1998). In addition, alachlor could significantly decrease circulating levels of T_4 after 7 days and return to control levels after 60 days in male Long-Evans rats at the concentration of 126 mg/kg (body wt/day) (Wilson et al., 1996). Therefore, the present data suggested that acetochlor can decrease circulating THs levels of fish at environmentally relevant concentrations.

In present study, the transcription of *trα* was up-regulated in livers of adults, whereas a non-linear dose-response relationship was observed at different doses of acetochlor in the transcription of *trα* of larvae and brains of adults. The changes of *trα* mRNA levels in larvae showed a significant positive correlation with what observed in brains of females. As was previously reported, the transcript levels of *trα* and *trβ* were increased in tail and brain of *Rana catesbeiana* tadpole after short-term exposure to 10 nM acetochlor (Helbing et al., 2006; Veldhoen and Helbing, 2001). However, the expression of both *trα* and *trβ* mRNA transcripts was not affected by

Fig. 2. Relative expression levels of *tra*, *d1*, *d2*, and *me* in livers of adults after 21 days of exposure to acetochlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. Data expressed as mean $\pm$ S.E. of each treatment (males or females, $n = 5$). * $p < 0.05$, relative to control.

48 h of acetochlor treatment in *X. laevis* tadpole tail tissue (Crump et al., 2002). One possible explanation for the transcription of *tra* levels could be that feedback systems attempt to respond to a disturbance of HPT axis homeostasis. Therefore, further studies are needed to discover the change of THs receptors transcription levels in a long-term exposure experiment.

Iodothyronine deiodinases are important regulators of circulating and intracellular THs levels in fish (Orozco and Valverde, 2005). In present study, the *d1* and *d2* mRNA levels were not significantly changed in the livers of adults (Fig. 2). As was previously reported, the hormone-dependent increase of *d3* expression was slightly attenuated with no effect on the expression of *d2* mRNA by acetochlor (10 nM) treatment in tail tissue of *X. laevis* tadpoles (Crump et al., 2002). These results might mean that T_3 homeostasis is not a compensatory mechanism by increasing *D2* expression. *D3* is of emerging significance for it inactivates both T_4 and T_3 by inner-ring deiodination (Orozco and Valverde, 2005). Although we were unable to measure the change of *d3* mRNA levels in this study, it is more likely that a reduction in the rate of T_3 inactivation may be manifested as a regulation of HPT axis in fish if the mechanisms in *X. laevis* could be applied to fish. In the current study, the *d2* mRNA levels were significantly down-regulated in larvae and brains of adults. Moreover, the profiles of *d2* mRNA levels in brains showed a positive correlation with plasma T_4 levels in females. In fish, hyperthyroidism suppresses *D1* and *D2* activities and expressions of their mRNA while hypothyroidism increases them (Orozco and Valverde, 2005; Van der Geyten et al., 2005). However, the effects of acetochlor treatment on *d2* mRNA and plasma THs levels in brains are contrary to what would be expected. It is more likely that the expression of *d2* mRNA may be a direct gene response in the brain, similar to the *d2* expression induced by T_3 treatment in the tadpole brain (Hogan et al., 2007). Similar results were also obtained in chick brains (Reyns et al., 2003), where *D2* showed a positive correlation with plasma T_4 levels. Therefore, these results indicated that exposure to acetochlor at environmentally relevant concentrations could decrease the gene expressions of

deiodinases in adult rare minnow, and the expressions were tissue-specific.

In this study, the expressions of *me* were not significantly induced in the livers of adults (Fig. 2), whereas decreased in larvae and brains of females (Figs. 1 and 3). Moreover, the profiles in brains showed a positive correlation with plasma T_4 levels in females. Previous studies have shown that hypothyroidism suppresses ME activities and expressions of their mRNA while restoring plasma T_3 increases them in males broiler chickens (Rosebrough et al., 2004). And enzyme activities and mRNA levels of malic enzyme were significantly up-regulated in the livers of rats by T_3 (Dozin et al., 1985). These results may support that malic enzyme is a well-characterised endpoint of THs action (Rosebrough et al., 2006). In addition, previous studies have demonstrated that *me* gene show a pattern of tissue-specific expression, such as in herring tissues (Biegniewska et al., 1990), and in chick embryo fibroblasts (Chung and Goodridge, 1999). The present data suggested that exposure to acetochlor at environmentally relevant concentrations can decrease the gene expression of *me* in adult rare minnow, and the expressions are tissue-specific.

Our studies found that the expressions of *nis* were significantly decreased at the end of acetochlor exposure in larvae and brains of females (Figs. 1 and 3). In extrathyroidal tissues, the highest mRNA levels of *nis* was in the brains of rare minnow (data not shown). In deer mice, the highest *nis* expression level was in the stomach, followed by testes, brain, and large intestine, whereas very low expression of *nis* was observed in the lung, kidney, heart, and liver (Cheng et al., 2007). In human and rat, the *nis* was also expressed and active in extrathyroidal tissues, such as salivary glands, gastric mucosa, brain, glands, and kidney (Dohan et al., 2003). To our knowledge, no data are available to compare with the changes of *nis* mRNA levels in fish. In the thyroid gland of *X. laevis* tadpole, the thyroid-stimulating hormone (TSH) stimulates NIS activity via up-regulation of *nis* mRNA (Opitz et al., 2006). Perchlorate did not significantly increase *nis* gene expression in the kidney and stomach through drinking water for 28 days in deer mice (Cheng et al.,

Fig. 3. Relative expression levels of *tra*, *d2*, *me*, and *nis* in brains of adults after 21 days of exposure to acetochlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. Data expressed as mean $\pm$ S.E. of each treatment (males or females, $n = 5$). * $p < 0.05$; # $p < 0.01$, relative to control.

2007). Present data suggested that exposure to acetochlor could decrease the gene expressions of *nis* in brains of females.

In present study, the observation that down-regulation of THs related genes expression in larvae were found may indicate that normal development of larvae may be affected at environmentally relevant concentration of acetochlor. For adults, the effects of acetochlor treatment on mRNA expression were tissue-specific, and a significant down-regulation was detected only in the brains with little or no effect on the livers. The expression of these genes in females was more sensitive to acetochlor than those of males. Thyroid hormone is essential for brain development. Present data suggested that exposure to acetochlor may affect normal brain development.

In our study, the effects on thyroid function in rare minnow have been throughout investigated after 21 days of exposure to acetochlor. Most researches focused on the short-term exposure, such as 1-day exposure (Veldhoen and Helbing, 2001) and 6-day (Helbing et al., 2006) for *R. catesbeiana* tadpoles, 3-day (Crump et al., 2002) for *X. laevis* tadpoles, and 7-day (Cheek et al., 1999) for *R. pipiens* leopard frog eggs and tadpoles, respectively. Therefore, little is known about the effect on thyroid function in fish at environmentally relevant concentrations of acetochlor long-term exposure, and further studies are needed to examine this issue.

5. Conclusion

The gene expressions of THs related genes in larvae were sensitive to acetochlor. These results indicated that normal development of larvae might be affected at environmentally relevant concentrations of acetochlor. Exposure to acetochlor can decrease circulating TH levels of adults, and result in tissue-specific alternations of THs related genes expression in adults. The present data suggested that exposure to acetochlor may affect normal brain development, especially in females. However, further studies are needed to examine

thyroid function and the effect of acetochlor on fish after long-term exposure.

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