Butachlor causes disruption of HPG and HPT axes in adult female rare minnow (*Gobiocypris rarus*)

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ABSTRACT

Butachlor is a chloroacetamide herbicide widely used in Asia, and may enter the aquatic environment through agricultural application. In this study, plasma VTG and hormone levels (E2, 11-KT, T3 and T4) were determined after the female rare minnow (*Gobiocypris rarus*) was exposed to butachlor at environmental relevant concentrations (0, 0.1, 1, and 10 µg/L) for 40 days. The mRNA levels of the HPG axis-related genes (*gnrh*, *erx*, *vtg*, *star*, *lhr*, *3β-hsd*, *cyp11a*, *cyp17*, *cyp19a* and *cyp19b*), and the HPT axis-related genes (*trx*, *dio1*, *dio2*, and *dio3*) were quantified after 20 and 40 days exposure to butachlor. For the HPG axis, the plasma 11-KT was increased at exposure concentration of 10 µg/L, and VTG was significantly decreased at 1 µg/L. Functional genes like *gnrh* and *cyp19b* in the brains, *star*, *lhr*, *cyp11a*, *3β-hsd*, and *cyp19a* in the ovaries, and *erx* and *vtg* in livers were up-regulated. For the HPT axis, the results showed that plasma T4 levels were significantly increased, the gene expression of *dio1* was up-regulated, *dio2* showed no significant variation, and *dio3* was down-regulated in the livers. These results indicated that butachlor may promote the accumulation of T4 in fish through inactive deiodinase type 3. The transcription of HPG axis-related genes could serve as an auto-regulation of hormone levels after exposure to butachlor. Furthermore, the activation of *gnrh* may play an important role as a feed-back mechanism in the regulation of hormone levels and crosstalk of endocrine axes.

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1. Introduction

The use of pesticides in agricultural areas may result in the contamination of surface water and groundwater and also poses a potential risk to aquatic organisms [1,2]. Pesticides have a wide range of acute and chronic health effects, including enzyme inhibition, oxidative damage, inhibition of neurotransmission, abnormal growth and development, and carcinogenic effect [3,4]. Physiological disorders caused by pesticides have been observed in wild fish including abnormal plasma hormone levels, retardant growth of gonads, and delayed sexual maturation [5,6]. Several pesticides have been found to be potential endocrine disrupting chemicals (EDCs) and to affect the normal endocrine system function in fish and their offspring [7,8].

Butachlor (2-chloro-2,6-diethyl-N-(butoxymethyl)-acetanilide) is a chloroacetamide herbicide that is widely used to control undesired weeds of rice fields in Asia. It is most frequently detected in agricultural soil, surface water, and sediment [9,10]. The concentrations of butachlor range from 0.1 to 1.4 µg/L in surface water

[11,12]. Previous investigations showed that butachlor is toxic to aquatic organisms, and the 48 h LC50 values ranged from 240 to 890 µg/L in fish [13]. Butachlor could induce oxidative stress by depressing the glutathione detoxification system in African catfish (*Clarias gariepinus*) [14]. Reproductive toxicity, carcinogenicity, genotoxicity and cytotoxicity have also been observed in aquatic organisms or mammalian cell lines after exposure to butachlor [15–17]. Butachlor also affects reproductive success and disrupts the sex and thyroid hormone levels in zebrafish [18]. However, little is known about its toxic mechanisms, especially the regulation of hypothalamic–pituitary–gonad (HPG) and hypothalamic–pituitary–thyroid (HPT) axes at environmental relevant concentrations.

The HPG axis regulates gonadal development and reproduction in fish [19]. The principal HPG axis neurohormone secreted by the hypothalamus is gonadotropin releasing hormone (GnRH). Luteinizing hormone receptor (LHR) has been shown to be expressed in the ovary during sexual maturation [20], and it relays the signal from the pituitary to the gonads to mediate the synthesis of sex steroid hormones [21]. Steroidogenic acute regulatory protein (StAR), which is the rate-limiting step in steroid hormone synthesis, regulates the transfer of cholesterol from the outer to the inner mitochondrial membrane, and has been detected in head kidney,

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gonad and other organs [22]. The cytochrome P450c11 (CYP11A) catalyzes the rate-limiting initial step in steroid biosynthesis, and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) is essential for the formation of sex steroids [23]. The cytochrome P450c17 (CYP17) and cytochrome P450c19 (CYP19) are the key enzymes to catalyze androgen biosynthesis, and transforms these androgens to estrogens, respectively [24]. Estrogens induce vitellogenin (VTG) production in the liver of fish through binding to estrogen receptors (ERs) [25]. Moreover, The HPT axis is responsible for maintaining homeostasis of the thyroid hormone (THs) by regulating the synthesis, secretion, transport and metabolism in teleosts. Iodothyronine deiodinases are important regulators of thyroid hormone action in teleosts [26,27]. Three deiodinases have been found in fish: type 1 (Dio1), type 2 (Dio2), and type 3 (Dio3), which control the conversion of T4 to the more active T3 or to the inactive metabolites rT3 and T2 [28]. The biological activity of THs are mediated by TH receptors (TRs) in fish [29]. Exposure to some EDCs can affect the hormones regulation of HPG or HPT axes and lead to a disturbance of hormone metabolism, hormone-regulated cellular or physiological processes in fish. Due to its small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates, rare minnow (*Gobiocypris rarus*) is considered to be an ideal model fish in the laboratory and an appropriate species for the assessment of endocrine-disrupting chemicals [30]. In this study, we detected the changes and regulation of *gnrh*, *star*, *3 β -hsd*, *lhr*, *cyp11a*, *cyp17*, *cyp19a*, *cyp19b*, *erx*, and *vtg* genes mRNA levels in the HPG axis, and *trx*, *dio1*, *dio2*, and *dio3* in the HPT axis of female rare minnow. Our studies aimed to examine the toxicity of butachlor in terms of growth, tissue indices, histological analysis, hormone levels, and mRNA expressions of HPG and HPT axes to elucidate the possible molecular mechanisms underlying toxic responses.

2. Materials and methods

2.1. Chemicals

Butachlor was purchased from Sigma–Aldrich Chemical Co. (USA). A stock solution of butachlor was prepared by dilution in HPLC-grade acetone. Working solutions were freshly diluted by the stock solutions with dechlorinated tap water. To obtain the final concentration for exposure, an appropriate amount of the solutions were added to the aquarium water via polytetrafluorethylene (PTFE) and isoversinic tubes (Abimed, Langenfeld, Germany). The solutions were mixed in vessels with the aquarium water, which flowed through the exposure chambers (18 L) at a rate of 4 L/h. The final concentration of acetone in the aquarium water was less than 0.001%.

2.2. Culture conditions and experimental design

The brood stock of rare minnow was raised in 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 $\pm$ 1 °C) with a photoperiod of 16:8 h (light:dark) and has been used for testing chemicals in our laboratory for more than nine years. Fish were fed with a commercial pellet food (Tetra, Germany) at a rate of 0.1% body weight per day and with newly hatched brine shrimp (*Artemia*) nauplii twice daily. Waste and residue were removed daily, and the test equipment and chambers were cleaned once a week.

Healthy five-month old adult female rare minnows that were offspring from the same pair of brood stock were randomly divided into four groups (30 fish in each group). Fish were exposed under flow-through conditions to various concentrations (control, 0.1,

1, and 10 μ g/L; nominal concentrations) of butachlor for 40 days; each treatment was conducted in triplicate. After 20 days of exposure, liver and ovary samples were taken in each group for isolation of total RNA (5 females). The remaining fish in each group were euthanized after 40 days of exposure. The blood was collected from each fish with a heparinized microcapillary tube and centrifuged immediately at 8000 $\times$ g for 10 min at 4 °C and the plasma stored at –80 °C until analysis. The ovary and liver of each fish were removed and weighed after the blood was collected. The gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as following: GSI = gonad weight (g)/body weight (g) $\times$ 100; HSI = liver weight (g)/bodyweight (g) $\times$ 100. In addition, the brains, livers and ovaries were flash-frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

2.3. Histological analysis

The liver and ovary were fixed in Bouin's solution, dehydrated and processed for paraffin wax embedding. Sections were cut at 3–4 μ m and stained with hematoxylin and eosin (H&E). Five randomly selected separate nonoverlapping microscopic fields were examined with a Zeiss Axioskop 2 mot plus microscope, and the digital images were viewed using Axiovision LE Version software. For ovary analysed, 100 oocytes were scored and the proportion of oocytes at different stage of perinucleolar oocytes, cortical alveolar oocytes, and vitellogenic oocytes calculated.

2.4. Plasma vitellogenin and hormone levels measurement

Plasma vitellogenin (VTG), 17 β -estradiol (E2), 11-ketotestosterone (11-KT), and thyroid hormones (T3 and T4) were measured using the enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Hufeng Chemical Industry Co., China), according to the manufacturer's instructions. All samples were analyzed in duplicate, and a separate standard curve was run for each ELISA plate. The assay detection limits were 30 ng/mL for VTG, 1.5 pg/mL for E2, 7.0 pg/mL for 11-KT, 0.65 ng/mL for T3, and 1.5 ng/mL for T4, respectively. All samples were measured at 450 nm using a Tecan infinite M200 plate reader.

2.5. RNA-isolation and reverse transcription

The total RNA was isolated from the brains, livers and ovaries (5 female fish) using the TRNzol-A⁺ reagent (TIANGEN, Beijing, China) and the manufacturer's protocol. Traces of DNA were removed by incubation with DNase-I (Promega, Madison, WI, USA). Then RNA samples were dissolved in ribonuclease-free water and stored at –80 °C. The reverse transcription reaction mixtures containing 10 μ L of total RNA and 2 μ L (0.05 μ g/ μ L) of Oligo(dT)15 primers were heated to 70 °C for 5 min and quickly chilled on ice. After cooling, 40 units of RNasin (RNase inhibitor; Promega), 200 U of Avian Myeloblastosis Virus Reverse Transcriptase (Promega), and buffer (Promega) were added to a total volume of 25 μ L 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.

2.6. Real-time PCR

Real-time PCR was performed in a MX3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 25 μ L 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 are listed in Table A.1. The thermal cycle parameters used were 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 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 in different tissues. Results were analyzed according to the delta-delta Ct method [31]. The *cyp19b*, *gnrh*, *star*, *lhr*, *3 β -hsb*, *cyp11a*, *cyp17*, *cyp19a*, *trx*, *dio1*, *dio2*, *dio3*, *erx* and *vtg* 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 of the amplification of the target gene only. Gene expression data are presented as the fold change relative to control fish within the same treatment period.

2.7. Statistics

All statistical analyses were performed with the SPSS (version 16.0; USA). All quantitative data were expressed as the mean $\pm$ SEM. A 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, and $p < 0.01$ was considered highly statistically significant.

3. Results

3.1. Mortality and growth

During the exposure period, mortality and malformation were not observed in the control and treatments. The body length was significantly decreased only at an exposure concentration of 10 μ g/L (Table 1). There were no statistically differences in the HSI and GSI between the control and treatments.

3.2. Histological analysis

Several pathological changes were observed in the livers and ovaries of fish after butachlor exposure (Fig. 1). The hepatocytes of control fish were compactly arranged (Fig. 1A). However, the hepatic tissue displayed disordered hepatocyte arrangement and vacuoles in the cytoplasm, cytoplasm degeneration and irregular nuclear shapes were observed in the 10 μ g/L treatment group (Fig. 1B). The ovaries of control rare minnow showed in process of gametogenesis with generations of ova in different developmental stages, and contained vitellogenic oocytes (17%), cortical alveolus stages (32%) and perinucleolar oocytes (30%) (Fig. 1C). In comparison, the major oocytes were vitellogenic oocytes (22%) and cortical alveolus stages (64%), and lower proportion of perinucleolar oocytes (14%) was observed at 10 μ g/L (Fig. 1D).

3.3. Plasma vitellogenin and hormone levels

At the end of the exposure period, plasma VTG levels were significantly decreased at 1 μ g/L (Fig. 2). There was no significant difference in the plasma E2 and T3 levels between treatment and control groups, whereas plasma 11-KT and T4 levels were significantly increased in the 10 μ g/L treatment group after 40 days of exposure to butachlor ($p < 0.05$).

Table 1

Growth, gonadosomatic index (GSI) and hepatosomatic somatic index (HSI) of female rare minnow after 40 days of exposure.

Con. (μ g/L)	Length (mm)	Weight (g)	GSI (%)	HSI (%)
Control	48.32 $\pm$ 3.16	1.36 $\pm$ 0.30	17.95 $\pm$ 3.52	1.95 $\pm$ 0.37
0.1	46.84 $\pm$ 3.26	1.26 $\pm$ 0.33	18.83 $\pm$ 6.23	2.35 $\pm$ 0.43
1	47.89 $\pm$ 3.36	1.32 $\pm$ 0.32	18.04 $\pm$ 6.33	2.34 $\pm$ 0.22
10	45.51 $\pm$ 2.71*	1.17 $\pm$ 0.27	17.42 $\pm$ 6.55	2.13 $\pm$ 0.62

Data expressed as mean $\pm$ SEM of each treatment ($n = 15$).

* Significantly different from the controls ($p < 0.05$).

3.4. Quantitation of genes mRNA in the brains

The expression of *cyp19b* and *gnrh* mRNA levels in the brains of female fish was determined after 20 and 40 days of exposure to butachlor (Fig. 3). The mRNA levels of *cyp19b* genes were significantly up-regulated for all the treatment groups after 20 days of exposure ($p < 0.05$), whereas there was no significant difference in *gnrh* mRNA levels between the treatment and control groups. At the end of exposure, the up-regulation of *cyp19b* and *gnrh* mRNA levels was observed in the brains only in the 10 μ g/L treatment group ($p < 0.01$).

3.5. Quantitation of genes mRNA in the ovaries

The expressions of *star*, *lhr*, *3 β -hsd*, *cyp11a*, *cyp17*, and *cyp19a* mRNA levels in the ovaries of female fish was determined after 20 and 40 days of exposure to butachlor (Fig. 4). The mRNA levels of *star*, *lhr*, *3 β -hsd*, *cyp11a*, *cyp17*, and *cyp19a* genes were significantly up-regulated for all the treatment groups after 20 days of exposure. After 40 days of exposure, the up-regulation of *star*, *lhr*, *3 β -hsd*, *cyp11a*, and *cyp19a* was observed in the ovaries only at 10 μ g/L, whereas the down-regulation of *cyp17* was observed at 0.1 and 1 μ g/L.

3.6. Quantitation of genes mRNA in the livers

The expression of *trx*, *dio1*, *dio2*, *dio3*, *erx*, and *vtg* mRNA levels in the livers of female rare minnow was determined after 20 and 40 days of exposure to butachlor (Fig. 5). The mRNA levels of *trx* and *dio3* genes were significantly down-regulated for all the treatment groups after 20 days of exposure, whereas the expression of *dio1*, *erx*, and *vtg* mRNA levels was significantly up-regulated after 20 days of exposure. There was no significant difference in the expression of *dio2* mRNA levels compared with the control after 20 and 40 days of exposure. At the end of exposure, the up-regulation of *dio1* and *erx* was observed in the livers of female in the treatment groups exposed to more than 1 μ g/L, whereas the down-regulation of *dio3* was observed at 10 μ g/L. Moreover, there were no significant changes in the *trx* mRNA levels after 40 days of exposure. The expression of *vtg* mRNA levels was still significantly up-regulated at all treatment groups after 40 days of exposure.

4. Discussion

The chloroacetamide herbicides include acetochlor, metolachlor, alachlor and butachlor. Some of chloroacetamide herbicides have been confirmed to possess endocrine disrupting properties [32]. For example, acetochlor significantly down-regulates thyroid hormone related genes in larval and tissue-specific alternative expressions in adult rare minnow [33], and metolachlor exposure has been demonstrated to disrupt the thyroid system in medaka [34]. Butachlor has a chemical structure, properties and metabolites that are similar to other chloroacetamide herbicides [35,36]. In this study, the effects of environmentally relevant concentrations of butachlor on the histological changes in the livers and gonads, plasma VTG and hormone levels, and expressions of HPG and HPT axis-related genes mRNA levels were investigated in adult female rare minnow under laboratory conditions.

In this study, no mortality or malformation was observed throughout the experimental period. However, the body lengths of fish at 10 μ g/L were less than controls after 40 days of exposure. Regarding growth, the toxic threshold of butachlor should be approximately 10 μ g/L, or at environmental levels. In previous work, exposure to butachlor at 100 μ g/L did not affect the HSI or GSI of female zebrafish after 30 days [18], and our results also

Fig. 1. Light micrographs of histology in adult female rare minnow after 40 days of exposure to butachlor, stained with hematoxylin and eosin: (A) normal female hepatic tissue; (B) female hepatic tissue in the 10 µg/L treatment group, it showed vacuoles in cytoplasm (black arrow), cytoplasmic degeneration (square) and irregular shaped nuclear (white arrow); (C) normal ovary showing ova in different development stages; (D) ovary from 10 µg/L butachlor treatment group showing abundant of Voc and Coc, and limited number of Poc. Voc, vitellogenic oocyte; Coc, cortical alveolus stage; Poc, perinucleolar oocyte.

showed that exposure to butachlor below 10 µg/L did not affect the HSI or GSI of female rare minnow after 40 days. The histopathological assessment of fish tissue has been used as a warning sign for diseases caused by exposure to environmental pollutions [37,38]. As shown in Fig. 1, vacuoles in the cytoplasm, cytoplasmic degeneration and irregular nuclear shapes were observed in the livers. Meanwhile a decrease in primary growth oocytes and an increase in maturing oocytes were observed at the 10 µg/L in the ovaries. These results suggested that butachlor could induce hepatic tissue damage and potentially have adverse effects on the fertility of the fish.

Moreover, sex steroid hormones play an important role in sex differentiation, sexual development, and normal reproduction in fish [39,40]. In the present study, plasma 11-KT levels were significantly increased in the 10 µg/L treatment group. There was a slight increase of plasma E2 in the exposure groups, but the increase was not statistically significant between the exposure and control groups. Previous studies have demonstrated that alachlor and acetochlor may partially mimic the primary endogenous estrogen and bind to estrogen receptors (ERs) [41,42]. According to these results, butachlor might imitate the function of estrogen in fish and lead to potential accumulation of E2. Moreover, estrogens induce vitellogenin (VTG) production in the liver of fish by binding to ERs and exerting its full biological effects [43]. In the current study, plasma VTG levels were significantly decreased in the 1 µg/L treatment group. It is reported that hepatically produced VTG is transported via the bloodstream and sequestered by the developing oocytes [44]. Whatever, the decrease of plasma VTG might be caused by the consumption during ova development. The up-regulation of *erx* and *vtg* mRNA levels was observed in all treatment groups after 20 and 40 days of exposure in the livers. It is reported that the expression of *erx* and *vtg* demonstrated a

significant relationship after E2 exposure in the liver of mud carp [45]. In female zebrafish, the increase of plasma E2 was accompanied by up-regulated hepatic *erx* and *vtg* expression after exposure to fluorotelomer alcohol (6:2 FTOH) [46]. Therefore, the present data suggested that butachlor could disrupt the sex steroid hormonal balance and function in fish at environmentally relevant concentrations.

Sex steroid hormones, testosterone and 17β-estradiol are produced from cholesterol through a series of reactions. The ovaries play a main role in producing steroid hormones for sexual and reproductive functions, as well as eggs produced for fertilization [47]. In the present study, the mRNA levels of *star*, *lhr*, *3β-hsd*, *cyp11a*, *cyp17*, and *cyp19a* in the ovaries, and *cyp19b* in the brains were significantly up-regulated for all the treatment groups after 20 days of exposure. The significant up-regulation of mRNA levels was observed only in the 10 µg/L treatment group after 40 days of exposure, not including *cyp17*, which was down regulated in the low-dose groups. Similarly, the expression of *cyp19b* was significantly increased after the exposure of zebrafish larvae to 10nM E2 [48]. The patterns of HPG axis gene expression suggested that butachlor may disrupt the sex steroid steroids synthesis of aquatic organisms at environmentally relevant concentrations.

THs are essential for the developmental and physiological functions in vertebrates. The plasma T4 levels at 10 µg/L were significantly higher than those of the control after butachlor exposure. Previous studies have shown that plasma T3 and T4 concentrations were increased after exposure to 100 µg/L butachlor in female zebrafish after 30 days of exposure [18]. Our data demonstrated that the up-regulation of *dio1* and down-regulation of *dio3* were observed in the livers, whereas there was no significant difference in the expression of *dio2* mRNA levels of females after 20 and 40 days of exposure. Similar results were obtained from the tail

Fig. 2. Plasma concentrations of hormone levels and vitellogenin after 40 days of exposure in female rare minnow.

Fig. 3. Relative expression levels of *cyp19b* and *gnrh* in the brains after 20 and 40 days of exposure to butachlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. * $p < 0.05$, # $p < 0.01$, relative to control.

tissue of *Xenopus laevis* tadpoles after acetochlor (10 nM) treatment, the hormone-dependent increase of *dio3* expression was decreased, and there was no effect on the expression of *dio2* mRNA [49]. The biological activity of THs is predominantly mediated at the level of gene expression by binding to TH receptors in fish. Our results showed that a down-regulation of *trα* mRNA levels occurred after 20 days of exposure but returned back to their normal levels, similar to those of control, at the end of exposure. As was previously reported, acetochlor does not bind to human TR β [50]. Taken together, these results indicated that butachlor could disrupt the homeostasis of THs via non-receptor pathway. The

accumulation of T4 in fish may be due to inhibit inner deiodination through inactive deiodinase type 3.

The HPG axis is the primary regulator of the mature reproductive system, and consists of neurons in the hypothalamus that secrete gonadotropin releasing hormone (GnRH). The expression of *gnrh* has been implicated in hypophysiotropic functions, development, and sexual behavior [51]. Previous studies indicated that there was a correlation between thyroid activity and GnRH levels [52]. In the present study, T4 and *gnrh* mRNA levels were up-regulated in the 10 µg/L treatment group after 40 days of exposure in the plasma and brain, respectively. It has been reported that

Fig. 4. Relative expression levels of *star*, *lhr*, *cyp11a*, *3β-hsd*, *cyp17* and *cyp19a* in the ovaries after 20 and 40 days of exposure to butachlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. * $p < 0.05$, # $p < 0.01$, relative to control.

Fig. 5. Relative expression levels of *trx*, *dio1*, *dio2*, *dio3*, *era* and *vtg* in the livers after 20 and 40 days of exposure to butachlor. The results were evaluated as the relative ratio of the expression level of each mRNA to that of β -actin. * $p < 0.05$, # $p < 0.01$, relative to control.

gnrh3 was significantly up-regulated in the brains of female rare minnow, whereas ovarian P450 aromatase was down-regulated in the 15 μg/L bisphenol A (BPA) concentration group after 35 days of exposure [53]. One possible explanation for the regulation between the HPT and HPG axes could be that the increase of T4 initiates the transcription of *gnrh* through the feed-back mechanism, which leads to alternation of sex hormone metabolism. The activation of *gnrh* results in changes in hormone levels and the transcription of HPG-related genes. The present data suggested that the activation of *gnrh* may play an important role as a feed-back mechanism in the regulation of hormone levels and in the crosstalk between endocrine axes. Moreover, the THs may also play critical

roles in normal reproduction, sex steroids levels, and expression of steroidogenic enzyme genes [54]. The previous studies have demonstrated that T4 administration in female catfish enhanced the growth rate of ovarian follicles and entry of oocytes into the maturational phase compared with controls in the preparatory phase [55]. In the present study, the increase of maturing oocytes accompanied by an increased in plasma T4 were also observed in the 10 μg/L treatment group. The mRNA levels of HPT or HPG axis-related gene were returned to the control levels at lower treatment groups after 40 days of exposure, which might indicate a self-adjustment of endocrine system in fish to butachlor exposure. Overall, the mechanisms for the endocrine system involve

complex arrangements of interacting axes, which are largely unknown and will require further experimentation.

5. Conclusion

In this study, the histological changes, plasma VTG, hormone levels, and the expressions of HPG and HPT axis-related gene mRNA levels were determined in adult female rare minnow after exposure to butachlor. The results indicated that butachlor could induce hepatic tissue damage and potentially have adverse effects on fertility of the fish at the tissue levels. Sex steroid synthesis, homeostasis, and regulation of the HPG axis could be disrupted in the fish. Butachlor may disrupt the thyroid hormonal balance of HPT axis via inactive deiodinase type 3. The activation of *gnrh* may play an important role as a feed-back mechanism in the regulation of hormone levels and the transcription of HPG-related genes. However, further studies should be performed with low-dose longer exposure to study the interaction between the endocrine axes.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The [Transparency document](#) associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cbi.2014.07.016>.

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