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Impact of herbicide pretilachlor on reproductive physiology of walking catfish, *Clarias batrachus* (Linnaeus)

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Abstract Herbicide pretilachlor is widely used in paddy fields to control annual weeds. The present study has been carried out in walking catfish, *Clarias batrachus*, to evaluate the impact of herbicide pretilachlor on reproductive physiology after chronic exposure. Based on the median lethal concentration value (96 h), fish were exposed to three nominal test concentrations of pretilachlor ((SL-I (1/20th LC_{50}), SLII (1/15th LC_{50}), and SL-III (1/10th LC_{50})) for 30, 45, and 60 days after which plasma sex steroid profile, plasma vitellogenin concentration, and gonadal aromatase activity were analyzed in both sexes. Plasma concentration of testosterone decreases in herbicide-exposed male fish. Significant increase in plasma 17β-estradiol, plasma vitellogenin concentration, and gonadal aromatase activity were observed in herbicide-exposed male fish. All these alterations in reproductive parameters in male fish are dependent on concentration and exposure duration of herbicide. On the other hand, significant decrease in plasma concentration of testosterone was observed in female fish which was also dependent on concentration and exposure duration of herbicide. No significant changes in plasma 17β-estradiol concentrations, plasma vitellogenin concentration, and gonadal aromatase activity were observed in female fish. Above findings clearly suggested that herbicide pretilachlor acts as endocrine disruptor in fish and affects overall reproductive physiology of fish, but its ability to induce reproductive toxicity in male and female differs considerably.

Keywords Pretilachlor · *Clarias batrachus* · Testosterone · 17β-estradiol · Vitellogenin · Gonadal aromatase activity

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Introduction

Herbicides are mostly used in agricultural fields to remove unwanted plants. From agricultural fields, they may enter nearby water systems and impose harmful effects on fish including altered reproductive physiology. Herbicide reduces the reproductive capacity of fish by creating hormonal imbalance (Shioda and Wakabayashi 2000), by disrupting sex steroid metabolism (Moore and Waring 1998) and by altering normal function of hypothalamic pituitary gonadal axis (Li et al. 2009).

The chloroacetamide herbicide pretilachlor is widely used in paddy field. It inhibits cell division in herbs by interfering with the normal process of fatty acid synthesis (Kaushik et al. 2006). Chloroacetamide herbicides have been detected in surface water (Hladik et al. 2008), soil (Chao et al. 2007), and sediments (Xue et al. 2005). Some of them are suspected carcinogens (Coleman et al. 2000). They are found to be toxic for fish by several authors from time to time. Butachlor disrupts thyroid and sex steroid endocrine systems in zebra fish (Chang et al. 2013), induces genotoxicity in *C. batrachus* (Ateeq et al. 2002), causes irregular protrusion of the

eyes and irregular swimming in *R. rutilus caspicus* and *S. lucioperca* (Mohammad and Hedayati 2017), and is responsible for the developmental toxicity, endocrine disruption, and immune toxicity in the zebrafish embryo (Tu et al. 2013). Pretilachlor induces 100% mortality in *Gambusia* at concentration of 25 mg/L (Sadeghi and Imanpoor 2013), increases plasma vitellogenin concentration in Chinese rare minnow (Zhu et al. 2014), induces oxidative stress and disrupts the endocrine system in zebra fish (Jiang et al. 2016), and alters the behavioral response of *Clarias batrachus* (Soni and Verma 2018). However, no work has been done so far to evaluate the reproductive toxicity of herbicide pretilachlor on walking catfish, *C. batrachus*, which is needed for environmental risk assessment.

Determination of plasma sex steroid level, plasma vitellogenin (VTG) concentration, and gonadal aromatase activity can be utilized to evaluate the reproductive toxicity caused by xenobiotics. Testosterone (T) and 17-β estradiol (E_2) play an important role in normal reproductive physiology of fish (Lubzens et al. 2010), so their changed plasma concentration can adversely affect the overall reproductive process. VTG is a yolk precursor protein which is synthesized in the liver under the influence of ovarian estradiol, and its increased concentration in male fish is an indication of their exposure to estrogenic endocrine disruptors (Flammarion et al. 2000). The enzyme aromatase converts T to E_2, and its altered activity may disrupt the normal reproductive physiology of fish (Dessì-Fulgheri and Lupo 1982).

Most of the guidelines recommend the use of measured or environmentally relevant concentrations of herbicides for toxicological analysis (OECD 1992; ASTM 2007). The measured concentration of pretilachlor has been reported up to 0.006 mg/L in aquatic systems (Nakano et al. 2004). But in the present study the nominal concentration of herbicide based on the obtained LC_{50} value (96 h) from our earlier study was used for toxicity evaluation (Soni and Verma 2018). The selection of such nominal concentrations is based on the fact that we want to establish walking catfish, *C. batrachus*, as an eco-toxicological model for evaluation of reproductive toxicity caused by pretilachlor and for which it has been suggested to carry out experiments with both measured and nominal concentrations (Raimondo et al. 2009). Walking catfish, *Clarias batrachus* (Linn.), has been selected for the present study because of its availability throughout season, its noninvasive nature, and its ability to easily acclimatize to laboratory conditions (Gupta and Verma 2020a, b). It is also distributed abundantly and of economic importance (Verma and Murmu 2010).

In view of the above, the present study has been carried out to evaluate the effect of nominal concentration of herbicide pretilachlor on plasma sex steroid levels (T and E_2), gonadal aromatase activity, and VTG production in walking catfish, *C. batrachus*.

Materials and methods

Experimental animal and chemicals

Walking catfish, *C. batrachus*, of an average weight 115 ± 4.5 g and average length 13 ± 3.5 cm were obtained from the local fish farms. They were treated with 0.05% potassium permanganate ($KMnO_4$) to remove skin infections and were acclimatized to laboratory conditions in glassy aquarium with dechlorinated water for 1 month. Physico-chemical characteristics of water were determined as per proper guideline (APHA 2012) and were maintained with suitable conditions (temperature = 28 °C ± 1.0 °C, pH 6.5 ± 0.2, total hardness = 28 ± 0.05 mg/L, ammoniacal-nitrogen = 2.12 ± 0.36 mg/L, dissolved oxygen = 6.6 ± 0.01 mg/L, salinity = 600 ± 20 mg/L, day and light hour = 12:12 hours). During acclimatization period, fish are provided with boiled eggs and water was replaced every 24 h due to accumulated excretory and remaining food materials.

Commercial formulation of pretilachlor (CAS No. 51218-49-6) with trade name 'Rifit' (50% EC) manufactured by Syngnta India limited, India, was used for toxicity testing.

Exposure of fish to sublethal test concentrations

The sublethal test concentrations selected for the present study are based on the obtained LC_{50} value (96 h) of pretilachlor (3.55 mg/L) in our earlier study (Soni and Verma 2018). Nine treatment groups of fish consisting of 12 specimens (6 male and 6 female) in each group were used for their exposure to predefined test concentration of pretilachlor (50% EC), i.e., SL-I (1/20th LC_{50}), SL-II (1/15th LC_{50}), and SL-III (1/10th LC_{50}) equivalent to 0.29 mg/L, 0.38 mg/L, and 0.58 mg/L for 30, 45, and 60 days. The concentrations of pretilachlor in water were determined by chromatographic method (high-performance liquid chromatography, UV detector

with 5 mm granulometry and 30 cm equivalent length, LOD: 0.05 mg L1 with recovery % of 99%). Measured concentration of pretilachlor (HPLC analysis) is shown in Table 1.

For each exposure, a group of fish consisting of 12 individuals (6 male and 6 females) was considered control (C). Experiments were carried out in triplicate. Fish from control and treatment groups were anesthetize using clove oil, and the blood was collected from the caudal vein. Collected blood samples were centrifuged at 10000×*g* for 15 min, and the resulting plasma was used for determination of plasma sex steroid level and plasma VTG concentration.

Determination of sex steroid level

Enzyme-linked immune-sorbent assay (ELISA) kits with specific detection range and sensitivity were used to determine plasma concentration of T (detection range: 100–20,000 ng/L and sensitivity: less than 50 ng/L), E_2 (detection range: 29.3–30000 ng/L and sensitivity: 28.5 ng/L), and VTG (detection range: 50,000–2,000,000 ng/L and sensitivity less than 50,000 ng/L) as per the manufacturer's instructions. Competitive inhibition enzyme immunoassay technique was employed during the assay. Assay standards were prepared. Standards and samples were added to the microtiter plate wells with specific antibody for T/E_2/VTG and horseradish peroxidase (HRP) conjugated T/E_2/VTG, as a result of which competitive inhibition reaction was started between labeled and unlabeled T/E_2/VTG. The plate was read at 450 nm in micro plate reader. Standard curve was plotted and the sex steroid hormone and vitellogenin concentration in each sample was calculated. Substrate solution was added and the color develops. The plate was read at 450 nm in micro plate reader. The optical density (OD) was used to plot the standard curve and calculation of sex steroid hormone and VTG concentration in each sample.

Table 1 Measured pretilachlor concentrations (mg/L) in exposure water (HPLC analysis)

Sl. No.	Nominal concentration	Measured concentrationa
01	0.29	0.28 ± 0.01
02	0.38	0.37 ± 0.01
03	0.58	0.57 ± 0.03

[a] Measured concentrations are expressed as mean ± standard deviation (2 replicates)

Determination of gonadal aromatase activity

After blood collection, fish were sacrificed and gonads were removed for measurement of aromatase activity by tritiated water assay as proposed by Lephart and Simpson (1991). After rinsing ovaries and testes in ice cold KCl (0.15 mol/L), 25 mg of the gonadal tissue was homogenized in homogenizing buffer (600 μL of ice cold 0.05 mol/L potassium phosphate buffer containing 1 mM ethylenediaminetetraacetic acid and 10 mM glucose-6-phosphate). The obtained homogenate was incubated with 0.5 1 U/mL glucose-6-phosphate dehydrogenase, 21.33 nM 3H-androst-4-en-3, 17-dione, and 1 mM NADP. The incubation was carried out at 37 °C in the presence of 5% CO_2 for 2 h. The released tritiated water was extracted and activity determined by liquid scintillation counting. The activity of enzyme aromatase was expressed as fmol of andrestenedione converted per hour per milligram protein. The specificity of the reaction was determined by nonlabeled androstenedione and aromatase inhibitor fadrozole. At its low concentration, fadrozole reduced aromatase activity, and at sufficiently high concentration, it completely inhibits the activity. On the other hand, nonlabeled androstenedione reduced tritiated water formation and on increasing concentration it came to the level as found in controls. Protein was estimated by Bradford assay (Bradford 1976).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to analyze the data obtained. Values of $p < 0.05$ were considered statistically significant, and Tukey's post hoc testing was done for intergroup comparisons.

Results

Male and female *C. batrachus* respond in a different way to herbicide pretilachlor. The response of male fish is more pronounced in comparison to females as all studied reproductive parameters were found to be altered in pretilachlor-exposed males while most of them showed insignificant variations in females. Obtained results of each studied parameter are described below.

Plasma sex steroid level

Significant changes in the plasma sex steroid profile were observed in the pretilachlor-exposed fish. Herbicide-

exposed male fish exhibited significant ($p < 0.05$) reduction in the plasma T level (Table 2). This reduction was dependent on sublethal test concentration of the herbicide as well as duration of its exposure. Maximum reduction was found in fish exposed to highest concentration of the herbicide and after maximum duration of exposure. For each selected test concentration, this reduction in plasma T level increases with increasing duration of exposure and becomes maximum at 60 days. Similar reduction in plasma T concentrations was found in herbicide-exposed female fish. This significant reduction ($p < 0.05$) increases with increasing concentration and exposure duration of pretilachlor (Table 2).

Significant ($p < 0.05$) increase in plasma E_2 levels was observed in male fish after herbicide exposure (Table 3). This increase was also dependent on test concentration of pretilachlor and duration of its exposure to fish. Maximum increase in plasma E_2 level was found in fish exposed to highest selected test concentration. For each selected sublethal test concentration, plasma E_2 level increases with increasing duration of exposure and becomes maximum at highest selected duration of exposure. Plasma E_2 concentrations were found almost constant in herbicide-exposed female fish, and no significant changes with that of control fish were observed at any selected test concentration of the herbicide after any duration of exposure (Table 3).

Plasma VTG concentration and aromatase activity

Considerable changes in the both plasma VTG concentrations and gonadal aromatase activity were noted herbicide-exposed male fish (Tables 4 and 5). After a particular duration of exposure, plasma VTG level and gonadal aromatase activity significantly increase ($p < 0.05$) with increasing concentration of the herbicide but remain constant with increasing duration of exposure.

In herbicide-exposed female fish, no such significant changes in plasma VTG level or gonadal aromatase activity were observed and it tends to remain constant with increasing concentration or exposure duration of the herbicide (Tables 4 and 5).

Discussion

Herbicides used in agricultural field may contaminate nearby aquatic system and create stressful condition for aquatic organisms. Fish is one among the most important inhabitants of aquatic system from both ecological and economical point of view. They quickly respond to toxicants (Cavas and Ergene-Gozukara 2005). Assessment of their physiological health is very much helpful in detection of level of toxicity caused by xenobiotics present in aquatic systems. Herbicides may change the overall reproductive physiology of fish by acting as endocrine disruptors; therefore, the present study has been carried out to access the reproductive toxicity caused by herbicide pretilachlor.

Differential reproductive response of male and female fish to herbicide pretilachlor was observed in the present study. Some earlier studies also reported such differential response of fish from time to time (Spano et al. 2004; Lee et al. 2006; Dong et al. 2013; Gupta and Verma 2020a).

In vertebrates, hormones control the overall process of reproduction (Lubzens et al. 2010). So to maintain normal reproductive physiology, it is necessary that they must be secreted within the range. Their out of range secretion may affect the survival of the species. In fish, environmental pollutants may act on hypothalamic pituitary gonadal axis and change the sex steroid level (Li et al. 2009).

T and E_2 are important hormones maintaining the normal reproductive process in fish (Lubzens et al. 2010). Therefore, change in plasma level of these hormone may adversely affect the reproductively physiology. We observed a reduction of plasma T level in herbicide-exposed male fish which is followed by a concomitant increase in plasma E_2 concentrations. These alterations in sex steroids are dependent on concentration and exposure duration of herbicide. Similar observations were made by Spano et al. (2004) in gold fish (*Carassius auratus*) exposed to atrazine and by Gupta and Verma (2020a) in *Clarias batrachus* exposed to pendimethalin. At the same time herbicide concentration– and exposure duration–dependent increase in gonadal aromatase activity was also observed in male fish. Similar increase in gonadal aromatase activity in fish after herbicide exposure was reported earlier by some workers (Bucheli and Fent 1995; Husoy et al. 1996; Dong et al. 2013). The function of the aromatase is to convert T into E_2. Thus, observed decrease in plasma T level with concomitant increase in E_2 in pretilachlor-exposed male fish is due to increased gonadal aromatase activity. Increase in plasma E_2 level in males may induce femaleness and adversely affect the process of gamete formation and maturation so finally decrease the reproductive success of fish.

Table 2 The level of plasma testosterone (ng/mL) in male and female *C. batrachus* exposed to different test concentrations of pretilachlor for 30, 45, and 60 days ($n = 6$)

Duration of exposure	Concentration (mg/L)			
	C (0.0 mg/L)	SL I (0.29 mg/L)	SL II (0.38 mg/L)	SL III (0.58 mg/L)
Male				
30 days	$11.25 \pm 0.642^{A,1}$	$8.01 \pm 0.587^{B,1}$	$7.00 \pm 0.331^{C,1}$	$6.54 \pm 0.642^{D,1}$
45 days	$11.66 \pm 0.589^{A,1}$	$8.00 \pm 0.338^{B,1}$	$6.12 \pm 0.287^{C,2}$	$4.18 \pm 0.201^{D,2}$
60 days	$11.34 \pm 0.439^{A,1}$	$6.25 \pm 0.411^{B,2}$	$4.52 \pm 0.267^{C,3}$	$3.79 \pm 0.148^{D,3}$
Female				
30 days	$6.01 \pm 0.387^{A,1}$	$5.11 \pm 0.189^{B,1}$	$3.56 \pm 0.302^{C,1}$	$3.41 \pm 0.303^{C,1}$
45 days	$5.87 \pm 0.264^{A,1}$	$5.00 \pm 0.247^{B,1}$	$3.50 \pm 0.176^{C,1}$	$2.32 \pm 0.230^{D,2}$
60 days	$6.01 \pm 0.331^{A,1}$	$3.54 \pm 0.213^{B,2}$	$2.55 \pm 0.199^{C,2}$	$1.69 \pm 0.186^{D,3}$

The alphabets represent a statistically significant difference ($p < 0.05$) between concentrations within exposure duration. The numerals represent a statistically significant difference ($p < 0.05$) between exposure durations within concentration

Reduced plasma T level was also observed in pretilachlor-exposed female fish. This reduction was herbicide concentration and exposure duration dependent. On the other hand, no significant change in plasma E_2 level and gonadal aromatase activity was observed in herbicide-exposed female fish. Thus, reduction in plasma T level is not because of increased conversion of T to E_2 as gonadal activity was found remained constant. Therefore, it may be due to histological disruption of T producing cells of ovary and needs further histological investigations.

VTG is synthesized in the liver of vertebrates under the influence of estrogen hormone (Sole et al. 2003). Both male and female fish are capable of producing VTG due to presence of receptors for estrogens on liver, but synthesis of VTG is restricted to female fish during normal conditions and genes are in inactive state in males (Kime 1999). Exposure to xenoestrogens activates these genes, and thus, synthesis of VTG in males is a useful biomarker to detect the estrogenic nature of xenobiotics (Kime 1999; Scholz et al. 2004; Reinen et al. 2010; Jiang et al. 2016). We observed an increased plasma concentration of VTG in male fish after their exposure to pretilachlor which simply indicates the estrogenic nature of this herbicide. The herbicide may bind to the estrogen receptor and resulted in increased production of VTG in male fish (Marlatt et al. 2008; Korkmaz and Dönmez 2017). Similar increase in VTG concentration was observed in rare minnow exposed to butachlor (Zhu et al. 2014) and in *C. batrachus* exposed

Table 3 Plasma 17-β estradiol level (ng/mL) in male and female *C. batrachus* exposed to different concentrations of pretilachlor for 30, 45, and 60 days ($n = 6$)

Duration of exposure	Concentration (mg/L)			
	C (0.0 mg/L)	SL I (0.29 mg/L)	SL II (0.38 mg/L)	SL III (0.58 mg/L)
Male				
30 days	$1.64 \pm 0.087^{A,1}$	$3.01 \pm 0.215^{B,1}$	$3.16 \pm 0.310^{B,1}$	$5.64 \pm 0.452^{C,1}$
45 days	$1.56 \pm 0.059^{A,1}$	$3.11 \pm 0.132^{B,1}$	$4.36 \pm 0.224^{C,2}$	$7.00 \pm 0.711^{D,2}$
60 days	$1.61 \pm 0.094^{A,1}$	$4.23 \pm 0.398^{B,2}$	$5.67 \pm 0.367^{C,3}$	$7.11 \pm 0.694^{D,3}$
Female				
30 days	10.69 ± 0.858	10.54 ± 0.990	10.64 ± 0.666	10.87 ± 0.597
45 days	11.01 ± 0.996	11.00 ± 0.861	10.56 ± 0.843	10.51 ± 0.847
60 days	10.56 ± 0.795	10.87 ± 0.679	10.82 ± 0.910	11.10 ± 0.996

The alphabets represent a statistically significant difference ($p < 0.05$) between concentrations within exposure duration. The numerals represent a statistically significant difference ($p < 0.05$) between exposure durations within concentration.

Table 4 Plasma vitellogenin level (ng/mL) in male and female *C. batrachus* exposed to different concentrations of pretilachlor for 30, 45, and 60 days ($n = 6$)

Duration of exposure	Concentration (mg/L)			
	C (0.0 mg/L)	SL I (0.29 mg/L)	SL II (0.38 mg/L)	SL III (0.58 mg/L)
Male				
30 days	70.12 ± 6.114$^{A\text{-}\cdot 1}$	101.58 ± 9.058^{B,1}	136.68 ± 15.119$^{C\text{-}\cdot 1}$	138.08 ± 12.186$^{C\text{-}\cdot 1}$
45 days	80.24 ± 6.111$^{A\text{-}\cdot 1}$	126.84 ± 9.221^{B,2}	148.85 ± 12.164$^{C\text{-}\cdot 2}$	168.47 ± 15.238$^{D\text{-}\cdot 2}$
60 days	76.29 ± 7.514$^{A\text{-}\cdot 1}$	130.88 ± 10.009$^{B\text{-}\cdot 2}$	150.37 ± 11.412$^{C\text{-}\cdot 3}$	201.07 ± 15.298$^{D\text{-}\cdot 3}$
Female				
30 days	526.28 ± 18.258	521.47 ± 10.269	522.67 ± 11.109	524.91 ± 9.561
45 days	535.31 ± 14.478	511.24 ± 9.746	519.82 ± 14.141	531.71 ± 12.964
60 days	542.19 ± 11.364	531.02 ± 13.025	534.00 ± 9.988	516.62 ± 16.102

The alphabets represent a statistically significant difference ($p < 0.05$) between concentrations within exposure duration. The numerals represent a statistically significant difference ($p < 0.05$) between exposure durations within concentration

to pendimethalin (Gupta and Verma 2020a). It has been reported that other chloroacetamide herbicides alachlor and acetochlor could also mimic the estrogens (Burow et al. 1999; Rollerová et al. 2000).

Conclusions

On the basis of results obtained, it can be concluded that herbicide pretilachlor affects the overall process of reproductive hormonal steroidogenesis and acts as endocrine disruptor in *C. batrachus*. But its effect is more pronounced in males in comparison to females. Walking catfish, *C. batrachus*, can be used as an ecotoxicological model for evaluation of reproductive toxicity caused by herbicide pretilachlor. It can also be concluded that among various biomarkers of reproductive toxicity, plasma E_2 and T level, gonadal aromatase activity and plasma VTG concentration can be used as early and reliable biomarkers for pretilachlor toxicity in male *C. batrachus*. The present study also indicated the xenoestrogenic nature of pretilachlor; therefore, its use in agriculture fields is a risk factor for human beings also.

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Author contribution statement S K Verma: conceptualization, supervision, methodology; R. Soni: writing–original draft preparation, investigation, writing–reviewing and editing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Table 5 Gonadal aromatase activity (f mol/h/mg protein) in male and female *C. batrachus* exposed to different concentrations of pretilachlor for 30, 45, and 60 days ($n = 6$)

Duration of exposure	Concentration (mg/L)			
	C (0.0 mg/L)	SL I (0.29 mg/L)	SL II (0.38 mg/L)	SL III (0.58 mg/L)
Male				
30 days	12.47 ± 0.781^{A,1}	26.11 ± 2.547^{B,1}	41.15 ± 3.254^{C,1}	52.14 ± 5.489^{D,1}
45 days	13.58 ± 0.695^{A,1}	28.02 ± 2.114^{B,1}	56.95 ± 4.147^{C,2}	57.17 ± 7.002^{C,2}
60 days	14.14 ± 0.888^{A,1}	39.34 ± 2.998^{B,2}	57.24 ± 6.149^{C,2}	65.85 ± 6.543^{D,3}
Female				
30 days	51.25 ± 4.257	52.78 ± 5.024	52.23 ± 4.687	54.09 ± 6.647
45 days	55.84 ± 5.011	51.14± 4.967	54.41± 4.548	50.34± 4.619
60 days	54.36± 4.981	56.64± 7.010	52.69± 3.997	52.51 ± 5.582

The alphabets represent a statistically significant difference ($p < 0.05$) between concentrations within exposure duration. The numerals represent a statistically significant difference ($p < 0.05$) between exposure durations within concentration

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