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Chronic toxicity of flucycloxuron in the mosquitofish, *Gambusia affinis*: acetylcholinesterase and catalase activities and pattern of recovery

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ABSTRACT

Andalin (25% EC), the trade name of flucycloxuron (FCX), is a chitin synthesis inhibitor belonging to the class of benzoylphenylurea. It was found a potent insecticide against mosquito larvae. In this present study we evaluate the toxicity of this insecticide on acetylcholinesterase (AChE) and catalase activities (CAT) in adult females of a larvivorous fish, Gambusia affinis (Teleosteen: Poeciliidae). This fish is extensively used in biological control of mosquitoes. The insecticide was added in rearing water at two concentrations corresponding to LC50 and LC90 previously obtained against fourth instars larvae of Culex pipiens (Diptera: Culicidae). The female adults were exposed to a continuous treatment for 28 days, and AChE and CAT activities determined at different exposure times (0, 7, 14, 21 and 28 days). FCX was found to cause inhibition in AChE activity and induction in CAT activity starting day 14. In a second experiment, exposed fish were transferred to clean water up to 8 days to asses the recovery pattern. Exposed fish gradually restored to the control values by day 4 and 8 in the LC50- and LC90-treated series, while CAT activity was recovered after 2 days for the LC50 and 4 days for the LC90. The overall data obtained suggested that G. affinis is a suitable biological model which can be used in toxicological and biomonitoring studies, and CAT activity is a good biomarker of oxidative stress induced by such insecticides. Moreover, it stimulated rapidly the antioxidant enzyme activities. The recovery pattern showed that this fish species was able to overcome relatively rapidly the toxic stress induced by this insecticide. Finally, this insecticide appears less toxic against *G. affinis* than the conventional insecticides.

Keywords: Flucycloxuron, Gambusia affinis, Insecticides, Biomarkers, Recovery

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INTRODUCTION

Pesticides play an important role in insect pest control [1]. However, the intensive utilisation of conventional insecticides caused side\secondary effects on the environment and subsequently alternative methods are developed [2]. Biological control of mosquito larvae with predators and other agents would be a more-effective and eco-friendly approach, avoiding the use of synthetic chemicals and concomitant damage to the environment [3]. In this context, there is search for new insect-selective insecticides with minimal ecotoxicological risks. Insect growth regulators (IGRs) seem promising because of their specific mode of action on insects and their lower toxicity against non-target organisms than conventional insecticides. Among these IGRs the benzoylphenylurea derivatives (BPUs) cause abnormal cuticular deposition and abortive molting in insects by interfering with chitin biosynthesis [4]. Andalin (25% EC), the trade name of flucycloxuron (FCX) is a new benzoylphenylurea derivative controlling mites and insects [5) by interference with chitin biosynthesis as demonstrated in insects such as *Spodoptera littoralis* [6] and *Tenebrio molitor* [7], and in a crustacean species *Penaeus kerathurus* [8]. This compound was also tested in insects such as *Ephestia kuehniella* [9 Bendjedou *et al.*, 1998] and Culex *pipiens* [10]. *Gambusia affinis* (Baird & Girard, 1853) (Pisces, Poeciliidae) are widespread and extensively used in mosquito biological control. In addition, this mosquito fish is a suitable model used in toxicological studies [11, 12]. Previous bioassays revealed that this fish is efficient against *C. pipiens* larvae [13], while FCX was found potent against mosquito larvae [10]. For environmental risk assessment, a comprehensive understanding on adaptation and/or a recovery is essential [13].

Several pollutants such as heavy metals and organic compounds increase the formation of reactive oxygen species and induce detoxification systems like antioxidant defence systems present in all aerobic cells [14, 15, 16]. Therefore, monitoring of adverse effects as well as modifications of cellular and molecular defence systems can both be used as biomarkers [17] Different antioxidative enzymes are commonly used to assess exposure to xenobiotics in animals [18]. Particularly, catalase (CAT) is an enzyme promoting the conversion of hydrogen peroxide to water and molecular oxygen and can be used as a biomarker of oxidative stress [19]. Acetylcholinesterase (AChE) has a key role in neurotransmission by hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses of the nervous system and is inhibited by several pesticides such carbamate and organophosphorous insecticides [20] and also by heavy metals [21]. The aim of this research was to investigate environmental stress following a flucycloxuron exposure by measuring CAT and AChE activity and to study the recovery pattern of these biological responses in *G. affinis*.

MATERIALS AND METHODS

Fish rearing

Gambusia affinis (Baired & Girard, 1845) were collected from Kherraza valley (4° 04’ N, 04° 02’ E) located at the west of Annaba city (Algeria) and acclimatized for at least 15 days before exposure. Adult females (mean length: 35.4 ± 3.2 mm, mean weight: 425.8 ± 4.8) were placed in 50-liter glass aquaria and fed commercial dry feed (Tetramin^{R}, Germany). The natural photoperiod of 14:10 (L: D) was maintained. Exposed and control fish were reared in aquaria (30 fish in each aquarium) under laboratory conditions: temperature 22.10 ± 0.48 °C; salinity 241.05 ± 32.75 mg/L; pH 8.10 ± 0.09; dissolved oxygen 2.84 ± 0.13 mg/L.

Insecticide and treatment

Andalin (25% Emulsifiable Concentrate: EC), the trade name of flucycloxuron, was kindly provided by Pr. G. Smagghe (Ghent University, Belgium). The compound was added to the rearing water at two final concentrations 35 ng/L and 1.9 µg/L corresponding respectively to the LC50 and LC90 on fourth-instars larvae of *Culex pipiens* [10]. Fish starved for 2 days were exposed to the insecticide for 28 days. Fish that survived after 28 day of exposure were transferred into clean water up to 8 days to evaluate the recovery pattern of environmental biomarkers. In each experiment, untreated fish were also used as controls.

Enzyme assays

At appropriate times fish were sampled from control and treated series, anaesthetized in 0.04 % MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate salt) and dissected; the brain and hepatopancreas were removed. The AChE activity was measured individually in brain [22] using acetylthiocholine as a substrate as previously described [23]. Catalase activity was determined in each hepatopancreas by the method of Aebi [24 1985]. Proteins were extracted [25] from sample and quantified by the Coomassie Blue method [26] with bovine serum albumin as standard. Enzyme activities were expressed as µM/min/mg protein.

Statistical analysis

The normality of data was verified using the Kolmogorov-Smirnov test, and the homogeneity of variances was checked by Levene's test. Data have been expressed by the mean ± standard deviation (m ± SD). Data were subjected to analysis of variance (ANOVA) followed by Tukey's test. All statistical analyses were performed using MINITAB Software (Version 13.31, PA State College, USA) and $p< 0.05$ was considered to be a statistically significant difference.

RESULTS

Enzyme activities

The chronic exposure of females of *G affinis* to the insecticide was followed by a recovery study up to 8 days. The AChE activity was estimated at various times in brain tissues. Results are presented in figure 1. In control fish, there was no significant change ($P >0.05$) in the AChE activity during the experiment. A significant decrease in AChE activity was recorded at the different times of exposure with a dose relationship effect. Two way ANOVA indicated significant ($p<0.001$) effects of both exposure time and concentration. The Tukey's test revealed that there was a significant difference in AChE activity between the two tested concentrations at 7, 14 and 21 days, respectively. The AChE activity decreased during the exposure time to reach a minimum at day 21 (LC50: 50 %; LC90: 60 %) which remained constant until the end of exposure (day 28). At the end of exposure, the inhibition of the AChE activity was 44 % for the LC50 and 55 % for the LC90.

The results of CAT activity are presented in figure 2. Data show that FCX at the two tested concentrations caused a marked and significant induction in CAT activity. CAT activity was induced in both time and concentration dependant manner ($P<0.001$). The values recorded increased until day 21 of exposure to reach a maximum of 40.40 ± 1.153 µm/min/mg proteins for the LC50 and 52.57 ± 4.61 µm/min/mg proteins for the LC90, and then declined. In addition, there was a significant difference between the two tested concentrations at day 14 and 21, respectively.

Recovery study

Fish were exposed to LC50 and LC90 of FCX for 28 days (day 0) then transferred to clean water up to 8 days, and AChE and CAT activities measured. The inhibition of AChE activity decreased during the recovery period (Fig. 3). At day 1, the inhibition was approximately 25% as compared to controls of the same exposure time. Exposed fish gradually restored to the control values by day 4 and 8 in the LC50- and LC90-treated series, respectively. In addition, there was no significant (P> 0.05) difference in the AChE activity between the two tested concentrations. When transferred to clean water, exposed fish exhibited rapidly a significant decrease in CAT activity (values recorded with the LC50 at the end of exposure: 35 vs 18 µm/min/mg proteins at day 1 during the recovery period; with the LC90: 40 at day 28 vs 26 µm/min/mg proteins at day 1 during the recovery period). This enzyme activity was restored to control values by day 2 with the LC50 and by day 4 with the LC90, respectively (Fig. 4).

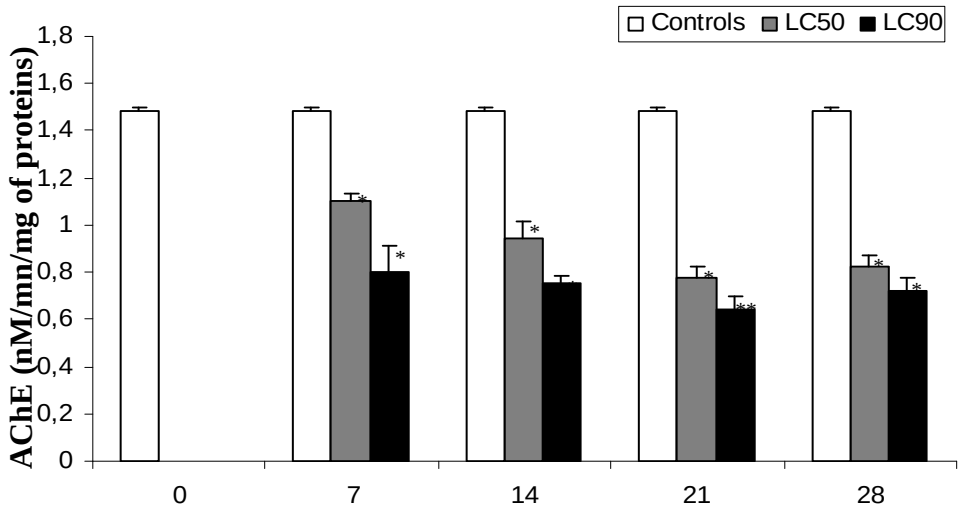

Fig 1. Activity of AChE (nM/mn/mg of proteins) in the females of *G. affinis* exposed to flucycloxuron at CL50 and CL90 (m ± s; n=5). Asterisks above treated series indicated a significant difference with controls of the same time (*: significant difference at p < 0.05; **: significant difference at p < 0.01; *: significant difference at p < 0.001).**

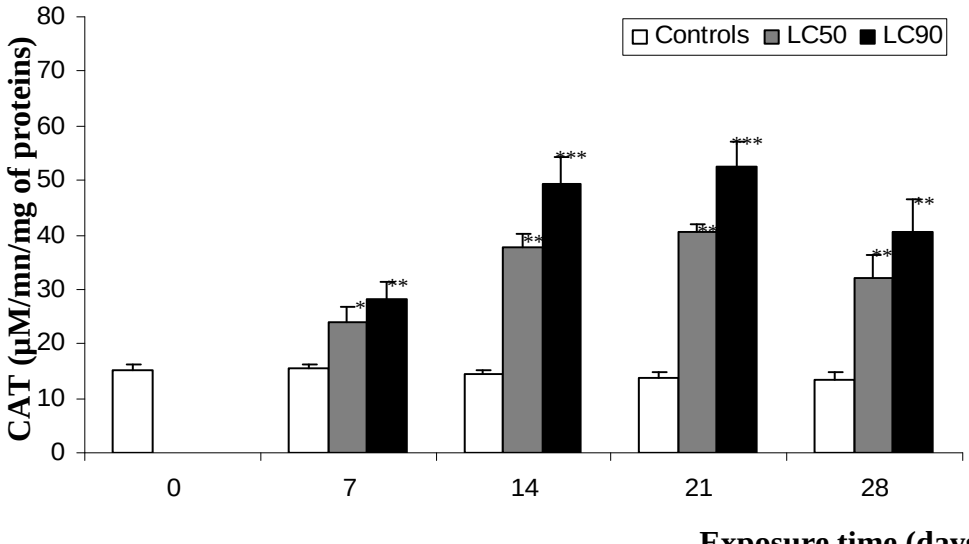

Fig. 2. Activity of CAT (µM/mn/mg of proteins) in the females of *G. affinis* exposed to flucycloxuron at CL50 and CL90 (m ± s; n=5). Asterisks above treated series indicated a significant difference with controls of the same time (*: significant difference at p < 0.05; **: significant difference at p < 0.01; *: significant difference at p < 0.001).**

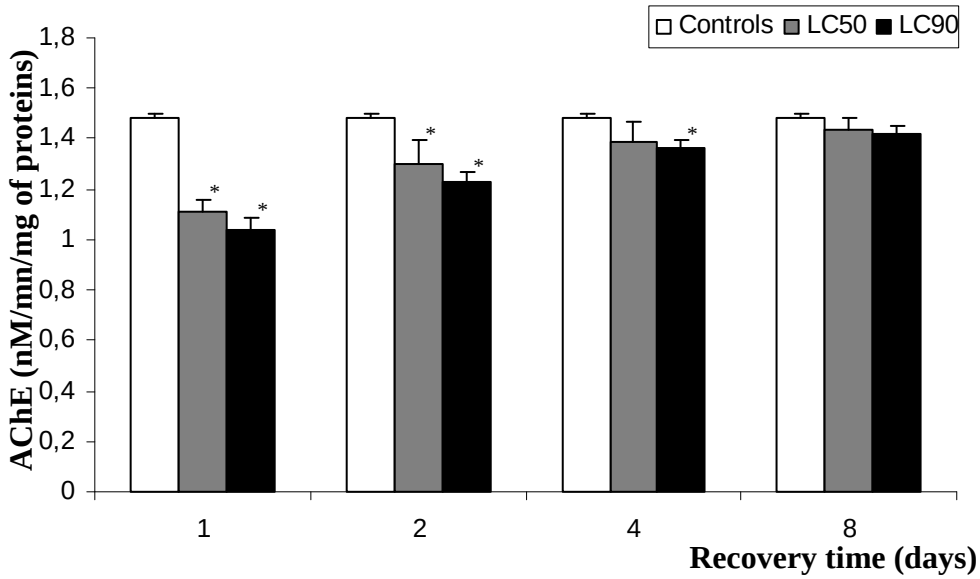

Fig 3. Activity of AChE (nM/mn/mg of proteins) in the females of *G. affinis* exposed to flucycloxuron at CL50 and CL90 for 28 days and its recovery response. (m ± s; n=5). Asterisks above treated series indicated a significant difference with controls of the same time (*: significant difference at p < 0.05; **: significant difference at p < 0.01; *: significant difference at p < 0.001).**

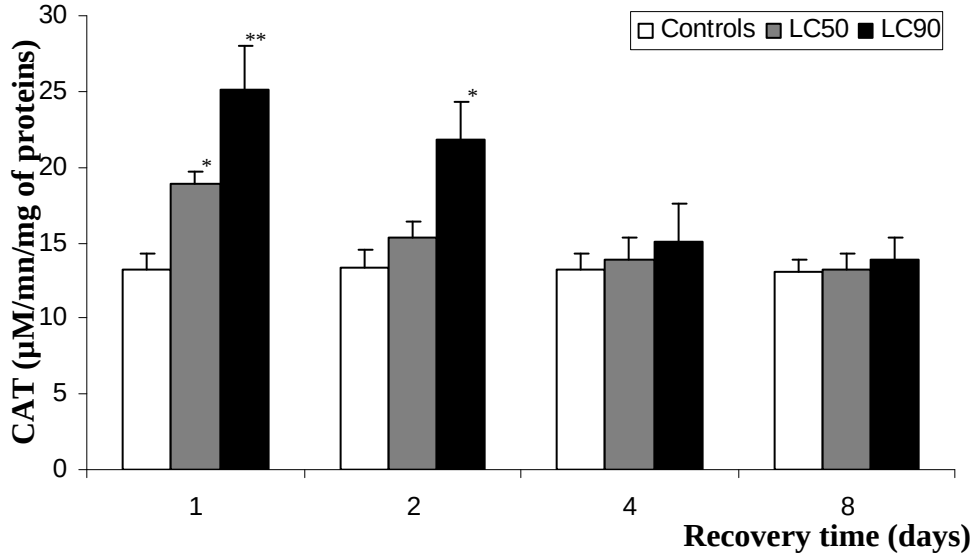

Fig. 4. Activity of CAT (µM/mn/mg of proteins) in the females of *G. affinis* exposed to flucycloxuron at CL50 and CL90 for 28 days and its recovery response (m ± s; n=5). Asterisks above treated series indicated a significant difference with controls of the same time (*: significant difference at p < 0.05; **: significant difference at p < 0.01; *: significant difference at p < 0.001).**

DISCUSSION

Mosquitoes are generally controlled by conventional insecticides such as carbamates and organophosphates. The potency of IGRs for mosquito control has been the subject of intensive investigations [10, 27]. However, the rational use of biological agents constitutes as a valuable alternative to chemical control [28], and *G. affinis* is the most potent agent [29]. In *G. affinis*, triflumuron, a chitin synthesis inhibitor (CSI), was found to reduce the number of juveniles per brood [8], while diflubenzuron, another CSI, was reported to affect their growth [30]. A recent

report revealed that halofenozide an ecdysteroid agonist, had no significant effects on growth, metric indexes and AChE activity in *G. affinis* [31].

The present study revealed that FCX exposure causes significant inhibition of brain AChE activity in *G affinis*. The observed effects varied as function the exposure time and the concentration. Indeed, the effect was relatively more marked with the highest concentration (LC90). The biological responses of exposed fish indicated that the AChE activity decreased as function the exposure time to reach a minimum at day 21 which remained constant until the end of treatment (day 28). This is probably to the degradation of the compound in the water and also to its stability in the body. Indeed, the toxicity of these IGRs can be related to a high retention and stability as active compound in the body [32, 33].

Similarly, an inhibition of AChE activity was observed in *L. macrochirus* exposed to diazinon [34] and to endosulfan [35], in *Gambusia affinis* exposed to chlorpyrifos [36] and in *Ghanna punctatus* exposed to metacid-50 and carbaryl [37]. This inhibition varied also according to the type of insecticide. It seems higher with OP insecticides like monocrotophos in *G affinis* [12] and fenitrothion in *Salmo salar* [38] as compared to the other classes of pesticides.

Like all aerobic organisms, fish are susceptible to the attack of reactive oxygen species and have developed antioxidant defences [39]. The cellular defence systems include the activity of certain enzymes, namely SOD, CAT, GPX. CAT is widely used as a biomarker of oxidative stress [40, 41, 42]. A number of studies reported an increase of CAT activities in bivalves [18, 40]. Our experiments showed that this pesticide induced an oxidative stress in exposed *G. affinis*. This, rapidly stimulate the antioxidant defences as evidenced by changes in CAT activity measured during the treatment. The maximum value was also recorded at day 21 and the response was more marked with the LC90. The increase of hepatic CAT activity was observed with various organic pollutants in *Anguilla Anguilla* [43] in goldfish [44] and in *Jenynsia multidentata* [45] Increased CAT has also observed in fish from polluted systems [46, 47, 48]. On the other hand, a decrease in the CAT activity during pesticides exposure was reported in *Oncorchynchus mykiss* with carbaryl and azinphos methyl treatment resulted in decrease in CAT activity [49], in *Channa punctatus* with deltamithrin [50].

For environmental risk assessment, a comprehensive understanding on adaptation and/or a recovery is essential [13]. In this study, exposed fish transferred to clean water recovered rapidly AChE activity after 4-8 days and CAT activity after 2-4 days according to concentrations. The recovery was influenced by the concentration but also by the insecticide used. Decreased AChE activity (79%) in *G affinis* after monochrotofos acute exposure was observed and the recovery period of 20 days are needed [12]. AChE activity in the atlantic salmon *Salmo salar* exposed to fenitrothion required periods of at least 6 weeks of recovery [38] while *Anguilla anguilla* exposed to thiobencarb for 96h, recovered brain AChE from the first hours in clean water [20]. In *G. affinis* exposed to monocrotophos for 96h, a period of 16 days was necessary to recover control CAT activity [12]. In comparison to earlier reports, FCX appears less toxic to this non-target species as compared to conventional insecticides. However, further experiments are needed to explain the AChE inhibition by this selective IGR.

CONCLUSION

The overall data obtained suggested that *G. affinis* is a suitable biological model which can be used in toxicological and biomonitoring studies, and CAT activity is a good biomarker of

oxidative stress induced by such insecticides. Andalin stimulated rapidly the antioxidant enzyme activities. The recovery pattern showed that this non-target fish species was able to overcome relatively rapidly the toxic stress induced by treatment. Finally, this insecticide appears less toxic than the conventional insecticides.

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