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Toxicity and perturbation of the metabolite contents by a chitin synthesis inhibitor in the mosquito larvae of *Culiseta longiareolata*

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ABSTRACT

Novaluron is an IGR of the benzoyl urea family, acting as a chitin synthesis inhibitor. The activity of a commercial formulation of Novaluron (10% EC) was tested, at different concentrations, ranging between 0.2 and 1.6 µg/l, against third and fourth-instar larvae of Culiseta longiareolata. (Diptera: Culicidae). The technical material showed a high level of activity with mortality recorded for both treated and following stages and happened after incomplete development. The LC_{50} values were 0.51 and 0.91 µg/l active ingredient for third and fourth instar larvae; and LC_{90} values were 2.32 and 4.30 µg/l respectively. In other experiments the compound was applied at LC_{50} and LC_{90} against the fourth instars larvae and its effects investigated on biochemical composition of larval body. Metabolite analyzes showed that novaluron affected significantly the amount of carbohydrates, lipids and proteins of fourth instars larvae starting from the day three following treatment. The carbohydrate and lipid amounts increased significantly whereas those of proteins decreased as compared with control series. For the same treated series a significant decrease was also recorded in the body weight with a decrease in development time. In the presence of chitin synthesis inhibitors, the last step of the chitin biosynthesis pathway is inhibited and the precursor is not converted in to chitin. It may either act on the hormonal level in the haemolymph to announce the synthesis, degradation or the inhibition of the metabolites. Than carbohydrates, lipids, proteins are under endocrine control and the exposure of the larvae to this xenobiotic product can modify the synthesis of these metabolites. The data obtained were discussed according to the mode of action of this insect growth regulator and the metabolism of lipids carbohydrates and proteins.

Key words: Novaluron, *Culiseta longiareolata*, Benzoylphenylurea, Biochemical composition, Toxicity.

INTRODUCTION

Culiseta longiareolata is considered among the most abundant mosquito species in Algeria, particularly in arid areas [1-3] and is controlled by conventional insecticides [1]; [4]. Because the use of neurotoxic insecticides for controlling insect pests has several disadvantages to various environmental aspects, including human health and economics, the 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.Thus, IGRs such as chitin synthesis inhibitors (CSIs) affect the hormonal regulation of molting and development processes [5]. Novaluron is a chitin synthesis inhibitor, belonging to the class of benzoylphenylurea, showing a high toxicity level and effectiveness against several dipteran species [6-9].

In insects, the principal forms of storage of energy are represented by carbohydrates and lipids and are closely related to the physiological events such as the flight, the moult and the reproduction. This phenomenon takes place however with the detriment of their structural or functional role since these biomolecules are not synthesized and are not stored with an aim of providing energy [10].

Recently, halofenozide, an ecdysteroid agonist belonging to benzoyl hydrazine IGRs, was reported to affect growth, molting hormone contents, cuticle secretion in *Cx. pipiens* [11], and the body contents of lipids, carbohydrates and proteins, *Cs. longiareolata* [3].

In the present study, we assessed the toxicity of Novaluron against third and fourth larval stages of *Cs. longiareolata*, and the duration of both stages were determined. In addition, its effects at LC_{50} and LC_{90} were studied on biochemical composition and on the body weight of the fourth larval stage in order to provide better insights in the physiology of its mode of action.

MATERIALS AND METHODS

2. 1. Toxicity bioassay

The susceptibility of laboratory-reared larvae of *Culiseata longiareolata* (Diptera: Culicidae) was determined, using a chitin synthesis inhibitor novaluron 10%. The larvae of *Cs longiareolata* were obtained from a stock colony of the laboratory. Novaluron 10% EC was serially diluted with distilled water and appropriate aliquots were added to treatment beakers to give the following final concentrations of 0.2, 0.4, 0.8 and 1.6 µg of active ingredient per liter . Newly ecdysed third and fourth-instars larvae of *Cs. longiareolata* were exposed to the different concentrations for 24 h [12]. The test units were beakers containing 25 larvae and each treatment and control was replicated three times. Larvae were daily fed with fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight), and water was replaced every four days. The bioassays were carried out in an insectary at 25-27 C°, 80-85% relative humidity and photoperiod 14:10 light and dark period. Assessment of larval mortality was made every other day, counting and removing dead larvae, pupae and adults (partially molted). The mortality percentage recorded at various developmental stages was corrected [13] and toxicity data were studied by probit analysis [14], and LC_{50} (50% lethal concentration), confidence limits (95%), and slope of the concentration-mortality lines were calculated [15].

2.2. Biochemical procedure

Biochemical bioassays were carried out on newly moulted fourth instar larvae of *Cs. longiareolata*, treated with two concentrations (LC_{50} = 0.91µg/l and LC_{90} = 4.40 µg/l) and sampled in different ages of larval development (1, 3, 5 and 7 days). The pooled sample (10 individuals, starting with 75) conducted on the surviving treated larvae were weighted and subjected to extraction in trichloracetic acid (TCA 20%) [16]. the supernatant of the first centrifugation, was used for the carbohydrates quantification, as described by [17], and to the pellet was used for a second centrifugation with a mixture of ether and chloroform (1V/1V) and the resulted supernatant 2 was used to quantify The lipids [18]. Therefore the protein bioassay was carried out from the dissolved pellet 2 in NaOH (0.1N) according to [19]. Data were

expressed in µg per mg whole body; means ± SD were analysed based on three replicates per treatments each using a Student's *t* test at p=0.05..

RESULTS

3.1. Insecticidal Activity

Novaluron showed a high level of activity against *Cs. longiareolata* and the dose-response relationship was determined on newly ecdysed third and fourth-instar larvae (Figs. 1 & 2). The third and instars of *Cs. longiareolata* expressed more susceptibility than the fourth instars. Novaluron caused mortality in larvae on ecdysis and delayed mortality also occurred in pupae and at the adult stage.

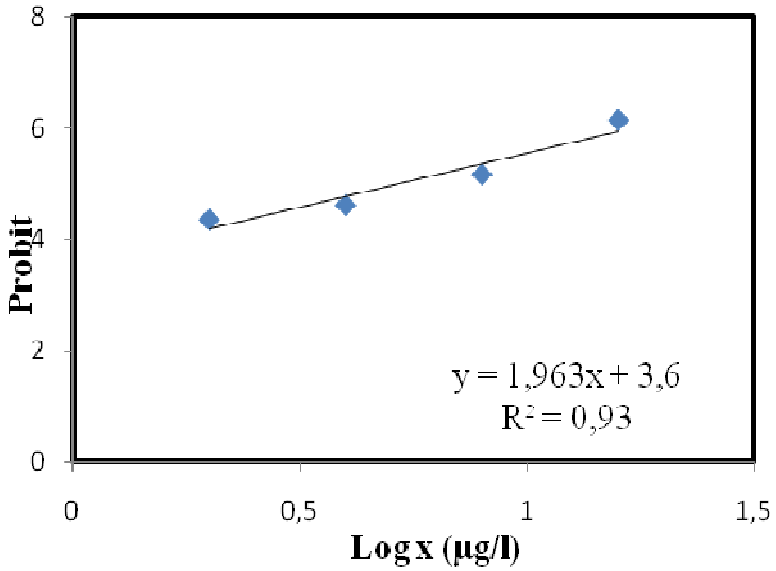

Fig. 1. Dose-response relationship for treatment of novaluron, applied for 24 h to newly ecdysed third- instar larvae of *Cs. longiareolata* (R^2 = coefficient of determination) (n=75).

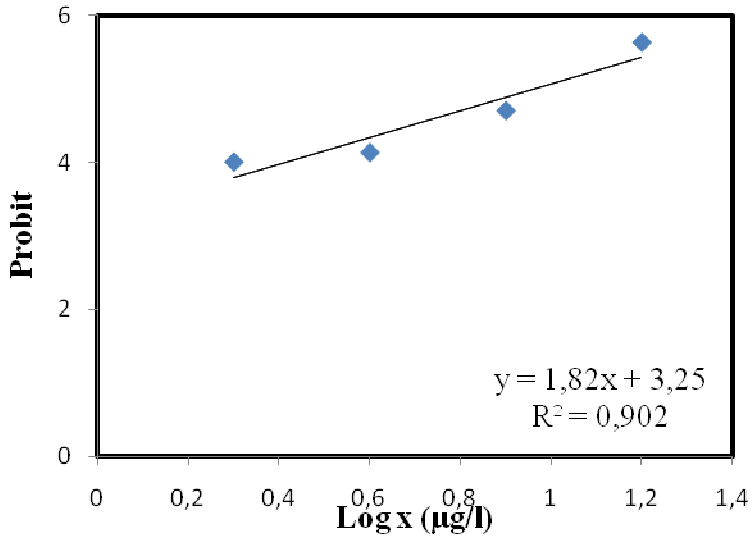

Fig. 2. Dose-response relationship for treatment of Novaluron, applied for 24 h to newly ecdysed fourth-instar larvae of *Cs. longiareolata* (R^2 = coefficient of determination) (n=75).

The product exhibited a larvicidal activity against the treated larval stages. With probit for the L3, the LC_{50} was calculated as 0.51 µg/l (95%; CL = 0.41-0.61 µg/l; Slope= 3.20) and the LC_{90} was 2.32 µg/l (95% CL = 0.41-0.61 µg/l), and for the L4 the LC_{50} was found as 0.91 µg/l (95%

CL = 0.69-1.19 µg/l; Slope= 3.34) and the LC_{90} was 4.30 µg/l (95% CL = 3.28-5.63 µg/l). After 2 days of treatment, intoxicated larvae showed a change in their behaviour by sinking to the bottom of the jar and remain stable until they died. Morphological examination of death larvae after treatment showed that most mortality occurred after an incomplete moult and as a consequence larvae or pupae died trapped within their old exuvium. The chitin synthesis inhibitor proved to affect growth and development in mosquito *Cs. longiareolata*.

3.2. Effects on larval development duration

The effect on development duration of the third and the fourth instars larvae treated with the different concentrations is presented in table1. The results show that novaluron interferes with growth by increasing the larval development duration. There was a significant difference between control and treated series with the highest concentrations (0.4, 0.8 and 1.6 µg/l) for the third instar larvae (P< 0.001), and with all tested concentrations for the fourth instar larvae (P< 0.01). With these concentrations the development duration of the third larval stage was recorded at 5.70 and 5.80 days, respectively, compared with control that was 3.66 (P> 0.05). However the effect, on the fourth instar larvae was significant (P>0.05) for the three higher concentrations (0.4, 0.8 and 1.6 µg/l) with a duration of 8.67, 9.33 and 10.67 days respectively, whereas for the control the age was 7.41 days.

Table 1: Effect of novaluron on the developmental duration (days), of the third and the fourth larval stages of *Cs. longiareolata*, treated with different concentrations (m ± s; n= 10). For each instar, mean values followed by the same letter are not significantly different (P< 0.05).

Developmental duration (day)		
Concentration (µg/l)	**Third instar larvae**	**Fourth instar larvae**
Control	3.66 ± 0.28 a	7.41 ± 0.81 a
0.2	3.60 ± 1.04 ab	8.00 ± 1.00 b
0.4	4.06 ± 0.61 c	8.67 ± 1.53 c
0.8	5.70 ± 0.60 d	10.67 ± 1.15 d
1.6	5.80 ± 0.80 d	12.33 ± 1.15 d

3.3. Effects on the larval body weight

The results, of the whole body weight measurement of *Cs. longiareolata* larvae, showed that the weight was affected under treatment with lethal concentrations (LC_{50} and LC_{90}) of the active material. In control, at one day age the larval body weight was 4.11 mg and increased to reach a maximum at day 7 with 6.60 mg weight coinciding with the pupal ecdysis (Table 2). In treated series, a significant decrease in the body weight was recorded from day 3 for the two tested concentrations, and the highest body weight recorded at day 7 was only 3.93 mg for LC_{50} (P= 0.012) and 3.85 mg for LC_{90} (P= 0.003) as compared with controls. ln addition, there was no significant difference (P> 0.05) in the weight of fourth instar larvae between the two tested lethal concentrations.

Table 2: Effect of novaluron administered at two concentrations (LC_{50}= 0.91 and LC_{90}= 4.30 µg/L) on the fresh body weight (mg) during the fourth larval stage development of *Cs. longiareolata* (m ± s, n= 10). For each time, mean values followed by the same letter are not significantly different (P< 0.05).

Time (days)	**Body weight (mg)**		
	Control	**LC_{50}**	**LC_{90}**
1	4.11 ± 0.58 a	4.05 ± 0.58 a	4.30 ± 0.49 a
3	4.68 ± 0.28 a	4.38 ± 0.56a b	4.26 ± 0.25 b
5	5.37 ± 0.38 a	4.03 ± 0.45 b	3.70 ± 0.60 b
7	6.60 ± 0.17 a	3.93 ± 0.75 b	3.85 ± 0.28 b

3.4. Effects on biochemical composition

The metabolite bioassays were carried out on the fourth instar larvae of *Cs. longiareolata*, using two concentrations (LC_{50} = 0.91µg/l and LC_{90} = 4.30 µg/l) of novaluron. The changes of metabolite amounts, carbohydrates, lipids and proteins, were estimated in the whole body of the larvae during different times of the developmental stage (1, 3, 5 and 7days) (fig.3).

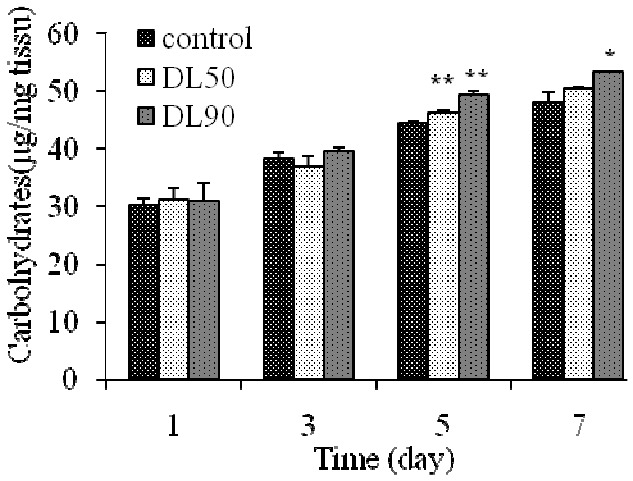

Fig. 3 A: The effect of novaluron, at two concentrations (LC_{50} = 0.91µg/l and LC_{90} = 4.30 µg/l), on the amount of the carbohydrates in the larval body (µg /mg body weight), during the fourth larval stage development of *Cs. longiareolata*.

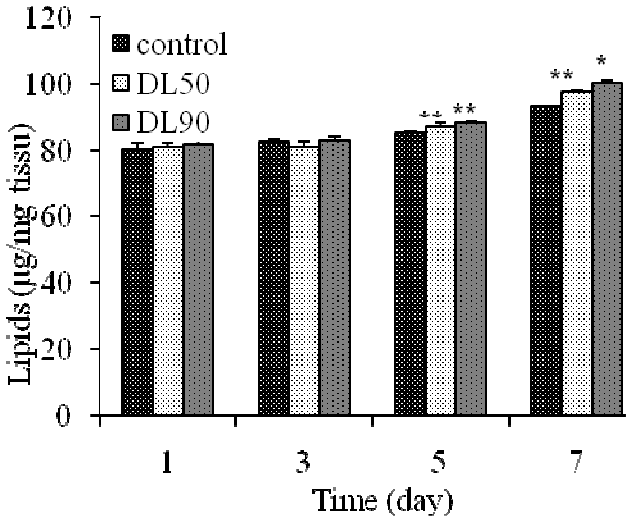

Fig. 3 B: The effect of novaluron, at two concentrations (LC_{50} = 0.91µg/l and LC_{90} = 4.30 µg/l), on the amount of the lipids in the larval body (µg /mg body weight), during the fourth larval stage development of *Cs. longiareolata*.

It was found that the carbohydrates and lipids level did not change at day 1 and 3 but an increase was recorded (P= 0.017 and P= 0.063) starting from the day 5 for the carbohydrates and lipids respectively for both concentrations. The differences in amounts were significantly higher (P=0.004) at day 7 compared to control for both concentrations (Fig. 3**A** & **B**). Whereas novaluron-treated larvae was found to undergo a high significant (P< 0.05), suppressed protein level, as compared to control, during the periods of bioassay 3, 5. For the day 7 decrease of protein amount was significant (P=0.018) only for CL_{90} compared to control series. In control, the changes in protein amounts presented a single peak that occurred at day 5 during the larval

development (Fig. 3**C**). The bioassay measurements of metabolites in the whole body of larvae revealed that Novaluron at the two tested concentrations shifted the moment of this peak (day 3), and it increased their values.

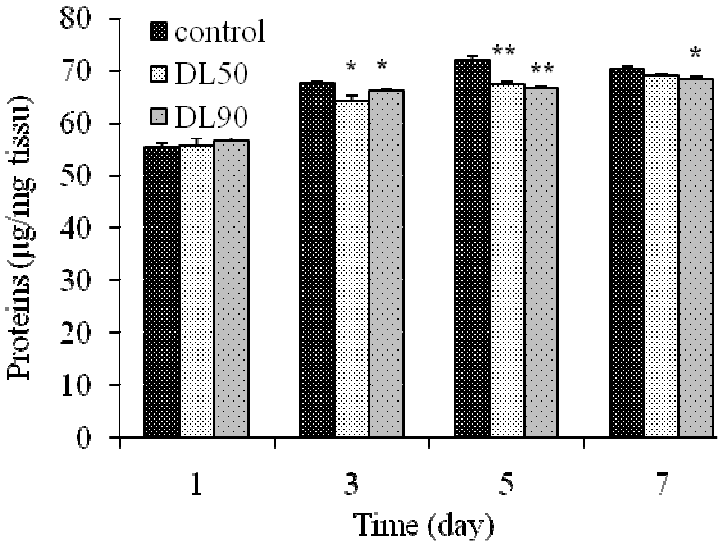

Fig. 3 C: The effect of novaluron, at two concentrations (LC_{50} = 0.91µg/l and LC_{90} = 4.30 µg/l), on the amount of the proteins in the larval body (µg /mg body weight), during the fourth larval stage development of *Cs. longiareolata*.

DISCUSSION

The potency of IGRs for mosquito control has been the subject of intensive investigations [2]; [20]; [11] [7&21]. A few reports documented the larvicidal efficacy of Novaluron under laboratory conditions against the larvae of *Aedes aegypti* [6] and *Culex* mosquitoes [22]; [23]. The present assays were focussed on the effects of an insect growth regulator, novaluron. In the study, Novaluron was found to disturb the growth and the development in *Cs. longiareolata* and this is similar to diflubenzuron when used against the same species [21]. The toxicity assays conducted under laboratory conditions on *Cs. longiareolata* larvae indicated that Novaluron exhibited a larvicidal activity when applied to newly ecdysed larvae for 24 h. The same results were found when the Novaluron was used against *Culex quinquefasciatus* [9] and *Culex pipiens* (Boudjelida, unpublished data). Novaluron is considered as endocrine disrupting compounds (EDC) for insect species. Up to now, very few EDC testing procedures are available for aquatic invertebrates. Therefore, new toxicity test guide lines for endocrine disruption with aquatic invertebrates have to be developed. The impact of toxic substances on the organisms is mainly evaluated at the ecotoxicological level and few are done at the molecular level. Therefore, it seemed of interest to us to find new endpoints at metabolites level of mosquito. Moreover, this information can explain the observations obtained with mortality testing. A link between ecotoxicological and metabolites data in response to novaluron exposure could enable a better understanding of the impact of toxic substances on this organism.

In insects, the haemolymph undergoes metabolic modification during the developmental stages [24] and the needs in energy are strongly under endocrine control [25]. The exposure of an organism to xenobiotic product can modify the synthesis of certain metabolite and disturb the functionality of the organisms [26]. Neuropeptides are the important regulators [27] of energy metabolism. Indeed, beside the exposure to a chemical product, any fluctuations would

happened, are related to the various physiological process of insect such as the moult, the nymphosis, the diapauses [28] and metamorphosis process [29].

The bioassays of the mean metabolites in this study reveal a modification of the amounts (carbohydrates, lipids and proteins) in the whole body of treated larvae of *Cs. longiareolata* compared to control during different times of larval development. Biochemical analyses performed on fourth instar larvae of *Cs. longiareolata* after treatment with two lethal concentrations (LC_{50} and LC_{90}) of novaluron reveal an increase in the contents of carbohydrates and the lipids in the treated larvae, compared to the control. Other growth regulators, like DFB, applied to the pupae or to the adult females of *Tenibrio. molitor* [30] affects with the same way the concentrations of carbohydrates and increases the concentration of the lipids [31]. Other studies show that the carbohydrate reserves vary in agreement with the different development stages of the insect. They increase during the rest periods, like metamorphosis and decrease during the growth periods like the stages of maturation of the gonads in insects. The quantity of lipids available for the reserves seems to be the result of a balance between the catch of food and the requests for reserves by processes such as reproduction, maintenance and growth, and this balance is disturbed by any toxic product [32]. The carbohydrates, as energy elements play a crucial role in the physiology of the insects [33] and are infected by the chitin synthesis inhibitor. The lipids represent the independent source of energy in insects and are transported via the haemolymph; from their synthesis site of storage [34] towards the user organs, in particular the vitellogenesis [28] and cuticular synthesis [35]. Indeed, these intense metabolic modifications are related to the various hormonal systems and neuro-secretion [36] modified under novaluron effects.

A biomarker represents a biological response of the impact or presence of xenobiotic in the organism, and not the direct evidence of this one. This response must be measured in an organism or its products and indicate a change compared to the normal state. The energy reserves are considered as biomarkers of effect that give information on the health condition of the organism [37]. I this case thee metabolite modification recoded is related to the presence of novaluron in the organism.

The high value of proteins recorded, during the larval stage at day 3 (Fig. 3C) is probably in relation with the transport of these proteins in the haemolymph, released from the mobilization of the reserves, destined for the synthesis of the new cuticle [38]. The proteins would also come, from the digestion of procuticular deep layers of the old cuticle and from an exogenic origin [39]. The proteins play a fundamental role in the organization of all known biological species living. They enter at various reactions such as the hormonal regulation and are integrated in the cell as a structural element at the same time as the carbohydrates and the lipids [40]; [24]. The modification in the concentration of the proteins results from the volume of haemolymph changed under stress of the insecticide [10]. This induction of proteins could be used to as biomarker of exposure which is the response to an interaction between a xenobiotic agent and a molecule or target cell.

Our results show that the total protein contents decrease after treatment by Novaluron during the tested larval stage of the mosquito species, *Cs. longiareolata*. A fall of the proteinemy is observed also at *L. decemilneata* after application of 20E, RH-5849 and the RH-5992 [41] and at *Spodoptera littoralis* after treatment with the RH-5849 [42]. In *Tenebrio molitor,* the application of growth regulators, KK-42 [43] and RH-0345 reduced with the same way the rates of proteins [44]. The observed drop in protein amounts in treated series of fourth instar larvae of *Cs. longiareolata* was similar to observation on effect of azadirachtin, a phytogenic insect growth

inhibitor, treatments in *Spodoptera litura* [45] and *Helicoverpa armigera* larvae [46]. Whereas, a topical application of a juvenile hormone analogue, methoprene, on fly *Bactrocera cucurbitae* exhibit an increase level of the proteins[47].

In fact the biochemical assays did not explain clearly the mode of action of the chosen substance and it remains unclear if the observed effects are linked to endocrine disruption. In the presence of chitin synthesis inhibitors, the final step of the chitin biosynthesis pathway is inhibited and the precursor is not converted in to chitin.

Haemolymph volume changes under insecticide stress resulting in alteration in protein concentration [10]. These arguments support and explain the decrease in the body weight of the treated larvae and extend the duration of development stages. Novaluron may either act on the hormonal level in the haemolymph to announce the synthesis, degradation or the inhibition of proteins or on the neuro-secretion cells which control endocrine glands. It can also change the behaviour feeding of larvae towards action to avoid food and to maintain the metabolism of the body to the expenditure of storage out of cellular proteins. In most insects the chitin synthesis inhibitor, Novaluron exhibit a toxic effect by preventing metamorphosis at each of the larval molts. In summary, the benzoylphenylurea have a good potential in formulating novel IGR-based control agents against mosquitoes in an environmentally-friendly manner to the aquatic ecosystem.

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