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Impact of a Chitin Synthesis Inhibitor, Novaluron, on the Development and the Reproductive Performance of Mosquito *Culex pipiens*

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Abstract: The management of disease vectors using conventional pesticides has failed because of the high reproductive ability, development of insecticide resistance of insect species and secondary effects on environment. These reasons are leading to a search for novel molecules. The Insect Growth regulators (I.G.Rs) were proposed as an alternative without any effect on non target organisms. The aim of this study was to assess the potential effects of an insect growth regulator belonging to the class of a chitin synthesis inhibitor, novaluron. A commercial formulation of this product (10% EC) was evaluated on several biological aspects (toxicology, development and potential of reproduction) of mosquito species, *Culex pipiens* under controlled laboratory tests. The results of the toxicological assays, against larvae and pupae developmental stage revealed an insecticidal effect with concentration-response relationship only for larval stage. The Mortality was observed occurring after precocious and incomplete emergence and the adult is trapped in the exuvial cuticle. Moreover, treatment with lethal concentrations caused a significant increase in the duration of the larval and longevity of adults and effected the reproduction, by decreasing number of laid eggs, percentage of hatching and fecundity.

Key words: Vector · *Cule*x · Insecticides · Development · Fecundity

INTRODUCTION

Mosquitoes are the most important class of insects well-known for their public health importance. They are vector of many diseases [1], like malaria, which is transmitted by the genus *Anopheles* and the Dengue by *Aedes*. The house mosquito *Cx. pipiens* is also a vector of a number of diseases, especially arboviruses (arthropod-borne viruses) and it is recognized as the primary vector of West Nile virus. *Cx. pipiens* represents the most interesting mosquito species in Algeria, particularly in urban areas [2, 3]. These vectors are generally controlled by conventional insecticides [4, 5]. Because of the secondary effects of the conventional neurotoxic insecticides on the environment and none target organisms, also the development of the insect resistance [6] the scientist works were focused on the search of novel molecules. They proposed the insect growth regulators (I.G.Rs) that seem to be promising because of their specific mode of action on insects [7, 8] and their lower toxicity against non-target organisms than conventional insecticides [9, 10]. In the last decades, the I.G.Rs compounds have shown promising results in controlling insects of agricultural, medical and veterinary importance [11, 13]. The present study was conducted to evaluate the toxicological effects of a chitin synthesis inhibitor, novaluron, on the development of the fourth newly exuviated larvae and pupae of the mosquito, *Cx. pipiens*. The disease spread depends directly on insect vector population and consequently, production of eggs by the insect could be a potential target for vector control. Actually few information are available concerning the effect of insect growth regulators on physiological aspects, like reproduction, in mosquito species. Therefore the effects, of the lethal concentrations (LC_{50} and LC_{90}) of novaluron were also investigated on the potential of reproduction after treatment during the fourth larval stage of the domestic mosquito *Cx. pipiens*, in order to provide better insights in the physiology of its mode of action.

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MATERIALS AND METHODS

Mosquito Rearing: The different developmental stages (larvae, pupae and adults) of *Cx. pipiens* (Diptera: Culicidae) were obtained from a stock colony of the laboratory. For assays, each 25 larvae were kept in Pyrex storage jar containing 500 ml of stored tap water and maintained at temperature between 25-27°C and a photoperiod of 14:10 (L:D) [2]. 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.

Toxicological Assays: Toxicological assays, against the new exuviated fourth instar larvae and ecdysed pupae, were carried out using different concentrations of novaluron. A series of concentrations of novaluron (10% EC, courtesy by Rohm and Haas, Spring House, PA) was prepared in distilled water. Appropriate aliquots (0.1-1 ml) were added to treatment beakers to give the final concentrations of 0.3, 0.5, 0.7 and 0.9 µg active ingredient per liter. The treated series of newly exuviated larvae and ecdysed pupae of *Cx. pipiens* were exposed to the product for 24 h according to WHO [14] and the control series were exposed to water only. After the exposure time, the larvae and pupae were removed and placed in untreated water. The tests were carried out with three replicates containing 25 larvae or pupae each. Mortality was registered daily until adult emergence. The duration of the larval and pupal developmental stage is determined after treatment with lethal concentrations LC_{50} and the LC_{90} and the longevity of the adults emerged was estimated. The results were compared to the control and analysed using Student's *t*-test. Toxicity data were analyzed by probit analysis [15] and LC_{50} (50% lethal concentrations) and 95% confidence limits (95% LC) were estimated.

Effect of Novaluron on Fecundity and Reproduction of *Cx. Pipiens*: The fecundity experiments were conducted on the eggs of *Cx. pipiens* collected from the breeding jars of the females emerged from treated fourth larval stage with the lethal concentrations (LC_{50} = 0.33 µg/l; LC_{90} = 0.75 µg/l) of the of mosquito species. For each concentration, 20 females and 20 males were kept in separate breeding cage. The laying eggs for each series were collected, counted and transferred to a new jar containing 500 ml of water and kept for larval hatching. Different parameters of mosquito reproduction; the number of egg laying, hatching rate, the fecundity, were studied. The fecundity was calculated by the number of eggs laid in ovitrap divided by number of females let to mate (The death of adults in the experiments was also considered). The obtained results were subjected to a statistical analysis using the *t*-test of student. Hatching Rate (HR) and reduction in fecundity (RF) were calculated according to the following formula:

$$HR = \frac{number\ of\ hatching\ eggs}{total\ number\ of\ eggs} \times 100$$

$$RF = \frac{number\ of\ eggs\ laying\ of\ control - number\ of\ egg\ laying\ of\ the\ treated\ females}{number\ of\ egg\ laying\ of\ the\ control\ females} \times 100$$

RESULTS

Insecticidal Activity: Concentration-response relationship was determined for novaluron applied to newly exuviated fourth instar larvae of *Cx. pipiens* and the mortality was recorded up to adult emergence (Fig. 1). Bioassays showed that novaluron have a high toxicity against the mosquito species, *Cx. pipiens*. The lowest concentration tested of 0.3 µg/l, caused 40% of mortality of *Cx. pipiens* larvae; however the highest concentration of 0.9µg/l caused more than 96% of mortality. The statistical analysis of the data reveals a very highly significant difference ($p < 0.01$) between concentrations. With probit analysis, the LC_{50} was estimated at 0.33 µg/l (95% CL = 0.23- 0.61µg/l; n= 75; slope = 1.20) and the LC_{90} was 0.75 µg/l (95% CL = 0.59 -0.90 µg/l). However the treatment of newly ecdysed mosquito pupae with the same concentration was not showing any significant mortality compared to control (Fig. 1).

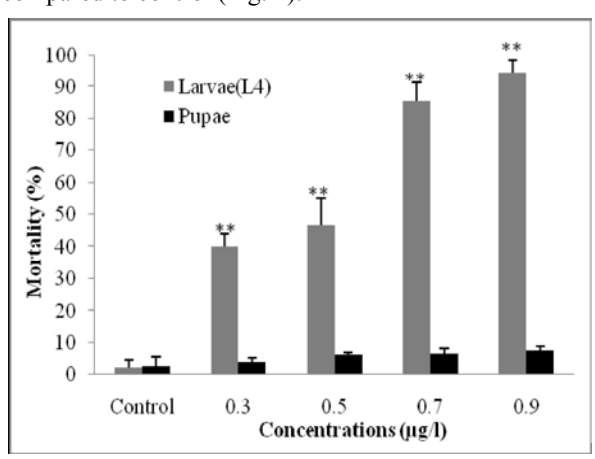

Fig 1: Concentration-response relationship for treatment of the novaluron applied to the newly exuviated fourth instar larvae and ecdysed pupae of *Culex pipiens*.

Table 1: Effect of the novaluron on the duration of the developmental stages and adult longevity after treatment of fourth instar larvae and pupae of *Culex pipiens,* with the lethal concentrations (LC_{50} = 0.33 µg/l and LC_{90} = 0.75 µg/l). Means followed by different letter indicate a significant difference (P<0.05), (n = 13-75).

	Duration and longevity of the developmental stages (day) of *Culex pipiens*					
	Treatment of the L4 stage			Treatment of the pupal stage		
Stage	Control	LC_{50}	LC_{90}	Control	LC_{50}	LC_{90}
L4	6.66[a]	7.67[b]	8.16[c]	-	-	-
Pupae	3.53[a]	3.66[a]	3.81[a]	3.53[a]	3.83[a]	4.13[b]
Adult	32.33 [a]	27.53 [b]	27.53[b]	32.33[a]	23.13[b]	23.53[b]

Table 2: Effect of lethal concentrations (LC_{50} = 0.33 µg/l and LC_{90} = 0.75 µg/l) of novaluron on reproduction of the females emerged from the treated fourth instar larvae of *Culex pipiens* (n= 20 females). Means followed by different letter indicate a significant difference (P<0.05).

Treatment	N° egg laid	Hatching rate (%)	Fecundity egg/female	Reduction in fecundity (%)
Control	1571[a]	97.41 ± 2.07[a]	66.6[a]	/
LC_{50} = 0.33 µg/l	1353[b]	92.05±4.82[b]	49.4[b]	15.32
LC_{90} = 0.75 µg/l	1304[c]	84.08±5.89[c]	34.8[c]	16.22

Effect of Novaluron on the Duration of Developmental Stages and Longevity of *Cx. Pipiens*: The effect of novaluron on the developmental duration and adult longevity of *Cx. pipiens* after the treatment with the lethal concentrations (LC_{50} = 0.33 µg/l and LC_{90} =0.75 µg/l) is presented in table 1. The results showed that the novaluron inhibits the development by increasing the stage duration. The increase in the larval development duration of the fourth instar larvae was significant (P<0.05) with a relation dose-response. The duration of the control was 6.66 days whereas the development duration of the treated series was 7.67 and 8.16 days for CL_{50} and CL_{90}, respectively. However, no significant difference was recorded in the duration of the following pupal stage for all used concentrations. Therefore the adult longevity was considerably reduced, by the treatment of novaluron, up to 27 days for the both lethal concentrations, whereas the longevity of the control series was estimated to 32 days (Table 1).

The application of novaluron with same lethal concentrations (LC_{50} = 0.33µg/l and LC_{90} = 0.75 µg/l) to newly ecdysed pupae revealed a significant effect (p<0.05) on the duration of development of the treated pupal stage. The duration of the pupal development was 3.44 days for control and increased to 4 days for the treated pupae with the LC_{90} (Table 1). The longevity of the adults emerged from the assays of the treated pupae was reduced compared to control. The longevity of 32 days of the mosquito adult of the control was reduced significantly to 23 days in the adults of the treated series for both lethal concentrations (Table 1).

Effect of Novaluron on Reproduction of the Females of *Cx. Pipiens*: The effect of novaluron on reproduction was evaluated on different parameters, of the females emerged from the treated fourth instar larvae of *Cx. pipiens* and the results are presented in Table 2. The number of eggs laid, by the females of *Cx. pipiens,* was reduced from 1571 to 1353 and 1304 according to the increase of the concentration, from LC_{50} to LC_{90}. For the same series of experiments the hatching rates was calculated and showed a significant decrease according to the treatment of novaluron (Table 2). When the hatching rate for the control was 96.84%, for the treated series was significantly reduced to 75.40% and 55.02% for the CL_{50} and CL_{90}, respectively. The number of eggs laid/female was inversely proportional to the concentration in treatment, when it was recorded 66 eggs for the non treated female only 49 and 34 eggs were counted for the treated females with CL_{50} and CL_{90} of novaluron, respectively. Fecundity of the mosquito was highly reduced after the treatment with the lethal concentrations of novaluron with a reduction of 15% for CL_{50} and 16% for CL_{90}.

DISCUSSION

Mosquitoes are known to be vector for a many diseases like malaria, dengue, West Nile, etc. The persistence of these diseases in many parts of the world [16, 17] has renewed the interest in re-evaluation of vector control strategies. Currently, the larvicides used for mosquito control are neurotoxic products. Beyond the pollution for the environment, these insecticides present a toxic effect to other class of animals like birds, fish, bees and mammals [18, 19]. The interest then has been focused on the development of new products that could be good alternatives for vectors control. Since, many molecules were proposed, such as botanical biopesticides [20, 21], medicinal plant extracts [22] and essential oils [23, 24].

During the last decays a new class of pesticides, acting via the development process on the insect, known the insect growth regulators (I.G.Rs) [25, 26], was suggested as an alternative to classical synthetic chemical pesticides because of do not present any effect on the environment and non target animals. The I.G.Rs were developed in order to inhibit or to disrupt the insect growth, the molt and or the metamorphosis [27, 28]. The potency of Insect growth regulators (I.G.Rs) for mosquito control has been the subject of intensive investigations [12]. A few reports documented the larvicidal efficacy of novaluron under laboratory conditions against the larvae of *Musca domestica* [29], *Culex* mosquitoes and *Aedes aegypti* [30, 31]. The present study was to evaluate the effects and the mechanism of an insect growth regulator novaluron, which is considered too as an endocrine disrupting compound (E.D.Cs) for insect species, on *Cx. pipiens*. The toxicity assays conducted under laboratory conditions on *Cx. pipiens* larvae indicated that novaluron exhibited a larvicidal activity with concentration-response relationship when applied to newly ecdysed larvae. The results showed that the novaluron exhibits toxic effects via the perturbation of the growth and the development of *Cx. pipiens* and this is similar to alsystin when used against the same species [4] and confirm a previous work, using novaluron on larvae of *Cx. pipiens* [13] and *Culex quinquefasciatus* and *Anopheles gambiae* [30] and *Aedes aegypti* [31]. This compound has been shown to manifest their activity via inhibiting the cuticular synthesis during the larval development of the target insects, where the larva undergoes premature apolysis, resulting to that the insect remains trapped in the molting process and dies slowly from starvation and desiccation. However, the treatment of newly ecdysed pupae of *Cx. pipiens* with the same concentrations, novaluron did not exhibit any toxicological effect. In the present study, novaluron treatment increased the larval duration, not the pupal and introverted the adult emergence. Those treated larvae escaped from mortality showed reduced longevity. The adult which emerged from treated larvae were morphologically normal but showed a great reduction in fecundity. These effects were confirmed by the reported results on *Spodoptera littoralis* under chlorfluazuron and lufenuron treatment [32, 33]. The same results were mentioned when the alsystin [4] and andalin [2] were tested on pupae of *Cx. pipiens*. In contrast, the use of the RH-0345 increased the pupal duration of *Cx. pipiens* [12] and *Tenebrio molitor* [34] and the same effect was observed when the RH-2485 and RH-5992 were applied against pupae of *Ephestia kuehniella* [35]. The influence of the novaluron on reproduction and fecundity of the female adults was recorded by the reduction of the number of laid eggs, hatching percentage and fecundity. These results are similar to those mentioned for *Tenebrio molitor* [36] and on *Argyrotaenia vellutinana* and *Choristoneura rasaceana* adults with RH-5992 and RH-2485 [37]. In general, sublethal effects caused by chitin synthesis inhibitors include delayed developmental rates [38, 39], reduced fecundity and fertility [25, 40, 41]; studies with application of other I.G.Rs including (methoxyfenozide, hexaflumuron, lufenuron, tebufenozide, pyriproxyfen and flucycloxuron) on other insect families reduced pupal weight, adult emergence, fertility and fecundity [42-44]. Molecular studies revealed that the effects of some I.G.Rs on insect molting and reproduction were due to the perturbation of gene expression in the hierarchy cascade of vitellogenesis and/or choriogenesis [45, 46]. Other works showed that the DFB (diflubenzuron, a chitin synthesis inhibitor) reduced longevity and weight of the adults [47], adult emergence [48], it also decreased significantly the numbers of ovocytes and the weight of the ovaries as well as the ovocyte bazal size in *Tenebrio molitor* [49] and *Cydia pomonella* [50].

In conclusion, the I.G.Rs effectively limit the pollution and control the risks associated with mosquitoes (usually insects), while respecting the environment and therefore constitute a good alternative to insecticides.

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