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Impact of an insect growth regulator on the development and the reproduction potency of mosquito

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Abstract

The present study was conducted under laboratory conditions and consisted to evaluate the lethal concentrations (LC₅₀ & LC₉₀) effects of an agonist of the molting hormone, the halofenozide (RH-0345), on several physiological and biological aspects (developmental duration, morphology, metabolites and potential reproduction) after treatment of newly ecdysed fourth instar larvae of mosquito species, *Culex pipiens*. The results showed that the RH-0345 induced a decrease of the developmental duration of the treated larval stage with morphological aberrations. The duration of control ones was 7.5 days whereas for treated series, it was 6.66 and 6 days for LC₅₀ and LC₉₀, respectively. The biochemical assays showed that the product has reduced significantly the amount of the metabolite contents; carbohydrates, lipids and proteins. The potential of reproduction of adult females emerged from treated fourth instar larvae, was affected and the number of laid eggs, hatching and fecundity were decreased compared to the control.

Keywords: Insect growth regulator, molting hormone, agonist and mosquito

1. Introduction

Mosquitoes are medically and veterinary important vectors, responsible for the transmission of many human and animal diseases, such as malaria, yellow fever, dengue and West Nile Fever [1]. The management of disease vectors using conventional neurotoxic pesticides has failed because of the high reproductive ability, development of insecticide resistance of insect species and secondary effects on none target organisms and environment [2]. These reasons are leading the scientists to focus on the search of novel molecules without secondary effects. They proposed the insect growth regulators (I.G.Rs) as new pesticide alternative, that seem to be promising because of their specific mode of action on insects and their lower toxicity against non-target organisms; specially vertebrate, than conventional insecticides [3, 4, 5]. In the last decades, the I.G.Rs compounds have shown promising results in controlling insects of agricultural, medical and veterinary field [6, 7, 8].

Culex pipiens (Diptera, Culicidae) is the most widely distributed mosquito in the world and carries a number of diseases [9]. According to its large distribution, this represents the most interesting mosquito species in Algeria, particularly in urban areas and is generally controlled by conventional insecticides [10]. 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. Therefore, the purpose of this study was to evaluate in laboratory, the effectiveness of an insect growth regulator, RH-0345, after treatment during the fourth instar larvae of the domestic mosquito *Culex pipiens*, and to provide better insights in the physiology of its mode of action.

2. Materials and methods

The experiments of the present study were conducted exclusively in the laboratory under controlled conditions.

2.1 Mosquito rearing

The fourth larvae of *Culex pipiens* were obtained from a stock colony of the laboratory. For essays 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) [11].

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 3 days. Whereas the adult were reared in cages contained a jar for laying eggs and were fed on honey of sucrose.

2.2 Ecdysone agonist bioassays

The bioassays were conducted using an insect growth regulator represented by an ecdysone agonist of the molting hormone; the halofenozide (RH-0345). The concentrations of halofenozide (23% EC, courtesy by Rohm and Haas, Spring House, PA) were prepared in distilled water. Appropriate aliquots (0.1-1 ml) were added to treatment beakers to give the final lethal concentrations; LC₅₀ = 12.58 and LC₉₀ = 28.58 µg/l [7]. The newly ecdysed 4th-instar larvae of *Cx. pipiens* were exposed to these lethal concentrations for 24h and controls were exposed to water only. The experiments were conducted according to WHO criteria [12]. After the exposure time of 24 h, larvae were removed and placed in clean water. For each lethal concentration the test was carried out with many replicates containing 25 larvae each, in order to arrive to the estimated number of surviving larvae / adult females. The surviving larvae were used to study the effect of the ecdysone agonist; the halofenozide on different parameters of the development (Stage duration and morphological observation) and the remaining larvae were followed until the emergence of adults, to assess the effects on reproduction (number of laid eggs, hatching and fecundity). The results were compared to the control and analyzed using Student's t-test.

2.3 Biochemical procedure

Newly ecdysed fourth instar larvae of *Cx. pipiens*, were treated with two concentrations LC₅₀ = 12.58 and LC₉₀ = 28.58 µg/l [7]. Each pooled sample contains 10 individuals, was weighted and subjected to extraction in trichloroacetic acid

(TCA 20%) [13] at 1, 2, 4 and 6 days. After a first centrifugation, the supernatant was used to evaluate the carbohydrates, as described by [16], and to the pellet was added a mixture of ether and chloroform (1V/1V) for making a second centrifugation. The lipids were quantified from the supernatant 2 [15]. Therefore the protein bioassay was carried out from the dissolved pellet 2 in NaOH (0.1N) [16]. Data were expressed in µg/mg whole body weight; means ± SD were analyzed based on three replicates per treatment each using a Student's t test.

2.4 Effect of the RH-0345 on fecundity and reproduction of *Culex pipiens*

The experiments on the fecundity were conducted on the eggs collected from the breeding jars of the mosquito females of *Cx. pipiens* emerged from treated fourth instar larvae with the lethal concentrations LC₅₀ = 12.58 and LC₉₀ = 28.58 µg/l [7]. For each concentration 10 females and 10 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 reproduction; the number of egg laying, hatching rate and the fecundity, were studied. Number of laid eggs was estimated by counting the number of eggs using a binocular microscope. 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. The hatching rate (H), reduction of hatching (RH), fecundity (F) and reduction of fecundity (RF) were calculated using the following formulas [17]:

$$H = \frac{\text{Number of hatched eggs}}{\text{Total number of eggs}} \times 100$$

$$RH = \frac{\text{Number of hatched eggs of controls} - \text{Number of hatched eggs of treated females}}{\text{Number of hatched eggs of control females}} \times 100$$

$$F = \frac{\text{Total Number of laid eggs}}{\text{Total Number of females}}$$

$$RF = \frac{\text{Number of laid eggs of controls} - \text{Number of laid eggs of treated females}}{\text{Number of laid eggs of control females}} \times 100$$

LC ₉₀ = 28.58 µg/l	6.00 ± 1.05 ***	3.33 ± 0.5
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3. Results

3.1 Effect of RH-0345 on the development of *Culex pipiens*

The application of RH-0345 with different lethal concentrations (LC₅₀ = 12.58 and LC₉₀ = 28.58 µg/l [5]) caused a very highly significant reduction (p<0.001) of developmental duration of treated stage or fourth instar larvae. The duration of fourth stage control was 7.5 days whereas for treated series, it was 6.66 and 6 days for LC₅₀ and LC₉₀, respectively. However, no significant difference was recorded in the duration of the following pupal stage for all used concentrations (Table 1).

Table 1: Effect of RH-0345 (LC₅₀, LC₉₀) on the duration of development (days) of larval and pupal stages of *Culex pipiens* (n=75).

Concentrations	Developmental duration (days)	
	4 th larval stage	Pupal stage
Control	7.5 ± 0.52	3.4 ± 0.51
LC ₅₀ = 12.58 µg/l	6.66 ± 0.69 **	3.4 ± 0.51

The ecdysone agonist proved to affect growth and development in mosquito *Cx. pipiens*. After about 2 days of treatment, intoxicated larvae showed a change in their behavior by descending to the bottom of the jar and they remained there motionless until they die. Morphological examination of intoxicated larvae revealed that the larvae did not shed the old head capsule nor completed ecdysis after 5 days following treatment with halofenozide and presents a doubled head (Fig. 1A). In some cases, even so the observations of intoxicated pupae showed the new cuticle was produced the ecdysis process was blocked and as a consequence such larvae or pupae died trapped within their exuvium (Fig. 1B). Further on in the development, halofenozide presented toxicity up to adult emergence because treated insects failed to ecdyse into pupae and adults and died within the respective exuvium (Fig. 1C).



Fig 1: The morphological aberrations observed on larvae and pupae after treatment (A: doubled head larva, B: Inhibition of the exuviation pupae-adult, C: The adult undergoes a partial pupal ecdysis limited to the anterior part)

3.2 Effect of RH-0345 on weight and metabolites of *Culex pipiens*

In control series the body weight of the fourth instar larvae increased with time. At 1 day the weight was 2.10 mg/larva and increase progressively to 4.30 mg/larva at the day 6. Therefore for the treated series, the larva weight decrease significantly for both lethal concentrations, where it was at the day 6, 3.59 mg/larva and 3.05 mg/larva for LC₅₀ and LC₉₀ respectively (Table 2).

Table 2: Effect of halofenozide (LC₅₀, LC₉₀) on the fresh body weight (mg/larva), of the fourth instar larvae of *Culex pipiens* (n=10). * = p<0.05, ** = p<0.01, *** = p<0.001.

Age (day)	Body weight (mg/Larva) of the 4 th instar larvae of <i>Cx. pipiens</i>		
	Control	LC ₅₀ = 12.58 µg/l	LC ₉₀ = 28.58 µg/l
1	2.10 ± 0.07	2.12 ± 0.05	2.09 ± 0.06

2	2.95 ± 0.07	2.62 ± 0.04 *	2.46 ± 1.06 **
4	3.97 ± 1.00	3.14 ± 0.05 ***	2.80 ± 0.07 ***
6	4.30 ± 0.03	3.59 ± 0.02 ***	3.05 ± 0.03 ***

Halofenozide was tested at two concentrations (LC₅₀ = 12.58 µg/l and LC₉₀ = 28.58 µg/l) on the changes of metabolite amounts; carbohydrates, lipids and proteins, of the fourth instar larvae of *Cx. pipiens* during different times of the developmental stage (1, 2, 4 and 6 days). The obtained results showed that the carbohydrates, lipids and proteins amounts of the control series increased significantly with throughout the stage (Fig. 2a, b, c). The results of the treated series revealed that carbohydrates, lipids and proteins level did not shift significantly at day 1 and 2. Therefore the metabolite amounts decreased significantly for both lethal concentrations starting from day 4 up to day 6 for carbohydrates, proteins and increased for lipids compared to control for the same periods.

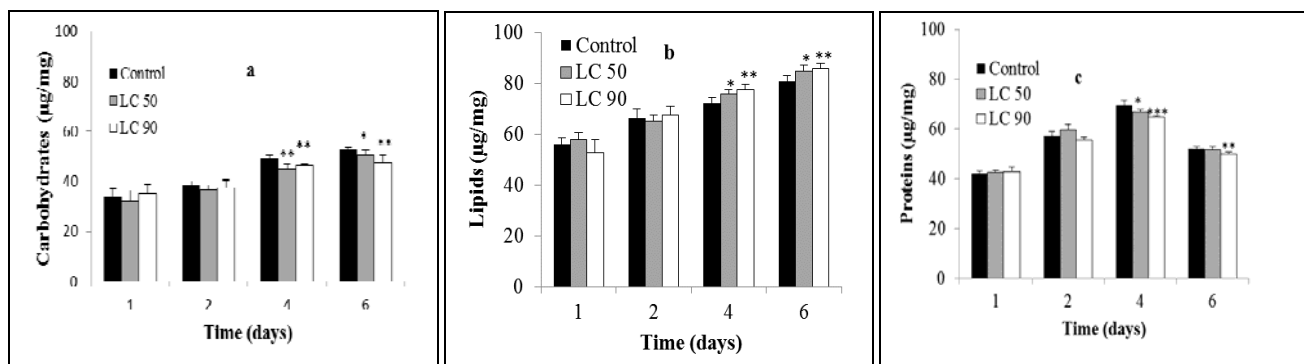


Fig 2: Effect of RH-0345 on the amount (µg/mg body weight) of the metabolites (a = carbohydrates, b = lipids and c = proteins) of treated fourth instar larvae of *Cx. pipiens* with the 2 lethal concentrations (LC₅₀ = 12.58, LC₉₀ = 28.58 µg/l).

3.3 Effect of RH-0345 on reproduction

The effect of halofenozide on reproduction was evaluated on different parameters, of the females emerged from the treated fourth instar larvae of *Cx. pipiens* (Table 3). The number of laid eggs of female controls was 2065 eggs. For treated series, the number of eggs was reduced to 1987 and 1738 eggs after treatment with the LC₅₀ and LC₉₀ respectively. The hatching

rate showed a high significant decrease in treated series by the two concentrations, which was 96.1% in controls and arrived to 92.84% for the treated series with LC₅₀ and, 85.03 for the treated one with LC₉₀. Halofenozide (RH-345) affected the fecundity; and this was more viewed with the highest concentration, with a reduction of 3.76% for LC₅₀ and 14.38% for LC₉₀ (Fig. 3).

Table 3: Effect of RH-0345 on some parameters of reproduction of adult females emerged from treated fourth instar larvae of *Cx. pipiens* (n=10 females, duration=30 days). *** = p<0,001.

Concentrations	Number of laid eggs	Hatching rate	Reduction of hatching (%)	Reduction of fecundity (%)
Control	2065	96.10 ± 1.34	/	/
LC ₅₀ = 12.58 µg/l	1987	92.74 ± 2.22 ***	6.84 ± 0.38	3.76 ± 1.57
LC ₉₀ = 28.58 µg/l	1738	85.03 ± 2.27 ***	23.56 ± 0.51	14.38 ± 1.13

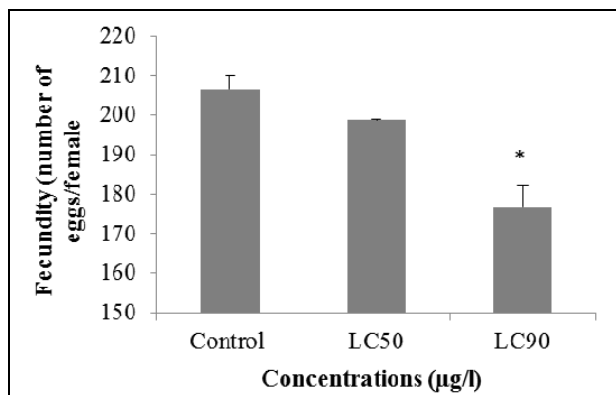


Fig 3: Effect of RH-0345 on fecundity (number of laid eggs / female) of females emerges from treated fourth instar larvae of *Cx. pipiens* (n=10 females; duration=30 days). * = $p < 0.05$.

4. Discussion

Mosquitoes are known to be the vector for a number of public health related diseases like malaria, dengue, Japanese encephalitis, West Nile, etc [1]. The development of the mosquito treated resistance has allowed the spread of the disease vector populations [18]. Instead of the secondary effects of the conventional pesticides, this was one reason why attention has been focused during recent years on the development of new alternatives for vector control, such as botanical biopesticides [19], essential oils [20] and insect growth regulators (I.G.Rs) [3] which exhibit an environmental safety and any mammalian toxicity. The I.G.Rs were developed to deliberately inhibit or disrupt insect growth, molt and metamorphosis. They act on insects by various mechanisms such as inhibition of chitin synthesis or by mimicking their hormones [6]. In this study, an agonist of the molting hormone, holofenozide (RH-0345) was tested on newly ecdysed fourth instar larvae of *Cx. pipiens* in order to evaluate its effects on development, biochemical aspect and reproduction potency. The obtained results showed that the $LC_{50} = 12.58 \mu\text{g/l}$ and $LC_{90} = 28.58 \mu\text{g/l}$ [7] caused a disturbance of development by the reduction of the developmental duration of the treated larvae of *Cx. pipiens*, in contrast to other results after treatment of *Cs. longiareolata* and *Cx. pipiens* larvae with novaluron which showed an increase in the development duration [8, 21]. However, no reduction in developmental duration was recorded for pupae. The same results were reported when andalin [10] and alsystin [11], were applied against pupae of the same species. The morphological malformations observed in treated series are represented with doubled head, some adults could not complete their ecdysis; inhibition of the exuviation pupae-adult and others undergo a partial ecdysis limited to the anterior part. Different abnormalities were observed by using other I.G.Rs on other insect species [22, 23].

The reduction in treated larval weight using RH-0345 confirmed previous studies when novaluron has been used against the larvae of *Cs. longiareolata* and *Cx. pipiens* [8, 21]. This could explain the reduction in the metabolic sources; such as carbohydrates that was induced by halofenozide on *Cx. pipiens* larvae. These perturbations can be understood by the ability of I.G.Rs to modify the synthesis of certain metabolites and disrupt the functionality of the organism [24] and a decrease in the trehalase activity or to their effects on the carboxylase activity.

The increase in lipids content induced by halofenozide on *Cx. pipiens* larvae was similar when novaluron was used against *Cs. longiareolata* and *Cx. pipiens* [8, 21]. This perturbation may

be explained by the interference of I.G.Rs with not only the synthesis of lipids but also their mobilization as promoted to convert into other metabolites or fatty acid. This could be also related to the various hormonal systems and under neuroendocrine control [25] and lipids are transported from the storage site (fat body) via the haemolymph towards the user organs, in particular cuticular synthesis [26] and vitellogenesis [27]. In addition, the odd case of lipid increasing may be attributed to the accumulation of carbohydrates which might lead to an inverse in their conversion rate to lipids as a reverse material [28].

The reduction in protein content induced by halofenozide on *Cx. pipiens* larvae was reported in other species [8, 21, 29]. This reduction was explained by a destructive effect of I.G.Rs on some of the cerebral neurosecretory cells of the brain responsible for secretion of the proteins of the treated larvae consequently DNA synthesis is inhibited.

The perturbation in reproduction parameters of *Cx. pipiens* was induced under the effect of RH-0345. The same effect was happened when the same species was treated with novaluron [30] and other insect orders with different insect growth regulators [31, 32, 33]. In general, sublethal effects caused by ecdysone agonists include delayed developmental rates [34], reduced larval and pupal weight [35], reduced fecundity and fertility [36].

5. Conclusion

The present molecules present a good alternative according to toxicity effects on mosquito larvae. They act by the perturbation of the hormonal system of the insects. Instead of their toxicological activities, its effect on the reduction of the metabolite quantities can be explained by the reduction of the development duration caused by the RH-0345 which functions as an ecdysteroid agonist by binding on natural receptors of molting hormone (20 E). Therefore it disrupts its content too, which caused a perturbation in a lot of biological aspects like weight, metabolites because all these aspects are regulated by 20E. The modification in biochemical composition may explain the disruption of reproduction parameters because the metabolites are necessary to the vitellogenesis, which is the process of their accumulation to ensure the egg maturation. These perturbations were due to the perturbation of gene expression in the hierarchy cascade of vitellogenesis.

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