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Ovicidal activity of an insect growth disruptor (methoxyfenozide) against *Culex pipiens* L. and delayed effect on development

Kaouther Hamaidia and Noureddine Soltani

Abstract
Current researches for mosquito control have focused on insect specific insecticides without environmental concerns. The present study was conducted to evaluate the Ovicidal activity of an ecdysone agonist (methoxyfenozide) on *Culex pipiens* L. (Culicidae: Diptera) under laboratory conditions using standard WHO protocol. Lethal concentrations (LC_{50}= 24.54 µg/L and LC_{90}= 70.79 µg/L) previously determined were tested against freshly laid eggs. The egg viability was examined and the deferred effects of treatment were also investigated on the development duration and the sex ratio. Data obtained show that egg hatching inhibition was dose dependent, with a reduction of 13.44% and 46.99% with LC_{50} and LC_{90}, respectively. In treated groups, the total development duration was significantly longer as compared to controls. The adult sex - ratio was significantly skewed in favor of males emerged from treated eggs with LC_{50} and LC_{90} of methoxyfenozide. Moreover, changes in the egg shell morphology and abnormal egg hatching pattern were observed. Morphologic abnormalities in larva were also recorded. Data from the current investigation clearly demonstrated that methoxyfenozide possess remarkable larvicidal and ovicidal activities against a medically important vector as ideal eco-friendly approach for mosquito's control.

Keywords: Methoxyfenozide, mosquitoes, *Culex pipiens*, eggs, ovicidal activity, development, morphological aberrations

1. Introduction
The world health organization has declared the mosquitoes as public enemy number one, since they act as vector for various diseases such as Zika [1], dengue fever [2], yellow fever [3], chikungunya [4, 5], malaria [6, 7] and Japanese encephalitis [8, 9]. In Algeria, mosquitoes have been the subject of several studies, including surveys in different areas [10, 11], biochemical composition [11, 12], blood meal feeding [13] and mosquito control by use synthetic insecticides [14, 15], biological agents [16], or biopesticides and plant extracts [17-19]. Mosquitoes are generally controlled by conventional insecticides such as organophosphate, carbamate and pyrethroid insecticides [20-22]. However, such insecticides have caused several environmental concerns [23, 24] and alternative strategies using eco-friendly products such the insect growth disruptors (IGDs) are developed [25, 26] for controlling pest populations.
The non-steroidal ecdysteroid agonists such as tebufenozide, halofenozide or methoxyfenozide constitute a new class of IGDs. These products mimic the action of the steroid insect molting hormone, 20-hydroxyecdysone (20E), and manifest their activity by binding to the ecdysteroid receptor complex in a manner competitive with ecdysteroids, inducing a precocious and incomplete lethal molt in several insect orders [27]. The ecdysteroid agonists like halofenozide and tebufenozide has been found potent against mosquito larvae [15, 28, 29]. The juvenile hormone (JH) and the molting hormone (ecdysteroids) plays an important role in insects controlling development, metamorphosis, reproduction, and behavior [30, 31]. Exogenous application of ecdysone agonists is effective only when endogenous levels of ecdysone in insects are low [32]. The ecdysteroid content is relatively low in freshly laid eggs [33-35] and the ecdysone agonists may disturb the embryonic development and produce an ovicidal activity [35]. The ovicidal activity was tested with several plant products, but few experiments were performed on IGDs as ovicide or on their delayed effects on development and sex-ratio [35-39]. Therefore, the objectives of this study were to evaluate the ovicidal toxicity of methoxyfenozide an IGD after treatment of freshly laid eggs of *Culex pipiens* L. (Diptera: Culicidae) and to examine its deferred effects on the developmental duration and the sex-ratio.

2. Materials and methods

2.1 Mosquito rearing

Cx. pipiens were reared in the Terrestrial and Aquatic Ecosystems Laboratory (Souk-Ahras University) since October 2014. The larvae were fed on fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast in the 3:1 ratio, as previously described [40], and dechlorinated water was replaced every three days Adults were fed on 10% glucose solution and adult females were fed with pigeon blood and the eggs raft produced were used for stock-rearing. Mosquitoes were held at 25 ± 2 °C, 70-85% relative humidity, with a photoperiod of 14 h light: 10 h dark.

2.2 Insecticide and treatment

Methoxyfenozide (23% Emulsifiable Concentrate: EC, Rohm and Haas, Spring House, PA) was kindly provided by Pr. G. Smagghe (Agrozoology Laboratory, Ghent University, Belgium). The product was dissolved in distilled water and added to treatment beakers at two final concentrations (24.54 µg/L and 70.79 µg/L) corresponding to LC_{50} and LC_{90}, respectively as previously determined with respect to the fourth-instar larvae *Cx. pipiens* (Diptera, Culicidae) [41]. Control experiments were conducted in dechlorinated tap water only.

2.3 Egg hatchability

Gravid females deposited directly their fresh eggs in an oviposition cage containing methoxyfenozide treated water. All experiments were performed in three replicates. Eggs were left in the water until hatching. The neonates (first instar larvae) were counted under a dissecting microscope before transferring them with a pipette to clean tap water. The egg hatchability (percentage of neonates that emerged from eggs) was recorded.

2.4 Duration of development and sex-ratio

After treatment of freshly laid egg rafts, the durations of the various immature stages have been calculated. Following hatch, first instar larvae were separated into three lots of 15 individuals for each experimental test, three replicas were made. After each molt, the larvae were transferred to a new container. The period of time from egg laying to hatching was considered as incubation period; from hatching till pupation was designated the larval period and from pupa formation till emergence of imago as pupal period. The adult sex-ratio was determined by counting the number of emerged males and females per egg raft.

2.5 Statistical analysis

Data are presented as the mean ± standard deviation (SD). Means were compared by one-way analysis of variance (ANOVA) and followed by post-hoc HSD Tukey test. All statistical analyses were performed using MINITAB Software (Version 16, PA State College, USA) and $p<0.05$ was considered to be a statistically significant difference. The number of individuals and repeats tested in each series is given with the results.

3. Results

3.1 Effects on egg hatchability

Data on egg hatchability are presented in table 1. Methoxyfenozide applied on freshly laid eggs caused a significant reduction in the egg hatchability with a dose response relationship. One way ANOVA revealed a significant effect of treatment ($F_{2, 27}= 109.90$; $p<0.001$). The egg hatchability inhibition was 13.44% with LC_{50} and 46.99% with the LC_{90} as compared with control series.

Table 1: Effect of methoxyfenozide on egg hatchability (%) against *Cx. pipiens* (mean ± SD, n = 3 repeats each containing 10 rafts; For the same parameter mean values followed by the same letter are not significantly different at $p>0.05$).

Groups	Number of laid egg/raft	Number of hatched egg /raft	Egg hatchability (%)
Control	66.60 ± 15.02a	66.60 ± 15.02a	100 ± 0.a
LC_{50}	62.20 ± 10.30a	54.20 ± 11.64b	86.55 ± 6.09b
LC_{90}	66.50 ± 11.08a	34.70 ± 6.86c	53.00 ± 11.08c

3.2 Effects on the duration of development

The effect of methoxyfenozide on the life-span was considered from treated newly laid eggs until adult emergence (Fig. 1). ANOVA revealed that methoxyfenozide affected significantly the development duration of all tested stages: incubation period ($F_{2, 27}= 96$; $p<0.001$), larval instars ($F_{2, 27} = 39.37$; $p<0.001$) and pupal stage ($F_{2, 27} = 16.41$; $p<0.001$). The compound also increased significantly the total development duration as compared to controls ($F_{2, 27}= 69.86$; $p<0.001$).

As shown in table 2, methoxyfenozide applied on eggs exhibits a delayed effect on both the number of males and females emerged/raft and the sex-ratio. ANOVA revealed significant effects of concentration on females ($F_{2, 27} = 167.07$; $p<0.001$) as well on males ($F_{2, 27} = 82.71$; $p<0.001$). Compared to control group, sex-ratio was significantly assigned in favor of males emerged from treated fresh eggs.

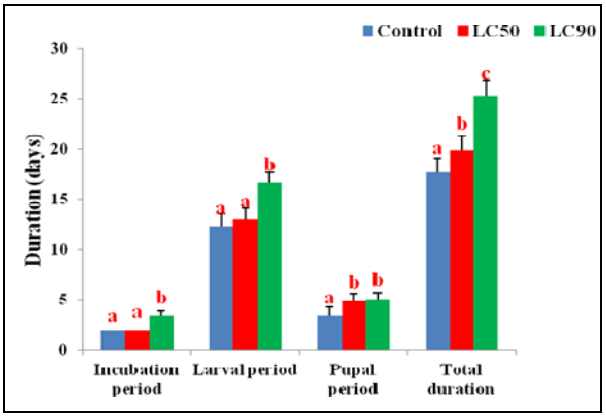

Fig 1: Effect of methoxyfenozide on development duration (days) of different immature stages of *Cx. pipiens* (n= 3 repeats each containing 10 rafts; for the same parameter mean values followed by the same letter are not significantly different at $p>0.05$).

Table 2: Deferred effect of methoxyfenozide applied on eggs of *Cx. pipiens* on the sex-ratio (mean ± SD, n = 3 repeats each containing 10 rafts).

Groups	Number of emerged females/raft	Number of emerged males/raft	Sex-ratio (M/F)
Control	35.70 ± 7.37a, A	30.90 ± 7.76a, A	0.78
LC_{50}	6.30 ± 2.45a, B	10.20 ± 3.52b, B	1.66
LC_{90}	1.80 ± 0.63a, C	3.70 ± 0.67b, C	2.21

Different capital letters indicate a significant difference between control and treated series of the same sex, while different small letters indicate a significant difference between sexes of the same group ($p<0.05$).

3.3 Morphological aberrations

Methoxyfenozide disturbs the growth and development and induced morphologic aberrations (Fig. 2). It dismantled egg raft of *Cx. pipiens* into individual eggs and induced larval emergence from the egg sidewall instead from operculum, and in some cases, we remarked hatching failure due to larval incapacity from going out from eggs. Moreover, several morphological abnormalities, altered larval body organization during embryo development were found (Fig. 2). Distended larval body. Appearance of larvae with two heads; normal one and another attached to the abdomen or in place of the saddle. Larvae with an inclined or segmented siphon or with eyes displaced. Furthermore, some adults showed abnormal wagging and restless movement for while then died. No abnormalities were recorded in the control group.

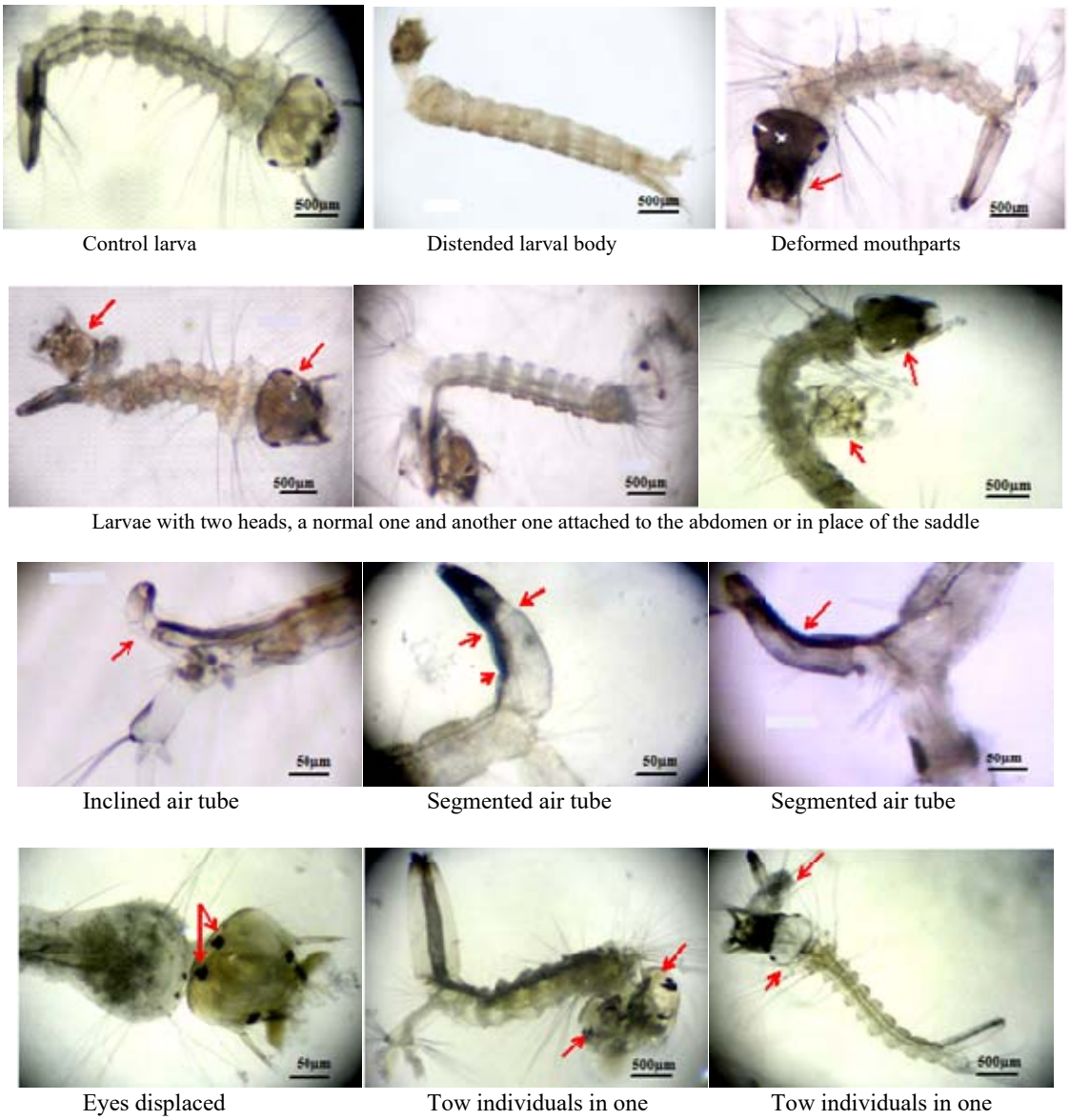

Fig 2: Morphologic aberrations observed in Cx. pipiens larvae following methoxyfenozide treatment of eggs.

4. Discussion

Exploring new pest management strategy by using insect growth disruptors represents an environmentally friendly option. These novel insecticides act on selective biochemical sites in insects such as chitin synthesis inhibitors, or juvenile hormone analogues and ecdysone agonists, which affect the hormonal regulation of different processes [42, 43]. Some insects exposed to such compounds may die due to abnormal regulation of hormone-mediated cell or organ development [44]. Methoxyfenozide, a bisacylhydrazine derivative, is a nonsteroidal ecdysteroid agonist that mimics the action of the molting hormones, because of their affinity for the ecdysteroid receptors (EcRs) [45, 46]. Contrary to conventional neurotoxic insecticides, methoxyfenozide have delayed activity, inducing mortality and morphogenetic abnormalities in stages beyond the one treated that impair insect survival, which means, it does not induce immediate mortality in the stage treated [47].

Our results showed that methoxyfenozide had significant direct ovicidal properties against *Cx. pipiens* which was due to the alteration in embryonic development caused by methoxyfenozide. The inhibition of *Cx. pipiens* egg hatch was higher in diflubenzuron treatment (21.8%) than pyriproxyfen and azadirachtin treatment [35]. Treated Spodoptera littoralis (Lepidoptera: Noctuidae) eggs with methoxyfenozide showed a concentration-dependent hatchability inhibition and significant mortality increase of larvae that hatched [48]. Furthermore, [49] confirmed its

outstanding ovicidal properties against *Operophtera brumata* (Lepidoptera: Geometridae). Moreover, methoxyfenozide significantly decreased egg fecundity [50] and fertility in *Ephestia kuehniella* Zeller [50, 51] while tebufenozide increased the duration of the pre-oviposition period [51]. In our study, the duration of total immature development was significantly prolonged by methoxyfenozide. Methoxyfenozide was also found to prolong significantly the timing of larval development of Spodoptera litura [52] and S. *frugiperda* [48, 53]. Similar observations were also reported in several insect species treated with other IGDs such as novaluron [12] chlorfluazuron [54] and pyriproxyfen [55].

Our data showed a several anomalies in egg hatching pattern and induced failure of larval emergence or their emergence from the egg sidewall instead of operculum with several morphogenetic aberrations. In a previous study, we found numerous abnormalities in Cx. pipiens larvae, intermediates larva-pupa and adult failure emergence after treated by kinoprene [56]. Similarly, changes in the Cx. pipiens egg shell morphology and abnormal egg hatching after treatment by diflubenzuron were reported [35]. Morphological and physiological variations in eggs may be the key factor to influence the ovicidal efficacy of IGPs.

Sex-ratio is a fundamental trait of population structure because it may govern reproductive potential [57] and variation in reproductive success, especially for males when females are the limiting sex [58]. Theoretically, extreme sex-ratio contributes to eliminate pest populations [59]. In our experiments, the sex-ratio was balanced in favor of males, which could have serious implications for the future population [59]. This effect could probably due to differential susceptibility between males and females to methoxyfenozide. This supports the hypothesis of increased female mortality [60]. Same results were observed in P. *idaeusalis* treated with tebufenozide an ecdysteroid agonist [47]. Moreover, offspring of Anopheles *gambiae* mosquitoes, genetically modified, presented 95% males which effectively suppress under laboratory conditions, the entire population of this vector [61].

5. Conclusion

The present results tested the ovicidal activity of methoxyfenozide on *Cx. pipiens*. In addition, this compound exhibited delayed effects on its growth and development. The development duration of immature stages was increased and several morphological aberrations observed. These effects are important from a practical point of view, because offspring can be reduced and as a consequence, the insect population can be maintained below a level of economic loss. Further experiments are needed to give additional information on the differential susceptibility to methoxyfenozide between males and females of *Cx. pipiens*.

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