Journal of Medical Entomology, 58(3), 2021, 1004–1011
doi: 10.1093/jme/tjaa260
Advance Access Publication Date: 28 November 2020
Research

Behavior, Chemical Ecology

Methoxyfenozide, a Molting Hormone Agonist, Affects Autogeny Capacity, Oviposition, Fecundity, and Fertility in *Culex pipiens* (Diptera: Culicidae)

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Subject Editor: Monika Gulia-Nuss

Received 15 July 2020; Editorial decision 19 October 2020

Abstract

The current study aimed to evaluate the effects of methoxyfenozide (RH-2485), an insect growth disrupter (IGD) belonging to molting hormone agonist class, against female adults of *Culex pipiens* L. under laboratory conditions. Lethal concentrations (LC_{50} = 24.54 µg/liter and LC_{90} = 70.79 µg/liter), previously determined against fourth instar larvae, were tested for adult female fertility, fecundity and oviposition after tarsal contact before mating and any bloodmeal. Methoxyfenozide was found to alter negatively their autogeny capacity and oviposition. A strong reduction of 56% and 72% ($P < 0.001$) in females' autogeny capacity was observed in both treated series, respectively. Alteration in oviposition were found to be higher with LC_{90} (OAI-LC_{90} = –0.62) than with the LC_{50} (OAI-LC50 = –0.42). Also fecundity and hatching rate (fertility) were significantly reduced in treated series as compared to controls. A significant reduction of 37.65 and 28.23% in fecundity and decrease of 56.85 and 71.87% in fertility were found, respectively in LC_{50} and LC_{90} treated series. Obtained data clearly demonstrated that methoxyfenozide have significant depressive effect on reproductive potential against medically important vector with minimizing ecotoxicological risks in mosquitoes management.

Key words: mosquito, insect growth disruptor, tarsal contact, fecundity, fertility

Most mosquitoes control programs are successively based on the use of chemical insecticides by outdoor spraying, impregnated nets, or indoor residual spraying (Nkya et al. 2013, Nwabor et al. 2017). Different classes of insecticides have been used. However, this strategy is threatened by development of insecticide resistance (Mouhamadou et al. 2019, Balaško et al. 2020, Ononamadu et al. 2020). Efforts to develop alternative tools to complement or even replace insecticide-based vector-control strategies must continue.

Metcalf & Horowitz (2014) provided a detailed review on the insect control with potential insect target-sites for development of novel specific and selective chemicals with minimal effect on environment. Unlike conventional insecticides, the class of insect growth disruptors (IGDs) possesses a specific activity spectrum with a novel insecticidal mechanism not based on a neurotoxic action (Dhadialla and Ross 2007).

IGDs are known as green chemistry compounds because of their spectrum of activity and selective toxicological profile (Dhadialla 2012). They are extremely efficacious due to disturbances in the endocrine mechanism (Roychoudhury 2016) at very low dose rates (Pener and Dhadialla 2012). All these characteristics make these insecticides important tools to manage insecticide resistance in integrated pest management programs (Dhadialla 2012). Methoxyfenozide (RH-2485) is a specific insecticide that belongs to a new class of IGDs, the non-steroidal ecdysteroid agonists. These chemical compounds mimic the action of the steroid insect molting hormone, 20 hydroxyecdysone (20E), by binding to the ecdysteroid receptor complex in a manner competitive with ecdysteroids, inducing a precocious and incomplete lethal molt in several insect orders (Dhadialla et al. 2005, Fahrbach et al. 2012, Smagghe et al. 2012).

In Algeria, several studies has been focused on alternatives to conventional insecticides by use IGDs (Hamaidia and Soltani 2014, Haouari-Abderrahim and Rehimi 2014, Hamaidia and Soltani 2016, Hamaidia et al. 2018, Hamaidia and Soltani 2019), biological agents (Boudjelida et al. 2008, Tabti and Abdellaoui-Hassaïne 2013) or plant extracts (Bouguerra et al. 2017; Dris et al. 2017a,b; Bouzidi et al. 2020).

In insects, ecdysteroids and juvenile hormone (JH) regulate simultaneously molting, metamorphosis, and reproduction (Truman and Riddiford 2019, Song and Zhou 2020). Their normal development

requires precise programming of rise and fall in a timely manner of hormones (Kang et al. 2017). A cross-talk between ecdysteroids and JHs was observed in *Drosophila melanogaster* (Liu et al. 2018) and *Schistocerca gregaria* Forskäl, 1775 (Orthoptera: Acrididae) (Lenaerts et al. 2019). JH ensures growth of larva, in same time preventing metamorphosis. Its level gradually decreases during development of insect allowing it to proceed to successive instars with each molt. Ecdysone brings the larvae to molt to the next larval stage if there is enough JH. If not, larvae initiate a molt to the adult stage (Song and Zhou 2020). The artificial activation of the ecdysone receptor by specific pesticides due to their agonistic capability induces an abnormal molt leading to death of the target (Pinto et al. 2019). Moreover, mating stimuli activated ovarian ecdysteroid biosynthesis (Ameku and Niwa 2016) indispensable for chorion formation (Lenaerts et al. 2019) and regulation of several cellular processes during early embryonic instars (Wang et al. 2018).

IGDs have been found to interfere with hormonal balance of target species by causing growth disruption, male sterility, and changes in biological fitness (Trostanetsky et al. 2015). These insecticides could be suitable for use in pest management programs (Rugno et al. 2016). IGDs with ecdysteroid activity have been reported to be effective against many insect pests like mosquitoes (Boudjelida et al. 2005, Hamaidia et al. 2018), cockroaches (Kilani-Morakchi et al. 2009, 2014), mealworms (Berghiche et al. 2007, 2008) or the Mediterranean flour moth (Soltani-Mazouni et al. 2012, Bakli et al. 2016).

The auto-dissemination approach aimed to spread insecticides by adults after tarsal contact to breeding sites. This method of pesticide 'self-delivery' has been shown effective in laboratory (Gonzalez and Harburguer 2020), semi-field environments (Swale et al. 2018), and open field trials (Chen et al. 2020). The strategy that we describe here exploits these findings.

Autogeny capacity is a key physiological trait in mosquito's abundance since they do not need bloodmeal for oviposition. In addition, it gives them a large ecological plasticity and omnipresence (Beji et al. 2017) even with less availability of vertebrate hosts (Culler et al. 2018).

Our study aimed to investigate for the first time if methoxyfenozide, an ecdysone agonist, may contaminate female adults of *Culex pipiens* L. from their larval sites via tarsal contact. For this, we investigated the application of methoxyfenozide by tarsal testing on virgin females before any bloodmeal, on oviposition activity index, autogeny capacity, fecundity, and fertility in order to obtain additional information on reproductive potential.

Materials and Methods

Mosquito Rearing

Culex pipiens were obtained from a stock colony of the Laboratory of Applied Animal Biology and kept as previously described (Rehimi and Soltani 1999). The mosquito species used is a strain collected in year 1995 from a protected nature reserve, the National Park of El Kala, far from anthropogenic sources of pollution. And since then, a rearing of this strain has been kept pure at the Laboratory Applied Animal Biology (Badji Mokhtar University, Annaba). Mosquitoes were maintained in an incubator at 25 ± 2°C under a photoperiod of 14:10 (L:D) h cycle and a relative humidity of 80%. Growing larvae were fed daily with fresh food consisting of a mixture of biscuit-dried yeast (75:25 by weight), and water was replaced every 3 d. Adults were held in 40 cm^3 cages and fed with 10% sucrose solution. The laid egg rafts were used for the following experiences.

Insecticide

In order to estimate the effects of an insect growth disruptor against *Cx. pipiens*, methoxyfenozide (23% EC, courtesy of Rohm and Haas, Spring House, Pennsylvania) kindly provided by Pr. G. Smagghe (Laboratory of Agrozoology, Ghent University, Belgium) has been evaluated. It belongs to the bisacylhydrazine insecticide class (BSH), reported as ecdysone agonists with molting hormone activity. The product was dissolved in distilled water and added to treatment cups at two final concentrations (24.54 µg/liter and 70.79 µg/liter) corresponding to LC_{50} and LC_{90} (i.e., the concentration causing mortality/effects in 50 and 90% of the treated insects, respectively) determined previously against fourth instar larvae (Hamaidia et al. 2018). The insecticidal activity of the tested product was characterized by these two reference lethal descriptors (LC_{50} and LC_{90}).

Tarsal Treatment

Mosquito legs, more precisely tarsi, are in contact with surfaces (Kong et al. 2015). Tarsi are the most relevant insect tissue for insecticide uptake (Balabanidou et al. 2019). Previously, we reported that females were more sensitive to methoxyfenozide than males (Hamaidia and Soltani 2016).

Tarsal testing was performed individually in small cages (10 cm^3) covered by nets (in which, the container containing the treatment takes the entire bottom) as follow: replicates of 15 virgin females before any bloodmeal (Gao et al. 2019) must rest on the treated LC_{50}- and LC_{90}-methoxyfenozide water surface for tarsal contact. When a mosquito tried to rest on the net; it was slightly disturbed to rest on the contaminated surface. After exposure time of 4 h, females were aspirated and introduced in new cages contained a cup with clean water, untreated males for mating and supplied with 10% sucrose solution. Untreated adults of both sexes were used in control series. Each test was repeated five times. Then, autogeny capacity, fecundity, and fertility were scored.

Determination of Autogeny Capacity, Fecundity, and Fertility

Fifteen females of *Cx. pipiens* were exposed by tarsal contact for 4 h to LC_{50}- or LC_{90}-methoxyfenozide before mating and any blood feeding, as described below. Control group was exposed to clean water. Each test was repeated five times. Then, each female was paired with one untreated male in an individual cage for mating. Overall, 75 pairs per treatment were investigated (15 pairs per repetition). After first oviposition without bloodmeal, autogeny capacity was estimated as the number of laying females. Then, egg's rafts were placed individually in separate recipients and fecundity (number of eggs per female) and fertility (percentage of newly hatched first instar larvae) were determined.

Oviposition Activity

Under the same laboratory conditions described above, tests were conducted in separate cages (40 cm^3). In each cage, three cups (7 cm diameter and 4 cm depth) were placed. The first cup contains clean water as control test, the second and the third contained LC_{50}- and LC_{90}-methoxyfenozide treated water, respectively. Each bioassay was performed with five replicates each containing 50 newly emerged (<8 h) untreated adults (25 females and 25 males). The number of egg's rafts (oviposition activity) in each treated cup was recorded daily until death of all adults. An oviposition activity index (OAI) was calculated according to Kramer and Mulla (1979) by the following formula: **OAI = (N Treatment – N Control) / (N Treatment + N Control)**

in which N is the number of egg rafts in each treatment. This index is only a coarse measurement of whether or not the females are influenced by a particular treatment. The OAI varies from −1 to 1 with 0 indicating no response. The positive sign means attractancy, and more eggs were deposited in the treated solution than control solution. And the negative sign means repellency; hence, more eggs were deposited in the control solution rather than treated solution.

Statistics

All statistical analyses were performed using MINITAB Software (Version 16, PA State College, United States) and $P \leq 0.05$ was considered to be a statistically significant difference. Results are presented as the mean ± standard error mean (m ± SEM). The normality of data was verified using the Kolmogorov–Smirnov test, and the homogeneity of variances was checked by Levene's test. Data were subjected to one-way analysis of variance (ANOVA) and mean values obtained separated by a post-hoc honestly significant difference (HSD) Tukey's test. In certain cases, the comparison of mean values was made by Student t test. The experiments were conducted using independent repeats. The number of individuals and repeats tested in each series were given with the results.

Results

Effects on Oviposition Activity

Effects of IGDs have been widely studied after water treatment; however, their effects on adults after tarsal contact have been overlooked. Oviposition activity following female's treatment by methoxyfenozide before mating and any blood feeding have been analyzed. In our experiments, *Cx. pipiens* females tended to lay a fewer egg's rafts in treated cups than in controls throughout the whole test duration. As shown in Table 1, from 25 females 13.80 produced their offspring in the control cups containing clean water, whereas only 5.60 and 3.20 rafts in LC_{50} and LC_{90} treated cups respectively. One-way ANOVA revealed a significant effect of treatment ($F = 272.59$; df = 2, 12; $P < 0.001$). Furthermore, the repellent effect of methoxyfenozide was estimated using an oviposition activity index (Table 1). Negative effects were found to be higher in LC_{90} ($OAI_{LC90} = -0.62$) than for the LC_{50} ($OAI_{LC50} = -0.42$) when compared with control group with concentration–response relationship (LC_{50} vs LC_{90}: $P = 0.0006$).

Effects on Autogeny Capacity

Many traits that influence reproductive success are physiologically interconnected. Thus, autogeny capacity of *Cx. pipiens* females was evaluated after tarsal contact with methoxyfenozide (Table 2) before mating and any blood feeding. A strong reduction of 56% ($q = 24.36$) and 72% ($q = 31.48$) in females' autogeny capacity was observed in LC_{50}- and LC_{90}-methoxyfenozide series respectively ($F = 272.59$; df = 2, 12; $P < 0.001$). Also, a significant difference ($P < 0.001$) between the two lethal concentrations tested was recorded.

Table 1. Effect of methoxyfenozide (LC_{50} and LC_{90}) on oviposition activity index (OAI) of *Cx. pipiens* (mean ± SEM, $n = 5$ repeats each containing 25 females, mean values followed by different lowercase letter are significantly different at $P \leq 0.05$)

Treatment	Laid rafts (m ± SEM)	OAI
Control	13.80 ± 0.64a	/
LC_{50}	5.60 ± 0.72b	−0.42
LC_{90}	3.20 ± 0.32c	−0.62

Table 2. Effect of methoxyfenozide (LC_{50} and LC_{90}) on autogeny capacity (%) of *Cx. pipiens* (mean ± SEM, $n = 5$ repeats each containing 15 females, means followed by different lowercase letter are significantly different at $P \leq 0.05$)

Treatment	Total number of females	Total number of rafts	Autogeny capacity (%)
Control	75	69	92 ± 2.13a
LC_{50}	75	28	37.33 ± 3.20b
LC_{90}	75	16	21.33 ± 4.26c

Effects on Fecundity and Fertility

Other reproductive traits (fecundity and fertility) have been assessing. Overall, 5,040, 991, and 557 eggs were produced in control and exposed groups to methoxyfenozide at LC_{50} and LC_{90} concentrations, respectively. Sterile eggs were often abnormal in appearance, being soft and semi-fluid, without a hardened or complete chorion (Fig. 1). Pictures were taken few hours after laying (≈3 h), after the white eggs (when first deposited) turned dark-brown, using Leica EZ4 (Leica Microsystems, France). The control eggs were stronger, difficult to dismantle and therefore easier to handle. Unlike those deposited by treated females, when we wanted to move them, the rafts scatter at the slightest touch. Results showed that methoxyfenozide reduced fecundity of *Cx. pipiens* (number of eggs per female) in both LC_{50} and LC_{90} series, with a reduction of 37.65 and 28.23%, respectively ($F = 140.20$; df = 2, 110; $P < 0.001$) (Fig. 2). However, there was no significant difference ($P > 0.05$) between the two tested concentrations.

Data on fertility are presented in Fig. 3. A significant reduction in female fertility (number of hatched eggs per female) was observed as well ($F = 222.64$, df = 2, 110; $P < 0.001$) after treatment with methoxyfenozide as shown after one-way ANOVA. The mean number of hatched eggs per female decreased from 72.67 in control group to 31.07 (reduction of 56.85%) and 20.25 (reduction of 71.87%), respectively in LC_{50} and LC_{90} series. In addition, there was a significant difference ($P < 0.05$) between the two tested concentrations.

Discussion

IGDs affect the embryogenesis (Hamaidia and Soltani 2016, Fiaz et al. 2019), physiology, molting process, reproduction, embryogenesis, and biosynthesis of the main biochemical contents (Hamaidia et al. 2018, Fiaz et al. 2019, Hamaidia and Soltani 2019). They decreased a crucial trait for mosquitoes; the body volume of emerged adults from treated larvae (Hamaidia and Soltani 2014, Hamaidia et al. 2018) and exhibited inhibition of adult emergence (Hamaidia and Soltani 2014, Hamaidia and Soltani 2019). Moreover, treatment with IGDs had significant behavioral (limits its displacement and speed), histopathological and cytotoxic alterations in the midgut (Fiaz et al. 2019).

Experiments to design insect repellents rationalizing the juvenile hormone activity is unknown (Bhattacharjee 2013), that's why juvenile hormone analogs (JHAs) have proven their effectiveness against pests on several levels; growth, development, biochemistry, reproduction…, but their repellent effect remains ambiguous (Ghoneim and Bakr 2018).

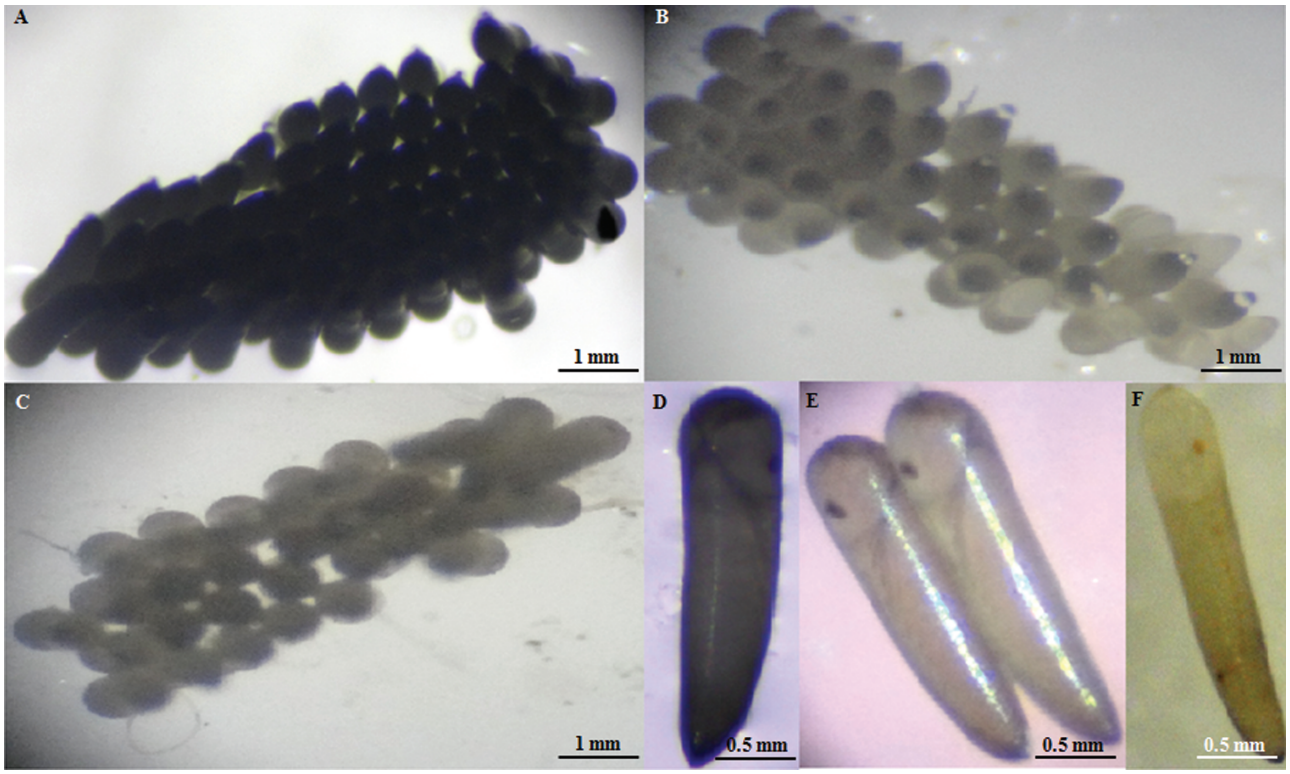

Fig. 1. *Culex pipiens* egg's rafts laid in control (A, D) and methoxyfenozide treated females by tarsal testing: rafts (A: control; B: LC_{50} series; C: LC_{90} series) and eggs (A: control; B: LC_{50} series; C: LC_{90} series).

Two reproductive traits, egg laying and mating success, can impact mosquito population size (Childs et al. 2016). Tarsal exposure to insecticide-treated surfaces was tested on mosquito by several works (Wong et al. 2012, Dennis et al. 2019, Spielmeyer et al. 2019, Williams et al. 2019, Gonzalez et al. 2020). Insecticides are commonly contains different additives, as adjuvants. These additives could improve insecticides effectiveness with full control potential (Melo et al. 2019). Risk assessment should not consider only insecticide active ingredients; otherwise important toxic effect will be missing which could lead to lack of toxicity estimation of the total chemical load (Mullin et al. 2016) which depended on the formulation/adjuvant combination (Preftakes et al. 2019).

The objective of our study was to evaluate for the first time if methoxyfenozide may contaminate adult females of *Cx. pipiens* after tarsal exposure and its effects on fecundity, fertility and oviposition. We discovered an alteration in oviposition. Females of *Cx. pipiens* tended to avoid treated water for the laying of their eggs. Also, we showed that treated females could not lay eggs or laid infertile ones. Same observations were made by Mbare et al. (2014) by application of pyriproxifen with tarsal exposure on *Anopheles*

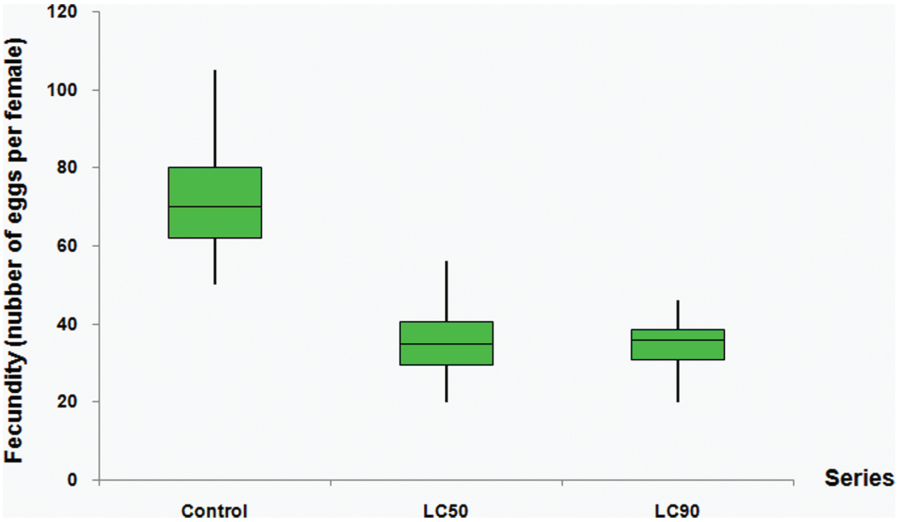

Fig. 2. Effect of methoxyfenozide on *Cx. pipiens* fecundity after female's tarsal contact before mating ($r = 5$ repeats each containing 15 females, mean values followed by different lowercase letter are significantly different at $P \leq 0.05$).

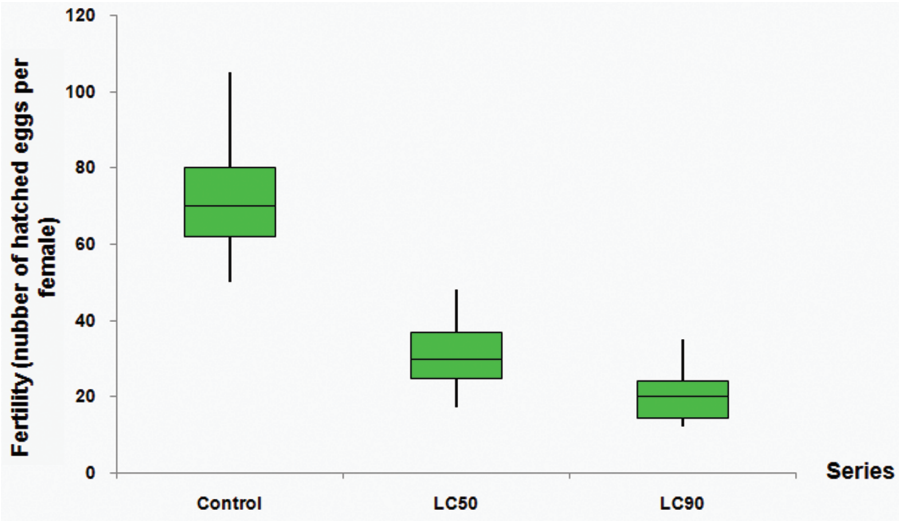

Fig. 3. Effect of methoxyfenozide on *Cx. pipiens* fertility after female's tarsal contact before mating ($r = 5$ repeats each containing 15 females, mean values followed by different lowercase letter are significantly different at $P \leq 0.05$).

gambiae s.s. (Giles) (Diptera: Culicidae) and *Culex quinquefasciatus* (Say) (Diptera: Culicidae).

The literature suggests that the decrease in fecundity observed was probably caused by an interference of ecdysone agonists with the female reproductive system. Shahout et al. (2011) reported that methoxyfenozide also applied by tarsal contact on *Spodoptera litura* (Noctuidae) significantly reduced both the total number of eggs laid and their hatching rate. Topical application of methoxyfenozide on newly emerged pupae of *Ephestia kuehniella* (Zeller) (Lepidoptera: Pyralidae) resulted an increase in pre-oviposition period, a decrease in oviposition period and in ovary growth parameters of adults which impact negatively fecundity and egg viability (Soltani-Mazouni et al. 2012). In addition, methoxyfenozide prevented, by topical test, insemination, egg production and reduction in oviposition rates due to impaired oogenesis in *An. gambiae* females (Childs et al. 2016).

Laid eggs are white and their smooth transparent endochorion turns dark less than 3 h after being laid, due to the process that produces eumelanin (Farnesi et al. 2017). Here, eggs oviposited by treated females with methoxyfenozide were soft and semi-fluid, without a hardened chorion. It was reported that tebufenozide an ecdysteroid agonist applied topically on adults induced smaller ovaries with fewer chorionated eggs in *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) (Salem et al. 1997) and exhibited a sterilizing action on *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) female and male adults (Smagghe et al. 2004).

It has shown that the third pair of legs of *Anopheles maculipennis*, (Meigen) (Diptera: Culicidae) because of its greatest contact with the treated surface, significantly contributed to the uptake of DDT (Ungureanu et al. 1961). Flexibility of tarsi arised important load-bearing ability (Kong et al. 2015). That's why pyriproxyfen treated autodissemination stations were efficient in reducing immature *Aedes albopictus* (Skuse) (Diptera: Culicidae) instars populating cryptic larval habitats within peridomestic environments (Unlu et al. 2017). Recently, it was demonstrated that *Anopheles* female mosquitoes of a resistant strain have structurally and functionally remodeled their legs as a resistance phenotype via improved deposition of cuticular proteins and chitin, resulting in substantially reduced penetration of insecticides (Balabanidou et al. 2019).

Our data provided further evidence that tarsal contact with methoxyfenozide may contaminate adult females of *Cx. pipiens* under laboratory conditions by affecting their reproductive potential. A significant proportion of females loosed their autogeny capacity and failed to oviposit compared to controls. Maybe they became sterile because they might have less or no mature eggs to lay (Bai et al. 2010). Recently, lufenuron (a chitin synthesis inhibitor) affected fertility, fecundity, and blood intake capacity in *Ae. aegypti* females after tarsal testing (Gonzalez and Harburguer 2020).

IGDs from the same family showed different efficacy, fenoxycarb was the fastest and potent toxic than methoprene and pyriproxyfen against *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) and *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) (El-Sheikh et al. 2016). Pyriproxyfen treated surfaces displayed significant reproductive disruption with drastic reduction in fecundity and fecundity in *An. gambiae*, *Cx; quinquefasciatus*, and *Ae. aegypti* before bloodmeal (Mbare et al. 2014, Yadav et al. 2019). In the same context, different classes of IGDs have shown different degrees of activity against natural enemies. Diflubenzuron and lufenuron were harmful to *Ceraeochrysa cincta* (Schneider) (Neuroptera: Chrysopidae), whereas buprofezin and methoxyfenozide were slightly harmful and tebufenozide and pyriproxyfen were harmless when sprayed on first-instar larvae (Rugno et al. 2016).

We presumed that methoxyfenozide might alter egg development or might affect mating process. These effects could be explained, in this study, by two hypotheses. First hypothesis concerned mating behavior. Mating deeply affects the reproductive physiology of mosquitoes. Opposing to other female insects after mating, *Cx. pipiens* loses her susceptibility to further copulation, so rely on sperm from only one male for a lifetime production of offspring (Tripet et al. 2003). Gabrieli et al. (2014) found that the male-transferred steroid hormone 20-hydroxyecdysone (20E) is a key regulator of oviposition in *An. gambiae*. According to our results, we could assume, that exposure of virgin females to methoxyfenozide switches them to an artificial mated status, triggering egg-laying and reducing susceptibility to copulation. Additionally, in *Ae. aegypti* methoxyfenozide is able to stimulate egg production; however, these eggs were unable to develop (Usry 2012). The second hypothesis is based on endocrine relationships. The ecdysteroid biosynthesis is stimulated by mating (Ameku and Niwa 2016) and bloodmeal (Koama et al. 2015). Ovarian ecdysteroids may indeed fulfill different functions as maternal

supply of hormone to the eggs, triggering of vitellogenesis, negative feedback control of gonadotropins, and triggering of pheromone biosynthesis (Smagghe 2009, Dhara et al. 2013). The maternal 3-dehydroecdysone, synthesized from ecdysone in female's ovaries, is converted into active ecdysone during the early embryonic development of offspring of silkworm. The down-regulated biosynthesis of these hormones, in females, lowered the 20E titer and hatching rate of their progeny without affecting their normal development (Wang et al. 2018). In our study, methoxyfenozide transferred by tarsal route in females of *Cx. pipiens* might play a negative feedback on ecdysteroids titers. Dibenzoylhydrazine (DBH), such as methoxyfenozide, disrupts steroid signaling pathways in mosquitoes (Morou et al. 2013) and leads to an impairment of ovarian maturation and oviposition (Lenaerts et al. 2019).

Conclusion

Importantly, the present work confirms the important role of insecticides uptake via legs when *Cx. pipiens* females contacted treated surfaces. It also showed an interference of methoxyfenozide with their reproduction process. Indeed, this ecdysone agonist applied by tarsal contact negatively affected the reproductive potential by reducing autogeny capacity, oviposition alteration, fecundity, and fertility. Conventional insecticides adversely affect biodiversity well beyond the immediate. A final, but crucial bottleneck, for restoring biodiversity and better vector-borne diseases management is to take a shift towards use of safer and sustainable compounds intended to modify the normal functioning of vectors without affecting environment. The class o IGDs is considered to be relatively safe to natural enemies (Bueno et al. 2017). Moreover, the EC formulation of methoxyfenozide contains many adjuvants that could interfere with the repellency (Mesnage and Antoniou 2017). Therefore, further experiments using the pure active ingredient are required to confirm the repellency effects observed.

Acknowledgments

We are grateful to Prof. G. Smagghe (Ghent University, Belgium) for the sample of methoxyfenozide. This research was supported by the Algerian Fund for Scientific Research of Algeria (Laboratory Applied Animal Biology to Pr. N. Soltani) and by the Ministry of High Education and Scientific Research of Algeria.

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