Comparative Effects of Two Moulting Hormone Agonists (Methoxyfenozide and Tebufenozide) on the Mediterranean Flour Moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae): Ecdysteroids Amounts of Testes and Reproductive Events

**H. Bouzeraa and N. Soltani-Mazouni**

Laboratory of Applied Animal Biology, Department of Biology, Faculty of Sciences, Badji Mokhtar University, 23000-Annaba, Algeria

**Abstract:** Methoxyfenozide and tebufenozide are lepidopteran-specific insecticides belonging to a new class of insect growth regulator, the non-steroidal ecdysteroid agonists. Their effects were evaluated on growth and biochemical composition of testes and reproductive events of the Mediterranean flour moth, *Ephestia kuehniella* Zeller, an important pest in stored products worldwide. Methoxyfenozide and tebufenozide were applied topically to newly ecdysed male pupae at their respective lethal dose 50 (0.01 µg/pupae for methoxyfenozide and 0.005 µg/pupae for tebufenozide). Treatment increased the size of the testes as compared with control series and the effect was more marked following methoxyfenozide treatment. In a follow-up experiment, the adults that survived from treated pupae were investigated for ecdysteroids quantification in testes and determination different reproductive parameters. Enzyme immunoassay measurements showed that the two tested compounds had no significant effect on the ecdysteroid amounts of the testes from the newly emerged adults. Moreover, methoxyfenozide significantly (p<0.01) decreased egg fertility while tebufenozide increased the duration of the pre-oviposition.

**Key words:** Insecticides • Toxicity • *Ephestia kuehniella* • Fecundity • Testes • Ecdysteroids

**INTRODUCTION**

Ecdysteroids are mostly secreted by prothoracic glands during larval development, though alternative sites of ecdysteroids production such as ovary [1], testes [2, 3] and abdominal integument [4, 5]. Testes may act as an endocrine gland and contribute to circulating pool of the ecdysteroids [6], that are stimulated by neurohormones, testes ec dysiotropine and ecdysteroids from the prothoracic gland and testes [7-10]. The testicular ecdysteroid play an important role in the development and maturation of the male reproductive tract of the moths [11, 12], spermatogenesis [13, 14] and testicular fusion [15, 16]. Moreover, the development of the male gonad and the maturation of the sperm take place during pupal stage [17] which require a quantitative and qualitative contribution of various metabolites such as proteins, carbohydrates, lipids and nucleic acid. The intensive research in the field of insect endocrinology has highlighted the importance of insect growth regulators (IGRs) based on insect hormones in controlling insect pests. They interfere with pest control growth and development [18]. The non-steroidal ecdysteroid agonists such as tebufenozide, methoxyfenozide or halofenozide represents a new class of IGRs. They induce precocious and incomplete molts in different insect orders [19, 20], with various morphogenetic types [21]. Methoxyfenozide and tebufenozide are lepidopteran-specific insecticides [20-23]. These products have a great potential for use as chemical preventive control in the stored product industry, especially against lepidopteran pests. Previously, methoxyfenozide, tebufenozide and halofenozide were found to decrease the ovarian weight, the thickness of chorion, the number of oocytes and the...
size of the basal oocyte of *Ephestia kuehniella* Zeller, which subsequently interfere in the reproductive parameters such as reduction of oviposition period, fecundity and egg fertility of female [24, 25]. In addition, these compounds affect the amounts of nucleic acids and some principal biochemical constituents (carbohydrates, lipids and proteins) of testes [26].

In order to extend these findings, the present study was designed to compare the activity of two ecdysteroid agonists (tebufenozide and methoxyfenozide), applied topically on the newly ecdysed male pupae of *E. kuehniella*, on the changes on morphometric measurements of testicles (size and volume) during the pupal stage, the ecdysteroids amounts measured in testes from the newly emerged adults and the reproductive events (duration of pre-oviposition and oviposition periods, fecundity and egg fertility) in untreated females paired with male adults that survived from the treated male pupae.

**MATERIALS AND METHODS**

**Insects: *Ephestia kuehniella* Zeller (1879) was reared on wheat flour at 27°C and 80% relative humidity in almost of testicular ecdysteroids is expressed in picograms (pg) continuous darkness as reported before [25]. Last instar larvae were collected from stock colony, separated according to their age in days from pupation.**

**Ecdysteroid Agonists and Treatment of Pupae:** Technical products methoxyfenozide (RH-2485, commercial formulation: Runner or Intrepid, Dow AgroSciences, USA) and tebufenozide (RH-5992, commercial formulation: Mimic or Confirm, Dow AgroSciences, USA), were kindly provided by Pr. G. Smagghe (Ghent University, Belgium). They were administered topically on the abdominal sternites of newly ecdysed male pupae (<8h old) at their respective LD50 previously determined: tebufenozide: 0.005 µg/insect [24] and methoxyfenozide: 0.01 µg/insect [25]. In the controls, pupae were dosed with 2 µl of acetone alone. The compounds were easily diluted in acetone, allowing better diffusion of the active ingredient throughout the cuticle.

**Determination of the Testes Size:** The duration of development of *E. kuehniella* male pupae is 10.02 ± 0.90 days for controls, 8.51 ± 1.50 days after treatment with methoxyfenozide and 9.20 ± 1.52 days after treatment with tebufenozide, as reported previously [21]. Pupae were sampled from controls and treated series at various times (1, 3, 5, 7 and 9 days) during the pupal development and dissected. The testes size was measured over time during pupal stage and the volume was determined according to Lambreas [27]. Ten males were used per dose for morphometric measurements of the testes.

**Enzyme Immunoassay for Ecdysteroid Measurements in Testes:** The testes were dissected from the newly emerged male adults that survived from treated male pupae. A pool of ten pairs of the testes was extracted with methanol by sonication and after centrifugation (5000g, 10 min), the supernatants were taken and evaporated. The extracts were resuspended in phosphate buffer (0.1 M, pH 7.4) and each sample was analysed by enzyme immunoassay (EIA) as previously described [28] using a conjugate of 20E coupled to peroxidase as the enzymatic tracer, tetramethylbenzidine as the colour reagent and a rabbit B polyclonal antibody that was 6 times more sensitive to ecdysone (E) than to 20-hydroxyecdysone [29]. The antibody and the tracer were supplied by Dr J.P. Delbecque (University of Bordeaux I, France). The amount of testicular ecdysteroids is expressed in picograms (pg) ecdysone equivalents/paired testes or equivalent ecdysone pg/ mg tissue. The number of repeats was four per series.

**Reproductive Aspects:** To determine potential effects of ecdysteroid agonists on the pre-oviposition, oviposition periods, fecundity and egg fertility of *E. kuehniella*, each newly emerged untreated female was immediately paired with one treated male in an individual plastic box containing food and placed in an incubator under laboratory conditions. Ten couples per series were investigated. The moment of first oviposition and the fecundity (number of laid eggs per female throughout its lifespan) were recorded. The egg fertility (percentage of neonates that emerged from eggs) was determined on four groups of 10 eggs per dose. For each series, freshly laid eggs were randomly collected at the beginning of the oviposition period from different tested pairs and the number of first-instar larvae was scored until egg hatching was complete in the control, i.e. 5 days after egg collection.

**Statistics:** Results are expressed as means ± standard deviation (SD). Comparison of mean values was made by Student’s *t*-test. All statistical analyses were performed using the Minitab Software (Version 16, PA State.
College, USA) and p< 0.05 was considered to be a statistically significant difference. The number of insects tested per series and the repeats were given with the results.

RESULTS

Effect on Morphometric Measurements of Testes: In *E. kuehniella* control the size of the testes evolve oscillatory during the pupal stage, i.e. the size gradually decreases from day 1 to day 3 and then gradually increases from day 3 to day 5. Thereafter, it gradually decreases until adult emergence (Table 1, 2 and 3 ; Fig. 1). After topical application of the tebufenozide and methoxyfenozide on the newly ecdysed pupae, the same profile was observed from day 1 to day 3, but it was higher compared to controls and that of methoxyfenozide was greater than that of tebufenozide. However, the size continued to gradually decrease from day 3 to day 7 compared to control. Furthermore, a variation was observed in both treatments: the methoxyfenozide slightly increased the size from day 7 to 9, while sublethal dose of tebufenozide decreased the size from day 7 to 9. Moreover, the methoxyfenozide significantly (p<0.001) increased the size at day 9 compared to tebufenozide and to the controls.

Effect on Amounts of Ecdysteroids in Testes: Enzyme immunoassay measurements of ecdysteroids were made on testes sampled at adult emergence from control and the treated series. In control, the amount of ecdysteroids is about 37.11± 2.81 pg/mg or 7.01 ± 0.40 pg/ testicles (Table 4). Treatment with the two tested compounds had no significant effect (p>0.05) on ecdysteroids in testes as compared to control series.

Effect on Reproductive Event Parameters: Treatment was made topically on newly ecdysed male pupae and the effects were investigated on the duration of pre- oviposition and oviposition periods, the fecundity and egg fertility of untreated female mated to surviving male adults from treated male pupae (Table 5, Fig. 2). In *E. kuehniella* laying started at 1.00 ± 0.00 day after mating and continued up 3.20 ± 0.40 days for an average production of 102.20 ±10.70 eggs/female. Treatment with tebufenozide resulted in a high significant (p<0.001) increase of the pre-oviposition period as compared to the controls and methoxyfenozide treated series. However, methoxyfenozide significantly reduced the egg fertility. We scored 34.7% of egg hatching rate against 82% for controls and 90% for treated with tebufenozide. Likewise, a significant difference between the two ecdysteroid agonists tested were observed in egg fertility (p<0.001).

Table 1: Effect of methoxyfenozide and tebufenozide applied topically to newly ecdysed male pupae on the length (µm) of testes during pupal development of *E. kuehniella*

<table>
<thead>
<tr>
<th>Time (Day)</th>
<th>Treatment</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2008±328.71a</td>
<td>1840±283.74a</td>
<td>1936±276.61a</td>
<td>1680±257.95a</td>
<td>1528±180.40a</td>
<td></td>
</tr>
<tr>
<td>Methoxyfenozide</td>
<td>2160±118.62a</td>
<td>1944±290.73a</td>
<td>1768±251.50a</td>
<td>1600±237.33a</td>
<td>1624±94.91b</td>
<td></td>
</tr>
<tr>
<td>Tebufenozide</td>
<td>2104±118.96a</td>
<td>1968±215.26a</td>
<td>1784±217.72a</td>
<td>1600±327.91a</td>
<td>1408±134.82a</td>
<td></td>
</tr>
</tbody>
</table>

mean ± SD, n = 10; values followed by the same letter are not significantly different, p >0.05

Table 2: Effect of methoxyfenozide and tebufenozide applied topically to newly ecdysed male pupae, on the width (µm) of testes during pupal development of *E. kuehniella*

<table>
<thead>
<tr>
<th>Time (Day)</th>
<th>Treatment</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1768±249.01a</td>
<td>1520±286.22a</td>
<td>1648±253.41a</td>
<td>1336±186.00a</td>
<td>1128±198.00a</td>
<td></td>
</tr>
<tr>
<td>Methoxyfenozide</td>
<td>1904±142.20a</td>
<td>1752±296.00a</td>
<td>1616±225.72a</td>
<td>1416±242.71a</td>
<td>1416±92.82b</td>
<td></td>
</tr>
<tr>
<td>Tebufenozide</td>
<td>1784±175.42a</td>
<td>1728±172.31a</td>
<td>1616±150.91a</td>
<td>1312±498.53a</td>
<td>1232±144.01a</td>
<td></td>
</tr>
</tbody>
</table>

mean ± SD, n = 10; values followed by the same letter are not significantly different, p >0.05
Table 3: Effect of methoxyfenozide and tebufenozide applied topically to newly ecdysed male pupae, on the volume (mm³) of testes during pupal development of *E. kuehniella*.

<table>
<thead>
<tr>
<th>Time (day)</th>
<th>Treatment</th>
<th>Control</th>
<th>Methoxyfenozide</th>
<th>Tebufenozide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3.26±0.0a</td>
<td>4.08±0.00a</td>
<td>3.49±0.00a</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>2.22±0.00a</td>
<td>3.11±0.00a</td>
<td>3.05±0.00a</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>2.74±0.00a</td>
<td>2.40±0.00a</td>
<td>2.42±0.00a</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>1.56±0.00a</td>
<td>1.67±0.00a</td>
<td>1.43±0.00a</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>1.01±0.00a</td>
<td>1.69±0.00b</td>
<td>1.11±0.00a</td>
</tr>
</tbody>
</table>

Mean ± SD, n = 10; values followed by the same letter are not significantly different, p > 0.05.

Table 4: Effects of methoxyfenozide and tebufenozide on ecdysteroid amounts in the testicles from newly emerged adults of *E. kuehniella* after treatment of newly ecdysed male pupae.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ecdysteroid amounts (pg/testicles)</th>
<th>Ecdysteroid amounts (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.01 ± 0.40a</td>
<td>37.11 ±2.81a</td>
</tr>
<tr>
<td>Methoxyfenozide</td>
<td>7.03 ± 1.10a</td>
<td>38.89 ± 5.32a</td>
</tr>
<tr>
<td>Tebufenozide</td>
<td>6.84 ± 1.02a</td>
<td>38.62 ± 8.31a</td>
</tr>
</tbody>
</table>

Means ± SD, n = 4; values followed by the same letter are not significantly different at p > 0.05)

Table 5: Effects of methoxyfenozide and tebufenozide on fecundity and egg fertility of *E. kuehniella* adults after treatment of male pupae.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Methoxyfenozide</th>
<th>Tebufenozide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecundity (eggs/female)</td>
<td>102.21 ± 10.70a</td>
<td>89.51 ± 11.71a</td>
<td>96.60 ± 10.51a</td>
</tr>
<tr>
<td>Eggs fertility (%)</td>
<td>82.00 ± 9.81a</td>
<td>34.70 ± 11.60b</td>
<td>90.00 ± 12.72a</td>
</tr>
</tbody>
</table>

Means ± SD, n = 5 couples; n=4 repeats, each containing 10 eggs, for each parameter values followed by the same letter are not significantly different, p > 0.05.

DISCUSSION

Recently, the efficacy of four ecdysteroid agonists was tested by topical application against *E. kuehniella* and their effects on reproductive events after treatment female pupae [25] and after male and female treatment [21] were evaluated. Based on the LD50 values, tebufenozide appeared to be the most effective ecdysteroid agonist against *E. kuehniella* pupae followed by methoxyfenozide, confirming previous reports on various lepidopteran, coleopteran and dipteran insects [28, 30-32].

In this study we investigated treatment of the males of *E. kuehniella* with tebufenozide and methoxyfenozide on different reproduction parameters after mating the treated male to untreated female. Results showed that methoxyfenozide caused a reduction of egg fertility. Injection of methoxyfenozide on male from the last instar larvae reduced fecundity of *Cydia pomonella* [33]. The effect of methoxyfenozide on egg fertility observed in *Helicoverpa zea* could be explained by its action on sperm [34]. In another bioassay, the fecundity and fertility were reduced by tebufenozide in different species: *Platynota idaeusalis* [35], *Choristoneura rosaceana* [36], *Choristoneura funebrana* [37] and *Spodoptera littoralis* [38]. In Lepidoptera, the production of eupyrene...
(nucleated) and apyrene (anucleated) are under spermatogenesis intensification [57] and simultaneously, ecdysteroids control [39, 40]. Injection of tebufenozide on increasing of the size of the testes [17]. Administration of male of the fifth instar larvae of C. rosaceana, reduced the ecdysteroid agonists on the male pupae at day 0 mass of spermatophore, its eupyrenes content and the (time of the fusion of the testes) showed no significant number of eupyrene in the female spermtheca [36]. effect on the size of the testes. The size and volume are greater but not significant, compared to the controls. Moreover, the two molecules decreased the size of the testes at day 5 compared to the controls, which may due to spermatogenesis reduction following treatment. Moreover, methoxyfenozide caused an increase of the size from day 7 to 9 compared to tebufenozide and to the controls. This increase might be due to the presence of sperm in the testes which normally in this time the sperm have already been released to the seminal vesicle according to Perveen [17].

The biological activities of the two compounds have demonstrated their tremendous potential in pest management programs in different insect orders. They affected the reproductive potential after perturbation of growth and development of testes in E. kuehniella. Future research such as the study of the fine structure of the testes in newly emerged adult, sperm count in the female spermatheca after mating should explore to better understand the mode of action of methoxyfenozide and tebufenozide on reproduction.

AKCNOWLDEGEMENTS

The authors are grateful to Pr. N. Soltani (Badji Mokhtar University, Algeria) for useful discussion and critical reading of the manuscript and Pr. G. Smagghie (University of Ghent, Belgium) for providing methoxyfenozide and tebufenozide. This research was supported by the Algerian Fund for Scientific Research and by the Ministry of High Education and Scientific Research of Algeria (CNEPRU and PNR projects to Pr. N. Soltani-Mazouni).

REFERENCES


