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Effect of methoxyfenozide on synthesis of major proteins in ovaries of *Ephestia kuehniella* Zell. (Lepidoptera: Pyralidae)

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Abstract

Insect growth regulators (IGRs) belong to a class of compounds which interfere with normal growth, development and reproduction of insects. Through greater selectivity of action IGRs have less undesirable effects on man, wild life and environment. Many of the IGRs mimic the action of insect hormones, ecdysone or juvenile hormone (JH). Methoxyfenozide is a potent non-steroidal ecdysone agonist developed as an insecticide and is effective against lepidopteran pests. The effects of methoxyfenozide (RH-2485) on reproduction of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), an important pest in stored products worldwide, were evaluated under laboratory conditions. Treatment at LD50 (0.01 µg/pupa) and LD90 (0.37 µg/pupa) was made by topical applications on newly ecdysed female pupae. Data showed that methoxyfenozide, significantly affected reproductive parameters in the ovaries in treated females such as amounts of proteins, vitellogenin and vitellin as compared to the control series. In addition, electrophoretic separation of ovarian proteins by sodium dodecyl sulfate polyacrylamide slab gels (SDS-PAGE) showed that the treatment with the ecdysteroid agonist resulted in reduction in the number and the intensity of some proteins bands compared to controls. The major proteins in the ovaries of lepidopteran insects are members of the storage protein family and as storage proteins are crucial for insect development they may be targeted for developing better insect control strategies.

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Introduction

In Lepidoptera such as *Ephestia kuehniella*, the process of vitellogenesis take place during the pupal stage and the oocyte accumulates and organizes yolk from precursors imported from hemolymph, while the cells of follicular epithelium synthesize additional components of the yolk as well as the vitellin envelope and chorion (Tefler, 2009). Ecdysone and juvenile hormone (JH) play a crucial role in the regulation of the growth, development and reproductive processes (Lafont *et al*., 2005).Juvenile hormones are a group of acyclic sesquiterpenoids that regulate many aspects of insectphysiology. It has a wide range of functions in regulating development and physiological processsuch as metamorphosis, caste determination, ovarian maturation, diapause and migration in insects (Riddiford, 1994). Ecdysone is a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone.Ecdysone is secreted from the prothoracic glands and it along with JH regulates the process of moulting and many other metabolic processes.

Insect growth regulator (IGR) insecticides are considered biorational pesticides that seem to be selective toward pests and show low or no toxicity to most natural enemies (Sadeghi *et al.,* 2009). These compounds are considered to be useful in pest control programs because they are target-specific, non-persistent, biodegradable and environmentally benign substances with less toxicity to non-target organisms. Methoxyfenozide is a lepidopteran-specific insecticide, belongs to the molt accelerating compounds (MACs) group within the (IGR) insecticides.

Its mode of action is to mimic the endogenous ecdysteroid hormone by binding to the natural hormone receptors and causing an anticipated lethal molt (Dhadialla *et al.,* 2005). Methoxyfenozide, and its related compounds such as halofenozide and Tebufenozide, are known to affect the reproduction of different insect orders, mainly by reducing sexual behaviour (Rodriguez-Enríquez *et al.,* 2010), fecundity and egg viability (Soltani-Mazouni *et al*., 2012).

Protein metabolism plays a key role in rebuilding adult structures during the transformation of larvae/pupae into adult. Vitellogenin is an important protein synthesized in the fat body transported in the haemolymph to the ovary (Kanost *et al.,* 1990; Vallae, 1993). This protein is a phospho glycolipoprotein (Engelmann, 1979). Vitellogenin is sequestered into the oocyte by receptor mediated endocytosis (Sappington and Raikhel, 1998).

Since the haemolymph protein pool acts as a reserve source of amino acid, transport of proteins, any change in the synthetic activity or utilization pattern, it is reflected in the haemolymph protein turn over. The last larval instar of holometabolous insects has been characterized by active synthesis of arylphorin (aromatic amino acids bearing storage proteins) and pupal storage proteins (Wyatt and Pan, 1978). During metamorphosis larval plasma proteins were hydrolyzed to free aminoacids and major part being incorporate into new adult proteins. Thus haemolymph proteins are crucial for insect development. The Fat body tissue plays a key role in storage proteins. Storage proteins increased during successive stages of development (Kanost *et al.,* 1990; Rajathi *et al.,* 2010). Proteins are synthesized in the fat body and released into the haemolymph to be incorporated later into various organ including ovaries (Vallae, 1993).

The present study was planned for investigating possible effects of the non-steroidal ecdysteroid agonists methoxyfenozide on the reproductive events of *Ephestia kuehniella* Zeller. *(Lepidoptera: Pyralidae)*. RH-2485 was applied topically on pupaes of *E. kuehniella* and its effects on abnormalities in the total protein content of the adult ovaries, and in the different ovarian protein bands are determined. In addition, we evaluated whether this IGR caused any measure ovarian vitellogenin and vitellin. The latter results should help in better understanding the mode of action of these dibenzoyl hydrazine insecticides, particularly in the adult stage.

Material and methods

Insect and breeding

E. kuehniella (Zeller), 1879 (Lepidoptera, Pyralidae) is reared, to the laboratory, in boxes containing flour at a temperature of 27°C and a relative humidity of 80% in total obscurity (Soltani-Mazouni *et al.*, 2012). Pupae are dated according to their age (days) from the pupal molting. Under our laboratory conditions the duration of pupal development is above 9 days.

Chemical and treatment of pupae

The concentrations of technical grade methoxyfenozide (Courtesy of Prof. G. Smagghe, Ghent University, Belgium) used in this study were 0.01 µg (LD_{50}) and 0.37 µg (LD_{90}) was prepared in acetone. Newly molted pupae (< 8h old) were topically treated (2 µl/pupae). The drug is easily diluted in acetone, allowing a better diffusion throughout the cuticle. In the control groups, pupae were treated with 2 µl of solvent (acetone). Three groups of 10 pupae per dose were used.

Determination of ovarian protein amounts

At appropriate times (0 days) after emergence, females were sampled from control and treated series. Then, individual ovary pairs were dissected, weighed and homogenized in 1 ml of trichloracetic acid (20%). All samples were stored at - 20° C until analysis. Extraction of proteins from ovaries was made as described by Soltani *et al.* (1996), and the final ovarian residue obtained was resuspended in 1 ml of NaOH (0.05 N). The total protein content in ovaries was determined in accord with Bradford (1976) using bovine serum albumin as standard.

Vitellin and vitellogenin quantification

The vitellogenins quantification was made, on ovaries samples collected at emergence. Ovaries, collected on newly emerged adult female controls and treated, were used for vitellin quantification. The extraction was performed using Tris buffer (0.5 M; pH 7.4) following the procedure of Postlethwait *et al.* (1980) and Fabre *et al.* (1992); then, the quantification was made according to Bradford (1976) using Coomassie brilliant blue G-250 (CBB) as a reagent and bovine serum albumin (BSA) as standard (Sigma). The absorbance was read at a wavelength of 595 nm.

Electrophoresis

Ovaries (poole of 8- 10 paired ovaries per series) were collected in controls and treated series and conserved in phenyl methyl sulfonyl fluide or PMSF (45mg/ml ethanol) at 0.1% in distilled water. SDS–PAGE was accomplished in slab gels according to Laemmli (1970). Soluble proteins were mixed with the sample buffer, boiled at 60 °C for 4 minutes. The protein content in each sample was determined before lyophilization by the method of Bradford (1976) using bovine serum albumin as a standard. Small amount (10 µg) of the boiled samples were cooled and loaded into 12% polyacrylamide gel. The gels were stained for 30 min in staining solution (0.025% Coomassie brilliant blue R 250 (Merck), 10% acetic acid, and 25% of 2-propanol). After 30 min, the gel was removed from the staining solution, rinsed with distilled water and distained in 10% acetic acid then placed in distaining solution contains (4.5% methanol and 10% acetic acid, 2.5% glycerol, 10% ethanol).

Statistical analysis

Results are presented as the mean ± standard deviation (SD). The significance between different series was tested using student's t test at 5% level. All statistical analyses were performed using MINITAB Software (Version 17, PA State, College, USA). The number of pupae tested in each experiment is given with the results.

Results and discussion

Effect on the proteins amounts

Methoxyfenozide was applied topically at two doses corresponding to LD_{50} and LD_{90} on newly ecdysed pupae. Quantitative evaluation of proteins recorded in each pair of ovaries from newly emerged adult females from treated pupae showed that methoxyfenozide reduced significantly the amounts of proteins ($p<0.05$) at both doses testes LD_{50} (0.01µg / 2µl) and LD_{90} (0.37µg / 2µl) (Fig. 1A). Data showed that the compound also caused a significant ($p<0.05$) reduction in the weight of ovaries as compared to the controls (Fig. 1B). Moreover, Resmitha *et al.* (2014) found a reduction in total protein concentration of the

methoxyfenozide treated 5th in star larvae of *Spodoptera mauritia*. Meskache and Soltani-Mazouni (2013) that methoxfenoside had any significant affect on protein amounts on newly ecdysed male pupae of *E. kuehniella*,

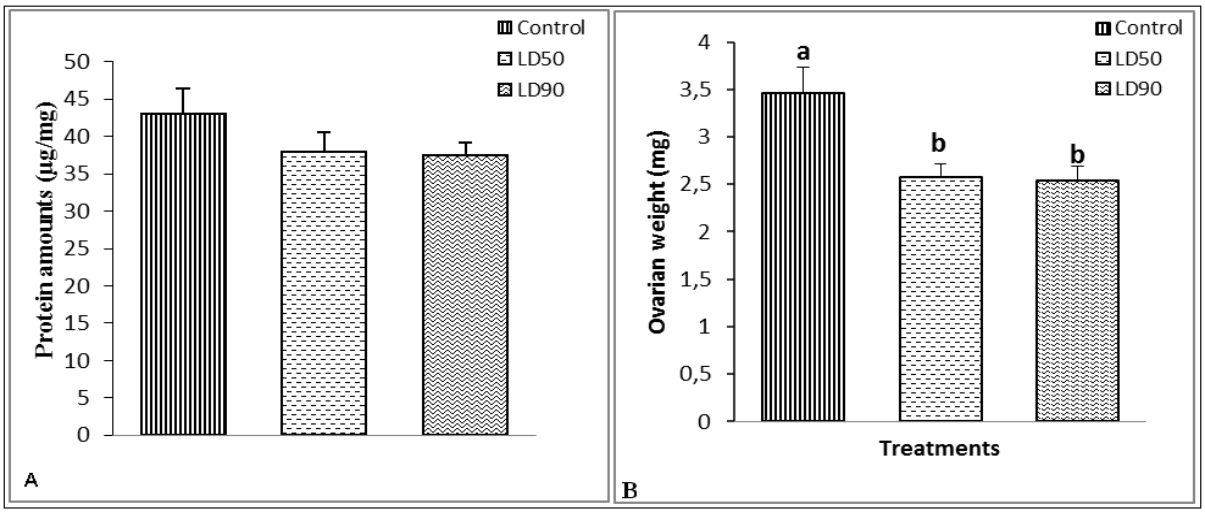

Fig.1. Effect of methoxyfenozide (RH-2485) (LD_{50} and LD_{90}) applied topically to newly ecdysed pupae on the protein (**A**) (µg /mg of ovaries) amounts and the weight (mg) of ovary (**B**), from newly emerged adult females of *E. kuehniella* (mean ± SD, n=6 females; for each component ; values followed by the same letter are not significantly different at p >0.05).

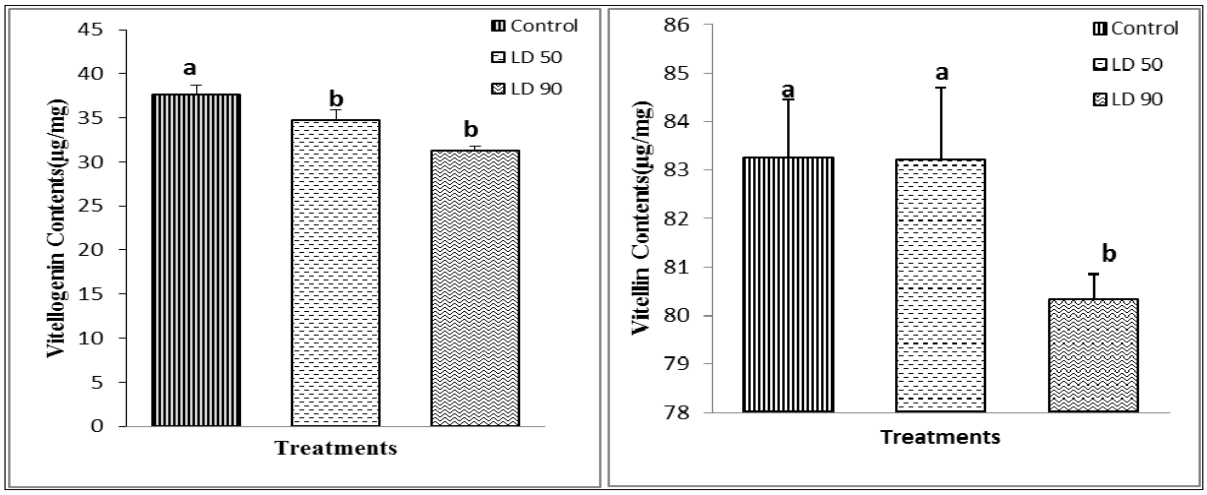

Fig. 2. Effect of methoxyfenozide topically applied on newly ecdysed pupae of *E. Kuehniella* on the content (µg/mg) of vitellogenin (**A**) and vitellin (**B**) in surviving adult females (Mean ± SD; n= 5 repeats; values followed by the same letter are not significantly different at p >0.05).

Effects on vitellogenin and vitellin amounts

Methoxyfenozide was administered topically on newly ecdysed pupae at two doses (LD_{50}, LD_{90}), and its effects were evaluated on vitellogenin and vitellin amounts in surviving adults from treated pupae. The compound reduced significantly the amounts of vitellogenins at the two tested doses. In addition, it acts with a dose-dependent effect at LD_{50} (p = 0.006), LD_{90} (p = 0.000) as compared to the controls. (Fig 2, A). The ovarian vitellin, in females of *E. kuehniella*, has values of 83.27 ± 1.2 µg in controls. Treatment with methoxyfenozide reduced significantly ($p<0.05$) the ovarian vitellin amounts only at the highest dose, as compared to the controls. (Fig 2, B).

This is in accordance with experiments conducted after administration of tebufenozidea related

compound of the same family of ecdysone agonists, on pupal case of female silkmoths. *In vivo* accumulation of vitellogenin (Vg) from the hemolymph was reduced in tebufenozide treated female ovaries as well as their ability to accumulate Vg *in vitro*. (Sridhara & Lee, 2013).

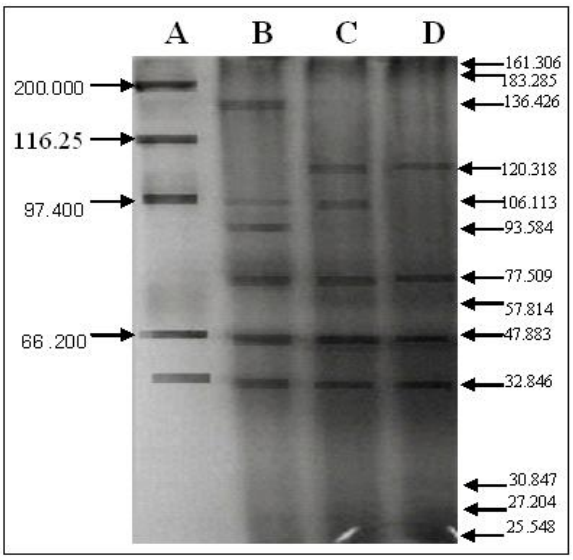

Fig. 3.SDS-PAGE patterns of ovarian proteins after topical application with RH-2485; LD_{50} (0.01µg / 2µl) and LD_{90} (0. 37µg / 2µl) from newly emerged adult females of *E. kuehniella* as compared to controls.**A**: Protein markers (a, Myosin; b, B-galactosidase; c, Phosphorylase b; d, Bovine serum albumin); **B**: Control; **C**: LD_{50} RH-2485; **D**: LD_{90} RH-2485.

Effect on quality of ovarian proteins: SDS-PAGE analysis

Ovaries were dissected from control and treated females at the adult life. Extracted ovarian proteins were analyzed by SDS-PAGE and results are given in Fig. 3. In controls, we could detect 13 protein bands in the ovarien protein pattern with a molecular mass of (183.285, 161. 306, 136.426, 120.318, 106.113, 93.584, 77.509, 57.814, 47.883, 32.846, 30.847, 27.204, and 25.548) kDa in 10% SDS-PAGE. When ovaries are treated, the different protein pattern revealed a differences in the intensity of some bands compared to controls. In protein profile of the methoxyfenozide treated there was a decrease in intensity and number of two major protein bands (93.584 and 136.426 kDa) with LD_{50}, and three protein bands (93.584, 106.113 and 136.426 kDa) with LD_{90}. The bands of 32.846, 47.883 and 77.509 kDa were more pronounced in controls while the bands 106.113 and 120.318 kDa were more important in treated series (Fig. 3).

Our results are in accord to the findings of Rajathi *et al.* (2010) in *Bombyxmori* that application of methoxyfenozide caused Significant changes in storage proteins (80 kDa) and 30 kDa proteins in the haemolymph at all three sublethal doses.

Conclusions

All these results gained lead us to understand that the methoxyfenozide affect significantly the process of reproduction, what is marked by the reduction of the weight of the ovaries, the drop of the protein concentrations, but also the change in the appearance of ovarian proteins, which disrupted the events of this complex phenomenon. Therefore, our results suggest that these negative effects observed in the adult female of *E. kuehniella* are due to a disturbance of the rate of ecdysteroides in presence of excess of methoxyfenozide after treatment of pupae newly exuviees, and those can be explained by that the administration of agonists of ecdysteroides can produce long-term toxic effects in adults of the same target species. Additional experiments on the impact of these agonists on the biochemical composition of the ovaries are needed to understand the mechanism of action of these molecules on the reproduction.

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