Long-Term Effects of Methoxyfenozide on the Adult Reproductive Processes and Longevity of *Spodoptera exigua* (Lepidoptera: Noctuidae)

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ABSTRACT The long-term effects of methoxyfenozide on the longevity and reproductive processes of beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), adults were assessed after exposure by ingestion. Methoxyfenozide significantly reduced adult male longevity compared with females by 1.1 and 1.5 d at 75 and 150 mg (AI)/liter, respectively. Fecundity decreased by >60% with both concentrations at 72 and 96 h after treatment, but at 48 h, no significant effect was observed. The carbohydrate, protein, and lipid content in the eggs were determined as representatives of the biochemical effects of methoxyfenozide associated with the disruption of reproductive processes. The content of carbohydrates in the eggs laid 48 h at treatment was similar to that of controls, but it increased by ≈1.5 and 2-fold in eggs laid after 72 and 96 h, respectively, compared with controls (15 μg per egg). Protein content was reduced ≈2.5 and ≈3-fold for each treatment concentration, respectively, compared with the controls (25 and 23 μg per egg for 75 and 150 mg [AI]/liter, respectively) in eggs collected 72 and 96 h after treatment. Lipid content significantly decreased by ≈1.6-fold in both treatment concentrations in eggs collected at 48 and 96 h after treatment compared with the controls (24 and 21 μg per egg for 48 and 96, respectively), but it was similar to controls (≈19 μg per egg) at 72 h (≈15 μg per egg) for both concentrations. The biochemical effects of methoxyfenozide on *S. exigua* egg formation detected in this work are consistent with the reduction in fertility observed, as reported previously.

KEY WORDS *Spodoptera exigua*, methoxyfenozide, carbohydrates, proteins, lipids

Over the past four decades, efforts have been made to develop insecticides with selective properties that act specifically on biochemical sites that are present in particular insect groups but that have properties that differ from other insecticides (Ishaaya et al. 2005, Berghiche et al. 2008). This approach has led to the discovery of the newest group of insect growth regulator (IGR) insecticides, the ecdysone agonists (Dhadialla et al. 1998). Tebufenozide, halofenozide, methoxyfenozide, and chromafenozide belong to this group of compounds, which have potential for the control of pest species from the insect orders of Lepidoptera, Coleoptera, and Hemiptera (Palli and Retnakaran 2001, Yanagi et al. 2006). Ecdysone agonists induce a premature and lethal larval molt by binding to nuclear ecdysteroid receptors, which is also the mode of action of the natural insect molting hormone, 20-hydroxyecdysone (20E) (Dhadialla et al. 1998, Smagghe et al. 2004). Due to the high specificity of their action in certain insect groups, they are considered to be environmentally friendly compounds (Palli and Retnakaran 2001). In addition, these compounds have been reported to be safer for beneficial organisms than conventional broad-spectrum chemical insecticides (Medina et al. 2001, Schneider et al. 2008), and as a result, they have been incorporated into many integrated pest management programs (Gurr et al. 1999, Smagghe et al. 2003a, Chapman et al. 2009).

The high effectiveness of ecdysone agonists against larvae of several important pest species has been widely documented previously (Biddinger et al. 2006, Yanagi et al. 2006, Berghiche et al. 2008, Osorio et al. 2008). In addition, previous studies have demonstrated that either by topical or oral administration, ecdysone agonists can produce long-term toxic effects on the adults of target species. For example, tebufenozide, methoxyfenozide, and halofenozide were

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found to negatively affect the fecundity and fertility of lepidopteran and coleopteran adults of the following species: *Choristoneura rosaceana* (Harris), *Cydia pomonella* L. (Lepidoptera: Tortricidae), *Spodoptera exigua* (Hübner), *Spodopera littoralis* (Boisduval) (Lepidoptera: Noctuidae), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), and *Tenebrio molitor* (Coleoptera: Tenebrionidae) (Farinós et al. 1999, Knight 2000, Sun et al. 2000, Taibi et al. 2003, Osorio et al. 2008, Pineda et al. 2009). However, the biochemical mechanisms involved in the reproductive processes of the target species of these chemicals and their relationship with reproductive parameters, such as fecundity and fertility, have been poorly investigated thus far. It is well known that the ovaries are sites of ecdysteroid synthesis. In addition, they may be a target of ecdysone agonists, which interfere in several processes associated with reproduction, such as vitellogenesis and oogenesis. Therefore, the altered levels of ecdysteroids due to the application of ecdysone agonists could result in abnormal oocyte growth and egg formation. These long-term effects could contribute to reducing population density by reducing the progeny of adults affected by these compounds (Sun et al. 2000).

Research on egg formation in Lepidoptera has been very well documented by Telfer (2009) and includes several different processes, from ovarian follicle development to egg maturation. The lepidopteran yolk consists of large protein-filled vesicles interspersed with smaller lipid droplets and particles of glycogen. The synthesis of these compounds is carried out by the fat body during immature stages. We hypothesized that methoxyfenozide could affect the biochemical processes implicated in egg formation after ovarian follicle development in *S. exigua*, thus having an influence on reproduction as well as on other adult parameters, such as longevity.

The aim of this study was to assess the long-term effects of methoxyfenozide on the reproductive processes, including biochemical processes, and adult longevity of adults of *S. exigua*, which is a highly polyphagous insect pest of various agricultural crops, including vegetables and ornamentals. For this, we examined the total content of carbohydrates, proteins, and lipids in eggs laid by exposed adults collected at different times after uptake of methoxyfenozide by ingestion.

Materials and Methods

Insects. Insects used in these tests came from a colony of *S. exigua* maintained during 21 generations in the Instituto de Investigaciones Agropecuarias y Forestales, Universidad Michoacana de San Nicolás de Hidalgo (Morelia, Michoacán, Mexico), and the colony had no history of insecticide exposure. The larvae were reared on a semisynthetic diet (Poitout and Bues 1974) in a controlled environmental chamber at 25 ± 2°C with 75 ± 5% RH and a photoperiod of 16:8 (L:D) h until the prepupal stage. Adults were released into 50 by 20 cm of brown paper bags for mating and egg laying and supplied with a 15% (wt:vol) honey solution made with water distilled. The paper bags were replaced every 2 d.

Bioassays. *Effects on Fecundity and Longevity.* To determine fecundity, a minimum of 24 and a maximum of 47 pairs of adults (<48 h old) were continuously offered a 15% honey solution containing 75 or 150 mg of active ingredient (AI)/liter of methoxyfenozide (Intrepid 2 F suspension concentrate, Dow Agrosciences, Zamora, Michoacán, Mexico). The 150 mg (AI)/liter treatment corresponds approximately to the maximal recommended field concentration of 144 mg (AI)/liter methoxyfenozide. Control insects were offered honey solution alone. Each adult pair was placed in a separate oviposition container (7.5 cm in diameter by 5 cm in height) lined with tissue paper. Methoxyfenozide was supplied for oral consumption using a moist cotton trough until the death of the moths. Insecticide solutions were replaced every 2 d to prevent fungal growth. The tissue paper was also replaced every 2 d, and fecundity was determined by counting the number of eggs laid by each female at 48, 72, and 96 h after the beginning of the study. Pairs that failed to reproduce were discarded. The effect of methoxyfenozide on the longevity of *S. exigua* adults was determined by checking moths daily until death occurred.

Effects on Biochemical Composition of Eggs. Eggs laid in the fecundity experiment were subjected to nutrient composition analyses. From the total number of eggs laid by females in each treatment and for each sample collection time, a minimum of three and a maximum of eight samples of 5 mg of eggs were selected at random (≈150 eggs for each sample). Each 5-mg sample was considered as a replicate. For nutrient analysis, the eggs were homogenized in 1 ml of trichloroacetic acid and centrifuged at 5,000 × *g* for 10 min at 4°C. The supernatant one was used for carbohydrate determination by the anthrone method (Soltani 1990). A 6-ml aliquot was taken from this supernatant and mixed with 1 ml of 0.1% anthrone solution (Sigma-Aldrich, St. Louis, MO) and then cooled in ice for 5 min and incubated in a water bath (Felisa, Guadalajara, Jalisco, Mexico) for 30 min at 80 ± 0.5°C. Carbohydrate quantification was performed at 625 nm by using trehalose (Sigma-Aldrich) as a standard.

The lipid content was determined according to the method of Goldsworthy et al. (1972). For lipid extraction, the precipitate of supernatant 1 was washed with 1 ml of ether + chloroform (1:1) and centrifuged as described above. The supernatant 2 was prepared by mixing 100 μl of this solution with 1 ml of concentrated sulfuric acid, followed by heating for 10 min at 100°C in a dry block heater (Lab-Line, Melrose Park, IL). After cooling, 2 ml of a 13 mM vanillin in 11.8 M phosphoric acid (J. T. Baker, Xalostoc, Estado de México, Mexico) solution was added. The absorbance levels of these solutions were then measured at 530 nm by using a standard lipid solution of 0.01 g/ml cholesterol (Química Saustes, Tlahuac, Mexico).

Proteins were extracted according to the method of Le Bras and Echaubard (1977). The precipitate of

Table 1. Effects of methoxyfenozide on the fecundity of *S. exigua* adults continuously treated by ingestion on three different dates of collection

Concn (mg [AI]/liter)	Duration of exposure (h)		
	48[a]	72[b]	96[c]
0 (control)	173 ± 27a	249 ± 24a	121 ± 12a
75	132 ± 22a	52 ± 6b	46 ± 19b
150	150 ± 30a	73 ± 10b	46 ± 7b

Within the same column, values followed by the same letter are not significantly different for comparisons between treatments (rows) within each time point (columns) ($P > 0.05$; LSD means separation).

[a] $F = 0.81$; df = 2, 49; $P = 0.45$.

[b] $F = 32.15$; df = 2, 64; $P < 0.001$.

[c] $F = 16.37$; df = 2, 41; $P < 0.001$.

supernatant 2 was suspended in 1 ml of distilled water and centrifuged as described above to produce supernatant 3. A volume of 800 μl of this supernatant was mixed with 200 μl of Bradford reagent (Sigma-Aldrich). This sample was maintained at room temperature for 10 min, after which, the protein concentration was determined at 595 nm by using bovine serum albumin (Sigma-Aldrich) as a standard.

Data Analysis. The effects of methoxyfenozide on fecundity and on the carbohydrate, protein, and lipid content of the eggs were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) mean separation using the Statgraphics software system (STSC Inc., Rockville, MD). In cases where assumptions of the ANOVA were violated and could not be normalized by transformation, a nonparametric Kruskal–Wallis test was applied. The results for insect longevity after continuous exposure to methoxyfenozide solution were subjected to ANOVA using a general linear models procedure with an LSD multiple range test ($P < 0.05$) to separate means (SAS/STAT version 8.1, SAS Institute, Cary, NC).

Results

Effects on Fecundity. At 48 h after the beginning of the experiments, methoxyfenozide treatment had no effect on adult fecundity (Table 1). However, the mean number of eggs laid per female had decreased compared with controls at 72 h by 71 and 79% for concentrations of 75 and 150 mg (AI)/liter, respectively, and at 96 h, it was decreased by 62% under both treatment concentrations compared with the controls.

Effects on Longevity. Methoxyfenozide treatment had a significant effect on the longevity of *S. exigua* males but not that of females (Table 2). The effect on longevity seemed to be dose dependent; in males, longevity was reduced by 1.11 and 1.54 d compared with females under treatments of 75 and 150 mg (AI)/liter, respectively. Both male and female control insects exhibited very similar adult life spans (8–9 d).

Effects on Biochemical Composition of Eggs. The total amounts of carbohydrates, proteins, and lipids present in the eggs of *S. exigua* were variable at each collection time. For eggs collected at 48 h, the total

Table 2. Longevity (mean ± SE) of *S. exigua* adults treated orally with methoxyfenozide

Concn (mg [AI]/liter)	Females	Males
0	9.08 ± 0.26aA	8.0 ± 0.46aA
75	8.85 ± 0.44aA	7.74 ± 0.33aB
150	9.00 ± 0.39aA	7.46 ± 0.34aB

Means within columns (lowercase letters) and within rows (uppercase letters) followed by the same letter are not significantly different ($P \leq 0.01$; LSD mean separation).

carbohydrate content was ≈15 μg per egg under both concentrations of methoxyfenozide and was not significantly different from that observed in the controls ($F = 1.32$; df = 2, 23; $P = 0.28$) (Fig. 1A). However, the carbohydrate levels increased significantly in eggs collected at 72 h ($K = 6.75$, $P = 0.034$) and at 96 h ($K =$

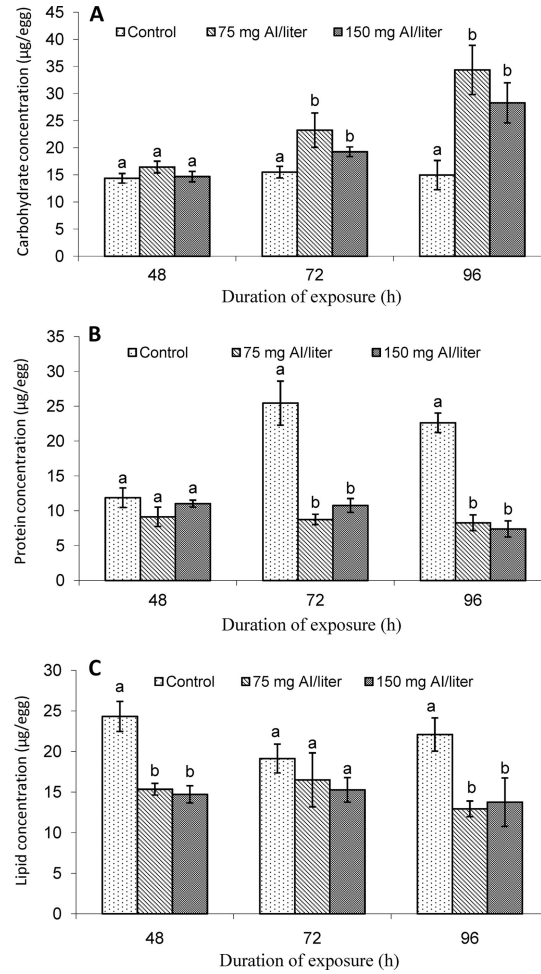

Fig. 1. Mean of the contents (micrograms per egg ± SE) of carbohydrate (A), protein (B), and lipid (C) in the eggs of *S. exigua* adults treated with methoxyfenozide. Bars in each group labeled with by the same letter are not significantly different from each other.

8.62, $P = 0.013$) posttreatment compared with the controls (15 μg per egg at both collection times).

The total amount of protein in the eggs collected 48 h after the beginning of the study was nine and 11 μg per egg for the 75 and 150 mg (AI) / liter treatments, respectively, and no significant differences were observed with respect to controls (12 μg per egg) ($K = 4.0$, $P = 0.13$) (Fig. 1B). However, the levels of protein had decreased significantly at 72 h (9 and 11 μg per egg for 75 and 150 mg [AI] / liter, respectively) ($K = 16.68$, $P < 0.001$) and at 96 h (8 and 7 μg per egg for 75 and 150 mg [AI] / liter, respectively) ($F = 35.39$; df = 2, 14; $P < 0.001$) after treatment. Both of the treatment concentrations that were bioassayed were significantly different compared with controls when eggs were collected at 72 h (25 μg per egg) and at 96 h (23 μg per egg) after treatment.

There was a significant decrease in the lipid content of eggs collected at 48 h (15.36 and 14.73 μg per egg for 75 and 150 mg [AI] / liter, respectively) ($K = 11.78$, $P < 0.01$) and at 96 h (12.94 and 13.77 μg per egg for 75 and 150 mg [AI] / liter, respectively) ($F = 6.73$; df = 2, 16; $P = 0.0076$) after the beginning of the study compared with the controls (24.33 and 22.1 μg per egg for 48 and 96 h, respectively) (Fig. 1C). At 72 h, the lipid content was $\approx$15 μg per egg in both of the concentrations bioassayed and was not significantly different from the controls (19.14 μg per egg) ($F = 0.77$; df = 2, 22; $P = 0.47$).

Discussion

Adults of several important lepidopteran pest species have often been reported to exhibit a reduction in fecundity after exposure to ecdysone agonists. The decline in fecundity of *S. exigua* observed in this study was similar in magnitude to that reported in previous studies in which tebufenozide and methoxyfenozide were applied topically or administrated orally to adult moths (Saenz-de-Cabezon et al. 2005, Pineda et al. 2006, Osorio et al. 2008).

Most studies on the toxicity of ecdysone agonists on lepidopteran pests have been conducted during larval stages, and relatively little has been published regarding their effects on the longevity of adults. In the current study, methoxyfenozide was found to reduce the longevity of *S. exigua* males compared with females by up to 17% at the higher concentration tested. Similarly, a reduction in longevity of 19 and 30% was observed on adults, regardless the sex, of *S. littoralis* (Pineda et al. 2009) and female of *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) (Reinke and Barrett 2007) when exposed to methoxyfenozide by ingestion or methoxyfenozide-treated surfaces, respectively. In contrast, no effects on longevity were observed when adults of *C. pomonella* and *Lobesia botrana* Denis et Schiffermüller (Lepidoptera: Tortricidae) were exposed to tebufenozide or methoxyfenozide (Pons et al. 1999, Saenz-de-Cabezon et al. 2005). The mechanism by which ecdysone agonists can affect adult longevity is unclear because the biochemical target sites for these compounds in adult Lepidoptera are unknown. Therefore, more information on the adult endocrine system is needed before a general interpretation can be formulated on the susceptibility of this life stage to ecdysone agonists.

The effects on reproductive parameters caused by ecdysone agonists have been well documented in several insects, but the mechanisms through which these effects might be exerted are poorly understood. It is well known that the maturation of insect eggs is dependent, among other factors, on the materials taken up from the surrounding hemolymph and on materials synthesized by the ovary in situ (Indrasith et al. 1988). These materials include proteins, lipids, and carbohydrates, all of which are required for embryogenesis (Kanost et al. 1990). In female adult insects, the fat body is the site of the synthesis of proteins that will later be secreted into the hemolymph, from which they will be absorbed by oocytes in the ovary through the follicular epithelium (Chapman 2004, Raikhel and Dhadialla 1992). If the titers of these constituents are altered, then the reproductive process can also be disrupted. Here, we showed that methoxyfenozide negatively affected the fecundity of *S. exigua* adults 72 and 96 h after treatment, and that protein content also had decreased at these two sample times (between 57 and 67%). The lack of significant effects observed in eggs collected 48 h after treatment suggests that the effects of methoxyfenozide on protein deposition increase with the duration of exposure to this compound. RH-5849 (the prototype compound of ecdysone agonists) and halofenozide were observed to result in a reduction of yolk protein synthesis, incorporation into the eggs of *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), *L. decemlineata*, and *T. molitor* females, or both, leading to lower yolk protein and total protein contents in the ovaries (Lawrence 1993, Farinós et al. 1999, Taibi et al. 2003). This decrease in the total amount of proteins could be explained, as in our study, by the interference of ecdysone agonists with the mechanisms controlling yolk deposition.

Other studies have reported that the levels of vitellogenins, which are the precursors of insect egg yolk proteins, in the hemolymph of *C. pomonella* and *L. decemlineata* females were significantly increased when these species were exposed to tebufenozide, methoxyfenozide, or halofenozide, either through contact with treated surfaces or by topical application (Sun et al. 2003, Farinós et al. 1999). These findings suggest that oocyte degeneration was followed by reabsorption of these proteins from the ovaries and that, consequently, an inhibition of oviposition occurred (Farinós et al. 1999).

The main source of energy for developing embryos in insects is usually lipids (Ziegler and Van Antwerpen 2006). Approximately 30–40% of the dry weight of the insect oocyte consists of lipids, mostly triacylglycerol (Chapman 2004). To our knowledge, there have been no reports published on the effects of ecdysone agonists on the patterns of lipid accumulation in insect eggs. However, a reduction of up to 1.5-fold in the lipid content of ovaries in newly emerged adult females was observed in *Spodoptera litura* (F.) (Lepidoptera: Noc-

tuidae) when individuals of this species were exposed as fifth instars to chlorfluazuron, an IGR belonging to the benzoyl phenyl urea group (Perveen and Miyata 2000). As in the current study, these authors also reported a significant reduction in the fecundity of this pest species. We observed that methoxyfenozide caused a decrease of ≈1.six-fold in the lipid content of eggs collected at 48 h and at 96 h after the beginning of the experiment for both of the concentrations bioassayed. It is well known that most of the lipids present in insect eggs during oogenesis originate in the fat body and are transported to the ovary by vitellogenin and lipophorin, which are the major insect hemolymph proteins (Lawrence and Chino 1974, Kawooya and Law 1988). During egg development, triacylglycerol is mobilized, transported through the hemolymph as diacylglycerol, and delivered to the maturing eggs, where it is converted to triacylglycerol (Kawooya and Law 1988). We presume that methoxyfenozide could have caused a reduction of the lipid content of *S. exigua* eggs through inhibition of fat body development because Salem et al. (1997) reported that decreases in egg development and maturation in tebufenozide-treated *Plodia interpunctella* (Hüner) (Lepidoptera: Pyralidae) females was related to smaller ovaries and to an apparent absence of fat bodies compared with controls.

In insect eggs, carbohydrates are involved in the formation of structures such as mandibular teeth and bristles and are used as a substrate for chitin formation during embryo development. Perveen and Miyata (2000) reported that chlorfluazuron decreased the carbohydrate titer in the ovaries of *S. litura* females. The lack of a significant effect found by these authors could be due to the fact that insects were exposed to the compound when they were in the fifth instar, which is a stadium at which insecticides are excreted more easily. In this study, the carbohydrate content in eggs from control insects remained constant (14–15 μg per egg) during the three times at which eggs were collected. However, in eggs from treated insects, carbohydrates increased significantly 72 and 96 h after treatment. The mechanism by which these effects might be exerted remains unclear; therefore, they merit further study.

The reproductive process in insects is under the control of the steroid 20E and the sesquiterpenoid juvenile hormone. Any interference with the homeostasis of these hormones by exogenous synthetic analogs (agonists or antagonists) can result in abnormal oocyte growth, egg formation, and embryogenesis (Smagghe et al. 2003b). In adult females, natural ecdysteroids are synthesized by the follicle cells in the ovaries and play a major role in ovarian development, vitellogenesis and egg maturation (Whiting et al. 1997, Lafont et al. 2005). The ovarian ecdysteroids, in both free and conjugated forms, are almost entirely taken up by and stored in the oocytes and may serve as a hormonal substrate for embryo development during embryogenesis (Berghiche et al. 2008). Although ecdysteroids were not measured in this study, it is reasonable to hypothesize that the effects that we observed on the fecundity of *S. exigua* also could be due to changes in ecdysteroid concentrations caused by methoxyfenozide in the eggs of this insect. If so, then vitellogenesis and embryogenesis were altered, as has previously been reported in *C. pomonella* (Sun et al. 2003). This hypothesis is supported by a study carried out by Berghiche et al. (2008) in which halofenozide was observed to increase the amounts of both free ecdysteroids and 20E in *T. molitor* eggs. These hormonal disturbances might also explain the reduction of egg viability reported previously in this important pest species when adults were treated with halofenozide (Taibi et al. 2003) and also could have plaid a role in the observed reduction of fertility in the F_1 generation when *C. pomonella* adults were exposed to methoxyfenozide treated surfaces (Sun et al. 2004). After adult exposure, methoxyfenozide can be accumulated within several insect tissues and in the reproductive system and eggs as well, as reported by Schneider et al. (2008) in the parasitoid *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae). Consequently, the agonist can be transferred, by both sexes through oogenesis and spermatogenesis, and incorporated into the eggs to cause the negative effects on fertility, which occurred in *S. littoralis* (Pineda et al. 2006). Moreover, as its metabolic stability is high (Smagghe et al. 1999), it can persist within the insect body during development up to the adult stage and cause the very long-term effect observed.

In conclusion, the results obtained in this study are the first demonstrating that methoxyfenozide interferes with the biochemical processes associated with vitellogenesis and oogenesis in *S. exigua* and reduces the amounts of some compounds essential for larvae hatching from eggs (proteins, carbohydrates, and lipids). This effect could explain the reduction in reproductive parameters reported previously in studies using ecdysone agonists in several insect pests (Farinós et al. 1999; Knight 2000; Sun et al. 2000, 2003; Taibi et al. 2003; Saenz-de-Cabezon et al. 2005; Pineda et al. 2006; Osorio et al. 2008,). Future studies should clarify where and how methoxyfenozide acts to cause the observed changes in the concentrations of carbohydrates, proteins, and lipids.

Acknowledgments

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