MUHAMMAD FIAZ

TOXICOLOGICAL AND MORPHOLOGICAL EFFECTS OF SQUAMOCIN AND TEBUFENOZIDE ON *ANTICARSIA GEMMATALIS* (LEPIDOPTERA: NOCTUIDAE) LARVAE

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
F442t
2019

Fiaz, Muhammad, 1986-
Toxicological and morphological effects of squamocin and tebufenozide on *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae / Muhammad Fiaz. – Viçosa, MG, 2019.
xi, 36 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: José Eduardo Serrão.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Lagarta-da-soja - Controle biológico. 2. Testes de toxicidade. 3. Ultraestrutura (Biologia). 4. Bioinseticidas. I. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. II. Título.

CDD 22. ed. 632.78

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APROVADA: 19 de fevereiro de 2019.

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DEDICATION

I dedicate this work to my parents, brother and sisters who gave me the motivation, encouragement throughout my studies. May ALLAH, The Almighty, have His mercy, forgive all their sins and accept all their good deeds.

ACKNOWLEDGEMENTS

First, I would like to bow and praise to ALMIGHTY ALLAH: Who is the eternal sole creator of whole universe, the most beneficent, the merciful, the gracious, and compassionate, whose blessings and exaltation flourished my thoughts and thrive my ambitions to have the cherish fruits of my modest efforts and enabled me to complete this PhD program. At the same time, I would also praise the perfectness and supremacy of the HOLY PROPHET HAZRAT MUHAMMAD (S.A.W) with great respect: Who is forever, a troch of guidance and blessing for the entire humanity.

I deem it my utmost pleasure to express my deepest feelings of regards and the sense of gratitude to my supervisor Professor Dr. José Eduardo Serrão, Departamento de Biologia Geral, Universidade Federal de Viçosa, who, despite of his busiest work routine, provided his precious advices, guidance, and valuable suggestions throughout these research activities. His continuous believe in my abilities, encouragement, and motivation always energized me whenever I felt stressed and exhausted.

I am grateful to the laboratory fellows, Pakistani and Brazilian friends, who were supportive and helpful throughout the years. They have been invaluable in finishing this project. At last but not the least, I would also delightedly acknowledge the financial and technical support provided by the CNPq and The World Academy of Science (TWAS) through a collaborative platform of TWAS-CNPq Fellowship Program (2014) for International PhD students. I also would like to express my gratitude to the Department of Entomology, the Postgraduate program and Universidade Federal de Viçosa for their support.

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ABSTRACT

FIAZ, Muhammad, D.Sc., Universidade Federal de Viçosa, February, 2019. **Toxicological and morphological effects of squamocin and tebufenozide on Anticarsia gemmatalis (Lepidoptera: Noctuidae) larvae**. Advisor: José Eduardo Serrão.

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) is the main defoliating pest of soybean (*Glycine max* L. Merrill, Fabaceae) in Brazil. There are a variety of plant products and synthetic insecticides used to control *A. gemmatalis*. The larval midgut is reported to be the 'front line' in creation of local immune defense and detoxification of xenobiotic compounds. These characteristics make the midgut cells to be the most affected by those xenobiotics. Squamocin from *Annona mucosa* and tebufenozide were evaluated to test their toxicity and their ultrastructural effects in midgut cells of *A. gemmatalis*. Toxicological results showed that A. gemmatalis was susceptible to both squamocin and tebufenozide. Larvae exposed to tebufenozide compromised larval fitness and its survivorship. LC_{50} and LC_{90} of squamocin and tebufenozide against *A. gemmatalis* were 37.14 mg/L^{-1}, 83.14 mg/L^{-1} and 3.86 mg/L^{-1}, 12.16 mg/L^{-1}, respectively. Squamocin and tebufenozide intake caused deformities in epithelial cells. Squamocin damage to midgut cells include enlarged basal labyrinth, highly vacuolated cytoplasm, damaged apical surface, release of cell protrusions to the gut lumen, autophagy and cell death. Ingestion of tebufenozide caused damage to striated border with release of protrusions to the midgut lumen, damaged nuclear membrane and nucleus with condensed chromatin and increase autophagic vacuolization. Both, squamocin and tebufenozide, damaged mitochondria and compromised respiration rate, while in case of tebufenozide, severe damage resulted in to modification of mitochondria into nanotunnels along with compromising respiration rate. Squamocin and tebufenozide are lethal to larvae, they compromised its fitness and induced severe morphological changes in midgut cells empowering control program against *A. gemmatalis*.

RESUMO

FIAZ, Muhammad, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Efeitos toxicológicos e morfológicos da esquamocina e tebufenozida nas larvas de *Anticarsia gemmatalis* (Lepidoptera: Noctuidae).** Orientador: José Eduardo Serrão.

Anticarsia gemmatalis Hübner (Lepidoptera: Noctuidae) é uma praga desfolhadora da soja (*Glycine max* L. Merrill, Fabaceae) no Brasil, sendo controlada por uma variedade de produtos naturais e inseticidas sintéticos. O intestino médio das larvas é reportado por fazer parte da defesa imune primária por participar da detoxificação de compostos xenobióticos. Estas características fazem do intestino médio o órgão mais afetado por tais xenobióticos. O composto esquamocina da planta *Annona mucosa* e tebufenozida foram avaliados para testar sua toxicidade e possíveis efeitos na ultraestrutura das células do intestino médio de *A. gemmatalis*. Resultados toxicológicos mostraram que *A. gemmatalis* foi suscetível tanto a esquamocina quanto a tebufenozida. Larvas expostas a tebufenozida tiveram comprometimento do fitness e sobrevivência. CL_{50} e CL_{90} de esquamocina e tebufenozida contra *A. gemmatalis* foram 37.14 mg/L^{-1}, 83.14 mg/L^{-1} e 3.86 mg/L^{-1}, 12.16 mg/L^{-1}, respectivamente. A ingestão de esquamocina e tebufenozida causaram danos nas células epiteliais do intestino médio como alargamento do labirinto basal, citoplasma altamente vacuolizado, superfície apical danificada, liberação de protrusões celulares no lúmen, autofagia e morte celular. A ingestão de tebufenozida causou danos na borda estriada com liberações de protrusões para o lumen do intestino, danos na membrana nuclear, núcleo com cromatina condensada e aumento de vacuolização autofágica. Ambos os compostos esquamocina e tebufenozida danificaram mitocôndrias e comprometeram a taxa respiratória, enquanto no tratamento com tebufenozida houve danos severos resultando na modificação das mitocôndrias em nanotúneis juntamente com o comprometimento da taxa respiratória. Assim sendo, esquamocina e tebufenozida são letais às larvas, comprometendo seu fitness e induzindo alterações morfológicas severas nas células do intestino médio, favorecendo o programa de controle contra *A. gemmatalis*.

INTRODUCTION

Soybean is principal food crop in Brazil, enlisting the country as the second largest producer in the world. Defoliation of soybean crops is caused by different insect species that can compromise yield and quality of production, especially when it occurs at reproductive stages (Begum & Eden 1965). Among those, *Anticarsia gemmatalis* Hubner 1818 (Noctuidae: Lepidoptera) is one of the major defoliating soybean pests in the western hemisphere (Bortolotto et al. 2015), which consumes leaves during all its larval instars, and consequently can cause complete defoliation of plants (Fugi et al. 2005) diminishing its productivity (Bortolotto et al. 2015).

Despite availability of several control methods, pest control is still largely based on the use of pesticides, in the sense of synthetic organic chemical-based ingredients that are applied on the crops, the commodity, or the household environment. In Brazil *A. gemmatalis* is controlled mainly by using synthetic insecticides (Navickiene et al. 2007; Panizzi 2013). Because of the fact, pesticide made possible a fastest and effective method to control weeds, diseases and insect in relatively short time (Arain et al. 2018).

Insecticide application is considered to be a necessary component in agricultural production (Tilman et al. 2002). In addition to have direct killing effect on pests, the application of pesticide also results in insect physiology, biology, behavior, fertility, fecundity, longevity and resistance in response to low or sublethal doses of insecticide over time (Guedes et al. 2016; Lee et al. 2000). Those sublethal effects vary depending upon insecticide class and targeted species. The effects impact on insect growth, development and fecundity, in addition to affect insect physiological and biochemical processes (Cao et al. 2017; Lee et al. 2000). Physiological and biochemical changes can be used to assess and predict pesticide efficacy and potency in context of controlling insect pests (Zhao et al. 2018).

Squamocin, also called anonin I, is derived from plant parts, which is an acetogenin (37 carbon atoms, α,β-unsaturated γ-lactone ring and adjacent bis-tetrahydrofuran (bis-THF) ring) (Rupprecht et al. 1990) is cytotoxic (Miao et al. 2016), larvicidal (Costa et al. 2014), antitumor (Chen et al. 2013), neurotoxic (Derbré et al. 2008) and inhibitor of mitochondrial complex I (Duval et al. 2006). The search of natural compounds for plant protection, especially in organic agriculture, has increased the interest in botanical insecticides (Martínez et al. 2015; Zanuncio et al. 2016). Squamocin is ascertained to be gut poisons and effective against insect pests such as *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Ribeiro et al. 2014) and *Aedes aegypti* L. (Diptera: Culicidae) (Costa et al. 2014, 2017).

Tebufenozide was first introduced in the early 1990s (Dhadialla & Jansson 1999), which mimics the biological function of the natural insect hormone JH and interacts with instar phase. Steroid molting hormone 20E and sesquiterpenoid juvenile hormone in insects plays a major role in growth regulation, development and reproductive processes (Riddiford 2012). Tebufenozide halts larval feeding when ingested and induces a premature molt, which is ultimately lethal (Wing et al. 1988).

The insect digestive system is divided in to three principal regions, foregut and hindgut, which are derived from the ectoderm, and the midgut, originated from endoderm is the main organ where digestion and absorption take places (Chapman 2013). In particular with ephemeropteran and lepidopteran midguts, so called invaginated shaped goblet cells with enclosed lumen filled with flocculent material are present which encloses a large extracellular lumen that is continuous with the midgut lumen, via a labyrinthine apical valve formed by the interdigitating microvilli (Lehane & Billingsley 2012). Among the organs tested against different insecticides, the medium intestine has been reported to be one of the most affected by chemicals (Gutiérrez et al. 2016; Catae et al. 2018).

In this work, we aimed to test the plant compound; squamocin from *Annona mucosa*, and chemical; tebufenozide against *Anticarsia gemmatalis* and evaluated their lethal and sublethal effects on survival and fitness of *A. gemmatalis* larvae. We report testing of squamocin and tebufenozide in context with ultrastructural and cytochemical aspects of midgut, suggesting these agents as tool in managing and controlling *A. gemmatalis*.

REVIEW OF LITERATURE

Researches are being carried out with crops to find lines and cultivars with relative levels of resistance to insects. Wild soybeans like, PI 171451, PI 227658 and PI 229358 are being used since early 1970s to cop with the resistance developed in insects like *Epilachna varivestis* (Van Duyn et al. 1972), *Trichoplusia ni* (Luedders & Dickerson 1977), *Diabrotica speciosa* and *Colaspis* spp. (Rezende & de Miranda 1980), *Pseudoplusia includens* (Beach & Todd 1988), *Spodoptera* spp. (Beach & Todd 1987) and *A. gemmatalis* (Oliveira et al. 1993). Piubelli et al. (2003) reported these PIs to possess moderately resistant against some seed sucking insects. When tested in field, PI 274454 lines were observed to be less defoliated than those cultivars tested as control (Rezende et al. 1980). Breeding program of the Instituto Agronômico (Campinas, São Paulo State, Brazil) released a cultivar IAC-100 having genealogy of PI 274454 and PI 229358 and was particularly resistant to sting bugs and other leaf feeders (Veiga et al. 1999).

Levy et al. (2004) reported the morphology of epithelial cells of *Anticarsia gemmatalia* larvae by light and transmission electron microscopy. The midgut of *A. gemmatalis* has three different regions: proximal, media and distal. The wall is formed by epithelial tissue which has four cell types, called columnar, goblet, regenerative and endocrine cells. Columnar cells are long and numerous in number, with the apical portion rich in microvilli and the basal portion invaginations forming in to a basal labyrinth. The goblet cells have a large central cavity encircled by cytoplasmic projections adjacent with mitochondria (Gomes et al. 2013). Regenerative cells show electron-dense cytoplasm with few organelles and endocrine cells exhibit electron-dense secretory granules, usually concentrated in the basal region of the cytoplasm.

Silveira et al. (2004) reported the virus infection of nucleopolyhedro virus in haemocytes of *Anticarsia gemmatalis* and characterized intrahaemocoelic infection by *A. gemmatalis* M nucleopolyhedrovirus (AgMNPV). Entire cells were phagocytosed by plasmatocytes only. Infected cells presented a common feature of necrosis, suggesting cytotoxic effect. There was an exuberant protrusion of budded viruses in granular haemocyte 1 as compared to other haemocytes. Infection to all types of haemocytes were observed, addition to have intense virus replication in these cells which is indication of importance of haemolymph for AgMNPV dispersal in natural host, which is a critical factor for permissiveness.

Knaak & Fiuza (2005) tested the efficacy of *Bacillus thuringiensis* and Nucleopolyhedrovirus and their effect on midgut epithelial cells of *Anticarsia gemmatalis.* They reported the efficacy of *Btk* up to 100% when used isolatedly, rather than using the association of AgNPV-Btk (98.68% of mortality). *Btk* exhibited changes in larval midgut after 6 hours of treatment.

Piubelli et al. (2005) tested effects of flavonoids from soybean genotypes on biology and physiology of *Anticarsia gemmatalis* and quantified those flavonoids. Higher mortality was observed in larvae fed on extracts of genotypes PI 274454, PI 227687, and “IAC-100” addition to negative negatively inclined initial larval growth and pupal weight and elongated larval cycle.

Levy et al. (2011) analyzed the peritrophic membrane of *Anticarsia gemmatalis* caterpillar resistant and susceptible to *A. gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV), in viral presence. They concluded that peritrophic membrane’s effectiveness in protection against pathogens is highly reliant on on the integrity of the epithelial cells and of the structural preservation of the peritrophic membrane, which is directly implicated in the resistance of *A. gemmatalis* larvae to AgMNPV.

Fiuza et al. 2013 reported lethal effects and receptors of *Bacillus thuringiensis* in the *Anticarsia gemmatalis.* They bound insecticidal crystal proteins to midgut epithelial cells of the *A. gemmatalis* larvae by using streptavidin-mediated detection and presented brush border as the binding cite of Cry1Aa and Cry1Ac along the entire length of midgut. Contrary to this, the binding cites of Cry1Ba were not regularly distributed in the microvilli of midgut. The data of binding cites demonstrated a correlation between receptors and toxicity of tested insecticidal crystal proteins of insect.

Almeida et al. (2014) reported the ultrastructural analysis of fat body and midgut of *Anticarsia gemmatalis* larvae treated with neem seed extract. They described swallow midgut cells of *A. gemmatalis* larvae, detachment of basal membrane and complete disruption in severe conditions in treatments. Lipid and protein reserves were observed to be depleted in treatments. Overall, *A. gemmatalis* exhibited negative effects on physiological and biological parameters when treated with neem seed kernel extract.

Costa et al. (2014) reported acetogenins as secondary metabolites which are exclusively produced by Annonaceae, having antitumor, cytotoxic, and pesticide properties. Data estimated with probit analysis suggested that squamocin induced mortality in *Aedes aegypti*. Concentrations of squamocin (50, 80, and 100 ppm) were effective in causing 50% mortality after 360 minutes while 6.4 ppm was effective in causing 50% mortality after 600 of application. Cytotoxic activity caused by 50 ppm of squamocin after 240 minutes resulted in midgut cells with low level of vacuolization in cytoplasm while 100 ppm of squamocin exhibited high level of vacuolization in cytoplasm, damage to apical surface and release of cell protrusion to lumen.

Schünemann et al. (2014) proposed *Bacillus thuringiensis* in management of velvetbean caterpillar and reported that *Bt* showed toxic activity to caterpillar. They emphasized that cultures those express *Bt* can be helpful in global agriculture system with the aim of reducing number of pests, which results in reduction of synthetic insecticide applications to control this pest, ultimately boosting final production.

Mushtaq et al. (2017) used insecticidal proteins from *Bt* to control *Anticarsia gemmatalis* and *Chrysodeixis includens*. High activity of Cry2Ac7 and Vip3Aa11 proteins were observed in *A. gemmatalis* and *C. includens* larvae. In addition to the above results, they also reported that Cry1Ie2 and Cry7Ab3 has anti-feeding activity in adults of *Ceratoma trifurcate*, which is an alternative pest of soybean.

Mageed et al. (2018) tested the efficacy of different chitin synthesis inhibitors (Flufenoxuron, Chlorfluazuron and Triflumuron) against *Spodoptera littoralis* (Boisd.). From all the tested chitin synthesis inhibitors, the highest efficacy was observed with flufenoxuron

(LC_{50} of 0.14 ppm) followed by chlorfluazuron (LC_{50} of 0.42 ppm) and triflumuron (LC_{50} of 1661.58 ppm). Variation in activities for each enzyme was observed against those chitin synthesis inhibitors. Upon ingestion these chitin synthesis inhibitors increase in activity was observed in AST (Aspartate Transaminase) while they decreased the activity of ALT (Alanine Transaminase).

Napoleão et al. (2018) reported a comprehensive introduction of insect midgut, its role in digestion, osmoregulation and defense system. They tested lectins and protease inhibitors on midgut and reported the interference of PIs with digestion processes, resulting in poor nutrient absorption and diminishing amino acid bioavailability. The effect of PIs intake can result in to development of deformities, delay in development and reduction in fertility. Ingestion of these PIs can also change the set of proteases secreted in insect gut, but this response is inadequate which can result in malnutrition status. Lectins are protein, known to interfere with glycoconjugates. The effect of these glycoconjugates on midgut include disruption of the brush border, peritrophic matrix and secretory cell layer. The effect also includes induction of oxidative stress and apoptosis, malfunctioning in nutrient absorption and damaging effects to symbionts.

Ristiati et al. (2018) determined the efficacy of seed extract of custard apple (*Annona squamosa*) against *Culex vishnui* mosquito larvae. Different concentrations had different mortality in *Culex vishnui* mosquito larvae. Custard apple seed extract of concentration 150 ppm was the most effective in causing highest mortality of 8.25.

Maciel et al. (2019) used seed extracts of *Annona squamosa* in microencapsulation to evaluate its lethal toxicity against *Tetranychus urticae.* Lethal toxicity was evaluated by pulverizing microencapsulation and the hexane and ethanol extracts on leaf disks of jack bean *Canavalia ensifomis* L. DC (Fabaceae). The hexane, microencapsulated and ethanol extracts had LC99 of 26.05, 45.26 and 53.27 g/L, respectively. In final consideration, from all tested formulations, the microencapsulation of *Annona squamosa* paved to be the efficient in causing mortality in *Tetranychus urticae*.

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CHAPTER 1

Squamocin induce histological and ultrastructural changes in the midgut cells of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

Ecotoxicology and Environmental Safety 156: 1-8

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Ecotoxicology and Environmental Safety 156 (2018) 1–8

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

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ARTICLE INFO

Keywords:
Acetogenin
Autophagy
Bioinsecticide
Respirometer rate
Ultrastructure
Velvet bean caterpillar

ABSTRACT

Annonaceous acetogenins (*Annona squamosa* Linnaeus) comprises of a series of natural products which are extracted from Annonaceae species, squamocin proved to be highly efficient among those agents. Squamocin is mostly referred as a lethal agent for midgut cells of different insects, with toxic effects when tested against larva of some insects. In present study, LC_{50} and LC_{90} of squamocin for *A. gemmatalis* Hübner (Lepidoptera: Noctuidae) were calculated using probit analysis. Morphological changes in midgut cells were analyzed under light, fluorescence and transmission electron microscopes when larvae were treated with LC_{50} and LC_{90} of squamocin for 24, 48 and 72 h. Results revealed that the maximum damage to midgut cells was found under LC_{90} where it showed digestive cells with enlarged basal labyrinth, highly vacuolated cytoplasm, damaged apical surface, cell protrusions to the gut lumen, autophagy and cell death. The midgut goblet cells showed a strong disorganization of their microvilli. Likewise, in insects treated with squamocin, mitochondria were not marked with Mitotracker fluorescent probe, suggesting some molecular damage in these organelles, which was reinforced by decrease in the respiration rate in these insects. These results demonstrate that squamocin has potential to induce enough morphological changes in midgut through epithelial cell damage in *A. gemmatalis*.

1. Introduction

The velvet bean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) is the main defoliating pest of soybean (*Glycine max* L. Merrill, Fabaceae) in Brazil (Sosa-Gomes, 2004; Panizzi, 2013). This species occurs from central region of the United States to Argentina and some Indian islands (Riffel et al., 2012). *Anticarsia gemmatalis* damages other crops also, such as peanuts, alfalfa, bean, pea, rice and wheat (Rahman et al., 2007).

In Brazil, *A. gemmatalis* occurs throughout the year, especially in the vegetative stage of the plants and it is controlled by using synthetic insecticides (Navickiene et al., 2007; Panizzi, 2013). These insecticides may cause side effects such as pest resistance, environmental pollution, toxic waste, emergence of new pests and reduction of beneficial insects (Bourguet et al., 2000; Nicholson, 2007). Hence, alternative methods to control *A. gemmatalis* (De Nardo et al., 2001; Navickiene et al., 2007) need to be developed. The integrated pest and ecological management aim to use safer products than synthetic chemicals for the safety of human health and environment (Matsumura, 2004; Pavela, 2007). The search of natural substances for plant protection, especially in organic agriculture, has increased the interest in botanical insecticides (Martínez et al., 2015; Zanuncio et al., 2016).

Annonaceous acetogenins comprised from a series of natural products extracted from Annonaceae species, among which squamocin proved to be the promising agent. Squamocin also called anonin I, is an acetogenin with 37 carbon atoms, α,β-unsaturated γ-lactone ring and adjacent bis-tetrahydrofuran (bis-THF) ring (Rupprecht et al., 1990). Use of single molecules are important for synthesis of new inseticdes, and computational approaches in designing new insecticide can be really helpful (Speck-Planche et al., 2011), by providing better understanding of physicochemical properties (Aschi et al., 2007), insecticidal activity (Liu et al., 2009; Khajehali et al., 2010; Sparks et al., 2001; Xue et al., 2007), and toxicological profiles (Eldred and Jurs, 1999).

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https://doi.org/10.1016/j.ecoenv.2018.02.080
Received 7 November 2017; Received in revised form 8 February 2018; Accepted 28 February 2018
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Acetogenins are cytotoxic (Miao et al., 2016), larvicidal (Costa et al., 2014), antitumor (Chen et al., 2013), neurotoxic (Derbré et al., 2008) and inhibitors of mitochondrial complex I (Duval et al., 2006). These compounds are gut poisons and effective against insect pests such as *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Ribeiro et al., 2014) and *Aedes aegypti* L. (Diptera: Culicidae) (Costa et al., 2014, 2017). Squamocin is mostly observed as an acting agent on midgut cells of different insects and is previously heavily tested against *A. aegypti* (Costa et al., 2014, 2017).

The digestive tract of insects is divided into three main regions: foregut and hindgut, which are derived from the ectoderm, and the midgut, the main organ where digestion and absorption take places, which has an endodermic origin (Chapman, 2013). Plant feeders usually contain short and enlarged midgut, allowing for a high throughput rate of food which is often abundant in quantity. Main functions of the midgut are digestion and absorption of food, regeneration of cells in case of any damage (Lehane and Billingsley, 2012) and creation of local immune defense by developing physical barrier which prevents dissemination of ingested pathogens in the gut (Silva et al., 2016) or physiological response for detoxification of xenobiotic compounds (Dow, 1987).

There are a variety of plant products that have toxicological properties used to control *A. gemmatalis* (Messiano et al., 2008; Mourão et al., 2014; Ribeiro et al., 2015); however, squamocin from *Annona squamosa* has been reported to be toxic gainst *A. aegypti* larva, but safer to its predators *Culex bigoti* Bellardi (Diptera: Culicidae) and *Toxorhynchites theobaldi* (Dyar & Knab) (Diptera: Culicidae) and to human cells (Costa et al., 2017) and might be an alternative to control *A. gemmatalis* caterpillars. We evaluated the toxic effect, histological and ultrastructural changes in the midgut cells of *A. gemmatalis* exposed to squamocin, in order to contribute for the development of new strategies for controlling this insect pest. Overall histological and ultrastructural changes of squamocin on tissue, cell and organelles under light, transmission and confocal microscopes are reported.

2. Materials and methods

2.1. Insects

Larvae of *A. gemmatalis* were obtained from mass rearing in the "Laboratório de Controle Biológico" of the "Instituto de Biologia Aplicada a Agricultura e Pecuária" (BIOAGRO, Universidade Federal de Viçosa) in Viçosa, Minas Gerais, Brazil. They were maintained at 26 ± 1 °C in 75 ± 5% relative humidity with a 12-h photophase. Larvae were placed in polystyrene boxes (15 × 9 cm) and fed on an artificial diet containing 10 g agar, 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein, 31.2 g minced beans, 12.5 g casein, and a 2.5 mL vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% thiamin and 0.004% HCl) (Greene et al., 1976).

2.2. Squamocin

The squamocin was obtained from the "Laboratório Químico de Produtos Naturais" of the Universidade Federal de Alagoas (Maceió, Alagoas, Brazil). Squamocin (CAS number: 120298-30-8) is a white solid wax obtained from a methanolic extraction of *Annona mucosa* Jacquin (Anonaceae) seeds following by a successive partition with chloroform (85.4 g; 57.3%) using a High Performance Liquid Chromatography (HPLC) (Costa et al., 2014). Squamocin with 97% of purity was pre-solubilized in 1% dimethylsulfoxide, dissolved in distilled water and solubilized in 2% Tween 20 to produce a stock solution of 998.85 mg/L.

2.3. Dose-response bioassay

Squamocin was diluted in 1 mL water to produce a stock solution by adjusting 1 g/L to obtain the required concentrations. Squamocin efficacy was determined by calculating the lethal concentrations (LC_{50} and LC_{90}) values under laboratory conditions. Six concentrations of squamocin besides the control (distilled water) were adjusted in 1 mL stock solution (treatments and distilled water) 31.16, 62.42, 124.85, 249.71, 499.42, 998.85 mg/L. For each treatment, aliquots were taken from the stock solution and mixed with distilled water in 2 mL glass vials. Different concentrations of the treatments were applied in 1 µL in the food of each individual of *A. gemmatalis*. Fourth instar larvae were used per concentration and placed individually in glass vials (2 × 15 cm) covered with a cotton lid and maintained in the dark. The number of dead insects in each vial was counted after squamocin exposure over 72 h. Each treatment was replicated three times in this bioassay.

2.4. Light microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 24, 48 and 72 h. Larvae from both treatment and control were collected, were dissected in saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) and the midgut regions divided in anterior, middle and posterior. The samples were transferred to Zamboni's fixative solution (Stefanini et al., 1967) for 12 h at 5 °C. Then, the samples were dehydrated in a graded ethanol series (70%, 80%, 90% and 95%), embedded in historesin Leica (Leica Biosystems Nussloch GmbH, Heildelberger, Germany) and sectioned at 3 µm thickness in Leica RM2255 microtome. Sections were stained with hematoxylin and eosin and analyzed using a Leica DMLS light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.5. Transmission electron microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 24, 48 and 72 h. The midgut of *A. gemmatalis* larvae from both treatment and control were dissected and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 containing 0.2 M sucrose for 4 h at room temperature. Then, the midgut was divided into anterior, middle and posterior region and fragments post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, followed by washing in the buffer and dehydration in a graded ethanol series (70%, 80%, 90% and 99%). The samples were embedded in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA) and ultrathin sections (70–90 nm) were obtained using a glass knife in a Sorvall MT2-BMT2-B ultramicrotome (Sorvall Instruments, Wilmington, DE, USA). Sections were stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963) and examined with a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Jena, Germany).

2.6. Fluorescence microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 72 h. The midgut of *A. gemmatalis* larvae from both treatment and control were dissected and transferred to sodium phosphate buffer (PBS). Diluted 1 mM MitoTracker® stock solution (Invitrogen, Waltham, MA, USA) to the 100 nM concentration in PBS buffer and the samples were then transferred to this solution for 2 h at 37 °C. After washing with buffer several times, midguts were transferred to PBS at 37 °C for 20 min. Samples were then transferred to iodide TO-PRO® – 3 (Invitrogen, Waltham, MA, USA) staining solution by diluting 1:1000 (1 µM) in PBS at 37 °C for 40 min. Carefully removed the medium/buffer covering the midguts, and replaced it with freshly prepared, pre-warmed buffer containing 2–4% formaldehyde. The samples were washed in PBS and mounted in slides with 50% sucrose

solution. The pieces were examined with a Confocal Laser Scanning Microscopes Zeiss LSM510 META (Carl Zeiss, Jena, Germany).

2.7. Respiration rate

Respirometry bioassays were conducted 24 h against control and treated larvae with squamocin extract, as previously detailed. The squamocin concentrations used were LC_{50} and LC_{90} and control with distilled water. Carbon dioxide (CO_2) (μL of CO_2 h^{-1}/insect) production was measured with a respirometer of the type CO_2 Analyzer TR_3C (Sable System International, Las Vegas, USA), using methodology adapted from previous studies (Plata-Rueda et al., 2017). Respirometers (25 mL) were used, each one holding three larvae of *A. gemmatalis* and connected to a closed system. CO_2 production was measured after the insects were acclimated in the chambers for a period of 12 h at 27 ± 2 °C. To quantify the CO_2 produced inside each chamber, compressed oxygen gas (99.99% pure) was passed through the chamber at a flow of 100 mL min^{-1} for a period of 2 min. This airflow forces all produced CO_2 molecules to pass through an infrared reader coupled to the system, which makes a continuous measurement of the CO_2 produced by the insects and held inside each chamber. After CO_2 measurement, the insects were removed from the chambers and then weighed using an analytical balance (Sartorius BP 210D, Göttingen, Germany). Respiration rate values were not normalized by body mass because this method masks the individual effects of the variables (Hayes, 2001). Five replicates were used for each treatment.

2.8. Statistics

The lethal concentration (LC_{50} and LC_{90}) and their confidence limits for squamocin was determined by logistic regression in dose-response assays based on the concentration Probit-mortality (Finney, 1971) using SAS user (v.9.0) program for Windows (SAS, 2002). Respiration rate were subjected to two-way analyses of variance (time × squamocin treatment) and Tukey's HSD (Honest Significant Difference) test ($P < 0.05$) when appropriate (PROC GLM). As the time interval was assessed in different insect samples, they are not pseudo replicates in time and therefore subject to regular two-way analyses of variance instead of repeated measures analyses of variance using SAS User software (Guedes et al., 2006).

3. Results

3.1. Toxicity

From the six concentrations of squamocin used to test mortality of *A. gemmatalis* (Fig. 1a), two different lethal concentration levels LC_{50} and LC_{90} were estimated by Probit with LC_{50} = 37.14 (32.33–41.66) mg/L and LC_{90} = 83.14 (72.41–100.21) mg/L (X^2 = 23.17; df = 5; $P < 0.001$) (Table 1, Fig. 1b). Mortality was always < 1% in the control.

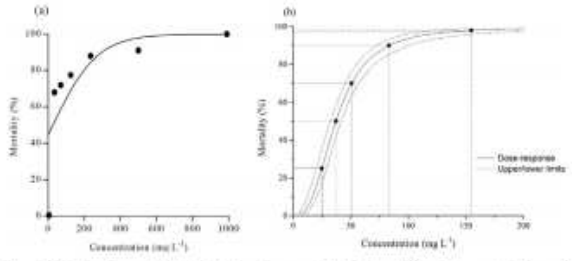

Fig. 1. (a) Larval mortality of *Anticarsia gemmatalis* fed on different concentrations of squamocin (31.16; 62.42; 124.85; 249.71; 499.42; 998.85 $mgl.^{-1}$). Black points represent different concentrations selected in probit analysis. (b) Estimated lethal concentrations of squamocin(LC_{50} and LC_{90}) for *Anticarsia gemmatalis* cartepillars (X^2 = 23.17; df = 5; P < 0.001). Lines denote 95% confidence intervals. Black points represent LC_{25}, LC_{50}, LC_{75}, LC_{90} and LC_{99} concentration, LC_{50} and LC_{90} were selected to evaluate histological and ultrastructural changes.

Table 1
Lethal concentration of the squamocin in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) caterpillars after 72 h exposure. X^2, chi squared value for the lethal concentrations and fiducial limits based on a log scale with significance level at $P < 0.001$.

Concentration	Estimated value (mg/L)	Fiducial limits		X^2
		Inferior	Superior	
LC_{25}	24.83	20.04	28.95	23.17
LC_{50}	37.14	32.33	41.66	
LC_{75}	55.57	49.76	62.83	
LC_{90}	83.14	72.41	100.2	
LC_{99}	200.30	153.85	296.29	

3.2. Histology

The three different regions of the midgut of *A. gammatalis* larvae from a control treatment showed a single layered epithelium of columnar digestive and goblet cells (Fig. 2a). The digestive cells showed

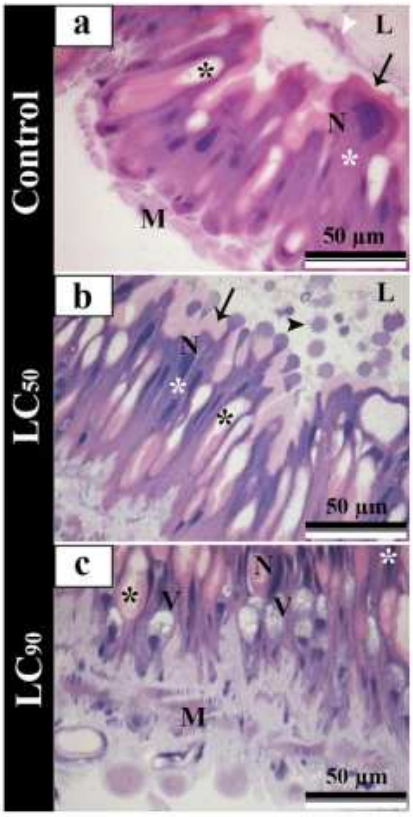

Fig. 2. Histological sections of *Anticarsia gemmatalis* midgut. [a]: control larvae showing epithelium with columnar digestive cells (white asterisk) with well-developed brush border (arrow) and nucleus (N). Note the presence of goblet cells (black asterisk), peritrophic matrix (white arrowhead) in the midgut lumen and the longitudinal and circular muscles (M), [b]: Larva fed on diet with LC_{50} of squamocin showing digestive cell with nucleus (N), goblet cell (black asterisk) and digestive cell (white asterisk). Note the apocrine secretions (arrowhead) released in lumen (L) and the brush border (arrow), [c]: Larva fed on diet with LC_{90} of squamocin showing vacuolization (V) in the digestive cells (white asterisk), goblet cells (black asterisk), nucleus (N) and longitudinal muscles (M).

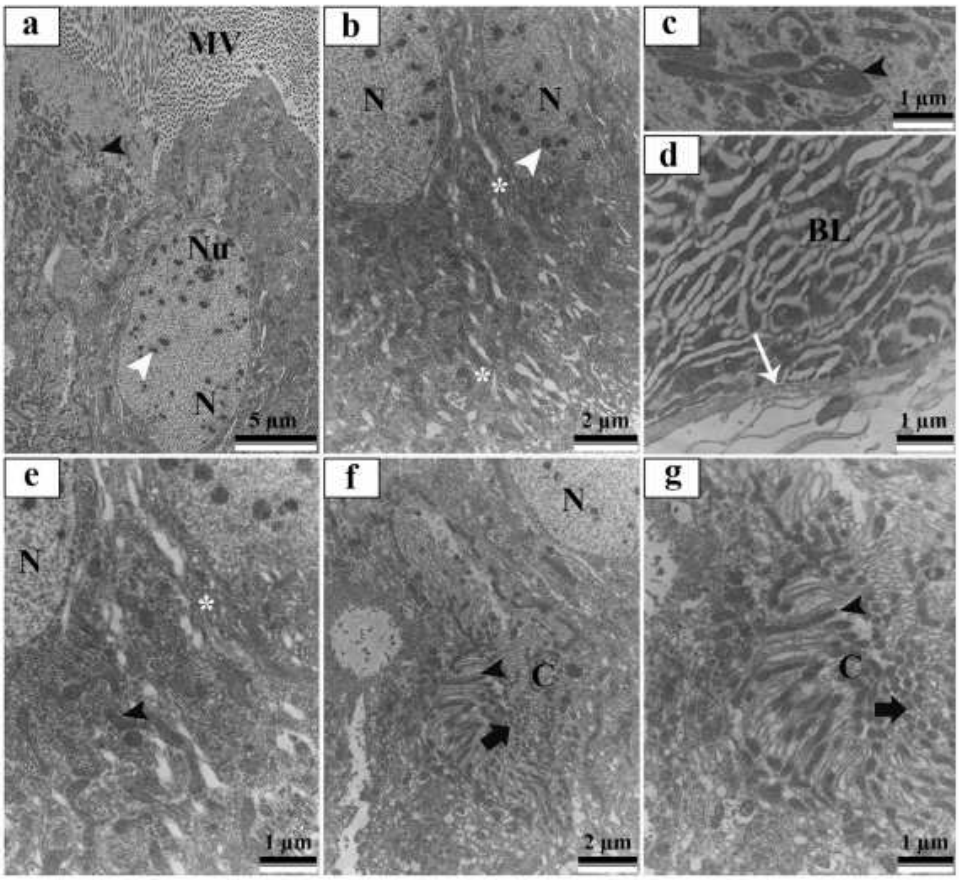

Fig. 3. Transmission electron micrographs of the midgut of *Anticarsia gemmatalis* control larvae. [a]: Median-apical region of digestive cell showing well developed nucleus (N) with few crumples of heterochromatin (white arrowhead) and nucleolus (Nu). Note apical well developed microvilli (MV) and many mitochondria (black arrowhead), [b]: Median-basal region of digestive cell showing nucleus (N) and cytoplasm with plasma membrane infoldings resulting in long and enlarged basal labyrinth (asterisks) [c]: Detail of mitochondria (black arrowhead) closely associated with basal plasma membrane infoldings, [d]: Detail of digestive cell basal cell region with enlarged labyrinth (BL) and the basal lamina (white arrow) and, [e]: Median region of digestive cells showing profiles of the basal labyrinth (asterisk) associated with mitochondria (black arrowhead), [f]: General aspect of the goblet cell showing the nucleus (N) and the internal cavity (C) with microvilli (black arrow), [g]: Detail of the goblet cell cavity (C) showing microvilli (black arrow) with mitochondria (black arrowhead).

spherical or elongated nucleus with decondensed chromatin and cytoplasm with some granules and vacuoles. The apical region of the digestive cells had a well-developed striated border, followed by the peritrophic matrix lining the gut content and well-developed circular and longitudinal muscle layers occurred externally to the midgut epithelium (Fig. 2a).

Histological changes in the midgut of *A. gemmatalis* were found after feeding larva with squamocin. Substantial effects occurred when larva was treated with LC_{50} concentration where they showed digestive cells with high vacuolation, mainly in the posterior midgut area (Fig. 2b), nucleus showed a slight increase in the amount of condensed chromatin. Apocrine secretions and secretory cytoplasmatic vesicles expelled from the epithelium were prominently visible in the lumen (Fig. 2b). A maximum effect was observed when larva was treated with LC_{90} concentrations where the midgut showed an irregular epithelium with high vacuolation in the cytoplasm (Fig. 2c) and secretory vesicles expelled from the epithelium to the lumen.

3.3. Ultrastructure

As showed previously in light microscopy, there are not differences among midgut regions of *A. gemmatalis*. The apical surface of digestive cell was rich in well-developed microvilli (Fig. 3a). The nucleus showed predominance of decondensed chromatin with few chromatin cumpling and a well-developed nucleolus (Fig. 3a, b). The perinuclear cytoplasm was rich in mitochondria (Fig. 3c) and rough endoplasmic reticulum. The basal cell region showing many plasma membrane infoldings forming an enlarged labyrinth (Fig. 3d) reaching to the median cell region (Fig. 3e) containing few openings to the hemocoel. In addition to digestive cells, the midgut of *A. gemmatalis* caterpillar had the so-called goblet cells characterized by an enlarged cavity filled with microvilli containing elongated mitochondria inside them (Fig. 3f, g).

Larvae fed on LC_{90} squamocin showed midgut cell with apical surface showing large electron-dense cell protrusions, which were released to the midgut lumen (Fig. 4a, b). The cytoplasm of digestive cells had

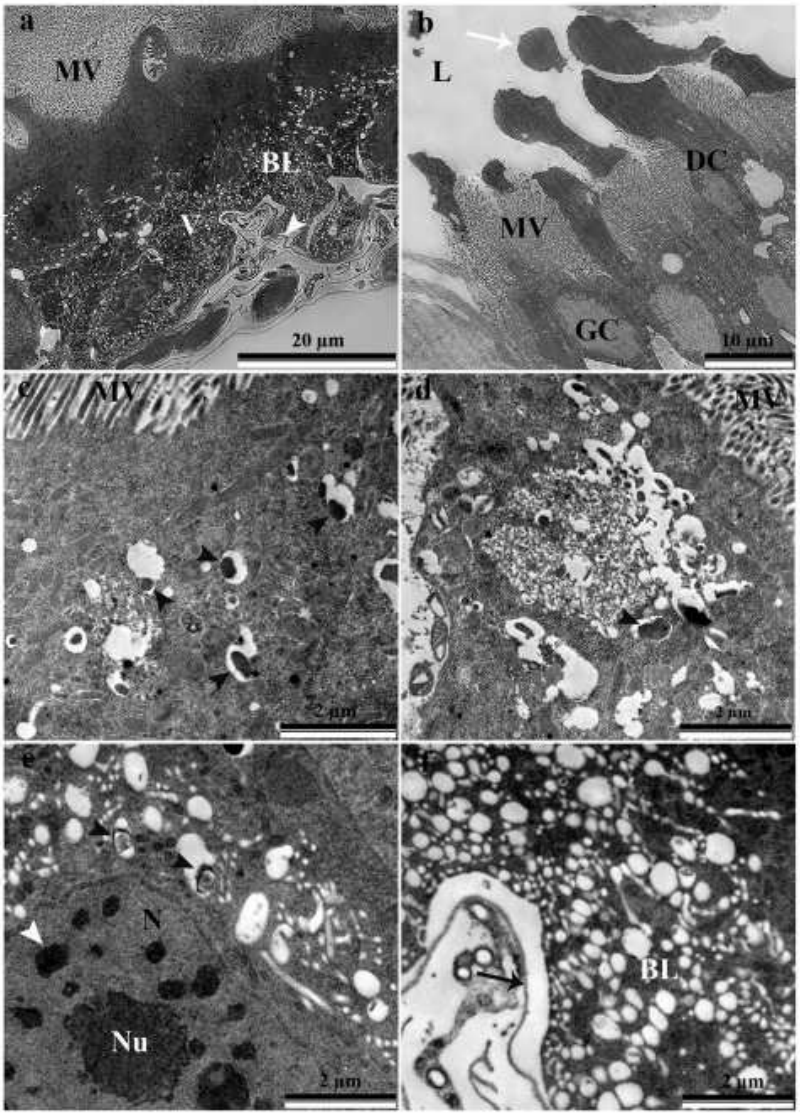

Fig. 4. Transmission electron micrographs of the midgut of *Anticarsia gemmatalis* larvae fed on squamocin. [a]: General aspects of epithelium showing cells with disorganized labyrinth (BL), cluster of vacuoles (V), microvilli (MV) and basal lamina (white arrowhead), [b]: General aspect of the midgut epithelium of larvae fed on LC_{50} squamocin showing digestive cells (DC) with large fragments of the apical cell region (white arrow) releasing to the lumen (L), disorganized microvilli (MV) and some goblet cell (GC), [c]: Apical region of digestive cell in larva fed on LC_{50} squamocin showing dying cell with many autophagic vacuoles (black arrowheads), [d]: Cytoplasm digestive cell in larva fed on LC_{90} squamocin showing autophagic vacuoles (black arrowhead) and an enlarged vacuole with organelle debris and disorganized microvilli (MV), [e]: Perinuclear region of digestive cell in larva fed on LC_{90} squamocin showing many autophagic vacuoles (black arrowheads), nucleus (N) with well-developed nucleolus (Nu) and condensed chromatin (white arrowhead), [f]: Basal region of digestive cell in larva fed on LC_{90} squamocin showing disorganized basal lamina (black arrow) and swelled basal labyrinth (BL).

many autophagic vacuoles (Fig. 4c, d, e). Some digestive cells showed disorganized microvilli, cytoplasm with enlarged regions filled with organelle debris (Fig. 4d) and damaged nuclear membrane. The nucleus had decondensed chromatin with some cumpling of condensed chromatin and well-developed nucleolus (Fig. 4e). The basal cell portion showed enlarged basal labyrinth with few openings to the hemocoel (Fig. 4f). In these insects, the goblet cell showed degraded microvilli with absence of mitochondria (Fig. 5a).

Regenerative cells had not ultrastructural changes in comparison with ones from control caterpillars, characterized by large nucleus and few cytoplasm organelles.

3.4. Confocal microscopy

Because squamocin is referred to inhibit mitochondrial complex I, we evaluated the occurrence of mitochondria in the digestive cells with MitoTracker probe which showed positive fluorescence for this organelle only in control group (Fig. 6a), while fluorescence results were negative in LC_{50} (Fig. 6b) and LC_{90} (Fig. 6c) squamocin treated larvae.

3.5. Respiration rate

The respiration rate of *A. gemmatalis* was significantly different for control and squamocin LC_{90} treated caterpillars ($F_{2,16} = 5.59$, $P < 0.001$), but was similar between control and LC_{50} squamocin ($F_{2,16} = 4.49$, $P = 0.459$) (Fig. 6). Respiration rate in *A. gemmatalis* caterpillar fed on squamocin extract LC_{90} for 1 and 3 h were different ($F_{2,16} = 5.13$, $P < 0.001$) with decrease in the respiration rate in 3 h treated caterpillars (Fig. 7).

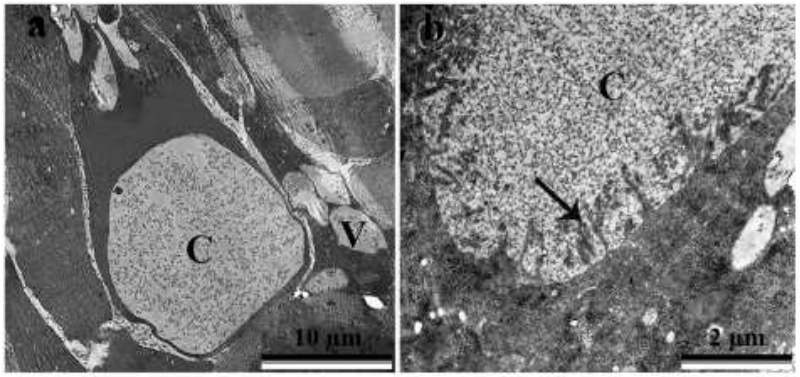

Fig. 5. Transmission electron micrographs of goblet cell in the midgut of *Anticarsia gemmatalis* fed on squamocin. [a]: General aspect showing multiple vacuoles (V) and the cell cavity (C) filled with flocculent material, [b]: Detail of cell cavity (C) showing disorganized microvilli (arrow) without mitochondria.

4. Discussion

Squamocin causes lethal effects in *A. gemmatalis* larvae as reported for other insect pests according to the concentration of this annonaceous acetogenins (Leatemia and Isman, 2004a; Colom et al., 2007; Souza et al., 2017). The susceptibility of lepidopteran defoliating pests such as *Helicoverpa armigera* Hübner (Noctuidae), *Plutella xylostella* L. (Plutellidae) and *Spodoptera frugiperda* (J.S. Smith) (Noctuidae) may vary with the exposure at squamocin oil applied to the body surface of these insects or by ingestion (Leatemia and Isman, 2004b; Ansante et al., 2015; Souza et al., 2017). In this study, the dose-response bioassay proves toxicity against *A. gemmatalis*, reaching a 90% mortality rate and increasing concentrations (LC_{50} = 37.14 mg/L and LC_{90} = 83.14 mg/L) of squamocin on this insect have shown immediate lethal effect within 72 h after exposure.

The idea of the trial was to test squamocin to evaluate its toxicity and affects on midgut of *A. gemmatalis*. Changes in the midgut cells of *A. gemmatalis* are induced for squamocin with damage to microvilli and intense cytoplasm vacuolization with autophagy in midgut cells. Our results were similar to those found by Costa et al. (2017) in the midgut digestive cells of *A. aegypti* larvae exposed to squmocin.

Gaban et al. (2015) have pointed out that midgut as heavily affected when *A. aegypti* larvae are treated with new chemistry insecticide showing intense secretory activity and droplets in the apical portion of the cell, released to the gut lumen. The histopathological features in the midgut of *A. gemmatalis* caterpillars fed on squamocin are similar to those found by Scudeler and dos Santos (2013) when they treated neem oil against *C. claveri*. However, there are no significant difference among different regions of *A. gemmatalis* midgut.

The midgut of *A. gemmatalis* treated with LC_{90} showed many autophagic vacuoles in comparision with midgut of caterpillars treated with LC_{50}. Same pattern of concentration dependent toxicity occurs in *A. aegypti* larvae exposed to squamocin (Costa et al., 2017) and *Ceraeochrysa claveri* (Neuroptera: Chrysopidae) treated with neem oil (Scudeler et al., 2017). Autophagy is a common feature in the turnover of cell cytoplasm (Mir et al., 2015), but it can be also a type of programmed cell death (Tsujimoto and Shimizu, 2005) by which body cleans out various debris, toxins and recycles damaged cell components (Levy et al., 2008; Gomes et al., 2013). Thus, the increase in autophagy in the midgut cells of *A. gemmatalis* caterpillars fed on squamocin may suggest the ultimate cell death.

MitoTracker probe is a mitochondrial-selective fluorescent label that selectively accumulates in the mitochondrial matrix by covalent bond to mitochondrial proteins by reacting with free thiol groups of cysteine residues (Presley et al., 2003). Previously it had been stated that *A. aegypti* larvae exposed to squamocin has effects on ion and water transport, which may result in digestive cell injuries (Costa et al., 2017). Derbré et al. (2008) stated that acetogenins from Annonaceae are not only strong inhibitors of mitochondrial complexes I and II, but also of cytosolic and endoplasmic reticulum enzymes. Our findings suggest the hypothesis that squamocin damages the thiol groups of proteins and peptides where MitoTracker bind, resulting in negative fluorescence in mitochondria. Our hypothesis of negative flouresnece of probe due to damage done by squamocin to mitochondrial targeting site is strongly supported by Yousefi et al. (2009) and Rochael et al. (2015). In their experiment they used MitoSOX probe for targeting mitochondria and concludes that flouresence signal of probe totally depends upon subsequent mitochondrial DNA binding. Thus, we detect a mitochondrial molecular damage which is not followed by a rapid morphological change, since at ultrastructural level, mitochondria show normal morphology.

An evidence of molecular damage to mitochondria is that squamocin compromises the respiration rate of *A. gemmatalis* up to 3 h after feeding exposure. Respiratory rate and body mass of insects represent the sum of the energy demands of the physiological processes of insects that are necessary to produce defense mechanisms against plant toxic compounds (Guedes et al., 2006; Correa et al., 2015; Plata-Rueda et al., 2017). Thus, low respiration rate is an indicator of physiological stress and plant toxic compounds may compromise insect respiration by impairing muscle activity, leading to paralysis (Guedes et al., 2006; Correa et al., 2015; Plata-Rueda et al., 2017). Annonaceous acetogenins have been reported to effectively disrupt the respiration process, inhibiting the mitochondrial respiration via complex I NADH: ubiquinone oxidoreductase, blocking mitochondrial oxidative phosphorylation resulting in cell death (Chen et al., 2011). In this study, the absorption of squamocin via ingestion by *A. gemmatalis* is negatively correlated with its respiration rate. Larvae of *A. gemmatalis* have low respiration rate caused by squamocin, possibly resulting in physiological costs due to

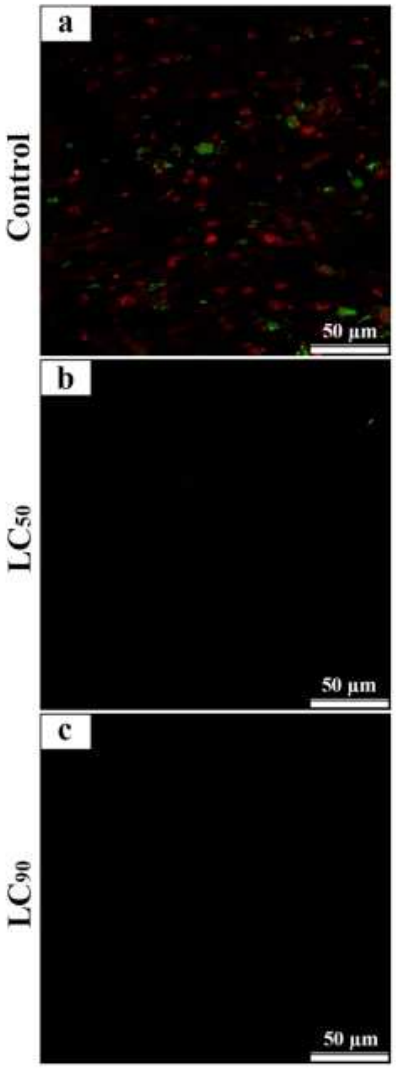

Fig. 6. Confocal micrographs of *Anticarsia gemmatalis* larval midgut stained with MitoTracker fluorescent probe. [a]: Control larvae showing mitochondria (green) and nucleus (red), [b]: Larvae fed on LC_{50} squamocin without mitochondria, [c]: Larvae fed on LC_{90} of squamocin without mitochondria. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

energy reallocation from other physiological processes. In particular, this favors the use of this toxic compound via ingestion and can cause significant negative effects on *A. gemmatalis*.

Our finding shows that squamocin induce histological and ultrastructural changes in the midgut cells of *A. gemmatalis*. The mortality caused for squamocin on *A. gemmatalis* may have advantages by their mode of action on this insect and may be a potential source as insecticidal compound. The potential of the squamocin against *A. gemmatalis* contribute to the establishment of alternative strategies in controlling of defoliating pest using botanical compounds that have low environmental impact compared with synthetic insecticides.

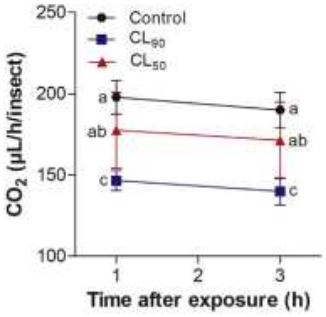

Fig. 7. Respiration rate (means ± se) of *Anticarsia gemmatalis* larvae after feeding to LC_{50} and LC_{90} squamocin application. Means with by different letters in the same time differ at by Tukey's mean separation test ($P < 0.05$).

Acknowledgments

The authors are grateful to The World Academy of Sciences TWAS (Italy), Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq (Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES (Brazil), Fundação de Amparo a Pesquisa do Estado de Minas Gerais FAPEMIG (Brazil) and Núcleo de Microscopia e Microanálise of Universidade Federal de Viçosa.

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CHAPTER 2

Toxicological and morphological effects of tebufenozide on *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae

Chemosphere 212: 337-345

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Chemosphere 212 (2018) 337–345

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

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HIGHLIGHTS

- The insecticidal effect of Tebufenozide was tested against *A. gemmatalis*.
- *A. gemmatalis* showed significant changes in histology and ultrastructure.
- Induction of mitochondrial nanotunnels by Tebufenozide.
- Tebufenozide compromises respiration and behavior of *A. gemmatalis*.

ARTICLE INFO

Article history:
Received 20 March 2018
Received in revised form
16 August 2018
Accepted 17 August 2018
Available online 20 August 2018

Handling Editor: Willie Peijnenburg

Keywords:
Autophagy
Insecticide
Respirometer rate
Survivorship
Toxicity
Ultrastructure
Velvet bean caterpillar

ABSTRACT

The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), is an important soybean pest in the Americas. Tebufenozide, a novel nonsteroidal ecdysone agonist is used to control this pest. Bioassays were conducted to assess tebufenozide toxicity and their ultrastructural effects on midgut of *A. gemmatalis*. The toxicity, survivorship, behavior response, and respiration rate for *A. gemmatalis* larvae after exposure to tebufenozide were evaluated. Also, *A. gemmatalis* larvae were treated with LC_{50} obtained from tebufenozide and changes were observed on their midgut cells after 24, 48 and 96 h. Tebufenozide was toxic to *A. gemmatalis* ($LC_{50} = 3.86$ mg mL^{-1} and $LC_{90} = 12.16$ mg mL^{-1}) and survivorship was 95% for adults that had not been exposed to tebufenozide, decreasing to 52% with LC_{50} and 27% with LC_{90} estimated value. Damage to midgut cells was increased with exposure time. These cells show damaged striated border with release of protrusions to the midgut lumen, damaged nuclear membrane and nucleus with condensed chromatin and increase in amount of autophagic vacuoles. Mitochondria were modified into nanotunnels which might be an evidence that tebufenozide induces damage to cells, resulting in cell death, proved by immunofluorescence analyses. This insecticide also caused paralysis movement with change in homeostasis and compromised larval respiration. Thus, sublethal exposure to tebufenozide is sufficient to disturb the ultrastructure of *A. gemmatalis* midgut, which might compromise insect fitness, confirming tebufenozide a possible controlling insecticide.

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1. Introduction

Searching for compounds to control pests is one of the main challenges for agriculture. Phytosanitary problems were initially dealt with natural insecticides from plant leaves, barks, flowers or nectar. Advancement of agricultural technology reduced natural insect control practices (Santos et al., 2017). Pesticide made possible a fastest and reliable method to control diseases, weeds and insect, pests are mostly controlled with chemical compounds

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https://doi.org/10.1016/j.chemosphere.2018.08.088
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(Cui et al., 2018).

Time trends in insects, after exposure to insecticides, have been thoroughly studied by various methods. Slow-acting, nonrepellent toxicants are a strategy to control insects (Su et al., 1982). Delayed toxicity is defined for *Solenopsis invicta* Buren (Formicidae: Hymenoptera) had <15% mortality at 24 h and >89% mortality at the end of bait toxicant experiment (Stringer et al., 1964). This technique was used with 90% mortality for fast acting compound at a fixed concentration, for *Conophthorus ponderosae* Hopkins (Coleoptera: Scolytidae) by Haverty and Dell (1984) to achieve, characterizing slow-acting compounds (Su et al., 1987). Suppression of treated populations reduces damaging potential and may provide long-term control. A successful slow acting insecticide will reduce further insecticide application quantity in comparison with fast acting insecticides to a certain limit.

Tebufenozide was introduced in the early 1990s to control lepidopteran pests (Dhadialla and Jansson, 1999). This chemical class mimics the biological function of the natural insect moulting hormone 20-hydroxyecdysone (20E) and interacts with ecdysteroid receptor (Fahrbach et al., 2012). Steroid moulting hormone 20E and sesquiterpenoid juvenile hormone in insects plays a central role in regulating growth, development and reproductive processes indicating as potential specific target sites for pest control. Fate of tebufenozide makes it unique by halting larval feeding when ingested and induces a premature molt, which is ultimately lethal (Wing et al., 1988).

The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) is one of the main defoliating pest of agricultural crops in the Americas, affecting mainly soybean fields (Haase et al., 2015). In field, the pest control system relies on conventional pesticides, including cyclodienes, organophosphates and pyrethroids (Baur et al., 2010), and biological control (Dudczak et al., 2017).

Pesticide consumption in Brazil is currently the highest in world (Pedlowski et al., 2012; Rigotto and Rocha, 2014; Bortolotto et al., 2015), despite having the world's richest and most abundant beneficial insect fauna (Dudczak et al., 2017). *Anticarsia gemmatalis* control demands an average of two-insecticide application during the season (Vincent et al., 2007). Broad-spectrum insecticides, such as organophosphates are highly toxic (Büyükgüzel, 2006) and even herbicides may cause side effects on non-target organisms (Rittman et al., 2013; de Saraiva et al., 2016). Although fungicides are usually non-toxic for insects, but they can have synergistic affects with other pesticides (Papaefthimiou and Theophilidis, 2001) leaving insect growth regulator insecticides as being one control measure considered safe for beneficial insects (Costa et al., 2014).

Toxic effects by tebufenozide on *Choristoneura orae* (Lepidoptera: Tortricidae) by aerial application reduced population of this pest with larval mortality, reduced mating success and fecundity of mated females (van Frankenhuyzen and Régnière, 2017). The toxic effects with histological and ultrastructural changes in midgut cells of *A. gemmatalis*, exposed to tebufenozide were evaluated. This can contribute to develop new strategies to control this insect pest. Toxicity, survivorship, locomotory behavior, histological and ultrastructural changes of tebufenozide on tissue and cell with light and transmission electron microscopes are reported.

2. Materials and methods

2.1. Insects

Anticarsia gemmatalis larvae were obtained from mass rearing of the "Laboratório de Controle Biológico" at "Instituto de Biologia Aplicada a Agropecuária" (BIOAGRO, Universidade Federal de Viçosa) in Viçosa, Minas Gerais, Brazil. They were maintained at 26 ± 1 °C in 75 ± 5% relative humidity with a 12-h photophase. Larvae were placed in polystyrene plastic containers (15 × 9 cm) and fed on an artificial diet containing 10 g agar, 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein, 31.2 g minced beans, 12.5 g casein, and a 2.5 mL vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% thiamin and 0.004% HCl) (Greene et al., 1976).

2.2. Insecticide

The insecticide tebufenozide (Mimic® 240 SC, Dow AgroSciences, Jacareí/SP, Brazil) 240 g L^{-1} was diluted in 1 mL water to produce a stock solution by adjusting 1 g L^{-1} to obtain the required concentrations.

2.3. Toxicity test

Tebufenozide efficacy was determined by calculating lethal concentrations (LC_{25}, LC_{50}, LC_{75} and LC_{90}) under laboratory conditions. Six tebufenozide concentrations besides control (distilled water) were adjusted in 1 mL stock solution (treatments and distilled water): 7.5, 15, 30, 60, 120, and 240 mg L^{-1}. For each treatment, aliquots were taken from the stock solution and mixed with distilled water in 2 mL glass vials. Different concentrations of treatments were applied in 1 μL on 0.25 g food for *A. gemmatalis* individual. Fourth instar larvae fed on diet with different insecticide concentrations were individualized in glass vials (2 × 15 cm) covered with a cotton lid and maintained in the dark. Mortality was assessed for four days. Bioassay had 6 larvae in each treatment and each treatment was replicated six times.

2.4. Survival analysis

The survival of third *A. gemmatalis* instar larvae were evaluated with 30 larvae per treatment. Treated group received artificial diet with different tebufenozide concentrations (LC_{25}, LC_{50}, LC_{75}, LC_{90}) determined by the dose-response bioassay while control group was fed only artificial diet. Larvae were individualized in plastic Petri dishes containing treated and control diet and their survivorship was monitored for 10 days. Survivorship was recorded every 12 h. Insects were recognized as dead when they were unable to move after being prodded with fine hair brush.

2.5. Behavior response

Larvae of *A. gemmatalis* were individually transferred to an open Petri dish arena (Ø 90 mm × 15 mm) lined with a filter paper (Whatman No. 1); the inner walls were covered with Teflon® polytetrafluoroethylene (PTFE) (Dupont, Wilmington, DE, USA) to prevent insect escape. The behavioral bioassays were carried out in arenas half-treated with tebufenozide dissolved in distilled water (control treatments were treated with distilled water only). Filter papers were sprayed with the insecticide (applied as 250 μL solution with the estimated LC_{50} and LC_{90} values) and placed on Petri dishes. One *A. gemmatalis* larva was left for 10 min in insecticide-treated arena, where they were released in its center. Twenty larvae (and arenas) were used for each compound following a completely randomized design. The movement of each insect within the arena during 10 min was recorded using a Canon® NTSC video camcorder (XL1 3CCD; Canon USA, Lake Success, NY) equipped with a 16× video lens (zoom XL 5.5–88 mm) and digitally transferred to a computer for subsequent analysis using a video tracking system (ViewPoint LifeSciences, Montreal, Quebec, Canada). The measurements taken with the tracking system included distance walked and resting time spent in each half of the arena.

The insects spending less than 1 s on the insecticide-treated half of the arena were considered repelled, while the ones remaining less than 50% of the time on such treated half were considered irritated. No insect mortality occurred during the behavioral bioassay.

2.6. Respirometry bioassay

Respirometry bioassays were conducted 24 h against the control and treated larvae with tebufenozide, as detailed. The LC_{50} tebufenozide concentration was used and control had only distilled water. Carbon dioxide (CO_2) (μL of CO_2 h^{-1}/insect) was measured with a CO_2 Analyzer TR_3C respirometer (Sable System International, Las Vegas, USA) with methodology adapted from previous studies (Plata-Rueda et al., 2017). Each respirometer (25 mL) had three *A. gemmatalis* larvae, connected to a closed system. CO_2 production was measured after the insects were acclimated in the chambers for 12 h at 27 ± 2 °C. Compressed oxygen gas (99.99% pure) was passed through a 100 mL min^{-1} flow chamber for two minutes to quantify the CO_2 per chamber. This airflow forces all CO_2 molecules produced to pass through an infrared reader coupled to the system making a continuous measurement of the CO_2 produced by the insects and held inside each chamber. After CO_2 measurement, the insects were removed from the chambers and weighed using an analytical balance (Sartorius BP 210D, Göttingen, Germany). Respiration rate values were not normalized per individual body mass because this method masks the individual variable effects (Hayes, 2001). Five replications were used per treatment.

2.7. Histopathology

Anticarsia gemmatalis larvae were exposed to LC_{50} lethal concentration of tebufenozide extract for 24, 48, 72 and 96 h. Five larvae from both treatment and control were collected and dissected in saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4). The samples were transferred to Zamboni's fixative solution (Stefanini et al., 1967) for 12 h at 5 °C. Samples were then dehydrated in a graded ethanol series (70, 80, 90 and 95%), embedded in historesin Leica (Leica Biosystems Nussloch GmbH, Heildelberger, Germany) and sectioned at 3 μm thickness in a Leica RM2255 microtome. Sections were stained with hematoxylin and eosin and analyzed using a Leica DMLS light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.8. Cytotoxicity

Anticarsia gemmatalis larvae were exposed to LC_{50} lethal concentration of tebufenozide for 24 h. The midgut of five *A. gemmatalis* larvae per treatment and the control were dissected and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 containing 0.2 M sucrose for 4 h at room temperature. Midgut fragments were post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, followed by washing in the buffer and dehydration in a graded ethanol series (70, 80, 90 and 99%). Samples were embedded in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA) and ultrathin sections (70–90 nm) were obtained using a glass knife in a Sorvall MT2-BMT2-B ultramicrotome (Sorvall Instruments, Wilmington, DE, USA). Sections were stained with 1% aqueous uranyl acetate and lead citrateand examined with a Zeiss EM 109 transmission electron microscope (Carl Zeiss, Jena, Germany).

2.9. Cell death

Third instar *Anticarsia gemmatalis* larvae were exposed to LC_{50} tebufenozide concentration for 24 and 48 h. Five midguts from each treatment and control were dissected in insect saline solution and transferred to fixative solution (0.1% Glutaraldehyde, 10% Formaldehyde, 1% $Cacl_2$ in 0.2 M sodium cacodylate buffer with pH 7.2) for 30 min following 10 s pulse in microwave oven. Samples were then washed in 0.1 M sodium phosphate buffer pH 7.2 plus 1% Tween-20 (PBST) for 40 min, incubated for 12 h in the primary antibodies: anti-cleaved-caspase-3 (1:500) (Trevigen Cat# 2305-PC-100, Temporary Antibody Registry: AB_2665453) in PBST for apoptosis detection. The samples were then washed in PBST and incubated for 12 h with a secondary antibody anti-rabbit IgG FITC-conjugated (1:500) (Sigma-Aldrich Cat# F0382, Antibody Registry: AB_259384). The whole midguts were mounted in slides and analyzed with Zeiss confocal microscope LSM 510 (Carl Zeiss) in fluorescence mode, at the Nucleus of Microscopy and Microanalysis at the Federal University of Vicosa (NMM/UFV).

2.10. Statistics

Lethal concentration (LC_{25}, LC_{50}, LC_{75} and LC_{90}) and their confidence limits were determined by logistic regression in dose-response assays based on the concentration Probit-mortality using SAS user (v.9.0) program for Windows. Survival bioassay was subjected to survival analysis using Kaplan–Meier estimator (Log-rank method) with Origin Pro (v.9.0) software (OriginLab Corporation, Northampom/MA, USA). The larvae surviving until the experiment end and emerged pupae were treated as censored data. Behavior response data were analyzed by one-way ANOVA and a Tukey's Honestly Significant test (HSD) was also used for comparisons of the means in the bioassays at 5% significance level. Respiration rate were subjected to two-way analyses of variance (time × insecticide treatment) and Tukey's HSD test ($P < 0.05$) when appropriate (PROC GLM). The time interval is not pseudoreplicates in time because they were assessed in different insect samples. Therefore, they were subjected to regular two-way analysis of variance instead of repeated measures analysis of variance using SAS User software.

3. Results

3.1. Toxicity

Bioassay technique used (treatment of artificial diet) and Probit analysis, showed tebufenozide toxicity against *A. gemmatalis*, determined from bioassays in laboratory condition. Larval mortality increased with tebufenozide concentration in the artificial diet with highest value obtained with 12.16 mg L^{-1} of this insecticide. Increase in mortality, decrease in food consumption, larval abnormalities after feeding on diet with different concentrations of tebufenozide were observed. Four lethal concentration levels LC_{25}, LC_{50}, LC_{75} and LC_{90} estimated by Probit were $LC_{25} = 2.17$ mg L^{-1}, $LC_{50} = 3.86$ mg L^{-1}, $LC_{75} = 6.85$ mg L^{-1} and $LC_{90} = 12.16$ mg L^{-1}, respectively, ($X^2 = 19.57$; df = 5; $P < 0.001$) (Table 1, Supplemental Fig. 1). Mortality was always <1% in the control.

Table 1
Lethal tebufenozide concentrations to *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae after 96 h exposure. X^2, chi squared value for the lethal concentrations and fiducial limits based on a log scale with significance level at $P < 0.001$.

Concentration (LC)	Estimated value (mg L^{-1})	Confidence limits		X^2
		Inferior	Superior	
LC_{25}	2.17	1.62	2.64	19.57
LC_{50}	3.86	3.28	4.41	
LC_{75}	6.85	5.97	8.16	
LC_{90}	12.16	9.88	16.59	

3.2. Survival analysis

Larvae fed on artificial diet treated at LC_{25}, LC_{50}, LC_{75} and LC_{90} tebufenozide concentrations showed different survival curves at different evaluated time (Supplemental Fig. 2). Survival analysis of data from *A. gemmatalis* larvae exposed to different lethal concentrations of tebufenozide indicated differences (Log-rank test, $\chi^2 = 38.05$, df = 4, $P < 0.001$). Survivorship was 100% in control, decreasing to 32.92% with LC_{25}, 27.16% with LC_{50}, 13.71% with LC_{75} and 3.85% with LC_{90}.

The LC_{50}, LC_{75} and LC_{90} tebufenozide concentrations induced intoxication symptoms in larvae, such as progressive paralysis and reduced food consumption along with slipping down of head capsule with 48 h after treatment (HAT), revealing double head capsules.

3.3. Behavior response

Representative walking tracks of *A. gemmatalis* larvae, when released on half treated arenas are exhibited in supplemental Fig. 3. It was observed that larvae tend to remain in untreated areas, however, no significant difference was observed in repellency. Equal walking response was observed in both LC50 concentrations and control group. High velocity walking with relentless movement was observed in LC_{90} concentrations as compared to other groups.

The distance traveled in control was equal in comparison with tebufenozide exposure with LC_{50} and LC_{90} concentrations. We also observed that, resting period was longer in control than LC_{50} and LC_{90} treatments. The distance traveled by *A. gemmatalis* was similar with insecticide treatments (LC_{50} and LC_{90}) in comparison with control ($F_{2,59} = 0.70$, $P = 0.802$ (Fig. 1a). With respect to the resting time, a variation on adult behavioral was observed in *A. gemmatalis* when exposure to tebufenozide ($F_{2,59} = 1.20$, $P < 0.001$) (Fig. 1b).

3.4. Respiration rate

The respiration rate of larvae treated with LC_{50} and LC_{90} concentrations was different from control group. Respiration rate was observed between 1 and 3 h for both treated and control larvae. Maximum decrease in respiration rate was observed in LC_{90} following with LC_{50} treated larvae (Fig. 2).

3.5. Histology

Morphological organization of the midgut epithelium in larvae was a monolayer of digestive and goblet cells (Fig. 3a). Goblet cells were characterized by the presence of an extracellular cavity that was partially connected to the lumen from a specific position along the axial length. Analysis of various sections showed well developed striated border (Fig. 3a). The single layered epithelium of digestive and goblet cells showed elongated nucleus with decondensed chromatin and cytoplasm with few vacuoles (Fig. 3a). A well-developed continuous layer of circular and longitudinal muscles supported the midgut along its whole extension (Fig. 3a).

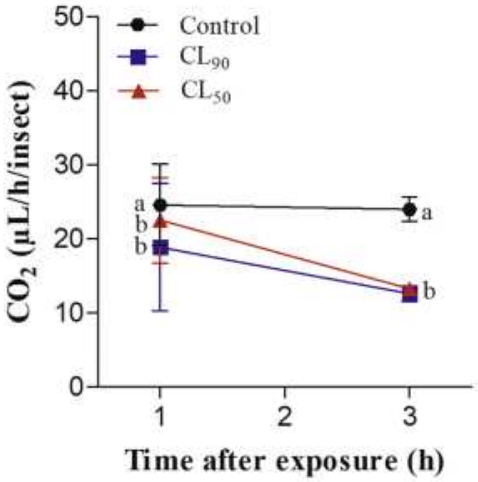

Fig. 2. Respiration rate of *Anticarsia gemmatalis* after exposed to LC_{50} and LC_{90} concentrations of tebufenozide on larvae. Lines followed by different letters differ at $P < 0.05$ (Tukey's mean separation test). Vertical bars represent the standard error of the mean.

Anticarsia gemmatalis fed on diets treated with LC_{50} tebufenozide concentrations showed morphological changes in the midgut when compared to control larvae. No difference was observed between 48 and 72 HAT. Swollen midgut cells were observed, which were detached from basal membrane at 48 HAT (Fig. 3c); meanwhile in 96 HAT midgut cells were more compact and elongated as compared to other treatments and control (Fig. 3b–d). There was an intensification of effects in a time-dependent way. Substantial destruction of cells in some regions of the midgut epithelium was found after four days, along with fragments of cells which were released to the midgut lumen (Fig. 3d). Similar way, less damage to striated border was observed in the midgut after 24 HAT (Fig. 3b) as compared to 96 HAT (Fig. 3d). Interestingly, midgut of treated group larvae of 96 HAT had fewer vacuoles (Fig. 3d) than 24, 48 HAT and control group (Fig. 3a–c).

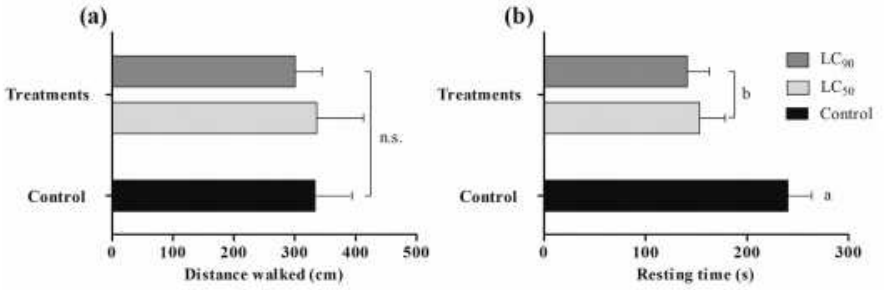

Fig. 1. Distance walked and resting time (Means ± SD) of *Anticarsia gemmatalis* larvae exposed to level LC_{50} and LC_{90} tebufenozide concentrations. (a) Distance walked (b) Resting time. Bars followed by different letters differ at $P < 0.05$ (Tukey's mean separation test). n.s. – non significant.

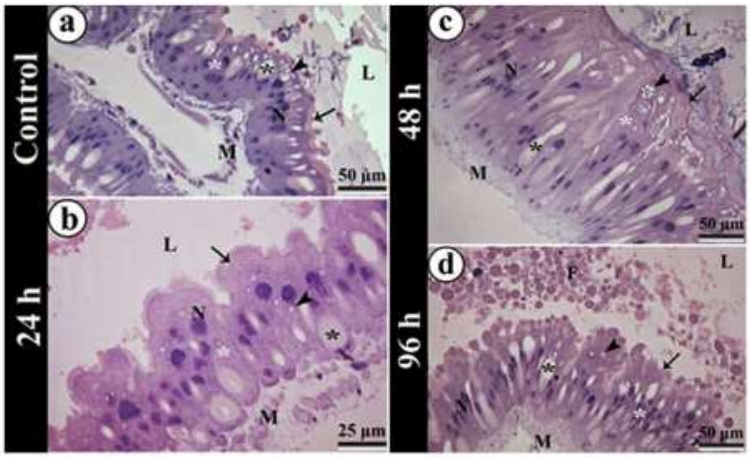

Fig. 3. Histological sections of *Anticarsia gemmatalis* midgut. **[a]**: control larvae showing epithelium with digestive cells (white asterisk) with well-developed brush border (arrow) and nucleus (N). Note the presence of goblet cells (black asterisk), abundance of vacuoles (arrow head), midgut lumen (L) and the longitudinal and circular muscles (M). **[b]**: Larva after 24 h fed on diet with LC_{50} of tebufenozide showing digestive cell with nucleus (N), goblet cell (black asterisk) and digestive cell (white asterisk). Note the lose attachment of brush border (arrow) and vacuoles (arrowhead). **[c]**: Larva after 48 h fed on diet with LC_{50} of tebufenozide showing abundance of vacuoles (arrowhead) in the digestive cells (white asterisk), goblet cells (black asterisk), nucleus (N) and longitudinal muscles (M). **[d]**: Larva after 96 h fed on diet with LC_{50} of tebufenozide showing digestive (white asterisk), goblet cells (black asterisk), nucleus (N), muscles (M). Note the damaged brush border (arrow) cell with few vacuoles (arrowhead) and abundance of cell fragments (F) released in the midgut lumen (L).

3.6. Ultrastructure

Upon high magnification analysis of *A. gemmatalis* midgut, it was found that apical region of digestive cells had long microvilli (Supplemental fig. 4a). The well-developed nucleus showed decondensed chromatin and nucleolus (Supplemental Figs. 4a and 4b). The perinuclear cytoplasm was rich in mitochondria which were ovoid or bean-shaped (Supplemental fig. 4c), rough endoplasmic reticulum (Supplemental fig. 4f), electron-dense granules (Supplemental Figs. 4b and 4d) and glycogen granules (Supplemental fig 4e). Basal cell region had prominent and enlarged plasma membrane infoldings.

The goblet cells in midgut of *A. gemmatalis* had a dense array of microvilli into the cavity (Supplemental fig. 5a). Into the microvilli there were elongated mitochondria (Supplemental fig. 5b). The nucleus had predominance of decondensed chromatin and the basal cell region had many plasma membrane infoldings forming and enlarged basal labyrinth (Supplemental fig. 5c).

Larvae fed on LC_{50} tebufenozide showed midgut digestive cells different from control. Apical surface revealed the presence of cell protrusions with different electron-densities, sometimes in close contact with luminal peritrophic matrix, which were released in lumen with microvilli disorganization throughout the cell (Fig. 4a and b). Nucleus had some crumpled chromatins but well-developed nucleolus (Fig. 4b and c). The electron-dense granules in the cytoplasm showed some degree of fusion resulting in big ones (Fig. 4a, b, 5b). Mitochondria were modified as nanotunnels (Figs. 4c and 5d). In these cells there were many autophagic vacuoles (Fig. 5a) with membranous content (5d) and organelles debris (5e, 5f). The basal plasma membrane was highly enfolded, forming irregular basal labyrinth (Fig. 5c).

In these tebufenozide treated larvae, goblet cells presented overall disorganization of microvilli with absence of mitochondria and cell cavity filled with flocculent content (Fig. 6).

3.7. Immunofluorescence

The overall intensity labeling was assessed for cleaved-caspase-3 in midgut of LC_{50} treated *Anticarsia gemmatalis* for 24 and 48 h to determine damage in the internal organs. Midgut cells cleaved-caspase-3 positive were found in both treatments of 24 h and 48 h as compared to control, which was negative (Supplemental Fig. 6).

4. Discussion

Larvae of *A. gemmatalis* exposed to tebufenozide by ingestion showed histological and ultrastructural alterations in the midgut that varied in intensity according to lethal concentration. Increase in mortality with different tebufenozide concentrations observed in *A. gemmatalis* were consistent with the effects of this insecticide in field populations against lepidopteron pest as *Grapholita molesta* Busck (Tortricidae), that caused up to 95% mortality (Silva et al., 2018), *Diatraea grandiosella* Dyar (Crambidae) (Trisyono and Chippendale, 1997), and *Spodoptera exempta* Walker (Noctuidae) (Smagghe and Degheele, 1994a; b). In this study, the best results were obtained with $LC_{50} = 3.86$ mg mL^{-1} and $LC_{90} = 12.16$ mg mL^{-1} demonstrates the effectiveness in low quantity of tebufenozide.

Survival analysis indicated that a significant proportion of variation in *A. gemmatalis* survival during our trials could be attributed to slow-action mode of tebufenozide. In particular, this insecticide required extended periods of time to achieve the mortality of *A. gemmatalis*. Possible explanation of longer lethal time and lower insecticidal potency might be the sublethal doses applied against this larva and its unique mode of action. Tebufenozide was less toxic than other insecticides used in integrated pest management and anti-resistance programs as compared to spinosad (5 µg/g of insect), thiamethoxam (2 µg/g of insect), and indoxacarb (0.6 µg/g of insect) (Maiza et al., 2013; Martínez et al., 2014).

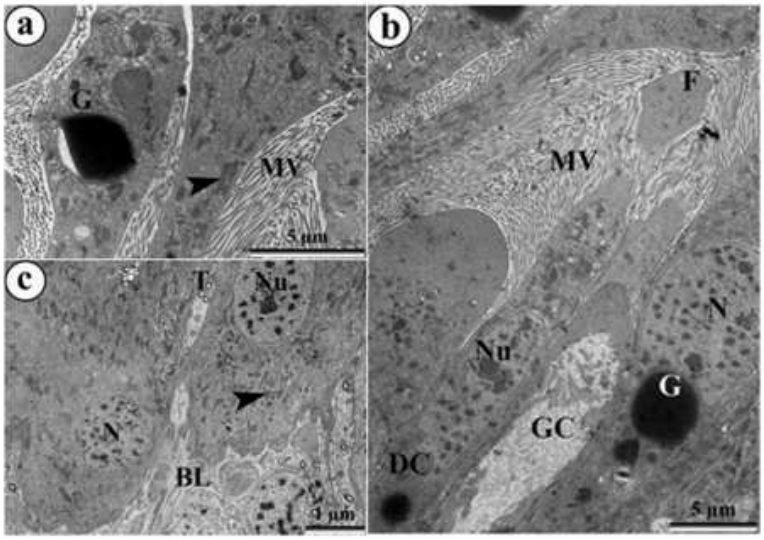

Fig. 4. Transmission electron micrographs of the midgut digestive cells of *Anticarsia gemmatalis* larvae fed on LC_{50} tebufenozide. **[a]**: General view showing apical region with disorganized microvilli (MV), large electron-dense granule (G) and mitochondria (arrowhead). **[b]**: General view of digestive cells (DC) showing disorganized microvilli (MV), nucleus (N) with nucleolus (Nu) and large electron-dense granule (G). Note the presence of cell fragment (F) released to the lumen. GC - goblet cell. **[c]**: Basal cell region showing nucleus (N), nucleolus (Nu), many mitochondria (arrowhead). T – trachea.

Our findings indicate that tebufenozide might have high impact on *A. gemmatalis* with extended exposure period. Survivorship results of *A. gemmatalis* is supported by those with insect growth regulators (pymetrozine, lufenuron, and pyriproxyfen) which caused moderate (≤35%) mortality to third-instar *Ferrisia dasylirii* (Cockerell) (Hemiptera: Pseudococcidae) nymphs after three days (Barbosa et al., 2018).

Sublethal effects of tebufenozide affect walking activity and food consumption of *A. gemmatalis*. Trisyono and Chippendale (1997) stated that tebufenozide inhibits larval growth of European corn borer, delays its pupation and decreases adult emergence. Lack of tebufenozide repellence to *A. gemmatalis* larvae made it potential insecticide. High doses of tebufenozide ingestion displayed rapid neurotoxic effects with hyperactivity and irritability (Corderiro et al., 2010). *Cydia pomonella* (Tortricidae: Lepidoptera) larvae treated with tebufenozide showed suppress feeding and weight gain reduction indicating larval stress (Smagghe et al., 2004). We cannot ensure increase enzyme activity after ingestion of tebufenozide, however, it is proved that intoxication changes the normal patterns of the abdominal intersegmental muscles contractions which are involved in circulation and respiration in insects (Slama and Miller, 1987; Slama, 1987). Neurotoxic activity may explain possible symptoms of such poisoning by blocking neuronal voltage dependent K^+ channels in muscles and nerves (Salgado, 1998). Similar results were found for *Blatta orientalis* Linnaeus (Blattodea: Blattidae) exposed to tebufenozide (Smagghe et al., 1996).

Respirometry results show that larvae treated with tebufenozide have lower respiration rate than control ones, which demonstrates that treated larvae have lower metabolic rate than untreated. Body mass and respiratory rate of insects represent sum of energy demands for physiological processes of insects which produce defense mechanisms against plant toxic compounds (Guedes et al., 2006; Correa et al., 2015; Plata-Rueda et al., 2017; Fiaz et al., 2018). Respiration is indirectly linked to the Krebs cycle (Glazier, 2015) suggesting the hypothesis that low respiratory rate could be the compromising activity of tebufenozide on Krebs cycle. Lower gas exchange cycles in insects are thought to be a water conserving mechanism (Kestler, 1980, 1984). This could be the evidence that one of the immediate and externally invisible side effects of tebufenozide was failure in gas exchange. Jõgar et al. (2006) demonstrated that cyclic gas exchange restricts water losses and supports hypothesis that discontinuous gas exchange cycles serve as water conserving mechanisms. According to their hypothesis spiracles are normally open for the shortest time necessary for efficient respiration, presumably to keep water loss from the tracheal system to a minimum. They claim that spiracle closure is the result of sustained contraction of the closer muscle, while opening commonly results from the elasticity of the surrounding tissue when the closer muscle is relaxed. We already demonstrated in present study that *A. gemmatalis* treated with tebufenozide showed paralytic effects with slow movement, which are under central nervous system in insects (Klowden, 2002).

Histopathological investigations show that after *A. gemmatalis* exposure to tebufenozide, adjacent cell groups could be detected indicating low morphological damage supporting survivorship results of having low mortality before 72 h. According to Lehane (1997), peritrophic matrix acts as barrier protecting midgut epithelium against mechanical and chemical damages. Cell fragments casting in to the lumen (Fiaz et al., 2018; Gonçalves et al., 2018) are supported by Gaban et al. (2015) that midgut cells of *A. aegypti* larvae showed intense secretary activity upon treating with new chemistry insecticides. Similar changes in cell morphology were found by Fiaz et al. (2018), when they treated squamocin against *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), like that found for tebufenozide.

A remarkable change in the midgut digestive cells is the occurrence of nanotunnel mitochondria, a morphological damage caused by tebufenozide. Nanotunnels may indicate normal or moderately impaired mitochondrial respiratory chain function

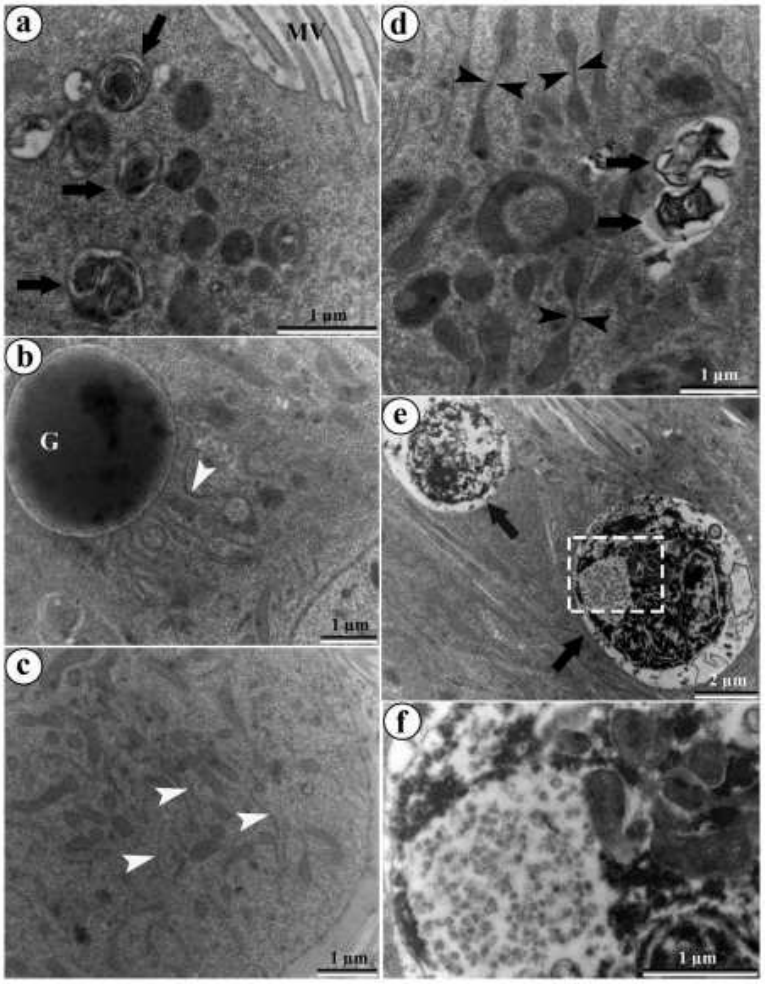

Fig. 5. Transmission electron micrographs of the midgut digestive cells of *Anticarsia gemmatalis* larvae fed on LC_{50} tebufenozide. **[a]:** Apical region showing many autophagic vacuoles (arrows) and microvilli (MV). **[b], [c]:** Median and basal region of the cell showing disorganized profiles of endoplasmic reticulum (white arrow head). Note the presence of a large granule (G) in median region. **[d]:** Median region of the cell and with autophagic vacuoles (arrow) and many mitochondrial nanotunnels (black arrowheads). **[e]:** Apical region with autophagic vacuoles (arrows). **[f]:** High magnification of dashed square at figure **e** showing autophagic vacuole filled with organelles debris.

(Vincent et al., 2016). Mitochondrial functions are fundamentally linked to their morphology and membrane ultrastructure. Decrease in respiration rate caused by the tebufenozide found here may be linked with mitochondrial damage, resulted in nanotunneling.

The increase in amount of autophagic vacuoles in midgut digestive cells of *A. gemmatalis* larvae treated with tebufenozide indicates that these cells undergo cytoplasmic reorganization (Alberts et al., 2014). Autophagy is a major proteolytic system for delivering cytoplasmic constituents to lysosome for degradation with the help of hydrolytic enzymes, which has a key role in cellular energy mobilization and homeostasis by clearing damaged organelles, aggregated proteins and pathogens (Levine and Kroemer, 2008). Stresses, such as nutrient or energy depletion, oxidative stress, hypoxia, mitochondrial damage, DNA damage, damaged organelles or pathogen infection can activate autophagy pathway (Kroemer et al., 2010). *A. aegypti* larvae (Costa el al., 2017) and A. gemmatalis larvae (Fiaz et al., 2018) exposed to squamocin have cellular detoxification by autophagy which seem to occur in this study.

Midgut cells cleaved-caspase-3 positive proves the occurrence of apoptosis in these cells of *Anticarsia gemmatalis* mediated by Tebufenozide, likely found in the honey bee *Apis mellifera* with Imidacloprid which induced apoptosis in the midgut cells (Rossi et al., 2013). Apoptosis is a fundamental feature of many important biological processes (Enzo and Davide, 2016) devoted to various biological processes such as development, tissue homeostasis, DNA damage response (Amundson et al., 1998) and immune response (Creagh et al., 2003). Possible explanation for apoptosis in midgut cells here observed might be because these cells cannot recover the damage caused by tebufenozide leading to the activation of apoptotic pathway, such as reported in midgut of *Spodoptera littoralis* (Lepidoptera: Noctuidae) treated with Spinosad

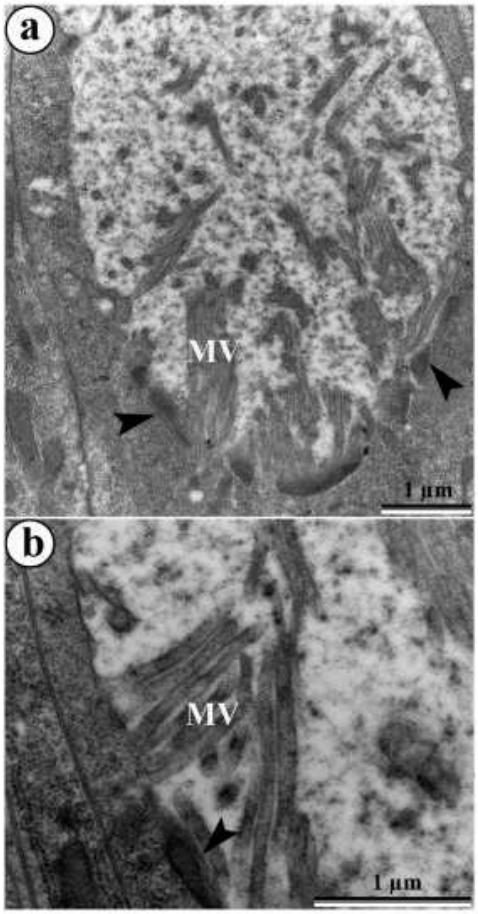

Fig. 6. Transmission electron micrographs of goblet cells in the midgut of *Anticarsia gemmatalis* fed on LC_{50} tebufenozide. **[a]**: General aspects showing cell cavity filled with flocculent material and disorganization of microvilli (MV). Note the absence of adjacent mitochondria (arrowhead) into the microvilli (MV). **[b]**: Detailing cell cavity and showing disorganized microvilli (MV) without mitochondria (arrowhead).

(Abouelghar et al., 2013), *Spodoptera exigua* treated with sublethal concentrations of Vip3 proteins (Hernández-Martínez et al., 2017) and *A. mellifera* (Hymenoptera: Apidae) treated with Spinosad (Lopes et al., 2018).

Overall, our toxicological data confirms that tebufenozide is highly efficacious against *A. gemmatalis*. Addition to have adverse effects on *A. gemmatalis* survivability, tebufenozide also change behavioral response. The tebufenozide activity alters midgut digestive cells mainly their brush border, vacuolation, fragment release into midgut lumen, mitochondria morphology, which results in cell death indicating that this compound can contribute to the enhancement of *A. gemmatalis* control.

Acknowledgments

We gratefully acknowledge The World Academy of Sciences TWAS (Italy), Conselho Nacional de Desenvolvimento Científico e Tecnológico CNPq (Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES (Brazil), Fundação de Amparo a Pesquisa do Estado de Minas Gerais FAPEMIG (Brazil) and Núcleo de Microscopia e Microanálise of Universidade Federal de Viçosa.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.08.088.

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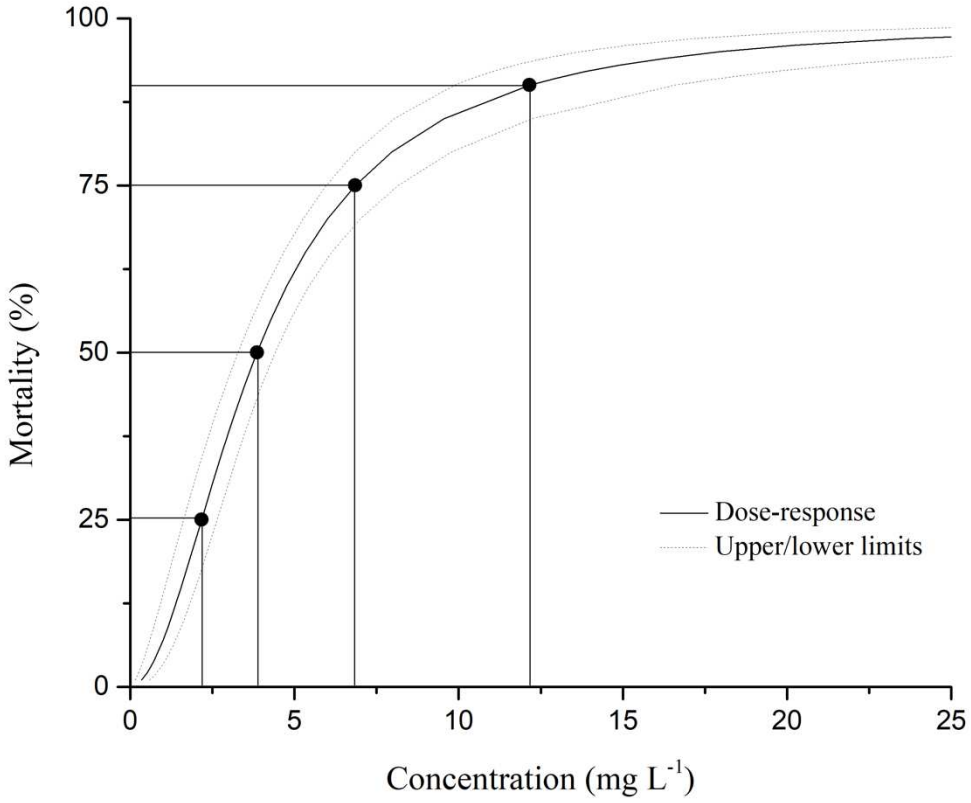
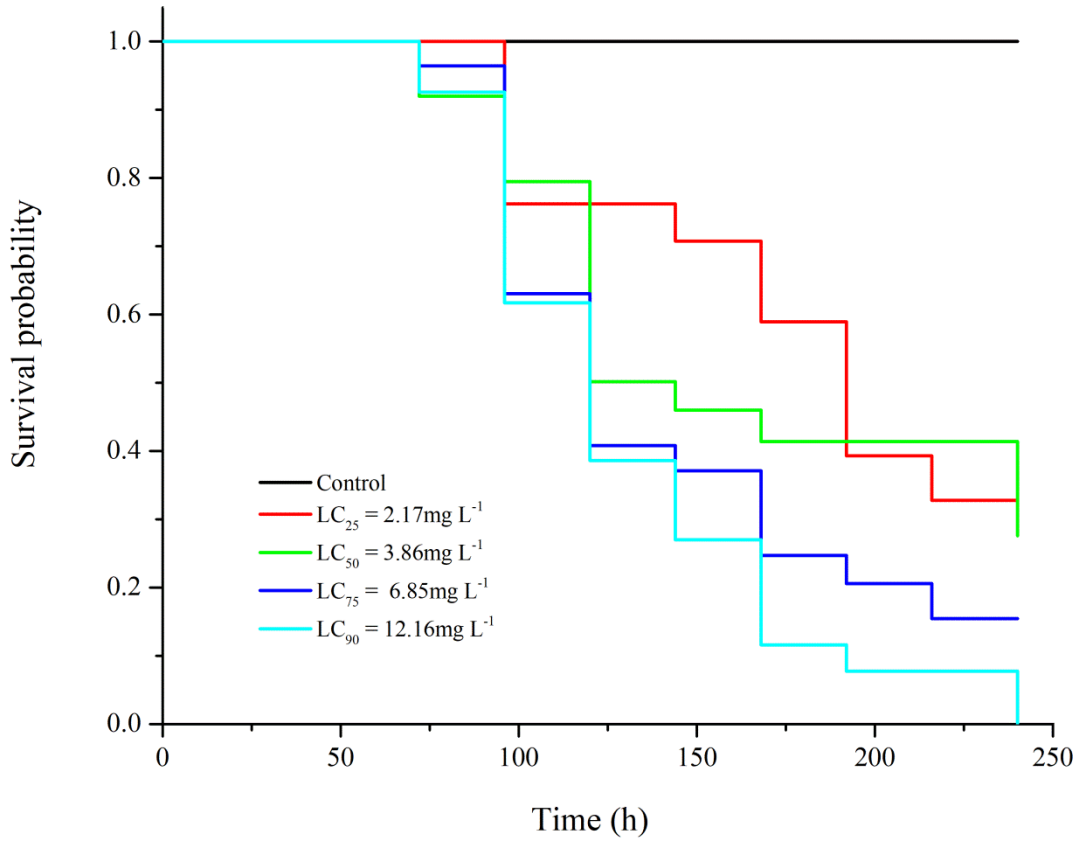
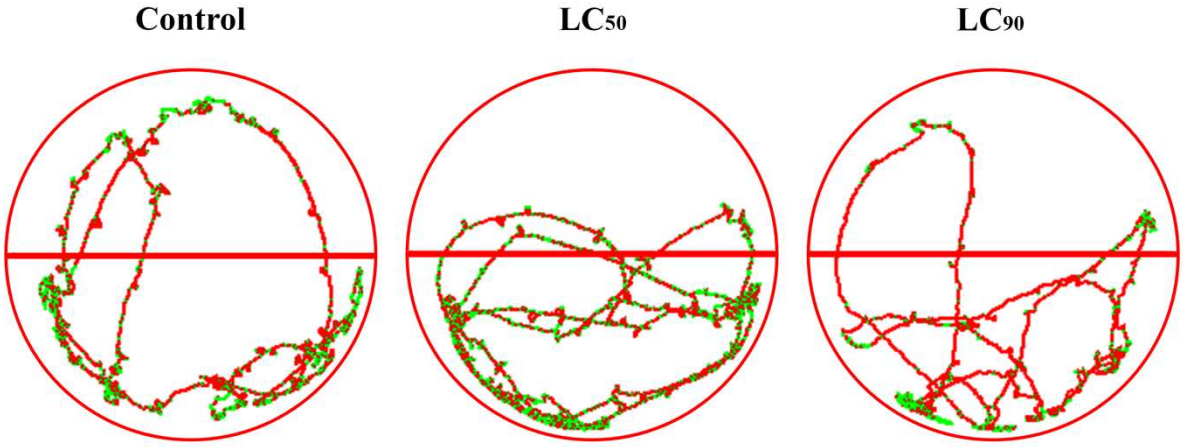
Supplemental figures



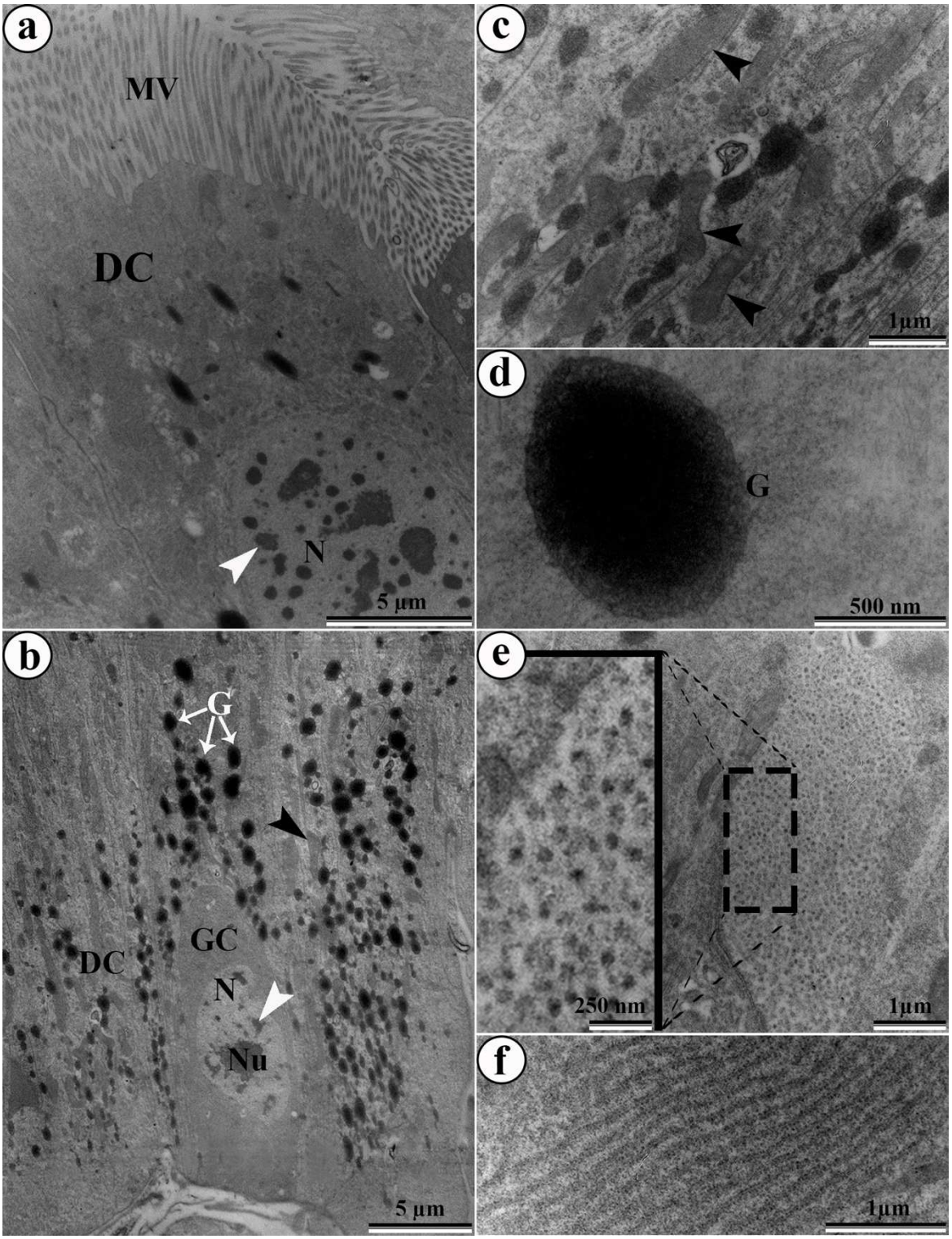
Supplemental figure 1. Larval mortality of *Anticarsia gemmatalis* caused by tebufenozide when fed with artificial diet treated at different concentrations (CL_{50} and CL_{90}) (X^2 = 19.57; df = 5; P <0.001). Lines denote 95% confidence intervals. Black point represents LC_{50} concentration selected to evaluate histological and ultrastructural changes.



Supplemental figure 2. Survivorship curves of *Anticarsia gemmatalis* larvae exposed at different lethal concentrations using the Kaplan-Meier method and compared using the log-rank test (X^2 = 38.05; P< 0.001).

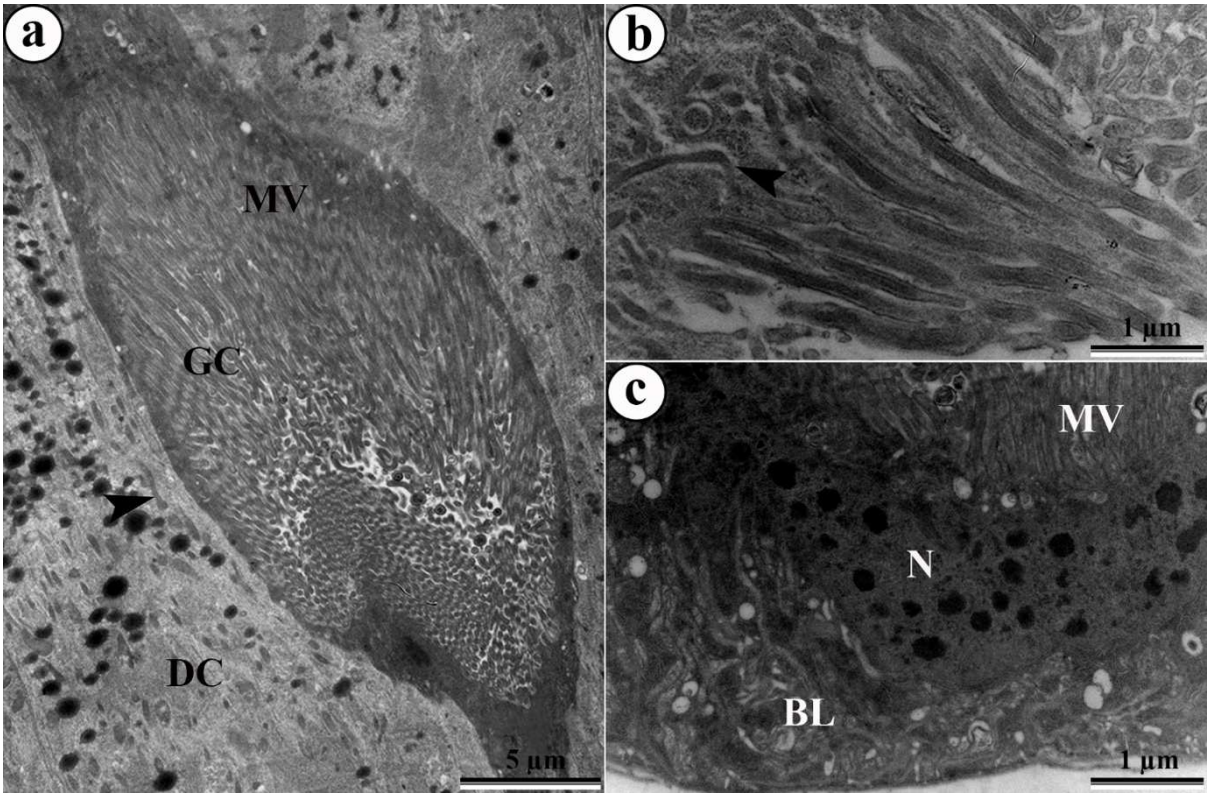


Supplemental figure 3. Representative tracks showing the walking activity of *Anticarsia gemmatalis*, over a 10-min period on paper-filter arenas (9 cm diameter) half impregnated with tebufenozide (upper half of each arena). Red tracks indicate high walking velocity; green tracks indicate low (initial) velocity.

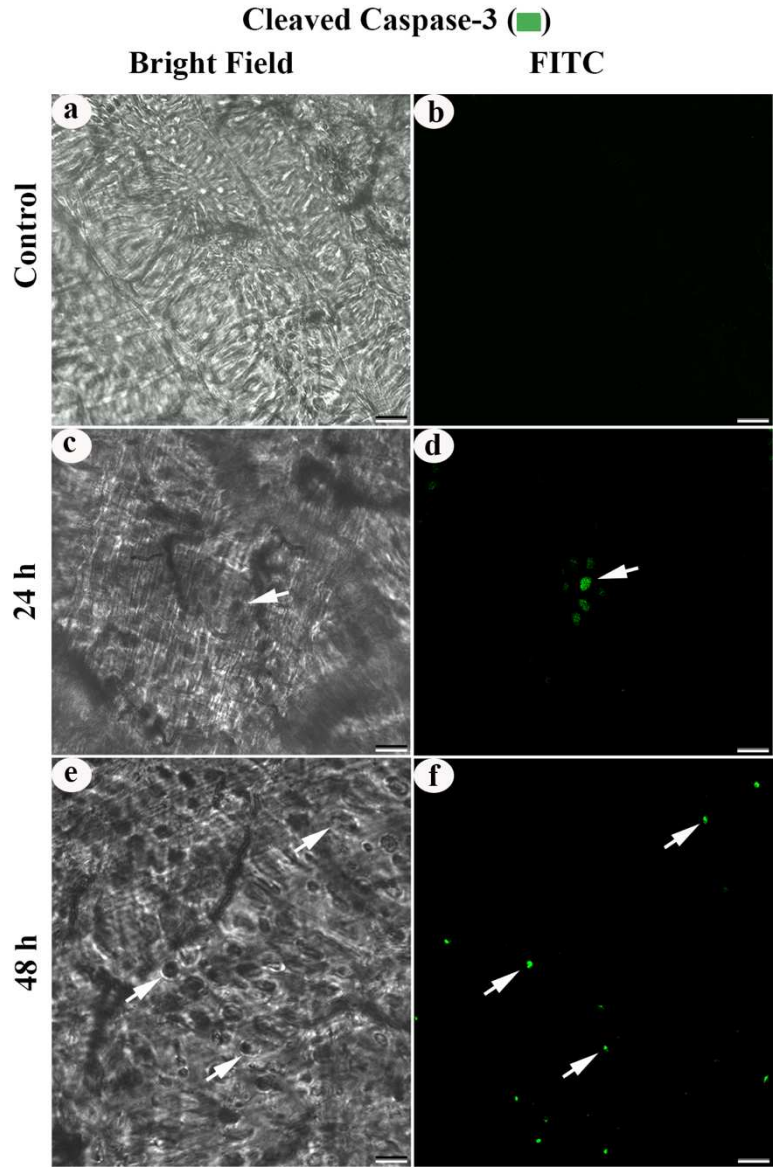


Supplemental figure 4. Transmission electron micrographs of the midgut digestive cells of *Anticarsia gemmatalis* control larvae. [a]: General view of digestive cell (DC). Showing apical region with microvilli (MV). Note nucleus (N) with decondensed chromatin and some cloths of heterochromatin (white arrowhead). [b] General view of digestive cell (DC) with mitochondria (black arrowhead) and electron-dense granules (G). Note well developed nucleus (N) with few crumples of heterochromatin (white arrowhead) and nucleolus (Nu). [c]: Detail

of mitochondria (black arrowhead). [d]: Detail of electron-dense granule (G) in cytoplasm. [e]: Glycogen granules. Insert: magnifying granules of glycogen. [f]: Rough endoplasmic reticulum cisterns.



Supplemental figure 5. Transmission electron micrographs of goblet cells in the midgut of *Anticarsia gemmatalis* control larvae. **[a]**: General aspect of goblet cell (GC) closed to digestive cell (DC) showing internal cavity with organized microvilli (MV) and adjacent cytoplasm with mitochondria (black arrowhead). **[b]:**) Detailing microvilli with mitochondria inside them (black arrowhead). **[c]:** Detailing basal region with well-developed nucleus (N), regular basal labyrinth (BL) and microvilli (MV).



Supplemental figure 6. Immunofluorescence staining for cleaved-3-caspase in the midgut of *Anticarsia gemmatalis* caterpillars. [**a-c-e**]: Bright field images. [**b**]: control larvae showing negative fluorescence. Apoptotic cells. [**d**]: larva after 24 h fed on diet with LC_{50} tebufenozide concentrations showing apoptotic cells (arrow). [**f**]: larva after 48 h fed on diet with LC_{50} tebufenozide concentrations showing apoptotic cells (arrows). Bars=20µm.

FINAL CONSIDERATIONS

Toxicological studies of both squamocin and tebufenozide were evident of having lethal and sublethal effects on *A. gemmatalis* proving these agents to be fundamental in controlling this insect pest. Despite being lethal to *A. gemmatalis* larvae, tebufenozide is harmless to non-lepidopteran and adult beneficial insects. Squamocin has already been proven to be inoffensive for human leukocytes and other beneficial insects (*Culex bigoti*), falling both of these agents in the category of integrated pest management (IPM) program.

Ingestion of both toxic substances induced alterations in midgut cells of *A. gemmatalis.* Those ultrastructural changes induced were dependent of exposure time and concentrations of squamocin as well as tebufenozide. Changes in ultrastructure induced by squamocin and tebufenozide presented apocrine secretions, cell protrusions, disorganization in microvilli and autophagic vacuoles.

Mitochondria in *A. gemmatalis* treated by squamocin and tebufenozide presented molecular and morphological damage, respectively. Molecular damage to mitochondria by squamocin was the evident of low respiration rate in larvae, ascertained by mitotracker bioassay. Tebufenozide had adverse morphological effects on mitochondria by reshaping them in to nano-tunnels ultimately affecting larval respiration rate resulting in death.

Behavioral responses of *A. gemmatalis* proved that tebufenozide was non-repellent to tebufenozide. However, tebufenozide induced irritability in larvae resulted in relentless movement while control larvae exhibited longer resting periods.

Autophagy induced in midgut cells of *A. gemmatalis* proved the apoptosis occurrence in these cells of *A. gemmatalis* mediated by both squamocin and tebufenozide. Apoptosis observed in midgut cells here might be because these cells could not recover the damage caused by squamocin and tebufenozide, leading to the activation of apoptotic pathway.

In summary, this study represents the evaluation of squamocin and tebufenozide toxicity against *A. gemmatalis* and their effects on behavior and midgut cells. The knowledge of ultrastructural and cytochemical features of midgut cells of *A. gemmatalis* enables the understanding of efficacy of action of these substances to control this defoliator pest. Therefore, the implications of these findings may have several applications in toxicology, biochemistry and physiology of insects for betterment of controlling this insect.