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# Neem oil (*Azadirachta indica* A. Juss) affects the ultrastructure of the midgut muscle of *Ceraeochrysa claveri* (Navás, 1911) (Neuroptera: Chrysopidae)

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ABSTRACT

Cytomorphological changes, by means of ultrastructural analyses, have been used to determine the effects of the biopesticide neem oil on the muscle fibers of the midgut of the predator *Ceraeochrysa claveri*. Insects, throughout the larval period, were fed eggs of *Diatraea saccharalis* treated with neem oil at a concentration of 0.5%, 1% or 2%. In the adult stage, the midgut was collected from female insects at two stages of adulthood (newly emerged and at the start of oviposition) and processed for ultrastructural analyses. In the newly emerged insects obtained from neem oil treatments, muscle fibers showed a reduction of myofilaments as well as swollen mitochondria and an accumulation of membranous structures. Muscular fibers responded to those cellular injuries with the initiation of detoxification mechanisms, in which acid phosphatase activity was observed in large vesicles located at the periphery of the muscle fiber. At the start of oviposition in the neem oil treated insects, muscle fibers exhibited signs of degeneration, containing vacant areas in which contractile myofilaments were reduced or completely absent, and an accumulation of myelin structures, a dilatation of cisternae of sarcoplasmic reticulum, and mitochondrial swelling and cristolysis were observed. Enzymatic activity for acid phosphatase was present in large vesicles, indicating that mechanisms of lytic activity during the cell injury were utilized but insufficient for recovery from all the cellular damage. The results indicate that the visceral muscle layer is also the target of action of neem oil, and the cytotoxic effects observed may compromise the function of that organ.


## 1. Introduction

Alternative tools that will help make crop protection more sustainable are currently being evaluated and are providing assistance in integrated pest management (IPM). We emphasize the use of natural products, such as biopesticides and biological control, including predatory insects. Evaluating the integration of non synthetic insecticides and biological control methods is critical to the success of an IPM (Chandler et al., 2011; Cloyd, 2012; El-Wakeil et al., 2013).

A biopesticide is a mass-produced agent manufactured from a living microorganism or a natural product and is sold for the control of plant pests (Bailey et al., 2010; Chandler et al., 2011). Natural products have been identified in plants and can be used as biopesticides. The botanic product that is most widely used is neem oil, an insecticidal chemical extracted from the seeds of the neem tree *Azadirachta indica* A. Juss (Meliaceae). Azadirachtin is the main active ingredient and is responsible for various actions against insects such as delayed growth and reductions in feeding, fecundity, fertility and repellency (Schmutterer, 1990; Mordue (Luntz) et al., 1998; Mordue (Luntz) and Nisbet, 2000; Morgan, 2009; Okwute, 2012; El-Wakeil et al., 2013).

The evaluation of the side effects that may be associated with the direct or indirect exposure of natural enemies to biopesticides is important for the implementation of the correct tactics in IPM. In spite of being widely used, neem products are being assessed, and their compatibility with natural enemies is being questioned (Medina et al., 2003; Aggarwal and Brar, 2006; Cordeiro et al., 2010; El-Wakeil et al., 2013; Scudeler and Santos, 2013; Scudeler et al., 2013, 2014, 2016a).

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One important method that ecotoxicological studies have been using to investigate sublethal effects on natural enemies is via morphological studies of organs that are in contact way during the exposure to the biopesticides. Morphological and ultrastructural changes in this organ can provide information on delayed effects on non-target organisms (Malaspina and Silva-Zacarin, 2006; Scudeler et al., 2016a). Furthermore, this type of analysis contributes to the improved understanding of the mode of action of the biopesticide in the organism.

During exposure via ingestion, the midgut, which is responsible for digestive and absorptive functions, becomes an excellent target organ in ecotoxicological studies (Malaspina and Silva-Zacarin, 2006; Catae et al., 2014; Scudeler et al., 2016a). Cytotoxic effects on epithelial cells of the midgut of the Neotropical green lacewing *Ceraeochrysa claveri* (Navás, 1911) caused by treatment with neem oil have been described for all stages of development of this predator (Scudeler and Santos, 2013; Scudeler et al., 2014; Scudeler et al., 2016a), but studies directed to the muscle layers that externally surrounds this organ are lacking. A network of circular and longitudinal striated type muscle fibers is responsible for distension and contraction, and provides the structural integrity of the digestive tract and peristaltic activity required for the function of this organ (Park and Shahabuddin, 2000; Secundino et al., 2005; Bernick et al., 2008). Loss of muscle tone and a reduction in gut motility after azadirachtin treatments (Mordue (Luntz) et al., 1985; Mordue (Luntz) and Nisbet, 2000) have been described, but the effects at the cellular level are unknown, especially at the ultrastructural level. This lack of knowledge is present in relation to synthetic pesticides or biopesticides, and little attention has been given to the muscle layer during the cytopathological analysis.

In order to check the occurrence of cell damage and a possible attempt recovery thereof, acid phosphatase is an important enzymatic marker which can act in the detoxification processes and muscle regeneration (Maltz and Oron, 1990; Amirmohammadi et al., 2012; Valizadeh et al., 2013).

With recognition of the importance of this tissue in the maintenance of the physiological functions performed by the midgut, we intend to describe the effect of neem oil intake on muscle cells of the predator *C. claveri*, thus contributing to a better understanding of the mechanism of action of this biopesticide on the midgut.

## 2. Materials and methods

### 2.1. Insects

The *C. claveri* larvae (0–12 h old) used in the bioassays were obtained from a colony of the Laboratory of Insects in the Department of Morphology at the Institute of Biosciences of Botucatu, Botucatu, Brazil. The *C. claveri* colony was reared with a natural diet of eggs of *Diatraea saccharalis* (Lepidoptera: Crambidae) during the larval stage and with an artificial diet (1:1 honey/yeast solution) in adulthood. The insects were maintained in an environmental chamber (B.O.D. incubator) with controlled climate conditions (25 ± 1 °C; relative humidity (RH) of 70 ± 10% and a light: dark photoperiod of 12L: 12D). Adults of *D. saccharalis* were provided by the CETMA Comércio de Agentes para Controle Biológico, Lençóis Paulista, São Paulo State, Brazil. The *D. saccharalis* larvae were maintained in the laboratory with an artificial diet under controlled conditions (26 ± 1 °C; 60 ± 10% RH; 14L: 10D).

### 2.2. Bioassays

Newly hatched larvae of *C. claveri* obtained from the stock rearing colony were randomly selected and placed individually in polyethylene boxes (2 cm height × 6 cm diameter). These larvae were exposed to a commercial formulation of neem oil emulsifiable via ingestion (Natuneem®, Natural Rural Ind. e Com. de Produtos Orgânicos e Biológicos Ltda, Araraquara-SP, Brazil) (organic product, certified by BCS OKO – Garantie, Doc. Natur – 9009/09.05/7331-BR), pure cold-pressed neem oil extracted from neem seeds containing 1500 ppm of azadirachtin A. Three concentrations (0.5, 1 and 2%) (v/v) were evaluated using the recommended label rate for its use in Brazilian agricultural fields and concentrations as previously evaluated for larvae, pupae and adults of *C. claveri* (Scudeler and Santos, 2013; Scudeler et al., 2014; Scudeler et al., 2016a).

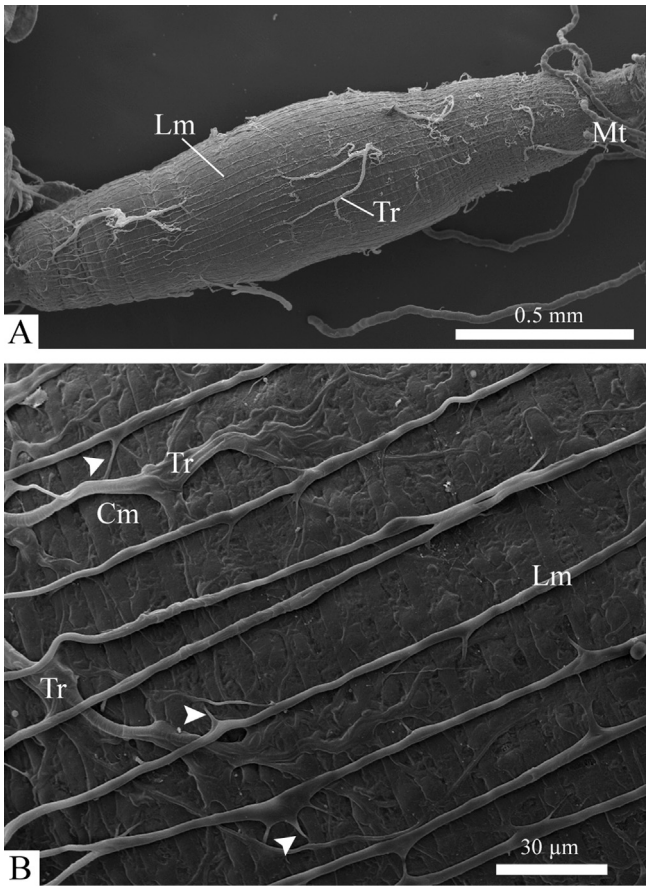


**Fig. 1.** SEM micrograph of the whole midgut of a newly emerged *C. claveri* adult (control). (A) Longitudinal muscle fibers (Lm) are present, and several tracheoles (Tr) running to midgut. Note also the Malphighian tubules (Mt) starting from the pyloric valve. (B) Muscle fibers are forming a muscle network over the midgut. Longitudinal muscle fibers overlap circular fibers (Cm) and tracheoles that insert this organ. Branching longitudinal muscle (*arrowhead*).

Neem oil solutions were prepared from the commercial formulation and were diluted in distilled water to 0.5% (5 mL/L), 1% (10 mL/L), and 2% (20 mL/L); solutions were made daily for use. Fresh egg clusters of *D. saccharalis* were collected and dipped once in neem oil solution for 5 s and air dried at room temperature for 1 h. For the control group, egg clusters were dipped into distilled water (Scudeler and Santos, 2013).

Larvae were randomly divided into four experimental groups (n = 30 per group), and each experimental group comprised five replicates. *C. claveri* larvae of treated groups (0.5, 1 and 2%) were fed with *D. saccharalis* eggs treated with neem oil *ad libitum*. Likewise, the control group was fed with eggs treated with water *ad libitum*. This intake of eggs that had or had not been treated with neem oil occurred throughout the larval period until pupation (on average, 13 days). Due to the sensitivity of the neem oil components to ultraviolet light, egg clusters not consumed were replaced every

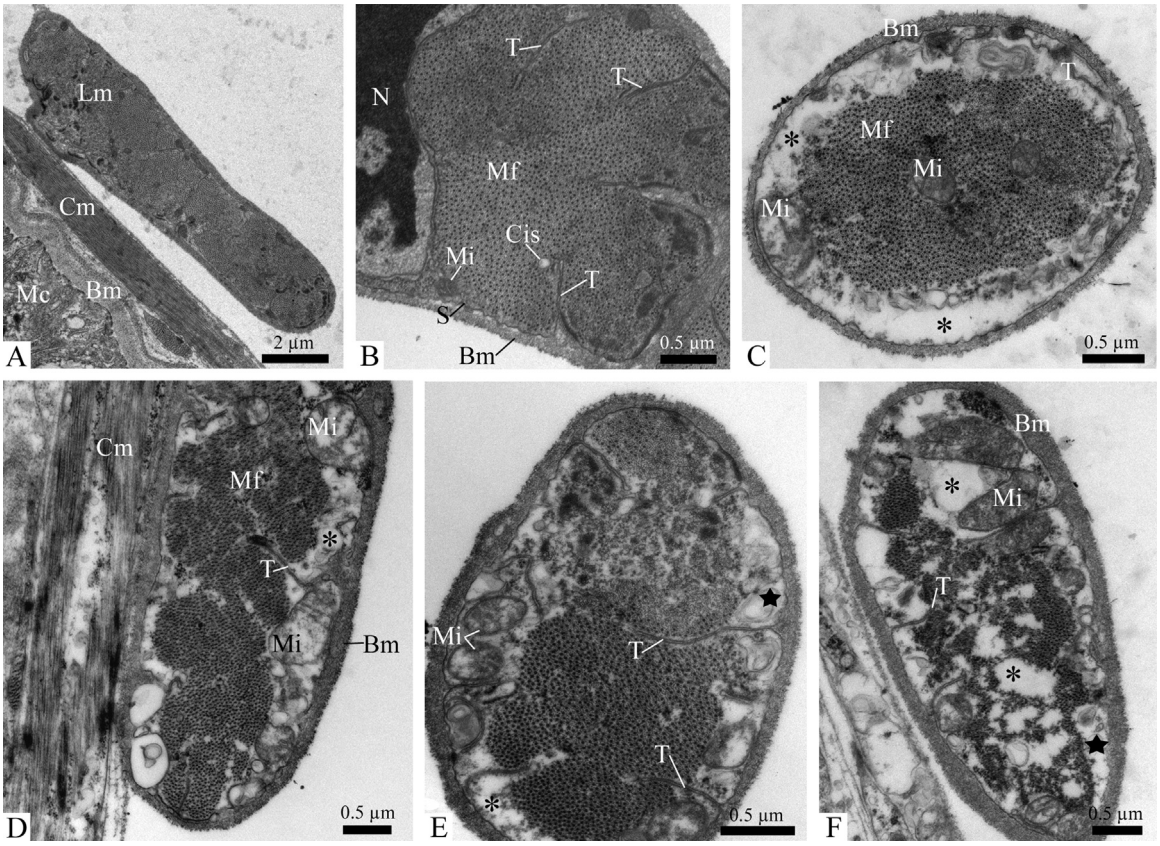


**Fig. 2.** Ultrastructure of *C. claveri* midgut muscle from control and neem oil-treated larvae, shown at the newly emerged stage of adults. (A and B) Control group. (A) Base of midgut epithelial cell (Mc) that is supported by a thick basement membrane (Bm) and is coated by both circular (Cm) and longitudinal (Lm) fibers of the gut musculature. (B) Cross-section through longitudinal muscle fibers showing a defined sarcolemma (S) that is surrounded by a basement membrane (Bm). Narrow invaginations of the sarcolemma form the T-system tubules (T). Cisternae of the sarcoplasmic reticulum (Cis) were observed in association with these tubules of the T-system. Note that T-system tubules divide the fiber incompletely into contractile fields containing myofilaments (Mf). (C) Neem oil 0.5%. (D and E) Neem oil 1%. (F) Neem oil 2%. Note that in the treatment groups, the reduction of myofilaments occurs mainly in the periphery of the contractile fields (*), as well as swollen mitochondria (Mi), and an accumulation of membranous structures (stars) is present. Nucleus (N).

3 days (Scudeler et al., 2016a). According to Schmutterer (1990), the residual effect of neem based products is restricted mostly around five to seven days.

Freshly emerged adults obtained from neem oil-treated larvae (0–12 h old) had the sex determined, and one freshly emerged female from the treatment groups was paired with two freshly emerged control males. This trio of insects was kept in a polyethylene box (9 cm height × 18 cm diameter) until the start the oviposition (on average 17 days). During this period, the adults were fed with an artificial diet (1:1 honey/yeast solution) and a cotton wick with distilled water every 5 days (Scudeler et al., 2016a). The entire experiment was conducted in the same environmental conditions as described for the insect rearing.

### 2.3. Electron microscopy

Adult females at two ages (0–1 day old and first day of oviposition) obtained from the control and treated groups were quickly cryoanesthetized, and their guts were dissected in saline solution for insects (0.1 M NaCl, 0.1 M $Na_2HPO_4$ and 0.1 M $KH_2PO_4$).

#### 2.3.1. Scanning electron microscopy (SEM)

Midguts obtained from adult females (n = 5 per age/group) were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 48 h at room temperature. Thereafter, the samples were washed in distilled water, post-fixed in 1% osmium tetroxide (Sigma Aldrich, St. Louis, Missouri, USA) diluted in distilled water for 30 min, dehydrated through a graded series of ethanol solutions, critical point-dried using liquid $CO_2$ (Leica CPD 030 critical point dryer) and gold-coated on a Baltec SCD 050 sputter coater. The samples were examined and documented using an FEI Quanta 200 scanning electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu.

#### 2.3.2. Transmission electron microscopy (TEM)

Midgut samples from five specimens of adult females of each age and experimental group were fragmented, fixed and processed for transmission electron microscopy as indicated below. All samples were analyzed and photographed in a Tecnai Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands) at the Electron Microscopy Center of the Institute of Biosciences of Botucatu.

**2.3.2.1. Conventional transmission electron microscopy.** The transmission electron microscopy procedures were performed in a manner similar to our previous studies on the *C. claveri* midgut (Scudeler and Santos, 2013; Scudeler et al., 2016a). The midgut fragments were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) solution in 0.1 M phosphate buffer (pH 7.3) for 24 h at room temperature, and then post-fixed in 1% osmium tetroxide in the same buffer for 2 h. After washing several times in distilled water, the samples were contrasted with an aqueous solution of 0.5% uranyl acetate (Electron Microscopy

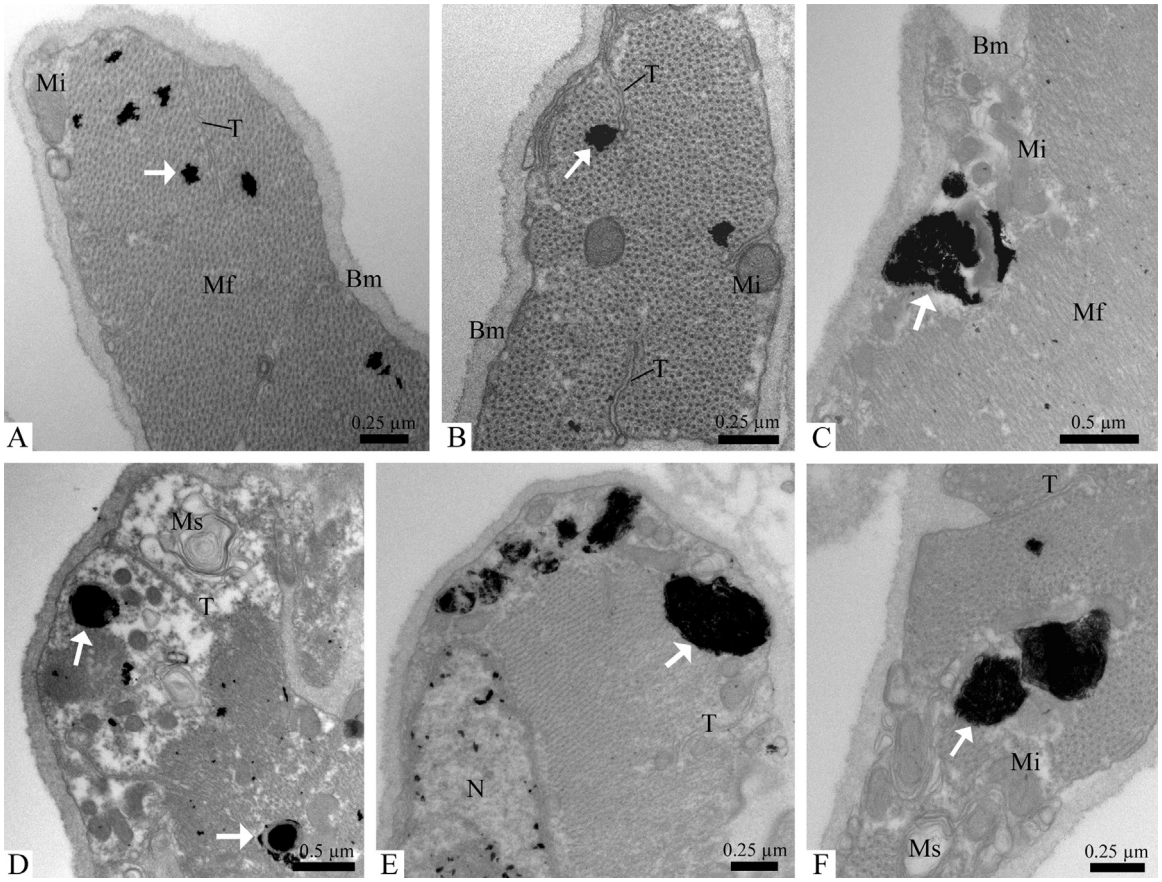


**Fig. 3.** Electron micrograph of *C. claveri* midgut muscle fibers that were incubated for acid phosphatase cytochemistry. These newly emerged adult insects developed from control and neem oil-treated larvae. Note the electron-dense deposits that are stained. (A and B) Control group. Small acid phosphatase-positive structures (*arrows*) are evident near the T-tubules (T) between the myofilaments (Mf). (C and D) Neem oil 0.5%. (E and F) Neem oil 1% and 2%, respectively. Acid phosphatase-positive lysosomes are present in the periphery of the muscle fiber between mitochondria (Mi) and areas that lack myofilaments. The detection of acid phosphatase activity was observed in large vesicles when compared with the control group. Basement membrane (Bm); nucleus (N); myelin structures (Ms).

Sciences, Hatfield, Pennsylvania, USA) for 2 h at room temperature, dehydrated in a graded acetone series, and embedded in Araldite resin (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Ultra-thin sections were contrasted with uranyl acetate and lead citrate (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) and then analyzed in a Tecnai Spirit transmission electron microscope.

**2.3.2.2. Ultrastructural cytochemical analysis.** The activity of acid phosphatase, a lysosomal marker with a possible effect on cellular repair, was examined in the tissues in a manner identical to that used by Scudeler et al. (2016a) in previous experiments on midgut epithelium of *C. claveri*. Midgut fragments were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose for 20 min, washed several times in sodium cacodylate buffer with 5% of sucrose and washed twice in 0.05 M acetate buffer (pH 5.0) with 5% of sucrose. Afterwards, the tissues were incubated in a solution of cytidine-5′-monophosphate (Sigma Aldrich, St. Louis, Missouri, USA) and 1% lead nitrate (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) in acetate buffer (pH 5.0) for 1 h at 37 °C (Pino et al., 1981). Post-fixation was performed in 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2) with 5% sucrose for 45 min and with 1% osmium tetroxide in cacodylate buffer for 1 h. Finally, the samples were dehydrated and embedded following the procedures for conventional analysis. Unstained ultra-thin sections were used for cytochemical analysis. The positive reaction in the cytochemical analysis is observed in the sections as electron-dense regions.

## 3. Results

The outer surface of the adult of *C. claveri* midgut is organized as a grid-like network composed of circular and longitudinal fibers. The longitudinal fibers were always positioned over the circular ones, and this orientation of the fibers was uniform throughout the midgut. The tracheoles that insert into this organ and the small branches of longitudinal fibers that result in the interconnection and fixation of fibers across much of the length of the midgut can be observed in SEM images (Fig. 1A and B).

Ultrastructural analysis of the basal region of the pseudostratified epithelium of freshly emerged *C. claveri* adults revealed that midgut epithelial cells rest on a basement membrane and are wrapped by muscle fiber bundles that form an internal circular tunic and another external longitudinal tunic (Fig. 2A). In the control group, muscle fibers showed a well-defined sarcolemma that was surrounded by a basement membrane. Narrow invaginations of the sarcolemma form the T-system tubules and sarcoplasmic reticulum cisternae were observed in association with these tubules. The nucleus and electron-dense mitochondria were observed in the periphery of the incomplete contractile fields filled by myofilaments that had been formed by invaginations of the T-tubules (Fig. 2B).

In the three neem oil treatment groups, we noticed severe ultrastructural changes. Muscle fibers showed a reduction of

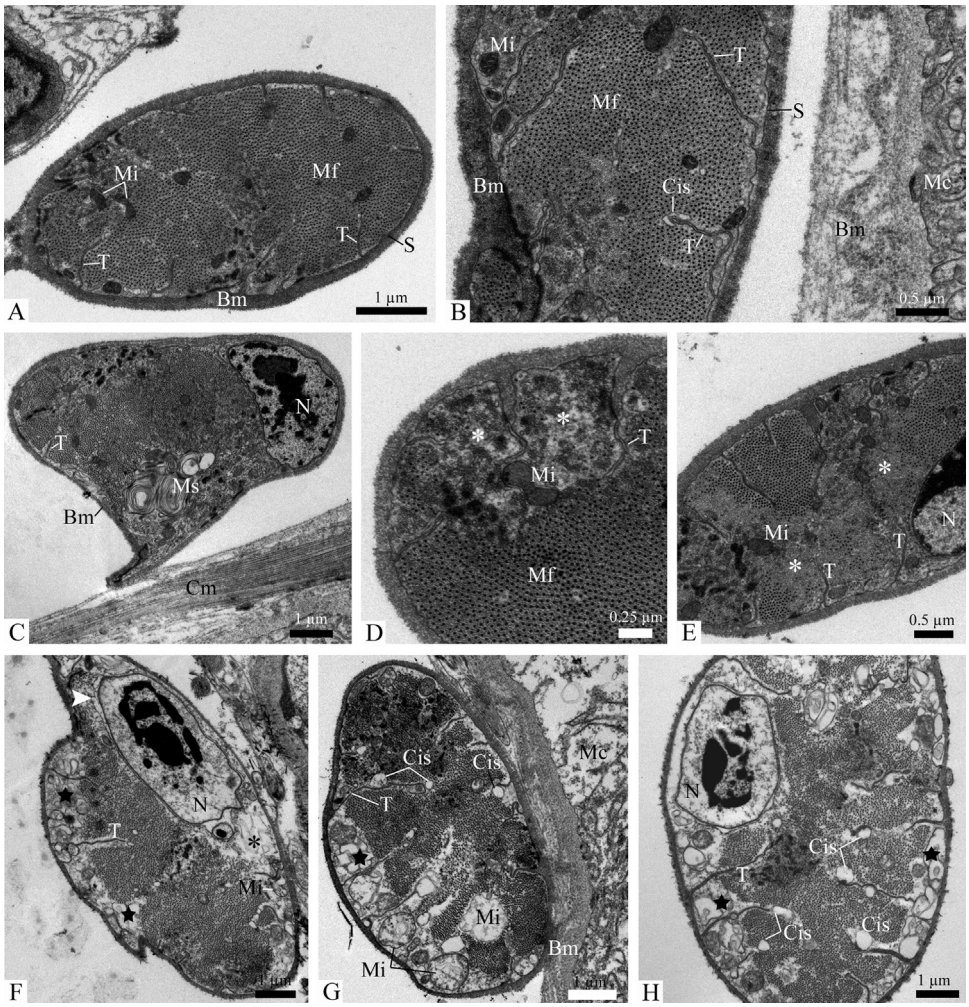


**Fig. 4.** Cross-section of *C. claveri* midgut muscle fibers from the control group (A and B) and the neem treatment groups (C–H) at the beginning of oviposition. (A and B) In this stage, the control group showed well preserved muscle fibers filled with myofilaments (Mf) and electron-dense mitochondria (Mi). (C) Neem oil 0.5%. (D and E) Neem oil 1%. (F–H) Neem oil 2%. Note the formation of myelin structures (Ms) inside the muscle fiber, the permanence of reduced myofilaments (*) and mitochondria (Mi) and the dilated cisternae of the sarcoplasmic reticulum (Cis). The nuclei showed clumping of chromatin (N) and dilatation of the perinuclear space (*arrowhead*). Membranous structures (stars) accumulated in the periphery of the fiber. Basement membrane (Bm); midgut epithelial cell (Mc); sarcolemma (S); T-system tubules (T).

myofilaments mainly in the periphery of the contractile fields, as well as swollen mitochondria and an accumulation of membranous structures near the sarcolemma (Fig. 2C–F).

The midgut muscle fibers, which were incubated for acid phosphatase cytochemistry, exhibited small acid phosphatase-positive structures near the T-tubules between the myofilaments in freshly emerged adults in the control group (Fig. 3A and B). In the neem treatment groups, acid phosphatase activity was observed in large vesicles present in the periphery of the muscle fiber between mitochondria and areas that lacked myofilaments (Fig. 3C–F).

At the start of oviposition, insects in the control group exhibited preserved muscle fibers with myofilaments, electron-dense mitochondria near the T-tubules and a sarcoplasmic reticulum associated with tubules (Fig. 4A and B). In the insects exposed to neem oil during the larval stage, muscle fibers still exhibited some degree of degeneration. Some muscle fibers contained vacant areas in which the contractile myofilaments were reduced or completely absent, and an accumulation of myelin structures, a dilatation of the cisternae of the sarcoplasmic reticulum and mitochondrial swelling and cristolysis were observed. The nuclei showed clumping of chromatin and a dilatation of perinuclear space. Membranous structures had accumulated in the periphery of the muscle fibers (Fig. 4C–H).

The acid phosphatase activity was localized in the control group to small areas present on the basement membrane and small vesicles in the periphery of the muscle fiber and near the T-tubules at the beginning of oviposition (Fig. 5A and B). Nonetheless, strong enzymatic activity was present in large vesicles localized in the periphery of the muscle fibers in insects that had received the neem treatments (Fig. 5C–F).

## 4. Discussion

We examined the midgut muscle organization and also provided an ultrastructural explanation of its morphology and the occurrence of ultrastructural changes caused by the ingestion of neem oil to better elucidate the mode of action of this biopesticide on this organ.

In *C. claveri,* the midgut visceral muscle presented with a similar organization as in other species of insects and was formed by a well-organized network of circular and longitudinal muscle fibers. The maintenance of visceral muscle function and integrity is important because visceral muscles provide peristaltic movements for the digestion and assimilation of ingested food (Park and Shahabuddin, 2000; Secundino et al., 2005; Bernick et al., 2007), and play an important role in homeostasis, physiology and regeneration

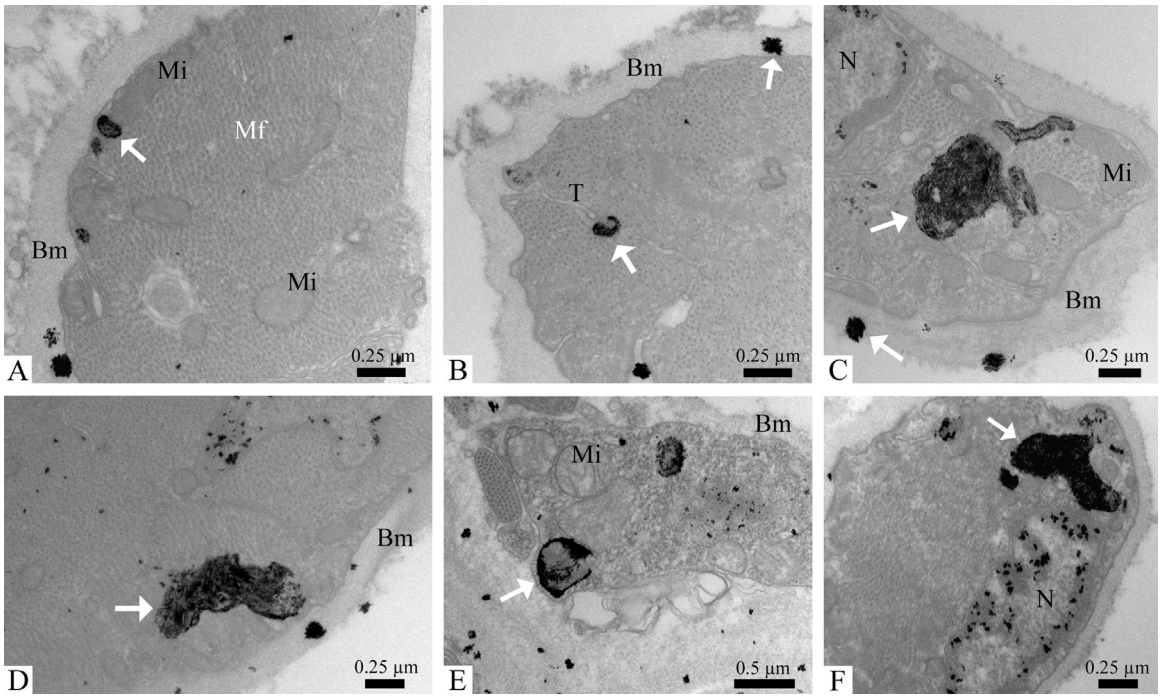


**Fig. 5.** Electron microscopic localization of acid phosphatase in *C. claveri* midgut muscle fibers from the control group (A and B) and the neem treatment groups (C–F) at the beginning of oviposition. In the control group, small amounts of reaction product (*arrows*) were localized on the basement membrane (Bm) and on small vesicles in the periphery of the muscle fiber and near the T-tubules (T). (C and D) Neem oil 0.5% and 1%, respectively. (E and F) Neem oil 2%. High enzymatic activity was present in large vesicles localized in the periphery of the muscle fiber. Nucleus (N); mitochondria (Mi); myofilaments (Mf).

in local tissue. In addition, the midgut muscles are an important component that can control the intestinal stem cell niche (Xu et al., 2011; Kux and Pitsouli, 2014).

Studies such as those conducted by Schaefer et al. (1967) and Bernick et al. (2007, 2008) included ultrastructural analyses that were performed to assist in the characterization of the muscle fibers, primarily to understand the organization of these components in these cells. The analysis of these components of the midgut is lacking, and many studies only describe the epithelium structure, neglecting this important element of the digestive system. Many articles have approached briefly changes caused by exposure to different types of insecticides in the midgut which impair the visceral muscle (Nasiruddin and Mordue (Luntz), 1993; Ling and Zhang, 2011; Ruiu et al., 2012; Almeida et al., 2014; Gutiérrez et al., 2016), consequently little information has been presented to readers.

Exposure to azadirachtin, the predominant component of neem oil, leads to the loss of muscle tone (Mordue (Luntz) and Nisbet, 2000), muscles become swollen and disrupted (Nasiruddin and Mordue (Luntz), 1993; Mordue (Luntz) and Blackwell, 1993) and a reduction in gut motility occurs after azadirachtin treatment(Mordue (Luntz) et al., 1985). Scudeler et al. (2016b) described the difficulty that some female *C. claveri* had in the elimination of the meconium from the alimentary canal, resulting in a shorter survival of these insects (only 53% for group treated with 2%) after neem oil ingestion in the larval instars. Changes in gut motility may affect the ability to eliminate meconium. A similar effect was observed in *Bombyx mori*. Munhoz et al. (2013) described larvae that had difficulty in eliminating excrement and exhibited a disorganization of the muscles fibers of the midgut, possibly due to the disarray of their myofibrils after the ingestion of insecticide chlorantraniliprole during the larval stage.

By means of ultrastructural analysis, we observed signs of cellular injury during the time period studied that included a reduction of myofilaments, swollen mitochondria and an accumulation of membranous structures near the sarcolemma. All of the evaluated concentrations induced cytotoxic effects, which were not concentration dependent. According to Scudeler et al. (2016b) the antifeedant and repellent effects could also explain this result, the low concentration of neem oil used in this experiment repelled fewer larvae from ingestion of the treated eggs, and therefore these larvae fed on large quantities of eggs and became more susceptible to the injurious effects of the neem. On the other hand, the high concentration of neem oil was strongly repellent to the larvae, but even the small amount of eggs consumed by this group resulted in damages. The malfunctioning of membranous structures that can lead to cellular vacuolation can be derived from the endoplasmic reticulum and mitochondria as a consequence of excessive cellular stress (Aki et al., 2012). Bernick et al. (2008) described this sign of degeneration that involved a deterioration of contractile elements in the midgut muscle during the larval-pupal transition in *Aedes aegypti*. Changes in mitochondria and membranes have been well documented in the midgut epithelium of *C. claveri* when exposed to neem oil (Scudeler and Santos, 2013, 2014; Scudeler et al., 2016a).

Although exposure to neem oil results in cytomorphological changes, cytochemical analysis showed that there was an increase in acid phosphatase activity in theses specimens; this enzyme plays a role in the detoxification process (Amirmohammadi et al., 2012; Valizadeh et al., 2013; Scudeler et al., 2016a). The increase in lytic activity is a mechanism for recovery after cell injury (Neerunjun and Dubowitz, 1977; Maltz and Oron, 1990; Cheville, 2009; Scudeler et al., 2016a). Acid phosphatase is a lysosomal enzyme, and during the cellular repair this lysosome activation acts to degrade cellular debris by autophagy. When the cell injury is significant, this lytic activity may not be able to process debris and therefore lead to accumulation of membranous structures derived of damaged organelles (Cheville, 2009).

Despite the finding that muscle fibers exhibited evidence of this detoxification mechanism, cell damage such as a reduced number of contractile myofilaments, an accumulation of myelin structures, a dilatation of cisternae of the sarcoplasmic reticulum and mitochondrial swelling and cristolysis were present in the oviposition period. The presence of injuries and the damages present in the midgut epithelium compromise the function of this organ, as highlighted by Scudeler et al. (2016a), in studies on *C. claveri* that were exposed to neem oil. Cytotoxic changes in the endoplasmic reticulum and mitochondria are some of the earliest signs of injury caused by treatments with plant-derived and insecticides in most of the cells of insects (Lü et al., 2010; Ling and Zhang, 2011; Qi et al., 2011; Catae et al., 2014).

Alterations at this cellular level clearly indicate that the visceral muscle layer is also the target of action of neem oil, which accounts for the symptoms associated with the effects the azadirachtin on muscle tone and gut motility that have been described previously in the insects (Mordue (Luntz) et al., 1985; Mordue (Luntz) and Nisbet, 2000). These results indicate that neem oil intake by *C. claveri* is cytotoxic to muscle fibers of the midgut, which compromises the function of this organ. These muscle fibers demonstrated a recovery attempt, but we found that it was inadequate due to the high level of cellular damage. Finally, we emphasize that the analysis of muscle layers cannot be neglected in the evaluation of cytopathological effects on the midgut of the insect because it is a critical component for the function of this organ.

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