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Alterations in the fat body and midgut of *Culex quinquefasciatus* larvae following exposure to different insecticides

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ABSTRACT

This study describes morphological alterations in the fat body and midgut of *Culex quinquefasciatus* larvae following exposure to different insecticides. To this end, both third and fourth instars of *C. quinquefasciatus* larvae were exposed for 30 and 60 min to organophosphate (50 ppb), pyrethroids (20 and 30 ppb), and avermectin derivates (1.5 and 54 ppb). Following incubation, pH measurements of the larvae gut were recorded. The fat body and midgut were also analyzed by light and transmission electron microscopy. These studies demonstrate a decrease in the pH of the larvae anterior midgut following exposure to all of the tested insecticides. Histochemical tests revealed a strong reaction for neutral lipids in the control group and a marked decrease in the group exposed to cypermethrin. Furthermore, a weak reaction with acidic lipids in larvae exposed to deltamethrin, temephos, ivermectin and abamectin was also observed. Insecticide-exposed larvae also exhibited cytoplasm granule differences, relative to control larvae. Finally, we noted a small reduction in microvilli size in the apex of digestive cells, although vesicles were found to be present. The destructive changes in the larvae were very similar regardless of the type of insecticide analyzed. These data suggest that alterations in the fat body and midgut are a common response to cellular intoxication.

1. Introduction

Adult female mosquitoes function as disease vectors for many vertebrate animals, and are therefore a focus of various control strategies to restrict their potentially devastating effects. However, these efforts require additional research pertaining to the biology of these organisms. Larval stage mosquitoes are much more abundant than adult mosquitoes and are also quite distinct in both form and function. An increased understanding of food ingestion, digestion and nutrient absorption by mosquito larvae has the greatest potential for new developments in mosquito control and population management (Linser et al., 2009).

The midgut of *Culex quinquefasciatus* (Say, 1823) larvae can be divided into four regions (i.e. cardia, gastric caeca, anterior and posterior midgut). In all of these regions, the epithelium is composed of a single layer of columnar digestive cells that consists of apical microvilli, cytoplasm with numerous mitochondria, and nucleus with polytene chromosomes. Regenerative cells are distributed alone or in groups scattered at the base of the digestive cells. The midgut epithelium is lined by a well-developed peritrophic matrix (PM) that separates it from the ingested food (Clements, 1996).

Immature mosquito larvae rely on the anterior portion of their midgut (stomach), which has a luminal pH of 10.5–11, for their initial digestive function (Dadd, 1975; Dow, 1984; Zhuang et al., 1999). In contrast, other organisms, such as vertebrates, utilize an acidic gut luminal pH for their digestive process (Linser et al., 2009). Changes in the pH or ion concentrations along the gut, or during digestion, can cause frequent changes in the amounts and molecular isoforms of PM proteoglycans, which can ultimately lead to alterations in physiologic features of the organism (Lehane, 1976).

Pyrethroids and DDT have been shown to affect sodium channels in mosquitoes (Brengues et al., 2003). Deltamethrin acts on the nervous system of these arthropods, which leads to rapid paralysis and death (Haug and Hoffman, 1990). Organophosphates inhibit acetylcholinesterase-mediated acetylcholine hydrolysis, resulting in an acute toxic effect due to hyperstimulation of muscarinic and nicotinic receptors (Gallo and Lawrk, 1991).

Exposure of *C. quinquefasciatus* larvae to 1.5 ppb of ivermectin leads to the development of ataxia and eventually death (Alves et al., 2004). This result suggests a connection between ivermectin and GABA receptors (Freitas et al., 1996).

The pyrethroids, deltamethrin and cypermethrin, are antagonists to ivermectin, and function by inhibiting sodium channels

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(Forshaw and Ray, 1990; Forshaw et al., 1993, 2000). The insecticidal activity of pyrethroids depends on their ability to modify the gating kinetics of voltage-gated sodium channels, thereby disrupting normal cellular communication (Soderlund, 1995). Voltage clamp techniques have been extensively employed to document the effects of pyrethroids on recorded sodium currents from invertebrate and vertebrate neurons (Narahashi, 1992, 1996). A delayed inactivation during the depolarization–repolarization cycle is a central effect of pyrethroid treatment. A recent study analyzed the effects of pyrethroid treatment on the depolarization of sodium channel gating under voltage clamp conditions at hyperpolarized membrane potentials. However, the insecticides sometimes lead to pH changes in the larval gut (Lehane, 1976).

Previous studies have shown that avermectins may affect other tissues in addition to the gut, such as the fat body (Strong and Brown, 1987; Alves et al., 2004). In mosquitoes, the fat body is distributed as a layer of cells close to the body wall, throughout the thorax and abdomen, and as lobes which extend into the body cavity, or as sheaths around certain organs (Clements, 1996). The insect fat body is the major organ of intermediary metabolism. In addition to lipid synthesis, most haemolymph proteins are also synthesized in the fat body which also stores proteins, lipids and carbohydrates (Chapman, 1998). The total carbohydrate and lipid content of young adult mosquitoes provides a measure of the energy reserve accumulated during the larval stage and protein synthesis for the haemolymph in larvae and adults. However, in adult females, the fat body produces the yolk polypeptides (Clements, 1996). Several studies have suggested that the fat body also plays a role in the immune response of the organism. Defensins are proteins that are produced in the fat body, released into the haemolymph, possess 3 or 4 disulfide bounds, and are active in the immune response against certain bacteria, yeast and viruses (Hoffmann et al., 1999; Zanetti et al., 1997).

Over the past decade, various studies regarding pesticide actions on the midgut and fat body morphology of the cloned Culicidae insect have provided new insight into the mode of action of insecticides. This study seeks to detect morphological changes in the fat body and midgut of *C. quinquefasciatus* larvae following exposure to different insecticides.

2. Materials and methods

2.1. Mosquitoes

C. quinquefasciatus larvae were obtained from a laboratory culture, as previously described by Gerberg (1979).

2.2. Insecticides and assay

In this study, both the third and fourth instars larvae of *C. quinquefasciatus* were exposed for 30 and 60 min to 50 ppb of the organophosphate, temephos (Fersol 500CE), 30 and 20 ppb of the pyrethroids, deltamethrin (Fersol 25CE) and cypermethrin (Fersol 200CE), respectively, and 1.5 and 54 ppb of the avermectin derivates {[ivermectin (Ivomec 1% p/v (Merial from Brazil))] and [abamectin (Vertimec 18CE (Syngenta))]}, respectively (LC_{50} – data not shown). For each insecticide tested, the larvae were divided into groups consisting of 10 specimens as well as the control group, which three replications each treatment. The temperature was maintained at 26 °C throughout all of the tests.

2.3. pH measurement inside the larvae gut of following insecticide exposure

Gut pH was measured *in vivo* by allowing larvae to ingest a variety of pH indicator dyes and observing the resulting color changes in their gut. The indicator dyes used were 0.5% bromothymol blue (pK_a 7), phenol red (pK_a 7.9) and thymol blue (pK_a 8.2).

To this end, a total of 180 third or fourth instar larvae of *C. quinquefasciatus* were exposed for 60 min to the insecticide concentrations described above. After 60 min of incubation, each larval group (30 specimens) was then maintained for an additional 60 min in 100 mL of insecticide solution for the treated groups, and 100 mL of water for the control group. The larvae were then washed and transferred to plastic containers containing food (murine chow, Labina – Purina™). The pH in each region was estimated by comparing the colors inside the gut to those observed with buffer solutions with a known pH and prepared with the same stains (Gontijo et al., 1998). Chloroform treatment (i.e. 3 drops) of the control tubes containing the control color patterns ensured that colors did not change throughout the course of the experiment. The non-dissected insects were observed under a stereomicroscope. Between 8 and 10 observations were made for each larval group exposed to the pH indicator.

2.4. Buffers

The following buffers were used in these assays: acetate/NaOH (pH 5 and 5.5); 2-[N-Morpholino] ethanesulfonic acid (MES)/NaOH (pH 6 and 6.5); N-[2-hydroxyethyl] piperazine-N0-[2-ethanesulfonic acid] (HEPES)/NaOH (pH 7); Tris(hydroxymethyl) aminomethane (TRIS)/HCl (pH 7.5, 8 and 8.5); borate/NaOH (pH 9, 9.5 and 10) and sodium carbonate/HCl (pH 10.5 and 11). The final concentration of each buffer used in the assays was 50 mM.

2.5. Histology and ultrastructure

After insecticide exposure, larvae were decapitated and the respiratory siphon was removed. The thorax and abdomen were fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Following fixation, the samples were dehydrated in graded ethanol series and embedded in butyl methacrylate and 2,4-dichlorobenzyl peroxide resin [electron microscopy sciences (EMS)]. Sections (4 μm thin) were stained with haematoxylin and eosin or 1% toluidine blue-Borax. Another set of slices from the same larvae were subjected to the following histochemical tests: total protein analysis by mercury bromophenol blue and lipid analysis by Nile blue sulfide (Pearse, 1968).

For transmission electron microscopy studies, the larvae were transferred to 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 5 h. The samples were then post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series, propylene oxide, and embedded in Epon 812 resin (EMS).

Ultra-thin sections were stained with 2% uranyl acetate for 30 min (Watson, 1958), and then incubated in lead citrate for 10 min (Reynolds, 1963). The samples were analyzed with a transmission electron microscope (Zeiss, EM 109) located in the Microscopy and Microanalysis Center at the Federal University of Viçosa.

3. Results

There were no differences in the morphological features studied among the third and fourth instar larvae of *C. quinquefasciatus*. Therefore, we provide a general presentation of the results, which were observed for both instars.

3.1. pH

The pH inside the midgut of *C. quinquefasciatus* larvae was determined to be alkaline (>9.5) in the control group. In contrast,

Table 1
pH of midgut of *Culex quinquefasciatus* larvae exposed to the different insecticides.

Insecticide	Ivermectin	Abamectin	Temephos	Deltamethrin	Cypermethrin	Control
pH	7.0–7.5	7.0–7.5	7.0–7.5	7.0–7.5	7.5–8.0	>9.5

insecticide treatment resulted in a decrease of the midgut pH to near neutral values (Table 1, Fig. 1).

3.2. Light microscopy

In the control group, the midgut epithelium consisted of a single layer of digestive cells exhibiting a well-developed brush border and cytoplasm with acidophilic regions (Fig. 2). Some digestive cells of the midgut also showed differentiated morphological features, such as an irregular apical surface and secretions which were surrounded by a strong peripheral basophilic region. The cells of the fat body possessed a nucleus with a variable size and shape that also demonstrated obvious areas of condensed and decondensed chromatin. Furthermore, these cells also exhibited large nucleoli and a cytoplasm rich in large clear areas which likely represent lipid inclusions and small protein granules (Fig. 3).

Pyrethroid-treated larvae possessed many midgut cells with a weakly stained cytoplasm as well as a nucleus with decondensed chromatin and evident nucleoli. In addition, some cells also presented secretory vesicles with a clear core surrounded by a more basophilic peripheral region. As shown in Fig. 4, larvae exposed to cypermethrin resulted in weakly stained apical surface midgut cells. In contrast, exposure of larvae to deltamethrin resulted in a cell apex with a short brush border (Fig. 5), and fat body cells with small protein granules, relative to the control group. Following treatment with temephos, many midgut cells possessed a cytoplasm that contained small vacuole-like structures, as well as a nucleus with decondensed chromatin and evident nucleoli (Fig. 6). This feature of the midgut differed not only from the control group, but also from the group exposed to pyrethroids. However, the fat body was similar to that observed with the control group.

Fig. 1. . Midgut of the larvae *Culex quinquefasciatus* from the group exposed to 20 ppb of cypermethrin for 60 min and posterior exposition an indicator of pH timol blue for 1 h. GC – gastric caeca, AMI – anterior midgut, PMI – posterior midgut.

The midgut and fat body of larvae exposed to avermectin derivates presented distinct morphological patterns. Larvae exposed to ivermectin exhibited longer digestive cells without secretory vesicles, a weakly stained cytoplasm, and a nucleus with decondensed chromatin and slightly evident nucleoli (Fig. 7). The fat body cells possessed protein-positive granules, with the smaller granules demonstrating the strongest reaction for the presence of protein (Fig. 8). In the larvae exposed to abamectin, the midgut digestive cells exhibited a well-developed brush border, cytoplasm and nucleus acidophil, with condensed chromatin and slightly evident nucleoli (Fig. 9). The cells of the fat body also showed small granules that were more acidophilic relative to the other larvae analyzed in this study.

The histochemical tests revealed a strong reaction for neutral lipids in the control group and a decrease in the cypermethrin-treated group. Furthermore, these tests also demonstrated a weak reaction for acidic lipids in larvae exposed to deltamethrin, temephos, ivermectin and abamectin. There were also differences in granules of the insecticide-exposed larvae compared to control larvae. The histochemical data are summarized in Table 2.

3.3. Ultrastructure

The ultrastructural analyses showed an intact peritrophic matrix in the midgut of all larvae. The digestive cells possessed large numbers of small secretory vesicles, well-developed microvilli and numerous mitochondria. The nucleus exhibited decondensed chromatin and a well-developed nucleolus.

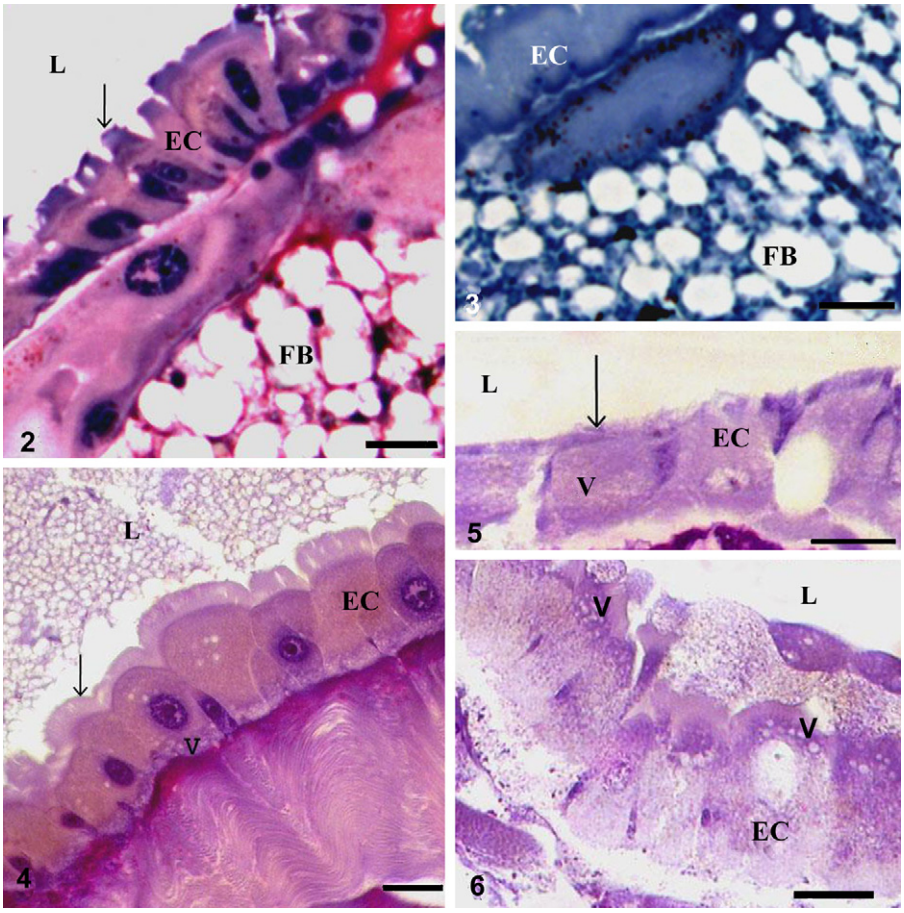
Exposure to pyrethroids and organophosphate resulted in larvae with a basal region of digestive cells with several plasma membrane infoldings and enlarged channels (Figs. 10 and 11). However, in larvae exposed to abamectin, the number of plasma membrane infoldings was significantly higher (Fig. 12)

In the apex of digestive cells, although the size of the microvilli was slightly reduced, vesicles were still apparent. Larvae exposed to deltamethrin exhibited a greater number of small, electron lucent vesicles in the apical part of the cytoplasm in the digestive cells. Smooth septate junctions, which joined two adjacent membranes of nearby epithelial cells, and numerous mitochondria in the apical cytoplasm, were both common in the midgut epithelial cells of the larvae (Figs. 10 and 11).

The trophocytes in the fat body were also observed to be intact. Furthermore, the nucleus possessed decondensed chromatin, while several lipid droplets were seen in the cytoplasm.

4. Discussion

This study clearly demonstrates a strong correlation between pesticide exposure of mosquito larvae and a decrease in the pH of



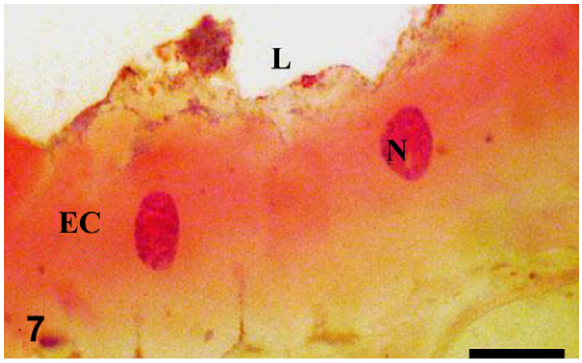
Figs. 2–6. Histological sections of third instar larvae or *Culex quinquefasciatus*. Fig. 2. Middle region of the midgut of control group showing cylindrical epithelial cells (EC) with microvilli (arrow). FB – fat body (HE stained). Fig. 3. Middle region of the midgut, showing cylindrical epithelial cells (EC). FB – fat body (bromophenol blue stained). Fig. 4. Larvae exposed to 20 ppb of cypermethrin for 60 min showing the middle region of the midgut with cylindrical epithelial cells (EC) with microvilli (arrow) and several vesicles (V) at the base of the cells (HE stained). Fig. 5. Middle midgut region of larvae exposed to 30 ppb of deltamethrin for 60 min. Note the cylindrical epithelial cells (EC) with short microvilli (arrow) and several vesicles at the base of the cells (V) (HE stained). Fig. 6 Larvae exposed to 50 ppb of temephos for 60 min showing the middle midgut region with cylindrical epithelial cells (EC) with a great number of vesicles (V) (HE stained). L – midgut lumen. Scale bars = 10 μm.

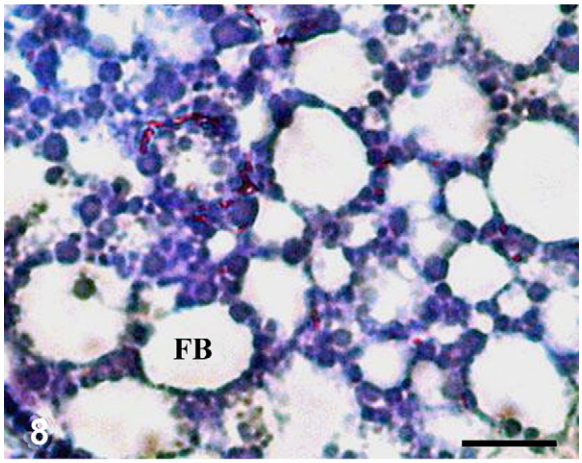
the larvae anterior midgut. The anterior midgut pH of mosquitoes varies in different species, but is generally maintained between 10.5 and 11 (Clements, 1996). A decrease in the midgut pH from an alkaline to a neutral value suggests an electrical imbalance, since the maintenance of alkaline pH is the main energy expense of the larvae. Alterations in midgut pH may result in a physiologic change in the haemolymph of the mosquito. To achieve an alkaline pH value of 11, strong cations (i.e. Na^+ or K^+) and weak anions (i.e. OH^-, CO_3^{2-}, or HCO_3^-) must accumulate in the lumen of the anterior midgut. Thus, the following two epithelial transport pathways are required for alkalinization of the midgut lumen: a cationic pathway, in which a strong cation replaces weak cations, and/or an anionic pathway, in which a weak anion replaces a strong anion (Boudko et al., 2001a). The Na^+ moves from the gut lumen to the haemolymph against the electrochemical gradient. The energy for this active movement is provided by V-ATPase pumps in the apical plasma membrane of the anterior midgut, and also by major Na^+/K^+ exchange pumps in the basal plasma membranes of rectal cells (Chapman, 1998). Moreover, because alkalinization in the midgut of mosquito larvae depends on V-ATPase activity (Boudko et al., 2001b), the proposed anion exchanger is likely to be driven by voltage since a transepithelial voltage is present (Clark et al., 1999, 2000) but Na^+ or K^+ gradients are not (Boudko et al., 2001a). Previous studies have shown that the pyrethroids, deltamethrin and cypermethrin inhibit sodium channels, which results in prolongation of the sodium current during membrane excitation (Forshaw and Ray, 1990; Forshaw et al., 1993, 2000; Soderlund, 1995). Thus, we suggest that the insecti-

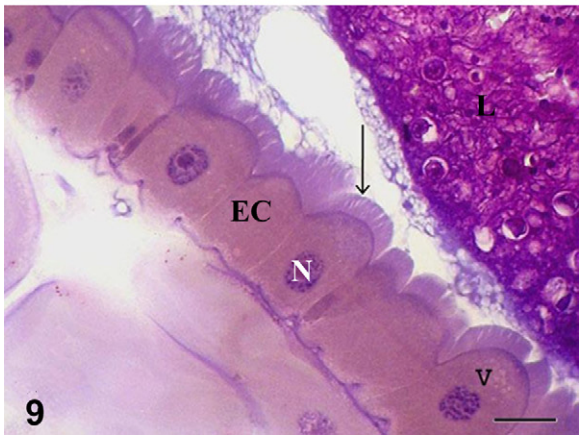
Table 2
Results of histochemical tests in the midgut and fat body of *Culex quinquefasciatus* larvae after exposure to insecticides during 1 h.

	Control			Cypermethrin			Deltamethrin			Temephos			Ivermectin			Abamectin		
	BB	NB		BB	NB		BB	NB		BB	NB		BB	NB		BB	NB	
		AL	NL		AL	NL		AL	NL		AL	NL		AL	NL		AL	NL
AIC	+++			++			+			+			+			+++		
NIC	+++			++			+			+			+			++		
SGT	+++	–	+++	+	–	+	++	+	–	+	+	–	+	+	–	++	+	–

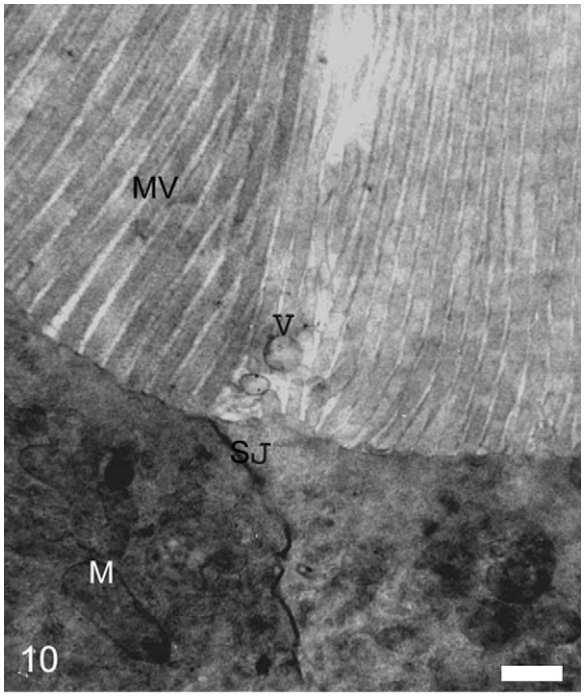
AIC = apex of the intestinal cells; NCE = nucleus of intestinal cells of the midgut; SGT = small granules in the trophocytes of the fat body; BB = bromophenol blue; NB = Nile blue; AL = acidic lipids; NL = neutral lipids; (+++) strong positivity; (++) mild positivity; (+) weak positivity; (–) negative.

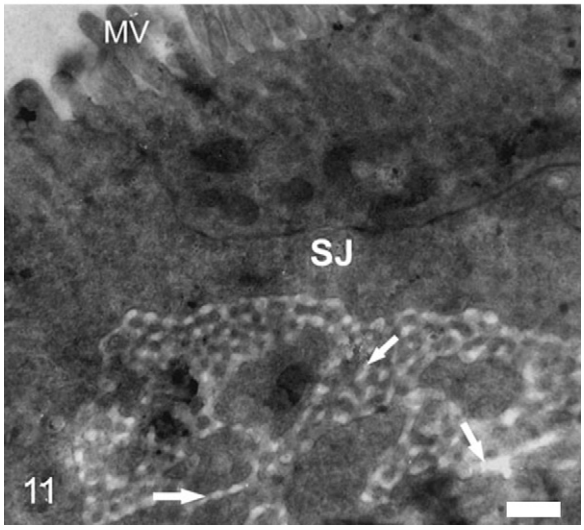






Figs. 7–9. Histological sections of third instar larvae or *Culex quinquefasciatus*. Fig. 7. Larvae exposed to 1.5 ppb of ivermectin for 60 min showing cylindrical epithelial cells (EC) with basophilic nucleus (N) (HE stained). Fig. 8. Fat body (FB) of the larvae showing positive reaction for protein in the cells with great quantity of vesicles (arrows) (bromophenol blue stained). Fig. 9. Larvae exposed to 54 ppb abamectin for 60 min showing midgut epithelial cells (EC) with brush border (arrow) and vesicles (V) (HE stained). L – midgut lumen. Scale bars = 10 μm.





Figs. 10–11. Transmission electron micrographs of the third instar larvae of *Culex quinquefasciatus*. Fig. 10. Cell apex of the midgut exposed to 20 ppb of cypermethrin for 60 min showing well-developed microvilli (MV), secretory vesicles (V) and smooth septate junctions (SJ). Fig. 11. Midgut cell of larva exposed to 50 ppb of temephos for 60 min showing short microvilli (MV), basal region with a large number of plasma membrane infoldings (arrows). M – mitochondria, SJ – smooth septate junctions. Scale bars = 1 μm.

cides tested in this study act on ion transporters in the midgut cells.

The midgut possesses well-developed microvilli in the cell apex because it is the main absorption area in the mosquito gut (Lacey and Federici, 1979; Ferreira et al., 1981; Lahkim-Tsror et al., 1983). Histochemical tests revealed a strong protein reaction in the cell apex of midgut cells in control, cypermethrin and abamectin exposed larvae. However, midgut from larvae exposed to temephos, deltamethrin and avermectin also presented positive reactions, suggesting the presence of protein in the cytoplasm, which has been previously demonstrated by Arruda et al. (2008). These results are not unexpected since the primary functions of the midgut include digestive enzyme production and nutrient absorption of digestion products (Snodgrass, 1935; King and Akai, 1984; Serrão and Cruz-Landim, 1995; Cavalcante and Cruz-Landim, 1999). However, stronger reactions were observed in the vesicles of some larvae which may be due to the different mechanisms of each insecticide. Taken together, these results suggest that the use of chemical larvicides, independent of the active ingredient, leads to physiological and morphological damage in epithelial cells of the midgut, which is likely where these compounds are absorbed. Regardless of the type of substance used, the similarity of detrimental changes in the organism indicates that these alterations are a common response to cellular intoxication.

Following pesticide treatment, several changes in specific organelles suggest that energy is necessary for vacuolation and

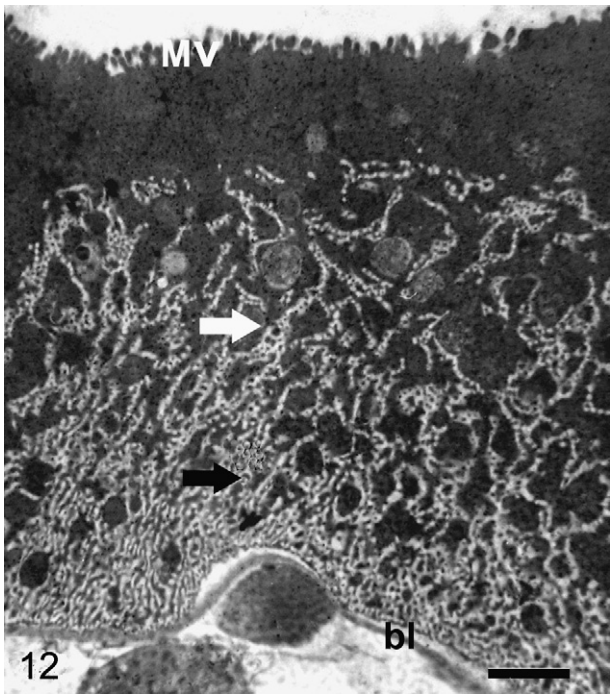


Fig. 12. Transmission electron micrograph of the midgut cells of the third instar larvae of *Culex quinquefasciatus* exposed to 54 ppb of Abamectin for 60 min showing short microvilli (arrow), basal region with great amount of plasma membrane infoldings (arrows) and electrondense cytoplasm. bl – basal lamina. Bar = 1 μm.

vesicle releasing, possibly in an attempt to detoxify cells. For example, exposure of larvae to pesticides results in an increase in the number of mitochondria in the digestive cells. This method of vacuolation may indicate that these cells are in a death process, possibly due to the presence of toxic substances that may alter microvilli size in the midgut of insecticide-exposed larvae, as described by Arruda et al. (2008).

The weak lipid reaction, mainly observed in larvae exposed to avermectin derivates which possessed fewer granules, is also associated with differences in granulation in fat body cells. This result corroborates previous results demonstrating a reduction in lipid size in fat body cells of *C. quinquefasciatus* larvae after exposure to 1.25 ppb of ivermectin (Alves et al., 2004).

Association of the histochemical tests with the quantity of secretory vesicles in the digestive cells of insecticide-exposed larvae indicates that insecticide metabolism may be due to the energy requirement and production of enzymes that generate the fat body. This study demonstrates that insecticides lead to structural damage in the midgut and fat body of mosquito larvae. Taken together these results corroborate the ability of these insecticides to alter both the physiology and development of mosquito larvae.

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