

## Site of action of hematoporphyrin (a photo-activated insecticide) in *Culex pipiens* larvae

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### ABSTRACT

The histopathological effect of hematoporphyrin on the mid-gut and the integument of the fourth larval instar of *C. pipiens* was studied using a transmission electron microscope. In this study *C. pipiens* larvae were treated with different concentration of hematoporphyrin. The concentration  $5 \times 10^{-4}$  M/L was found to produce 100% mortality after half-hour exposure to the 400 W/M<sup>2</sup> artificial lights using a solar simulator. The ultrastructural examination showed that the normal lamellate cuticle was heavily affected, taking the shape of amorphous cuticular region. The endo- and exo-cuticle were not distinguishable. It was found that the epidermal cells underneath the cuticle were distorted. The mitochondria of these cells exhibited irregular shapes. The fat body underneath the integument could be detected with visible vacuolisation. Muscle cells revealed the degenerated sarcomeres with gaps and vacuoles. The Z discs have irregular shape and are distorted. Mid-gut cells appeared with cytoplasmic vacuoles. The Golgi body is fragmented into small bodies. The rough endoplasmic reticulum is broken-down into separate vascular structures. These cytopathological observations confirm the insecticidal efficiency of hematoporphyrin against *C. pipiens* larvae.

**KEYWORDS:** histopathology, mid-gut, integument, insect larvae, TEM

### INTRODUCTION

It is now believed that photoinsecticides may provide the basis of a new generation of pesticides as reported by Lenke *et al.* (1987), Ben Amor *et al.* (1998), Salama (2000a) and Salama (2000b). The use of hematoporphyrin in insect control exhibits several advantageous features compared with conventional insecticides, insect growth regulators and biological control agents for insect control management. This photoinsecticide can be directly administered in aqueous solution and in association with an attractant; its photophysical/ photosensitizing properties have been determined in a variety of media and have been shown to be particularly efficient as reported by Jori (1985). The relatively high water solubility of hematoporphyrin, the ascertained lack of photomutagenic activity as stated by Jori & Spikes (1983) and its widespread clinical use as a phototherapeutic agent against solid tumours and other diseases as reported by Jori (1986), although there are some potential hazards and limitations (Lenke *et al.* 1987). Photoinsecticide is also very rapidly photobleached upon exposure to UV or visible light as reported by Jori & Spikes (1983).

There is a shortage of literature dealing with the fine structure of insect cells treated with photoinsecticide; most of the literature deals with normal insecticides or those treated with additives. Hematoporphyrin appears to be mainly accumulated in the midgut, Malpighian tubules, adipose tissue and cuticle as reported by Ben Amor *et al.* (1998) with the aid of fluorescence microscopy analysis of *Ceratitis capitata* flies after 48 hours exposure.

The present study examines the ultra-structural changes induced by a photoactivated insecticide, hematoporphyrin, on the midgut and the integuments of the fourth larval instar of *C. pipiens*.

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## MATERIALS AND METHODS

A colony of *C. pipiens* was obtained from the Entomology Department, Faculty of Science, Zagazig University, Benha branch. This colony has been maintained in the laboratory for four year. Larvae were maintained in the laboratory under natural photoperiods, a temperature of  $25 \pm 2$  °C and  $70 \pm 5\%$  relative humidity. About 250-300 larvae were reared in white enamel bowls (35 cm in diameter and 10 cm in height), half -filled with distilled water and covered with muslin. A mixture of bread, dried yeast and dried milk in a 2:1:1 ratio respectively were ground, sieved and used as larval food. The amount of food added daily was increased with larval age; excess food was avoided to prevent scum formation. The bowls were kept constantly aerated. Exuviae with any scum were removed daily. A field strain of *C. pipiens* collected from Qalyubia Governorate, Egypt, was also used in the preliminary experiments. Different concentrations of hematoporphyrin were prepared to test the susceptibility of the fourth instar larvae. A group of 50 larvae was used for each test, and every dilution had six replicates. For examination under the microscope, larvae were chosen from the concentration that had induced 100% mortality after 30 minutes' exposure to the  $400 \text{ W/m}^{-2}$  artificial light using a solar simulator. Control experiments were tested similarly but without exposure to the artificial light and kept in the darkness. Moribund larvae (i.e. those that responded weakly to stimulation) rather than dead larvae were chosen. The selected larvae were washed in distilled water before being cut, pre-fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 at 4°C. The specimens were post-fixed in 1% osmic acid (prepared by mixing 2%  $\text{OSO}_4$  in  $\text{H}_2\text{O}$  and 0.2 M Na cacodylate in a 1:1 ratio) for 2 hours at 4°C. The specimens were then washed in 0.1 M cacodylate buffer (at 4°C for 30 minutes). After fixation, the larval samples were dehydrated by soaking in ethanol/water mixtures of progressively increasing ethanol concentration. Dehydration continued with 1:1 ethanol:propylene oxide for five minutes, followed by 100% propylene oxide for two minutes. Infiltration was initiated in 1:1 solution of propylene oxide and then the embedding mixture (araldite resin CY 212; 24.2g, hardener “ DDSA “ Hy 964; 18.4 g, MNA “ methyl nadic anhydride “; 2.5 ml and accelerator “ by 0.64, 0.8 ml). After embedding, the capsules were polymerised at 60 °C for 24 hours. Sections were cut at  $1\mu$  by LKB ultramicrotome using glass knives. After examination of the semi-thin sections, ultra-thin sections were cut from the chosen region and were mounted on copper grids, and stained with uranyl acetate and lead citrate on a Reichert ultrastainer (Leica). The ultra-thin sections (silver section) were finally examined and photographed by Geol 100 CX transmission electron microscope.

## RESULTS

The susceptibility of the laboratory and field strains to  $5 \times 10^{-4}$  M/L concentration of hematoporphyrin is presented in table 1 and typical larvae are shown in figure 1. The obtained data indicated that the mortality was total in the laboratory colony, but lower in the field strain. Figure 1 showed that the cuticle of the *C. pipiens* field strain was darkened more than in the laboratory strain. Pupae of *C. pipiens* were shown to be healthy and not affected by hematoporphyrin treatment.

Table 1: Susceptibility of the fourth instar larvae of *C. pipiens* laboratory colony and the field strain to hematoporphyrin after half-hour exposure to the  $400 \text{ W/M}^2$  artificial lights.

Concentration	Averages of mortality %	
	Lab. colony	Filed strain
$5 \times 10^{-4}$ M/L	$100 \pm 0.0$	$85 \pm 0.31$

### Ultrastructural changes

**The integument:** Ultrastructural examination of untreated *C. pipiens* larvae (Figs. 2 and 3) reveals the normal lamellate cuticle. The cuticle consists of chitin microfibrils embedded in a protein matrix. The three main types of cuticle can be detected and are laminated in structure. Each lamina consists of a sheet of microfibrils all of which are oriented in a single preferred

direction. The microfibrils of successive sheets are oriented at a slight angle to each other. The angle changes progressively in one direction. Such architectures referred as Helicoidal structure (Fig. 2), and was clearly damaged in treated larvae (Fig.4).

As in most arthropods the integument consists of a single layer of epidermal cells. This epidermis is seen in full only when the new cuticle is being laid down; at other times it is exceedingly attenuated (Fig. 3). The nucleus is relatively large in size with peripherally located chromatin. The plasma membrane is an essentially permeable barrier that controls the passage of molecules and ions between the cytoplasm and the surrounding medium. There are a few mitochondria scattered throughout the cytoplasm of the cell. Two membranes create an external limiting membrane, forming the outer shape and an inner membrane from which arise the cristae. The mitochondria of the hypodermis are slightly less electron-dense. Fig. (4) shows the destruction of the integument and fat body vacuolisations. The exo- and endocuticle are not distinguishable, and the epidermal cells underneath the cuticle are distorted. The leakage of lysosomes can be seen with the adjacent vacuoles, which is responsible for cell lysis. In Fig. (5) only the exocuticle is present to identify the integument boundary. Ultrastructural changes to the mitochondria show deformation, marked coalescence and inner damage (fig. 6).

**List of Abbreviations for the figures:**

Ep= Epicuticle; Fb=Fat body; Ex= Exocuticle; Mb= Muscle bundles; En=Endocuticle; Z = Z line; H= Hypodermis; M= Mitochondria; N=Nucleus; Pm= Plasma membrane; Ch= Chromatin; Ne= Nuclear envelope; Cy=Cytoplasm; Ct= Connective tissue; Bp=Basal plasma membrane; Lc= lysed cytoplasm; Rer = Rough endoplasmic reticulum; G = Golgi body



Fig. (1): A photograph represents the laboratory strain of *C. pipiens* larva at (left) and the field strains at (right). Note the darkening of the integument of the field strain.



Fig. (2): Ultrastructure of integument in untreated 4<sup>th</sup> *C. pipiens* larva showing the normal structure (× 40000).

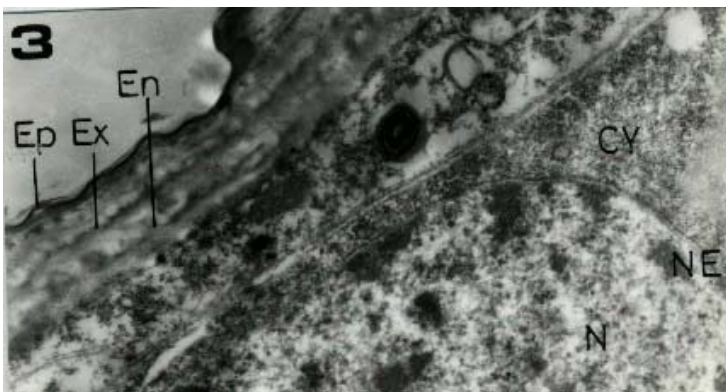


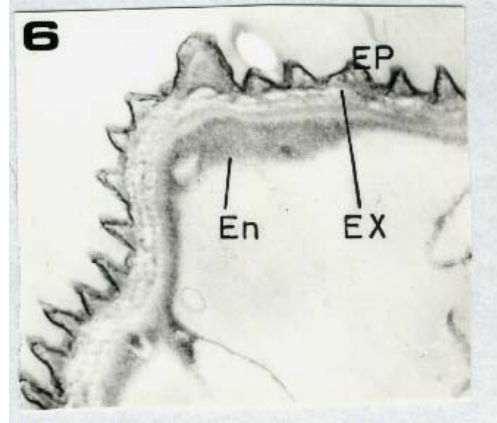
Fig. (3): Photomicrograph showing the normal *C. pipiens* larva illustrating the epidermal layer (× 20000).



Fig. (5): Ultrastructural changes in mitochondria of *C. pipiens* larva treated with the photoinsecticide ( $\times 27000$ ).



Fig. (6): Electron micrograph of a *C. pipiens* larva in advanced stage of the photoinsecticide treatment ( $\times 40000$ ).



**The fat body:** The ultra-thin section of the fat body cell of an untreated *C. pipiens* larva (Fig. 7) shows the nucleus of fat cell contains heavily electron dense chromatin condensations; the cytoplasm also contains numerous mitochondria. After treatment with the photoinsecticide it seemed to be highly vacuolated with necrosis, as observed in (Fig.8). Hematoporphyrin treatment resulted in the lysis of these cells.

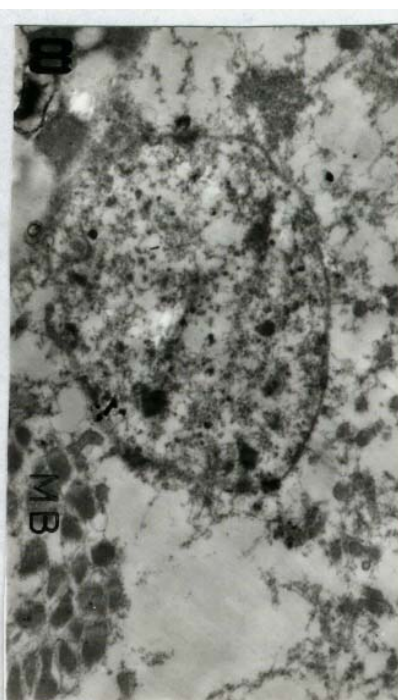


Fig. (7): Ultrastructure of fat cell of untreated *C. pipiens* larva ( $\times 27000$ ).

Fig. (8): Ultrastructural changes of fat cells of a photoinsecticide treated *C. pipiens* larva ( $\times 20000$ ).



**The Muscles:** The muscles of *C. pipiens* larva seem to be made up of striated fibres (Fig. 9). Each fibres consists of a number of parallel fibrillate or sarcostyles. The fibrils are threadlike with visible differentiation, the Z line or *Zwischenscheibe* is clearly detected. *C. pipiens* larvae are characterized by the absence of H-band (Henson's line). The fine structure of the myofibrils shows the presence of thick, apparently tubular, myosin filaments and fine actin filaments. In contractions, these actin filaments slide between the myosin unity. Ultrastructural examination of the hematoporphyrin treated larva (Fig. 10) revealed atrophied muscles, resulting in disorganization of their components as compared to the previously mentioned normal muscles. The Z discs had irregular shapes and were distorted. Gaps and vacuoles appeared in the sarcomeres.

**The Midgut:** The fine structure of the midgut epithelial cell of an untreated *C. pipiens* larva (Fig. 11) shows that the basal membranes of the cell, adjacent to the haemocoel, are enfolded with a few opening to the haemolymph so that the extra cellular spaces, which they enclose, are relatively isolated. The cell generally contains mitochondria and extensive endoplasmic reticulum with ribosomes assumed to be concerned with the synthesis of digestive enzyme. The nuclear chromatin is clumped into patches of varying densities; there is also abundance of lamellated rough endoplasmic reticulum. The majority of the elements of the endoplasmic reticulum in this case exist in the form of lamellar structure or flattened cisternal vesicles. This type of endoplasmic reticulum is characterized by the presence of numerous minute granules bordering the outer surface of the membranes of the reticulum. These particles are rich in RNA and proteins and hence they are known as the "ribonucleoprotein particles or ribosomes". Similar particles are also dispersed in the cytoplasmic matrix. Electron micrograph also shows the existence of peritrophic membrane lining the internal midgut cavity.

Ultrastructural examination of photoinsecticide treated larva (Fig. 12) showed the appearance of cytoplasmic vacuoles. Some mitochondria are swollen with irregular shapes, while others are greatly elongated with prominent cristae. The two mitochondrial membranes are not demarcated. The Golgi body is fragmented into small particles, which are thinned out and gradually disappear (Fig. 13). The rough endoplasmic reticulum (Fig. 14) is broken down into separate narrow vascular structures without any clear connection with the nuclear membrane. The rupture of lysosomal membranes resulted in the release of its enzymes causing destruction of the cellular constituent and complete lysis of the cell. The peritrophic membrane was also affected by the treatment (Fig. 15).

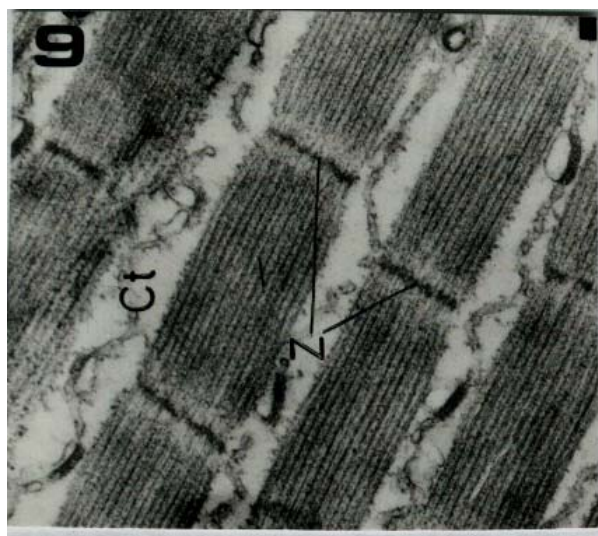


Fig. (9): Ultrastructure of muscles in an untreated *C. pipiens* larva ( $\times 27000$ ).

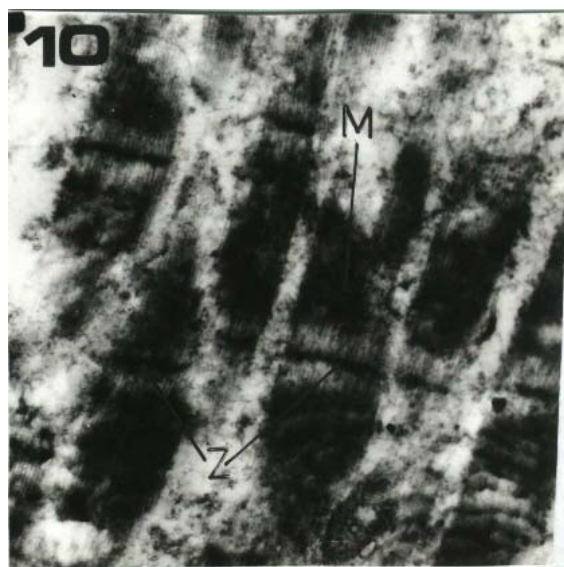


Fig. (10): Ultrastructural changes in muscles of *C. pipiens* larva treated with hematoporphyrin ( $\times 27000$ ).

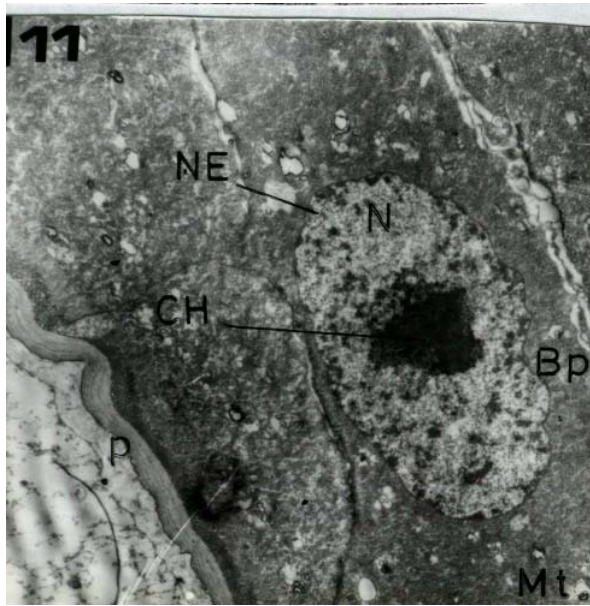


Fig. (11): Ultrastructure of midgut cell of untreated *C. pipiens* larva ( $\times 27000$ ).

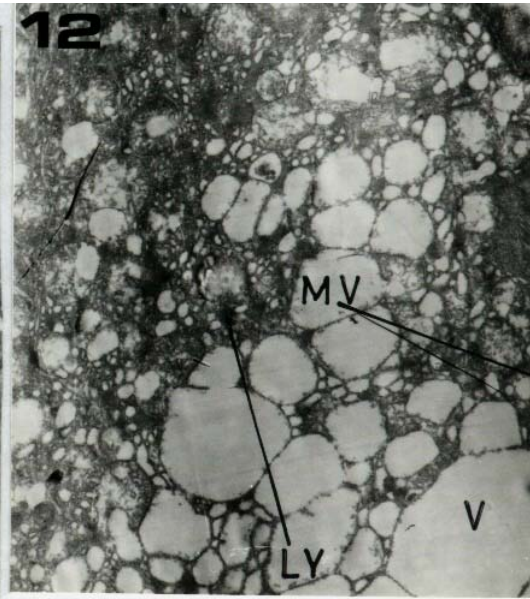


Fig. (12): Ultrastructural changes in midgut cell treated with hematoporphyrin ( $\times 27000$ ).

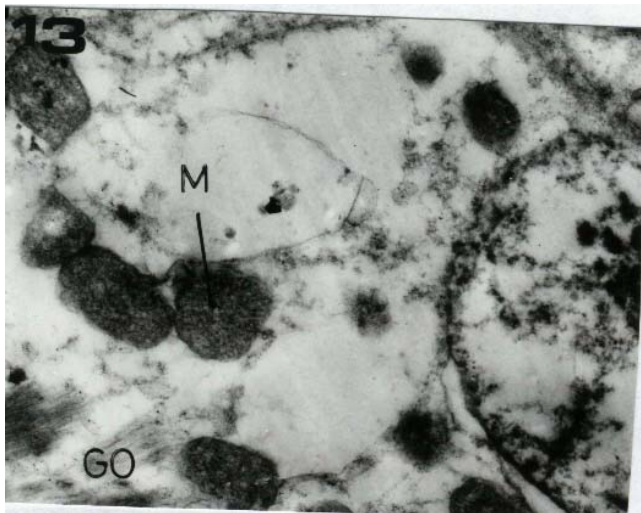


Fig. (13): Ultrastructural changes in Golgi body after treatment ( $\times 27000$ ).

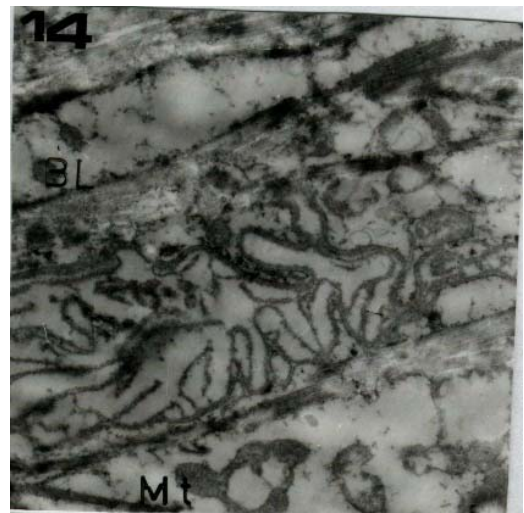


Fig. (14): Ultrastructural changes in the rough endoplasmic reticulum after treatment ( $\times 20000$ ).



Fig. (15): Ultrastructural changes of the peritrophic membrane of *C. pipiens* larva treated with the photoinsecticide ( $\times 27000$ ).

## DISCUSSION

These results show that hematoporphyrin is highly toxic to *C. pipiens* larvae. This confirms the findings of Jori & Spikes (1983) who concluded the rapid insecticidal action of hematoporphyrin can be correlated with its mode of photoinducing irreversible damage to biological systems. The results agree well with Ben Amor *et al.* (1998) who pointed out that porphyrins may represent a class of useful photoinsecticidal agents; in particular, hematoporphyrin appeared to be very active against at least two fly species, namely *Ceratitis capitata* and *Bactrocera oleae*, known to induce severe damage in various agricultural areas worldwide. Our results indicated that the photoinsecticide larvicidal activity depends on the integumental transparency of the tested larvae. The results obtained showed that the tested *C. pipiens* pupae with the hematoporphyrin were found to be healthy and this may be explained by the photoinsecticide are mainly act as a stomach poison. Ultrastructural data concerning the normal larvae structure agreed with the findings of Bakr *et al.* (1997).

Ultrastructural examination of fat cells showed that the nucleus contains heavily electron-dense chromatin condensation. Also, the cytoplasm contain numerous mitochondria. This agrees with Salama (1994).

The ultrastructure of *C. pipiens*' muscle fibres is in general similar to that of other insects. Each muscle fibre consists of a number of parallel fibrillate or sarcostyles. The fibrils are threads like with visible differentiation, and the Z line could be detected, in accordance with Nagai & Graham (1974). However, the median disc Henson's line (H- band) could not be detected, this is in accordance with Salama (1994), and also with Rossi-Durand (1991) who examined the sarcomere lengths and fine structure in three histochemical fibre types of antennal muscles in the rock lobster. He demonstrated that these fibres deviated from the typical fast structure in having long sarcomeres and in having some unusual ultrastructural characteristics (absence of the H-band and presence of Z-tubules). The ultrastructural data revealed that a delicate peritrophic membrane lines the midgut; this is in accordance with Salama (1994).

Our results showed that the normal lamellate cuticle was heavily affected in the treated larvae taking the shape of amorphous cuticular region. The endo- and exo-cuticle were not distinguishable. It was found that the epidermal cells underneath the cuticle were distorted. The nuclear envelope remains reasonably intact. The cytoplasmic material was markedly deteriorated. The mitochondria exhibited a change of its appearance with irregular shapes. The fat body cells underneath the cuticle could be detected with visible vacuolisation. Muscle cells revealed the degenerated sarcomeres with gaps and vacuoles. The Z discs are irregularly shaped and are distorted. Unfortunately no supporting literature is available on the effect of photoactivated insecticides on insect cuticle.

Mid-gut cells of treated larvae appeared with cytoplasmic vacuoles. The Golgi body was found to be fragmented into small particles. The rough endoplasmic reticulum is broken down into separate vascular structures. Similar changes were observed with insect growth regulators by Bakr *et al.* (1997).

The fat body cells of treated larvae could be seen with visible vacuolisation. This may be due to the lipophilic nature of the photoinsecticide. According to Kessel & Chou (1983), the photoinsecticide damage should especially occur at the level of the membranous systems owing to the lipophilic nature of hematoporphyrin.

The muscle cells revealed the degenerated sarcomeres with gaps and vacuole. The Z discs have irregular shape and are distorted. These findings are in agreement with Bakr *et al.* (1997) using insect growth regulators against insect muscles. Hafez (1991) used fenitrotheion and d-phenothrin on *Schistocerca gregaria* and Salama (1994) used sumicidin on *C. pipiens* larvae. The mitochondria appeared with irregular shapes, obviously impairing their respiratory function. This finding was supported by Ulrich Schliiter (1980) who reported that the enlargement and deformation of mitochondria are presumably caused by starvation. Hafez



(1991) reported that the mitochondria coalesced, fused and became deeply stained after fenitrothion treatment; Salama (1994) showed that mitochondria swell following Sumicidin treatment.

Mid-gut cells of treated larvae appeared with cytoplasmic vacuoles. Vacuole formation is a cellular defence mechanism against cytotoxins which segregates the substances in vacuoles and prevents them disrupting cellular metabolism. The appearance of vacuoles in pathological conditions have been also reported by Pilat (1935) who found that poisons caused the appearance of vacuoles in locusts; in these vacuoles various substances accumulated, and this represented the initial stage of disintegration. The rough endoplasmic reticulum then breaks down into separate vascular structures, and the Golgi body fragments into small particles. The peritrophic membrane is also affected by the photoinsecticide. Similar results were reported by Charles & de Barjac (1983) who reported ultrastructural changes in the midgut of *Aedes aegypti* larvae treated with *Bacillus thuringiensis* var. *israelensis* crystals. Ingestion was followed by mid-gut epithelium disruption. Earliest changes consist of an enlargement of intra-and intercellular spaces in the basal region of the cell. Endoplasmic reticulum disintegrates by forming spherical structures, which increase in size during intoxication. Mitochondria at first condensed, and then become swollen with the disappearance of internal cristae. In the cardia cells, which secrete the peritrophic membrane, the Golgi apparatus may produce electron-dense secretion vesicles; in this event, the peritrophic membrane assumes an abnormal configuration. Before complete breakdown a cellular hypertrophy is observed. Davidson (1979) showed that the rapid loss of cellular integrity in *C. pipiens* larvae having ingested *Bacillus sphaericus* indicated that mosquito larvae digested those bacteria. The outer cells wall layer and cytoplasmic ground substance disappeared rapidly. Digestion probably released the toxin from bacterial cells. A refractory period of 7-10hr occurred between ingestion of cells and the appearance of major histological changes. All bacteria were confined within the peritrophic membrane until after host death. Comparing the effect of photoinsecticide on *C. pipiens* larvae with simple insecticide, insect growth regulators and the microbial control agents reveals that in fact their mode of cell destruction is strikingly similar.

The results confirm the insecticidal efficiency of hematoporphyrin (a photoactivated insecticide) against *C. pipiens* larvae. Thus, its use may be recommended in pest-control management strategies to reduce costs and reduce the pesticide impact in the environment.

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