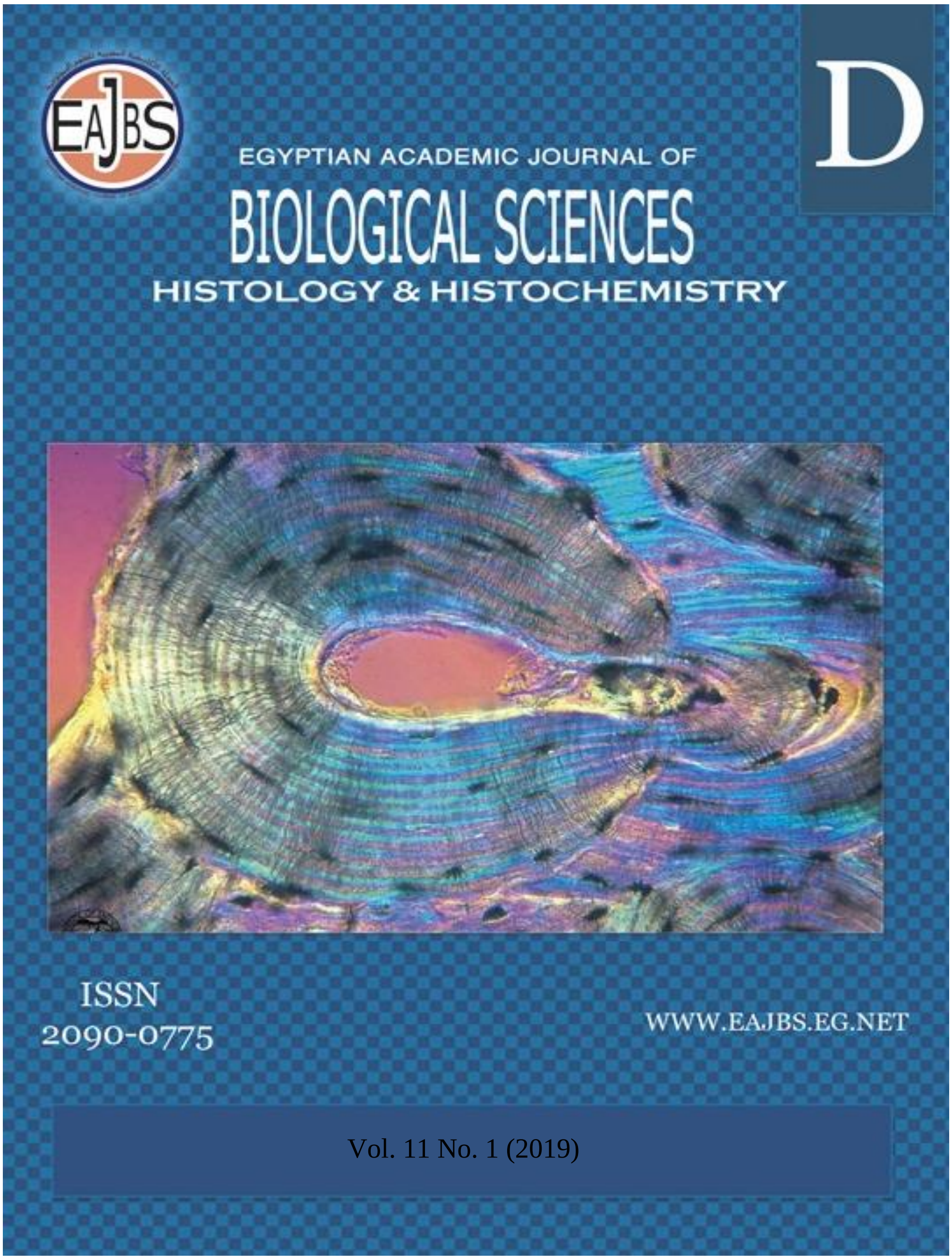

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Lufenuron–Induced Ultrastructural Changes in the Larvae of *Musca domestica* (Diptera: Muscidae)

Shimaa S. Ahmed; Amira E. Abd elhamid;and Shaimaa A.A. Mo'men*
Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt
#E.Mail : shaimaa_momen@hotmail.com

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ABSTRACT

The present work designed to assess the effect of Chitin Synthesis Inhibitor (CSI) Lufenuron against the larvae of the housefly, *Musca domestica* L. (Diptera: Muscidae). Lufenuron was applied at different concentrations (10, 100, 1000 and 2000 ppm) to larval diets. Results recorded a high toxic effect and the determined sub-lethal doses (LC_{50}) were 254.379, 124.108, and 70.109 ppm for the first, second and third larval instars, respectively. The ultrastructure investigations of normal and treated *Musca domestica* larvae were studied to evaluate the effect of tested compounds on cuticle, muscles, and mitochondria. Cuticle structure in untreated larvae consists of the epicuticle exo, endocuticle and epidermis layer. Muscles are made up of a number of fibers; each fiber is bounded by the sarcolemma. The whole fibers appeared to be transversely striated. The essential features of these patterns are the Z, H, A bands and I. Mitochondria are a doubled-membrane-bounded body-containing matrix. While Lufenuron-treated larvae revealed reduced size of both endocuticle and epithelial layer was not arranged in a single layer. The epicuticle exo, endocuticle, was not obviously distinguished. Disorganization of muscle components as compared with normal muscles were the bands and zones are less defined.Mitochondria showed irregular shapes or become disintegrated. These outcomes recommended using Lufenuron in *Musca domestica* control programs combined with the programs of integrated pest management.

INTRODUCTION

The house flies transporter a lot of human and animal intestinal diseases, including protozoan, bacterial infections, other than viral and rickettsial infections. Flies also transfer eye diseases and infectious wounds or skin diseases (Greenberg, 1965). To control *M. domestica* many insecticides have been used directly or indirectly. Houseflies have developed resistance to such insecticides. Consequently, it is important to study alternatives and more satisfactory methods of insect control. The use of bioinsecticides which depend on bacteria, viral, fungi, botanical pesticides, and insect growth regulators (Rao *et al*., 1990; Mourad *et al*., 2008, and Atwa *et al*., 2010).

Insect growth regulators (IGRs) are recognized as an insect developmental inhibitor that prevents normal metamorphosis of immature stages to the adult stage. For example, a) chitin synthesis inhibitors as (Buprofezin, Hexaflumuron, and lufenuron) b) ecdysone agonist as (Tebufenozide) c) juvenile hormone analog as (pyriproxyfen). Several studies have examined their effect on several insect pests (Wang & Tian 2009, Abo El-Mahasen et al. 2010, Gelbic et al., 2011, Abdel Rahman 2017, Muhammad *et al*, 2019).

Some (IGRs) interfere in inhibiting chitin synthesis of insect cuticle; cuticle gives support and protection through its rigidity and hardness thus consider as a target organ for controlling. Chitin is a constituent of arthropods, principally the body wall. It is a polymer of β-1,4-N-acetyl-D-glucosamines (GlcNAc). The Polymerization of its monomer (GlcNAc) into chitin is catalyzed by chitin synthase, using Uridine 5′-diphospho acetylglucosamine (UDP-GlcNAc) as a substrate (Merzendorfer and Zimoch, 2003). Some composites have been synthesized and formulated for commercial use in the control of insect pests (Tomlin, 2000; Dhadialla *et al.*, 2005, Abdel Rahman 2017). This offer alternative compounds to the conventional larvicides known as Chitin synthesis inhibitors (CSIs). These compounds are highly biodegradable safe for non-target organisms; action is target pest-specific (Sontakke *et al*., 2013). The use of CSIs has been widely observed to weaken reproduction and development of insect species (Arthur and Hartzer 2018; Arthur *et al*., 2009; Malik *et al*., 2017; Muhammad, *et al*., 2019). These compounds have been investigated for their ovicidal effects against several stored grain insects by treating either adult or the commodities in which eggs were placed (Trostanetsky and Kostykovsky 2008; Trostanetsky *et al*., 2015).

These compounds suppress the development of the whole life cycle on insects (Verloop and Ferrel, 1977 and Gelbic *et al.*, 2011). However, these compounds disturb the hormonal balance in insects, by causing physiological disorders, such as alteration of carbohydrates (Ishaaya and Ascher, 1977); inhibition of DNA synthesis (DeLoach *et al.*, 1981); cuticular lipids (Salama *et al*., 1976), and increase in phenoloxidase activity (Deul *et al*., 1978).

Lufenuron (Match 10%) is an insect growth regulator of the benzoylphenylureas (BPUs) group basically a chitin synthesis inhibitor (CSI). It has low toxicity and persistence against non-target organisms, therefore, studies of effects of CSI against insects can influence application of synthetic insecticides and reduce unsafe effects. It has the properties of juvenile hormone (JH) as well as ecdysteroid agonists. Moreover act on the incorporation of N-acetyl glucosamine monomer into chitin in the integument, causing the formation of abnormal new cuticle and death of the insect (Nakagawa *et al*., 1996, Nakagawa and Matsumura 1993, 1994). It has been applied successfully against many pests owing to its classic larvicidal effect and its ability to inhibit the chitin biosynthesis and consequently disturbs the integrity of the insect cuticle (Pener and Dhadialla 2012, Abdel Rahman 2017, Muhammad, *et al*.,2019)

The present study was carried out to examine the ultrastructural malformations obtained when Lufenuron was applied against a serious public health pest, *Musca domestica* larvae as an applicable tool in programs of integrated pest management (IPM).

MATERIALS AND METHODS

Insect Rearing:

Musca domestica L. colony was obtained from the Medical Insect Research Center, Dokki, and Giza. The adults were allowed free access to sugar and cotton pads soaked in milk powder dissolved in water. Larvae were reared according to the method described by (Pavela, 2008) on a mixture of sterilized bran (38 g), milk powder (2g) and water (60 ml), and maintained at 27 ± 2 °c and 70±5 % RH.

Tested Compound:

Lufenuron (Match 10 % EC), N[2,5, dichloro-4-(1,1,2,3,3,3-hexafluoro proxy)- phenyl/amino]-2,6-difluorobenzamide.

Biological Study:

All tests were carried out in laboratory conditions of 27 ± 2 °C and 70 ± 5 % relative humidity. Larvae were allowed to feed on medium containing different concentrations (10, 100, 1000 and 2000 ppm) of Lufenuron (CSI). Control groups were fed on normal medium. Each concentration of the tested CSI and the control group were replicated 5 times each containing 20 larvae of the 1st instar. Mortality was recorded until pupation. The selected concentrations were determined according to (Abo El-Mahasen *et al*., 2010).

Ultrastructure Study:

To determine the effects of the IGR (Lufenuron) on chitin synthesis, muscle and mitochondria the following experiments were conducted. Histopathological and Ultrastructure effects in the chitin synthesis, muscle tissue induced in last larval instar of *Musca domestica* resulting from 1st instar larvae treated with Lufenuron. The experiments were performed in two groups of laboratory reared house fly larvae. The first groups were fed by Lufenuron-medium mixture at LC_{50} concentrations of Lufenuron. The second group was fed on a normal medium at the same time. They were held in an incubator at 27°c. At the last larval instar (72 hr after treatment), numbers of larvae were transected, fixed as soon as possible 3%phosphate buffered glutaraldehyde (PH 7.3) for 2 hr. After two rinses in the buffer (for a period of 4 hours) the specimens were postfixed in 1% buffered osmium tetraoxide for 1 hr at 4°c. The tissue pieces were washed twice in buffer for 30 minutes. The specimens were then dehydrated in ascending grades of ethanol, 50 %, 70%, 80 %, 90% and absolute. Then were cleared in toluene for 10 minutes. The specimens were embedded in the resin of choice Epon. Semi-thin sections were cut from these blocks ‘’stained with toluidine blue’’ and were examined by the light microscope (Radwaan *et al*., 2008).

Statistical Analysis:

Data obtained from the susceptibility test were estimated using "LdPLine®"software, [http://embakr.tripod.com/ldpline/ldpline.htm].

RESULTS AND DISCUSSION

In the present study, the larval mortality of *M. domestica* was monitored through the larval duration (1st, 2nd, and 3rd instar larvae) following treatment by feeding using different concentrations of Lufenuron (10, 100, 1000 and 2000 ppm). Results represented in (Table 1) revealed that larval mortality was dose-dependent. The larval mortality was significantly increased by increasing the dose of the treatment. Concentrations of 2000 ppm resulted in over 85% larval mortality through larval duration. Our investigation is in accordance with (Abdel Rahman 2017, and Muhammad *et al*.,2019).

Many studies have been tried to apply different types of IGRs against the housefly (Webb and Wildey, 1986, Howard and Wall 1995, and Huseyin *et al*., 2019). Medina *et al*. (2002) reported that the differences in the toxicity of IGRs depend upon penetration through the cuticle, distribution inside the insect body and excretion. Chitin synthesis inhibitors (CSI) is a category Insect growth regulators that have been found to be very effective for killing immature stages of a wide variety of insect pests (Huseyin *et al*., 2019). Lufenuron (CSI) has lipophilic properties enables it to interfere with the exoskeleton chitin by contact.

The recorded values of LC_{50} were decreased significantly from instar to the subsequent one. Usually, the earlier instars were found to be highly sensitive to the tested compounds than older ones. The obtained low values of slope reflect the similar response of the subsequent instars to the different concentrations of Lufenuron; this observation was in the agreement (Badr 2000, Culter *et al*., 2005, Han *et al*., 2006, and Bakr *et al*., 2010).

Table 1: Effect of different concentrations of Lufenuron on the larval mortality of *M. domestica* through the larval duration.

Concentration (ppm)	Percent mortality (%) of *M. domestica* larvae		
	1st instar larvae	2nd instar larvae	3rd instar larvae
Control	0.0	0.0	0.0
10	17.06	22.76	27.53
100	39.25	44.10	53.74
1000	50.82	66.18	70.41
2000	87.04	91.42	96.91
Slope	0.7197±0.0789	0.7688±0.0788	0.7819±0.0795
$\chi 2$	20.153	11.444	17.156
LC_{50}	254.379	124.108	70.109
g**	2.2394	1.1142	1.6432
P*	0	0.003	0.0002

N = 5 replicates per test.
* P < 0.05 means significantly differed
** Means g > 0.4, so lower and upper limits were not calculated.

Our data obviously indicated that Lufenuron may be promising for the larval control of *M. domestica*. Furthermore, it was found that the feeding application method had high larvicidal activity at the higher concentrations and can be used in housefly control programs.

Ultrastructure study of the normal larval cuticle (Fig. 1a) showed that it is differentiated into two major regions, an inner region which contains chitin and forms the bulk of the cuticle and the thinner outer epicuticle which contain no chitin. The cuticle is a secretion of the epidermis. The chitinous cuticle as it is first secreted is known as procuticle, but later the outer part becomes tanned and sclerotized to form exocuticle and the inner undifferentiated parts are called endocuticle. Although, the cuticle of the last larval of *Musca domestica*, resulted from 2-days old larvae fed on Lufenuron –medium mixture at LC_{50} concentration (Figs. 1b, & c). Revealed darkening and blackening in epicuticle and exocuticle, reduced size of endocuticle and epithelial layer not arranged in single layer. In some larvae the epi and exocuticle are not distinguishable. Our observations are in agreement with (Hegazy and Degheele, 1992) where *Musca domestica* larvae fed on a diet treated with Diflubenzuron exhibited disruption in the formation of chitin microfibres and the procuticle lacked lamellae.

Muscles of the normal larvae (Fig. 2a) are made up of a number of fibers, each fiber is bounded by the sarcolemma which comprises the plasma membrane of the cell plus the basement membrane. The characteristic features of muscle cells are the presence of myofibrils embedded in the sarcoplasm and extending continuously from one end of the fiber to the other. All filaments in the fiber tend to be aligned so that the whole fiber appears to be transversely striated. The essential features of these striations are the Zdisc which runs across the fiber at regular intervals. Other striations are the isotropic (I band) which is bisected by (Z-line), and anisotropic (A- band) is the denser and is bisected by narrow light band the (H band). Ultrastructure examination of the muscles of treated larvae showed degenerated muscles, resulting in disorganization of components as compared with normal muscles where the bands and zones are less defined (Fig. 2b).These investigations were similar to that of (Al-Zeeb *et al*., 2018).

Mitochondria are considered as the powerhouse of the cell. It is a doubled-membrane-bounded body containing a matrix. Ultrastructural examination of Mitochondria of normal larvae (Fig. 3a) shown that they are always in close contact with fibrils. This organelle is bounded by two membranes; appear as oval or spherical organelle. After Lufenuron treatment they appeared in irregular shapes or become disintegrated leaving only remnant (Figs 3b, & c).

These histopathological changes agree with those results on other insect species such as *Agrotis ipsilon* (Abdel-Aal, 1996), *S. littoralis* (Hassan, 2009). Similarly, benzoylphenyle ureas induced a great disturbance in cuticle deposition of *S. littoralis* larvae (Hegazy, 1990). Histopathological changes of the integument revealed the effect of chitin synthesis inhibitors (CSIs) in various insect species belonging to several orders (Bakr *et al*., 1997; Sokolova *et al*., 2003, Hassan 2009, Al-Zeeb *et al*., 2018).

The chitin synthesis inhibitor Teflubenzuron disrupted the cellular structure of the integument and component of the cuticle in treated desert locust 5th instar nymphs (Al-Mokhlef *et al*.,2012). *Leptinotarsa decemlineata* larvae treated with the Diflubenzuron exhibited thinner lamellae and a procuticle thickness less than half of that of the untreated larvae, with no lamellar appearance to the procuticle, (Hegazy *et al*., 1989). In *Chrysodeixis chalcites* larvae treated by Tebufenozide, a dramatic increase in endoplasmic reticulum, increase in the volume of nucleus,and the presence of numerous oval and elongated mitochondria was observed (Smagghe *et al*. 1997). Spherical structures and extra layers were observed between the endocuticle and epidermis cells in *Tenebrio molitor* and *Mythimna separate* larvae treated with DFB (Ren *et al*., 1988).

In conclusion, it is recommended to use Lufenuron in *Musca domestica* control programs in coordination with the programs of integrated pest management

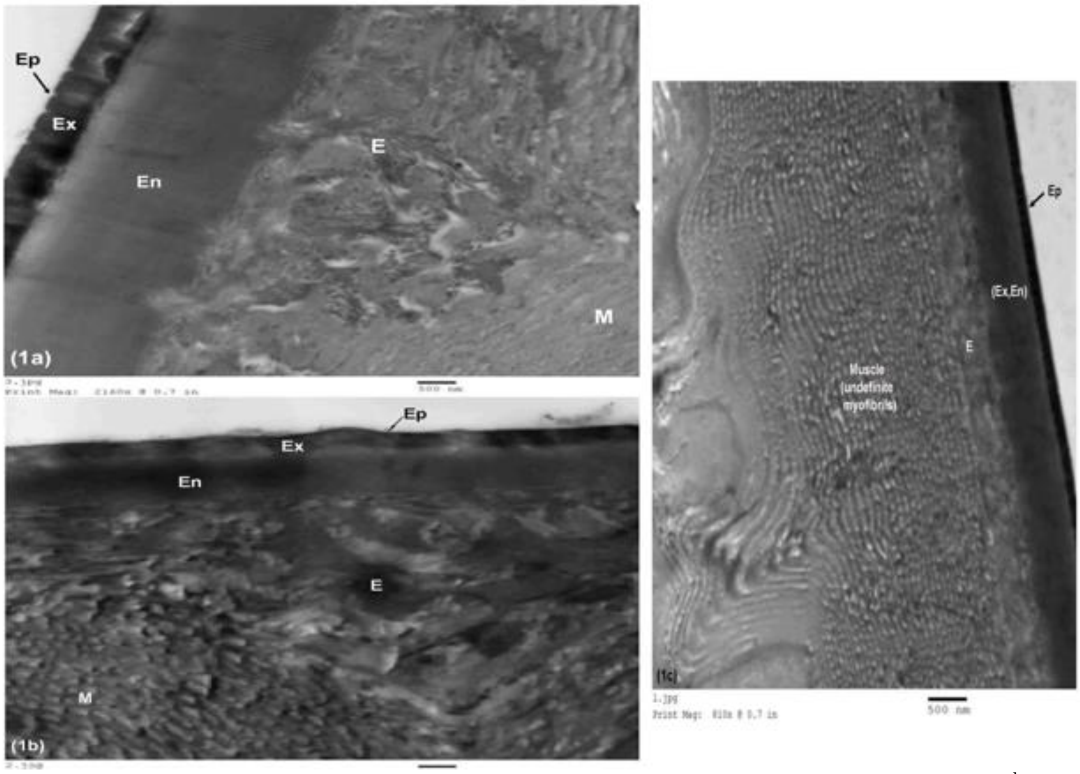

Fig.1: **(1a)** Electron micrograph of longitudinal section of the cuticle of untreated 3rd larval instar of *Musca domestic*; (Ep) Epicuticle, (Ex) Exocuticle, (En) Endocuticle , (E) Epidermis layer, (M) Muscle, appeared in their normal shape.(500nm)

(1 b, c) Electron micrograph of longitudinal section of the cuticule of Lufenuron treated 3rd larval instar of *Musca domestic*; (Ep) appeared dark and black. (Ex) and (En) reduced in size. (Ep) and (Ex) are not distinguishable. (E) are not arranged in single layer, (M) presented as undefined myofibrils.(500nm)

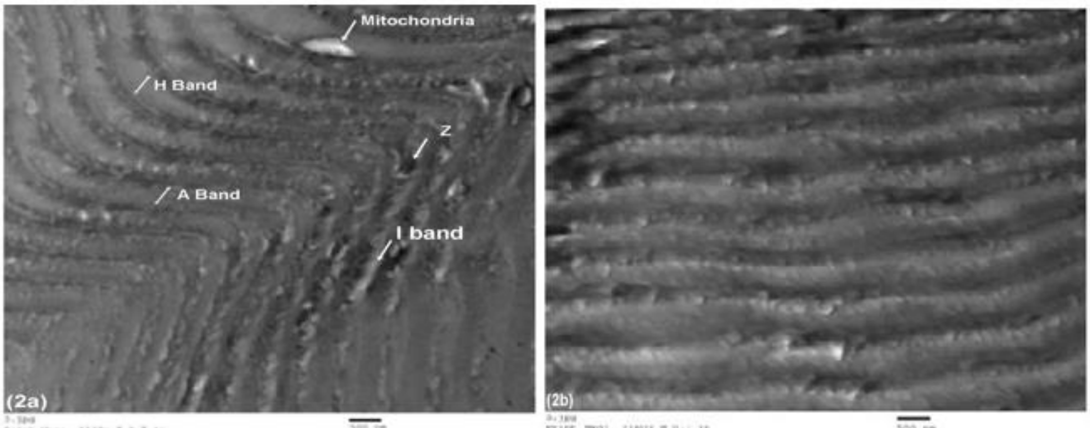

Fig. 2: **(2a)** Electron micrograph of longitudinal section in the muscle of untreated 3rd larval instar of *Musca domestic*; showing (I band), (Z-line), (H band) and (A band).

(2b) Electron micrograph of the longitudinal section in the muscle of Lufenuron treated 3rd larval instar of *Musca domestic*; presented in disorganization of components, the bands, and zones are less defined.

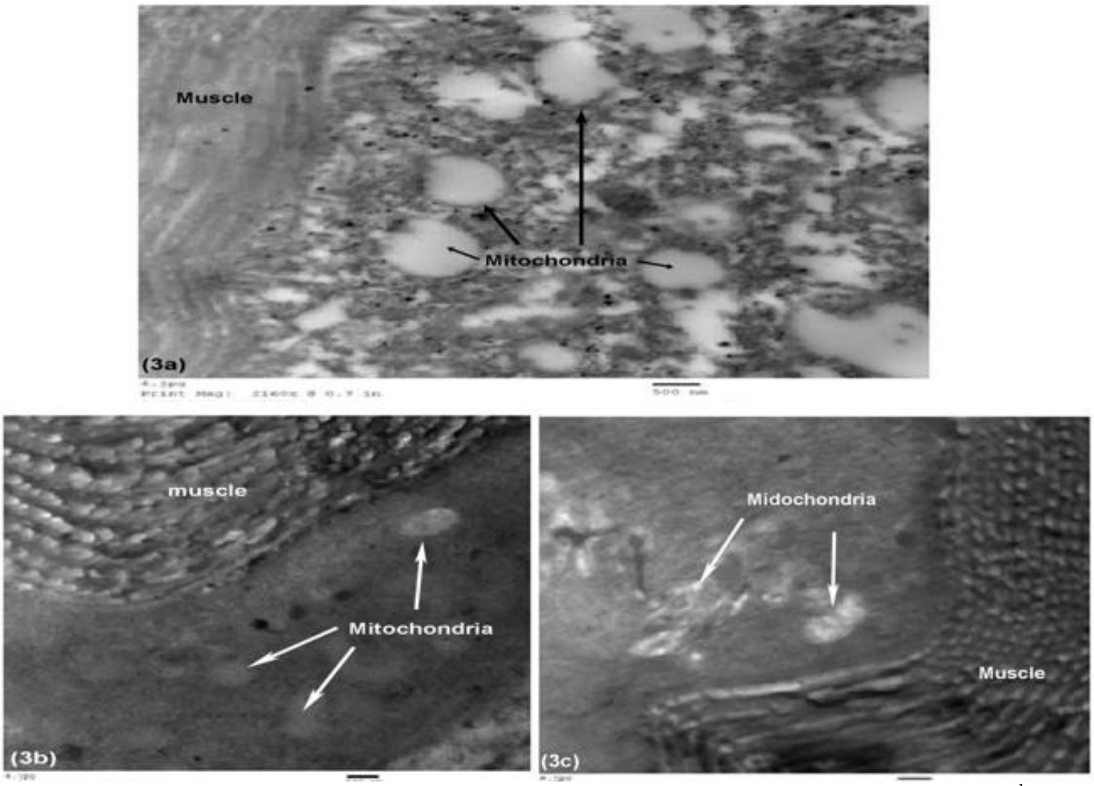

Fig. 3: **(3a)** Electron micrograph of longitudinal section of the muscles of untreated 3rd larval instar of *Musca domestic*; Mitochondria are in close contact with fibrils, appeared as an oval or spherical organelle. (500 nm)

(3 b, c) Electron micrograph of longitudinal section of the muscle of Lufenuron treated 3rd larval instar of *Musca domestic*; Mitochondria appeared in irregular shapes and disintegrated leaving the only remnant. (500 nm)

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ARABIC SUMMARY

التغيرات المُستحدثة باليوفينرون في التركيب الدقيق ليرقات
***الماسكا دومستكا* (ديبترا: ماسكيدي)**

شيماء صلاح أحمد ، أميرة السيد عبد الحميد ، شيماء أحمد أحمد مؤمن

[1] قسم علم الحشرات، كلية العلوم، جامعة عين شمس، القاهرة، مصر.

تم تصميم هذا العمل لتقييم تأثير مثبط تخليق الكيتين (CSI) ضد يرقات الذبابة المنزلية ، *الماسكا دومستكا* (ديبترا ماسكيدي)، تم إختبار نشاط الوفينورون كمبيد لليرقات بإستخدام طريقة التغذية خلال مرحلة الطور اليرقي. تم تطبيق الوفينورون بتركيزات مختلفة (10 ، 100 ، 1000 و 2000 جزء في المليون) على الوجبات الغذائية اليرقات لمدة 72 ساعة. سَجلت النتائج تأثيرا عالي السُمية وتم تحديد الجرعات النصف المميتة (LC_{50}) 70.109 ؛124.108 ؛254.379جزء في المليون ، على التوالي خلال طور اليرقات. تسبب هذا العامل الحيوي الذي تم إختباره في العديد من االمتغيرات النسيجية. تمت دراسة التركيبات الدقيقة ليرقات *الماسكا دومستكا* الطبيعية والمُعالجة لتقييم تأثير المركب المختبر على الهيكل والعضلات والميتوكوندريا. يتكون هيكل اليرقات غير المعالجة من وطبقة داخلية (اندو) وخارجية (ايكزو) وطبقة البشرة. تتكون العضلات من عدد من الألياف ، وتحد كل ألياف بساركولما. يبدو أن الألياف بأكملها مخططة بشكل مستعرض. الميزات الأساسية لهذه الأنماط هي نطاقات Z-band و I و H و A. الميتوكوندريا عبارة عن جسم مزدوج الغشاء محاط بمصفوفة. في حين ، اظهرت اليرقات التي تم معاملتها بالوفينورون إنخفاض حجم كل من بشرة إندو والطبقة الظهارية ، ولم تكن مرتبة في طبقة واحدة. لا يمكن التمييز بوضوح بين الطبقات ، إضطراب مكونات العضلات مقارنة بالعضلات الطبيعية ، حيث كانت الالياف و النطاقات أقل تميزًا. كما ظهرت الميتوكوندريا في أشكالًا غير منتظمة أو متتفككة. أوصت هذه النتائج بإستخدام لوفينورون في مكافحة*الماسكا دومستكا* بالتضامن مع برامج المكافحة المكتاملة للافات.