Ultrastructural Observations on the Gonads and Neurosecretory Cells of Schistocerca gregaria After Treatment with Lufenuron (CGA-184699)

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Source: Journal of Orthoptera Research, 21(2) : 141-148

Published By: Orthopterists' Society

URL: https://doi.org/10.1665/034.021.0202

Ultrastructural observations on the gonads and neurosecretory cells of *Schistocerca gregaria* after treatment with lufenuron (CGA-184699)

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Abstract

Topical application of a series of concentrations of lufenuron, a chitin-synthesis inhibitor, on the neck membrane of newly molted fifth-instar desert locusts, *Schistocerca gregaria,* gave various ultrastructural changes in ovarioles, including a disintegrated follicular epithelial cell layer, vacuolated cytoplasm and the appearance of lysosomal bodies. In males, electron micrographs showed loosely disrupted testicular tissues with vacuolated testicular epithelia. In both sexes the neurosecretory cells in the pars intercerebralis exhibited trapped neurosecretion without normal liberation into their connecting nerves and the mitochondria appeared to be losing their cristae.

Key words

S. gregaria, ovaries, testes, neurosecretory cells, ultrastructure, lufenuron, chitin synthesis inhibitors

Introduction

Use of insecticides to control insect pests has many environmental, human health and economic disadvantages. Much research has been undertaken on possible alternatives, such as insect growth regulators (IGR), which are generally considered to be environmentally acceptable because they only affect systems unique to insects and certain other arthropods.

Benzoylphenyl ureas are known to be highly effective IGRs against many agricultural pests and to have a relatively low toxicity to mammals and natural enemies (Degheele 1990, Ishaaya 1990). Lufenuron (Fluphenacurn™, Match™ or CGA-184699™) is one of a group of chitin-biosynthesis inhibitors widely assessed against various harmful insect species. Lufenuron was used in Egypt against *S. gregaria* (Bakr *et al*. 2008, 2009) and in other countries on other pests: such as fruit tortrix (Charmillot *et al.* 1991, Ioriatti *et al.* 1993), cat flea (Hink *et al.* 1991, Jacobs *et al.* 2001) and the red palm weevil, *Rhynchophorous ferrugenius* (Tanani 2001). Lufenuron was shown to cause gross changes and DNA polymorphism in *D. melanogaster* (Abd-Alla *et al.* 2003). It also has an effect on *Musca domestica* (Ghoneim *et al.* 2004), *Drosophila melanogaster* (Bogwitz *et al.* 2005) and on *S. gregaria* (Bakr *et al.* 2008).

Though there have been a few studies dealing with the effect of lufenuron on the reproductive system, most concern the role of lufenuron as a chitin-synthesis inhibitor, affecting growth and development of immature stages in different insect species via stage prolongation, with inhibitory action on development, blocking of chitin synthesis, of morphogenesis and reduction of adult longevity (for reviews see Post & Vincent 1973, Slama 1974, Staal 1975, Retnakaran *et al.* 1985, Hoffman & Lorenz 1998, El-Sheikh 2002, Tunaz & Uygun 2004).

The desert locust *Schistocerca gregaria* (Forskål) causes severe damage to crops considered staple foods for humans and animals. It remains necessary to develop an effective preventive control strategy against this species, one relying upon early warning to suppress locust multiplication and prevent outbreak of the mobile swarms through an effective control against nymphal instars. The present study takes a new approach by considering the possible application of lufenuron as a disruptor of the ovaries, testes and neurosecretory cells in this significant agricultural pest.

Materials and methods

Adults and nymphs of the desert locust *S. gregaria* have been reared for several years in our laboratory at Cairo University. Laboratory stocks were housed in electrically heated wooden cages (25×25×25 cm) at a constant temperature of 30±1°C, with moderately fluctuating relative humidity (50-70%). Insects were fed on fresh clover (*Trifolium alexandrinum*) from November to May and thereafter fresh leaves of *Sesbania sesban.* Cages were supplied with suitable pots containing moistened sand for egg deposition. Hatched hoppers were transferred to 20×25-cm cylindrical glass jars. After the fourth moult, hoppers were released into large cages.

To test the effect of lufenuron on the gonads, we collected newly molted fifth-instar nymphs. A series of lufenuron concentrations was prepared (50, 100, 200, 300 and 400 ppm). For each concentration, 30 insects were tested in five replicates of six grasshoppers each. At the beginning of the stadium, lufenuron solutions (5ml) were topically applied to the neck membrane of the test insects. LC_{50} values were determined according to Finney (1971).

For TEM observations, treated ovarioles, sperm tubes and part of the brain (including the neurosecretory cells in the pars intercerebralis at the mid-dorsal line) were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.3) at 4°C for 24 h; they were then washed in three changes of the fresh buffer. Specimens were fixed in 1% osmium tetraoxide in the same buffer for 2h at 4°C, then washed in the same buffer and dehydrated in alcoholic series up to absolute alcohol. They were then removed to absolute alcohol and acetone, followed by pure acetone for half an hour each. Infiltration took place in acetone and resin (Epon 812) 2:1 and 1:1 for 4 h each. Specimens were then placed into pure resin overnight and finally, into resin blocks for 3 d at 50°C.

Semithin sections (1 nm thick) were prepared using a glass knife, then stained with toluidene blue. The specimens were examined under a normal light microscope and photographed. Ultrathin sections (90 nm thick) were obtained using a diamond knife. Sections were placed on a copper-grade mesh and stained with uranyl acetate and lead citrate for half an hour; they were then examined with a Joel 1200ExII (Japan) transmission electron microscope

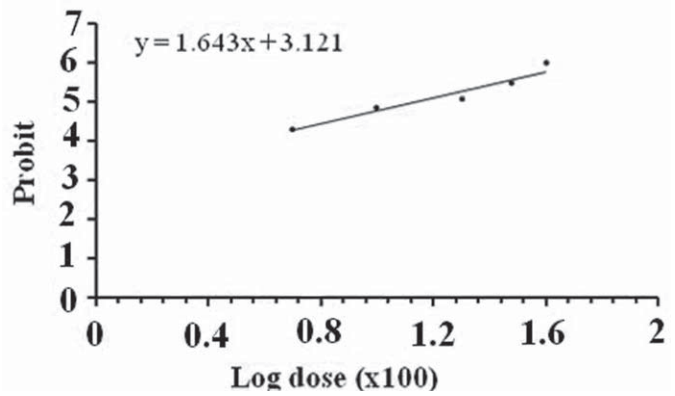

Fig. 1. Regression representing the LC_{50} value of Lufenuron concentration in the fifth nymphal instar of *S. gregaria*.

and photographed in the Electron Microscopy Unit at the Faculty of Agriculture, Cairo University.

Probit analysis (Finney 1971) was used to transform the number of insects killed by log dose of lufenuron, applying per cent mortality to the area under a normal curve and obtaining a regression line. Statistical analysis took place using Microsoft Excel©.

Results

The LC_{50} was estimated to be 138.9 ppm (Fig. 1).

Histopathological examination of treated ovarioles of adult female *S. gregaria* showed a faint yolk deposition in the oocyte, with many vacuoles extending far within it. The follicular epithelial cell layer appeared small and disintegrated: it was vacuolized with loosely arranged septa between cells, these having lost their compact shape (see semithin section of the oocyte, Fig. 2). At lower concentrations (50 ppm) the cytoplasm of the follicle cell appeared nonhomogenous and vacuolated, with many lysosomal bodies,

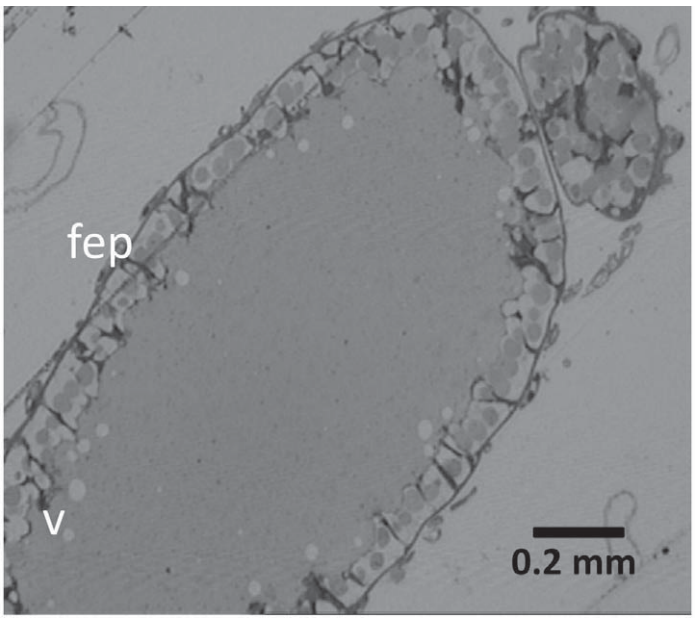

Fig. 2. Semithin section in the treated oocyte showing disorganized follicular epithelial cells (fep) with many spaces in between, appearance of many vacuoles (v) and disappearance of follicular septa from some regions.

perhaps indicating the beginning of cell lysis. Mitochondria were disintegrated, with loosely arranged or absent cristae (Fig. 3). At intermediate concentrations (100 ppm), the nuclei were enlarged, the cytoplasm shrunken, with splitting of follicular epithelia that had folded in on itself (Fig. 4). The nucleus appeared (Fig. 5) with dense accumulations of chromatin; the splitting nonhomogenous cytoplasm showed an accumulation of many differently shaped mitochondria lacking their cristae; large vacuoles also appeared.

The vacuolated follicular epithelium was broken and detached from the follicle cells, with the appearance of patches of abnormal

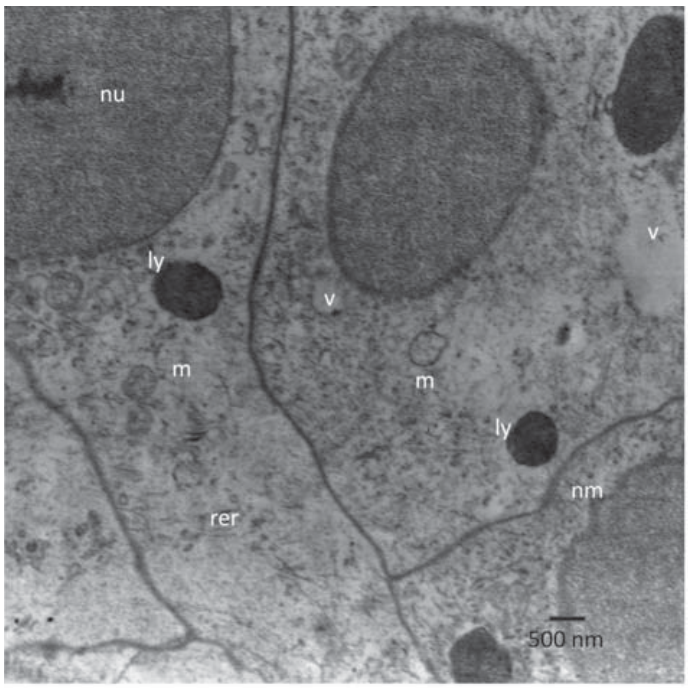

Fig. 3. Electron micrograph of treated ovariole showing nonhomogenous cytoplasm with many lysosomal bodies (ly), vacuoles (v), disintegrated rough endoplasmic reticulum (rer), mitochondria with loosely formed cristae (m) and the nucleus appearing with an irregularly folded nuclear membrane.

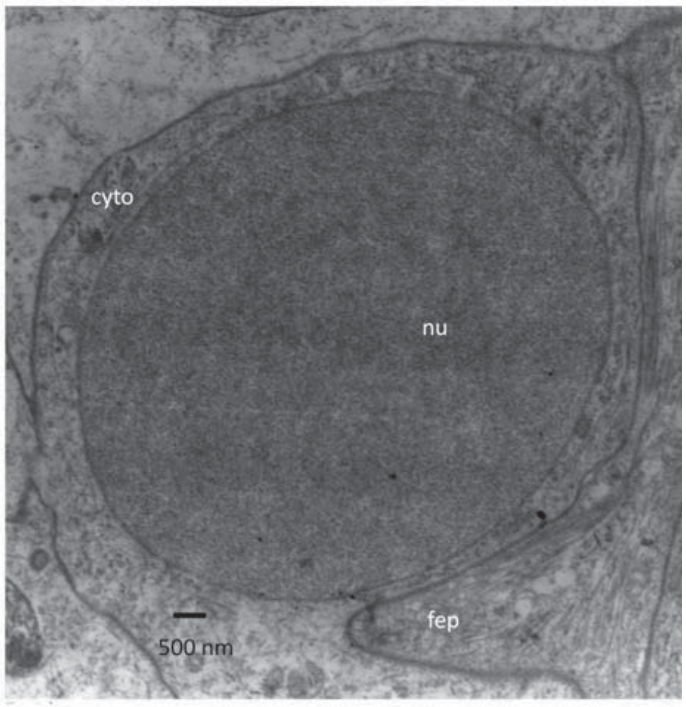

Fig. 4. Electron micrograph of treated follicle cell showing enlarged nucleus (nu) with shrunken cytoplasm (cyto) and follicular epithelium (fep) appearing folded on itself.

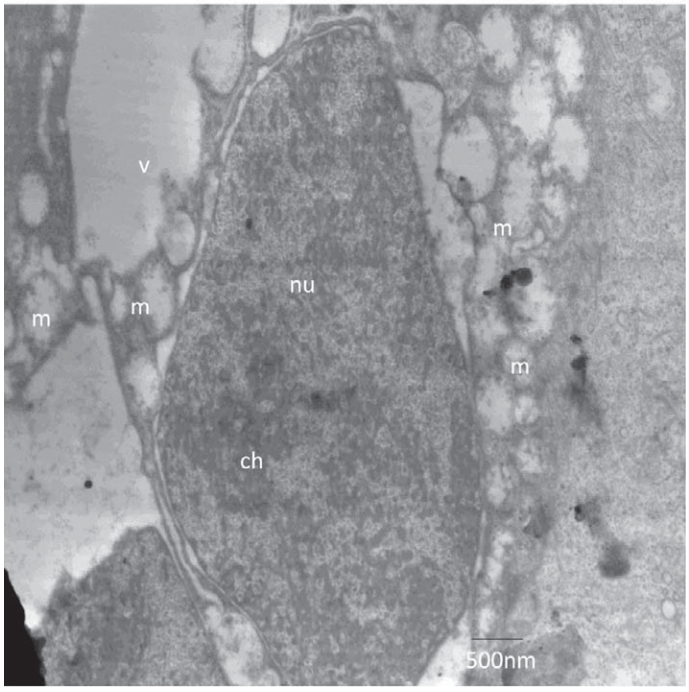

Fig. 5. Electron micrograph illustrating accumulation of chromatin in nucleus of treated oocyte; cytoplasm appears vacuolized and degenerate, with accumulation of many mitochondria losing their cristae.

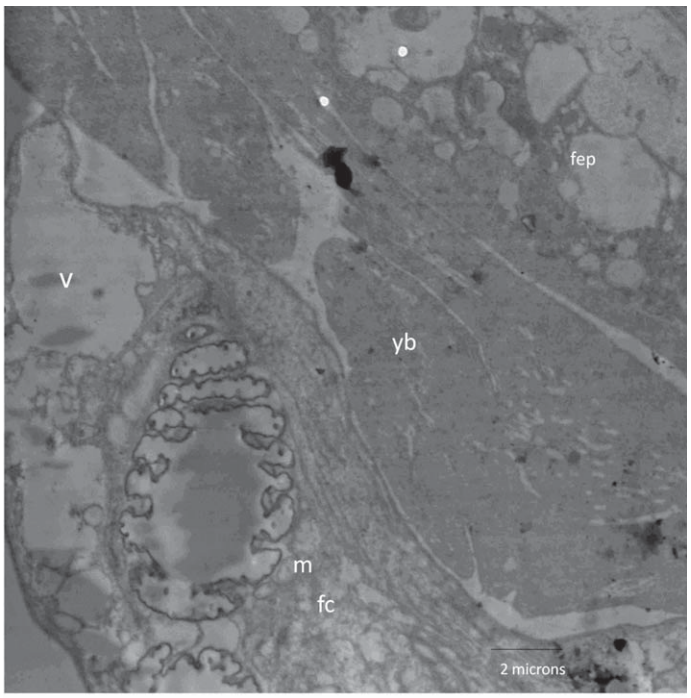

Fig. 6. Electron micrograph of treated oocyte showing vacuolized follicular epithelium (fep). Follicle cells (fc) appear disintegrated, losing their contents, with vacuoles and mitochondria without cristae (m); large yolk bodies (yb) appear detached and fractured.

yolk-shaped bodies with splits and granular lines: these bodies indicate altered integration and processing of yolk, attesting to defective and asynchronous vitellogenesis. Follicle cells degenerated and the cytoplasm appeared loose (Fig. 6) due to heavy vacuolation. The follicular epithelium failed to remain as an integral layer: follicle cells burst, scattering their contents and exhibiting large vacuoles. Lipid droplets also appeared and the mitochondria lost their cristae; the mitochondria disintegrated and less coherent smooth endoplasmic reticulum appeared (Fig. 7).

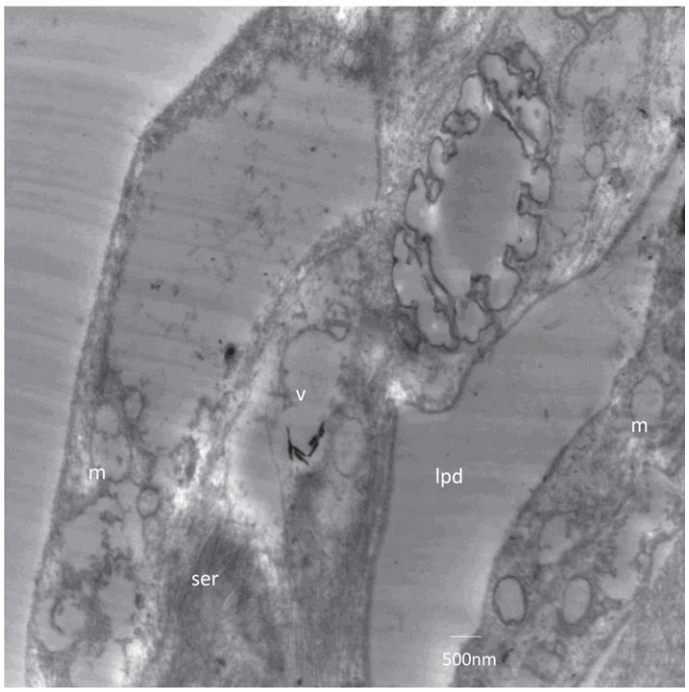

Fig 7. Electron micrograph of treated oocyte at higher concentrations of lufenuron (100 ppm) showing the bursting of follicle cells, discharging their contents and with large lipid droplets apparent instead. Many organelles appear disintegrated: mitochondria (m) and smooth endoplasmic reticulum (ser).

Ultrastructure of sperm tube treated with lufenuron.—Histopathological examination of sperm tubes of treated *S. gregaria* males showed a disorganized and loose spermatocyst. Some cells were destroyed and their size in the central part was smaller than cells at the edges. Many vacuoles appeared, testicular tissues were disrupted and loose. Spermatocytes failed in their transformation into normal spermatids which appeared disorganized (Fig. 8). Cytoplasm was disintegrated with scattered abnormally shaped organelles; mitochondria appeared with traces of cristae, Golgi bodies disintegrated with collapsed vesicles, and vacuoles and lysosomal bodies also appeared (Fig. 9). The testicular epithelia became thickened, vacuolated and detached from the peritoneal sheath and testicular debris increased. The tissues appeared to lose their consistency, with clear cell damage and total lyses (Fig.10). In the maturation zone, sperm bundles appeared degenerated and scattered spermatids were thinned out, with incomplete transformation into mature spermatozoa. Some of them appeared without organized division into head and tail and with vacuoles scattered. Some spermatids appeared in an incomplete state (Fig. 11).

Ultrastructural effects upon the cerebral neurosecretory cells treated with lufenuron.—Neurosecretory cells in the central nervous system control many major physiological events in the postembryonic life of insects. Histopathological examination of the neurosecretory cells found a neurosecretion trapped inside the cells, despite it having passed to their axons. This secretion appeared in two forms (Fig. 12): minute vesicles each surrounded by a membrane or as a large body arising from the heavy accumulation of neurosecretory material inside the neurosecretory cell: cells may be separated into these two groups.

Cells appeared loosely associated and irregular in shape. The

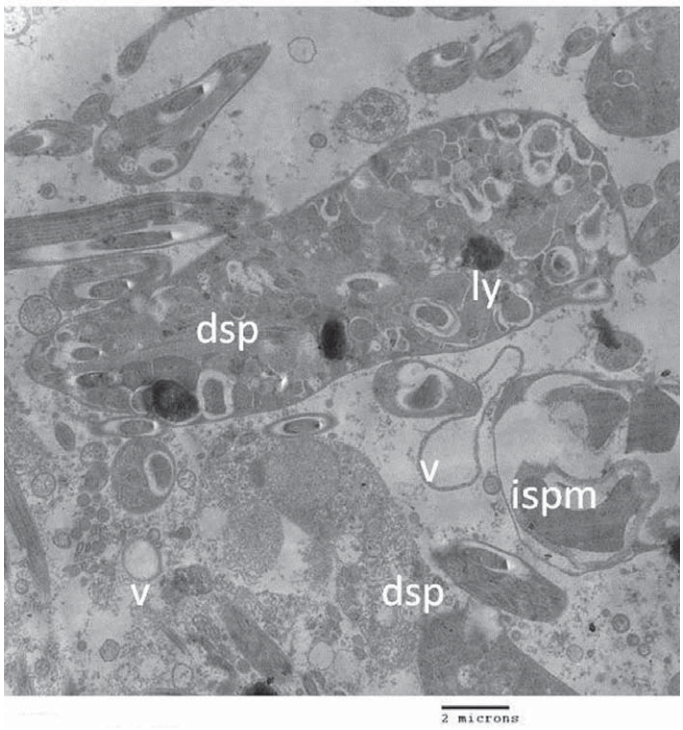

Fig. 8. Electron micrograph of treated male showing appearance of disorganized spermatocyst (dsp) with incomplete formation of spermatids (ispm).

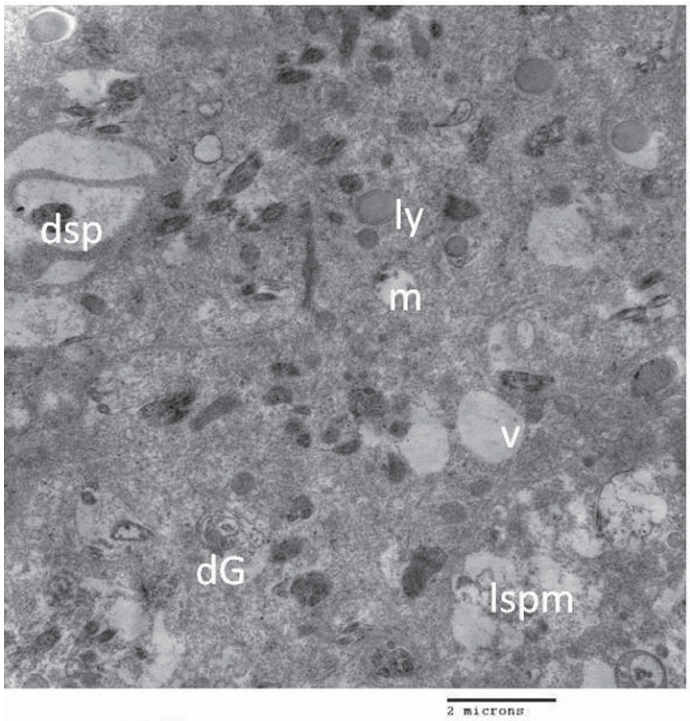

Fig. 9. Electron micrograph of treated spermatocytes showing loose (lspm) and disorganized (dsp) spermatocysts and disintegrated Golgi complex (dG), mitochondria (m) losing cristae, formation of vacuoles and lysosomal bodies (ly).

effect of lufenuron on cells may decrease their ability to transport to their axons (Fig. 13), so that neurosecretions accumulate inside the cells in large amounts, while their axons appear cleared, with neurosecretory material not passing to the corpus cardiacum in the normal pathway. Large vacuoles were also obvious. Mitochondria, losing their crescent cristae, took on rod shapes, enclosed by a rough endoplasmic reticulum. Golgi bodies appeared disorganized, with many scattered lysosomal bodies; these bodies are the first indication of the beginning of neurosecretory cell death (Fig. 14).

Discussion

Ultrastructure of ovarian follicle treated with lufenuron.—Of the effects of lufenuron on cell development, chromatin condensation in cell nuclei is the initial sign of cell death (Patricio & Cruz-Landium 2007). Histopathological changes in treated ovaries showing similar results were recorded by Lim and Lee (1982). They treated the grasshopper *Oxya japonica* with diflubenzuron and found a retardation of ovarian development causing an increase in the receptor tyrosine kinase present on the adjacent follicle cells. This localized signal from the oocyte to the follicle cells normally leads to dorsal follicle cell delimitation and orientation of the future dorsoventral axis of the embryo.

Similarly, Polivanova and Triseleva (1989) fed nymphs of *Locusta migratoria* in the laboratory on extract of *Ageratum houstonianum*: they found completely suppressed oocyte development.

A slight depression of oogenesis was reported in *Drosophila melanogaster* when fed a diet containing lufenuron before vitellogenesis development: egg hatching is strongly inhibited (Wilson & Cryan 1997).

The palm weevil *Rhynchophorus ferrugineus*, treated with flufenoxuron, exhibited ovarian developmental retardation, manifest as oocyte resorption, delay of follicle and oocyte development and destruction of the follicular epithelium. Delay of oocyte development gave abnormal distribution in oocyte size and shape, and resulted in the accumulation of yolk granules. This would presumably cause a decrease in fecundity and egg viability. In this weevil destruction of follicular epithelium involved degeneration, hyperplasia and necrosis in the follicular cells (Ghoneim *et al.* 2003).

Similar results were observed following exposure of *Tribolium castaneum* to another BPU, novaluron (Kostyukovsky & Trostanetsky 2006). In adult females of *S. gregaria,* Hussein *et al.* (2008) found a degeneration of ovarioles and oocytes, disintegration of mitochondria, enlarged vacuoles and fractured yolk bodies, mostly into two halves. This was after feeding the 5th instar on fresh food dipped in several concentrations of Cascade™ (flufenoxuron) and Karate™ (pyrethroids).

Observation revealed that monocrotophos mainly affected earlier stages of development in testes and ovaries of *Chrotogonus trachypterus*, which were severely damaged after a prolonged time, exhibiting contraction of ooplasm and nuclear membrane (Shakeet & Bakshi 2009). Fragmentation in the nuclear and chromatin material was also observed; the nuclei of follicular epithelial cells showed pycnosis and damaged yolk bodies. El Boki *et al.* (2010) indicate destruction of follicular epithelium involving degeneration, hyperplasia and necrosis in follicle cells, with regression in accumulation of yolk granules and with vacuolization of cytoplasm in male *Rhyncophorus ferrugineus* after treatment with chitin synthesis inhibitors flufenoxuron and botanical extract of neem. Lufenuron has a dramatic effect on the ovarian follicle, leading to a reduction in oocyte size, and in the number of chorionated oocytes; it decreases the number of eggs laid, modifies egg shape and color, reduces viability of eggs and induces the process of oosorption in *Rhodnius prolixus* (Mansur *et al.* 2010).

Ultrastructure of sperm tube treated with lufenuron.—This study is the first dealing with lufenuron as a spermatogenesis inhibitor, showing

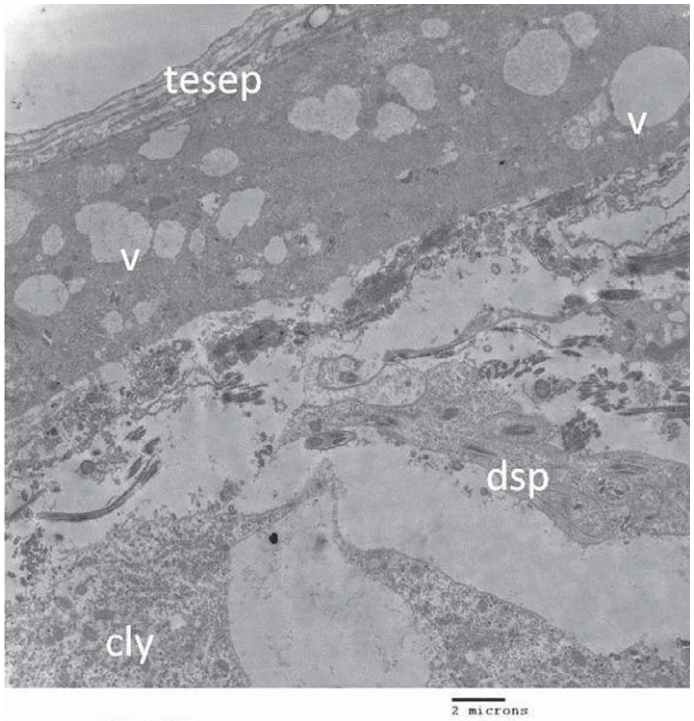

Fig. 10. Electron micrograph of treated male showing degenerate testicular epithelia (tesep) leaving many vacuoles (v) and disassociated cells (dsp), lacking formation of spermatids, with total lyses (cly) and cell damage.

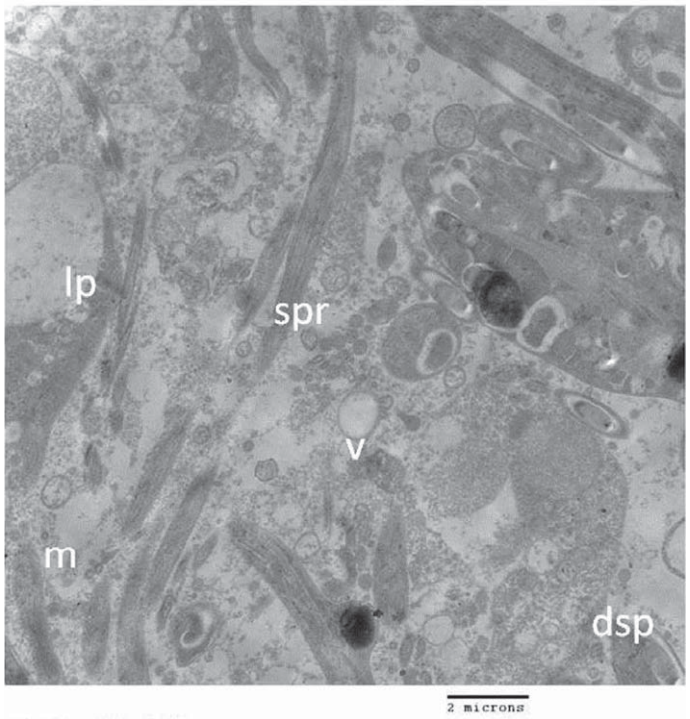

Fig. 11. Electron micrograph of treated male showing tissue losing consistency, with incomplete spermatid (spr) formation and degeneration of spermatocytes (dsp), appearance of vacuoles (v), and lipid droplets (lp).

its histopathological effects on the sperm tube of the treated and newly molted penultimate instar.

Janake (1992) found that pycnosis occurred in spermatogonia and spermatocyte cells of *Poecilocerus pictus* when gonads were treated with endosulfan. Lufenuron is genotoxic to the germ-line cells of male *Drosophila melanogaster* (Abd-Alla *et al*. 2003) and exhibits different degrees of genotoxic potential in the three successive stages (spermatozoa, spermatids and spermatocytes). Disintegration of the germ cells and degeneration of the sperm bundles in the testes were also observed by Abdel-Rahman *et al*. (2004) after treatment of the male *Pectinophora gossypiella* (Lepidoptera: Gelechidae) with azadirachtin.

Effects of gamma rays on intact spermatocyte cells, with complete cessation of the spermatogenesis process, was reported on peach fruit flies (Shehata *et al.* 2006). Swelling of the dividing cells, vacuolation in the cytoplasm, clear separation of the basement membrane from the peritoneal membrane and disintegrated spermatids were observed in *Heteracris littoralis* after azadirachtin treatment (Ghazawi *et al.* 2007).

Cytoplasm shrank and became vacuolated, some spermatogonia became hypertrophied, forming giant cells, while spermatozoa formed elongated cells containing spaces and vacuoles in male *C. trachypterus* after monocrotophos treatment (Shakeet & Bakshi 2009). Bakr *et al.* (2010) saw damage and vacuoles in zones of reduction and transformation, with degeneration and necrosis appearing in spermatids and spermatozoa after Consultat™ treatment of male *S. gregaria.* Degeneration and necrosis of testicular germ cells, spermatogonia, spermatocytes, spermatids and spermatozoa, together with inhibition in sperm bundle formation was apparent after Lufox™ treatment; El Boki *et al.* (2010) observed shrunken testicular follicles, degenerated germ cells, displaced testicular cysts, depopulation of germ cells and reduced numbers of spermatogonia in the male palm weevil *Rhyncophorus ferrugineus* when treated with a chitin synthesis inhibitor (flufenoxuron).

Due to the reproductive abnormalties appearing in adults of *S. gregaria,* it is suggested that lufenuron should be further evaluated for use in pest management programs.

Ultrastructural effects of the cerebral neurosecretory cells treated with lufenuron.—The part played by neurosecretory cells in the pars intercerebralis during moulting and metamorphosis is well known (Wigglesworth 1954) and in some insects these cells are also involved in ovarian development and oviposition (Thomsen 1952, Nayar 1958). Differences in their histological appearance at different times have often been used as evidence that neurosecretory cells are involved in developmental events (Dupont-Raabe 1952, Arvy & Gabe 1952, Highnam 1958).

While some workers have dealt with ultrastructural effects on the neurosecretory cells in the *pars intercerebralis* region of the brain, this study is the first concerned with this point under the treatment of lufenuron. Neurosecretory proteins in rapidly maturing females of *L. migratoria,* have been compared with similar aged azadirachtin-treated females. This revealed an accumulation of stainable material in the corpora cardiaca and neuropilar storage areas of the brain neurosecretory system in treated insects; this is associated with a lack of ovarian development (Rembold *et al.* 1983). The accumulation of these neurosecretory proteins was attributed to the slow rate of their transport to the neurosecretory reservoirs.

Rembold *et al.* (1984) suggest that azadirachtin interferes with the neuroendocrine system controlling ecdysone and JH synthesis, leading to a high accumulation of stainable neurosecretory material in the corpus cardiacum of *L. migratoria.*

Subrahmanyam *et al.* (1989) demonstrated that azadirachtin-treated *L. migratoria* have a very slow transport rate of neurosecretory proteins to the corpus cardiacum. On the other hand, the transport of labeled protein from the brain to the corpus cardiacum and its

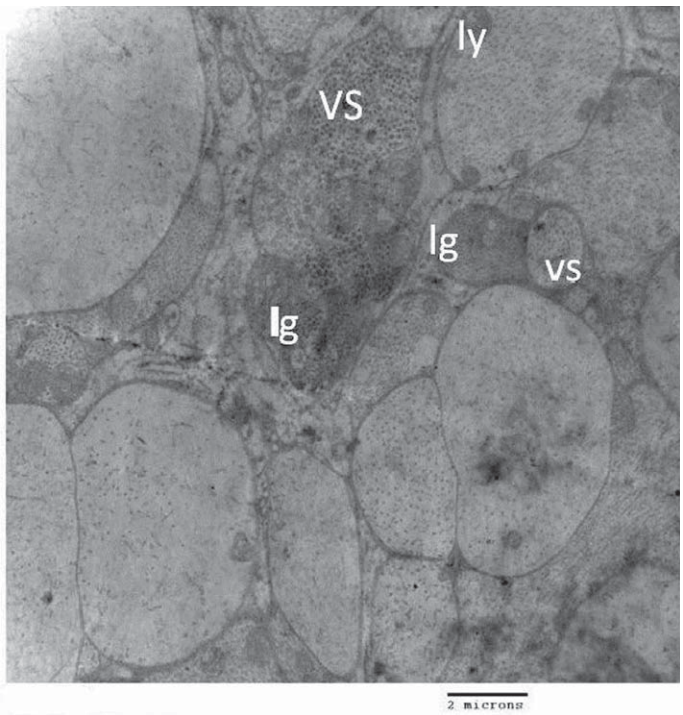

Fig. 12. Electron micrograph of treated brain showing neurosecretory cells filled with secretion in two forms: large bodies (lg) and minute vesicles (vs), and with the appearance of lysosomal bodies (ly).

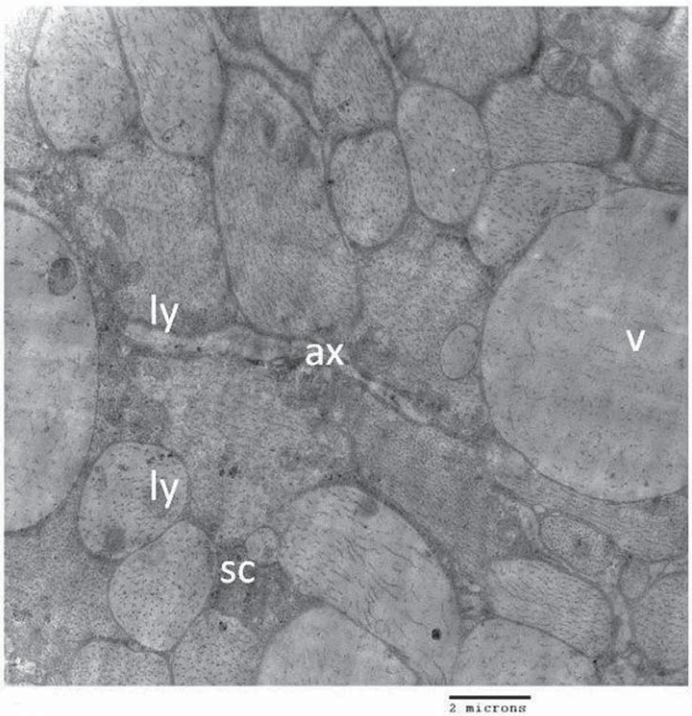

Fig. 13. Electron micrograph of treated brain showing accumulation of neurosecretion in the neurosecretory cells (sc), with the appearance of many lysosomal bodies, vacuoles (v), and with axons filled with neurosecretion (ax) (middle part only).

release has been observed at a very low level in azadirachtin-treated *L. migratoria* (Highnam & West 1971).

Guiqiang *et al.* (1997) showed that in migratory locusts, *L. migratoria*, treated with the insect growth regulator Cascade, the number of cerebral neurosecretory cells in which were scattered more large vacuoles, with aberrant and amplificate axon separation from cell bodies, decreased significantly. The membrane and inner carinulae in deformed mitochondria were absent, and the neurosecretory particles under transportation in the axoplasm decreased markedly.

The bursting of the neurosecretory cells may be due to the accumulation of the neurosecretory materials, as in *Heteracris littoralis* nymphs under azadirachtin control (Ghazawi *et al.* 2005), the inside of the treated neurosecretory cell showing globules of secretions in a densely accumulated manner.

Consulat (a chitin synthesis inhibitor) caused an inhibition of total haemolymph protein in adults of one-day old treated 5th nymphal instars of *S. gregaria*: this reduction in protein levels may be due to the destructive effect of tested compounds on some cerebral neurosecretory cells of the brain of treated nymphs. Thus insufficient stimulation of neurosecretory activity led to protein deficiency and left the amount of protein absorbed from the haemolymph insufficient for oocyte development (Bakr *et al.* 2009).

It appears that lufenuron decreases the release of neurosecretory material from the neurosecretory cells in the *pars intercerebralis* and also reduces transport to the corpora cardiaca, resulting in the accumulation of neurosecretory materials. From another perspective, lufenuron exerts its effect on the organelles inside the cell, not only leading to accumulation of neurosecretion, trapping these insect growth regulators inside their cells, but also having a drastic effect upon the cell contents. Lufenuron interferes with the neuroendocrine system, causing a 'loose' appearance in cells and reducing the turnover of the neurosecretory proteins, which leads to densely accumulated neurosecretory materials in the system.

Our results show that the damage is dose dependent; dose increases damage (Fig. 15). The gonads are more affected than the neurosecretory cells ($P \leq 0.05$).

Acknowledgements

The author is thankful to Dr. Khaled Mohammed Abdel Rahman, associate professor in the Entomology Department, Faculty of Science, Cairo University, for providing laboratory facilities.

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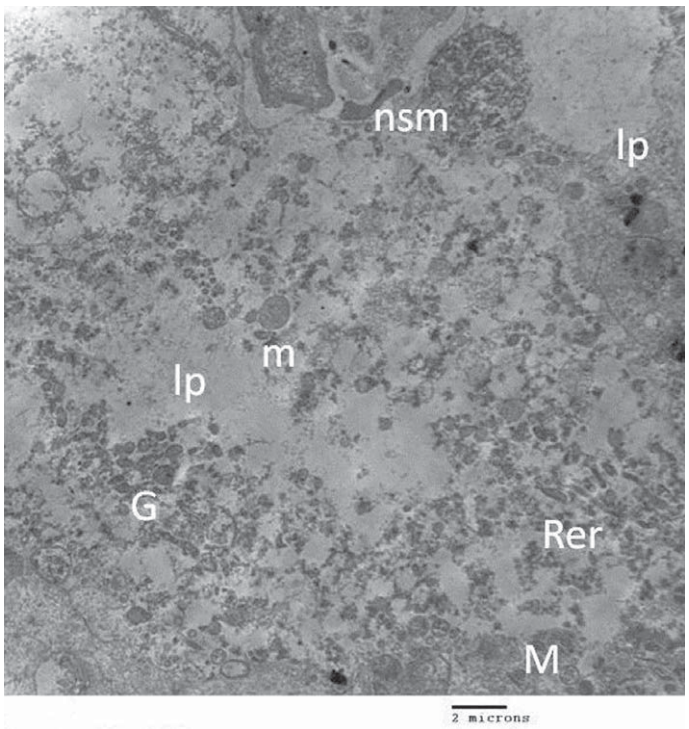

Fig. 14. Electron micrograph of neurosecretory cells in the treated brain showing accumulation of neurosecretory material (nsm) inside the cell, with the appearance of disorganized mitochondria (m) and a loose golgi complex (G); rough endoplasmic reticulum (Rer) encloses rounded and rod-shaped mitochondria (M) without cristae; lipid droplets are observed.

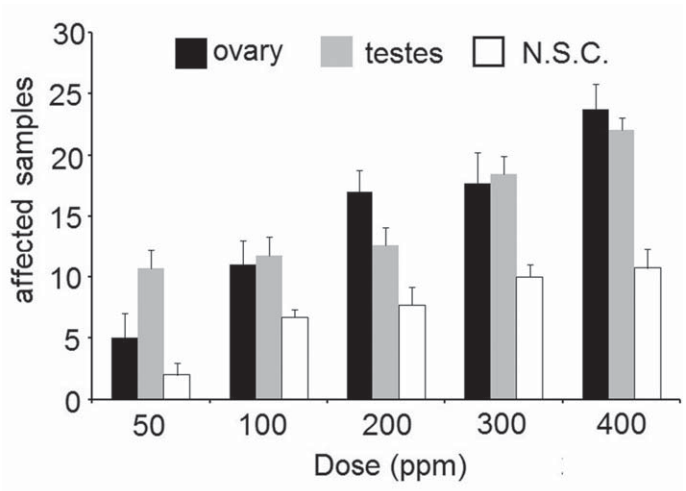

Fig. 15. Effect of different doses on target tissues, showing means and standard deviation.

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