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ORIGINAL ARTICLE

Gonadal Ultrastructure effects of some Insecticidal agents against Red Palm weevil *Rhynchophorus ferrugineus*

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ABSTRACT
The red palm weevil Rhynchophorus ferrugineus is the most important insect pest for the date palm trees in the middle east and Gulf states. The objective of this study was to investigate the pathogenicity of sublethal doses of a natural plant extract (neem) and a synthetic insect growth regulators (IGRs) (flufenoxuron) on the ultrastructure of testis of the red palm weevil. Prepupae were grouped and treated with neem extract (Neem J, 50 ppm) and a synthetic insect growth regulator (flufenoxuron, 0.05 ppm). Ultrastructure observations of spermatogenesis confirm the histopathological findings and suggest that the two IGRs act at the cellular level. Collectively, these investigation suggest that IGRs alter germ cells functions in Rhynchophorus ferrugineus by acting at one or more stages of maturation pathway during spermiogenesis in male.In spermatozoa, IGRs induced aberration in acrosomal development which causes failure in acrosomal reaction and egg envelope penetration and consequently in fertilization process. Also, inhibition of chromatin condensation, mitochondrial degeneration and disorganization of flagellar elements resulted from IGRs treatment perturb the function, viability and mortality of spermatozoa.
Keywords: *R. ferrugineus*, IGRs, spermatogenesis, spermatzoa.

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INTRODUCTION

The red palm weevil (RPW) *Rhynchophorous ferrugineus* (Oliv.) is the most important pest of the date palm in the MiddleEast [1]. It was first reported in Egypt in 1992. It infested the governorates Ismailia and Sharkia, an area with an estimated one million palm trees [2]. Although they have been known as major pests for a long time, efficient and acceptable methods of controlling them are still lacking in many cases.

The information about the histological and ultrastructural features of the different internal systems of *R. ferrugineus* is scarce. No available data were provided in the literature about the histology or ultrastructure of gonads (ovary & testis) or about the gametogenesis of this pest. Such information might be valuable in finding a suitable insecticide against this pest. The development of germinative cells in Coleoptera, as in the majority of insects, takes place within cysts, contained in the testicles [3].The classic spermatozoa of Coleoptera are characterized by enormous accessory bodies and large and almost fully crystallized mitochondrial derivatives in the tail region. The head region is formed by an acrosome (three-layered) and nucleus [4,5,6,7]. Effect of phytochemical azadirachtin on the morphology and cytology of the testis follicle of the Indian grasshopper, *Melanoplus sanguinpes* showed alterations in the cytology of apical part, transformation zone, maturation zone and terminal zone cells as well as reduction in cell size in the apical part, disintegration of testicular epithelium and degeneration of spermatids in the transformation zone, disintegration of epithelial layer in maturation zone and disintegration of sperm bundles in the terminal zone of sperm follicle [8].

The effects of phytopesticides, Piperidine showed considerable changes in the scanning electron micrograph such as an entire tubule was appeared to be shrunken with thick and prominent myoepithelial cells and the rope like structure of the plasma membrane was found to be highly pycnotic and disintegrated with columnar epithelial cells. The muscular layer also thickened and highly pycnotic than the control insect *Odontopus varicornis* (heteroptera: pyrrhocoridae) [9]. This study aimed to provide information about the Ultrastructure histopathological changes of spermatogenesis and

testicular spermatozoon of *R. ferrugineus* is based on transmission electron microscopic observation occurred in the testes of the adult male developed from treated larvae with sublethal doses of two compounds of IGRs ; fluefenoxuron (chitin synthesis inhibitor) and natural neem extract.

MATERIALS AND METHODS

The experimental insect

Prepupal stage of the red palm weevil, *Rhynchophorus ferrugineus* were collected from diseased date palm tree at Al-Manaief Al-Gharbia region, Ismailia governorate. They were kept in the laboratory in small plastic cages approximately 5x10x13 cm. The insects were incubated at laboratory temperature of (28±2ºC) and relative humidity (80±10% R.H.). Soon after collection, the prepupae were randomly assorted in groups and topically treated individually with different doses of the plant extract (Neem J) or the insect growth regulator (flufenoxuron).

Bioassay and administration of chemicals:

Two insect growth regulators (IGRs) were used in the present study. One of them is a natural plant extract obtained from the neem tree *Azadirachta indica* (Meliaceae). Azadirachtin is one of the most important active ingredients of neem seed kernel of this tree. The other IGR used is the chitin synthesis inhibitor (fluefenoxuron), with the formula of $C_{21}H_{11}ClF_6N_2O_3$. Its IUPAC name is 1-[4-(2-chloro-α,α,α-trifluoro-*p*-tolyloxy)-2-fluorophenyl]-3-(2,6-difluorobenzoyl) urea.

Dose level were used from neem : 50 ppm. On the other hand, dose level were prepared from fluefenoxuron: 0.05 ppm. For each treatment, prepupae were topically treated with 10 µl of the plant extract or the synthetic IGR. These insecticides were topically applied onto the dorsum of the prothorax. Control insects were topically applied with acetone only. All treated and control insects were checked daily until pupation and then adult emergence. Adults were provided daily with portions of fresh sugarcane stem tissue approximately 0.5x2x2cm.

Electron microscopy

The testes were dissected out and fixed with 2.5% buffered gluteraldehyde in 0.1 M phosphate buffer, pH. 7.4 for 45 minutes at 4°C. The tissues were then washed several times with the same buffer and post-fixed with 1% buffered osmium tetroxide (OsO_4) for 60 minutes at 4°C. They were dehydrated in an ascending ethanol series, embedded in Epoxy resin and sectioned with an ultramicrotome. After double electron staining with uranyl acetate and lead citrate, the sections were examined and photographed with the Jeol electron microscope Jem 100 CX at the electron microscopy unit, Faculty of Science, Alexandria University.

RESULTS AND DISCUSSION

Description of spermatogenesis and spermatozoa of untreated specimens:

The following description of spermatogenesis and testicular spermatozoon of untreated *R. ferrugineus* is based on transmission electron microscopic observation. The histological structure of the testes revealed that spermatogenic cells, in different stages of spermatogenesis, including spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa occur in cysts. These cysts consist of a group of germ cells at the same developmental stage.

Spermatogonia and spermatocytes:

Spermatogonia are usually located at the periphery of the testicular follicles. The spermatogonial cells of the normal *R. ferrugineus* are round in shape with a large central nucleus which contains small clumps of chromatin and one or two nucleoli. Clear cytoplasm with numerous irregularly distributed mitochondria, free ribosomes, and some cisternae of the rough endoplasmic reticulum were also detected (Fig.1).

The primary spermatocytes with a nucleus that contains clumps of condensed chromatin within diffused nuclear matrix. The chromatin are heterogeneously distributed along the periphery of the nucleus. In addition to few mitochondria, large amounts of free ribosomes were detected in the cytoplasm as shown (Fig.2).

Secondary spermatocytes possess a smaller nucleus, granular in appearance and large clumps of condensed chromatin are shown at one pole of it. The nucleus is surrounded by a sparse clear and electron-dense cytoplasm (Fig.2).

Spermatids and spermiogenesis

During spermiogenesis, remarkable nuclear elongation, acrosomal development, formation of nuclear fossa and centriolar complex, flagellar development, elongation of mitochondria and cytoplasmic changes take place.

In early spermatids, spherical nuclei, with diffuse chromatin, were surrounded by asymmetrically distributed cytoplasm. As spermiogenesis proceeds, the process of chromatin condensation can be

described as gradual growth from the periphery of the nucleus towards the centre. So, a central core of electron-lucent area was showed (Fig.3).

An oval-shaped acrosomal precursor filled with material of moderate electron density was detected at the anterior pole of nucleus in more advanced (mid) spermatids. Elongated mitochondria, centriolar complex and flagellar precursor were observed in this stage of spermatids differentiation (Fig.4).

In late spermatids, the mitochondria were transformed to mitochondrial thread called the nebenkern derivatives (term of insect sperm) [10], and arranged parallel to the axoneme (Fig.5).

When nuclear elongation progresses, the acrosomal precursor transforms into a smaller and deeper granules embedded in shallow invagination at the anterior pole of nucleus. The centriolar complex, consisting of proximal and distal centrioles in a mutually perpendicular arrangement, connected the initial segment of flagellum with a shallow depression at the posterior pole of nucleus (Fig.5).

Finally, the granular residual cytoplasm was streamed backward leaving only thin sheath around the nucleus.

Testicular spermatozoon:

Ultrastructural examination of testicular spermatozoon of normal *R. ferrugineus* showed that spermatozoon has an anterior–posterior sequence, elongated head region (comprising a conical–shaped acrosome and long nucleus) and tail or flagellum. Figure 6 shows a normal spermatic cell with clear plasma membrane and absence of the mid–piece.

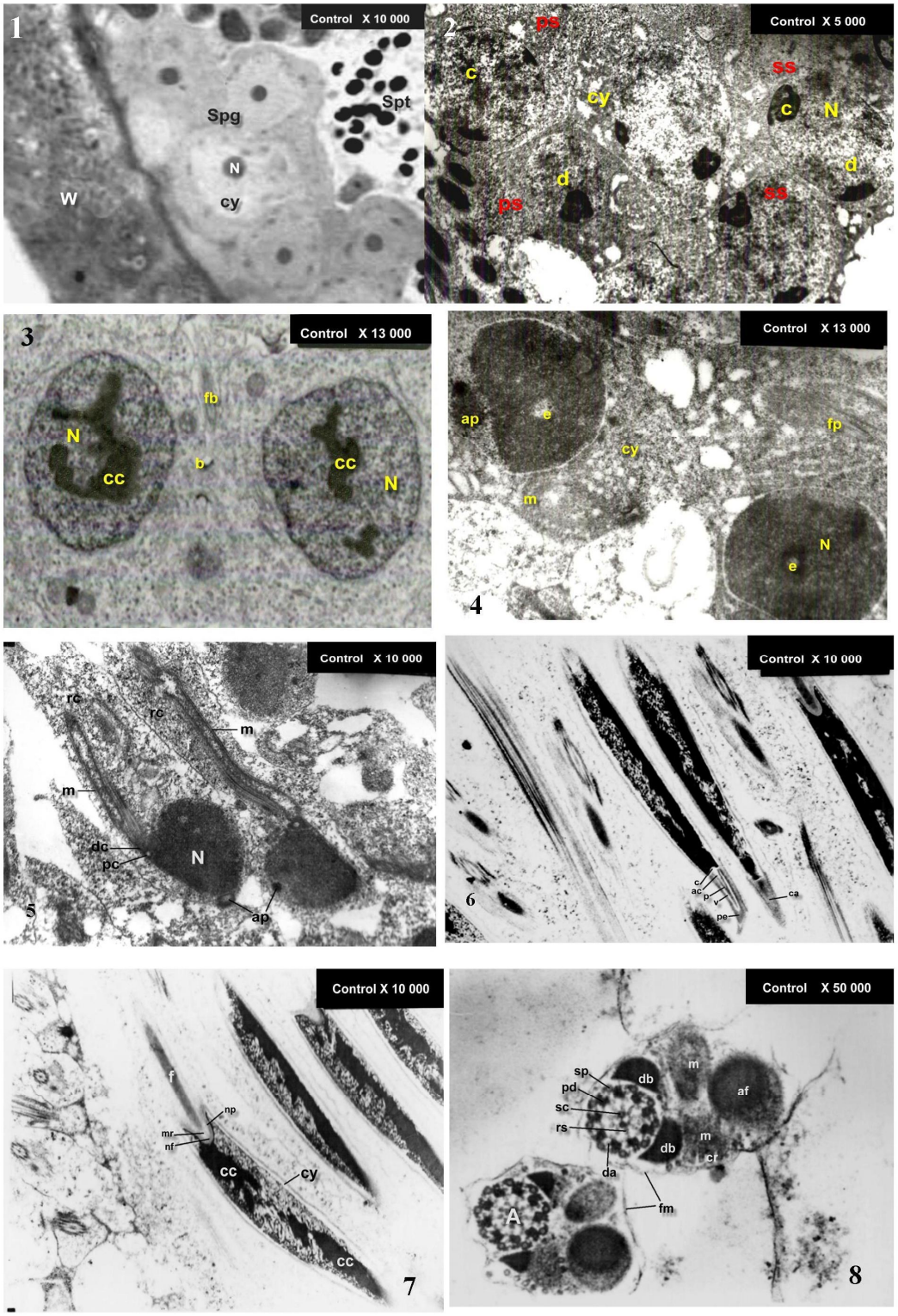
The acrosome: The conical shaped acrosome, that embedded in extra cellular cap, has an anterior pointed end, and blunt basis, with a diameter equal to the diameter of the anterior end of the nucleus, rested on the apical end of the nucleus. A clear matrix involving acrosome triangular depression and shallow nuclear depression was recognized. This clear matrix represents the mode of attachment of the acrosome. The acrosomal material has a layered appearance in longitudinal section. A well–developed membrane–bound vesicle of moderately electron dense material, and sub–acrosomal chamber within which there is a conical shaped perforatorium were identified (Fig.6). This perforatorium was slightly more electron–dense than the acrosomal vesicle. This structure is typical in many coleopteras as in *Sitophilus zeamais* and *Sitophilus oryzae*, the acrosome has three layers: an extra-acrosomal amorphous layer, an acrosome, and a inner cone or perforatorium. [4,5,6]. The inner cone or perforatorium has a filamentous substructure, which may play a cytoskeletal role during the process of sperm-oocyte interaction and fertilization. A similar structure has been reported for the featherwing beetle acrosome [11].

The nucleus: The elongated nucleus was surrounded by a narrow strips of cytoplasm with no organelles. The nucleus shows heterogeneous chromatin condensation, thus highly condensed chromatin strands at the periphery and electron–lucent areas in the core were observed (Fig. 7). Posteriorly, the nuclear fossa penetrates deeply in the nucleus and the two sides of nuclear fossa appeared overlapping the mitochonderial derivatives and the short mid–region. Both the mid–region and the derivatives were separated from the nucleus by a singly, continuous, thin post–nuclear plate (Fig.7).

The flagellum: Transverse section through the flagellum showed the flagellar organisation. The flagellum comprises the axoneme, two unequal mitochondrial derivatives, two deltoid accessory bodies (between the axoneme and mitochondrial derivatives), and large axial fibre (Fig.8). This pattern similar to most Coleoptera, in *S. zeamais* and *S. oryzae*, the flagellum has two accessory bodies of equal size that are adjacent to the axoneme and lateral to the mitochondrial derivatives [12]. In *S. zeamais* and *S. oryzae*, the flagellum morphogenesis has the same pattern as that described for other Coleoptera, which involves the formation of the axoneme, two mitochondrial derivatives, and accessory structures [13,14].

Both longitudinal and transverse sections through the mitochondrial derivatives provide evidence of transverse regular cristate structure and moderate electron dense matrix. The mitochondrial derivatives were parallel to the long axis of flagellum and the larger one has a more electron dense area (Figs.8).

The axoneme has the typical 9+9+2 arrangement of microtubules described for many insects. The 2 central axonemal singlets have electron–lucent core surrounded by protofilaments. The nine peripheral microtubular doublets had 2 subunits; A and B. The outer and inner dynein arms and radial spoke were projected from the microtubular wall of subunit A (Fig.8). Nine peripheral microtubular singlets, accessory microtubules, with less electron dense core than the peripheral doublets and electron–dense fibrous arms between them were observed (Fig.8).This pattern of axoneme are similar to that found in *S. zeamais* and *S. oryzae* [12].



Figures 1-8 Transmission electron micrographs of normal *R. ferrugineus.***(1)** through spermatogonia (spg). **(2)** through primary and secondary spermatocytes. **(3)** through early spermatids. **(4)** through mid-spermatids. **(5)** through late spermatids. **(6)** L. S in spermatozoon. **(7)** L. S in spermatozoon. **(8)** T. S in felagellum of spermatozoon. **Abbreviations:** Cy, cytoplasm; N, nucleus; Spt, spermatids; W, cyst wall; c, clumped chromatin; d, diffuse chromatin; ps, primary spermatocytes; ss, secondary spermatocytes ; b, cytoplasmic bridge; fb, flagellar precursor; cc, condensed chromatin; ap, acrosome precursors; e, electron-lucent area; m, mitochondrial thread; f, flagellum; dc, distal centriole; pc, proximal centriole; rc, residual cytoplasm; ac, acrosomal core; c, sub-acrosomal chamber; ca, extracellular cap; p, perforatorium; pe, pointed end of acrosome; v, acrosomal vesicle; mr, mid-region; nf, nuclear fossa; np, nuclear plate; A, axoneme; af, axial fiber; cr, cristate structure; da, dynein arm; db, deltoid accessory bodies; fm, flagellar membrane; pd, peripheral doublets; rs, radial spoke; sc, central singlets; sp, peripheral singlets.

Ultrastructural features of the testis of treated specimens.

Spermatogonia and spermatocystes

Both spermatogonia and spermatocytes from testis of flufenoxuron-treated weevil revealed ultrastructural aberrations similar to that showed in neem-treated weevil. These ultrastructure aberrations detected as spermatogonia appeared normal, their mitochondria exhibited moderate degeneration (Fig.9). Some vacuoles were also detected in the cytoplasm. Primary spermatocytes exhibited a degree of cellular alterations, including low staining affinity, degeneration of cytoplasmic organelles and absence of nuclear architecture. Few spermatocytes revealed small clumps of condensed chromatin at the central area of nucleus (Fig.9). Secondary spermatocytes shows prominent reduction in chromatin condensation and disorganized cytoplasm, several vacuoles and vesicles were showed near some chromatin-containing membranes indicating nuclear envelope reformation failure (Fig. 9).

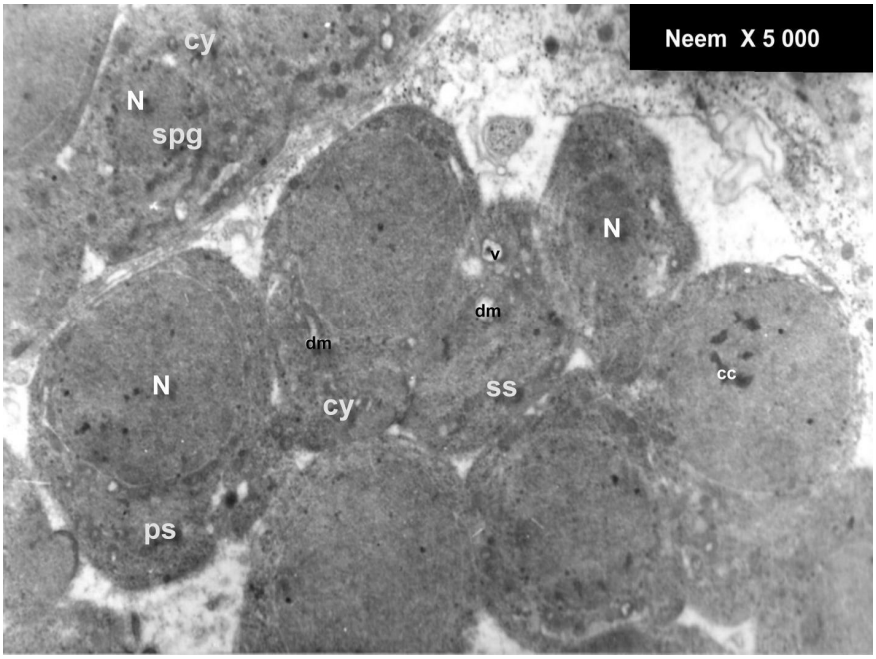


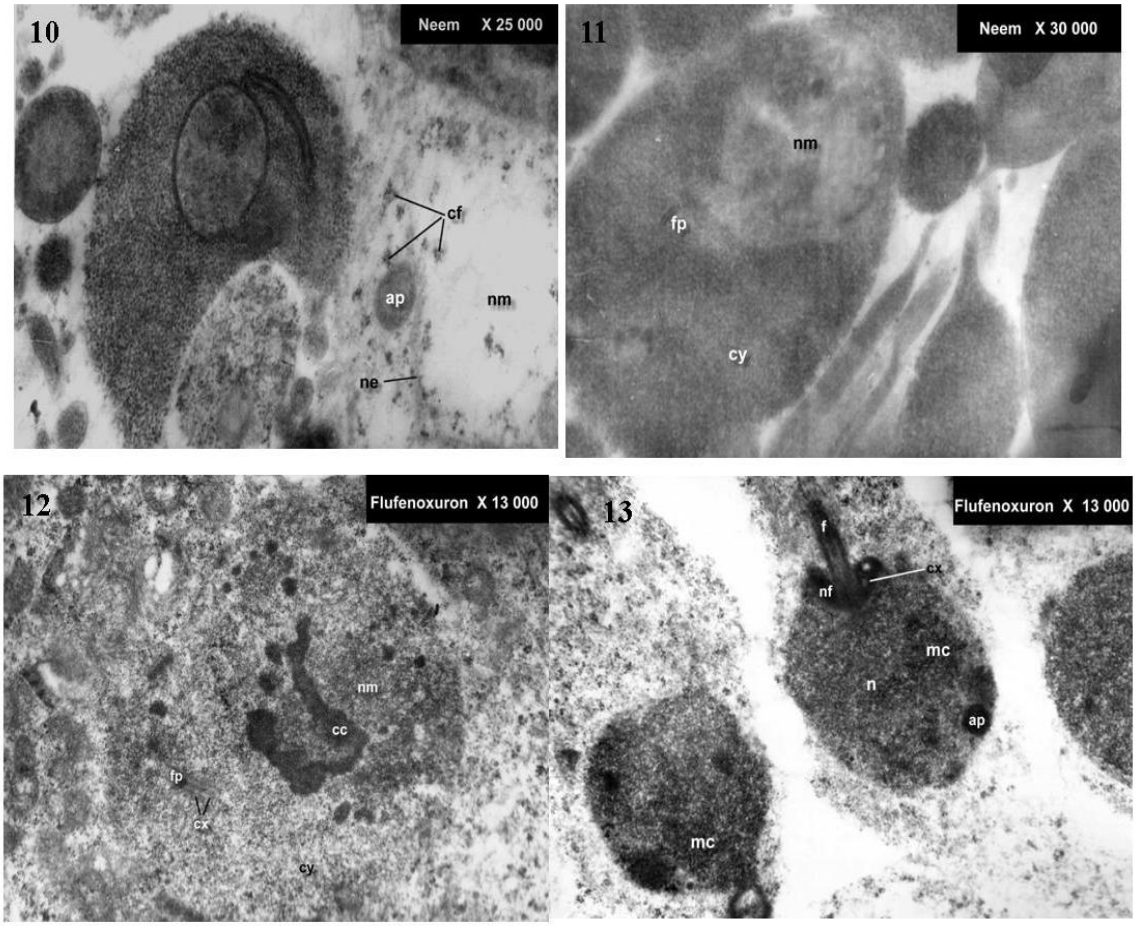
Figure (9) Transmission electron micrographs of neem-treated *R. ferrugineus.*(9) through spermatogonia (spg) and 1ry (ps) and 2ry (ss) spermatocytes. **Abbreviation:** dm, degenerated mitochondria.

Spermatids and spermiogenesis

In neem-treated weevil, more advanced spermatids showed a characteristic appearance of cell death (necrosis). Cell death characterized by disappearance of nuclear matrix and fragments of chromatin distributed at the periphery of the nucleus and outside the disrupted nuclear envelope were seen in Fig.(10). Retardation in spermatid differentiation characterized by degeneration of nuclear matrix, centriolear complex, flagellar precursor and other cytoplasmic organelles were observed in latter stage of spermatids (Fig.11).

In flufenoxuron-treated weevil mid-spermatid cell reveals degeneration of mitochondria and centriolear complex, disappearance of nuclear envelope and delayed chromatin condensation (Fig.12). The flagellar precursor appeared normal within granular cytoplasm.

In late spermatids, moderate homogenous chromatin condensation, acrosome precursor, centriolear complex, well-developed nuclear fossa and degenerated flagellum were observed (Fig. 13).



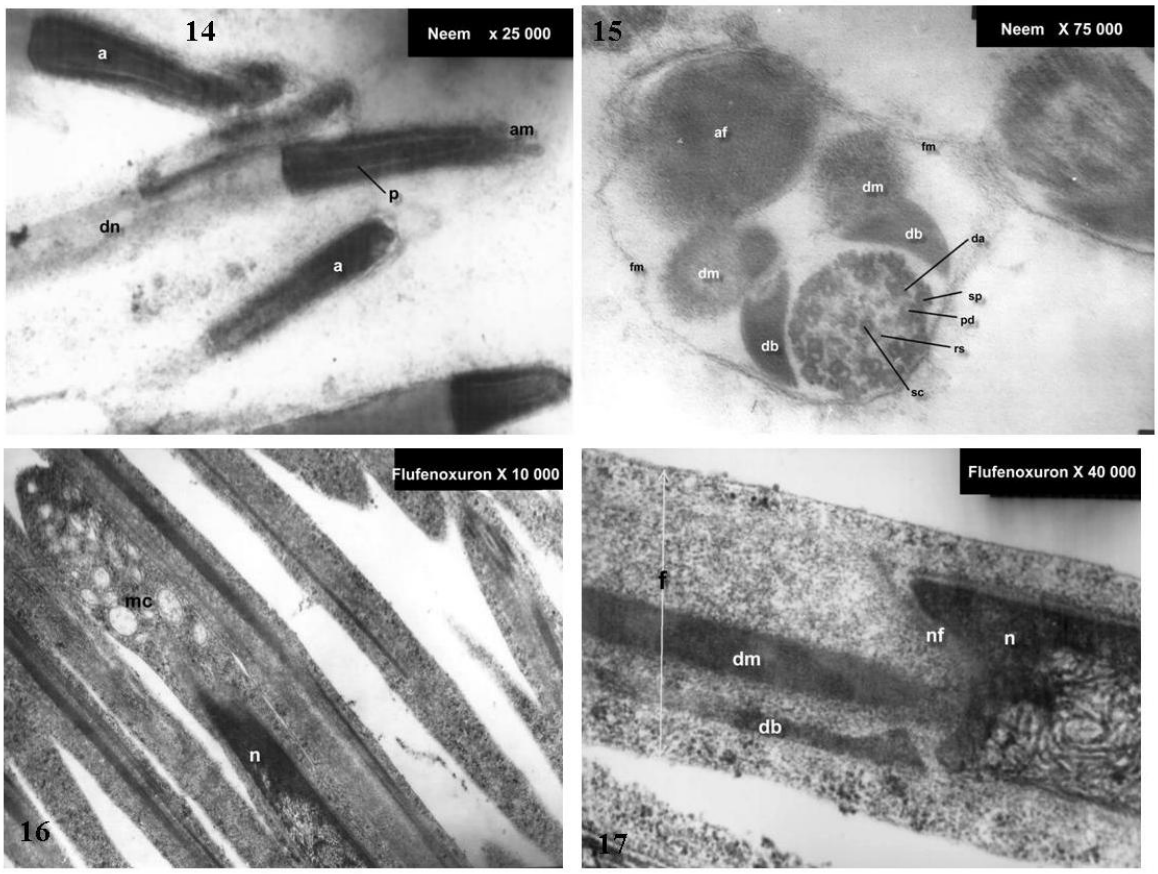
Figure(10-13) Transmission electron micrographs of (fig.10&11) neem-treated and (fig.12&13) flufenoxuron-treated *R. ferrugineus.*(10&12) mid spermatid. (11&13) late spermatid. **Abbreviations:** fp, flagellar precursor degenerated; nm, nuclear matrix disrupted; cf, chromatin fragments; ne, disrupted nuclear envelope; nm, disrupted nuclear matrix; ap, acrosome precursor; cx, centriolear complex; fp, flagellar precursor; nm, nuclear matrix. ap, acrosomal precursor; cx, centriolear complex; f, flagellum; mc, moderate homogeneous chromatin condensation.

Spermatozoa

In neem-treated weevil,the demonstrated spermatozoon abnormalities were excessive elongation of both the acrosome and nucleus, abnormal shape of perforatorium, absence of acrosomal core and anterior nuclear depression, acrosomal membrane blebbing and massive nuclear degeneration (Fig.14). In transverse section through the flagellum, flagellar membrane degeneration, degeneration in radial spoke within the axoneme, homogeneous electron-dense appearance of axial fiber and absence of cristal structure of mitochondrial derivative were illustrated in Fig. (15).

In flufenoxuron-treated weevil, The prominent ultrastructural abnormalities in spermatozoa of flufenoxuron-exposed male weevils are the failure in acrosomal differentiation and massive mitochondrial degeneration. The abnormal acrosome exhibited dilated membranous component filled with vesicles and moderate granular matrix. Fig. (16) also shows hemilateral absence of nuclear fossa. Highly condensed chromatin strands at the periphery of spermatozoal nucleus was showed in Figs. (16&17).

It is evident from the present study that the TEM drastic changes in spermiogenesis and spermatozoon. In spermatozoa, IGRs induced aberration in acrosomal development which causes failure in acrosomal reaction and egg envelope penetration and consequently in fertilization process. Also, inhibition of chromatin condensation, mitochondrial degeneration and disorganization of flagellar elements resulted from IGRs treatment perturb the function, viability and mortality of spermatozoa which intern affects the process of reproduction.



Figures 14-17 Transmission electron micrographs of neem-treated (fig.14&15) and flufenoxuron-treated (fig.16&17) *R. ferrugineus.*(14) L.S. of spermatozoon. (15) T.S. of flagellum of spermatozoon. (16&17) L. S. of spermatozoon. **Abbreviations:** mc, abnormal acrosome with dilated membranous compartment; dm, degenerated mitochondria ; db, deltoid accessory bodies.

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