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Effects of azadirachtin on mortality rate and reproductive system of the grasshopper *Heteracris littoralis* Ramb. (Orthoptera: Acrididae)

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Abstract

Male and female nymphs of *Heteracris littoralis* were topically treated with serial concentrations of azadirachtin. Effects on mortality, development, oogenesis and spermatogenesis were observed. Mortality was dose-dependent; fourth and fifth instars died about the time of ecdysis. Overaging took place at low concentrations. Ovaries in treated adult females showed complete shrinkage with abolished oocyte growth, and the number of deposited egg pods/female decreased from 4 to 9 in normal females, to 1 to 3 pods in treated insects. Deformation in sperm tubes was observed in treated males. Electronmicrographs revealed disintegration and destruction in follicular cells and mitochondria in females. In males the testicular epithelia and the spermatids completely disintegrated. Treatment with higher doses inhibited cyst formation around the spermatogonia.

Key words

azadirachtin, LC_{50}, fecundity, oocyte, sperm, ultrastructure

Introduction

Increasing problems of pest resistance to pesticides enhanced interest in botanical insecticides during recent decades. Extracts from the neem tree *Azadirachta indica*, of which azadirachtin is the most important active principle, received the attention of many research workers. It was found to exhibit deterrent, antiovipositional, antifeedant, growth-disrupting (growth-regulating), fecundity and fitness-reducing properties on many insect species.

The disruption of insect development and behavior by azadirachtin was observed in Orthoptera (Schmutterer *et al.* 1993, Linton *et al.* 1997), Mallophaga (Habluetzel *et al.* 2006), Hemiptera (Nisbet *et al.* 1992), Isoptera (Grace & Yates 1992), Coleoptera (Steets 1976, Klocke & Barnby 1989), Lepidoptera (Nathan *et al.* 2006 a, b), and Diptera (Lucantoni *et al.* 2006).

In addition, azadirachtin is an effective sterilant. After uptake of the active material, females of some insect pest species were sterilized to various degrees, sometimes completely (Steets 1976, Schmutterer 1987), where juvenoids and ecdysteriods strongly affected fecundity and/or egg sterility if applied during sensitive growth phases of target insects (Karnavar 1987, Tanzubil & McCaffery 1990). On the histological level, azadirachtin was found to inhibit oogenesis (Ghosh *et al.* 1999, Medina *et al.* 2004) and spermiogenesis (Linton *et al.* 1997, Abdel-Rahman *et al.* 2004).

The aim of the present work is to investigate some effects of azadirachtin on histological and ultrastructural levels of the gonads in *H. littoralis*. The economic importance and the pest status of this species have been documented by El-Shazly (1991).

Materials and Methods

Adults and nymphs of *Heteracris littoralis* Ramb. were collected from Abou Rawash district (Giza Governorate), Egypt. A laboratory stock was reared in electrically heated wooden cages (25×25×25×25 cm at a constant temperature of 30±1°C, with fluctuating relative humidity (50 to 70%). Insects were fed clover, *Trifolium alexandrinum*, from November to May and then fresh leaves of *Sesbania sesban*. Cages were supplied with suitable ovipositional pots for egg deposition which were kept moistened. Hatched hoppers were transferred to 20×25-cm cylindrical glass jars. After the fourth or fifth moult, hoppers were released in the large cages.

To test the effect of azadirachtin on mortality of 4^{th}, 5^{th} and 6^{th} female nymphal instars, a series of concentrations was prepared (50 to 400 ppm). For each concentration, 20 to 30 insects were tested in five replicates of 5 to 6 grasshoppers each. At the beginning of the stadium, azadirachtin preparations (5 µl) were topically applied to the neck membrane of the tested instars. Control groups were treated with 5µl acetone. The end point was determined 24 h after treatment by counting dead and moribund individuals in each jar. The average weight was determined for each group, to calculate toxicity/mg body weight. LC_{50} values were determined according to Finney (1971) and mortality corrected according to Abbott's formula (Abbott 1925).

To study the effect of azadirachtin on life span and egg production of the adult females, groups of 20 newly emerged females were topically treated with different concentrations of azadirachtin (25 to 1000 ppm). Five µl of azadirachtin preparation were applied to the neck membrane of the tested insects. The experiment was repeated three times. Insects were reared in groups of ten females and left to mate with normal males in 25×25 × 25-cm cages. Insects were offered suitable amounts of food and ovipositional pots, which were checked daily for egg deposition. Longevity was calculated for all groups.

To study the effect of azadirachtin on the morphology of the ovary and sperm tubes, 10 d-old females and newly emerged males were topically treated with azadirachtin (10 µl of 25 ppm). Treated insects were dissected after 10 days and the ovaries, ovarioles and sperm tubes measured using a stage micrometer and an ocular piece. The measurements were taken three times with 5 replicates each of 5 insects each.

In insect groups used to study azadirachtin effects on the histology of the ovaries and testes, females were treated with 10 µl of 25 ppm, 10 days before their oviposition period (mid-preoviposition period), while males were treated after their imaginal molt with two doses: 25 and 200 ppm. Treated insects were examined 7 d after application. Parts of ovarioles, and sperm tubes of treated

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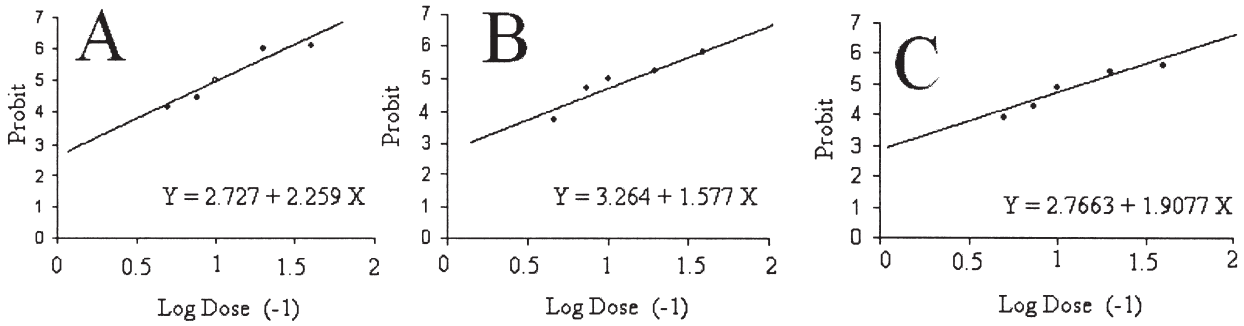

Fig. 1. Regression lines representing the mortality rate of 4th (A) 5th (B) and 6th (C) female instar nymphs of *H. littoralis* with increasing concentration of azadirachtin.

insects and control groups were fixed in Carnoy's Formula I for 6 h. Specimens were washed with absolute alcohol, then hydrated with a descending alcoholic series and preserved in Formal Saline solution (Abdel Rahman 1995). Specimens were dehydrated in ascending alcoholic series and cleared in xylene for a few seconds, infiltrated in three changes of paraffin wax (melting point 58°C) each lasting 20 min. Specimens were sectioned serially at 6µ thick using a rotatory microtome (American Optical) and stained with Ehrlich haematoxylin and eosin.

For TEM observations, ovarioles and sperm tubes were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.3) at 4°C for 24 h, then washed in three changes of the fresh buffer. Specimens were post-fixed in 1% osmium tetraoxide in the same buffer for 2 h 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, then into pure resin overnight. Finally, the specimens were put in resin blocks for 3 days at 50°C. Semi-thin sections (1µ thick) were prepared using a glass knife and were stained with toluedene blue for a few seconds. The specimens were examined under a normal light microscope and photographed. Ultra-thin sections (90 nm thick) were obtained using a diamond knife. Sections were put on a cupper grade mesh and stained with uranyl acetate and lead citrate for half an hour, then examined with a Joel JEM JE 1200EXII (Japan) transmission electron microscope and photographed.

Results and Discussion

Effect of azadirachtin on mortality rates and molting

Treated 4th, 5th and 6th instars of female *H. littoralis* displayed dose-dependent mortality rates, as demonstrated in Fig. 1 and Table 1. Females of the 4th instar were significantly more sensitive, as shown by the LC_{50} values in Table 1. According to the values of the moments (m_1, m_2), differences in the lethal doses between instars were significant at 95% confidence limits. Nymphs of the fourth and fifth instars died at about the time of ecdysis of the control individuals. At 50 ppm, 6th instar nymphs survived for a long period as over-aged individuals. The last instar in control groups lasted 9.8 ± 0.3 d, while over-aged nymphs survived for 15.16 ± 2.35 d (6 ± 1.5 d more than the control).

Azadirachtin inhibited molting when applied within the first two days after molt onto 6th instar nymphs. Nymphs were unable to undergo or terminate ecdysis. Treated nymphs remained in this condition, unable to shed their old cuticle successfully. Weak ecdysial movements that could last for several hours led to rupturing of old skin along the dorsal midline of the meso- and metathorax and over the pronotum and head capsule, but the shedding was not continued, leading finally to death. When insects were treated 3 to 5 days after the last molt, few adults were able to ecdyse, but shrinkage of wings was observed in emerging individuals.

These results agree with those of Sieber and Rembold (1983) on the molting and mortality of *L. migratoria: a* dose of 2 µg/g body weight completely prevented molting and prolonged intermolt period, which ranged between 8 and 60 days, compared to 6 (4th

Table 1. Effect of different concentrations of azadirachtin on the mortality rate of fourth, fifth and sixth female instar nymphs of *H. littoralis*.

Concentration (ppm)	Percent dead		
	Fourth instar	Fifth instar	Sixth instar
400	86.66	80.00	73.33
200	84.61	60.00	66.67
100	50.00	50.00	46.67
75	30.00	37.50	23.07
50	20.00	20.00	14.28
Control	0.00	0.00	0.00
LC_{50} (ppm)*	101.50^{A}**	126.12^{B}	148.22^{C}
Toxicity in mg/g body weight	7.51^{A}	15.76^{B}	28.00^{C}
Moments (m_1-m_2) at 95% confidence	6.05 – 9.31	10.33 – 17.09	20.21 – 33.21
SE Slope	0.14	0.16	0.12

*LC_{50} was estimated according to Abbott (1925) and Finney (1971).

**LC_{50} values followed by different letters are significantly different from each other based on 95% confidence limits.

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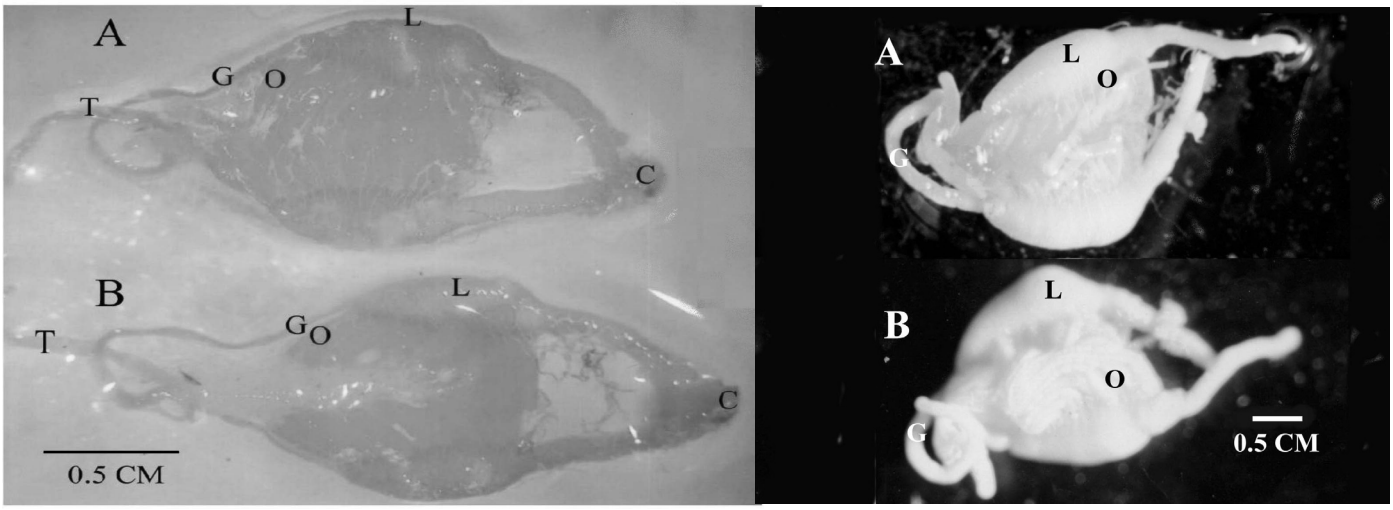

Fig. 2. Normal (left) and azadirachtin-treated ovary (right) of *H. littoralis*. A, Ventral view; B, Dorsal view; C, Common oviduct; G, Accessory gland; L, Lateral oviduct; O, Ovariole; T, Terminal filament.

instar) and 9 days (5th instar) in the control groups. At lower doses of azadirachtin (1.7 mg/g body weight) adults with curled wing tips and reduced longevity were produced. Other authors support these findings: Pener & Shalom (1987), Pener *et al.* (1989), Van der Host *et al.* (1989).

On the other hand treatment with higher doses (2.9 mg/g) resulted in death during the imaginal molt. Doses ranging between 6 and 7.3 mg/g body weight caused either death prior to the molt or greatly extended instar duration. Doses of 80 mg/g body weight resulted in death within 24 h [Mordue (Luntz) *et al.* 1985].

Also, nymphs of *S. gregaria* treated with azadirachtin survived beyond 40 days without molt and generally died during ecdysis (Rao & Subrahmanyam 1986, Nicol & Schmutterer 1991). Other grasshoppers treated with neem had abnormal nymph-adult molts as in *Zonocerus variegatus* (Olaifa *et al.* 1991).

It could be concluded that treatment of *H. littoralis* 4th, 5th and 6th instar nymphs with high concentration of azadirachtin resulted in high dose-dependent mortality rates during the period of ecdysis in comparison with control groups. At lower concentration, it caused prolongation of the stadium, leading finally to death. In some few cases malformed adults were able to emerge (Table 1).

Effects of azadirachtin on the fecundity and fertility of *H. littoralis*

Treated female suffered from decrease in number of egg pods/female in a dose dependent manner (Table 2). The average number decreased from 6.47 ± 2.21 (control group) to 2.00 ± 0.82 (females treated with 25 ppm). At higher doses (100 ppm and above) no egg-pods were deposited.

Such was also the case observed in azadirachtin-treated *L. migratoria* adults (Rembold and Sieber, 1981) as well as in *Oncopeltus fasciatus* (Heteroptera: Lygaeidae) (Dorn *et al.* 1987), *Spodoptera exempta* (Lepidoptera: Noctuidae) (Tanzubil and McCaffery, 1990), and in *Sesamia calamistis* (Lepidoptera: Noctuidae) and *Eldana saccharina* (Lepidoptera: Pyralidae) (Bruce *et al.* 2004).

Effects of azadirachtin on the female reproductive system

Effects of azadirachtin on the ovary of H. littoralis.—Female *H. littoralis* (10-d old adults) topically treated with azadirachtin (10 µl of 25 ppm) showed shrinkage in the whole ovary, besides a partially abolished oocyte growth, as only a few eggs were developed (Fig. 2). The whole organ seemed like the developing ovary of a nymph (3.5±0.3 cm in length, compared to the control 7.4±0.2 cm), while the average length of ovarioles in treated insects was 1.5±0.12 mm, compared to 8.0±0.58 mm in the control females.

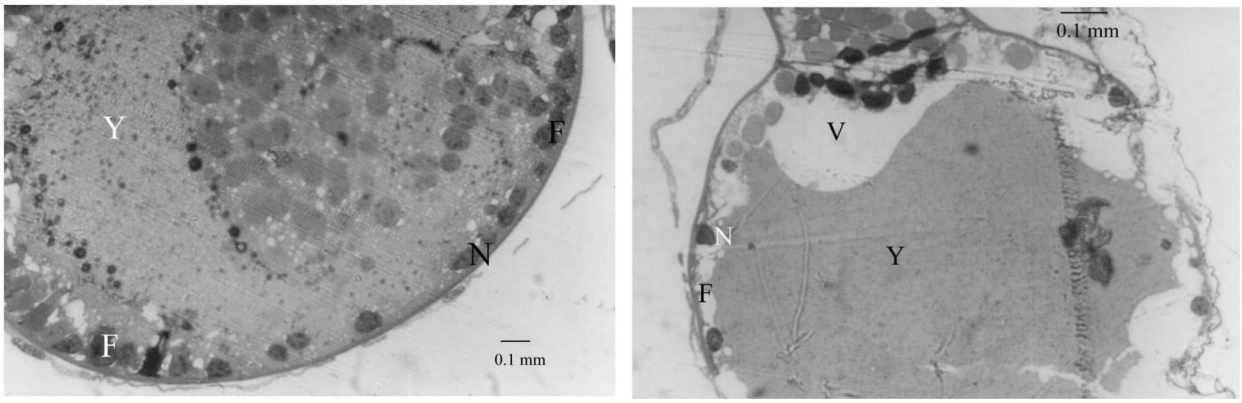

Fig. 3. Transverse section in normal oocyte (left) with homogenous yolk and with regular follicular epithelium; treated (right) showing yolk mass separated from follicular epithelium as well as follicular epithelium totally destroyed. F, Follicle cells; N, Nucleus; V, Vacuole; Y, Yolk.

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Table 2. Effect of different doses of azadirachtin on the longevity and egg production of *H. littoralis* (mean ± SD).

Dose Ppm	Longevity (days)				No. egg-pods/ female	No. eggs/ pod
	Pre-ovip. Period	Ovip. Period	Post-ovip. period	Total		
Control	20.50 ±3.14 (15 – 25)	42.60±4.61[a] (36 – 61)	6.30 ± 2.11[a] (3 – 11)	70.21±3.11[a] (62- 90)	6.47 ± 2.21[a] (4 - 9)	31±6.0[a] (26 – 45)
25	22.50 ±2.96 (17 – 28)	8.70 ± 6.40[b] (1 - 21)	2.70 ± 1.10[b] (1 - 4)	36.10 ± 5.60[b] (18 – 53)	2.00 ± 0.82[b] (1 - 3)	29 ± 4.0[b] (21 – 33)
50	22.60 ±4.58 (17 – 27)	7.60 ± 6.40[b] (1 - 18)	2.20 ± 1.10[b] (1 - 3)	33.20 ± 6.40[b] (18 - 48)	1.85 ± 0.69[b] (1 - 3)	27±3.6[b] (24 - 31)
75	23.70 ±4.06 (18 – 28)	6.80 ± 5.40[b] (1 - 15)	2.40 ± 1.30[b] (1 - 5)	33.60 ± 5.90[b] (19 - 48)	1.64 ± 0.67[b] (1 -2)	19±2.4 [c] (17 – 24)
100	30.30±2.60[x] (24-36)	-	-	30.30 ± 2.60[x] (24-36)	-	-
200	25.20±1.20[x] (20 – 27)	-	-	25.20 ± 1.20[x] (20 - 27)	-	-
400	22.50±1.10[x] (18 – 24)	-	-	22.50 ± 1.10[x] (18 - 24)	-	-
1000	21.30±3.30[x] (15 – 25)	-	-	21.30 ± 3.30[x] (15 - 25)	-	-

Values followed by different superscripts (a, b, c) in the same column are significantly different ($P < 0.05$).
[x] individuals died without laying eggs. ovip., ovipostion.

Effects of azadirachtin on the histological components of the ovarioles.— A normal ovariole in female *H. littoralis* is surrounded by a peritoneal coat of connective tissue which contains a reticulum of muscle fibers. The walls are formed of a layer of compact cuboid epithelial cells, resting upon a basement membrane (the follicular cells) (Fig. 3) and containing a homogenous yolk material. The pronucleus is more or less located in a central position. The matrix contains large yolk spheres and clear spaces near the apices of the follicular cells. At the end of the maturation stage, the follicular epithelial cells provide the vitelline membrane and chorion around the egg. Thus in *H. littoralis* the follicular cells play an important role in incorporation of yolk granules into the oocytes during vitellogenesis.

Treatment of 10 d-old adult females with 25 ppm of azadirachtin affected the above histological components as follows: the oocytes were degenerate, with faint yolk deposition. The follicular cell layer was folded on itself and the cells partly destroyed, their compact shape lost as compared with control specimens. The yolk material was no longer homogenous and contained many vacuoles. The follicular epithelia were irregularly developed, showing clear separation of yolk. In a few cases the oocyte succeeded in reaching the egg stage, but large vacuoles appeared between the yolk spherioles, and the eggs failed to complete their development as compared with the normal compact yolk spherioles in the control groups.

Electron micrographs showed that normal follicle cells appeared with homogenous ooplasm (Fig. 4). Inside the cytoplasm, the mitochondria contained normal cristae, indicating a well-developed capability for follicle-cell protein synthesis and secretion throughout vitellogensis (Fig. 6). During vitellogenesis the nucleus and the nuclear membrane appeared normal and the cytoplasm was homogenous and rich in mitochondria (Fig. 8).

Many changes were observed in the histological structure of treated female ovarioles as shown by electron micrograph. During vitellogenesis the follicle cells appeared degenerated, with the presence of many vacuoles (Fig. 5). The contents of mitochondria were totally disintegrated without any cristae inside (Fig. 7). The ooplasm was no longer homogenous; the nucleus appeared with many lysosome-like bodies, indicating the beginning of cell lysis (Fig. 9).

Schmutterer *et al.* (1981, 1993) reported severe damage to the female ovarian follicles of *Locusta*, *Nomadacris*, and *Schistocerca* after treatment with azadirachtin. Also, Rembold (1987) reported delayed vitellogenin synthesis in azadirachtin-treated *L. migratoria*, causing severe damage to the oocytes. Shalom *et al.* (1988) showed inhibition of oocyte development and oocyte resorption in female *L. migratoria* after treatment with azadirachtin The inhibition of ovarian follicle development was also reported by Ghosh *et al.* (1999) on *Gesonula punctifrons* (Orthoptera: Acrididae) treated with azadirachtin.

The effect of azadirachtin on histological components of the reproductive system, in insects belonging to orders other than Orthoptera, reveals different degrees of damage. Schulz and Schlüter, (1983) showed that neem caused dissolving of mitochondria inside the ooplasm in the ovarioles of *Epilachna varivestis* (Coleoptera: Coccinellidae). Autophagic vacuoles were formed and the prefollicular epithelium was partly destroyed and folded on itself. Jagannadh and Nair (1997) reported that azadirachtin induced a considerable reduction in the ovariole size and a delay in previtellogenic differentiation of oocytes, in addition to apparent degeneration of ovarioles in larvae, pupae and adults of *Spodoptera mauritia* (Lepidoptera: Noctuidae). Medina *et al.* (2004) reported that azadirachtin affected the ovarioles of *Chrysoperla carnea* (Neuroptera: Chrysopidae): growing follicles in treated females were significantly smaller than those of controls.

It seems that when azadirachtin is administered at an appropriate time, it can cause severe damage to the oocytes, probably as a result of interference with the vitellogenesis process. This may explain the blocking of the developmental process of ovarioles and consequently the shrinkage of the ovary (Fig. 2). Vitellogenesis is a rather complicated process, involving the deposition of yolk in the oocyte, resulting in a very rapid increase in size. Azadirachtin may inhibit vitellogenin synthesis or absorption which eventually leads to: inhibition of both oogenesis and ovarian ecdyosteroid synthesis (Rembold & Sieber 1981), inhibition of ovarian development (Karnavar 1987), delaying of the vitellogenin synthesis process (Rembold 1987) and interference with vitellogenin synthesis and its absorption by the follicles (Schmutterer *et al.* 1981, 1993; Ghosh *et al.* 1999).

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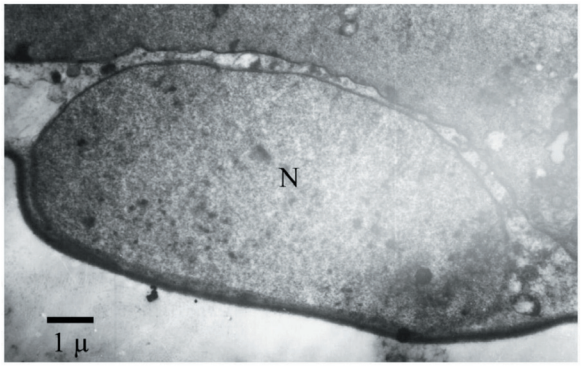

Fig. 4. Electron micrograph of normal follicle cell during middle vitellogenesis showing the homogenous ooplasm. N, nucleus.

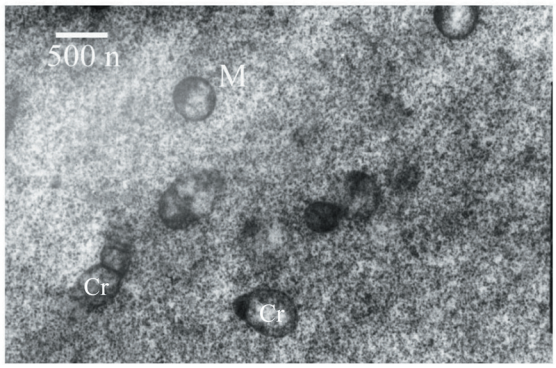

Fig. 6. Electron micrograph of normal follicle cell near the end of vitellogenesis showing homogenous ooplasm and abundant mitochondria (M) with normal cristae (Cr).

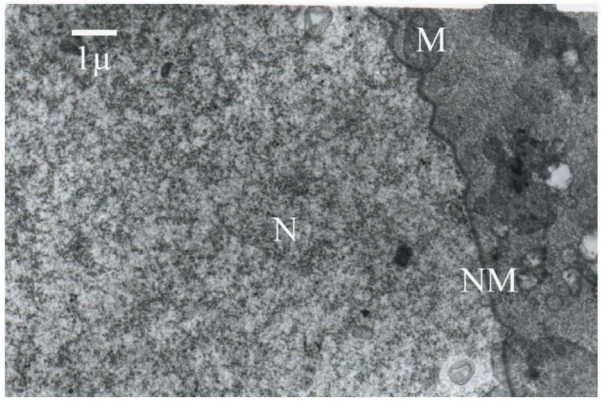

Fig. 8. Electron micrograph of normal follicle cell during vitellogenesis showing a homogenous cytoplasm, rich in mitochondria (M). N, nucleus; NM, nuclear membrane.

The effects of azadirachtin application may resemble those of juvenile hormone and its analogues, in respect to ovarian development, time of application and vitellogenin synthesis. Several authors have reported the effects of juvenile hormone analogues/mimics on the histology of the reproductive system as well as oogenesis in different insect groups. Tobe and Pratt (1975) established a link between JH and ovarian maturation in *S. gregaria*, with a peak of JH synthesis at the onset of the previtellogenic period. Previtellogenesis occurred on days 5 to 6 in adult females and vitellogenesis occurred on days 7 to 8. Application of the JH mimic was timed with respect to the gonadotrophic cycle of individual insects.

Feyereisen and Tobe (1981) showed that oogenesis and vitellogenesis are inhibited by other JH analogues such as precocene in

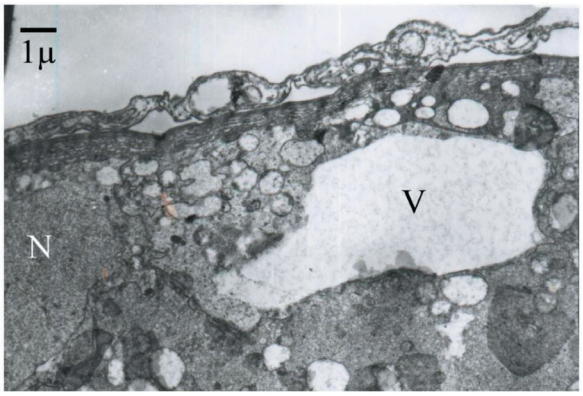

Fig. 5. Electron micrograph of follicle cell in the treated insects during middle vitellogenesis; showing bursting of the follicle cell, disappearance of its contents and appearance of many vacuoles. N, nucleus; V, vacuole.

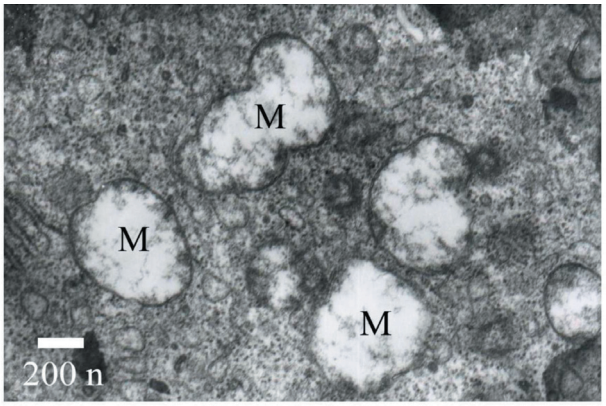

Fig. 7. Electron micrograph of follicle cell in the treated insect near the end of vitellogenesis showing nonhomogenous cytoplasm with damaged mitochondria (M) without cristae inside.

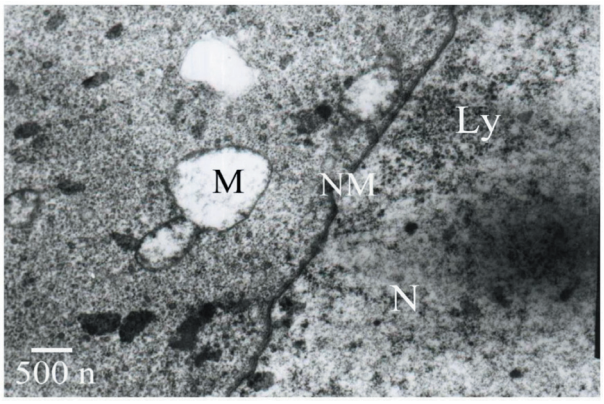

Fig. 9. Electron micrograph of follicle cell in the treated insect showing damaged mitochondria. Appearance of nucleus with many lysosome-like bodies (Ly). M, mitochondria; N, nucleus; NM, nuclear membrane.

many insects. Eid *et al.* (1988) showed that ovaries of the female *S. gregaria* treated with precocene did not exhibit the vitellogenic stage, while in the normal females vitellogenesis started earlier. Ahi (1988) used another JH mimic (aldrin) on *Poekilocerus pictus* and mentioned abnormal fragmentation of oocytes with degeneration of the follicular epithelial cells. Polivanova and Triseleva (1989) also observed inhibition of vitellogenesis and sterilization of *L. mi-*

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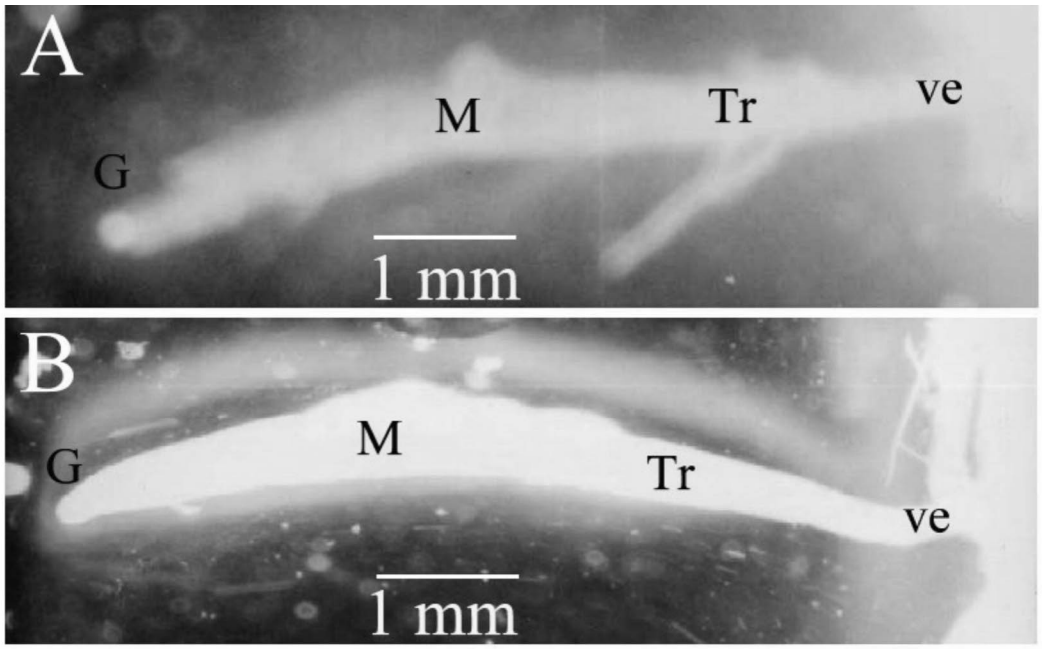

Fig. 10. A, Normal sperm tube B, Treated sperm tube. G, germarium; M, maturation zone; Tr, transformation zone; ve, vas efferens.

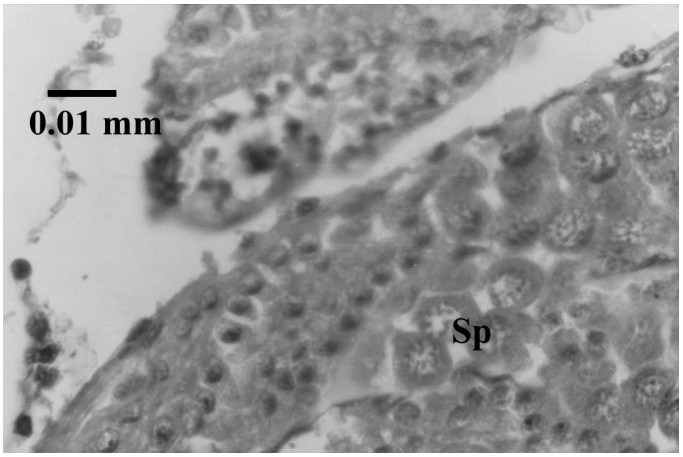

Fig. 11. Sagittal section of a normal sperm tube showing spermatocyte maturation (Sp).

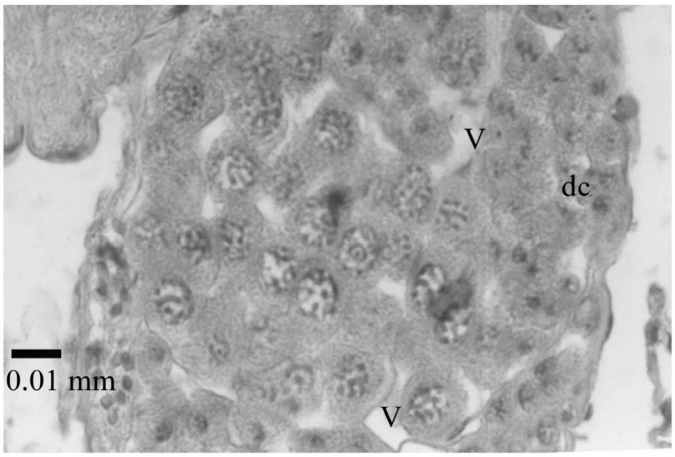

Fig. 12. Sagittal section in treated sperm tube in the apical zone showing nonhomogenous matrix and appearance of vacuoles and cell swelling. dc, degenerating cells; V, vacuoles.

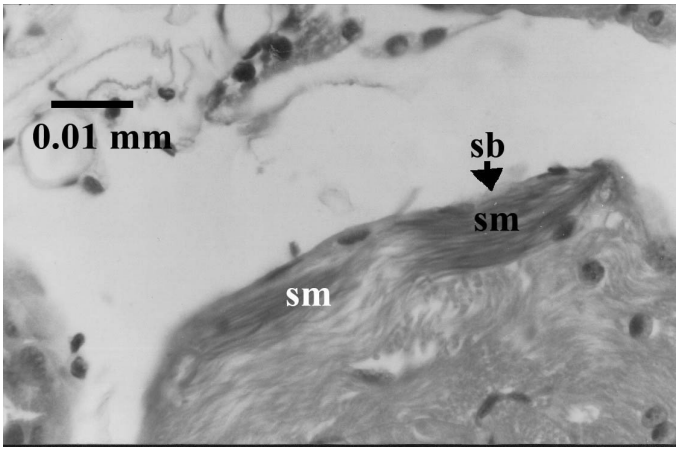

Fig. 13. Sagittal section of a normal sperm tube showing the transformation of spermatides into sperm (sm); sperm bundle (sb) (arrow).

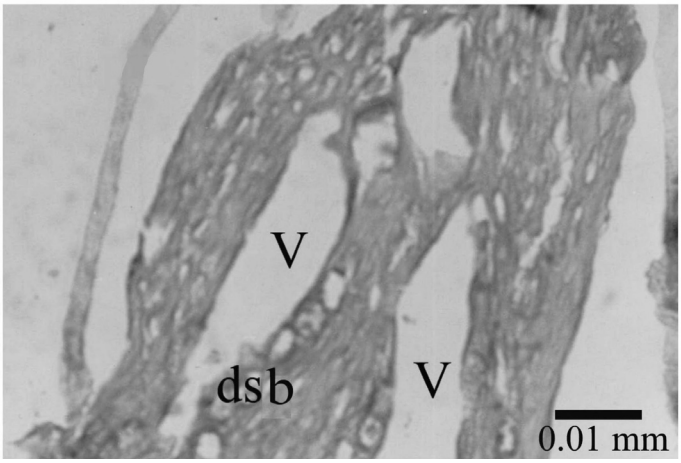

Fig. 14. Sagittal section in treated sperm tube in the transformation zone showing degeneration of the sperm bundles (dsb), and appearance of large vacuolated areas (V).

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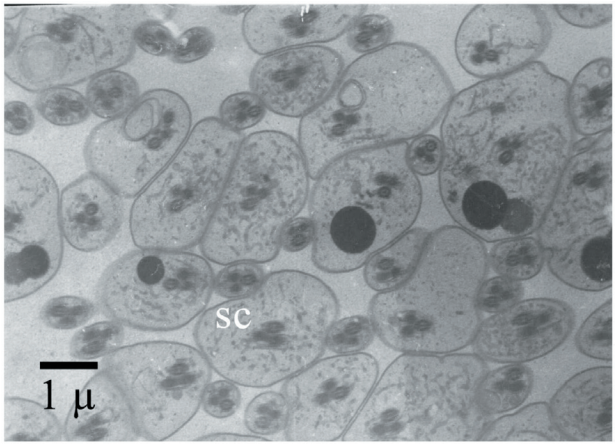

Fig. 15. Electron micrograph in the maturation zone of a normal sperm tube of *H. littoralis* showing division of germ cells into spermatocytes (sc).

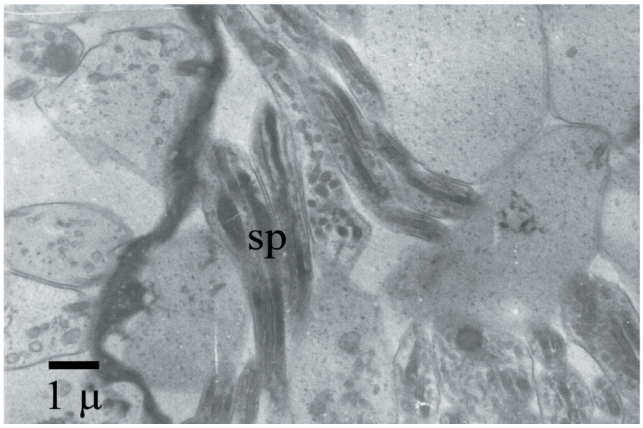

Fig. 16. Electron micrograph in the transformation zone of a normal sperm tube of *H. littoralis* showing the differentiation of spermatocytes into spermatids (sp).

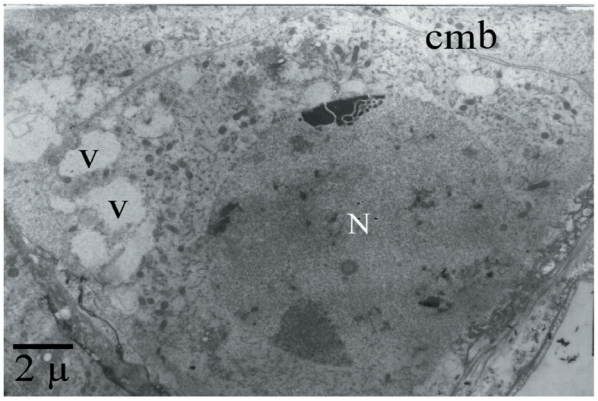

Fig. 17. Electron micrograph in the maturation zone of a sperm tube in the treated insect showing division inhibition of the germ cells and appearance of vacuoles (V), nucleus (N), cell membrane (cmb).

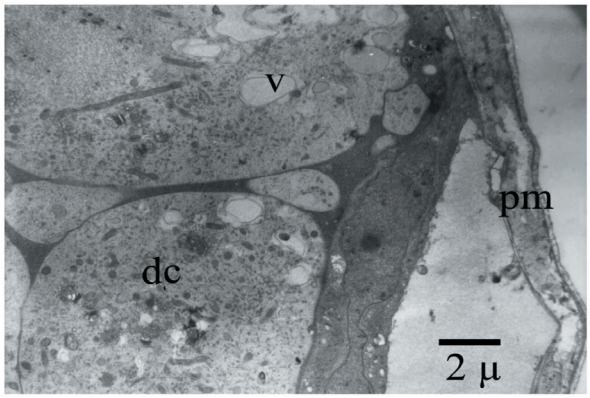

Fig. 18. Electron micrograph in the maturation zone of a sperm tube in the treated insect showing the detachment of the peritoneal membrane (pm) from the basement membrane and appearance of many vacuoles (V), degenerated cell (dc).

gratoria when last instars were fed on treated food with precocenes. Application of precocene and methoprene to *Phormia regina* (black blow fly) inhibited oocyte development and lowered the amount of vitellogenin, respectively (Yin *et al.* 1989). The follicular epithelium surrounding the vitellogenic oocytes of *L. migratoria* treated with the JH analogue, methoprene, was found to develop large spaces between the cells, with an eventual decrease in their volumes (Davey *et al.* 1993). Pinto *et al.* (2000) tested pyriproxyfen on *Apis mellifera* and reported the inhibition of vitellogenesis.

Effects of azadirachtin on the male reproductive system

Effects of azadirachtin on the sperm tubes of H. littoralis.—Treated sperm tubes showed a swelling in the zone of growth with a slight reduction in its length (Fig. 10b). A follicle in treated males measures 5.15 ± 0.11 mm in mean length while its mean width is 0.55 ± 0.13, these values were significantly larger than the control which measures 6.25 ± 0.21 mm in average length, its average width at the maturation zone being 0.3 ± 0.02.

Effects of azadirachtin on the histological components of the sperm tubes.—Histologically, sagittal and transverse sections in the apical part of a sperm tube of males treated with low doses of azadirachtin (25 ppm), showed swelling of the dividing cells; some cells were destroyed and cells in the central part were smaller in size as compared with the control group (Figs 11, 12). In the transformation zone there was clear cell damage, total lyses, and the tissue lost its consistency with the occurrence of many vacuoles. The testicular epithelia in the transformation zone were completely disintegrated; the spermatids were degenerated leaving vacuolated areas. The control group formed normal sperm (Figs 13, 14). In the maturation zone there was disintegration of the epithelial layer, while the matrix appears with many vacuoles and totally damaged cells. In the terminal zone there is degeneration of the sperm bundles and the peritoneal membrane has become finer, with the appearance of large vacuolated areas.

Electron micrographs of follicles from untreated males showed that the spermatocytes undergo their division and development normally (Fig. 15) then complete their differentiation and transformation till sperm is finally liberated (Fig. 16). In insects treated with 200 ppm azadirachtin, the cyst formation around the spermatogonia in the germarium was inhibited without any further development to spermatocytes and with the appearance of many vacuoles (Figs 17, 18). The electron micrographs also showed a clear separation of the basement membrane from the peritoneal membrane.

Schistocerca gregaria treated with azadirachtin suffered from arrested spermatogenic meiosis at Metaphase I (Linton *et al.* 1997). In *Epilachna varivestis* (Coleoptera: Coccinellidae) degeneration of

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the sperm bundles without sperm formation has been reported by Schulz and Schlüter (1983). 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. Shimizu (1988) showed that azadirachtin caused degeneration of spermatocytes in males of *Mamestra brassicae* (Lepidoptera: Pieridae), suggesting a direct effect of azadirachtin on the testicular membrane, rendering the tissue incapable of developing spermatocytes.

A review of the literature concerning the effects of some other insect growth regulators on insect gonads showed that aldrin caused pycnosis of germ cells leading to incomplete spermiogenesis in *Poekilocerus pictus* (Ahi 1988).

However, it should be pointed out that the factors which regulate spermatogenesis are not well understood (Dumser 1980). There is no strong evidence to indicate that hormones are generally involved (Engelmann 1970), but in some moths, the molting hormone could facilitate the process by increasing the permeability of the wall of the testis to some macromolecular factors (Wilde & Loof 1973).

It seems that at higher concentrations azadirachtin prevents the spermatogonia from cyst formation and therefore the spermatocytes fail to make their normal divisions and complete spermiogenesis. On the other hand, at lower concentrations the process of spermatocyte formation proceeds till the appearance of the sperm bundles, but with aberrations and degenerations.

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