DYNAMICS OF SPERMIOGENESIS IN *SPODOPTERA MAURITIA* BOISD. (LEPIDOPTERA : NOCTUIDAE)

Thesis submitted
to the University of Calicut
in partial fulfilment of the requirements for the Degree of
DOCTOR OF PHILOSOPHY
in Zoology

By

MANOGEM E.M., M.Sc.

DEPARTMENT OF ZOOLOGY, UNIVERSITY OF CALICUT
KERALA, 673 635, INDIA

2002

DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT

Fax : 0494 400269
Grams : UNICAL
E-mail : nair@unical.ac.in

Dr. V. S. K. NAIR, Ph.D.
PROFESSOR

Off : 0494 401144
Phone Extn.604,419,420
Resi : 0494 400031

CALICUT UNIVERSITY P. O
673 635, KERALA, INDIA

CERTIFICATE

This is to certify that this is an authentic record of the research work carried out by Miss. E.M. MANOGEM, M.Sc. from October, 1997 to March, 2002 as a part-time student in partial fulfilment of the requirements for the Degree of **DOCTOR OF PHILOSOPHY** under the Faculty of Science of the University of Calicut under my supervision and guidance. No part of this thesis has been presented before for any other degree. I also certify that she has passed the Ph.D. qualifying examination of the University of Calicut, held in December, 1999.

DR. V. S. K. NAIR, M. Sc. PH. D.
PROFESSOR
DEPARTMENT OF ZOOLOGY
UNIVERSITY OF CALICUT
KERALA - 673635
INDIA.

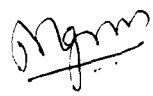
(V.S. KRISHNAN NAIR) 27.3.02

Residence: C-22, Calicut University Campus, Kerala 673635, India.

DECLARATION

I do hereby declare that the present work is original and it has not previously formed the basis for the award of any degree or diploma.

Calicut University,
March, 2002.



MANOGEM E.M.

ACKNOWLEDGEMENTS

With immense pleasure, I wish to express my profound sense of gratitude and indebtedness to Dr. V.S. Krishnan Nair, Professor, Department of Zoology, for his supervision and invaluable guidance throughout the period of my work.

Funding for this study has been provided by the United States Department of Agriculture (USDA), Washington in the form of a major research project sanctioned to Dr. V.S. Krishnan Nair. I worked as a Junior Research Fellow in this project. I thank USDA and Dr. Krishnan Nair for allowing me to present the unpublished data collected during the investigation for my Ph.D. thesis. I thank Dr. Y. Ramamohan, Professor, Department of Neuropathology, NIMHANS, Bangalore for permitting me to utilize the Electron Microscope facilities. I also thank Dr. M. Gayathri (Assistant Professor, NIMHANS), Mrs. B.N. Hemavathy (Senior Technician, NIMHANS) for technical assistance.

I record my sincere thanks to Dr. U.V.K. Mohamed, Professor and Head of the Department of Zoology, University of Calicut for providing the necessary laboratory facilities.

I express my sincere thanks to my colleagues, Miss. A. Sindhu, Mrs.P.M.Mona, Dr. T.M. Benny, Mrs. K.V. Nisha Suresh Babu and Mr.K.Sarafulla for their help and co-operation at various phases of the present work. I also acknowledge the help received from Mr. K. Narendran of the Department of Art and Photography Unit, University of Calicut.

I would also like to thank Mr. V. Lohithakshan, our laboratory assistant for his help in the rearing of the insect colony and Mr. K. Balu for carefully typing the manuscript.

Manogem E.M.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1. Insect Spermatogenesis : a review

In recent years, studies on the chemical or biological control of insect pests or the genetic manipulation of insect population through sterile/semisterile male technique are often hampered due to paucity of information on the timing and sequence of insect gametogenesis especially spermatogenesis. More attention has always been paid to the study of the process of oogenesis and its regulatory factors in females, when compared to similar studies conducted in male insects. This is because females are considered to be more important from the point of view of reproductive strategies and have always been the focus of research in the biological and chemical control of insects. The advent of the sterile male techniques for insect control have aroused a renewed interest in the study of male reproductive systems of insects. The literature on insect spermatogenesis is extremely large, although here also this is caused by the large number of species investigated rather than by relatively broad coverage. The testicular development and spermatogenesis have been extensively studied in many lepidopterans (Chaudhury and Raun, 1966; Retnakaran, 1970; Holt and North, 1970; Chase and Gilliland, 1972; Salama, 1976; Numata and Hidaka, 1980; Lai-Fook, 1982; Sridevi *et al.*, 1989; Venugopalan *et al.*, 1994).

Spermatogenesis provides a useful model system to study the profound morphological changes that take place during the terminal differentiation of a cell. The internal reproductive system of the male, including the testes has been studied in varying degrees of detail in many orders of insects (Phillips, 1970; Roosen-Runge, 1977). In most insects, spermatogonia and spermatocytes develop in the pupal and nymphal stages and the testes of the imago contain only spermatids and spermatozoa. Further, insect spermatogenesis provides a classical example of clonal, encysted development of germ cells (Roosen-Runge, 1977).

In insects, spermatogenesis is initiated by a species – specific number of mitotic division in the gonial cells. These cells orginate from a single spermatogonium. Division of gonial cells produce spherical spermatocytes, which undergo meiosis and differentiation to form spermatozoa. The germ cells which develop into spermatocysts differentiate more or less synchronously and are surrounded by a capsule of somatic cells. It is generally agreed that each cyst houses a single clone of germ cells. The testes of insects are usually paired organs, but in many species testes fuse into single median organ. The testes may lie above or below the gut in the abdomen and are often close to the midline. Usually, each testis consists of a number of follicles ranging in number from one in certain beetles to over 100 in grasshoppers. In Lepidoptera, the follicles are incompletely separated from each other whereas the testis of Diptera consist of a simple, undivided sac which may be regarded as single follicle. In certain insects the follicles are grouped together into several separate lobes. In the cerambycid, *Prionoplus*, for example, each testis comprises 12 to 15 lobes each with 15 follicles. The testes of Apterygota are often undivided sacs, but it is not certain in this case whether they are strictly comparable with the gonads of other insects since the germarium occupies a lateral position in the testis instead of being terminal. The wall of a follicle is a thin epithelium, consisting of two layers of cells, resting on a basal lamina. In some Hymenoptera and Lepidoptera, the two testes are bound together by a peritoneal sheath. In most Lepidoptera, the paired testes seen in the larval stages fuse to form a single median structure later in larval development.

1.1.1. Testicular sheaths

Cholodkovsky (1905) distinguished in Lepidoptera and Diptera, an outer layer of varying thickness and inner membraneous sheath or tunica propria. Trachea are plentiful in the outer sheath and penetrate to the inner sheath. Tunica

propria consists of two layers, a pigmented epithelial stratum and an inner lining which is translucent and nucleated. Zick (1911) considers the elongate cells in the outer layer of tunica propria as pathways of nutrition for inner one. The tunica propria grows and differentiates with the advancement of spermatogenesis. When spermatogenesis ceases, the tunica propria dwindles and many of its degenerating cells may be extruded into the follicles. Electron microscopic study of Bairati (1967) conducted in *Drosophila*, revealed that the testicular wall consists of an external layer of pigment cells and an internal layer of myoid cells. These membranes had an average thickness of 0.4 μm. The pigment layer has two functions. It protects the germ cells from radiation and osmotic changes. The function of myoid layer is considered to be tonic. According to him, testicular wall is a membrane containing and enveloping the germinal tissue without entering into stable association with it. In *Locusta*, each testicular tubule is bounded outside by compact fibrous connective tissue layer forming the basement membrane which supports the germ tissue (Viswanath *et al.*, 1974).

In *Heliothis virescens*, the sheath contains 4 membranes. Each wedge shaped follicle of larval testis is bounded by 'capsula lobuli'. Inner to this, the 'tunica interna' is seen. The four follicles of each testis are covered by 'membrana communis' and also an epithelial tissue layer. When the testes fuse, this epithelial layer is lost. In the prepupal period, the membrana communis remain as a thin layer. The tunica externa develops over the membrana communis to envelope the testis. The tunica externa and tunica interna are thin (1 μm) structureless membranes. Membrana communis is 3 to 6 layered connective tissue made up of irregularly spaced cells. The thickness of membrana communis decreases with age (Chase and Gilliland, 1972).

In gypsy moth *Porthetria dispar*, each testis consists of 4 follicles covered with a sheath of 3 membranes and an epithelial sheath. The structure of testicular

membranes is similar to that of *Bombyx mori* and *Heliothis virescens* (Salama, 1976). In *Spodoptera litura* each of the larval testicular lobes is made up of 4 follicles. The testicular lobes are enclosed within two thick, double layered peritoneal sheaths, each of which is composed of two layers of epithelial cells. The external sheath form a common envelope to all follicles and it is made up of lightly stained cuboidal cells, resting on a basement membrane. Inner sheath is made up of more darkly stained elliptical cells (Sridevi *et al.*, 1989).

In *Anagasta kuehniella*, the testis wall is formed by an external cell layer which surrounds the testis as a whole and by an internal cell layer that extends inward to enclose each chamber. A thin basement lamina lies over the outer surface of testis while a some-what thicker one marks the inner limit of the wall (Szollosi *et al.*, 1980). In the external layer, most cells show a sparse endoplasmic reticulum and few inclusions other than glycogen. Some cells, contain a more extensive system of rough endoplasmic reticulum. They are preferentially located between the inner and outer layers of the wall. Cells of the inner layer have a dense cytoplasm, containing glycogen rich areas, lipid droplets, and pigment granules (Szollosi *et al.*, 1980).

A nutritive function for testicular sheaths has been suggested by several authors. In *Dieliphela euphorbiae* at the time of maturation of spermatids in the late larval and early pupal period a maximum number of granules and droplets are observed in the cells of the inner lining whereas in the final phases of spermatogenesis, the sheath cells degenerate and products of their dissolution appear (Buder, 1917). Omura (1936) found that the fatty substances of membrana communis diminish during the pupal stage of *Bombyx mori* and the cells constituting the membrana communis appear collapsed in the adult stage. Thus he considers membrana communis to be a source of nutrients for the testes. Shimizu and Yagi (1978, 1982) showed that the testis wall of diapausing pupae of *Mamestra*

brassicae was necessary for *in vitro* elongation of meiotic cysts. Giebultowicz *et al.*, (1987) suggest that testis sheath releases factors which stimulate meiosis. Further the sheath can also provide nourishment or other conditions for survival of cysts, thus allowing meiosis to occur in germ cell differentiation.

1.1.2. Interstitial Cells

In early stages, cysts are usually tightly packed. Later they seem to float in a fluid-filled cavity (Zick, 1911). They may also lie suspended in a loose, interstitial network of "trophocytes" continuous with the inner lining of the follicle as in milkweed bug (Bonhag and Wick, 1953). In still other cases, the cysts remain densely crowded throughout spermatogenesis and no obvious interstitium develops (Meves, 1907).

In Diptera, cysts are not obvious. Cholodkovsky (1905), recognised in *Leptis* that the interior of the follicle contained some interstitial cells derived from the epithelial lining of the inner tunica. These appeared to partition the germ cells into groups. In *Dolichopus* and *Volucella*, he described 'yolk containing' vacuolated cells, which he regarded as nurse cells, distributed among spermatocytes and spermatids. In *Laphira*, he observed cyst-like structures, but did not mention any investment. Between the spermatogonial and spermatocytic region which he called the 'cyst zone', he found a vacuolated and granular mass staining with carmine. He speculated this as 'conglomerate of fused nurse cells'. Lomen (1914) mentioned no 'cysts' in *Culex pipiens*. He noticed large cells among cellular debris in the spermatozoal zone and suggested that these might be derived from germ cells. *Drosophila* has been investigated thoroughly by number of investigators (Cooper, 1950). Aboim (1945) came to the conclusion that interstitial cells somewhat tenuously delimit groups of germ cells which in other insects are enclosed in definite cysts. These cells are connected with the tunica propria of the testis, as in many other Dipteran species. Later, Aboim's experiments with agametic testes

showed that interstitial cells are not derived from this layer, but from a mesodermal group of cells, the 'apical cells'. Aboim expressed the conviction that they delimit germ cell territories. According to Bairati (1967) interstitial cells are easily recognised in electron micrograph by their volume, characteristic shape and richness in organelles. They are less electron-dense than the germ cells.

The interstitial cells, termed as 'cyst cells' (Snodgrass, 1935; Lee *et al.*, 1982), have also been reported in insects by Rule *et al.* (1965). Some other workers have called certain similarly placed cells as the 'sustentacular cells', which arise from follicular epithelium and, meandering between the spermatids, regulate the development (Smith, 1968). In the larval testis of *Pieris rapae* the 'cyst cells' are not very electron-dense and encircle the highly electron-dense germ cells (Kim *et al.*, 1982). In this insect, these cells also enclose the sperm bundles and their organelles are on an increase in pupae (Lee *et al.*, 1982). In the testis of *Tenebrio molitor* connective tissue sheath cells have been observed (Menon, 1969).

Saxena and Tikku (1989) reported an ultrastructural investigation of the interstitial cells in *Dysdercus koenigii*. These cells are spindle shaped with long cytoplasmic extensions going around the spermatocysts and usually 2-3 cells surround one cyst. The extensions are either single layered or branched and the cytoplasm is enriched with organelles like rough endoplasmic reticulum, polysomes, mitochondria, Golgi complex and secretory vacuoles. The major part of interstitial cell is occupied by a prominent nucleus and in transverse section the nucleus appears to be surrounded by cytoplasm forming a sort of frilly border. The rough endoplasmic reticulum is believed to be mostly associated with an active protein synthesis and in interstitial cells, its cisternae penetrate every course of the main body of the cell and its branches. The mitochondrial aggregates near the channels are indicative of their hyperactivity and the electron dense material in

channels as well as in vacuoles at the time of spermiogenesis emphasize upon the secretory nature of these cells.

1.1.3. Cysts and Cyst Cells

The greatest part of sperm development in insects occurs in "spermatocysts," in which germ cells develop more or less synchronously surrounded by a capsule of somatic cells. Cysts begin to be formed during the spermatogonial stage and by the time spermatocyte have differentiated, a lumen appears. A fully grown cyst is a hollow sphere of 32 – 64 germ cells surrounded by an envelope consisting of one or more cyst cells. Generally, each cyst contains one clone of germ cells derived from a single primary spermatogonium (Snodgrass, 1935). In Lepidoptera, Ammann (1954) observed that cysts are formed around groups of 8 or 16 interconnected spermatogonia.

Demandt (1912) found in *Dytiscus* that the apical region of the testis is filled with cells varying widely in nuclear size and chromatinic pattern. They represent a mixture of germ cells in various stages of development and somatic cells. Cell borders are indistinct. The spermatogonia continue to multiply within the cysts. Somatic cell nuclei of cysts become much larger than the nuclei of germ cells. The flagella of developing spermatids first point towards the hollow centre of the cyst, later towards the center of the testis. An important cytological and cytochemical study of cyst-wall formation was conducted by Anderson (1950) in the Japanese beetle *Popilia japonica*. He reports that during spermatogenesis, the cyst cells are large and flat and forms an envelope which closely follows the outline of spermatid bundles. During the process of nuclear condensation the sperm head turn towards the wall of the cyst and push deeply into cyst cells. The cyst is propelled through the crowded follicles into the funnel of vas deferens. Spermatozoa are released from the cysts after they entered the vas efferens and the abandoned cyst cells degenerate. Glycogen serves as nutrient for the spermatozoa in the seminal

receptacle. Edwards (1961) demonstrated that cyst cells do not leave the testis, but break down and dissolve while still in the testicular cavity.

1.1.4. Germ Cells

Germ cells of insects develop as clones. The normal course of spermatogenesis in insects was first studied by Verson (1889) in *Bombyx mori*. Later many others also investigated the cytological changes in the male germ cells during their differentiation to spermatozoa. Insects have cystic spermatogenesis. The germ cells develop by synchronous division within a sac formed by a single layer of cells believed to be of mesodermal origin (Hannah-Alava, 1965). Spermatogenesis is initiated with the formation of primary spermatogonia. Spermatogonia develop into spermatocytes which undergo meiotic divisions to give rise to the spermatids. Spermatids then differentiate to spermatozoa. The number of spermatogonial generation varies in different species.

In each follicle it is not known whether one or few stem cells give rise to later generation of spermatogonia or the later generation of spermatogonia themselves can function as stem cells (Hannah-Alava, 1965). The concept of a germ line is indeed an important one. A quasi-dichotomous division of primary germ cell, which give rise to primary spermatogonium and another stem cell appears to exist in *Drosophila melanogaster*, *Bombyx mori* and several Acrididae (Hannah-Alava, 1965). However, in other species little is known about this phenomenon.

The testicular follicles are the sites of the mitotic/meiotic cell division of germ cells and their differentiation to spermatozoa. The entire process is referred to as spermatogenesis. This process usually occurs during the last larval instar or pupal stage and in some species continues in the adult stage. Each follicle contains a large apical cell or complex of cells that serve as a trophic function, providing

nutrients for the developing spermatogonia. In several insect species, each follicle is divided apically to basally into zones that represent the different stages of spermatogenesis. Apically, the germarium or zone of spermatogonia is composed of the germ cells (spermatogonia) and somatic mesodermal cells. The next region is the zone of growth or zone of spermatocytes. The spermatogonia undergo several mitotic divisions, forming primary spermatocytes that become encysted in somatic cells. The primary spermatocytes undergo meiosis and produce haploid daughter cells in the next region, the zone of maturation and reduction. With the first and second meiotic division the primary spermatocytes become secondary spermatocytes and spermatids respectively. In the basal zone of transformation, the secondary spermatids become transformed into flagellated spermatozoa.

In general, all the cells in a cyst are derived from a single primary spermatogonim. The number of sperms which a cyst ultimately produces depends on the number of spermatogonial divisions and this is fairly constant for a species.

Sridevi *et al.*, (1989) observed in *Spodoptera litura* that spermatocytes begin maturation and elongation in the mid-last larval instar. Spermatocytes within a spermatocyst divide synchronously and sperm in various stages of development can be seen in both the pupal and adult testis.

The time taken for the completion of spermatogenesis varies, but in *Melanoplus* the period is about 28 days, the spermatogonial division occupying eight or nine days and spermiogenesis ten (Muckenthaler, 1964). In most insects, meiosis is complete before the final moult and in insects which do not feed as adults spermatogenesis is brought to completion before adult emerges.

Synaptonemal complexes

Spermatocytes that enter prophase of the first meiotic division are recognized by a structure, the synaptonemal complex. Homologous chromosomes

pair at this stage, they align themselves along their entire length and homologous regions in the chromosomes become juxtapositioned to each other. The chromosomes become synapsed by a tripartite structure that extends from telomere to telomere between the chromosomes.

Not all insect species form synaptonemal complexes during spermatogenesis. In Hymenoptera males, such as ants, bees, wasp and *Drosophila* have no synaptonemal complexes. Those of hydroptilid Trichoptera are multiple and quite conspicuous polycomplexes (Dallai and Afzelius, 1995) as are those of Orthoptera (Wolf and Mesa, 1993). In the Lepidoptera, *Lymantria dispar* the spermatocytes of eupyrene prophase I are characterised by large nuclei with synaptonemal complexes, diffuse chromatin and dense perinuclear sheathing (Garvey *et al.*, 2000).

1.1.5. Spermiogenesis

The spermatid which is formed after meiosis is typically a rounded cell containing the normal cell organelles. Subsequently it becomes modified to form the sperm and this process of spermiogenesis entails a complete reorganisation of the cell.

Intercellular bridges

During the division of spermatocytes to spermatids the daughter cells do not separate but remain connected by intercellular bridges. The cell membrane is strengthened by an electron dense material at the intercellular bridge. The intercellular bridges are extensive in Diptera and in some other insects and interconnected spermatids are aligned side by side (Phillips, 1974).

Nucleus and Chromatin structure

In the early spermatid the nucleus appears to have a typical interphase structure with the chromosome fibrils unoriented. In *Chorthippus*, the nucleus becomes very long and narrow and as it does so, the chromosome fibrils become

aligned more or less parallel with its long axis. The fibrils appear to form an anastomosing net work when the nucleus is seen in cross section. As the nucleus elongates and narrows, the nucleoplasm between them is progressively reduced until finally the whole of the nucleus appears to consist of a uniformly dense material (Das and Ris, 1958). During spermatid maturation the homogeneous mesh work of thin filaments may aggregate to form flat lamella that may take a concentric arrangement as in *Thermobia, Anurida maritima* (Dallai, 1970) and *Chortophaga* (Bloch and Brack, 1964), or form intricate labyrinths as in *Melanoplus* (Fawcett *et al.*, 1971). The aggregates also form hexagonally spaced chromatin fibres as in *Tettigonia* (Sjostrand and Afzelius, 1958) or condense further to large dense blocks as in *Acheta domestica* (Kaye, 1962). Nuclear condensation may start centrally, as in dragonfly, (Kessel, 1970) or start peripherally as in Homoptera. In some insect spermatozoa the chromatin consists of scattered condensed regions within an empty looking mass of dispersed chromatin (Dallai *et al.*, 1997; Fernades *et al.*, 2001).

Post-meiotic transcription

Post-meiotic transcription is virtually absent; transcription of genes coding for the proteins needed during spermiogenesis is said to be restricted to the spermatocyte stage (Erickson, 1990). Bloch and Brack (1964) have found that meiosis is followed by a cessation of RNA synthesis and that excess RNA is eliminated from the nucleus. During the sloughing off of residual cytoplasm that accompanies cell elongation, most RNA is lost from the cell. As a consequence of this shape of the spermatozoan is determined by the genes of the diploid male producing it, rather than by the genes in its own haploid nucleus.

Acrosome formation

The acrosome is derived, from Golgi material, which in spermatocytes is scattered through the cytoplasm in the form of dictyosomes. There may be 30 or 40

of these in the cell and they consist of several pairs of parallel membranes with characteristic vacuoles and vesicles. After the second meiotic division the dictyosomes in *Acheta* fuse to a single body called the acroblast, which consists of 6-10 membranes forming a cup with vacuoles and vesicles both inside and out.

In the later spermatid a granule, called the pro-acrosomal granule, appears in the cup of the acroblast and increases in size. The acroblast migrates so that the open side faces the nucleus and then the granule, associated with a newly developed membrane, the interstitial membrane, moves towards the nucleus and becomes attached to it. As the cell elongates the acroblast membranes migrate to the posterior end of the spermatid and are sloughed off together with much of the cytoplasm and various other cell inclusions. The pro-acrosomal granule then forms the acrosome, becoming cone-shaped and developing a cavity in which an inner cone is formed (Kaye, 1962). In *Gelastocoris* (Heteroptera) the proacrosome is formed from the fusion of granules in the scattered Golgi apparatus and no acroblast is formed. This may also be the case in Acrididae.

The function of the acrosome is to aid in the penetration of the eggs by the sperm; it may furnish enzymes which dissolve the egg membranes. However, this explanation may not be sufficient for all species since in several species of tiger beetles no acrosome could be identified (Nath, 1956). Also, in several Thysanura, the acrosome is located in the neck region of the sperm posterior to the nucleus (Nath, 1956; Bawa, 1964).

Mitochondrial transformation

In the spermatid the mitochondria fuse to form a single large body, the nebenkern. Pratt (1962) has described the several steps in nebenkern formation in the hemipteran *Murgantia*. The filamentous mitochondria aggregate in late telophase of the second meiotic division; and the mitochondria fuse to form longer

units and approach each other closely. They also anastomose with their neighbours. Then a network is formed that consists of two unconnected interlocked network of rings. In a cross section the nebenkern looks like a jigsaw puzzle with many mitochondrial profiles that form two halves, each consisting of several concentric layers (Tokuyasu, 1975). Finally the two halves of the nebenkern extend to be elongated mitochondria which extend in parallel along the flagellum.

Pratt (1962) assumes that the process of nebenkern formation may be one that will divide the mitochondrial material equally in two parts to maintain symmetry and that which will organize the mitochondrial material for its specific role in the mature spermatozoon. The two mitochondrial derivatives in many species have unequal diameters. The furrow, which divides the nebenkern into two equal halves is in alignment with the axoneme (Tokuyasu, 1975). Another possible function of the nebenkern is that its many concentric shells store the large membranous area that is needed in the formation of the elongated mitochondrial derivatives of the spermatozoon (Tokuyasu, 1975).

The two mitochondria formed during spermiogensis extend along the flagellum in most species and differ from their equivalents in somatic cells in three respects:

1) their length and size are relatively enormous, in some species they occupy by far the largest part of the spermatozoon with a length of several millimetres (Afzelius *et al.*, 1976; Mazzini, 1976; Pitnick *et al.*, 1995). 2) the mitochondrial cristae tend to be regularly spaced and to be orientated perpendicularly to the longitudinal axis (Phillips, 1970, 1974) 3) the mitochondrial matrix contains a conspicuous crystalline material which occupies most of the mitochondrial space or part of it, in the latter case it is close to the mitochondrial membrane at the side bordering the flagellar axoneme. The crystalline material has been isolated and contain two main polypeptides of 52 and 55 kilodaltons molecular weight and they contain a high

percentage of proline (Baccetti *et al.*, 1977). The order Heteroptera is characterised by having two or three crystalline bodies inside each of its two mitochondrial derivatives, rather than a single one (Dallai and Afzelius, 1980). Mitochondrial DNA has been demonstrated at various stages of *Drosophila* spermiogenesis up to the stage of deposition of a crystal (Bairati *et al.*, 1980). The mitochondrial DNA is apparently transferred to the egg at fertilization and is possibly used by the embryo (Kondo *et al.*, 1991).

Flagellar growth

The early insectan spermatid has a single centriole (Friedlander and Wahrman, 1966, 1971), whereas other animals generally have a diplosome (two centrioles in a perpendicular orientation). In most animals, the centrioles replicate after the first meiotic division, which gives the first spermatocyte, four centrioles. According to Friedlander and Wahrman (1971) no such replication occurs in insects. However it is of interest, that the primary spermatocytes of *Spodoptera littoralis* (Godula, 1985). or *Drosophila melanogaster* (Rasmussen, 1973) are reported to have four flagella.

The centriole of the early spermatid moves to the cell surface and then retracts towards the nucleus, bringing a membranous vesicle with it. The centriole then sprouts a short flagellum, that extends into the vesicle (Szollosi, 1975). The centriole is recognized by its nine triplet microtubules and also by a high electron density. It appears straight and rigid. When acting as a flagellar basal body the centriole consists of nine doublets rather than triplets.

The centrioles of some Neuroptera are unusual in that they are exceedingly long (Friedlander and Wahrman, 1966). They are longest known centrioles and are hence 'giant centrioles.' Centrioles in Cecidomyiid and Sciarid spermatozoa have many doublets rather than nine (Phillips, 1970). Centrioles in insect

spermatozoa generally have doublets, rather than triplets (Phillips, 1974). It is generally considered that centrioles act either as basal bodies of flagella (or cilia) or as poles of the mitotic spindle, but cannot have both functions at the same time.

The flagellum has a central core of nine microtubular doublets surrounding two central singlet microtubules, the well known 9 + 2 microtubular arrangement. The wall of the two central microtubules contain 13 protofilaments. The lumen of these tubules appear clear or electron-dense, depending on the species (Dallai and Afzelius, 1990). During spermiogenesis, in heteropteran bugs (Danilova *et al.*, 1984), and other species, there are large amounts of amorphous pericentriolar matter, which assist in establishing an unusual nuclear pattern.

The centriole of the mature spermatozoon is surrounded by a material termed 'centriolar adjunct.' In most insects it has a homogeneous or finely granular appearance, but in gall midges it contains globular inclusions (Dallai *et al.*, 1996). The centriolar adjunct consists mainly of proteins, although the presence of RNA has also been demonstrated (Cantacuzene, 1970). Its function remains unknown.

The flagellum grows by elongating at the free tip, where a distal swelling is seen (Wolf, 1996 a). This swelling contains a tubulin reserve, in order to ensure an uninterrupted growth even when tubulin is needed for other processes within the cell body (Wolf, 1996b). The newly formed flagellum has a simple 9+2 axonemal structure, but eventually accessory tubules develop (peripheral singlets). They appear as outgrowths from the B-tubules of the axoneme (Dallai and Afzelius, 1993) and are organized in the same way as other microtubules, except that the number of protofilaments usually is higher than 13. Because of the presence of accessory tubules in most insect orders, the axoneme is represented with the short hand formula 9+9+2 (nine accessory tubules, nine doublets and two central microtubules). The sperm tail in insects thus has several categories of microtubules.

1.1.6. Structure of mature spermatozoa

The mature sperm of most insects are filamentous in form, often about 300 μm long and less than a micron in diameter. The length of a spermatozoan can vary from 1.7 μm as in termites *Reticulitermes lucifugus* (Baccetti *et al.*, 1981) to 58,000 μm as in *Drosphila bifurca* (Pitnick *et al.*, 1995). The head and tail of the sperm are of approximately the same diameter. The cell wall of the sperm is a typical three-layered membrane, but in some species it is coated on the outside by a layer of glycoprotein known as glycocalyx. It is made up of rods at right angles to the surface of the sperm. Lepidopteran sperms have a series of projection running along their length. These projections are made up of thin laminae stacked parallel with the surface membrane. They become rearranged in the ejaculatory duct to form a complete coating all round the sperm (Baccetti, 1972).

The greater part of the head region is occupied by the nucleus. The DNA is apparently arranged in strands parallel with the long axis of the sperm. In front of the nucleus is the acrosome. In most insects this is a membrane bound structure of glycoprotein with, a granular extra acrosomal layer and an inner rod or cone. Sperm of Neuroptera have no acrosome and occasional species with no acrosome occur in other orders. The acrosome is concerned with attachment of the sperm to the egg and also with lysis of the egg membrane, thus permitting sperm entry.

Mitochondria become progressively longer and narrower during the course of spermiogenesis. The mitochondria of mature insect sperm generally extend from the base of the nucleus to the end of sperm tail. The sperm of Pterygota have two mitochondrial derivatives which flank the axial filament. Within these, the cristae become arranged as a series of lamellae which project in from one side of the derivative and are found to be at right angles to the mitochondrial long axis. The matrix of the derivative is occupied by a para crystalline material. Spermatozoa of

Mecoptera and Trichoptera have only one mitochondrial derivative, while phasmids have none at all. In this case respiration is entirely anaerobic.

Immediately behind the nucleus, the axial filament or axoneme arises. In most cases this consists of two central tubules with a ring of nine doublets and nine accessory tubules on the outside. The central tubules are surrounded by a sheath and are linked radially to the doublets. Some unusual exception to this 9 + 9 + 2 arrangement occur. It is presumed that the axial filament causes the undulating movements of the tail which drive the sperm forwards.

In Kalotermitidae and Rhinotermitidae, there is no flagellum at all. The sperm of *Reticulitermes* is spherical with no acrosome, but it has a few normal mitochondria. This sperm is non-motile. Non-motile sperm also occur in the dipteran family Psychodidae and in Protura.

Sperms are grouped together in bundles in a number of insects for at least some part of their existence and sometimes the bundles persist even after transference of the sperm to the female. Five different sperm types have been recorded from Hymenopteran, *Dahlbominus fuscipennis* (Lee and Wilkes, 1965). Pairs of sperms also occur in some Coleoptera. Coleopterans produce spermatozoa with diploid or tetraploid sperm nuclei (dipyrene or tetrapyrene spermatozoa) besides the normal haploid ones.

Sperm dimorphism in Lepidoptera

Lepidopteran males display dichotomous spermatogenesis, producing eupyrene (nucleate) and apyrene (anucleate) spermatozoa. This unconventional type of spermatogenesis is an evolutionary novelty of Lepidoptera as

a) It occurs throughout the order, including Zeugloptera which is the most primitive systematic group of Lepidoptera.

b) but is absent from the closely related order of Trichoptera, with which they form together the super-order Amphiesmenoptera (Friedlander, 1983a). Eupyrene and apyrene spermatozoa reach together the spermatheca of the inseminated females. However, only eupyrene ones fertilize the eggs (Friedlander and Gitay, 1972), although according to species 50-90% of the sperm transferred to the female are apyrene (Silberglied *et al.*, 1984).

i) Eupyrene sperm

Lepidopteran eupyrene are distinguished from apyrene sperm not only for possession of a nucleus and lacinate appendage, but also by the presence of an acrosome, larger size of the two mitochondria, and electron dense centres in the peripheral and central singlets as reported in *Lymantria dispar* (Garvey *et al.*, 2000).

Phillips (1971), in a survey of 18 lepidopteran species, describes that the acrosome of eupyrene sperms consist of homogeneous electron-dense material and usually forms a cup-shaped cap anterior on the nucleus. In *Plodia interpunctella* the acrosome in cross-section is polygonal in appearance which suggest a para crystalline composition (Ashrafi and Roppel, 1973).

In *Pieris brassicae*, the head of eupyrene sperm is 10 μm long at least 1 μm wide, which includes anteriorly the acrosome complex consisting of an extracellular dense band, an intercellular dense lamina, and a tubular structure which incises and parallels the surface of the dense nucleus. The extracellular dense band which continues on to the axoneme is considered to be the reticular body and therefore a non-acrosomal structure.

In eupyrene sperm, the nuclei are long and thin with highly condensed chromatin which in cross section are circular, semicircular, oval, polygonal or heart shaped (Phillips, 1971) or helment shaped posteriorly and flattened anteriorly

(Riemann, 1970). That of *Bombyx mori* is crescentic in cross section; the posterior part of the nucleus is very flat and is compressed between the cell membrane and either the centriole or anterior part of the axoneme (Friedlander and Gitay, 1972).

In mature eupyrene, one mitochondrial derivative is always somewhat larger than the other. In *Plodia interpunctella* the two unequal derivatives almost completely fuse to form a single body in which the larger of the two is cristate (Ashrafi and Roppel, 1973). This corresponds with a description drawn from *Bombyx mori, Ephestia cautella* and *Ectomyelois ceratoniae* (Pyralidae) and *Hyalophora cecropia* which are stated to have two apposed mitochondrial derivatives, which fuse into one structure having a crescent shape in cross section (Friedlander,1983 b). However, *Bombyx* have two subequal, apposed derivatives (Friedlander and Gitay, 1972). In *Trichoplusia ni* (Riemann, 1970) and *Spodoptera frugiperda* (Riemann and Gassner, 1973) one derivative is very rudimentary and in *Heliothis virescens* and *Heliothis zea* in which the disparity in size is only moderate, the smaller is embedded in the larger (Riemann and Gassner, 1973).

Of two centrioles present in the spermatid, one persists within a long lateroposterior excavation of the nucleus. Friedlander and Wahrman (1971) state that only one centriole is present in the spermatid of *Bombyx mori* though acknowledging the occurrence of two centrioles in the spermatocytes.

Phillips (1970) states, for 195 insect species, in 15 orders, that no centriole persists in the mature spermatozoon. Investigation of testicular eupyrene of *Heliothis punctigera* appears to confirm this absence. At the anterior end of the axoneme the dense accessory tubules (peripheral singlets) surround a cylinder. Behind this the cylinder is absent and two central singlets and nine doublets take their place within the peripheral singlets, giving a normal 9+9+2 axoneme. No transitional region possessing the triplets of a true centriole has been detected and no true, triplet centriole is seen.

The axoneme of the lepidopteran sperm is of the classical insectan type with the exception that the peripheral singlets which have 16 protofilaments in the wall (Dallai and Afzelius, 1990). Features common to spermatozoal axonemes in most insect groups, include linkage of the peripheral singlets to the doublets, presence of two dynein on each A subtubule of the latter; spokes and spoke heads; and two central singlets linked by two convex lines, comprising the sheath. A similar structure is indicated for *Bombyx mori* (Danilova and Vereiskaya, 1969) and *Pieris brassicae* (Zylberberg, 1969). The central and peripheral singlets usually have solid centres (Phillips, 1970, 1971).

Lacinate appendages have been demonstrated in eupyrene sperm of *Pieris brassicae* (Andre, 1959); *Bombyx mori* (Friedlander and Gitay, 1972); *Ephestia cautella* (Friedlander, 1976) and indeed in all 18, mostly unspecified lepidopteran species examined (Phillips, 1971). They extend from the plasma membrane overlying the acrosome nearly to the posterior end. They are usually very large near the anterior end and gradually taper along the length of the cell. The shape varies with the species, as columnar, trapezoidal, clavate, pyramidal or rectangular. The number varies considerably among neighbouring sperm. The substructure of the appendage is the same in all species investigated. In cross section they appear to be composed of alternate electron-lucid and electron-dense zones with a periodicity of 90°A (Phillips, 1970, 1971, 1974). In *Ephestia cautella* each appendage originates in the spermatid, from a radial row of intra-cellular microtubules in single file. The original plasma membrane lying externally to the tubules, is discarded and is replaced by a definitive plasma membrane. The newly formed plasma membrane develops from the flat membranous cisternae and lies internal to the tubules (Friedlander, 1976). Recently Garvey *et al.* (2000) reported that eupyrene spermatozoa undergo additional morphogenesis upon leaving the testes, by loosing their lacinate appendages and gaining an extra cellular sheathing.

An additional appendage projecting perpendicular to a line joining the two central singlets also exist. This is the reticular appendage. It lies on the side of the sperm occupied by the larger mitochondrial derivative, consistently adjacent to doublet 6. It is morphologically dissimilar to the others, (Phillips, 1970, 1971) and its origins are distinct from the lacinate appendages. The reticular appendage of *Euptoleta hegesia* has a paracrystalline core and extends to the distal tip of the spermatozoon (Mancini and Dolder, 2001).

In transverse sections reticular appendage consists of a honey comb of lucid and dense regions (Phillips, 1971). It develops as an extracellular conglomerate of small spheres embedded in electron-opaque material, and connected by thin lamellae to the body of the sperm, these lamellae or septa are seen for *Heliothis punctigera*. The posterior region of the axoneme in *Trichoplusia ni* is attached to the plasma membrane by a septa at a point between the axoneme and the smaller mitochondrial derivative. Further, anteriorly it moves to a position below the nucleus and assumes the form of a broad crescent (Riemann, 1970).

ii) Apyrene sperm

Apyrene sperm is smaller and completely lacking in the nuclear material. Like eupyrene sperm, the apyrene sperms are produced in large numbers, usually comprising over half the total sperm complement. They are transferred to the female during copulation, and migrate to the sperm storage organ (spermatheca).

Apyrene sperm develop later and more rapidly than nucleated sperm. Maturation divisions that occur in the larva give rise to eupyrene sperms. Then just before pupation, most cysts of spermatocytes remain small and undergo rapid, asynaptic meiosis. During the pupal stage, these new cells elongate and discard their nuclei thus becoming apyrene sperm (Leviatan and Friedlander, 1979; Lai-Fook, 1982).

In *Ephestia cautella*, the spermatids which give rise to these anucleate sperm have each several micronuclei. These are degraded by DNAase in the spermatid and after anucleation, by lysosomes of the somatic testicular cells (Friedlander and Miesel, 1977). In *Pieris brassicae*, however, the transient nucleus is not subdivided (Zylberberg, 1969).

Apyrene sperms have no lacinate or reticular appendages. In *Lymantria dispar* the maturation of apyrene spermatozoa involves the morphogenesis of the axoneme and mitochondrial derivatives in the absence of differentiating nuclei and acrosome (Garvey *et al.*, 2000). The mitochondrial derivatives are small and are equal in diameter and length (Phillips, 1971; Riemann, 1970). In cross section they form a 'V', cradling the axoneme in *Bombyx mori* (Friedlander and Gitay, 1972) and extend from a little posterior to the anterior cap, nearly to the posterior end of the cell. Each is paracrystalline usually with 50A° hexagonal densities in cross sections (Phillips, 1971). Two approximately equal mitochondrial derivatives are known for apyrene sperm of *Plodia interpunctella* (Ashrafi and Roppel, 1973) and for *Heliothis punctigera* (Jamieson, 1987). The peripheral and central singlets of the axoneme of the apyrene sperm do not display the dense contents seen in eupyrene though the peripheral singlets may have a slight electron density (Phillips, 1971; Riemann and Gassner, 1973).

Apyrene sperms are produced in great numbers which comprise 50% of the sperms transferred in *Spodoptera litura* (Etman and Hooper, 1979). Apyrene sperms are immediately activated during ejaculation. In a variety of Lepidoptera, apyrene sperms are rendered motile by a secretion from the male tract (Shepherd, 1975; Herman and Peng, 1976). In *Manduca sexta*, apyrene become motile in the spermatophore, in a process involving proteases from the male duct (Friedlander *et al.*, 2001). Apyrene sperm make the complicated journey through the female reproductive tract from the bursa to the spermatheca (Holt and North, 1970;

Katsuno, 1977 b; Etman and Hooper, 1979). In several insect species, the anucleate sperm accumulate in a diverticulum of the spermatheca where they degenerate within 5 hours (Katsuno, 1977 b).

Eupyrene meiotic divisions are regular and lead to the development of eupyrene mononucleated spermatid. But apyrene meiotic metaphases and anaphases are highly irregular and majority of telophase chromosomes remain isolated instead of clustering at the spindle poles and the resulting spermatids contain numerous micronuclei of two types, one of which is small and with electron dense chromatin and the second with diffuse chromatin. The second type get filled with electron opaque chromatin at a later stage of development. The volume of this type of micronuclei varies considerably both within the cell and among spermatids throughout spermiogenesis. During the maturation of apyrene spermatids, the micronuclei are extruded from the cells (Friedlander and Miesel, 1977).

Nuclear elongation is not causally related to nucleoprotein transitions as transitions occur in the eupyrene spermatids after nuclear elongation. Replacement of the nucleoprotein occurs in the eupyrene sperm in a polarized manner. The cytoplasm of the eupyrene spermatids show an increasing amount of cytoplasmic lysine-rich proteins, while no such phenomenon occurs in apyrene spermatids. This differential protein distribution may reflect functional differences between the two types of spermatids and is related to the regulation of dichotomy in lepidopteran spermatogenesis (Friedlander and Hauschteck-Jungen, 1982).

iii) Functions of apyrene spermatozoa

The function of the apyrene spermatozoa is still unclear. All the theories concerning this question are based on structural observations or biochemical analyses only. None of them is sustained by convincing experimental approaches.

Among the several feasible theories concerning the function of apyrene spermatozoa proposed are:

(a) they provide nutrients for the eupyrene spermatozoa within the female genital tract (Riemann and Gassner, 1973);

(b) the DNA of their dicarded nuclei is metabolized and serves as a substrate for glycogen biosynthesis (Sugai, 1965);

(c) they facilitate acquisiton of motility by the eupyrene spermatozoa within the female tract (Osanai *et al.*, 1990);

(d) in polyandrous species, they play a role in the competition among the spermatozoa of the different inseminating males within the female genital tract (Siberglied *et al.*, 1984)

(e) they help in transporting the eupyrene spermatozoa within the female genital duct (Iriki, 1941)

(f) they make way for the migration of the eupyrene spermatozoa across the testicular 'basement membrane' (Katsuno, 1977a).

Active apyrene sperms have been shown to be necessary for separation of eupyrenes from their bundles (Kramer *et al.*, 1983). Activated apyrene spermatozoa promoted digestion of amorphous masses from the vesicula seminalis which was probably necessary for sperm maturation. Apyrene spermatozoa must be activated in the spermatophore to stir the contents and promote dissociation of eupyrene bundles and separation of each individual eupyrene spermatozoan both mechanically and by biochemical reactions. The flagellating apyrenes also cause digestion of the soft plug, a proximal part of the spermatophore, resulting in opening to the ductus seminalis, which help in the migration of eupyrenes. Kawamura *et al* (2000) reported a peristaltic phenomenon in both eupyrene and

apyrene sperm bundles in *Bombyx mori*. Through peristaltic action, cytoplasm of the eupyrene sperms and both cytoplasm and nuclei of the apyrene sperms are discarded from the posterior end of the sperm bundles thus eliminating the irregular nuclei of apyrene sperm while preserving the nuclei of eupyrene sperm.

Eupyrene spermatogenesis is highly sensitive to genetic and other experimental manipulations, while apyrene spermatogenesis generally resists these manipulations. Sublethal rearing temperatures are found to disrupt eupyrene spermatogenesis in *Plodia interpunctella* and *Ephestia cautella* (Lum, 1977). Inherited sterility of male progeny of gamma irradiated *Ephestia cautella* (Riemann, 1973) is characterized by highly irregular eupyrene spermatogenesis but regular apyrene spermatogenesis. Abnormal eupyrene spermatogenesis but normal apyrene spermatogenesis occur in both infertile hybrids of interspecific crosses (Richard *et al.*, 1975) and induced polyploid males (Katsuno and Tamazawa, 1979).

1.2. Hormonal regulation of spermatogenesis in insects

The male insect maintains a level of differentiation within the germ cell line in pace with its somatic development. This ability must be due to the existence of some rate regulating mechanisms or regulatory points, in the developmental sequence of male germ cells. Fukuda (1944) first suggested that the development of male germ cells of silkworms is controlled by hormones. When silk worms in the early third or fourth instar stage are deprived of their corpora allata, the larval state is not maintained but the allatectomised larvae will mature and metamorphose precociously. Even in small sized moths produced by allatectomy, fertilizable spermatozoa were produced. These observations led Fukuda (1944) to suggest that hormones released by corpora allata regulate spermatogenesis in insects.

1.2.1. Juvenile hormone

Wigglesworth (1936) was the first among the many investigators to note that in *Rhodnius prolixus* mature sperm could be produced in the absence of the hormone produced by corpora allata, the juvenile hormone (JH). He concluded that JH was not necessary for spermatogenesis, but did not exclude the possibility of involvement of other hormones. Studies on *Bombyx mori*, (Fukuda, 1944; Takeuchi, 1969; Yagi and Fukushima, 1975), *Spodoptera littoralis* (Metwally and Gelbic, 1974), *Dysdercus cingulatus* (Ambika and Prabhu, 1978) and *Ectomelois ceratoniae* (Leviatan and Friedlander, 1979) showed that corpus allatum hormone or its analogue can alter or inhibit the process of spermatogenesis or can induce the production of defective sperms.

JH acts as a division rate inhibitor. It abolishes the ecdysone stimulated gonial mitosis in *Rhodnius* (Dumser and Davey, 1974). In *Periplaneta* removal of endocrine glands, testis transplantation and hormone injections showed that ecdysone accelerated testis development but JH inhibited it (Blaine and Dixon, 1976).

In some insects opposite effects were observed after implantation of corpora allata or JH/JH analogue (JHA) application. Apparent stimulatory effects upon spermatogenesis, particularly spermiogenesis, have been found in *Mamestra brassicae* (Yagi, 1975), *Papilio xuthus* (Nishiitsutsuji-Uwo, 1961) and in *Pterostichus nigrita* (Ferenz, 1963). In all these species spermatogenesis was accelerated by application of JH which simultaneously breaks their diapause. In *Eurygaster integriceps*, JHA treatments during prediapause accelerated spermiogenesis which however remained incomplete (Shinyaeva, 1981). JH is involved in the spermatogenesis arrest in diapausing larva and spermatogenesis renewal in postdiapausing larva of codling moth (Friedlander, 1982). Elongation of eupyrene sperm nuclei of *Ectomyelois ceratoniae* is triggered by a decline in

endogenous JH titre, and could be inhibited by maintaining a high JH titre. But the elongation of the flagella is unaffected by high exogenous titre of JH mimic (Leviatan and Friedlander, 1979).

1.2.2. Ecdysteroids

Spermatogenesis is found to occur in the last larval instar or during the pupal stages of many insects. The role of ecdysteroids secreted by prothoracic glands and other tissues in promoting spermatogenesis has been well documented in *Bombyx mori* (Takeuchi, 1969). *Chilo suppressalis* (Yagi *et al.*, 1969), *Hyalophora cecropia* (Kambysellis and Williams, 1971a), *Ephestia kuehniella* (Nowock, 1973), *Mamestra brassicae, Spodoptera litura* (Fukushima and Yagi, 1975) and *Rhodnius* (Dumser and Davey, 1975). In the presence of 20-hydroxyecdysone there was an increase in the rate of cell divisions in the spermatogonial cells of *Rhodnius* (Dumser and Davey, 1975). This cell division occurs in the last larval instars and requires ecdysteroids in the absence of JH (Dumser and Davey, 1974).

In *Manduca sexta*, the development of spermatocytes in the last larval instar depends on the release of 20-hydroxyecdysone during the wandering stage of larva. It was found that the spermatocytes stopped developing if the abdomens were isolated before the release of this peak. The development is restored by the exogenous application of 20-hydroxyecdysone (Friedlander and Reylonds, 1992). By means of ligation and transplantation experiments combined with the administration of ecdysteroids, it was demonstrated that ecdysteroid promotes the fusion and torsion of testis in *Ephestia kuehniella* (Nowock, 1973) and *Spodoptera mauritia* (Benny and Nair, 1999). In *Tenebrio*, the synthesis of testicular proteins is also stimulated by ecdysteroids (Alrubeai and Gorell, 1981).

The presence of ecdysone in the ovary and the discovery that ecdysteroids are essential for spermatogenesis led to the hypothesis that ecdysteroids might also

be present in the testis. Large amounts of 20-hydroxyecdysone was found in the testis of *Calliphora vicina* (Koolman *et al.*, 1979). Shimizu and Yagi (1978) reported that larval testis itself could promote spermatogenesis *in vitro*, replacing the effect of exogeneously treated 20-hydroxyecdysone. Loeb *et al.* (1982) provided evidence that the testis is, a source of ecdysteriods. They found that the testis sheath of larval *Heliothis virescens* secrete several immuno-detectable ecdysteroids *in vitro*. The synthesis and release of testis ecdysteroids is regulated by a brain peptide, the testis ecdysiotropin. Testes as the source of ecdysteroids have been reported in other insects also (Loeb *et al.*, 1982, 1986, 2001; Gelman *et al.*, 1989; Jarvis *et al.*, 1990).

1.2.3. Macromolecular factors

Studies on the regulatory factors of spermatogenesis became much more complicated by the discovery of a blood-borne high molecular weight substance: the macromolecular factor in certain lepidopteran insects (Kambysellis and Williams, 1971b, 1972). The origin of the macromolecular factor appears to be in the haemocytes. 20-hydroxyecdysone is hypothesized to alter the permeability of both the testicular wall and the cyst wall allowing the macromolecular factor to enter the spermatocysts. Macromolecular factor like activity was also demonstrated to be present in mammalian serum (Kambysellis and Williams, 1971b).

1.2.4. Neurohormonal factors

Neurohormonal factors produced by the brain and segmental ganglia also seem to regulate testicular development and spermatogenesis. A brain peptide testis ecdysiotropin is necessary to stimulate testis sheath to produce ecdysteroids (Loeb *et al.*, 1987). Neurosecretory factors from *Drosophila* brain have also been implicated in regulating rate of the mitotic division of spermatogonia (Garcia-

Bellido, 1964). In *Heliothis virescens*, the rate of development of spermatocysts in larvae appears to be modulated by stimulatory factors from the brain and inhibitory factors from sub-oesophaeal ganglion (Loeb *et al.*, 1985).

1.2.5. Apyrene spermatogenesis inducing factors

Lepidopteran males produce two kinds of sperm, eupyrene (nucleate sperm) that are capable of fertilizing the ovum, and apyrene (anucleate) which may affect the motility and behaviour of eupyrene sperm. A few studies have demonstrated that apyrene spermatogenesis is promoted by haemolymph factors of unknown origin as well as a factor from the brain (Friedlander and Benz, 1981, 1982; Jans *et al.*, 1984; Gelman and Borkovec, 1986). Gelman *et al.* (1988) have shown that in *Ostrinia nubilalis* apyrene spermatogenesis is stimulated by ecdysteroids. This effect is dependent on the larval age and concentration of the ecdysteroid used.

1.3. Insect growth regulators

Insect growth regulators (IGRs) belong to a class of compounds naturally occurring or synthetically prepared which interfere with normal metamorphosis and reproduction in insects. There are three categories of IGRs (1) compounds which directly or indirectly influence the hormones regulating metamorphosis, reproduction, behaviour etc. (eg., juvenile hormone analogues, ecdysone agonists, anti juvenile hormone agents, anti ecdysteroids, neurohormone analogues or their antagonists)

(2) compounds which inhibit cuticle formation through an effect on cuticle synthesis (eg; benzoylphenyl ureas), and (3) compounds with miscellaneous mode of action (e.g., Azadirachtin). Several such compounds are known and their effects on metamorphosis and reproduction in a number of insect species have been extensively studied and reviewed (Retnakaran *et al.*, 1985; Darvas and Varjas, 1990; Nair, 1993; Dhadialla *et al.*, 1998).

The discovery of compounds with hormonal and antihormonal activities in insects have greatly facilitated studies in Insect Endocrinology since hormones control many life processes of insects such as metamorphosis, development, metabolism and reproduction. These compounds (Anti hormonal agents, hormone analogues) which induce either hormone deficiency or hormone excess in treated insects are excellent probes to analyse the role of hormones in metamorphosis and reproduction. Further, these compounds have great potential as insect control agents in integrated pest management programmes (Nair, 1993). Effects of these compounds on the female reproductive system have been extensively studied. Unfortunately similar studies in male insects are rather fragmentary.

1.3.1. IGRs based on insect hormones

i) Juvenile hormone analogues

From the time of the discovery of the chemical nature of juvenile hormone (JH) it was suggested that JH could be used as specific control agents of insects (Williams, 1967). This led to the discovery of juvenile hormone analogues (JHAs). Numerous analogues with JH activity have been studied. The most active ones, such as methoprene and hydroprene, however, lack the epoxide function present in JH (Staal, 1982). More recently, several highly active compounds (e.g. Fenoxycarb and Pyriproxyfen) have been synthesized. Some biological and insecticidal effects of fenoxycarb and pyriproxyfen have been reviewed (Grenier and Grenier, 1993, Miyamoto *et al.*, 1993).

Changing the haemolymph JH titre by treatments with JHA at critical periods during the life cycle of an insect will interfere with normal metamorphosis and reproduction. JHAs even interfere with the activity of other hormones. Methoprene inhibits the secretion of prothoracicotropic hormone in *Mamestra brassicae* (Hiruma *et al.*, 1978). In *Spodoptera mauritia* hydroprene has a direct

inhibitory effect on the secretory activity of prothoracic glands early in the last instar larval development (Balamani and Nair, 1992).

ii) Antijuvenile hormone agents

The limited scope of JHAs as insect control agents necessitated the discovery of compounds with anti JH activity. Anti JH agents disrupts normal development of early larval instars and inhibits JH dependent reproductive activities (Sam Mathai and Nair, 1984a; Santha and Nair, 1986; 1988; 1991, Santha *et al.*, 1987; Nair, 1993). Some well known examples of anti JH agents are precocenes, Fluoromevalonolactone (FMev), ETB, EMD, compactin, piperonyl butoxide, Allylic alcohols, Bisthiolcarbamate etc. Anti JH agent : precocene causes JH defeciency in treated insects by selectively destroying the parenchymal cells of corpora allata (Unnithan *et al.*, 1977). Treatment of precocene to pupae and reproducing females is found to inhibit ovarian development and induce sterility.

iii) Ecdysone agonists

Ecdysone mimics or ecdysoids are compounds which are structurally similar to ecdysteroids and possess moulting hormone activity in insects. They are classified into four groups: zooecdysoids, phytoecdysoids (extracted from plants), synthetic ecdysoids (steroids with moulting hormone activity) and non-steroidal agonists.

The first biacylhydrazine ecdysteroid agonist was discovered by Rohm and Hass Company in 1983. Subsequent chemical modification of this compound led soon to the discovery of a slightly more potent analogue, RH-5849 (Wing, 1988). Treatment of insects with minute doses of RH 5849 interferes with normal feeding activity in larval lepidopterans and insects belonging to other orders, by forcing a lethal, premature moult (Wing *et al.*, 1988; Sakunthala and Nair, 1995). Later another non-steroidal ecdysone mimic RH 5992 (tebufenozide) was discovered and this compound is more potent than RH 5849 in lepidopteran larvae.

In the spruce bud worm, *Choristoneura fumiferana* RH 5992 induces an incomplete moult (Palli *et al.*, 1995). Tebufenozide was also shown to disrupt normal spermatogenesis and other reproductive activities of several lepidopteran species (Carpenter and Chandler, 1994; Friedlander and Brown, 1995; Smagghe and Degheele, 1997). RH 5992 acts similar to 20-hydroxyecdysone by binding to the ecdysone receptor (Smagghe and Degheele, 1997).

iv) Anti ecdysteroid Agents

Since ecdysteroids play a critical role in insect development, reproduction, and embryogenesis, anti ecdysteroid agents which alter ecdysteroid titre have great potential as insecticides. The normal growth and development of *Manduca sexta* larvae can be inhibited by two vertebrate hypocholesterolaemic agents, triparanol and 22, 25 di-azacholesterol, by blocking the conversion of β-sitosterol to cholesterol which is a precursor of ecdysone synthesis (Svoboda *et al.*, 1972).

Azadirachtin, a tetranortriterpenoid extracted from the seeds of neem is found to disrupt growth, moulting and oogenesis of insects. A few studies indicate that azadirachtin treatments might also interfere with JH production or with the synthesis or release of eclosion hormone. However, several observations indicate that azadirachtin-induced morphogenetic effects are due to delayed or suppressed ecdysteroid titres (Jagannadh and Nair, 1992, 1993).

v) Neurohormones as IGRs

Neurons are found to produce and release peptide hormones which act as chemical messengers controlling growth, development and reproduction in insects. Moreover, neuropeptides are found to regulate the secretion of other hormones like JH and ecdysteroids. This led to the idea of manipulating the neurosecretory system for insect control purposes. Proctolin, a pentapeptide, which acts on the contraction of cockroach hindgut was the first fully characterised neuropeptide.

The neurohormones are found to interact with the receptor of the target cell thus causing hormonal inbalance in insects.

1.4. Objectives of the investigation

From the foregoing review it is evident that spermatogenesis in insects is initiated by a species-specific number of mitotic divisions in the gonial cells originating from a single spermatogonium. Division of gonial cells produce spermatocytes which undergo meiosis and differentiation to form spermatozoa. In Lepidoptera, the sequential steps in spermatogenesis have been studied by several workers using histological and histochemical techniques. However, ultrastructural studies on spermatogenesis have been comparatively few in case of lepidopteran insects. Hence it was thought worthwhile to examine in detail the testicular development and spermiogenesis during larval-pupal development and metamorphosis of *S. mauritia* Boisd. (Lepidoptera: Noctuidae) utilizing histological and ultrastructural techniques. This lepidopteran insect is a sporadic pest of paddy in the state of Kerala, in India. This species was chosen because of the availability of a sizeable background data from this laboratory on the effects of insect growth regulators with hormonal and anti-hormonal activity on larval development, metamorphosis and reproduction of this insect (Nair, 1981, 1993; Sam Mathai and Nair, 1983, 1984a,b,c,d; Santha and Nair, 1986, 1987, 1988; Santha *et al.*, 1987; Nair and Rajalekshmi, 1989; Pradeep and Nair, 1989; Balamani and Nair, 1989, 1991, 1992; Jagannadh and Nair, 1992, 1993; Sakunthala and Nair, 1995; Venugopalan *et al.*, 1994; Benny and Nair, 1999). Further several studies have shown that hormones like juvenile hormone (JH) and ecdysteroids control insect spermatogenesis. However a detailed investigation has not yet been carried out on the role of hormones in spermiogenesis of *S. mauritia*. Hence, one of the major objectives of the present investigation is to analyse the role of hormones on the spermatogenesis in *S. mauritia*. For this, the classical approach extensively utilized

in insect endocrinological laboratories has been employed. This involves studying the effects on spermatogenesis by ligaturing (neck/thorax) larvae to eliminate the endogenous hormonal sources. Further, the effects on spermatogenesis by exogenous treatments of ligated larvae with hormone analogues/agonists to restore hormonal balance were analysed by histological and ultrastructural studies. These studies have been greatly facilitated by the availability of compounds with hormonal activities in insects. These compounds (JH analogues, Ecdysone mimics) which rescue the hormone deficiency in ligated larvae or cause hormone excess in treated insects are excellent probes to analyse the role of hormones in insect metamorphosis and reproduction. Further these compounds have great potential as insect control agents in integrated pest management programmes (Nair, 1993). It is hoped that the results of this investigation will not only lead to a better understanding of the neurohormonal control of insect spermatogenesis, but also will provide valuable information concerning the potential of hormone analogues/agonists in pest control strategies.

Chapter 1 deals with a detailed review of insect spermatogenesis with special emphasis on spermiogenesis and the role hormonal factors controlling this process.

Chapter 2 provides basic information on the pest-status and a detailed account of the rearing and maintenance of *S. mauritia* Boisd (Lepidoptera: Noctuidae), under laboratory conditions.

Chapter 3 examines the different phases of germ cell and somatic cell differentiation during larval-pupal-adult development of *S. mauritia* utilising histological and ultrastructural techniques.

Chapter 4 deals with the role of hormones and the effects of hormone analogue/ agonist on spermatogenesis of *S. mauritia*.

CHAPTER 2

PEST STATUS, REARING AND BIOLOGY OF *SPODPOPTERA MAURITIA* BOISD. (LEPIDOPTERA: NOCTUIDAE)

2.1. Pest status

Spodoptera mauritia Boisd. (Lepidoptera : Noctuidae) popularly known as rice swarming caterpillar, or army worm is a sporadic pest of *Oryza sativa*. The pest attack status of this insect occasionally assumes serious dimension and cause considerable damage especially when there is good start of a monsoon followed by a prolonged dry spell. This insect is distributed all over India and usually occurs on paddy from July to September. In caterpillar stage, the pest has a tendency to migrate from field to field in large swarms. Appearing suddenly in swarm of thousand, they destroy the whole field of paddy completely and marches on to the next field. Hence, the pest is referred to as the army worm. Nursery and early growing stages of the crop are most susceptible to the caterpillar attack. In the nursery, the seedlings are cut and completely eaten up as though grazed by cattle. Larval feeding progresses from the leaf margins leaving behind the leaf midribs thus completely destroying the young paddy seedlings. If the out break is severe, serious damage results in heavy loss of crop. Loss in yield caused by larval infestation ranges from ten to twenty percent. The pest status of *Spodoptera mauritia* is further complicated by their ability to migrate to alternate host plants (for e.g., *Ischaemum aristatum)* during off-season periods at the end of which they make a full-scale come back on the nursery stages of paddy. *Spodoptera* species have been widely utilized in physiological, biochemical and endocrinological research, its position being tenth in the group of insect species often used for research.

2.2. Rearing and maintenance of the larvae

The adult moths, attracted to the light during night were collected using a sweeping insect net. In the laboratory, these moths were transferred to glass chimneys closed at both ends with muslin cloth. Adult moths were allowed to feed on 10% solution of honey. Cotton swabs soaked in 10% solution of honey were

kept in the glass chimneys for this purpose. The females laid eggs on the cloth or on the sides of the chimneys, from which the first instar larvae hatched after 3 days.

Initially, the larvae were reared in glass chimneys. The larvae were supplied with fresh tender leaves of the grass *Ischaemum aristatum* collected from paddy fields. The food material was changed every day. Uniform rearing conditions were provided to the larvae and the containers in which the larvae were kept were placed away from intense light. The larvae were maintained at room temperature, RH 90 $\pm$ 3% and 12:12 light: dark photoperiod regime. As the young larvae grew in size, they were transferred to large plastic troughs. During summer days the cloth covering the trough was wetted frequently. The pupae were kept separately in beakers for adult emergence.

2.3. Biology

Under these rearing conditions, *S. maurita* larvae developed at a uniform rate and underwent 6 larval instars before pupating.

First instar larva

The newly hatched larvae congregated on the cloth covering of the chimney. The first instar larvae were characterised by the presence of a large, black head shield and light green coloured body. On each segment of the body setigerous, small, wart-like dark pigmented tubercles were present which were arranged in a cross-wise row. The larvae moved in a characteristic leaping manner. They descended by means of silken threads to the tender grass leaves supplied for feeding. Newly hatched larvae did not feed immediately. The first instar larvae measured about 1 mm in length and 0.5 mm in width. First instar larvae moulted to second instar after 2-3 days.

Second instar larva

In second instar larvae, three white longitudinal lines appeared on the dorsal surface of the body extending from the prothorax to the last abdominal segment. The body of larvae is green in colour with two pairs of white, longitudinal, lateral stripes, one pair being more prominent. On each segment, setigerous, small, wart-like, dark tubercles were present. The second instar larvae also descended using silken threads. Newly moulted second instar larvae measured about 2.5 mm in length and 0.5 mm in width. The duration of this instar was 2-3 days.

Third instar larva

The larvae no longer used the silken threads to descend to the grass leaves. The third instar larvae possessed three white longitudinal stripes on the dorsal side and two pairs on the lateral side of the body extending from anterior to the posterior end. These larvae also had reddish black supraspiracular stripes extending from anterior to posterior end. The newly moulted third instar larvae measured 6 mm in length and 1 mm in width. After 2-3 days larvae moulted to fourth instar.

Fourth instar larva

The three, dorsal longitudinal stripes became dull white in colour. Two lateral reddish black stripes were present, one on each side of the body. Body became greyish in colour. Black intermittent dots appeared on each segment dorsolaterally which broadened towards the later stages of the instar. The dorsum of the larva was paler than the supraspiracular area and was overlaid with strands and fleck of brown. The newly moulted larva measured about 11.5 mm in length, and 2 mm in width. The fourth instar larval period extended upto 2-3 days (Pl. I: Fig. 2).

Fifth instar larva

The fifth instar larva had a duration of 3±0 days. The larvae were characterized by double rows of black, triangular markings present on the dorsolateral side bordered with narrow white stripes. The triangular markings became more prominent than the fourth instar larval stage. Three dorsal longitudinal stripes were present. The paired supraspiracular stripes became transparent and pink in colour. The integument was partially transparent so that internal structures were visible. During this instar the developmental events were more marked and the growth of the larvae exhibited a specific pattern. The newly moulted larvae measured about 22 mm in length and 4 mm in width. The fifth instar larvae fed voraciously and grew quickly (Pl. I: Fig. 3).

Sixth instar larva

Under the laboratory conditions, the period of development of sixth instar larvae was 6 ± 0 days. Newly moulted sixth instar larva measured about 30 mm in length and 6 mm in width. The larvae fed voraciously during the first three days after ecdysis and attained maximal body weight on day 3 (Pl. I: Fig. 4). The fully grown larvae stopped feeding and the body colour changed from dark green to grey. Within a few hours of the cessation of feeding, there occurred a massive excretion of fluid faeces. This behaviour will be hereafter referred to as "gut purge" which marks the commencement of post-feeding stage. Shortly after completion of gut purge, larvae starts wandering behaviour which lasts for 24 h. During this period, the larvae measured 24 mm in length and 5 mm in width. These larvae wandered about at the bottom of the rearing trough. At the later stages of wandering, the body of larvae shrunk gradually and the movements reduced as the abdominal prolegs got retracted. The larvae then got transformed into prepupal stage. The prepupal stage was characterised by a highly wrinkled larva which

underwent larval-pupal apolysis after 24 h. The prepupal stage measured 20 mm in length and 5 mm in width.

Pupal instar

Pupae were of the obtect type, dark brown in colour and measured 16 mm in length and 5 mm in width (Pl. I: Fig. 5). Adults emerged out of the female pupae after 7 days. Male pupae took 8 days for adult emergence.

Adults

Adults were medium-sized moths and had a conspicuous spot on the forewings, which had wavy pattern on the fringe. Insects measured about 15 mm in length and had a wing span of 30-35 mm. Adult moths exhibited sexual dimorphism in their morphological characters. Males were dark greyish with white markings on forewings and were provided with large tufts of hairs on the forelegs. Females lacked both these white markings and tufts of hairs (Pl. I: Fig. 6). Mating took place in the night within 24 h after emergence. Egg laying commenced 24 h after mating. Eggs were laid in masses of 100-500 each and were covered with buff coloured silken hairs (Pl. I: Fig. 1). On the whole, egg period lasted 2-3 days, larval period 19-23 days and pupal period 7-8 days.

2.4. Experimental animals

The larvae/pupae used for various experiments were collected from the laboratory colony reared and maintained as described above. Newly moulted larvae were isolated from the stock culture and reared in separate containers to facilitate the synchronous development of larvae and also for the precise determination of the age of larvae used for experimental work. Larvae which showed head capsule slippage were kept isolated on the day prior to the moulting. The newly moulted larvae which had pale colour were separated on the day of

PLATE I

Fig. 1. Egg Mass

Fig. 2. Fourth instar larvae

A. Day 1 larva
B. Day 3 larva

Fig. 3. Fifth instar larvae

A. Day 1 larva
B. Day 3 larva

Fig. 4. Sixth instar larvae

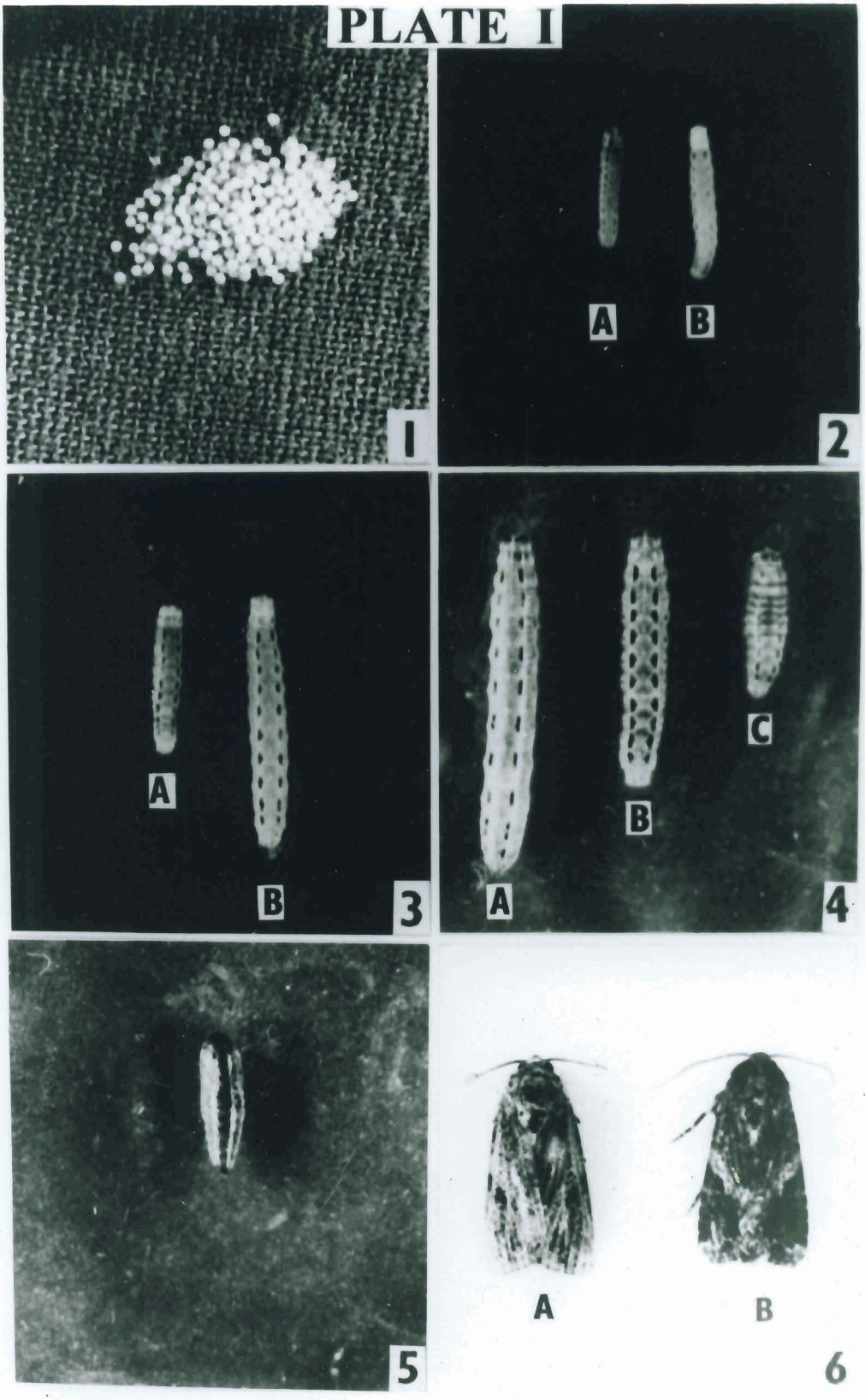
A. Day 3 larva
B. Day 4 larva (wandering stage)
C. Prepupa

Fig. 5. Pupa

Fig. 6. Adults

A. Female
B. Male

PLATE I



ecdysis itself. In the case of sixth-instar larvae, to observe the determination and timing of gut purge, the experimental larvae were placed in separate containers the bottom of which was lined with filter paper in order to absorb the fluid faeces. Gut purged larvae were separated by using the criteria of loss of weight, colour change and the observation of fluid faeces in the filter paper. Sexing was possible in the late fifth instar stage when the undifferentiated testes became clearly visible through the transparent cuticle of male larvae. The experimental insects were reared and maintained as in the case of stock culture.

Prepupae were separated from the stock culture to obtain newly moulted male pupae. Newly ecdysed (day 0) pupae were easily recognised as they appeared pale cream/green coloured. Soon tanning of the pupal cuticle commenced changing the colour to light brown and finally dark brown.

The age of larvae and pupae were abbreviated to day *n* where day 0 indicates the day of ecdysis to this stage. Newly ecdysed larvae/pupae were treated as day 0, larvae/pupae 24 h old as day 1 and so on.

CHAPTER 3

TESTICULAR DEVELOPMENT AND SPERMIOGENESIS

3.1. Introduction

In Lepidoptera, the sequential steps in spermatogenesis have been studied by several workers using histological and histochemical methods (Chaudhury and Raun, 1966; Retnakaran, 1970; Holt and North, 1970; Chase and Gilliland, 1972; Salama, 1976; Numata and Hidaka, 1980; Lai-Fook, 1982; Sridevi *et al.*, 1989; Venugopalan *et al.*, 1994). However, ultrastructural studies on the spermatogenesis in lepidopteran insects have been comparatively few. In a few studies that are available, the sequential structural modifications of germ cells during spermiogenesis have been correlated with the morphological changes occurring during postembryonic development and metamorphosis (Yasuzumi and Oura, 1964; Danilova and Vereiskaya, 1968a, b; 1970; Phillips, 1970; King and Akai, 1971 a, b; Danilova, 1973a, b; Leclercq-Smekens, 1978a,b). Spermiogenesis or differentiation of lepidopteran sperm, involves dramatic morphological and structural changes. The changes accompany an extensive remodelling of the nucleus and all of the subcellular organelles such as mitochondria (Phillips, 1970). Further lepidopteran species exhibit a peculiar mode of dimorphic sperm differentiation; the spermatocytes differentiate into eupyrene (nucleate) and apyrene (anucleate) sperms. Regular meiotic divisions result in the formation of eupyrene spermatozoa which later undergo differentiation and only these spermatozoa possess the ability to fertilize eggs. On the other hand apyrene spermatozoa are the outcome of irregular divisions and they are not able to fertilize eggs. Eupyrene spermatozoa have the haploid number of chromosomes, while apyrene ones have none. Although majority of germ cells develop into apyrene sperms; their biological role is not known. The structural and ultrastructural characterisation of spermatozoa are very important to programmes directed towards the biochemical control of insect pests and genetic manipulation of insect population through sterile and semi-sterile males (Dumser, 1980). In order to analyze the mechanism of spermatogenesis it

was thought worthwhile to examine in detail the ultrastructural differentiation of the two sperm types in *Spodoptera maurita* Boisd. (Lepidopera : Noctuidae) and to trace the morphofunctional events which take place during spermiogenesis.

3.2. Materials and Methods

3.2.1. Animals

Our earlier studies have demonstrated that spermatogenesis commences during late fifth instar larval stage. Further the process of spermiogenesis is initiated in the sixth (last larval instar) instar larvae and is brought to completion in pupal stage (Venugopalan *et al.*, 1994). Since spermiogenesis takes place in the sixth instar (last) larvae and pupae the testes of these stages were utilized for ultrastructural studies. The last instar larvae and pupae of the required stage were obtained from laboratory stock culture, reared and maintained in separate containers as mentioned earlier (Chapter 2). The males could be distinguished in the fifth instar larval stage itself, when the testes became clearly visible through the transparent cuticle. Fifth instar larvae were kept in separate containers for obtaining newly ecdysed (day 0) sixth instar larvae.

3.2.2. Dissections

Sixth instar larvae/pupae were mildly anaesthetised in specimen tubes having a wad of cotton soaked in ether at the bottom. The larvae/pupae were taken out from these specimen tubes and pinned dorsal side up in a wax-lined petri dish. The larvae were then kept immersed in insect Ringer solution (Ephrussi and Beadle, 1936). A longitudinal cut was made on the dorsal surface of the larvae/pupae. The left and right cuticular flaps were then pinned laterally on to the wax tray and the testes were taken out using a fine forceps.

3.2.3. Histological techniques

The testes were fixed overnight in Bouin's fluid. The tissues were then washed in distilled water, dehydrated through alcohol series, infiltrated with paraffin wax and finally embedded in wax. Serial sections of thickness 5 μm were cut using a Reichert Precision Rotary Microtome. Sections were stained in Heidenhain's haematoxylin-eosin observed and photographed under Zeiss Universal microscope.

3.2.4. Electron microscopy

The testes of sixth instar larvae and pupae were dissected in insect Ringer solution and transferred immediately into 3% gluteraldehyde fixative in 0.1 M phosphate buffer (pH 7.4). The fixation was pursued in the cold (4°C) and continued overnight. The tissues were rinsed in Sodium cacodylate buffer (pH 7.4), post-fixed in 1% buffered osmium tetroxide for 1-2 h and dehydrated in graded alcohols. Tissues were stained *en bloc* by immersing in a 2% solution of Uranyl acetate for 1 h. Tissues were then transfered to propylene oxide and then to a mixture of Propylene oxide and Araldite mixture (1:1) and kept over night in the rotator at 48°C. The Araldite mixture contained Araldite Cy212 (10g), Dodecenyl succinic anhydride (DDSA; Hy964, 10g) as hardner, Dibutylphthalate (1g) as plasticizer and Tridimethylaminomethyl phenol (DMP 30; Dy 064, 0.6 g) as accelerator. The tissues were then transferred to Araldite mixture for infiltration at 48°C for 6 h and finally embedded in the Araldite mixture in beam capsules and kept for polymerization at 48°C for 48 h.

After polymerization semithin and ultrathin sections were cut with a glass knife using a Reichert-Jung-Ultracut-E microtome. Semithin sections (0.5 - 1 μm thick) were transferred from the knife's trough to a drop of distilled water on a clean

glass slide. Optimum drying was obtained by putting the slide on a high temperature hot plate for 30 sec. to 1 min. This procedure also promoted a vigorous adherence of section to the slide. These semithin sections were stained with a solution of Toluidene blue (1 g/100 ml) in distilled water containing 0.5% Sodium carbonate. The staining was performed by adding few drops of stain on top of sections and the slides were kept at 80°C for 20 sec. Then the excess stain was drained, the slides were rinsed in distilled water and again dried. The stained sections were observed under a light microscope. After observing the appropriate area of the tissue from the semithin sections, blocks were trimmed for ultrathin sectioning. The ultrathin sections included only the preselected area of the material. Ultrathin (10-100 nm thick) sections were collected on mesh grids by placing the grids underneath the sections floating in the trough liquid and then slowly raising the grids to avoid folding of the sections. These sections were stained first in a saturated solution of Uranyl acetate in 50% methanol for 1 h by immersing the grids in the staining solutions. After thorough rinsing in distilled water the sections were stained in a 2% Lead citrate solution. This was done by floating the grid-mounted sections for 5 min on a drop of the staining solution placed on a piece of parafilm kept in a petridish. The stained sections were examined and electron micrographs were taken with a JEOL 100c x II Transmission Electron Microscope operating at 80 kv.

3.3. Results

3.3.1. Anatomy and histology of testis

Larval testes of *S. mauritia* are paired organs, kidney-shaped and contains four pyriform follicles or lobuli. The testes are situated dorsolateral to the alimentary canal. In the last larval instar (sixth), the volume of the testis increases progressively, reaching a maximum in day 3 larvae. During the pharate pupal

(prepupal) stage the paired testis fuses in the mid-dorsal line into a single median spherical structure. The spherical shape of testis is maintained throughout pupal period. From early pupal stage onwards the fused testis shows a remarkable increase in volume. In the young pupae the fused testis undergoes torsion. In the pupal stage from day 2 onwards the volume of testis is found to decrease. The four follicular nature of the testis is lost in the late pupal stages. The testis is covered with a sheath of membranes.

i) Histological studies

In general, histological studies have shown that testicular follicles of fifth instar larvae (penultimate larval instar) of *S. mauritia*, contain only spermatogonial cells. The germ cells later become enclosed within a layer of non-germinal cells, forming cysts. The membraneous covering of individual cyst which covers the germ cells will be hereafter referred to as the cyst envelope. The spermatocyte cysts appear during the phagoperiod (day 0 – day 3) of sixth instar (last larval instar) larval development. Even in day 0 larvae, spermatocyte cyst cells are evident, though their number is considerably less. Spermatidal cysts develop in the prepupal and pupal stage. Further the initiation in the differentiation of sperm bundles take place on day 4 and day 5 of sixth instar larval development even though their number is considerably small. In *S. mauritia* as in other Lepidoptera spermatogenesis is dichotomous, spermatids differentiated into typical eupyrene and atypical apyrene (anucleated) sperm bundles. Well differentiated sperm bundles are seen in the pupal stage. The four follicles of larval testes are not present in pupa. The fused testis is divided into eight longitudinal compartments by a membrane. Towards the end of pupation the entire testicular interior becomes a homogeneous structure.

ii) Ultrastructural studies

Ultrastructural studies were conducted on the testis of sixth instar larvae and pupae of *S. mauritia*. During spermiogenesis distinct changes occur in the nucleus, acrosome and mitochondria. The ultrastructural changes occurring in cellular organelles of germ cells during different developmental stages are reported. Testes utilized for these studies were those of sixth instar larvae and pupae of different age groups.

3.3.2. Ultrastructure of the testes of sixth instar larva

i) Testicular sheath

The larval testicular wall or sheath consists of two cellular layers; the internal, the tunica interna and the external, the tunica externa. The internal tunica folds inwards separating the follicles by septae made up of two layers of tunica interna. The tunica externa is unfolded and is absent along the interfollicular septa. However, membrana communis, the external layer which covers tunica externa seen in pupal testes is altogether absent in the larval testis sheath. The testicular sheaths are lined by two basal laminae, a thick membrane lies over the outer surface of the testis forming the outer basal lamina (Pl. II: Fig. 7). The testicular lobes are separated from the haemolymph by an acellular outer basal lamina. A somewhat thinner one marks the inner surface of the testicular sheaths, forming the inner basal lamina. The inner basal lamina also covers the two surfaces of the interfollicular septa, and also covers the inner surface of tunica interna facing the cysts containing the germ cells (Pl. II: Fig. 7).

Generally, tunica externa is thinner and more electron opaque than the tunica interna. The cells contain rough endoplasmic reticulum and numerous vacuoles

(Pl. II: Fig. 7). However, pigment granules and glycogen particles are absent. The cytoplasm of the outer layer is coarsely textured.

In tunica interna, the cells are flat and contain numerous electron dense pigment granules and rough endoplasmic reticulum. Mitochondria are abundant (Pl. II: Fig. 7). The cytoplasm of the inner layer is homogeneous. The nuclei of the inner layer are large and egg shaped and occupy a central position within the cell. Chromatin clumps are seen scattered through out the lumen of the nuclei (Pl. II: Fig. 7)

Mitochondria have a dense or dark matrix and possess a few flat cristae. In the cytoplasm, few tracheoles, multivesicular bodies, clusters of glycogen particles, numerous vacuoles, lipid droplets, large spherical proteinaceous bodies and few pigment granules are evident (Pl. II: Fig. 7).

ii) Interstitial cells

Towards the peripheral region of follicular interior, surrounding the spermatogonial cells, elongated interstitial cells are often observed (Pl. II: Fig. 9). The interstitial cells are spindle shaped with long cytoplasmic extensions. The cytoplasmic extensions in certain cases are seen growing around the germ cells. At certain places these extensions send off finger like processes which appear to enter the lumen of cyst (Pl. II: Figs. 9, 10). A high nucleo-cytoplasmic ratio is observed. The cytoplasm is enriched with organelles like smooth endoplasmic reticulum, mitochondria and polysomes throughout its length. Mitochondria are seen in large numbers (Pl. II: Fig. 8). Cytoplasm also contains a large number of secretory vacuoles. The major part of the interstitial cell is occupied by a prominent nucleus with a conspicuous nucleolus (Pl. II: Figs. 9, 10). The nucleus, like the cellular outline is spindle shaped. The nucleolus in many cases has a lobbed outline. The

nucleoli in certain cases assume signet ring configuration. The cytoplasmic extensions at certain regions are seen to possess secretory material. In the nucleus, chromatin is evenly distributed. Vacuoles laden with some dark secretions are observed at the branched location of the cytoplasmic extensions, but some of the vacuoles are empty (Pl.II: Figs. 8, 9, 10).

iii) Germ cells

Germ cells of the larval testes comprises of spermatogonia, spermatocytes and spermatid cyst cells.

iv) Spermatogonia

The testes removed from day 0 larva of *S. mauritia* contain spermatogonia, which always lie close to the wall of the follicle or in the centre. Spermatogonia is characterized by the possession of large nuclei, which occupy most of the area of the cell (Pl. II: Figs. 9, 10). Hence the nucleo-cytoplasmic ratio area is quite high. The shape of the nucleus is almost spherical with a conspicuous nucleolus (Pl. II: Figs. 9, 10; Pl. III: Fig. 11). The chromatin is dispersed uniformly or evenly in the nucleus. Nuclear envelope is regular. The cytoplasm contains few mitochondria, rough endoplasmic reticulum, a small Golgi apparatus, lipid inclusions and a cluster of granulo-fibrillar dense bodies. The mitochondria are of pleomorphic type having varying shapes and sizes. They are rounded, elongated or tubular with tubular or lamellar cristae. Short stretches of extensive rough endoplasmic reticulum are also observed (Pl. II: Fig. 9; Pl. III: Fig. 11).

v) Spermatocyte

As mentioned earlier meiotic divisions are initiated during day 2 - day 3 of sixth instar larval stadium. The size of primary spermatocyte is small which might be due to the preceding repeated mitotic division of the spermatogonia. During the

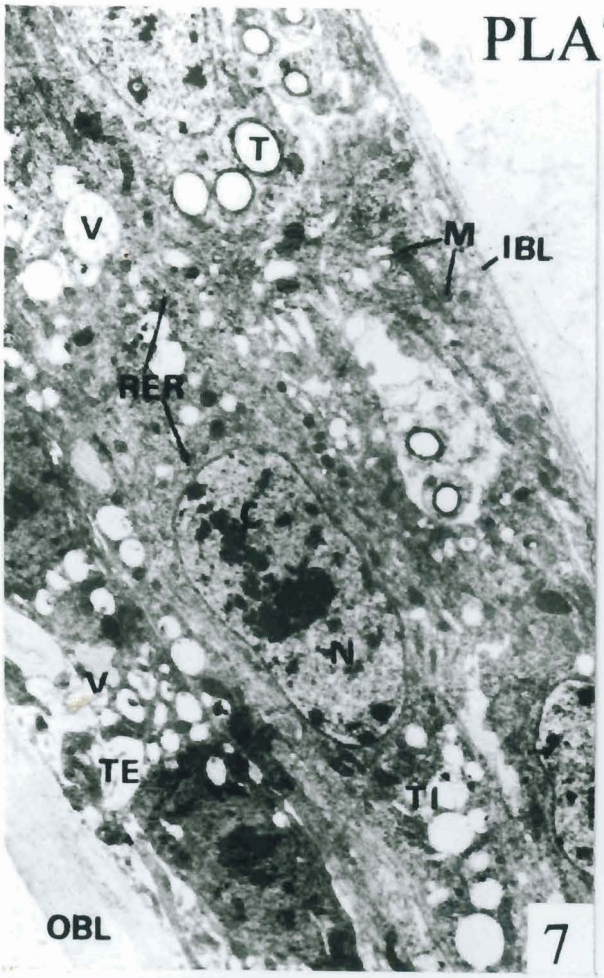
PLATE II

Fig. 7. Testicular sheath of day 0 sixth instar larva, showing cells of tunica externa (TE) with numerous vacuoles (V) and cells of tunica interna (TI) showing a prominent nucleus (N) with chromatin clusters(C), numerous mitochondria(M), rough endoplasmic reticulum (RER), vacuoles (V) and tracheoles (T). Outer (OBL) and inner (IBL) basal lamina can be distinguished. X 14,100

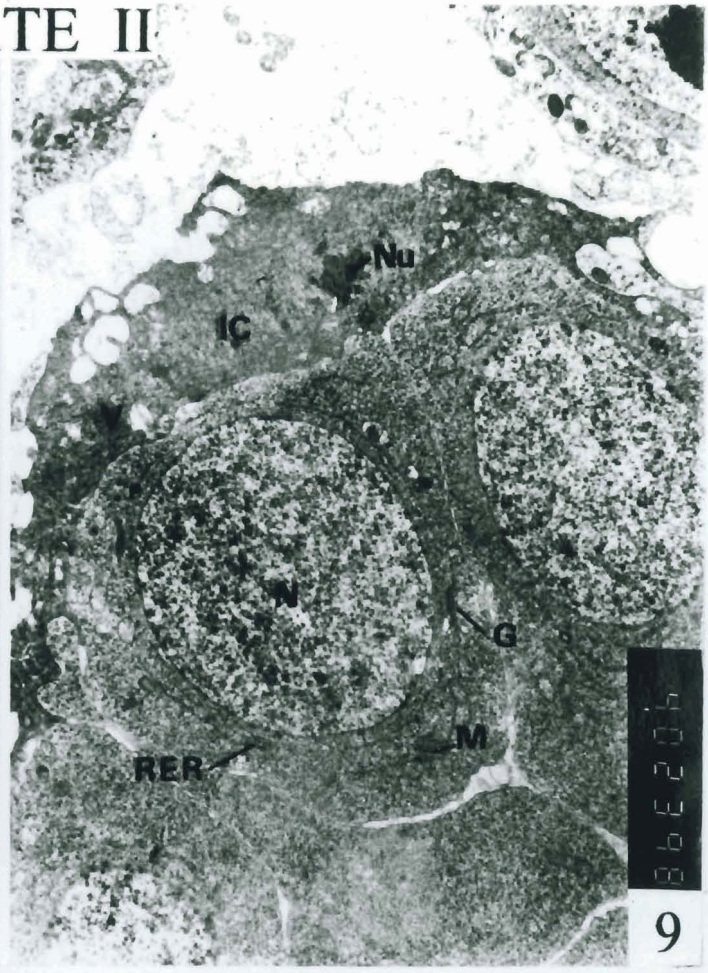
Fig. 8. Interstitial cell (IC) of day 0 sixth instar larva showing elongated nucleus (N) and cytoplasm rich in mitochondria (M), rough endoplasmic reticulum (RER) and vacuoles (V). X 7500

Figs. 9 and 10. Spermatogonial cells and the surrounding interstitial cell (IC) of day 0 sixth instar larva. Interstitial cell cytoplasm shows numerous mitochondria (M), vacuoles (V) and a spindle shaped nucleus (N) with nucleolus (Nu). Spermatogonial cell contains a prominent nucleus (N) with nucleolus (Nu) and the cytoplasm enriched with numerous mitochondria (M), rough endoplasmic reticulum (RER) and Golgi apparatus (G). X 6900

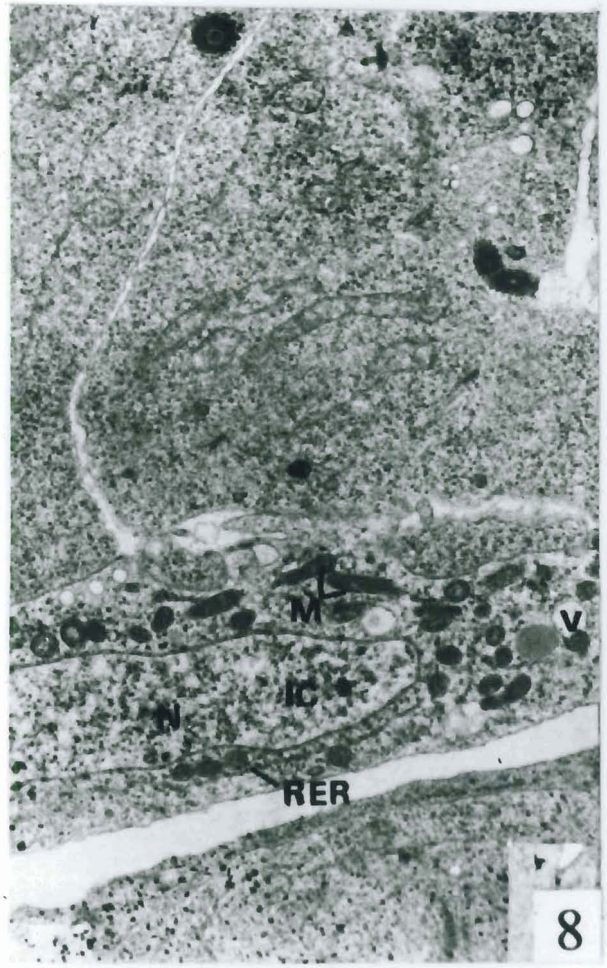
PLATE II



7



9



8

10

growth of spermatocytes of *S. mauritia* the cell diameter increases. The cells that enter the prophase of the first meiotic division, mostly in the zygotene stage can easily be identified since they possess fully developed synaptonemal complexes within the spherical nuclei (Pl. III: Fig. 12). A compact nucleolus embedded in a cloud of chromatin is also visible in the nucleoplasm. Clumping of chromatin is observed. In certain preparations moderately condensed chromatin interspersed with small dense particles is seen arranged around the synaptonemal complexes. The nucleolemma (nuclear envelope) is characterized by numerous pores (Pl. III: Fig. 12). The cytoplasm of the spermatocytes contain small amount of mitochondria, but no typical Golgi stacks are seen. Instead small vesicles are present. The small dense vesicles are formed from golgian activity, which may sometimes be found in the neighbourhood of a few typical Golgi cisternae. These small vesicles actually represent the proacrosomal granule which later coalesce to form the acrosomal vesicle. Two basal bodies or centrioles are evident in close proximity to one another in the cell's periphery.

In the day 3 sixth instar larvae, spermatocytes that enter the diplotene stage, is characterized by the absence of synaptonemal complexes and the presence of ill-defined accumulations of dense material in the nuclear periphery. The nucleoli have lost their compact appearance and consists of dense threads (Pl.III: Fig. 13). The cytoplasm contains few mitochondria with less dense matrix and few vacuoles.

After the completion of first meiotic division, the spermatocyte nucleus is irregular in shape and the nuclear envelope has numerous pores (Pl. III: Fig. 14). The size of the nucleus becomes reduced and the cytoplasm gets enlarged. The spermatocyte cytoplasm contains typical stacks of Golgi cisternae, elongated mitochondrial aggregates, few vacuoles and vesicles. Basal bodies are also evident. As is typical of the meiotic prophase, the two centrioles move to opposite poles of

PLATE III

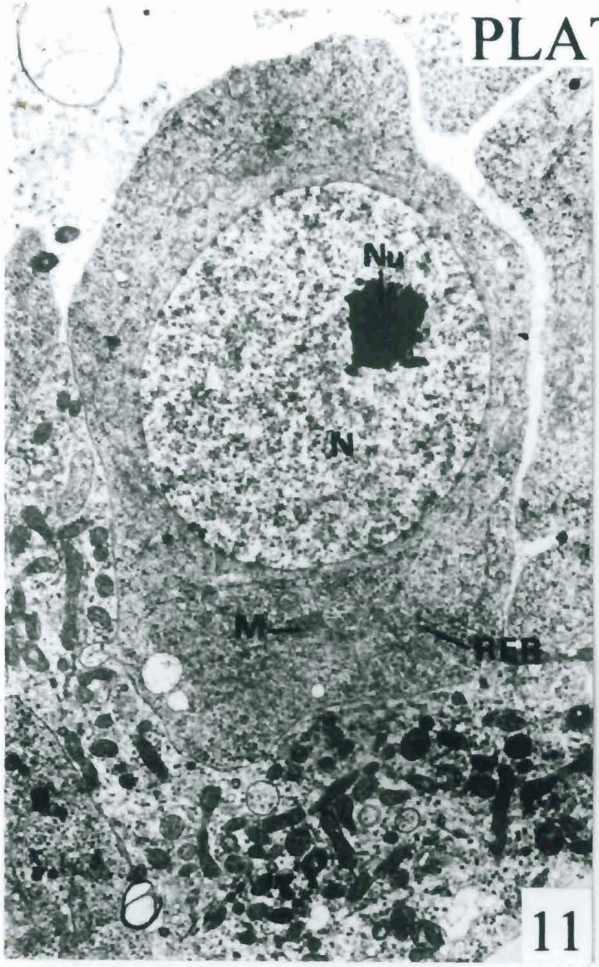
Fig. 11. Spermatogonial cell of day 0 sixth instar larva showing a prominent nucleus (N) and nucleolus (Nu) together with its cytoplasm containing rough endoplasmic reticulum (RER) and mitochondria (M). X 7200

Fig.12. Spermatocyte of early sixth instar larva showing nucleus (N), synaptonemal complex (SC) and nucleus with nuclear envelope having nuclear pores (Np). X 11000

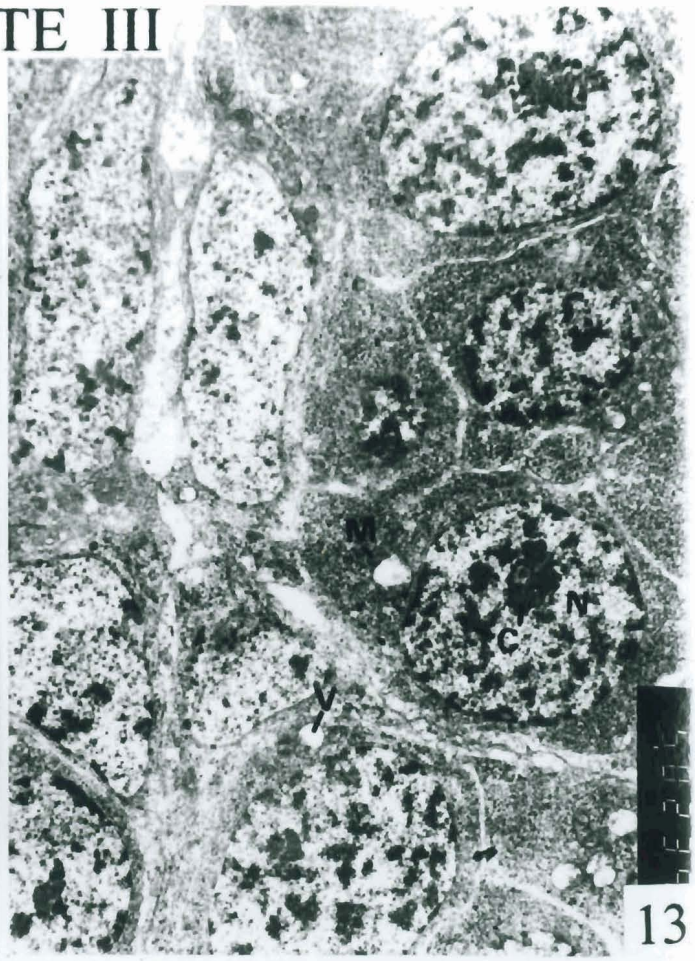
Fig. 13. Spermatocytes showing prominent nucleus (N) with dense chromatin threads (C) and cytoplasm having mitochondria (M) and vacuoles (V). X 8600

Fig. 14. Advanced stage of spermatocyte showing irregular nucleus (N), with numerous nuclear pores (Np) in the nuclear envelope. Cytoplasm is seen enriched with numerous mitochondrial accumulation (M) and vacuoles (V). Golgi apparatus (G) shows the presence of vesicles. X 11500

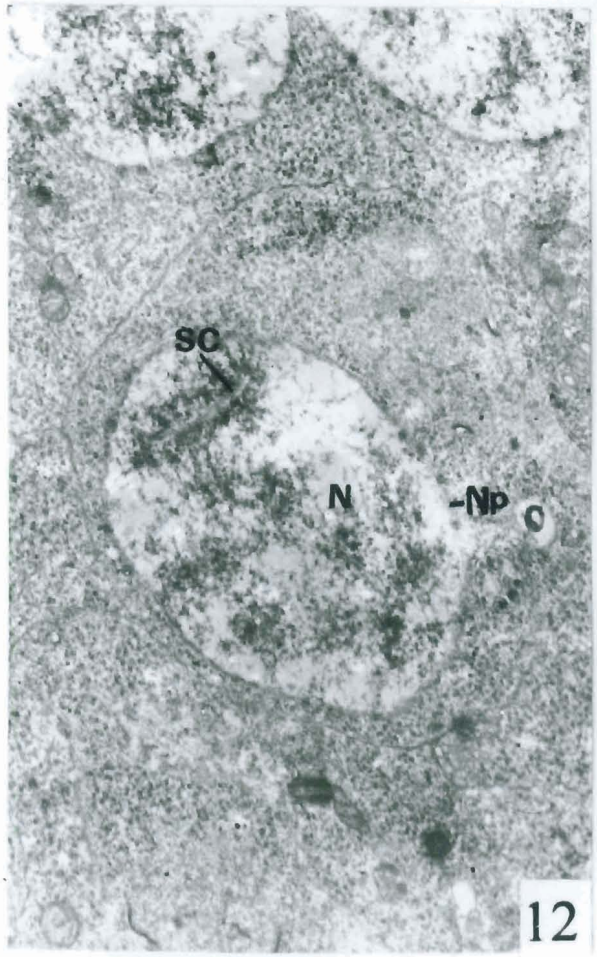
PLATE III



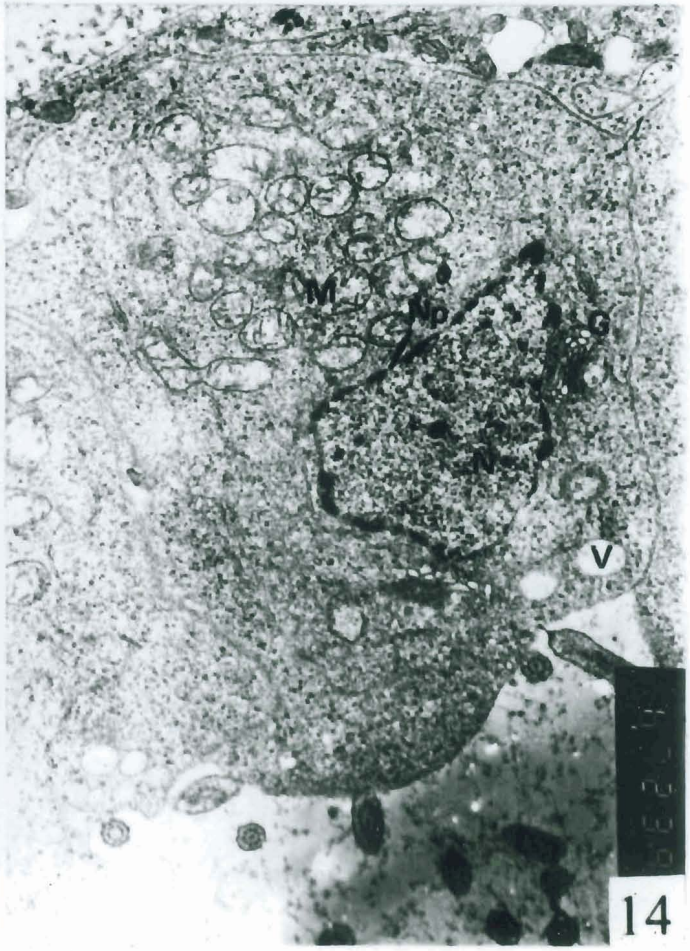
11



13



12



14

the nucleus to form centriole pairs or diplosomes. The number of mitochondria considerably increases. The mitochondria rearrange and fuse into a large spherical mass, the nebenkern. Also noted that the tips of flagella contains numerous axonemes.

vi) Spermatid cysts

Some elongating spermatid cysts are also observed in the day 3 and day 5 (prepupal) larvae of *S. mauritia*. When the spermatids starts elongation, the entire cysts which was round in shape earlier, gets elongated. The cytoplasm contains numerous mitochondrial accumulations with an elongating nucleus and an axoneme. Fusion of numerous mitochondria into single nebenkern is also observed. Acrosomal bag gets lengthened and becomes closely apposed to the nucleus (Pl.IV: Fig. 15).

3.3.3. Ultrastructure of the pupal testes

i) Testicular sheaths

The testicular sheath of pupa is similar to that of larval testes consisting of two cellular layers, an external cell layer or tunica externa, which surrounds the testes as a whole and by an internal cell layer or tunica interna that extends inwards. The testicular sheath is lined by two basal laminae, an inner basal lamina covering the tunica interna and the outer basal laminae of tunica externa facing membrana communis (Pl. IV: Fig. 16). The inner basal lamina made of two closely apposed layers of fibrous extracellular material, covers the tunica interna facing the cysts containing the germ cells. The inner basal lamina also cover the interfollicular septa. The inner basal laminae show numerous cytoplasmic processes or interdigitating finger like processes (Pl. IV: Figs. 16, 18). The inner layer comprises numerous glycogen droplets, few lipid inclusions and vesicles. The

cytoplasm is electron dense. Intercellular spaces are observed in the inner layer of the wall. These spaces constitute a network of channels filled with a flocculent material (Pl. IV: Figs. 16, 18). Beneath the inner layer of the testis sheath lies numerous spermatid cysts and their size is smaller compared to the sheath cells. In the inner layer, the nuclei of cells are elongated and occupy a central position within cells. Clumps of chromatin are dispersed within the cell. Few mitochondria with stacks of lamellar cristae are also observed in the inner layer (Pl. IV: Figs. 16, 18). Usually the external layer, or tunica externa is thinner than the tunica interna. The thickness of the external layer is comparatively reduced in the pupal testis. In the external layer, most cells shows a sparce endoplasmic reticulum and the mitochondrial accumulation is reduced to few numbers.

In addition to tunica interna and tunica externa, there is another layer of testicular sheath, the membrana communis which is present towards the exterior of tunica externa (Pl. IV: Figs. 16, 17). Membrana communis is present only in the pupal testis. Membrana communis consists of large rounded cells. The cytoplasm is somewhat homogeneous with dark inclusions which probably represent pigment granules. In this layer, most cells show a sparce endoplasmic reticulum, but abundant glycogen droplets and numerous mitochondria. Mitochondria have a dark matrix and possess lamellar cristae. The membrana communis is comparatively thicker than the internal and external layer. The cells observed in this layer are larger in size with a prominent nucleus. Clumps of chromatin are distributed irregularly in the nuclei (Pl. IV: Figs. 16, 17).

ii) Interstitial cells

In the day 1 pupal testis spindle shaped interstitial cells are evident as in the larval testis. In certain cases the long cytoplasmic extensions are seen to get extended towards the germ cells, the spermatid cysts. Clumps of chromatin are

PLATE IV

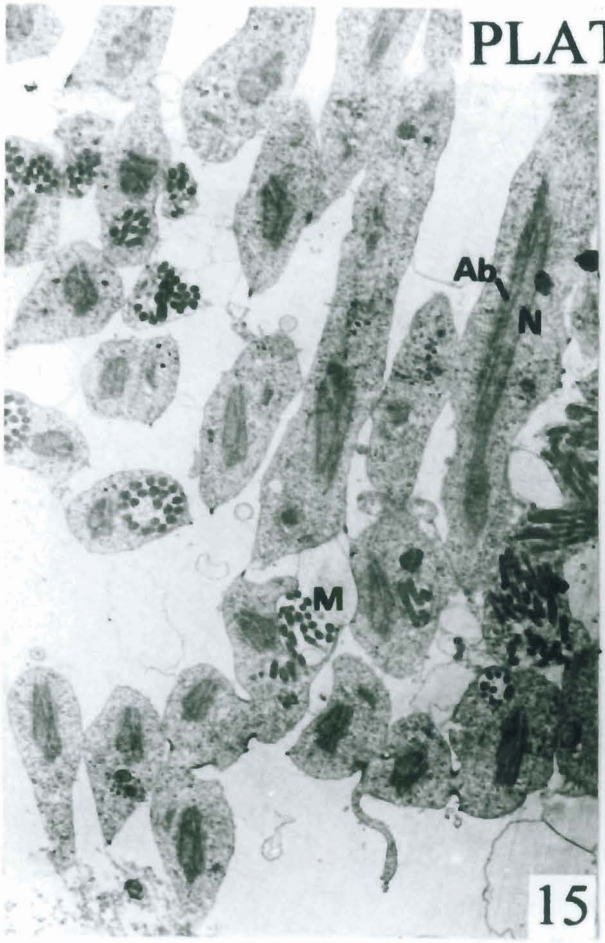
Fig. 15. Spermatid cysts of day 5 sixth instar larva (pharate pupa) showing elongated nucleus (N) and adjacently placed acrosomal bag (Ab). Mitochondria (M) are seen in clusters. X 4320

Fig. 16. Testicular sheath of day 7 pupa showing tunica interna (TI) and membrana communis (MC), both having prominent nuclei (N) The cytoplasm contains mitochondria (M). Tunica externa (TE) lies between tunica interna and membrana communis. The inner basal lamina (IBL) shows cytoplasmic projections (CY). X 4700

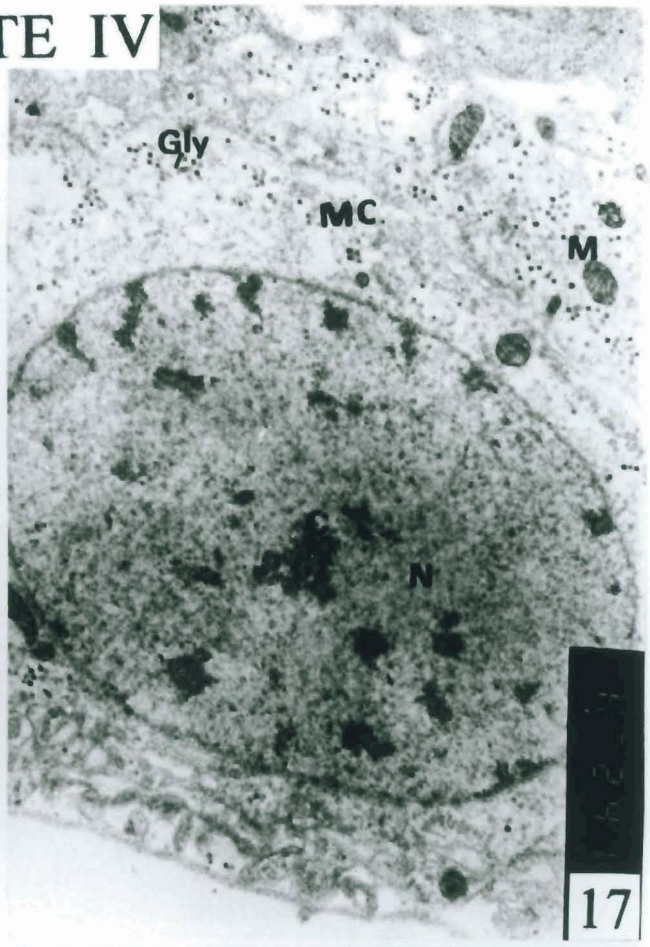
Fig. 17. Higher magnification of the membrana communis layer (MC) showing nucleus (N) with chromatin clumps (C) and its cytoplasm containing glycogen droplets (Gly) and mitcohondria (M). X 12500

Fig. 18. Higher magnification of tunica interna (TI) showing nucleus (N) and cytoplasm containing mitochondria (M), numerous glycogen particles (Gly), lipid droplets (L) and vesicles (Vs). The inner basal lamina show cytoplasmic projections (CY). X 12700

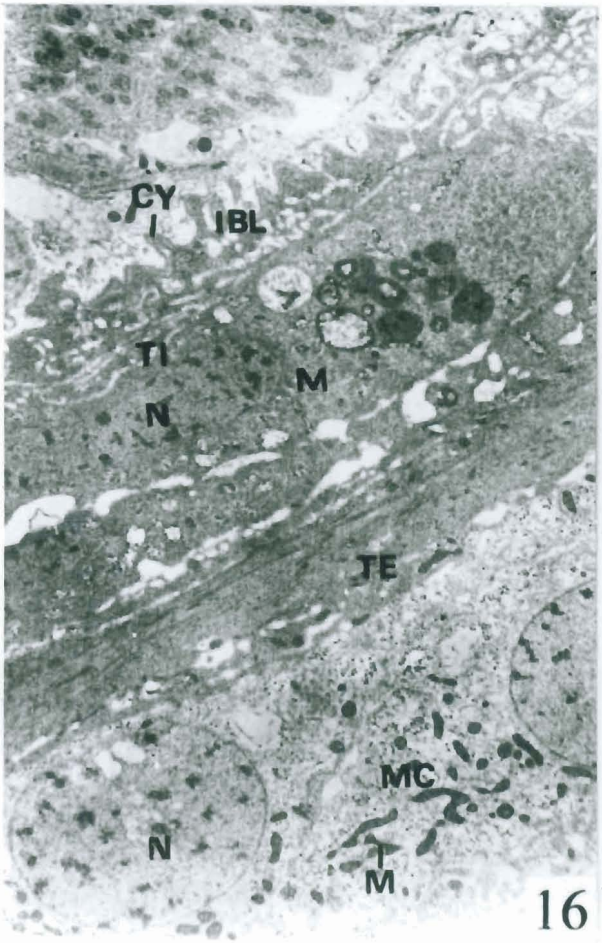
PLATE IV



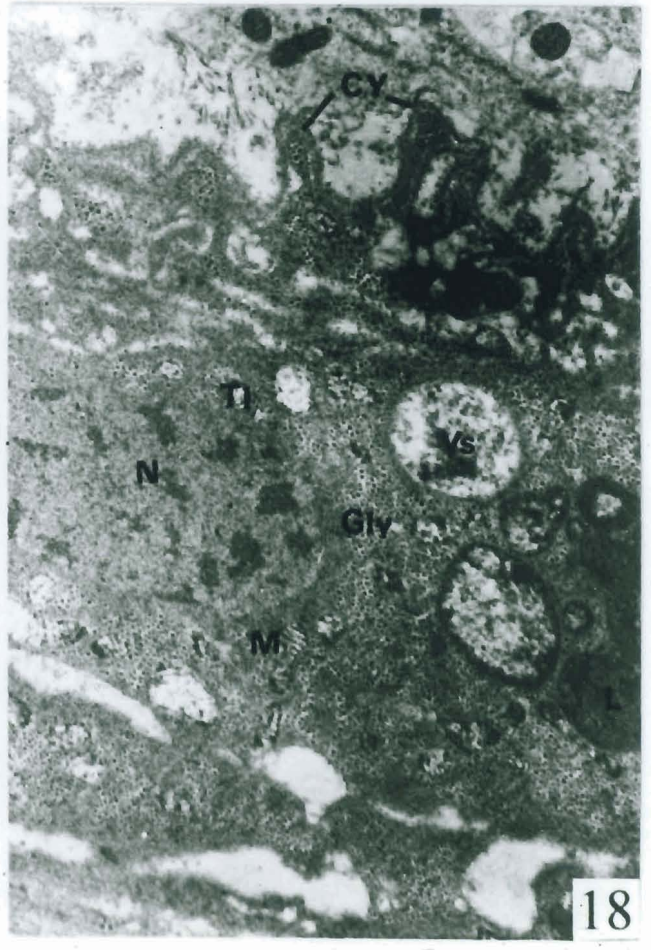
15



17



16



18

dispersed regularly in the nucleus and the cytoplasm is often enriched with organelles like mitochondria, smooth endoplasmic reticulum and few secretory vacuoles (Pl. V: Fig. 19).

iii) Germ cells

The day 1 pupal testis contains mostly of early spermatids and some elongating cysts. The male germ cells get organized into cysts. In these cysts all the cells develop synchronously. In the early eupyrene spermatid cysts, fusion of numerous mitochondria into a single nebenkern is observed (Pl.V: Fig. 21). Division of the nebenkern into two mitochondrial derivatives of unequal size with an axoneme are evident. Reorganization of the internal structures of the mitochondria are seen, so that cristae are formed in the inner surface. As the eupyrene spermatids begin to elongate, the nebenkern divides into two mitochondrial derivatives of unequal size, with one usually extending further posteriorly than the other. Elongation of nebenkern derivatives begins even before they move into their definitive position adjacent to the flagellum and it continues during most of the remaining period of sperm maturation. The resulting two mitochondrial derivatives of unequal size possess outer and inner membrane. Later they elongate and coil in the sperm tail. Cristae develop on the inner membrane (Pl.V: Figs. 19, 20, 21).

Examination of cross section through eupyrene spermatids in pupal testis of *S. mauritia* reveals the presence of nucleus, two mitochondrial derivatives of unequal size, an axial filament or axoneme, multivesicular bodies and Golgi complexes (Pl. V: Figs. 20, 21). The axial filament or axoneme has a typical 9+9+2 microtubular arrangement (i.e., 9 singlet tubules, 9 doublets and 2 central micro tubules are present in the axoneme). The axoneme is present along most of the length of the sperm tail.

PLATE V

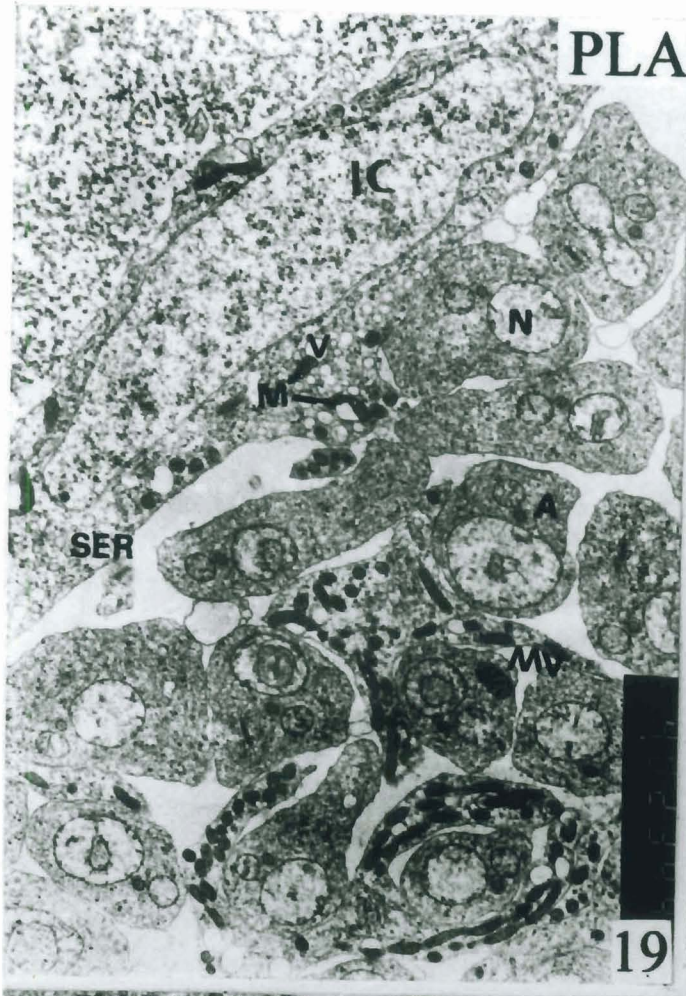
Fig. 19. Spermatid cysts and the interstitial cell (IC) from day 1 pupal testes. Cytoplasm of interstitial cell is rich in smooth endoplasmic reticulum (SER), mitochondria (M), and secretory vacuoles (V). Spermatid cysts contain nucleus (N) mitochondrial derivatives (MD), axoneme or axial filament (A) and multivesicular body (MV). X 6400

Fig. 20. Higher magnification of the spermatid cyst showing nucleus (N) mitochondrial derivatives (MD), axoneme (A), Golgi apparatus (G) and multivesicular body (MV). X 13900

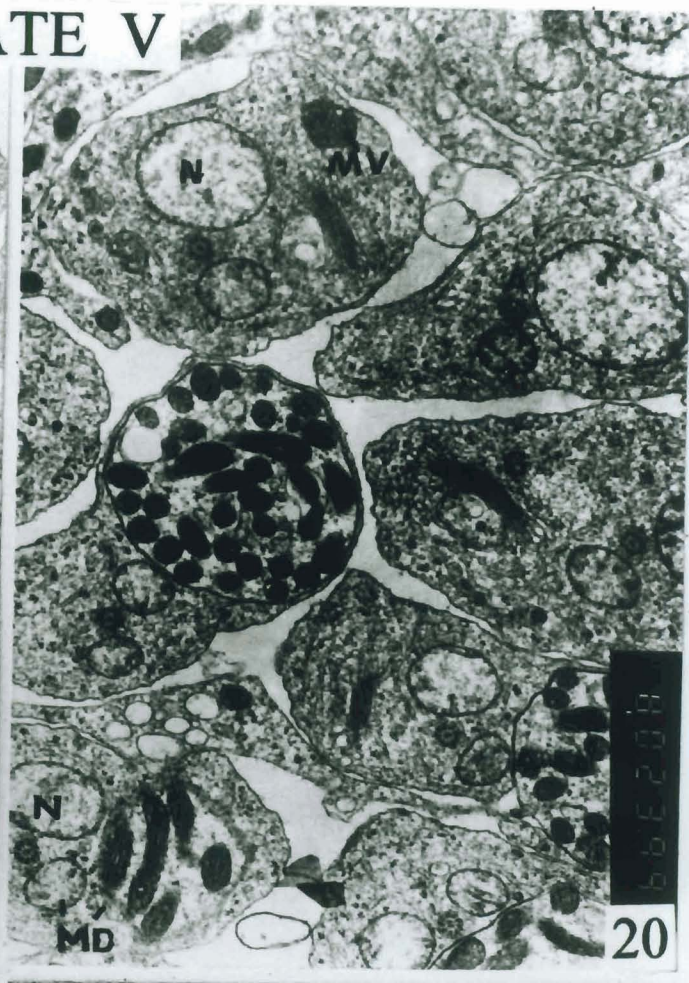
Fig. 21. Cross section of eupyrene spermatid cysts from day 1 pupal testis showing nebenkern (Nb), mitochondrial derivatives (MD), axoneme (A) and Golgi complex (G). X 11200

Fig. 22. Late eupyrene spermatid with elongating nucleus (N), acrosomal bag (Ab) and axoneme (A). X 4100

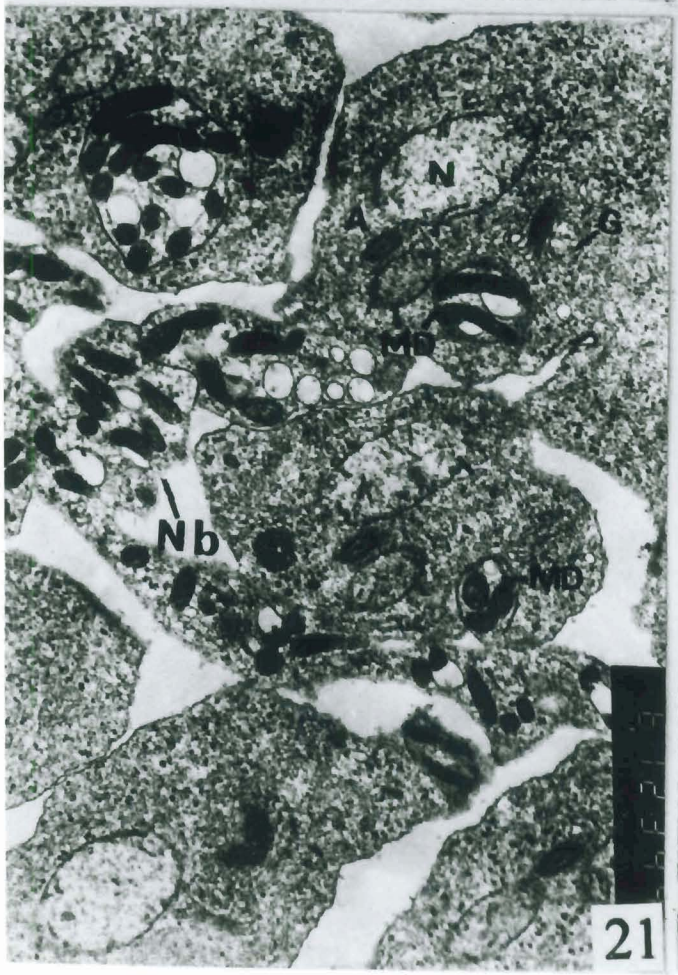
PLATE V



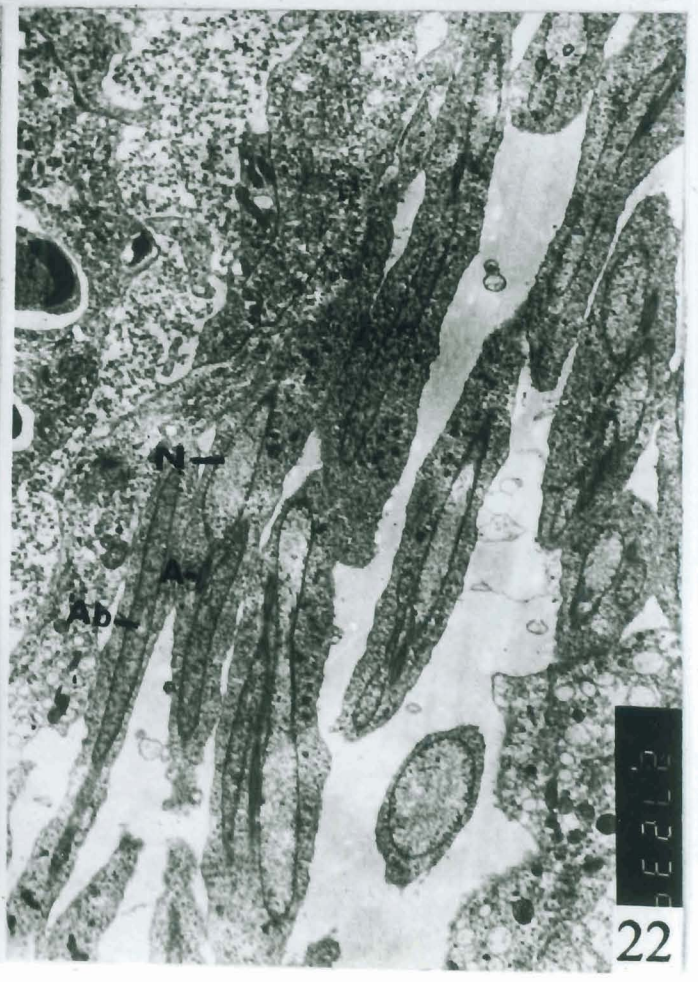
19



20



21



22

Eupyrene meiotic divisions are regular and leads to the development of eupyrene mononucleated spermatids. In these spermatids certain drastic changes have been observed. The nucleus elongates together with the acrosome and axoneme (Pl. V: Fig. 22). When the cell elongates, the nebenkern divides into two mitochondrial derivatives. The mitochondrial derivatives get twined around the elongating axoneme. Then the basal bodies get separated from the nucleus and forms the basal corpuscle for the development of axoneme. An electron-dense acrosome is formed near the Golgi-complex, which gets attached to the nuclear membrane. Initially the newly formed eupyrene spermatids are round and pass through several stages before becoming long thin spermatozoa. The main nuclear changes are an overall alteration in shape from round through conical, to that of a short spindle. In early eupyrene spermatids, the Golgi apparatus is responsible for the formation of acrosome. Initially, a spherical body, termed the proacrosomal granule appears in the Golgi complex. As this granule becomes larger, it becomes intimately associated with the nucleus, forming the acrosomal vesicle. Further, the basal body which remained separate earlier, gets again reattached to the nuclear membrane. In later phases of spermatid differentiation the acrosomal vesicle lengthens and becomes closely apposed to the nucleus (Pl. VI: Fig. 23).

Examination of the testes of day 7 pupa contains mostly of eupyrene and apyrene spermatozoa which differ profoundly at the ultrastructural level. Eupyrene spermatozoa have an elongate nucleus and two apposed mitochondrial derivatives that fuse into one structure having a crescent shape in transverse sections (Pl. VI: Figs. 24, 25). The sperm flagella is characterized by the presence of certain cell surface modifications, particularly, the lacinate and reticulate appendages. From the cell surface protrude 26 numbers of lacinate appendages, which appear in transverse sections as rays of alternating electron-opaque and electron-lucid bars. The reticular appendage persists, as an extra-cellular conglomerate of small spheres

PLATE VI

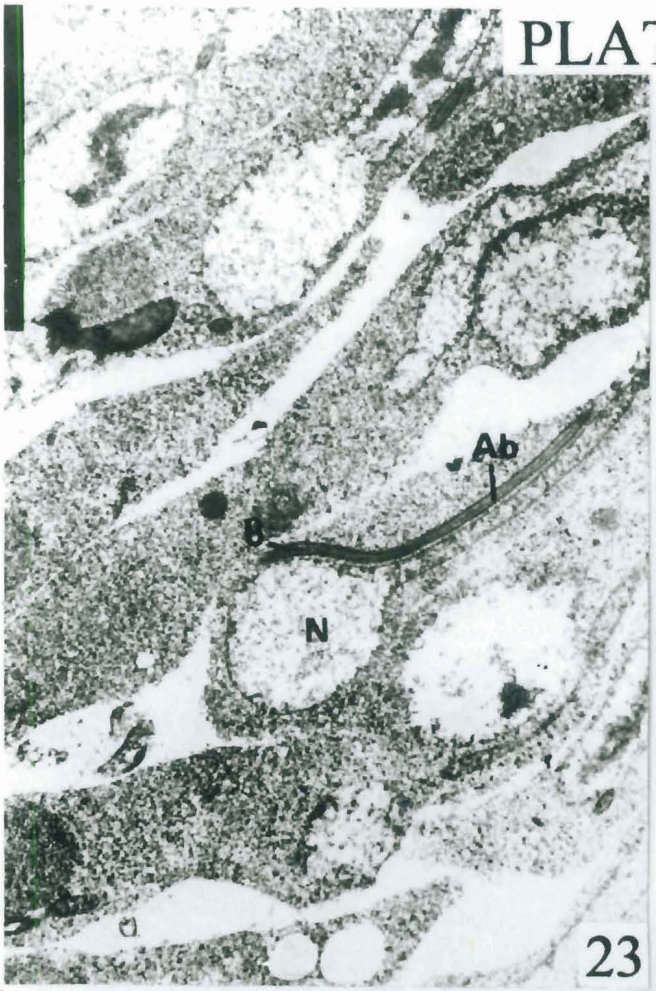
Fig. 23. Late eupyrene spermatid showing nucleus (N) with adjacently placed acrosomal bag (Ab) and basal body (B). X 10000

Fig. 24. Transverse section through the head region of eupyrene spermatozoa showing the nucleus (N) which form a crescent shaped structure. Lacinate (LA), reticulate (RA) appendages and cytoplasmic bridges (Cb) are visible. X 11200

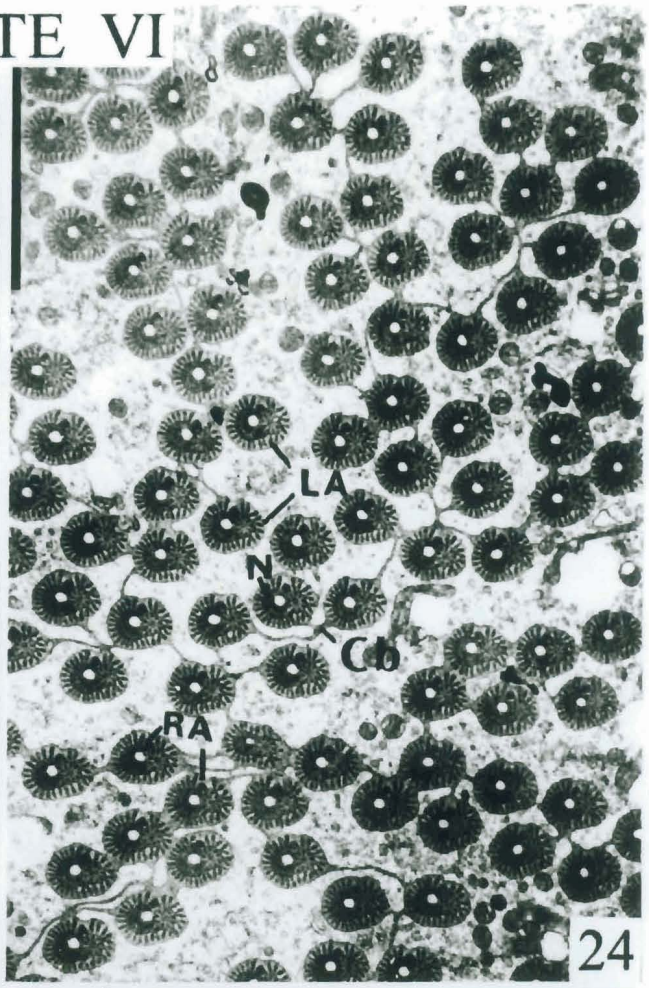
Fig. 25. Higher magnification showing the nucleus (N), mitochondrial sheath (M) and axoneme (A). The lacinate appendages (LA) and reticulate appendages (RA) are also visible. X 36000

Fig. 26. Longitudinal section of eupyrene spermatozoa from day 7 pupa. Nucleus (N). X 4200

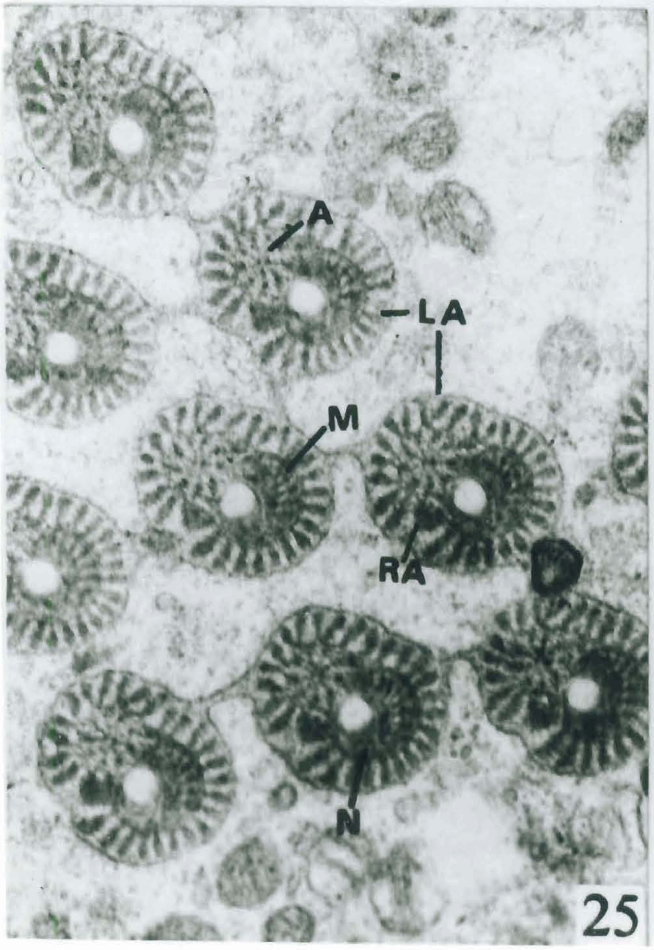
PLATE VI



23



24



25

26

embedded with electron-opaque material. They extend from the plasma membrane overlying the acrosome near to the posterior end. The reticular appendage is located near the posterior side of the centriole and above the crescent shaped mitochondria. In transverse sections, it consists of a honey comb of lucid and dense regions. The axoneme has two central singlets, nine doublets, and 9 peripheral singlets, establishing a normal 9+9+2 axonemal configuration. The eupyrene spermatozoa are covered by a sleeve and connected to each other by cytoplasmic bridges (Pl. VI: Figs. 24, 25). Comparatively, in longitudinal sections, the mature eupyrene spermatzoa are larger than the apyrene ones (Pl. VI: Fig. 26; Pl. VII: Fig. 27).

In the day 1 pupal testis of *S. mauritia* young apyrene spermatid cysts are observed. In these apyrene spermatids also, formation of nebenkern is observed (Pl. VII: Figs. 28, 29). In the later stage of apyrene sperm development nucleus is seen located in the centre. One of the characteristic feature of apyrene spermiogenesis of *S. mauritia* is that acrosome formation is not detected. Further, the mitochondrial derivatives are located close to the axoneme which become quite slender. During apyrene spermiogenesis, the basal body is not attached to the nucleus as in eupyrene ones. Axoneme is also not attached to the nucleus. Further the apyrene spermatozoa are not connected by intercellular bridges.

Transverse sections of the flagella of apyrene spermatozoa of *S. mauritia* reveal the presence of two separate mitochondrial derivatives forming a 'V' shaped configuration. The mitochondrial derivatives are small and are equal in diameter, length or size (Pl. VII: Figs. 30, 31). The apyrene sperms bear no lacinate or reticular appendages. A small cap extending anteriorly beyond the flagellum replaces the nucleus and acrosome. In the apyrene sperm, the peripheral and central singlets of the axoneme are located above the two mitochondrial derivatives and the

PLATE VII

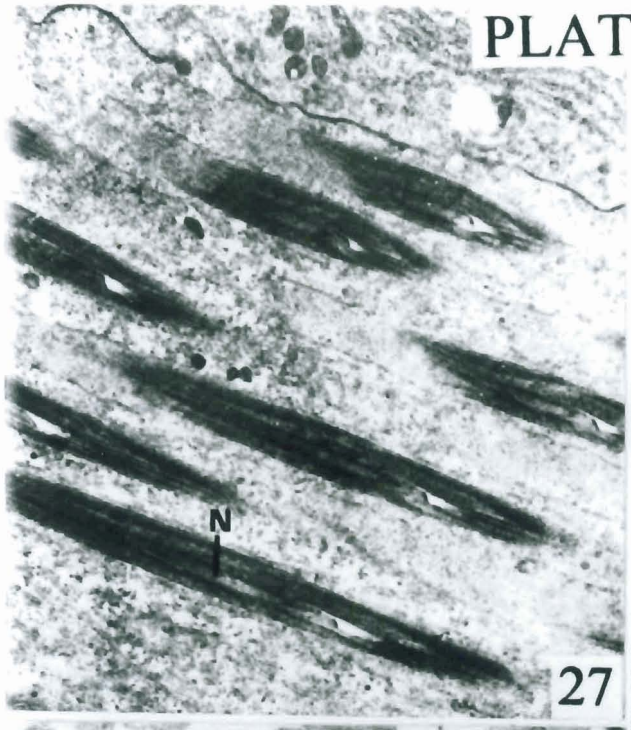
Fig.27. Higher magnification of eupyrene spermatozoa N - Nucleus. X 12000

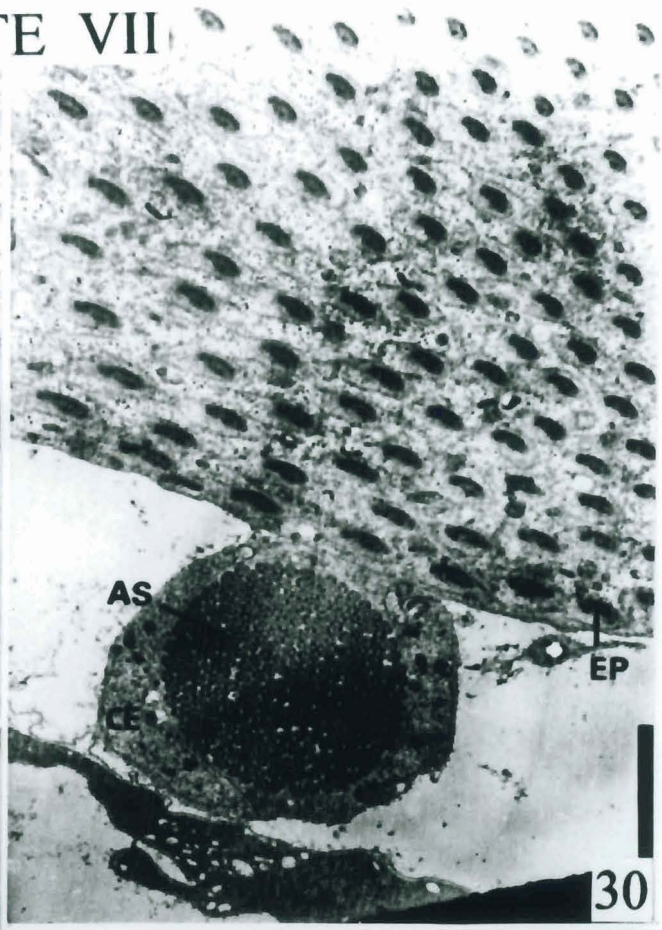
Figs. 28 and 29. Early apyrene spermatid from day 1 pupa showing the nebenkern (Nb). Fig. 28 X 2500; Fig. 29 X 3900

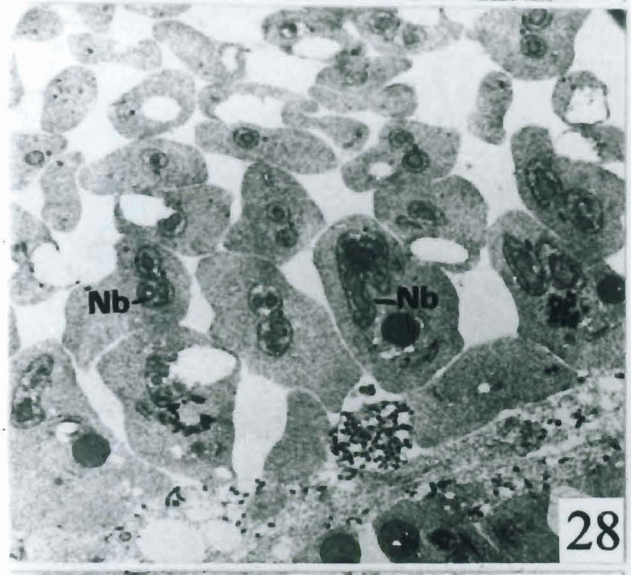
Fig. 30. Transverse section through eupyrene (Ep) and apyrene spermatozoa (AS), CE-Cyst envelope. X 4300

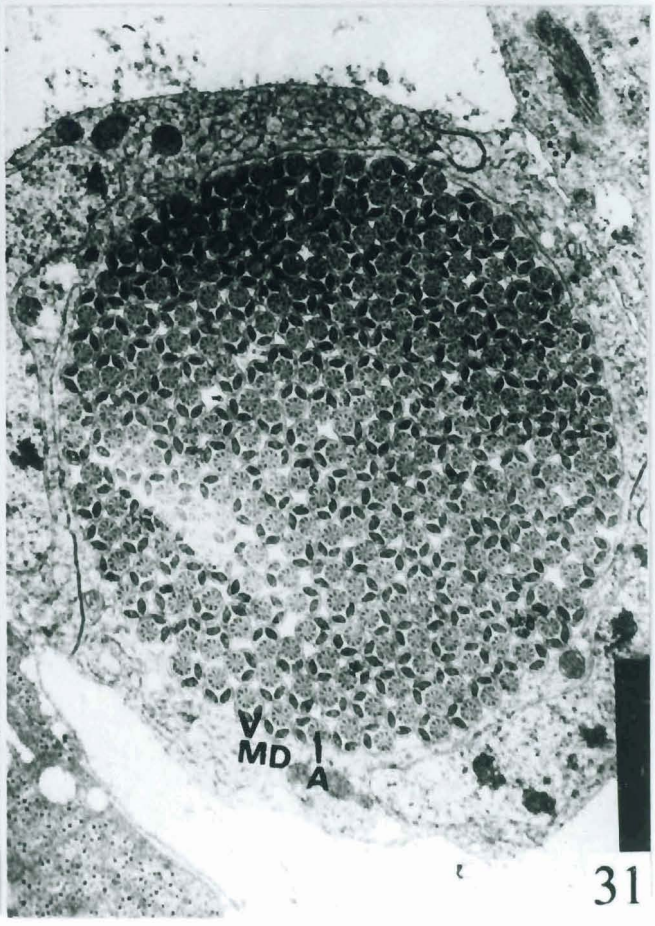
Fig. 31. Higher magnification of apyrene spermatozoa showing mitochondrial derivatives (MD) and axoneme (A). X 10600

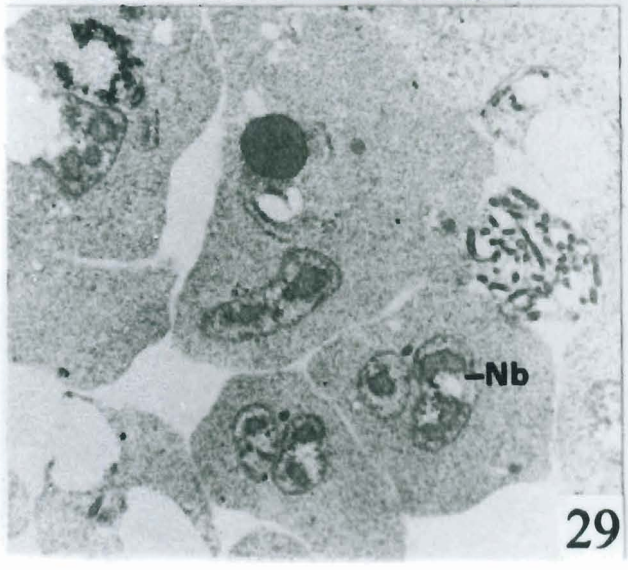
PLATE VII











contents of the peripheral singlets are dense. The size of apyrene sperms is small when compared to eupyrene ones (Pl. VII: Figs. 30, 31).

iv) Cyst envelopes

Eupyrene and apyrene sperm bundles are enclosed within cysts whose envelopes are composed of large cells. In cysts enclosing apyrene spermatozoa the cells of cyst envelope have large nuclei. The cytoplasm consist of long stretches of rough endoplasmic reticulum, mitochondria, vacuoles and dark granules (Pl. VII: Fig. 31). The cyst envelope of eupyrene spermatocyst also possess cells with a large amount of mitochondria, vacuoles and rough endoplasmic reticulum (Pl. VI: Fig. 26). In the cyst envelopes sinous intracellular septae could be distinguished extending from external surface of cyst (Pl. VII. Fig. 31).

3.4. Discussion

The testes of *S. mauritia* resemble those of other lepidopteran insects in location and gross morphology. The present studies have shown that the testes of *S. mauritia* grow in size gradually throughout the sixth larval instar and reaches maximum volume in day 2 pupae. The volume of testes then decreases gradually. Fusion of the larval testes occurs along the mid-dorsal line and form a single median structure in the pharate pupal (day 5 larva) stage. Finally the fused testes undergoes torsion in young pupae.

Testicular sheaths

The testicular sheaths of *S. mauritia* are similar in most respects to those of other lepidopteran insects, as in *Bombyx mori* (Omura, 1936), *Heliothis virescens* (Chase and Gilliland, 1972), *Cydia pomonella* (Friedlander, 1989), *Anagasta kuehniella* (Szollosi *et al.*, 1980) and *Phragmatobia fuliginosa* (Wolf, 1993). Lepidopteran testes have a covering of several layers called by different names in

the literature. In the larval and pupal testes sheaths of *S. mauritia* only two layers are seen, an external sheath, the tunica externa and an internal one, the tunica interna. The two layers are bounded by an outer basal lamina and an inner basement lamina. In other insects it has been reported that a single layered sheath exists in the beetle *Ips confusum* (Bhakthan *et al.*, 1969) and two continuous layers in *Dytiscus marginalis* (Demant, 1912), *Drosophila melanogaster* and *Dacus oleae* (Baccetti and Bairati, 1965). In *Locusta migratoria* a discontinous inner layer has been reported and this is external to the basal lamina (Szollosi and Marcaillou, 1977). In the pupal testes of *S. mauritia* there is a layer external to tunica externa, the membrana communis.

The electron microscopic studies on the testes sheaths of *S. mauritia* indicate that the tissue of the inner layer is structurally different from that of the outer layer. The cells of the inner layer contains lipid and glycogen deposits, abundant mitochondria, centrally located nuclei with dense chromatin clusters and pigment granules. The inner layer also contains large amounts of proteinaceous bodies. A network of swollen channels containing flocculent material has also been observed. Similar channels containing flocculent material has been observed in *Locusta migratoria* (Szollosi and Marcaillou, 1977) and *Anagasta kuehniella* (Szollosi *et al.*, 1980).

Utilizing horse radish peroxidase as an electron dense tracer experimental studies on both species have shown that the protein molecules freely penetrates the testes sheaths and readily pass through the channels into the follicles. In these insects the channels are normally filled with haemolymph carrying nutrients and hormones to the spermatogonia. However, in *Locusta migratoria* (Szollosi and Marcaillou, 1977) *Schistocerca gregaria* (Jones, 1978) and in *Anagasta kuehniella*

(Szollosi *et al.*, 1980) it seems that there exists a blood-testes barrier in the cyst envelope.

Testes of *Heliothis virescens* and *Lymantria dispar* secrete ecdysteroids *in vitro* during mid and late last instar larval stage and mid to late developmental period of the pupal stage (Loeb *et al.*, 1982, 1984, 1988). Testes of *Ostrinia nubilalis* was also found to synthesize ecdysteroids *in vitro* especially during late last larval instar (Gelman *et al.*, 1989). It has also been suggested that exogenous ecdysteroids are needed to initiate (Loeb *et al.*, 1988; Friedlander, 1989) or boost (Loeb *et al.*, 1986) endogenous ecdysteroid production of the testes *in vitro*. Immunocytochemical studies have indicated that the inner layer of testicular sheath synthesize and release ecdysteroids (Loeb, 1986). However, as Loeb (1986) herself has suggested this study does not rule out the possibility that the immunoreactive steroids observed in the testes sheath cells were sequestered from haemolymph or from the lumen of the testes into the inner layer of the sheath for later processing and/or release.

Transmission electron micrographs of the testicular sheaths of *S. mauritia* show that tissue of tunica interna exhibits high secretory activity. The tissue of inner layer is structurally different from that of the outer layer and contains glycogen deposits, abundant mitochondria and a peculiar network of swollen channels containing flocculent material. These features suggest that the tissue contain steroid secreting cells. However, the presence of rough endoplasmic reticulum and large proteinaceous bodies do indicate that these cells might be involved in the synthesis of proteins which might have a critical regulatory role in spermatogenesis. In fact there is a large body of evidence in literature that testes sheaths synthesizes certain proteins and liberates them into follicles and testicular lumen. These proteins promote spermatogenesis in *Mamestra brassicae* (Shimizu

and Yagi, 1982). In *Heliothis virescens* and *Lymantria dispar* testes sheath and fat body of developing male pupae synthesize soluble growth factor like substances when exposed to ecdysteroids. These growth factors are needed for meiosis to occur in spermatocytes cultured *in vitro*. When growth factors couple to their receptors, the gene activation and transcription which follow induce cell division, differentiation and development (Loeb, 1994). Giebultowicz *et al.* (1987) report that testes sheath of *Heliothis virescens* release factors which stimulate meiosis in cultured spermatogonial cysts and the effects of testes sheath is dose dependent and varied with donor's age.

Interstitial cells

The present investigations in *S. mauritia* have revealed the presence of interstitial cells existing mostly amongst gonial cells of larval and pupal testes. They have extremely complicated organisation as evidenced by their cytoplasmic extensions. The cellular organelle occupy even the smallest of cytoplasmic extensions. The size and nature of the interstitial cell is comparable in larval and pupal stages. Prominent nuclei are present occupying major portion of the interstitial cells. The cytoplasm contains rough endoplasmic reticulum, round mitochondria with complex system of internal tubular cristae, abundant membranes of smooth endoplasmic reticulum, few lipoproteins and a large number of vacuoles. The ultrastructural features of interstitial cells suggest that they are steroid secreting. The rough endoplasmic reticulum and their associated ribosome seemed to be associated with protein synthesis, required for maintenance and renewal of cytoplasmic constituents including steroidogenic enzymes. The mitochondrial aggregates are indicative of their hyperactivity. Studies on the interstitial cells in insects are relatively few when compared to those of mammals. A few studies have been reported in the larval testes of *Pieris rapae* in which the interstitial cells

encircle the high electron dense germ cells (Kim *et al.*, 1982). In *Tenebrio molitor* these cells are observed in the connective tissue of sheath cells (Menon, 1969). Also in the testes of *Dysdercus koenigii*, interstitial cells exist with their cytoplasmic extensions covering the spermatocysts (Saxena *et al.*, 1977).

Intercellular bridges

Ultrastructural studies on the germ cells of *S. mauritia* have clearly shown the existence of intercellular bridges. Such intercellular bridges are observed in mammalian testes (Guraya, 1998). Further developing germ cells can be considered as syncytia since germs cells of both sexes are generally connected by cytoplasmic bridges (Gondos, 1984). The presence of intercellular bridges provides the basis for the synchronisation of multiplication and differentiation of germ cells. Such intercellular bridges are clearly visible in the eupyrene sperm bundles. These cellular interconnections develop by incomplete cytokinesis during germ cell mitosis, and are involved in the synchronization of spermatogenic maturation and communication between cells (Guraya, 1998). Further it has also been suggested that the syncytial nature of germ cells facilitates their movement within the testicular lumen and for the mechanism of sperm release (Guraya, 1998).

Cyst envelope

Studies have demonstrated that in contrast to testis wall the cyst envelope appear to be impermeable to exogenous molecules (Szollosi and Marcaillou, 1977; Szollosi *et al.*, 1980). These studies suggest that the first site of action of macromolecular factors from haemolymph would be the cyst cells rather than the germ cells themselves. It seems that in lepidoptera the cyst fluid in which the germ cells mature differs from the testicular ones.

Germ cells

Histological studies of spermatogenesis of *S. mauritia* have revealed that in the early sixth instar larvae (day 0 - day 2), the spermatogonia aggregate into cysts (spermatocysts) in which mitotic division occur resulting in the formation of spermatocytes. These spermatocytes undergo meiotic divisions to form spermatids, which appear on day 3 of the sixth instar larvae. Differentiation of eupyrene and apyrene sperm bundles is initiated in the prepupal phase of sixth instar. Fully differentiated eupyrene and apyrene sperm bundles appear for the first time during the early pupal period. Maximum number of eupyrene and apyrene bundles is found in day 6 pupa. The spermatogonial and spermatocyte cysts disappear during the late pupal period and are no longer visible in the testes of the adults (Venugopalan *et al.*, 1994). Generally in insects having a prolonged adult life span, spermatogenesis continues to the adult stages. On the other hand, in insects having a short life span as in *S. mauritia*, the spermatogenesis is completed in the pupal stage itself. The time table of spermatogenesis in *S. mauritia* as observed in the present ultrastructural studies to a large extent supports and supplements the earlier histological observations.

Spermatogonia

The present ultrastructural investigations show that the testes of early last instar larvae (day 0 - day 3 sixth instar larva) of *S. mauritia* are characterized by the presence of large nuclei with a prominent nucleoli, and the chromatin distribution uniform. The cytoplasm is enriched with cellular organelles, like pleomorphic mitochondria, rough endoplasmic reticulum and Golgi-apparatus. The spermatogonia undergo repeated mitotic divisions. In the early sixth instar larvae, the spermatogonia aggregate and clump into cysts (spermatocysts). Spermatogonia later differentiate resulting in the formation of spermatocytes.

Hannah-Alava (1965) suggested that the main reason for the relatively little information in the literature on spermatogonia or spermatogonial stages, is probably because most of the investigations were on the meiotic and post-meiotic stages. Hannah-Alava (1965) also reported that in insects, spermatogonia are of 2 types, primary and secondary, and the primary spermatogonia are of two types, pre-definitive and definitive. In *S. mauritia*, such classification of spermatogonia is not possible.

Several authors have reported that proliferating spermatogonia are already present in the early larval instars and spermatogonial proliferation continues uninterruptedly and quasi-independently throughout the rest of the insect's life. (Leviatan and Friedlander, 1979; Gelbic and Metwally, 1981). The factors controlling the transformation of spermatogonia into spermatocytes in insects are still unknown. The transformation, like proliferation of spermatogonia, appears to be a pre-determined automatic process. It has repeatedly been observed that the number of spermatogonial divisions required for the process is specific for each species resulting in a spermatocyst containing a defined number of cells (Phillips, 1970).

Spermatocyte

A stable spermatocyte pool is maintained through continuous spermatogonial mitosis. In the testicular follicles of *S. mauritia* spermatocytes have been recognized by the presence of a characteristic structure: the synaptonemal complex, during the meiotic divisions. Ultrastructural features of spermatocytes of *S. mauritia* resemble those reported in other Lepidoptera (Wolf, 1990), Orthoptera (Wolf and Mesa, 1993) and Trichoptera (Dallai and Afzelius, 1995). A few authors also noted an increase in the number of mitochondria during spermatocyte differentiation (Andre, 1959; Hoage and Kessel, 1968).

The time table for appearance of the first clearly recognizable spermatocyte varies among species. In *S. mauritia* spermatocytes seem to appear during the early stages of the last larval instar. Distinct primary spermatocytes at pachytene stage are present in the second of the five larval instars of the carob moth, *Ectomyelois ceratoniae* (Leviatan and Friedlander, 1979), but they may appear at later larval stages in other species (Holt and North, 1970). As reviewed by Friedlander (1997) the early spermatocytes form a permanent pool which is maintained by an uninterrupted recruitment of meiotic cells derived from mature spermatogonia, proliferating throughout insect's life. Subsequently, spermatogenesis advances uninterruptedly and automatically, until the diffuse stage, after which spermatogenesis becomes a discontinuous process punctuated by predetermined stations.

Progression from one station to the next is under hormonal control. The hormonal cues controlling the advance to the next station, are correlated to the developmental status of the individual. In *Manduca sexta*, the spermatocytes develop, uninterrupted until mid-meiotic prophase, but its advancement to metaphase is solely dependent on the post wandering peak of 20-hydroxyecdysone. The meiotic division is interrupted in the absence of 20-hydroxyecdysone, leading to lysis of cell before metaphase, which can be reinitiated by exogenous application of 20-hydroxyecdysone (Friedlander and Reynolds, 1992). Furthermore, under experimental conditions, early spermatocytes withstand drastic treatments, which cause lysis of more advanced spermatogenic cells (Friedlander,1982).

Observations on *S. mauritia* reveal significant morphological changes in the spermatocysts of the testes during development of the insect and these are directly related to the process of differentiation of spermatocytes into mature spermatozoa,

like in other Lepidoptera (Lai-Fook, 1982; Kasuga *et al.*, 1985; Scheepens and Wysoki, 1985; Osanai *et al.*, 1986).

Spermatids

From the observations of pharate pupae and day 1 pupa of *S. mauritia* it appears that the spermatids undergo the early stages of spermiogenesis during this period. In the eupyrene spermatids, axial filament forms. Numerous mitochondria fuse to form the nebenkern. The nebenkern then divides into two mitochondrial derivatives. Structure of organelles in the spermatids has been described in several insects, but function of organelle during sperm differentiation is not well understood. The mitochondrial derivatives are good examples. Similiar observations are noted in the post-meiotic eupyrene sperm maturation of *Heliothis virescens* (F) (Lachance and Olstad, 1988). The elongated mitochondrial derivatives extend from the nuclear to near the caudal end in most insect spermatids. However, they are complex, highly differentiated structures, yet little is known about how and where biochemical functions are partitioned in these structures (Lachance and Olstad, 1988).

The present electron microscopic studies indicated that the process of nebenkern development and dissolution in *S. mauritia* are similiar to that of *Pieris* and *Murgantia* (Phillips, 1970). The two mitochondria of the nebenkern, although more complex in form appear to be functionally as well as structurally similar to that of other mitochondria. Numerous small mitochondria transform into large mitochondrial derivative, which occupies most of the volume of mature insect spermatozoa. Mitochondrial derivatives becomes longer and narrower during the course of spermiogenesis. As evidenced, in other lepidopterans the two mitochondrial derivatives are unequal in size. During spermiogenesis, the mitochondrial cristae of nebenkern assume, evenly spaced parallel folds disposed

perpendicular to the long axis of the cell. The final realignment of the two mitochondrial derivatives of the nebenkern after they separate from each other is related to the growth of the spermatid flagellum.

It is reported that the unique phenomenon of mitochondrial aggregation and fusion to form nebenkern followed by dissociation of two mitochondria, and their elongation and internal reorganization is without a parallel in the biology of somatic cells (Phillips, 1970). The functional significance of this remarkable series of transformations remains a mystery. The two mitochondrial derivatives with periodically spaced parallel cristae extends into the lumen from one side of the mitochondrial derivative, and the matrix is packed with paracrystalline materials. The mitochondrial derivatives assume precise, species-specific shapes, so that in certain cases in transverse section, they present highly characteristic and sometimes bizarre profiles.

However, in other organisms, there is evidence of localized activity in certain portions of mitochondrial derivatives. Kreb cycle enzymes were reported confined to the secondary helices of glycogen together with phosphorylase activity (Personne and Anderson, 1969; Favard and Andre, 1970). But, in insects, similar studies were not reported although respiratory enzymes are present in the mitochondrial derivatives and axial filament of insects (Bigliardi *et al.*, 1970).

During spermiogenesis a complex process of cell differentiation occurs and the spermatids undergo several structural changes. In *S. mauritia* spermiogenesis is initiated in the prepupal stage and continue to the adult. As reported, the process of spermiogenesis from spermatids to sperm includes axoneme elongation, formation of a nebenkern and mitochondrial derivatives. Studies in *Locusta migratoria* (Szollosi, 1975) and in *Drosophila melanogaster* (Fuller, 1993) have highlighted the changes in the structure of each cell organelle during spermiogenesis. In

Lepidoptera as in other insects (Fox *et al.*, 1974; Henning and Kremer, 1990) the RNA that is involved in spermatid differentiation is synthesized during the meiotic prophase and, the protein content of the late primary spermatocytes differs between the eupyrene and apyrene lines. The spermiogenesis of silkworm revealed different characteristics from those of other insect species (Yamashiki and Kawamura, 1997). One of the specific phenomena in the silkworm, as a member of Lepidoptera, is that the spermatocytes at prophase 1 are already equipped with 4 flagella protruding from the cell and thereafter the axoneme of the flagellum penetrated deeply into the cell by the spermatid stage. Such characteristics, common among lepidopteran species, were observed in *Bombyx mori* (Friedlander and Wahrman, 1971), in *Spodoptera littoralis* (Godula, 1985) and in *Ephestia* (Wolf and Kyburg, 1989). Similar observation are also observed in the testes of *S. mauritia* and a single flagellum is present in each spermatid. Young spermatids of insects contain two centrioles, one of which serves as a basal body for the growing flagellum, while other is oriented at right angles to the first. In all species of insects, both centrioles dissappear during spermiogenesis. The axoneme of spermatid contained a variety of tubular elements which persisted in the spermatozoa. This formed a typical 9+9+2 arrangement with the central pair of microtubular and nine peripheral doublets and nine singlets. It is presumed that the axial filament causes the undulating movements of the tail, which drives the sperm forwards (Phillips, 1970).

During spermiogenesis of *S. mauritia*, in day 1 pupa, the spermatids undergo specific modifications involving nuclear elongation, chromatin condensation, acrosomal formation and flagellar development with axoneme and accessory structure formation. The sperm nuclear development is characterized by a change from a spherical to an elongated shape. The acrosome is produced by the Golgi

complex, as described in other insects (Phillips, 1970; Baccetti, 1972; Yasuzumi, 1974; Baccetti and Afzelius, 1976; Bao *et al.*, 1989; Fernandes and Bao, 1996).

Eupyrene sperm bundles

The sperm bundles are formed as a result of maturation and differentiation of spermatids. In *S. mauritia*, spermatogenesis is dichotomous, the spermatids differentiated into two types of spermatozoa: eupyrene and apyrene spermatozoa. Eupyrene meiotic divisions appear to be regular and lead to spermatids having a spherical nucleus which undergo transformation to eupyrene spermatozoa during spermiogenesis. Eupyrene and apyrene spermatogenesis differ profoundly in their respective patterns and time table. Both kinds of cells appear to be derived from the same kind of bipotential spermatocytes. Polymorphism among spermatozoa is a common phenomena in many insect species. The highest degree of sperm variation is seen in a wasp, *Dahlbominus fuscipennis*, in which five types are known (Lee and Wilkes, 1965).

Eupyrene spermatozoa of *S. mauritia* are characterised by the possession of nucleus, lacinate appendages, acrosome, two large sized mitochondria and peripheral singlets and central singlets usually having solid centres (Andre, 1961; Phillips, 1970). The axoneme is of 9+9+2 type with accessory tubules having 16 protofilaments (Dallai and Afzelius, 1990; Mediros and Silveria, 1996).

The nucleus of eupyrene sperm in *S. mauritia* is long and thin with highly condensed chromatin, but in cross section they are semi-circular. Phillips (1971) reports that the nuclei of eupyrene sperm of the several insects are of different shapes circular, semicircular, oval, polygonal and heart shaped. In *Bombyx mori* the nucleus is crescentic in cross section and the posterior part of the nucleus is very

flat, and is compressed between the cell membrane and either the centriole or the anterior part of axoneme (Friedlander and Gitay, 1972).

One of the notable feature of the eupyrene sperm of *S. mauritia* is that the two apposed mitochondrial derivatives, fuses into single structure having a crescentic shape. This corresponds to the earlier description drawn from *Bombyx mori*, *Ephestia cautella* and *Ectomyelois ceratoniae* and *Hyalophora cecropia* (Friedlander, 1983b). However, in most cases one of the mitochondrial derivatives is larger than the other and disparity varies considerably. Observations reported from the testicular eupyrene sperm of *Trichoplusia ni* (Riemann, 1970) and *Spodoptera frugiperda* (Riemann and Gassner, 1973), showed that one of the derivatives becomes rudimentary, where as in *Heliothis virescens* and *Heliothis zea* (Riemann and Gassner, 1973) the disparity in size is only moderate.

Certain cell surface modifications are observed in the eupyrene sperm of *S. mauritia*. They are the lacinate appendages and reticular appendages. These appendages were first described by Andre (1959, 1961) in sperm of two butterflies, *Pieris* and *Macroglossum*, and have been termed as "appendices laciniae." In the eupyrene sperm of *S. mauritia*, 26-27 numbers of lacinate appendages have been demonstrated. Similar studies on the lacinate appendages have been reported in eupyrene sperm of *Pieris brassicae* (Andre, 1959; Dallai and Afzelius, 1990). *Bombyx mori* (Friedlander and Gitay, 1972), *Ephestia cautella* (Friedlander, 1976), *Euxoa, Tetracis, Hydriomena, Archips, Desmia funeralis* and in almost of all 18, unspecified lepidopteran species examined (Phillips, 1971). Lacinate appendages have also been reported in *Heliothis virescens* (Richard *et al.*, 1975) and in *Heliothis punctigera* (Jamieson, 1987). Earlier different terms like 'radial spokes' in *Plodia interpunctella* (Ashrafi and Roppel, 1973); laminate projections in *Trichoplusia ni* (Riemann, 1970); 'radial mantle' in *Bombyx mori* (Danilova and

Vereiskaya, 1969) and in *Anagasta kuehniella* (Riemann and Thorson, 1971) and 'radial projections' in *Hyphantria cunea* (Trandaburu *et al.*, 1976) have been used to describe these structures. Lacinate appendages usually extend from the plasma membrane like blades and extend from the anterior tip posteriorly along most of the length of the tail of lepidopteran spermatozoa. These appendages have a striated appearance in cross sections. When sperm of *S. mauritia* are viewed in transverse sections, all but one of the radial surface projections appear to consist of regularly spaced thin laminae with a 90A° periodicity. The shapes and number of the blade like appendages, when viewed in cross sections vary considerably along the length of the spermatozoa. It has been reported that these appendages disappear during the passage to and through the vas deferens, and instead in their place, a prominent coat of extracellular material is present. The functional significance of the appendages and coats are unknown.

Transverse sections of testicular eupyrene sperm of *S. mauritia* also revealed the presence of reticular appendage, external to the definitive plasma membrane. The reticular appendage is dissimilar to the lacinate appendage. Lacinate appendage are seen as broad columns composed of evenly disposed light and dense bands. When observed in transverse sections, the reticular appendage appear as a honey comb of electron-lucid and dense regions. 'Reticular appendages' have also been reported in *Pieris brassicae* (Andre, 1959; Dallai and Afzelius, 1990), *Bombyx mori* (Friedlander and Gitay, 1972) and *Ephestia cautella* (Friedlander, 1976). Different terms have been attributed for reticular appendage, they are the 'unpaired organ' of *Macroglossum stellatarum* (Andre, 1961), the 'Satellite body' of *Trichoplusia ni* (Riemann, 1970), *Anagasta kuehniella* (Riemann and Thorson, 1971), *Hyphantria cunea* (Trandaburu *et al.*, 1976) and the 'clear band' of *Bombyx mori* (Yasuzumi and Oura, 1964) and *Plodia interpunctella* (Ashrafi and Roppel, 1973). The reticular appendage also dissappear during the passage to and through

vas deferens and a coat of extra cellular material replaces the area. The role of the reticulate appendages is still unknown. In *Bombyx mori*, the remnant of the reticular appendage appears to protrude as a strip of poorly resolved membrane through an indentation or slit in the sleeve. A narrow electron-dense ribbon, in the inner face of the sleeve, is found on each side of the slit (Friedlander and Gitay, 1972).

Apyrene sperm

The apyrene sperm of *S. mauritia* can be easily distinguished from eupyrene ones. Some of the characteristic features of apyrene sperm of *S. mauritia* are that they have neither lacinate or reticular appendages, nor any other extracellular coating material. The mitochondrial derivatives are small and equal in diameter and length. Similar mitochondrial derivatives are also known for the apyrene sperm of *Plodia interpunctella* (Ashrafi and Roppel, 1973); and *Heliothis punctigera* (Jamieson, 1987). The axoneme consisting of the peripheral and central singlets do not have dense contents. In *Heliothis punctigera*, the contents of the peripheral singlets are dense, with 16 protofilaments in their tubular wall (Medeiros and Silveira, 1996).

The apyrene spermatozoa of *S. mauritia* are similar to those described in the silkworm, *Bombyx mori* (Sado, 1963). Apyrene sperms are smaller and are about half the diameter and length of eupyrene sperm. Transmission electron micrographs of apyrene sperms show them to be little more than simple flagella, accompanied by paired mitochondrial derivatives. The eupyrene spermatozoa have nuclei at the anterior end, where as the apyrene spermatozoa have granular nuclei in the central part. The two types of spermatozoa appear to derive from the same kind of bipotential primary spermatocytes (Leviatan and Friedlander, 1979; Friedlander and Benz, 1981). In eupyrene spermatid, differentiation follows the generalized scheme leading to spermatozoa having the regular type of elongated nuclei, which is found

in many species of insects (Baccetti and Afzelius, 1976). In apyrene spermiogenesis, the meiotic division are irregular and the spermatid nuclei never elongated. Nuclei are eventually discarded from these cells which subsequently develop into anucleate apyrene spermatozoa. During this process, the chromatin remains in a condensed condition and does not undergo the characteristic transformation occuring in the corresponding eupyrene spermatids (Friedlander and Wahrman, 1971; Friedlander and Miesel, 1977).

Eupyrene and apyrene spermatozoa together reach the spermatheca of the inseminated females and only the eupyrene ones fertilize the eggs (Friedlander and Gitay, 1972). But, majority of the sperm transferred to the female are the apyrene spermatozoa (Siberglied *et al.*, 1984). The eupyrene sperm are immotile until activated by alkaline phosphatase in the female bursa copulatrix, whereas the apyrene are active in the semen (Kinefuchi, 1978). The function of apyrene spermatozoa is still unclear. However, there has been several feasible theories, which states that, apyrene sperm helps in the transportation of eupyrene sperm to the female genital tract, (Iriki, 1941; Riemann, 1970; Friedlander and Miesel, 1977; Kinefuchi, 1978) and are activated during ejaculation by a polypeptide which apparently works in a hormone like manner (Shepherd, 1975). But persistence of the apyrene sperm in the spermatheca led Riemann (1970) to suggest that they have an additional, unknown function. Eupyrene sperm mature before apyrene and the apyrene sperm migrate out of the testes before eupyrene sperm (Katsuno, 1977a). Katsuno (1977a) suggested that the apyrene sperm act as a trail blazer for the eupyrene sperm by making holes in the layer of cells which separates the lumina of the testicular follicles from those of the vasa deferentia. Apyrene sperm may play, at least additionally a role in competition between rival sperm deposited by different males. They may either eliminate by displacement or inactivation the eupyrene sperm from previous mating or prevent or delay further mating by the female.

Katsuno (1977c) also suggested that apyrene sperm are themselves activated by seminal plasma from the copulatory pouch, the activating factor being a secretion from the male ejaculatory duct. The activated apyrene sperm are necessary for the separation of the eupyrene sperm from their bundles and the inactive apyrenes are ineffective. This shows the possibility that apyrene sperm aid in transportation of the eupyrene or play some other mechanical role. Further Katsuno (1978) suggest that the action of the apyrene sperm in releasing eupyrene may be mechanical.

3.5. Summary

1. Ultrastructural and histological investigations were conducted on the testes of sixth instar larvae (last larval instar) and pupae of *S. mauritia* to study the dynamics of spermiogenesis.

2. In the larval stages of *S. mauritia*, testes are seen as paired, kidney shaped structures located dorso-lateral to the alimentary canal. Each testis consists of four follicles. The volume of testis progressively increases reaching a maximum in the day 3 larva.

3. During the pharate pupal stage, the paired testes fuses into a single median structure. In the young pupa, the fused testis undergoes torsion and increases in volume. The volume of testis is found to decrease from day 2 pupa onwards.

4. Ultrastructural studies have demonstrated that the larval testes are covered by a sheath consisting of two cellular layers, the outer tunica externa and the inner tunica interna. The two layers are bounded by an outer basal lamina and an inner basal lamina. Tunica externa is thinner than tunica interna, contains numerous vacuoles and rough endoplasmic reticulum. The tunica interna is homogeneous and contains numerous glycogen particles, lipid droplets, abundant mitochondria, numerous vacuoles, few pigment granules and centrally located nuclei with dense chromatin clusters. The cytoplasm also contains long stretches of rough endoplasmic reticulum and large spherical proteinaceous bodies. Ultrastructural features suggest that the cells of tunica interna are the source of certain proteins which might have critical regulatory role in spermiogenesis.

5. In the larval and pupal testes, elongated, spindle shaped interstitial cells exist among the gonial cells. These cells possess long cytoplasmic extensions.

Major portion of the interstitial cell is occupied by a prominent nucleus. The cytoplasm is enriched with many cellular organelles like mitochondria, polysomes, few lipoproteinaceous granules and smooth endoplasmic reticulum. Cytoplasm also contains a large number of secretory vacuoles. Ultrastructural features suggest that these cells are steroid secreting.

6. The germ cells develop more or less synchronously in cysts, surrounded by a capsule of somatic cells. Major part of sperm development occurs in these spermatocysts. Spermatogonial and spermatocyte cysts appear in the day 0 - day 3 sixth instar larvae. Spermatidal cysts develop in the prepupal and pupal stage. Further, the initiation in the differentiation of sperm bundles occurs in day 4 - day 5 of sixth instar larval development. Fully formed sperm bundles are seen in the pupal stage.

7. In *S. mauritia*, spermatogenesis is dichotomous; the spermatids differentiated into typical eupyrene (nucleated) and atypical apyrene (anucleated) sperm bundles.

8. The spermatogonial cells are characterized by the presence of spherical nuclei, having conspicuous nucleoli: Chromatin is uniformly distributed in the nucleus. The cytoplasm is enriched with cellular organelles like pleomorphic mitochondria with tubular or lamellar cristae, short stretches of rough endoplasmic reticulum, Golgi-apparatus and few lipid inclusions.

9. Spermatocytes are characterized by the possession of fully developed synaptonemal complexes which possibly represent the zygotene stage of meiosis. The nuclei contain nucleoli with chromatin clumps. Nuclear envelope possesses numerous nuclear pores. Cytoplasm contains a small amount of mitochondria but no typical Golgi stacks are seen. Instead, small vesicles are present which are formed from golgian activity. These vesicles contain small granules which represent the proacrosomal granule. Later, these vesicles fuse to form the acrosomal vesicle containing the acrosomal granule. In the diplotene stage, the spermatocytes are characterised by the absence of synaptonemal complexes. During the completion of meiotic division, the spermatocyte nucleus becomes irregular in shape with numerous nuclear pores.

10. In the pupal testes, the membrana communis covers the tunica externa, but in larval testes sheath this layer is altogether absent. The cells of membrana communis layer are larger in size with a prominent nucleus containing clumps of chromatin. Cytoplasm is homogeneous with numerous glycogen droplets and mitochondria. Reduction in thickness of the external layer is

seen with sparce endoplasmic reticulum and few mitochondrial accumulations. The tunica interna contains abundant glycogen deposits, few lipid inclusions, mitochondria and network of channels filled with flocculent material. The cytoplasm is electron-dense. Tissue of tunica interna exhibit high secretory activity. The testicular sheath is lined by two basal laminae, an inner basal lamina, covering the tunica interna and an outer basal lamina of tunica externa facing membrana communis.

11. Early eupyrene spermatid cyst of day 1 pupae comprise mainly of nucleus, an axial filament or axoneme, multivesicular bodies and Golgi complex. Numerous mitochondria fuse to form a structure called nebenkern which later on divide to form mitochondrial derivatives which are unequal in size. The axoneme possesses a typical 9+9+2 micro tubular arrangement. In the eupyrene mononucleated spermatid, elongation of the nucleus together with acrosome and axoneme are seen.

12. Eupyrene spermatozoan of *S. mauritia* consists of an elongated nucleus, with two apposed mitochondrial derivatives fusing into a crescent shaped structure and with certain cell surface modifications, the lacinate and reticulate appendages. The lacinate appendages are 26 in number and appear as rays of alternating electron-lucid bars, whereas the reticular appendage consists of a honey comb of lucid and dense regions. An axoneme with 9+9+2 configuration persists. Spermatozoa are interconnected by cytoplasmic bridges.

13. Apyrene spermatid cysts in day 1 pupa are characterized by the formation of nebenkern. During later stages of apyrene spermiogenesis, acrosome formation is not detected, and the basal body is not attached to the nucleus. The flagella of apyrene spermatozoa comprises of two separate small mitochondrial derivatives of equal size, and an axoneme consisting of 9+9+2 arrangement. Lacinate appendages, reticular appendages and intercellular bridges are absent. Apyrene sperm is small, when compared to eupyrene ones.

14. Cyst envelope is present in both eupyrene and apyrene sperm bundles. In the apyrene cysts the envelope possesses cells with large nuclei with their cytoplasm containing short stretches of rough endoplasmic reticulum, mitochondria, vacuoles and dark granules. Sinuous intracellular septae are also distinguished in the cyst envelope.

CHAPTER 4

ROLE OF HORMONES AND EFFECTS OF HORMONE ANALOGUES ON SPERMIOGENESIS

4.1. Introduction

Although there are many reports on the normal course of spermiogenesis in lepidopteran insects, only few investigators have studied about its regulatory factors. The available information on the regulatory factors in insects spermatogenesis is incomplete and contradictory. It has been proposed that in insects, spermatogenesis may proceed as a sequential auto-differentiative process which is not affected by alterations in the titre of morphogenetic hormones. This hypothesis is supported by several studies which show that 1) the spermatocytes are able to undergo autonomous meiosis and spermatid differentiation *in vitro* in the absence of any hormone added to the medium (Lender and Duveau-Hagage, 1963) (2) Spermatogenesis proceeds normally in larvae which have been ligated between the thorax and abdomen, thus effectively isolating the testes from the cerebral and thoracic endocrine glands (Loeb *et al.*, 1985) and (3) the timetable of meiosis remains unchanged in larvae which are induced to undergo precocious pupation by endocrine manipulation (Friedlander *et al.*, 1981). Several studies, however, do not support this view. Ever since, the pioneering work of Goldschmidt (1917) (who first induced the *in vitro* differentiation of spermatocytes cultured with pupal haemolymph), many reports have been published concerning factors which regulate the development of insect sperm. According to Loeb and Birnbaum (1981) in *Heliothis virescens* changing the osmotic pressure of the haemolymph influences the development as well as degeneration of sperm. In the same species, factors of neural origin seem to influence the rate of spermatogenesis (Loeb *et al.*, 1985). Due to these conflicting results and the probable existence of unknown parameters, a credible model describing the hormonal regulation of spermatogenesis has not been forth coming.

Several studies have shown that hormones do control insect spermatogenesis (reviewed by Dumser, 1980; Koeppe *et al.*, 1985). The role of juvenile hormone (JH) in insect spermatogenesis remains unclear. Wigglesworth (1936) was the first to demonstrate that removal of JH producing organs corpora allata (CA) has no effect on spermatogenesis in *Rhodnius*. Similiar results were obtained for *Locusta migratoria* (Girardie and Vogel, 1966; Cantacuzene, 1967) and *Schistocerca gregaria* (Cantacuzene, 1967). JH acts as an inhibitor of testicular development, but the exact nature of action is still unknown. Inhibition can be removed and spermatogenesis accelerated by allatectomy (Fukuda, 1944; Nowock, 1973) or imposed by implantation of supernumerary corpora allata (Metwally and Gelbic, 1974; Leviatan and Friedlander, 1979). Decline in JH titre triggers elongation of eupyrene sperm nuclei whereas artificially maintaining JH at a high level inhibits the elongation process (Leviatan and Friedlander, 1979). In *Rhodnius* JH inhibits spermatogenesis by abolishing ecdysone stimulated gonial mitosis (Dumser and Davey, 1975). In some insects, JH acts as a stimulator of spermatogenesis. In *Mamestra brassicae* (Yagi, 1975); *Papilio xuthus* (Nishiitsutsuji-Uwo, 1961) and *Eurygaster integriceps* (Shinyaeva, 1981) spermatogenesis is acelerated by JH treatment.

Ecdysteroids have a stimulatory effect on spermatogenesis and this has been well documented in several insect species both in *in vitro* and *in vivo* experiments. In *Hyalophora cecropia* and *Samia walkeri*, the prothoracic gland hormone has an accelerating effect on spermatogenesis in *in vitro* culture (Schmidt and Williams, 1953). Later Nishiitsutsuji-Uwo (1961) also found that in *Papilio xuthus*, spermatogenesis was accelerated by prothoracic gland hormone. *In vitro* studies by Yagi *et al.*, (1969) showed that ecdysteroids stimulate testicular development in *Chilo suppressalis*. These observations were also confirmed by Kambysellis and

Williams (1971a, b) in several species of saturniids and by Fukushima and Yagi (1975) in *Spodoptera litura* and *Mamestra brassicae.*

Several investigators have shown that testes contain ecdysteroids and that these are secreted by the testis sheath (Koolman *et al.*, 1979; Loeb *et al.*, 1982; 1986, 1987; Gelman *et al.*, 1989; Jarvis and Rees, 1990). Testis ecdysteroids may control meiotic and/or mitotic divisions during the early stages of spermatogenesis; interact with the haemolymph macro-molecular factor (Kambysellis and Williams, 1971b, 1972) to induce spermatocyte differentiation and influence the development of apyrene sperm (Dumser, 1980; Hoffmann and Behrens, 1982; Gelman *et al.*, 1989).

Serum factors were found to regulate spermatogenesis in certain lepidopteran insects. Studies by Kambysellis and Williams (1971a,b, 1972) showed that the response in intact testes requires two factors, the ecdysteroids and the macromolecular factor produced by the haemocytes. A few studies on the dichotomous spermatogenesis in lepidopteran insects have demonstrated that the shift in commitment of the spermatocyte from eupyrene to apyrene spermatogenesis is related to an apyrene spermatogenesis inducing factors of unknown origin as well as a factor from the brain (Jans *et al.*, 1984; Gelman and Borkovec, 1986).

In the present investigation, the sixth larval instar of *S. mauritia* is found to be the most suitable developmental stage to study the role of major hormones (JH and ecdysteroids) on spermiogenesis. To evaluate the role of hormones in spermiogenesis, the classical endocrinological approach on the effects of ligation, coupled with treatment of insect growth regulators (IGRs) like Juvenile hormone analogue, ecdysone agonists on spermatogenesis have been analysed in the present study.

4.2. Materials and Methods

4.2.1. Experimental animals

The animals used for various experiments were the sixth instar larvae (last larval instar) and pupae of *S. mauritia*. The larvae and pupae of the required stage were obtained from laboratory stock culture reared and maintained in separate containers as described previously (Chapter 3). In all experiments, developmentally synchronous larvae were used.

4.2.2. Ligation

Sixth instar larvae of *S. mauritia* were mildly anaesthetised with diethylether and tightly ligated between the head and prothorax using a cotton thread, thereby effectively separating the cephalic endocrine structures (the brain, corpora cardiaca and corpora allata) from the rest of the body. These larvae which lacked brain, corpora cardiaca and corpora allata but possessed prothoracic glands were designated as neck-ligated larvae.

Ligations were also carried out between pro- and mesothorax. These larvae which lacked brain, corpora cardiaca, corpora allata and the prothoracic glands will be, hereafter, referred to as thorax-ligated larvae.

4.2.3. Histological techniques: (See Chapter 3).

4.2.4. Electron Microscopy Techniques: (See Chapter 3).

4.2.5. Chemicals

i) Juvenile hormone analogue

The juvenile hormone analogue (JHA) hydroprene (ethyl 3, 7, 11-trimethyl dodeca-2,4-dienoate) was a gift, from Dr. G.B. Staal, Zoecon Corporation, Palo

Alto, California, U.S.A. The compound was dissolved and diluted in acetone to obtain 5 μg /5 μl of the solvent.

ii) RH 5992

The non-steroidal ecdysone agonist RH 5992 (Tebufenozide, 1,2-dibenzoyl-1-tert-butyl hydrazide) was obtained as a gift from Rohm and Haas Company, Spring House, Pennsylvania, U.S.A. It was dissolved in acetone and diluted to obtain the required concentration.

4.2.6. Treatments

Larvae and pupae were treated topically with different doses of hormone analogue/agonist using a Hamilton microsyringe as described below.

i) Treatment of JHA on ligated larvae

Neck/thorax-ligated sixth instar day 1 larvae were treated topically with daily doses of 5 μg JHA on abdominal tergites. The first dose was given 30 minutes after ligation and the second and third doses were given on the subsequent days at almost the same time of the day. Neck/thorax ligated day 4 larvae were treated with a single dose of 5 μg JHA in a similar manner. Larvae kept as controls were treated with 5 μl of acetone. Testes were removed from the treated and control larvae and subjected to histological and ultrastructural studies as described earlier.

ii) Treatment of RH 5992 on ligated larvae

Neck-ligated day 1 or day 4 larvae were treated topically with a single dose of 5 μg RH 5992. Control larvae were treated with 5 μl of acetone. Testes of treated and control larvae were removed on different days and subjected to histological (P. 43) and ultrastructural (P. 43) studies.

iii) Treatments of JHA/RH 5992 on day 0 pupae

Newly ecdysed (day 0) pupae of *S. mauritia* were treated topically with a single dose of 5 µg JHA or 10 µg RH 5992. Pupae kept as controls were treated with 5 µl of acetone. Experimental and control pupae were kept in separate beakers covered with muslin cloth. The pupae were checked daily for mortality, moulting, duration of pupal instar and morphological abnormalities. Testes were then removed from treated pupae after seven days of treatment and subjected to electron microscopic and histological studies.

4.3. Results

4.3.1. Effects of ligations of sixth instar larvae and treatments of hormone analogues/agonists on spermatogenesis

This study was undertaken to investigate the role of cerebral factors and prothoracic gland secretions on spermatogenesis of *S. mauritia*. In order to analyse the role of hormones/effects of hormone analogues/agonists on spermatogenesis, the classical approach of neck/thorax-ligation to eliminate the endogenous source of hormones and the hormone replacement therapy have been employed. Sixth instar day 1 and day 4 larvae were used for experiments. Larvae of these age groups were selected because eupyrene/apyrene spermatid differentiation take place during these days of larval-pupal transformation (Venugopalan *et al.*, 1994). In one set of experiments, sixth instar day 1 or day 4 larvae were 'neck-ligated' to remove the endogenous source of cerebral neurosecretory factors and JH. In another set of experiments, sixth instar day 1 or day 4 larvae were 'thorax-ligated' to eliminate the endogenous sources of both head factors (cerebral neurosecretory factors and JH) and prothoracic gland secretions. Further the effects of treatments of ligated larvae with hormone analogues/agonists on spermatogenesis were looked into.

Table 1. Effects of ligation of sixth instar larvae on morphogenesis and testicular fusion

Day of ligation	Nature of ligation	*n*	Days of survival	% of surviving larvae which showed			Nature of testes lobes
				No change	Gut purge	Pupation	
Day 1	Neck-ligated	10	7 ± 1	30	70	--	Unfused
Day 1	Thorax-ligated	10	7 ± 1	30	70	--	"
Day 1	Unligated (control)	10	6 ± 0	--	100	100	Fused

i) Effects of neck/thorax ligation of day 1 larvae on morphogenesis and spermatogenesis

In order to analyse the role of various hormonal factors on spermatogenesis, effects of neck/thorax-ligation of day 1 sixth instar larvae on morphogenesis and growth and differentiation of germ cells were looked into.

The duration of unligated normal sixth instar larvae was 6 ± 0 days. Sixth instar larvae at the termination of the phagoperiod i.e. on day 4 purged their guts and subsequently exhibited wandering behaviour. These larvae underwent pupation 48 h after the gut purge i.e., on day 6. Seventy percent of neck/thorax-ligated day 1 larvae purged their guts. They survived up to 7 ± 1 days, but failed to pupate. Their testes lobes remained unfused (Table 1). Testes were dissected from these larvae on day 5 and processed for histological and electron microscopic studies.

Histological studies on the testes of the ligated larvae, showed that differentiation of various types of spermatocysts was completely blocked and the testes contained only an undifferentiated mass of tissue. Spermatogonial and spermatocyte cells were not seen.

The electron micrographs revealed that the spermatogonial and spermatocyte cysts were either absent or they could not be distinguished in the undifferentiated mass of tissue (Pl. VIII: Fig. 32). Most of the cells appeared to be abnormal and non-functional. In most cells, numerous autophagic vacuoles, numerous darkly stained small granular bodies and multivesicular sac remnants were present (Pl. VIII: Fig. 33). Pycnosis was observed in some cysts. The demarcation of individual cyst or cells seemed to be absent. The cellular membraneous envelope was seen

Table 2. Effects of treatments of ligated larvae with JHA on metamorphosis and testicular fusion

Nature of ligation	Dosage	*n*	% of surviving larvae which showed			Nature of testes lobes
			No change	Gut purge	Pupation	
Neck-ligated	5 μg JHA	20	100	--	--	Unfused
Control	5 μl acetone	10	30	70	--	Unfused
Thorax-ligated	5 μg JHA	20	100	--	--	Unfused
Control	5 μl acetone	10	30	70	--	Unfused

ruptured at many places. Few threads of endoplasmic reticulum were evident, but the ribosomes were lacking.

Necrotic or degenerating cells with numerous membraneous whorl like structures, autophagic vacuoles and few mitochondria were seen (Pl. VIII: Fig.34). The mitochondria sometimes formed aggregates and were with less dense matrix and few cristae (Pl. VIII: Figs. 33, 34). Spermiogenesis was suppressed and the dimorphic sperms, eupyrene and apyrene present in the normal spermiogenesis was absent in the ligated larvae.

4.3.1.1. Effects of treatments of ligated day 1 larvae with juvenile hormone analogue (JHA) or ecdysone agonist on morphogenesis and spermatogenesis

The above study has clearly demonstrated that neck/thorax ligation of day 1 larvae inhibits not only larval development and metamorphosis, but also testicular development and spermatogenesis, particularly spermiogenesis. Therefore, it was thought worthwhile to investigate whether treatments of neck/thorax-ligated larvae with JHA, hydroprene or ecdysone agonist, RH 5992 has any effect on metamorphosis and spermatogenesis.

i) Effects of treatments of neck-ligated larvae with JH analogue

Sixth instar day 1 larvae were neck-ligated and treated with repetitive daily doses of 5 μg JHA for 3 days. The ligated larvae kept as controls were treated with an equivalent amount of acetone. Effects of these treatments on morphogenesis and spermatogenesis were carefully studied. Testes were dissected out on day 5 and processed for histological and electron microscopic studies.

None of the JHA treated or control larvae pupated. Also, these larvae did not show the premetamorphic behaviour of gut purge, while 70% of the control

PLATE VIII

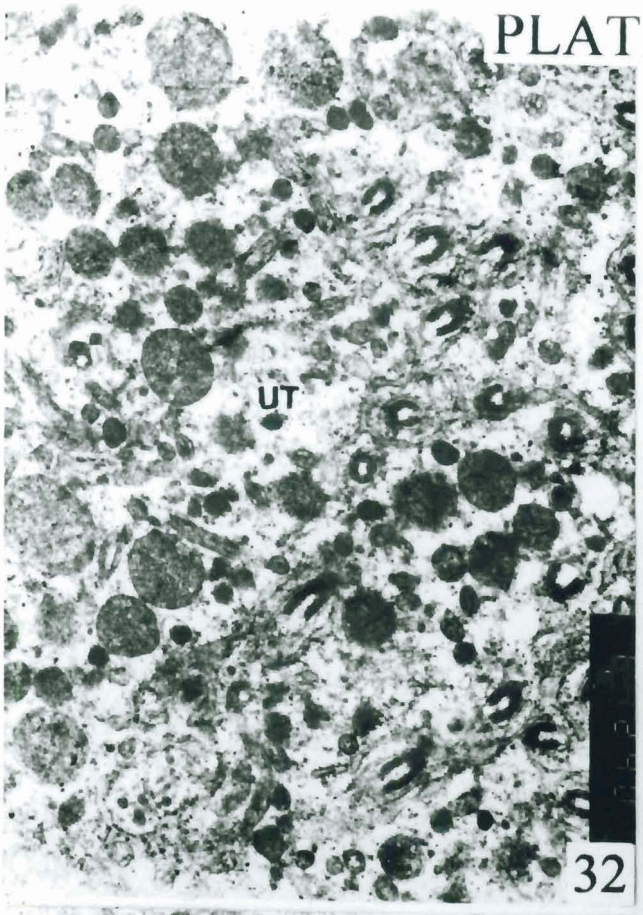
Fig. 32. Testicular tissue of larva neck-ligated on day 1 showing its undifferentiated nature (UT). X 9300

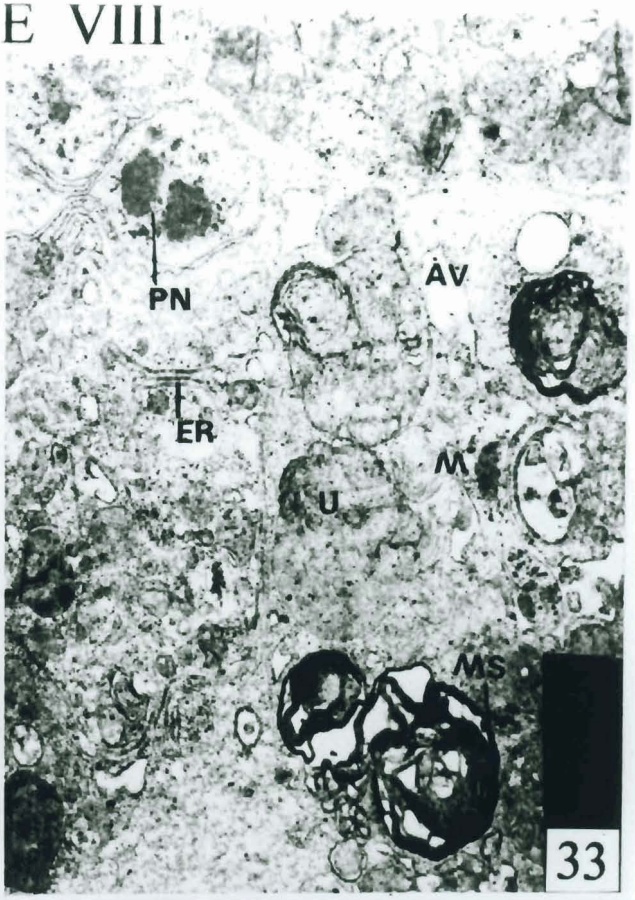
Fig. 33. Degenerating germ cells of larva neck-ligated on day 1 showing pycnotic nucleus (PN), numerous autophagic vacuoles (AV), multivesicular sac (MS), endoplasmic reticulum (ER), mitochondria (M) and undifferentiated cell (U). X 12000

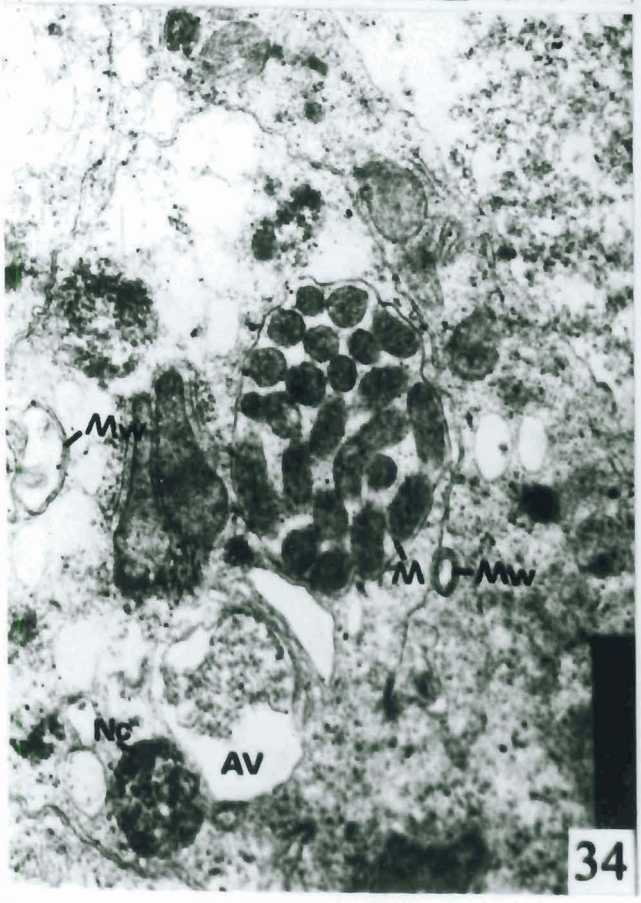
Fig. 34. Degenerating gonial cells of larva neck-ligated on day 1 showing necrotic cells (Nc), autophagic vacuoles (AV), membraneous whorls (Mw) and abnormal mitochondria (M). X 15400

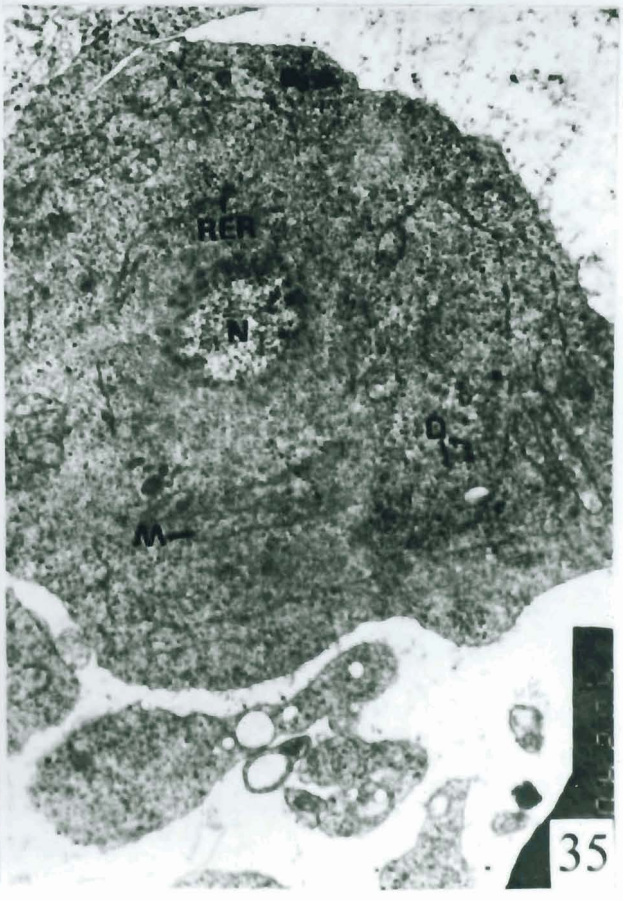
Fig. 35. Undifferentiated early germinal cell of neck-ligated day 1 sixth-instar larva treated with JHA, showing highly condensed nucleus (N) and cytoplasm with elongated mitochondria (M), rough endoplasmic reticulum (RER) and darkly stained dense bodies (D). X 7900

PLATE VIII









larvae exhibited gut purge after 4.5 $\pm$ 0.5 days (Table 2). The testes of the treated and control larvae were unfused. The testicular volume of JHA treated larvae was much reduced, when compared to that of control larvae. The testes lobes were flat and poorly pigmented.

Histological studies clearly revealed the presence of large empty spaces in testicular tissue. The interior of testis gave the appearance of an unorganised mass and the germinal cells were necrotic and apparently degenerating.

Electron microscopic studies demonstrated that even though a few of the germinal cells were necrotic and degenerating, majority of the germ cells had a normal appearance (Pl. VIII: Fig. 35; Pl. IX: Figs. 36, 37). However, the germinal cysts were almost indistinguishable. They were not recognizable as spermatogonial or spermatocytal cysts in certain areas. In a few germ cells, the nucleus appeared abnormal and reduced in size without a nuclear envelope (Pl. VIII: Fig. 35). The boundary of nuclei was also seen disrupted at several places. Few elongated mitochondria without cristae, short stretches of endoplasmic reticulum with dispersed ribosomes and some darkly stained dense bodies were evident in the cytoplasm of the abnormal germ cells. Cytoplasm showed high electron opacity (Pl. VIII: Fig. 35).

In certain areas, a few germinal cells were recognizable although, they appeared abnormal, when compared to normal unligated larvae (Pl. IX: Figs. 36, 37). One of the noticeable abnormality was the reduction of cytoplasm of the germ cell and the irregularly shaped nuclei. In the nuclei, a clumping of chromatin was seen to occur, with a reduction of aggregates of nuclear chromatin and their distribution. Large irregular heterochromatic bodies were seen. Germinal cells were separated from each other by large empty spaces (Pl. IX: Figs. 36, 37). Another notable feature was the disintegration of the nuclei of gonial cells (Pl. IX.

PLATE IX

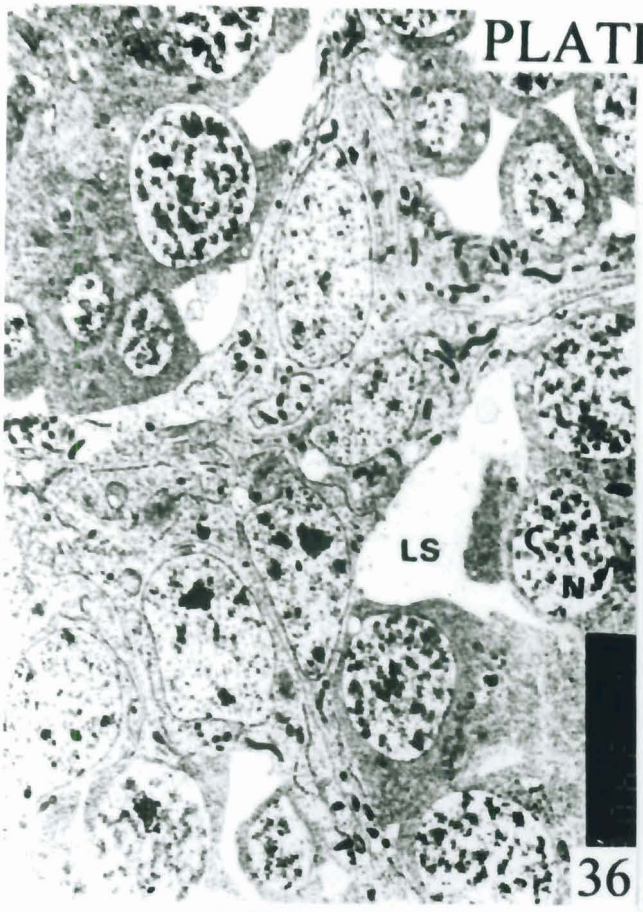
Fig. 36. Germinal cells of neck-ligated larva treated with JHA showing large empty spaces (LS) between the germinal cell nuclei (N) containing condensed chromatin (C). X 3500

Fig. 37. Gonial cells of JHA treated neck-ligated larva with disintegrated nuclei (DN) and condensed chromatin (C). Empty spaces (LS). X 6500

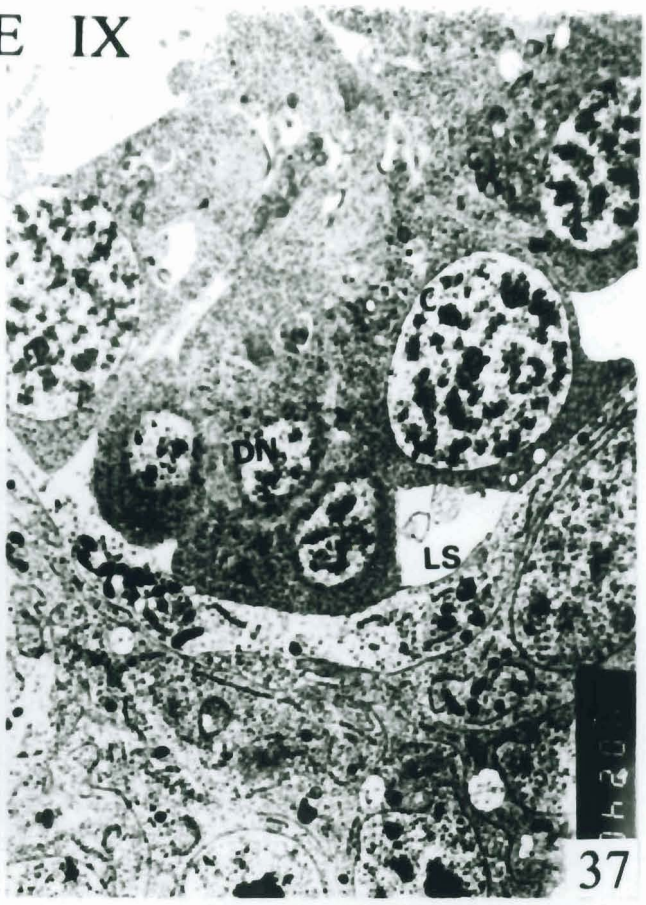
Fig. 38. Gonial cell of JHA treated neck-ligated larva under high magnification showing disintegrated nucleus (DN) having nuclear pores (Np) and condensed chromatin (C). Cytoplasm contains mitochondria (M) without cristae and few vacuoles (V). X 20000

Fig. 39. Germinal cells of thorax-ligated larva treated with JHA showing abnormal spermatocysts separated by large spaces (LS). The germ cell shows necrotic nucleus (NC) and cytoplasm with large autophagic vacuole (AV). X 9900

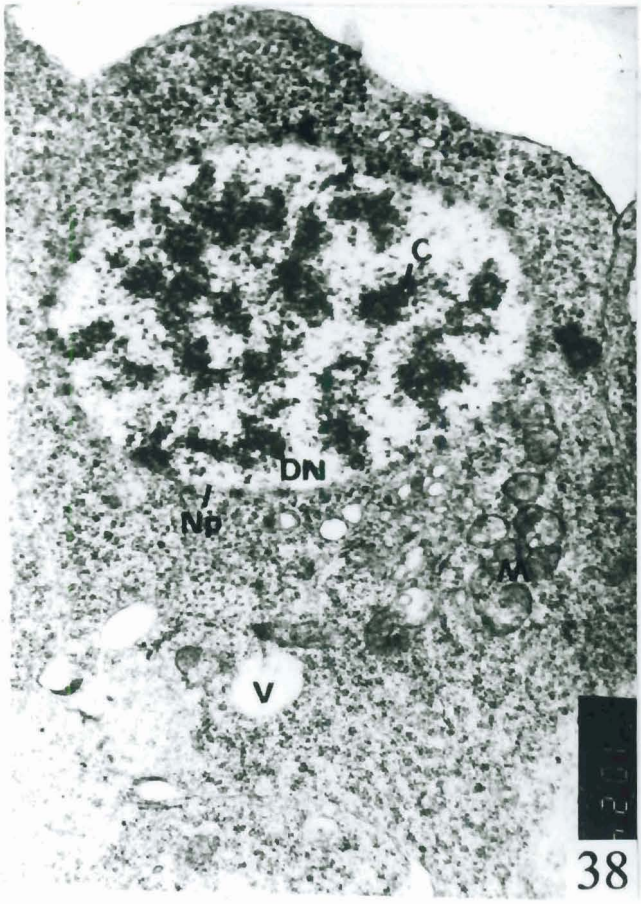
PLATE IX



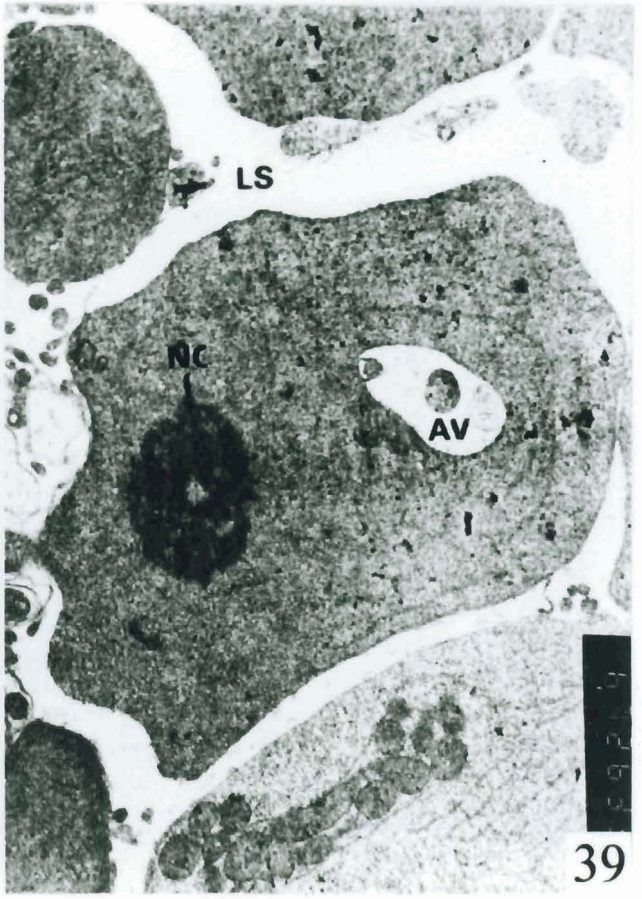
36



37



38



39

Fig. 37). In these cells, the nuclear envelope was absent and few nuclear pores were evident. The cytoplasm contained empty vacuoles and the few mitochondria present were electron luscent and without cristae (Pl. IX: Fig. 38). Differentiation of eupyrene/apyrene sperm bundles was completely absent.

ii) Effects of thorax-ligation and treatment of JHA

A group of sixth instar day 1 larvae were thorax-ligated and treated with repetitive daily doses of 5 µg JHA for three days. Ligated larvae kept as controls were treated with an equivalent volume of acetone. The effects of these treatments on morphogenesis and spermatogenesis were carefully studied. Testes were reclaimed on day 4 and processed for histological and electron microscopic observations.

The JHA treated or control larvae failed to pupate and these larvae did not show the premetamorphic behaviour of gut purge. Treated larvae survived for 4 to 5 days. The testes of both treated and control larvae remained disjuncted (Table 2). The testicular volume of JHA treated larvae was considerably less when compared to that of control larvae.

Histological studies of the treated larvae showed that most of the germinal cells were necrotic and degenerating. However, the testes of control, ligated larvae contained only an undifferentiated mass of tissue.

Ultrastructural observation revealed that the germinal cells though present were abnormal. Most of them were not distinguishable into various spermatocysts, i.e., spermatogonial, spermatocytal or spermatid cysts. Large spaces were seen between individual germ cells. The germ cells had necrotic and degenerating nuclei. Nuclear envelopes were absent in the apparently disintegrating nuclei. Autophagic vacuoles were present in the cytoplasm (Pl. IX: Fig. 39). Cellular

organelle were relatively few and most of them were abnormal. Most of the germ cells possessed pycnotic nuclei (Pl. IX: Figs. 40, 41). Few elongated mitochondria without cristae were seen. These mitochondria were electron lucid. Short stretches of rough endoplasmic reticulum were evident, but ribosomes were lacking. Degenerating interstitial cells were observed. In some areas dense lipid droplets were also present in the intercellular spaces and in the interstitial cells (Pl. X: Figs. 40, 41). The nuclei present in the interstitial cells were elongated and the cytoplasm contained rounded mitochondria and numerous vacuoles. The mitochondria were with less dense matrix. The nuclear envelope was ruptured and the chromatin distribution seemed to be reduced (Pl. X: Fig. 41). These observations showed that there is a complete inhibition of spermatogenesis.

Testicular sheath

The testicular sheath of the treated animals displayed the presence of intercellular spaces. Most of the cellular organelles were lacking, particularly the mitochondrial aggregates and rough endoplasmic reticulum. Distribution of glycogen particles and lipid droplets were altogether absent as compared to the testicular sheath of untreated normal larvae. Basal lamina varied in thickness in different regions of testicular sheath. In certain preparations vacuoles and empty areas were present in testicular tissues. The elongated nucleus with dispersed chromatin so often observed in the sheath cells of normal testicular sheaths were also lacking. The mitochondrial system was poorly developed and consisted of scarce globular units. The cells of both tunics (internal and external) lacked the electron-opacity and polarized distribution of organelles (Pl. X: Fig. 42).

iii) Effects of treatment of neck-ligated larvae with RH 5992

Day 1 sixth instar larvae were neck-ligated and treated with a single dose of 5 μg RH 5992. Neck-ligated larvae kept as controls were treated with an equivalent quantity of acetone. Testes were reclaimed on day 4 and were subjected to histological and electron microscopic studies.

The ligated larvae treated with RH 5992, survived for about 4 - 5 days, while larvae kept as controls survived for 9 ± 1 days. However, both failed to pupate. RH 5992 treated larvae, exhibited the pre-metamorphic behaviour of gut purge after 2.5 ± 0.5 days. Seventy percent of the control larvae also exhibited gut purge behaviour after 4.5 ± 0.5 days (Table 3). An imperfectly formed new larval cuticle was also observed beneath the partially shed, ruptured old cuticle. The testes of these larvae as well as those of controls remained disjuncted.

Histological observations showed the presence of well differentiated spermatocysts. The testes contained mostly of spermatid cysts and eupyrene sperm bundles. Spermatogonial and spermatocytal cysts were also present, but their number was greatly reduced. On the other hand, the control ligated larvae appeared to be an unorganised mass and the germinal cells were necrotic.

Electron microscopic studies have demonstrated that there is an acceleration of spermatogenesis in the RH 5992 treated larvae. Eupyrene spermiogenesis had advanced to a large extent. Spermatids exhibited nuclear and acrosomal elongation. Mitochondrial aggregates were observed in the cytoplasm. Spermatogonial and spermatocytal cysts were not evident. However, numerous empty vacuoles and other cellular remnants were evident (Pl. X: Fig. 43; Pl. XI: Fig. 44). Apyrene sperm bundles were absent. In the testes of larvae kept as controls, spermiogenesis was completely inhibited.

PLATE X

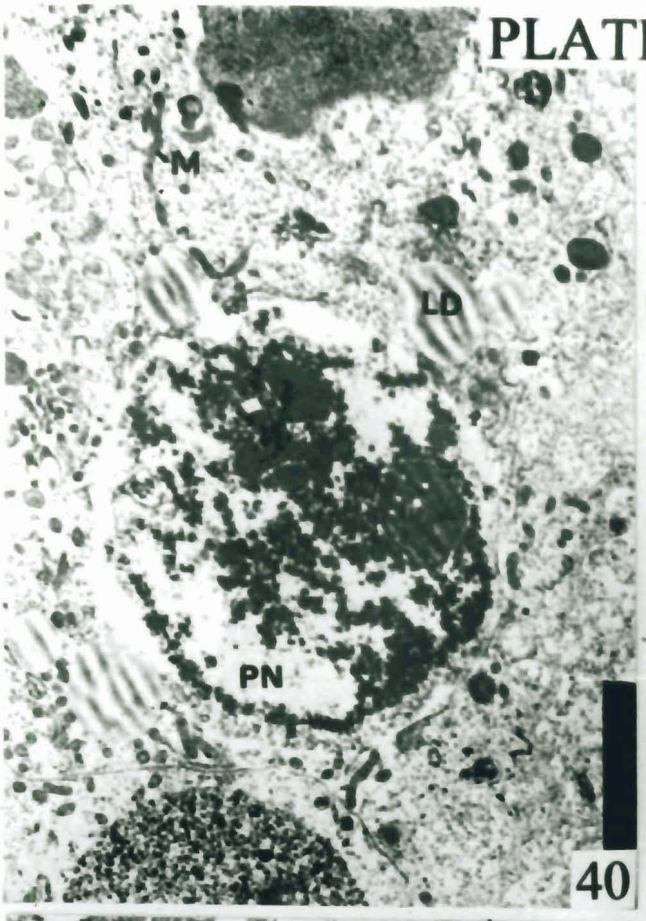
Fig. 40. Degenerating germ cells of thorax-ligated larva treated with JHA showing pycnotic nuclei (PN). Cytoplasm show dense lipid droplets (LD) and mitochondria (M). X 6000

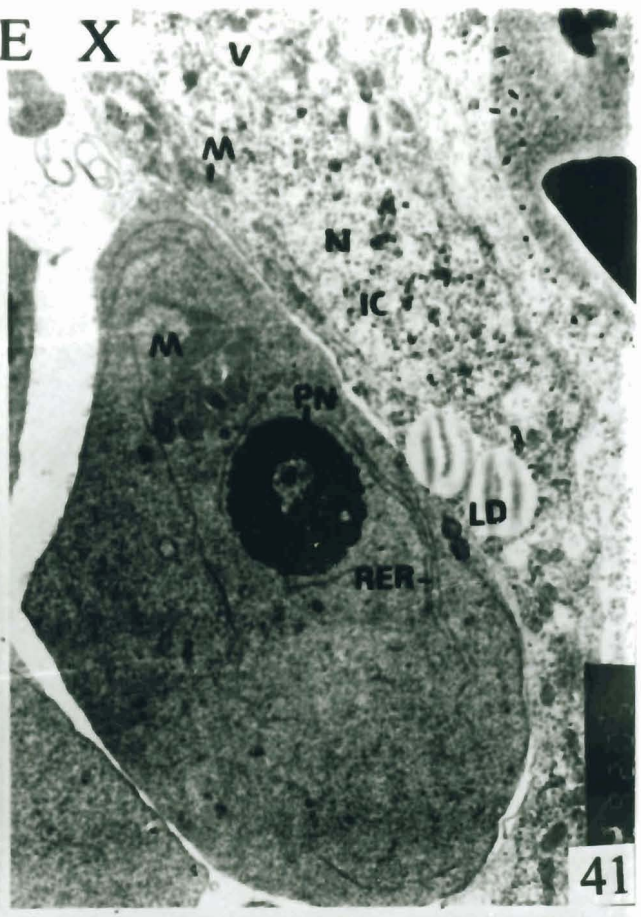
Fig. 41. Abnormal germ cell and interstitial cell (IC) of thorax-ligated larva treated with JHA. Germinal cell show pycnotic nucleus (PN) and cytoplasm with mitochondria (M) and short stretches of rough endoplasmic reticulum (RER). Interstitial cell (IC) shows elongated nucleus (N) and cytoplasm shows mitochondria (M), vacuoles (V) and few lipid droplets (LD). X 7500

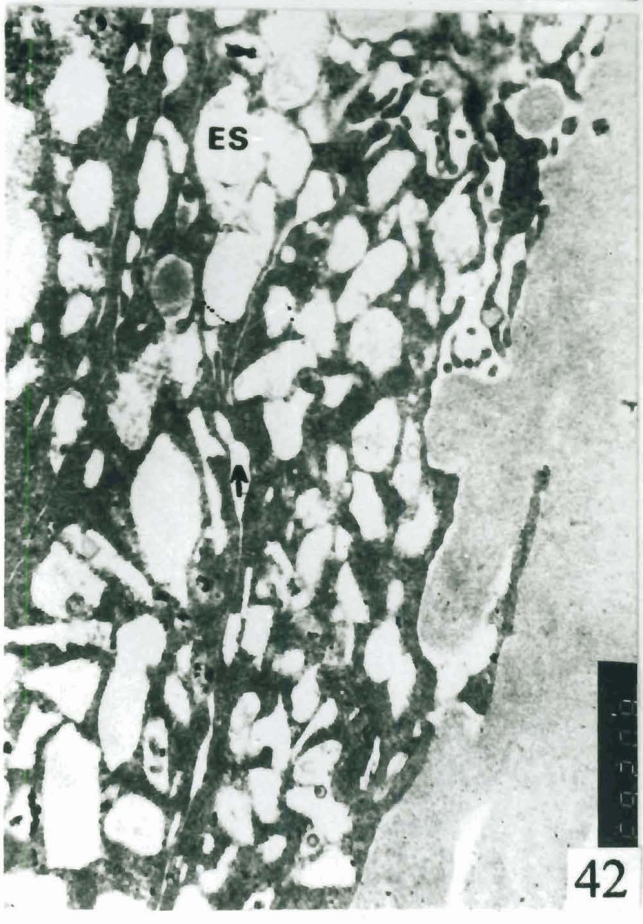
Fig. 42. Testicular sheath of thorax-ligated larva treated with JHA, showing empty regions (ES) without organelles. Arrow represents the intercellular spaces. X 12300

Fig. 43. Section through the testis of day 1 neck-ligated sixth instar larva treated with RH 5992 showing eupyrene spermatids with elongated nucleus (N) and acrosome (A). Mitochondrial cluster (M) is evident. X 4200

PLATE X







4.3.1.2. Effects of treatments of neck-ligated day 4 larvae with JHA or RH 5992 on morphogenesis and spermiogenesis

Studies conducted on the normal spermatogenesis in *S. maruritia* show that final stages of differentiation of eupyrene spermatozoa take place during day 4/ day 5 of sixth instar larval period. Further the differentiation of apyrene spermatozoa commences during this period (Venugopalan *et al.*, 1994). To analyse the involvement of hormonal factors and the effects of hormone analogues on spermiogenesis (eupyrene/apyrene), the undermentioned experiments were conducted. Day 4 (Wandering) sixth instar larvae were neck-ligated and then treated topically with either a single dose of 5 µg JHA or 5 µg RH 5992. Day 4 larvae kept as controls were neck-ligated and treated with the same volume of solvent. The effects of these treatments on morphogenesis and testicular development were studied. The testes were dissected two days after treatment in RH 5992 treated larvae along with their controls. From JHA treated and control larvae the testes were dissected on the fourth day after treatment. The testes from treated and control larvae were processed for histological and ultrastructural observations.

The day 4 neck-ligated larvae which were kept as controls showed a complete inhibition of pupation. These larvae survived for 4 ± 1 days and died without pupating (Table 4). The testes lobes remained unfused. Histological and ultrastructural studies showed that the testes appeared to be unorganized mass and most of the spermatocysts were not clearly distinguishable. Mostly spermatid cysts appeared and they seemed to be abnormal.

Table 4. Effects of treatments of JHA to day 4 ligated larvae on metamorphosis and testicular fusion

Nature of ligation	Dosage	*n*	% of surving larvae which showed		Nature of testes lobes
			No change	Pupation	
Neck-ligated	5 μg JHA	20	20	80	Fused
Control	5 μl acetone	10	100	--	Unfused

i) Effects of neck-ligation and treatment of JHA

Neck-ligated day 4 larvae treated with 5 µg JHA accelerated the moulting process. The treated larvae underwent pupal development. Eighty percent of the ligated larvae treated with JHA transformed into headless pupae. These pupae survived for about three days. These pupae had fused testes lobes (Table 4). The testes of day 2 headless pupae were then processed for histological and ultrastructural studies.

Histological studies of the testes of headless pupae revealed that most of the spermatogonial, spermatocyte and spermatid cysts were degenerating. However, eupyrene/apyrene sperm bundles were formed, but the number was reduced and some of the sperm bundles were abnormal.

The ultrastructural studies of testes of headless pupae revealed that most of the spermatocysts were necrotic and degenerating. Degeneration or cellular autolysis of the germ cells were the most predominant feature. The spermatogonial cells, spermatocyte cysts and spermatid cysts were not easily recognizable (Pl. XI: Figs. 45, 46). The size of the nuclei got reduced and necrosis of the nuclei was observed. The cytoplasm contained abundant mitochondria and short stretches of rough endoplasmic reticulum. Golgi apparatus and multivesicular bodies were absent. The nuclei disintegrated and eventually lost their shape and much of their chromatin substances. Nuclear membrane deteriorated (Pl. XI: Fig. 45). In advanced stages of degeneration the nuclei were irregularly shaped and had lost much of the internal contents. In the irregulary shaped nuclei condensed chromatin clumps appeared which suggest a pycnotic process. Even though the cell boundaries were maintained by membranes, an overall shrinkage of cells was quite often observed. Nuclei and nucleoli also seemed to have shrunken in size. Numerous areas of cytoplasmic lysis were observed. Mitochondrial population of

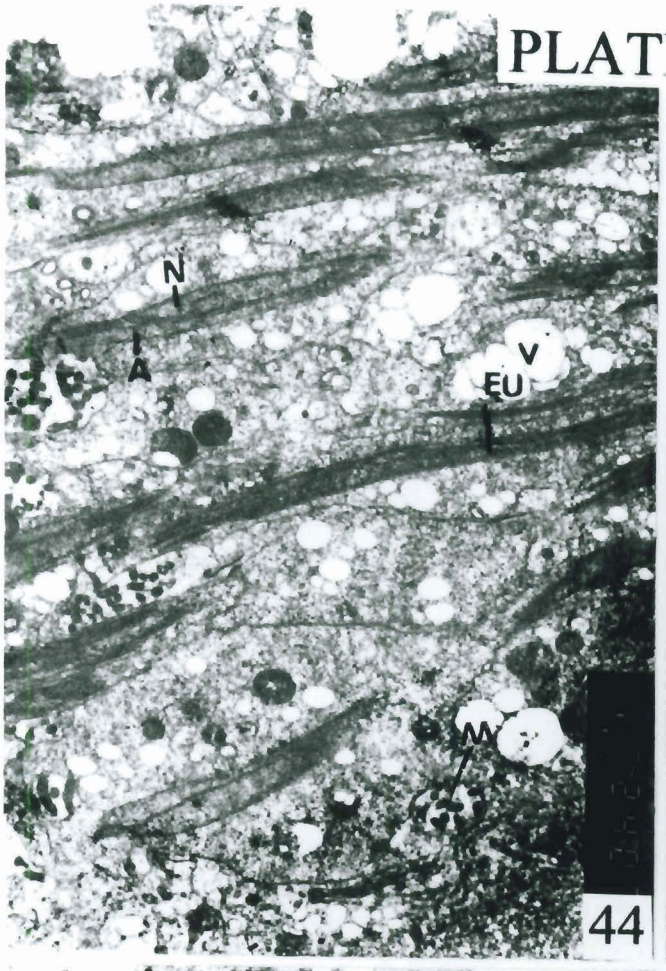
PLATE XI

Fig. 44. Higher magnification of eupyrene spermatids (EU) of day 1 neck-ligated larva treated with RH 5992. Elongated nucleus (N), acrosome (A), mitochondrial clusters (M) and vacuoles (V) are seen. X 4600

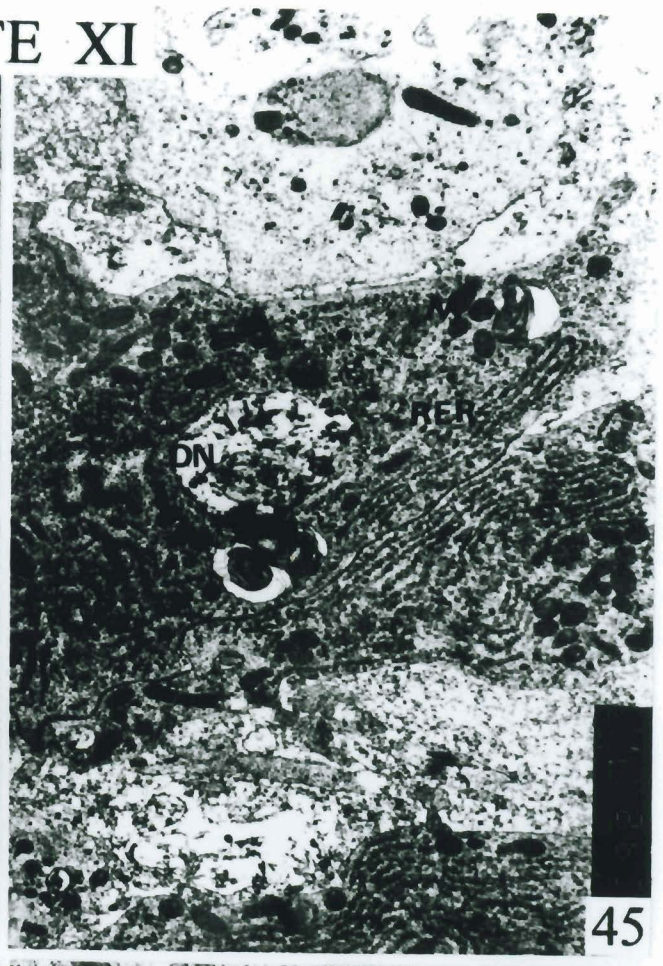
Figs. 45 and 46. Degenerating germ cells of headless pupa showing disintegrated nucleus (DN) and cytoplasm with rough endoplasmic reticulum (RER) and mitochondria (M). Fig. 45 X 8100; Fig. 46 X 12500.

Fig. 47. Cross section through the head region of eupyrene sperms (ES) of headless pupa. X 10400

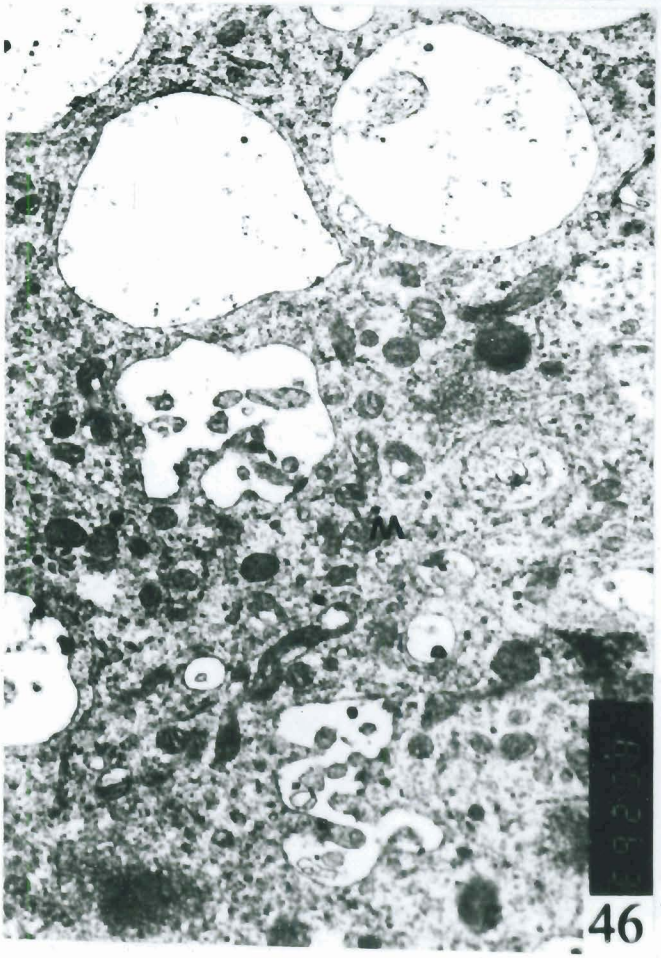
PLATE XI



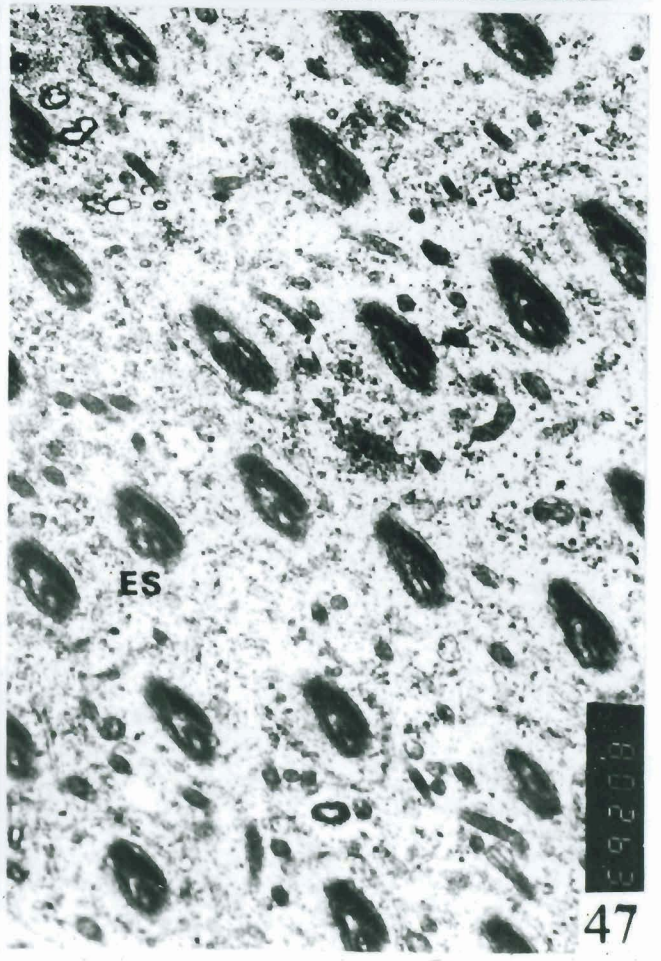
44



45



46



47

different sizes was also observed. In addition, there was a remarkable accumulation of cell fragments. Most of the cellular organelles were vacuolised (Pl. XI: Fig. 46). Degeneration occurred mostly in the early germ cells. Necrosis did not affect the eupyrene spermatozoa (Pl. XI: Fig. 47).

Ultrastructural studies on the testicular sheaths revealed that no clear distinction could be made between the internal and external tunics. The inner and outer basal lamina were not evident. The spherical nuclei contained clumps of heterpycnotic bodies of chromatin. The nuclear envelope was seen ruptured at many places. A few evenly dispersed glycogen particles were also evident. Vacuoles were predominant with numerous mitochondrial population of different sizes, most of them were elongated with less dense matrix. Their cristae were not evident. Long stretches of rough endoplasmic reticulum seen in the sheath cells of larvae kept as controls were lacking. Intercellular channels were also absent (Pl. XII: Fig. 48).

ii) Effects of neck-ligation and treatment of RH 5992

Neck-ligated day 4 larvae treated with a single dose of 5 μg RH 5992 moulted into larval-pupal intermediates. The intermediates had thoracic legs which were larval in appearance. However, they had highly sclerotised pupal cuticle in the thoracic tergum and abdominal region. The abdominal region was entirely pupal showing even the presence of pupal cremasters. These intermediates survived for three days (Table 5).

The testes lobes of larval-pupal intermediates were found to be fused. The volume of testes was slightly larger than that of the unfused testes of the control. Testes were subjected for histological and ultrastructural observations.

Table 5. Effects of treatment of RH 5992 to day 4 ligated larvae on metamorphosis and testicular fusion

Nature of ligation	Dosage	*n*	% of surviving larvae which showed			Nature of testes lobes
			No change	Pupae	Larval pupal intermediates	
Neck-ligated	5 µg RH	20	20	--	80	Fused
Control	5 µl acetone	10	100	--	--	Unfused

Table 6. Morphogenetic effects of treatment of day 0 pupae with JHA

No. of treated pupae	Dosage	% of Mortality	No. of pupae transformed into normal adults
30	5 µg JHA	56	--
10 (Control)	5 µl acetone	--	9

Histological studies showed the presence of few spermatogonial cysts and spermatocyte cysts. Spermatid cysts were present in large numbers. Although, the presence of eupyrene sperm bundles were seen, their number was drastically reduced.

Ultrastructural observations showed mostly the presence of spermatid cysts, which consisted mainly of nucleus, mitochondrial derivative with axoneme and multivesicular body. Fusion of numerous mitochondria into a single nebenkern was seen, as observed in the day 1 normal pupa. Some of the spermatid cysts possessed few empty vacuoles and the mitochondrial derivatives were abnormal. Golgi apparatus and rough endoplasmic reticulum were absent. Most of the germ cells were apparently degenerating and appeared to be abnormal (Pl. XII: Figs. 49, 50, 51).

The testicular sheath displayed an extraordinary increase of glycogen deposits throughout the sheath. No clear distinctions could be made between the internal and external tunics. Similar cells filled with glycogen particles were found situated throughout the sheath. Glycogen particles were numerous and formed unevenly distributed clusters. The sheath possessed nucleus with unevenly distributed chromatin. Few mitochondria were present. Tracheoles were also evident. The sheath varied considerably in thickness and displayed hypertrophic zones, 3-4 times thicker than other zones (Pl. XIII: Figs. 52, 53).

4.3.2. Effects of treatments of JHA/RH 5992 on pupae

i) Effect of JHA treatment on pupae

Newly ecdysed day 0 pupae were treated with a single dose of 5 µg JHA. Control pupae were treated with an equivalent quantity of solvent acetone. High mortality was observed in the JHA treated pupae (Table 6). The surviving pupae

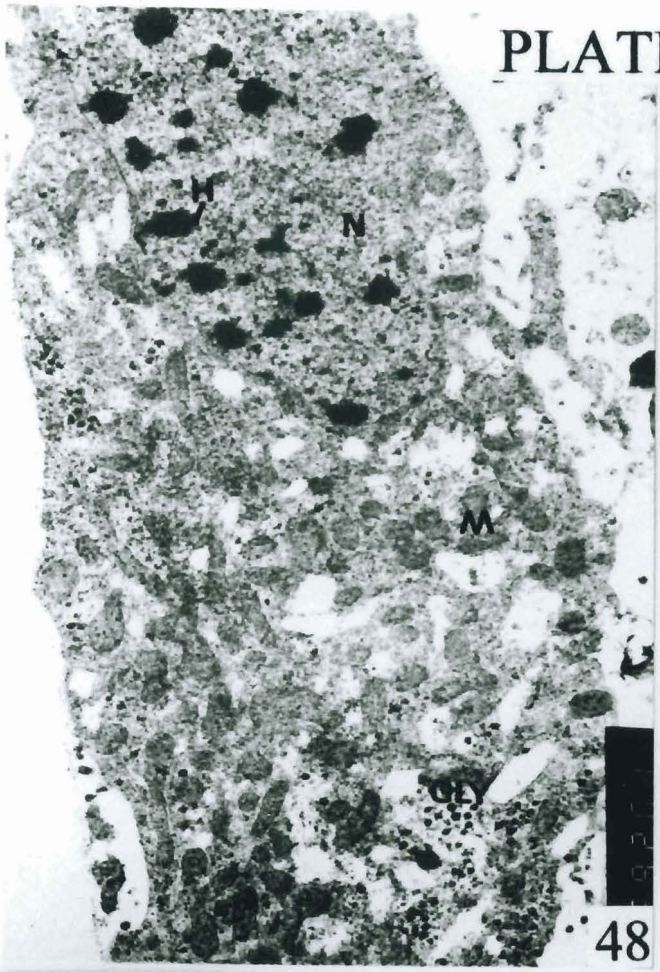
PLATE XII

Fig. 48. Testicular sheath of headless pupa showing lack of demarcation of internal and external tunics. Nucleus (N) contains clumps of heterpycnotic bodies (H) of chromatin. Glycogen particles (GLY) and mitochondria (M) are seen scattered in the cytoplasm. X 12900

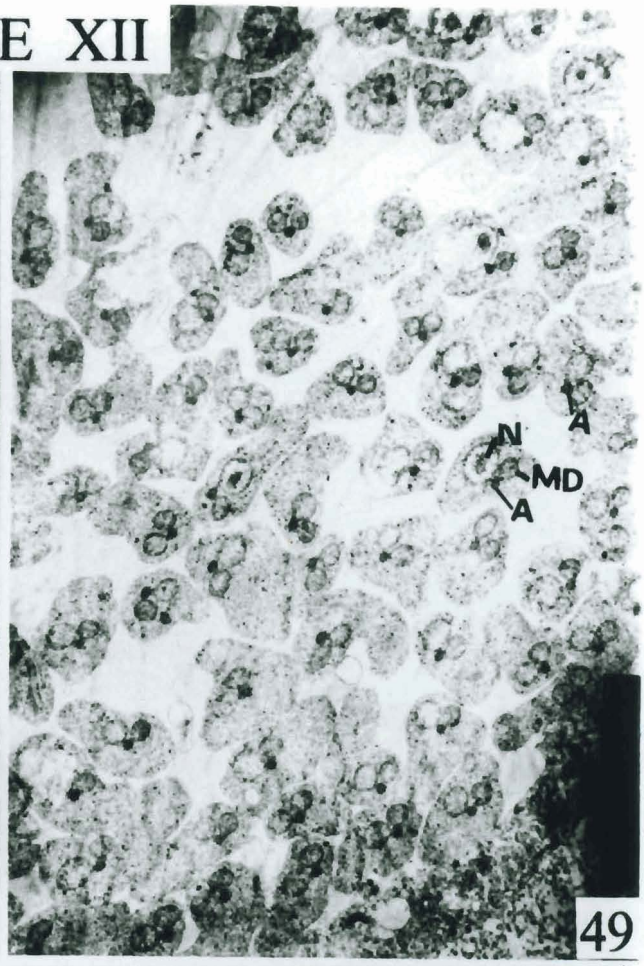
Fig. 49. Testis of larval-pupal intermediate obtained after RH 5992 treatment showing spermatid cyst with nucleus (N), mitochondrial derivative (MD) and axoneme (A). X 4300

Figs. 50 and 51. Testes of larval-pupal intermediate obtained after RH 5992 treatment showing spermatids with nucleus (N), multivesicular bodies (MV), mitochondrial derivative (MD), axoneme (A) and few vacuoles (V). Fig. 50 X 4300; Fig. 51 X 11600.

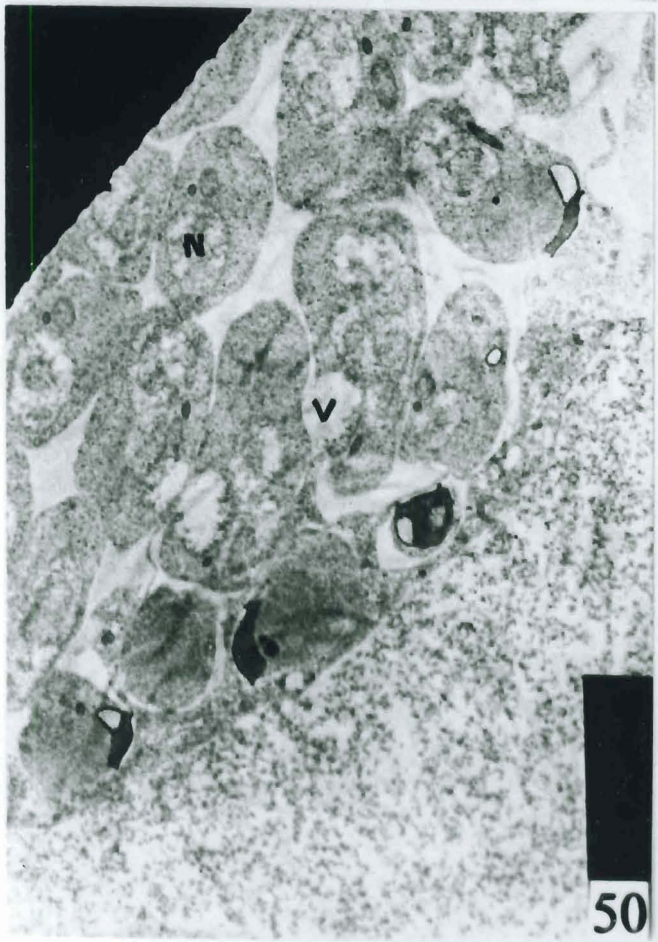
PLATE XII



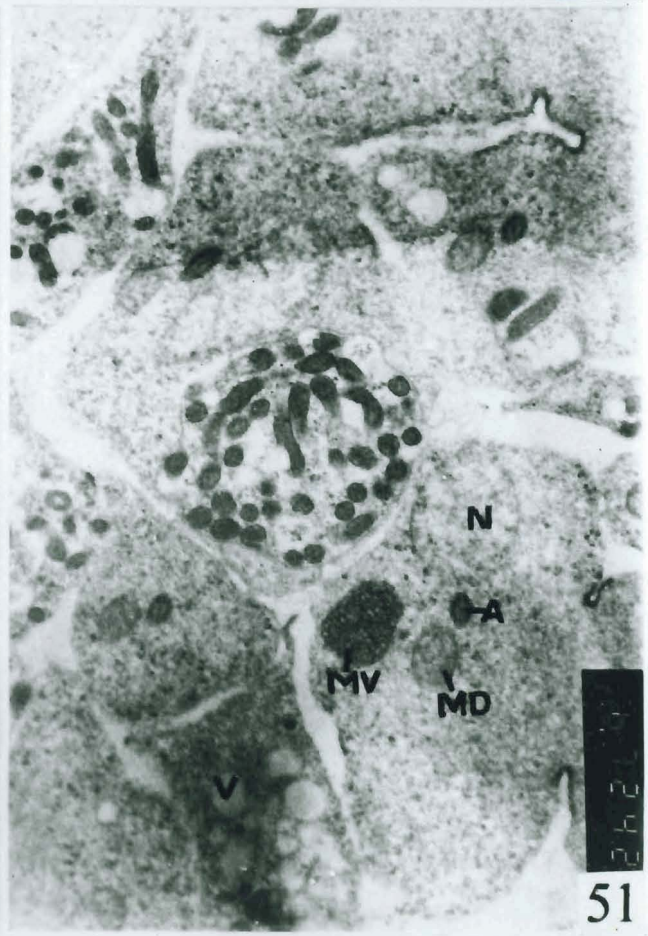
48



49



50



51

showed various abnormalities in pupal-adult development. Most of the treated pupae failed to emerge as adults. However, emergence of abnormal adults was also observed. The adults emerged had malformed forewings and antennae. In the case of unemerged pupae, when the pupal cases were removed, they contained adultoids. Head and thorax showed normal imaginal differentiation, and the wings were well developed, though unstretched.

Testes were dissected out from treated/control pupae on day 7 and subjected to histological and electron microscopic studies. The volume of the testis was less when compared to that of the controls.

The testes displayed reduced sperm bundles. Most of the cells were necrotic and degenerating. The degenerating spermatid cyst cells looked like round loosely arranged cells. Eupyrene and apyrene sperm differentiation were not recognizable. However, a few malformed sperm bundles were seen. Large spaces among the germ cells were also seen.

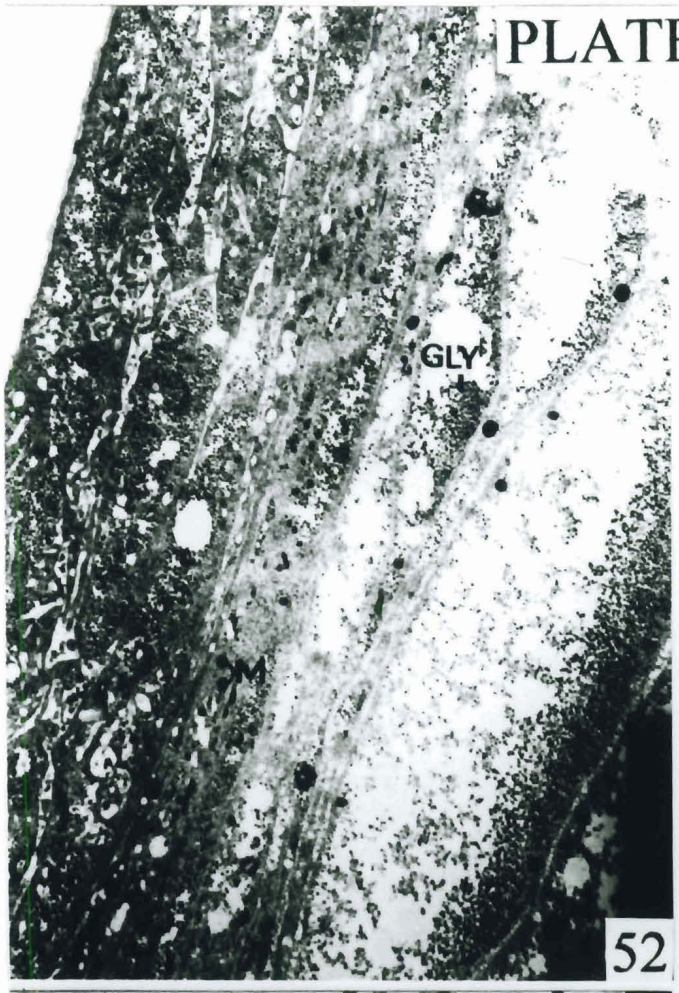
Electron microscopic studies revealed that spermiogenesis progresses, but the spermatozoa formed were abnormal or malformed. The spermatocysts were indistinguishable. Degeneration occurs mostly in the spermatogonial and spermatocytal cysts. In these cells disintegration of nuclei occurs and the nuclei lose their shape. As degeneration progresses the nuclei became irregular in shape with their chromatin substances and other cellular organelles seemed to under deterioration. Most of the cell membranes were either absent or ruptured (Pl. XIII: Figs. 54, 55). Some cells displayed pycnotic nuclei. In addition, numerous myeloid structures and shrunken cells were observed (Pl. XIII: Fig. 55). Accumulation of numerous cellular remnants was also observed. Mitochondria were relatively few in number and their tubular cristae were not evident. Compared to the normal day 7 pupal testis, the treated pupal testis displayed malformed eupyrene spermatozoa.

PLATE XIII

Figs. 52 and 53. Testicular sheath of larval-pupal intermediate obtained after RH 5992 treatment showing the presence of glycogen particles (GLY) and scattered mitochondria (M). Note the presence of a prominent nucleus (N) and tracheole (T). Fig. 52 X 4300; Fig. 53 X 4500.

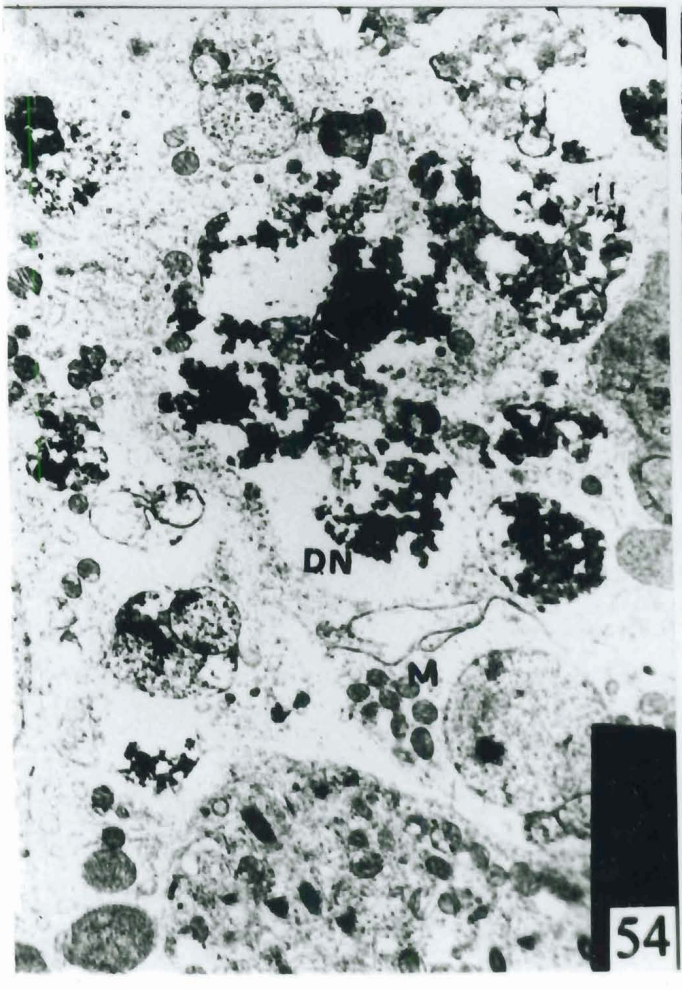
Figs. 54 and 55. Testes of day 7 pupa after JHA treatment, showing germ cells with disintegrating nucleus (DN), mitochondria (M) and myeloid structures (My). Fig. 54 X 7300; Fig. 55 X 6000.

PLATE XIII

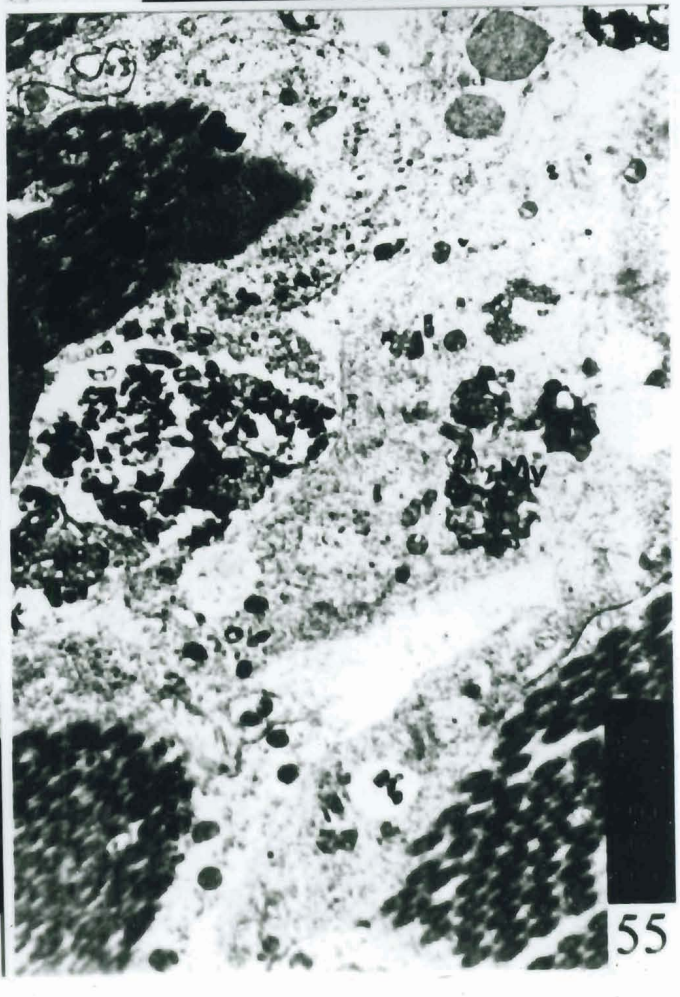


52

53



54



55

Table 7. Morphogenetic effects of treatment of day 0 pupae with RH 5992

Dosage (μg)	*n*	% of Mortality	% of Adult emergence	Days of survival
10 μg RH	24	41.6	16.6	9-10
Control 10 μl acetone	10	--	9	8

Transverse section of eupyrene spermatozoa showed lacinate appendages. Spermatozoa were connected by cytoplasmic bridges as in the case of normal germ cells. Mitochondria were numerous (Pl. XIV: Fig. 56).

The testicular sheath of the JHA treated pupal testis varied considerably in thickness. The testicular sheaths lose their characteristic structural features and no clear distinction could be seen between external and internal tunics. Throughout the sheath the cells appeared to be hyperactive. Some of the features of the sheath were the absence of glycogen droplets. The nuclei of sheath cells were irregular in shape and the contents were completely lacking. Numerous mitochondria of varying shapes and of less dense matrix were present. Numerous short stretches or scattered well developed rough endoplasmic reticulum were evident. Large deposits of spherical proteinaceous granules were also present. Intercellular channels were absent (Pl. XIV: Figs. 57, 58).

ii) Effects of RH 5992 treatment on pupae

Newly ecdysed day 0 pupae were treated with a single dose of 10 µg RH 5992. Control pupae were treated with an equivalent quantity of acetone (Table 7). High mortality was observed towards the end of pupal period. Most of the pupae died on or before the day of adult emergence. The treated pupae survived for 9 ± 1 days, but failed to emerge as adults. However, few pupae emerged as normal adults. In the case of unemerged pupae, when the pupal cases, were removed, they were found to contain adultoids. Adults with well developed, but unstretched wings and antennae were observed. On the other hand, moths which emerged from the control pupae were normal and healthy.

PLATE XIV

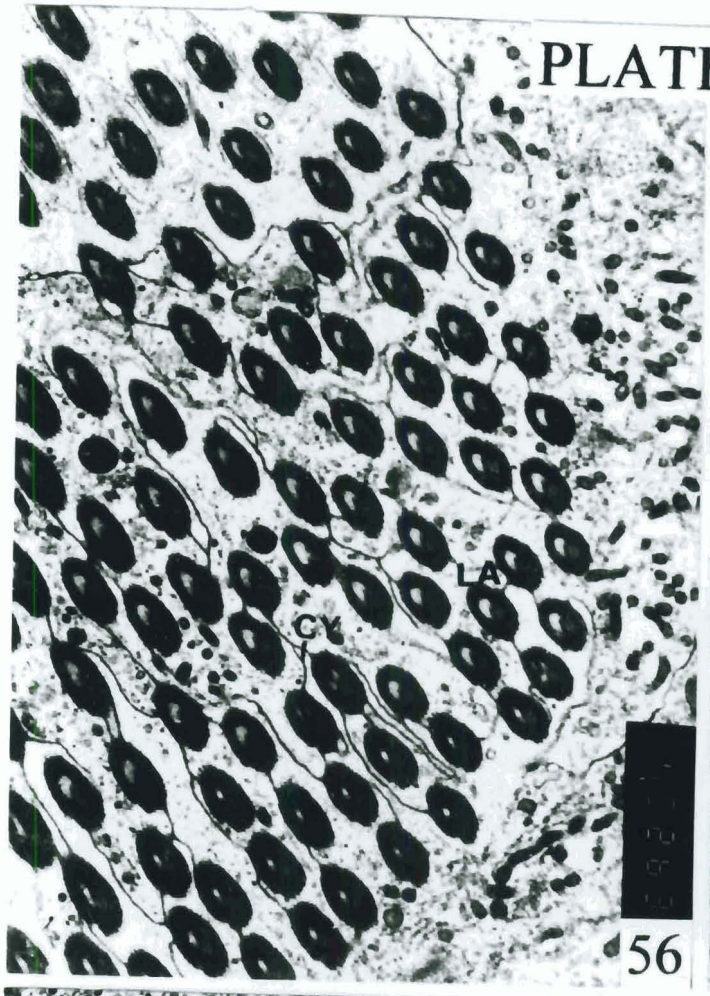
Fig. 56. Testis of day 7 pupa after JHA treatment showing malformed eupyrene spermatozoa with lacinate appendages (LA) interconnected by cytoplasmic bridges (Cy). X 7100

Figs. 57 and 58. Testicular sheath of day 7 pupa after JHA treatment showing irregular nucleus (N). Cytoplasm show mitochondria (M) and scattered rough endoplasmic reticulum (RER). Fig. 57. X 3900; Fig. 58 X 10450

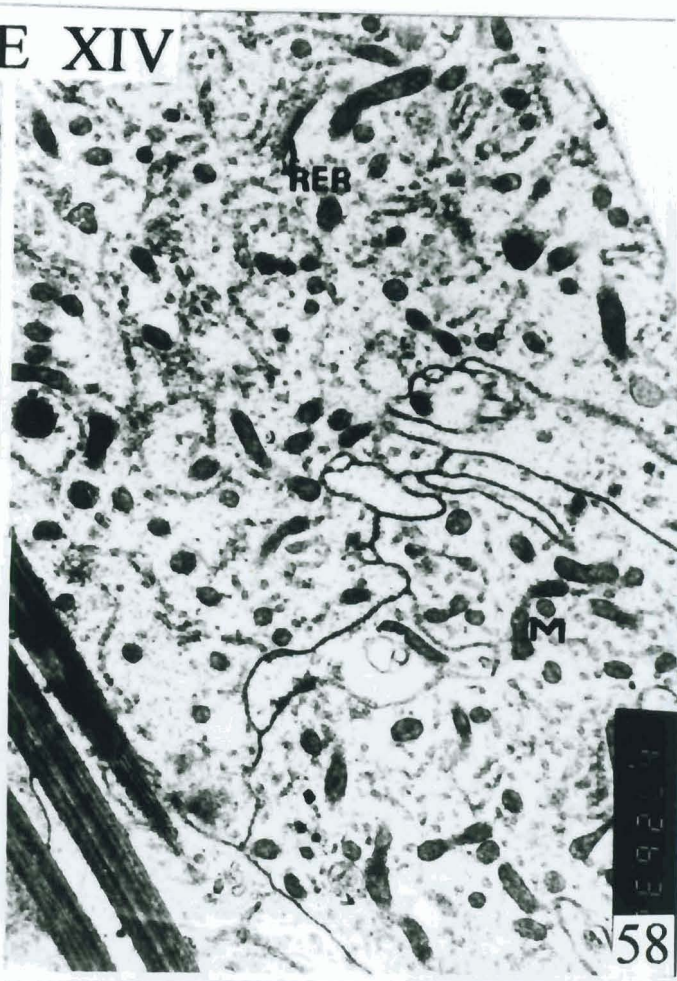
Fig. 59. Light micrograph of testis of day 7 pupa showing elongating spermatid cysts (EC) and eupyrene (EP), apyrene (AP) sperm bundles. X 750

Fig. 60. Light micrograph of testis of day 7 pupa after RH 5992 treatment showing elongating spermatid cysts (EC) and apyrene sperm bundles (AP). X 800

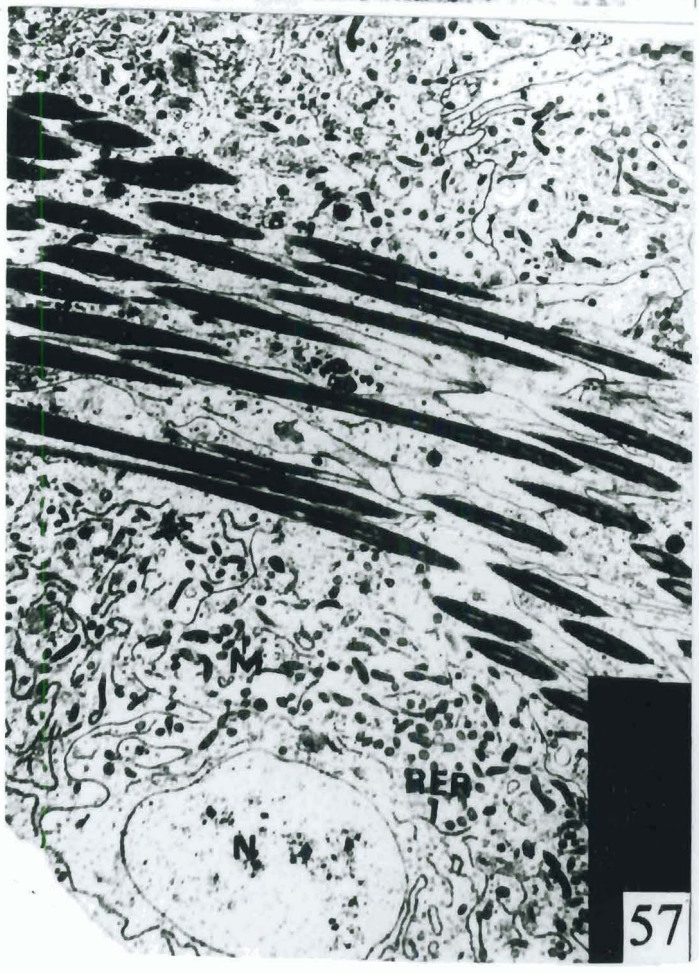
PLATE XIV



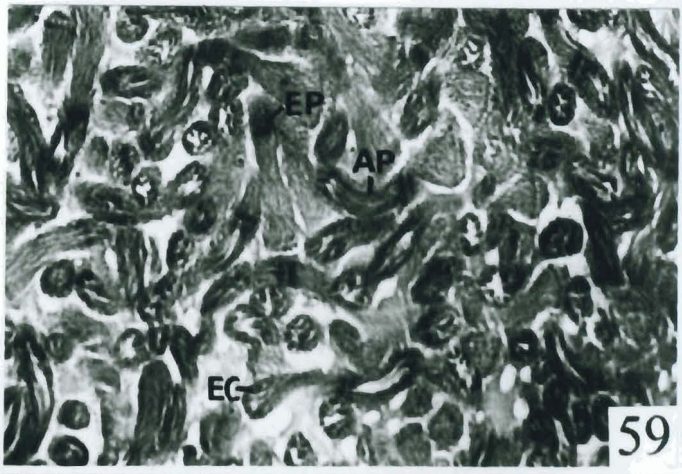
56



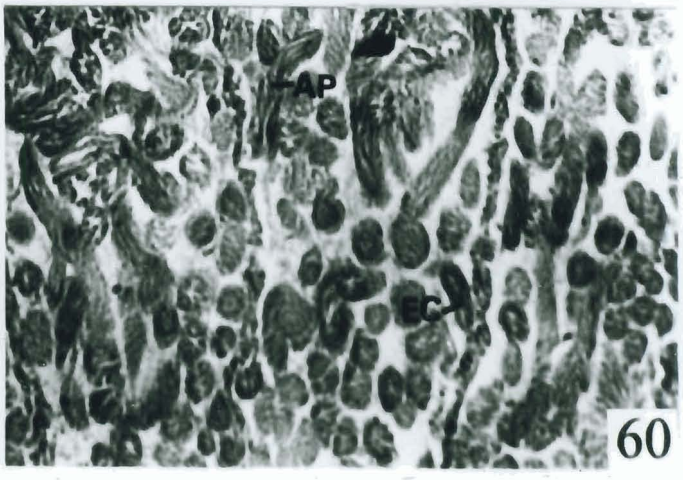
58



57



59



60

Testes were dissected out from pupae on day 7 and were subjected for histological studies. The volume of the testes of treated pupae was almost comparable to that of the controls.

Histological studies of the control pupae revealed the presence of numerous sperm bundles. Eupyrene and apyrene sperm bundles could be recognized (Pl. XIV: Fig. 59). The treated pupae also showed an abundance of sperm bundles in the testicular follicles but were less when compared to control. Elongating spermatid cysts were present in large numbers and seemed to be normal (Pl. XIV: Fig. 60).

4.4. Discussion

Developmental profile of morphogenetic hormones during larval-pupal-adult transformation of Lepidoptera: an overview

In lepidopteran insects the developmental changes that take place during metamorphosis occur in a sequential order as has been demonstrated in *Bombyx mori* (Kiguchi *et al.*, 1985). These sequential events of larval-pupal and pupal-adult metamorphosis have been demonstrated to be under the control of ecdysteroids, juvenile hormone (JH) and brain hormones (Gilbert *et al.*, 1996). During larval-pupal metamorphosis of all Lepidoptera examined there are two peaks of haemolymph ecdysteroid titre, one at the transition from feeding stage to post-feeding wandering larva and the other in association with pupal cuticle formation (Bollenbacher *et al.*, 1975; Fujishita *et al.*, 1982). In the last larval instar the high level of JH early in the instar declines to undetectable levels midway through the stadium. This drop in JH titre is permissive to the release of first pulse of brain hormone, the prothoracicotropic hormone (PTTH) which activates the prothoracic glands causing an initial small peak in the concentration of ecdysteroids. Ecdysteroids thus released (the so called commitment peak of ecdysteroids) have

been implicated in the genetic switchover in the epidermal commitment from larval to pupal type (Truman *et al.*, 1974; Riddiford, 1976) and in the induction of gut purge, wandering behaviour and other prodromal symptoms of pupation (Truman and Riddiford, 1974; Gilbert *et al.*, 1981; Nagata *et al.*, 1987). In the last larval instar of Lepidoptera a brief increase in the haemolymph titre of JH in the prepupal phase has been observed. The major role attributed to this prepupal JH peak along with the second pulse of PTTH is to activate the prothoracic glands to release sufficient ecdysteroids needed for larval-pupal transformation (Cymborowski and Stolarz, 1979; Gruetzmacher *et al.*, 1984; Ohtaki *et al.*, 1986). Several studies have suggested that pupal-adult transformation in Lepidoptera occurs in the absence of JH. The ecdysteroids on the other hand increase to a major peak in the haemolymph during pharate adult stage. This increase in ecdysteroids promote pupal-adult metamorphosis. Our studies have demonstrated that the developmental profile of hormones in *S. mauritia* is consistent with the currently acceptable model applicable to other Lepidoptera (Santha and Nair, 1987; Balamani and Nair, 1989, 1991, 1992; Mona, 2001).

Larval-pupal metmorphosis of *S. mauritia*: Effects of ligation and treatments of JHA and RH 5992

The present study confirms and supplements many of the earlier findings on the role of brain factors and prothoracic gland secretions on the endocrine regulation of larval-pupal metamorphosis of *S. mauritia*. Seventy percent of neck/thorax ligated day 1 larvae exhibited the premetamorphic behaviour of gut purge. In the last instar larvae of lepidopterans it is the so-called commitment peak of ecdysteroids which induces the gut purge (Truman and Riddiford, 1974; Fujishita *et al.*, 1982). The promotion of gut purge in the ligated larvae of *S. mauritia* shows that the first pulse of PTTH has already been released in these

larvae which stimulates the prothoracic glands to secrete the commitment peak of ecdysteroids. The ligated larvae however do not pupate which suggest that the low titres of ecdysteroids which promote gut purge may not be sufficient to induce pupation. Further the second major surge of ecdysteroids is not occurring in these larvae. Neck/thorax ligation of day 4 larvae prevented pupation while the control (unligated) larvae pupated on day 6. As explained earlier, the major surge of ecdysteroids occurs in the prepupal phase of last instar larvae of lepidopterans, the role of which is to promote pupal cuticle secretion and pupation (Truman and Riddiford, 1974). Last instar larvae of *S. mauritia* ligated on day 1 and day 4 failed to show pupal cuticle secretion because the second major surge of ecdysteroids is not occurring in these larvae.

Neck/thorax-ligated day 1 larvae treated with JHA did not exhibit the premetamorphic behaviour of gut purge. This may be due to inhibitory effects of JH on ecdysteroid biosynthesis. Even though PTTH is considered as the principal hormone involved in the activation of prothoracic glands, JH also appears to function as an interendocrine regulator of PTTH-prothoracic gland axis. The regulatory effect of JH on this axis causes a depression in the secretory activity of prothoracic glands early in the instar (Safranek *et al.*, 1980; Sakurai, 1983, 1990; Watson and Bollenbacher, 1988; Sakurai *et al.*, 1989; Balamani and Nair, 1992).

Treatments of ecdysone agonist RH 5992 to neck-ligated feeding (day 1) larvae induced gut purge and precocious lethal moulting. These results support some of the reports of Smagghe and Degheele (1992) which state that the *in vivo* mode of action of ecdysteroid agonists (RH 5849 and RH 5992) is similar to that of endogenous ecdysteroids. Possibly both bind to the ecdysteroid receptors and stimulate epidermal cells to undergo apolysis and synthesize new cuticle. They also demonstrated that malformation in the new larval cuticle was due to the

disappearance of several cuticular and haemolymph proteins in the treated larvae. In the present experiment synthesis of larval cuticle rather than pupal cuticle indicate that endogenous titre of JH is high at the time of treatment.

Treatments of ecdysone agonist RH 5992 or JHA to neck-ligated post-feeding (day 4) larvae induced pupal cuticle secretion. Treatments of ecdysone agonist to day 4 ligated larvae result in the production of larval-pupal intermediates. Larval-pupal intermediates with scleroticed pupal cuticle in the abdominal region and thoracic tergum were produced. These larval-pupal intermediates had fused testes lobes. However larval thoracic legs were retained. Formation of larval-pupal intermediates after treatments of last instar larvae with ecdysone agonists has been reported in other lepidopterans also (Smagghe and Degheele, 1994).

On the contrary, treatments of JHA to neck-ligated day 4 larvae induced to a large extent normal pupation. These results are consistent with our earlier observations in *S. mauritia* (Balamani and Nair, 1989, 1991) and in other lepidopterans (Cymborowski and Stolarz, 1979; Hiruma, 1980; Gruetzmacher *et al.*, 1984) which demonstrate that the presence of JH in the prepupal stage is critical not only to activate the prothoracic glands to their maximal rate of secretion but also to promote normal pupal morphology (Kiguchi and Riddiford, 1978; Hiruma, 1980; Gruetzmacher *et al.*, 1984). Further it has been suggested that prepupal JH increase promotes the formation of normal pupal features by preventing precocious adult metamorphosis (Hiruma, 1980). Thorax ligated larvae treated with JHA failed to show pupal cuticle secretion obviously due to the lack of prothoracic glands.

Pupal-adult metamorphosis of *S. mauritia*: Effects of treatments of JHA and RH 5992

Treatments of JHA or RH 5992 to newly ecdysed pupae of *S. mauritia* caused high mortality. The surviving pupae failed to emerge as normal adults. When such unemerged pupae were dissected, they were found to contain adultoids which possessed pupal cuticle in the abdominal region. In lepidopterans, during pupal-adult metamorphosis, the prothoracic glands undergo programmed cell death or apoptosis. Treatments of JHA to newly ecdysed pupae of *Manduca sexta* prevent apoptosis (Dai and Gilbert, 1997) and the endogenous ecdysteroid titre is maintained at a high level (Dai and Gilbert, 1998). Our earlier studies have demonstrated that treatments of newly ecdysed pupae of *S. mauritia* with JHA considerably increased endogenous ecdysteroid titres (Mona, 2001). The failure of emergence of adults from JHA or RH 5992 treated pupae of *S. mauritia* might be due to the maintenance of high titre of endogenous ecdysteroid titres since it is well accepted that eclosion hormone could be released only after a drop in ecdysteroid level (Truman, 1971; Riddiford, 1985). The development of pupal-adult mosaics might be due to the differential sensitivity of different tissues to ecdysteroids.

Effects of ligations on spermatogenesis

The results of the present study show that neck/thorax ligation of day 1 / day 4 larvae result in a complete inhibition of spermatogenesis. The testes of both neck/thorax-ligated larvae contained an undifferentiated mass of tissue. Most of the early germ cells were not distinguishable. Degenerating germinal cells with pycnotic nuclei, numerous autophagic vacuoles and abnormal membranous whorls were observed. Reduced endoplasmic reticulum and a few mitochondria were also seen. Eupyrene and apyrene sperm bundles were completely absent. Obviously the suppression of spermatogenesis in ligated larvae is due to the lack of cerebral

factors and/or prothoracic gland secretions. The stimulatory effect of ecdysteroids on spermatogenesis has been well documented in several insect species (Schmidt and Williams, 1953; Yagi *et al.*, 1969; Dumser and Davey, 1975). Neurohormonal factors produced by the brain and ganglia also regulate testicular development and spermatogenesis. A brain peptide testis ecdysiotropin induce testis sheaths to produce ecdysteroid which in turn stimulate spermatogenesis (Loeb *et al.*, 1987).

Effects of juvenile hormone analogue on spermatogenesis

The present histological and ultrastructural observations reveal that in day 1 neck-ligated larvae treated with juvenile hormone analogue (JHA), spermatogenesis has progressed to a limited extent. On the other hand in day 1 thorax-ligated larvae treated with JHA, there is an inhibition of spermatogenesis and the partially differentiated germ cells undergo degeneration and lysis. It may be recalled that neck-ligated larvae possess prothoracic glands and hence some amount of spontaneous secretion of ecdysteroids will be taking place. It is well accepted that cellular response to ecdysteroids is enhanced in the presence of JH (Denlinger, 1979). Hence somewhat normal development is observed in a few germ cells of neck-ligated larve treated with JHA. However majority of germ cells in these larvae as well as those of thorax-ligated larvae treated with JHA show degeneration. Present ultrastructural studies have shown that treatments of JHA to pupae caused degeneration and necrosis of early germinal cells and formation of malformed sperm bundles.

Degenerative, irregular and inhibitory effects on spermatogenesis are due to the high titre of JH circulating in the haemolymph. These findings are consistent with earlier observations in *Bombyx mori* and *Lasperesia pomonella* (Yagi and Fukushima, 1975; Friedlander and Benz, 1982) which showed that JH when present in high titre directly inhibits spermatogenesis and causes sperm lysis.

Further when JH was excluded from *in vitro* culture, the spermatids are found to differentiate normally in the codling moth *Cydia pomonella* (Friedlander and Benz, 1982). Exogenous application of JHA inhibit spermatogenesis in *Spodoptera littoralis* (Yagi and Kuramochi, 1976), *Corcyra cephalonica* (Deb and Chakravorty, 1981), *Bombyx mori* (Yagi and Fukushima, 1975) and *Leptocoris coimbatorensis* (Kaur *et al.*, 1987). In *Ectomyelois ceratoniae* due to high titre of JH the elongation of nuclei of spermatids during the formation of spermatozoa are found to be inhibited (Leviatan and Friedlander, 1979). In *S. mauritia* the inhibitory effects of JHA were more conspicuous when this hormone analogue was applied to late last instar larvae or pupae when the endogenous titre of JH is low. An excess of JH or its analogues might block the cell division and differentiation of gonial cells. It seems that JHA may block the differentiation of germ cells in a similar way as its well known inhibitory effects on morphogenesis.

One cannot rule out the possibility that the inhibitory effects of JHA on spermatogenesis as observed in the present study are due to the high titres of ecdysteroids circulating in the haemolymph. As seen in the present study the inhibitory effects of JHA on the spermatogenesis is most conspicuous when treatments are made in the late phases of sixth instar larva and on newly ecdysed pupa. As mentioned earlier juvenile hormone has a prothoracicotrophic effect during these developmental phases of *S. mauritia* (Balamani and Nair, 1992; Mona, 2001) as well as in other lepidopterans (Sakurai *et al.*, 1989; Dai and Gilbert, 1998). The high titres of ecdysteroids circulating in the haemolymph will result in hyperecdysonism. This might very well derange the sequential steps of spermatogenesis. It may also be pointed out that JHA treatments mostly affect the early phases of spermatogenesis. Eupyrene/apyrene sperm bundles are not affected.

Effects of ecdysone agonist on spermatogenesis

As detailed earlier, neck/thorax ligation of day 1 larvae of *S. mauritia* completely blocked spermatogenesis. On the other hand, treatment of neck/thorax ligated day 1 larvae with ecdysone agonist RH 5992 induces a renewal of spermatogenesis. Histological and ultrastructural studies on the testes of treated larvae revealed the presence of well differentiated spermatogonial, spermatocytal and spermatid cysts. These studies concur with the earlier findings of several workers that endogenous ecdysteroids or ecdysteroid mimics treated exogenously accelerate spermatogenesis. Ecdysteroids stimulate mitotic and/or meiotic divisions during early stages of spermatogenesis (Kambysellis and Williams, 1972; Takeda, 1972; Dumser and Davey, 1975; Friedlander and Reynolds, 1988) interact with haemolymph macromolecular factor to induce spermatocyte differentiation (Kambysellis and Williams, 1972) and influence the development of apyrene sperm (Hoffmann and Behrens, 1982; Loeb *et al.*, 1982; Gelman *et al.*, 1989). Evidently the mode of action of tebufenozide (RH 5992) is similar to that of endogenous ecdysteroids in stimulating spermatogenesis. RH 5992 is thought to act through the ecdysteroid receptor and persist in tissues much longer than 20-hydroxyecdysone as this compound is not easily metabolized *in vitro* (Talbot *et al.*, 1993).

The present observations show that neck-ligated day 4 larvae treated with RH 5992 moulted into larval-pupal intermediates. Histological and ultrastructural studies of testes revealed that germ cells are apparently degenerating. The neck-ligated larvae possess prothoracic glands and hence secretion of ecdysteroids is taking place endogenously. Treatment of these larvae with RH 5992 may result in hyperecdysonism. Many of the coordinated processes in cell differentiation depend on a critical ecdysteroid titre. The large amounts of ecdysteroid agonists circulating in the haemolymph will disrupt the differentiation of spermatids. In experiments

using *Drosophila* cell lines Wyss (1976) reported that low levels of ecdysone stimulated cell divisions and cell differentiation whereas high levels had an inhibitory effect.

Ultrastructural changes in testicular sheath following RH 5992 and JHA treatments

As mentioned earlier the testicular sheaths occupy a strategic location controlling ionic and molecular exchanges between the germ cells and the haemolymph. Further, testicular sheaths of lepidopterans are the source of ecdysteroids which have a critical stimulatory role in spermatogenesis (Loeb *et al.*, 1984; Gelman *et al.*, 1989; Jarvis and Rees, 1990). Several studies have also demonstrated that testicular sheath itself is the source of macromolecular growth factor like proteins which are released into the intratesticular fluid and these proteins might have a regulatory role in spermatogenesis (Shimizu and Yagi, 1982).

Ultrastructural studies on the testes sheaths of RH 5992 treated larvae clearly showed an extraordinary increase of particulate glycogen in the cells which appear to be metabolically active. Diapausing testes of *Cydia pomonella* cultured in the presence of 20-hydroxyecdysone promotes *in vitro* spermatogenesis renewal by sustaining functional integrity of the sheath cells and by enhancing their glycogen metabolism (Friedlander, 1989). In *S. mauritia* the promotion/acceleration of spermatogenesis in RH 5992 treated larvae is possibly mediated through a similar mechanism. The ecdysone agonist RH 5992 or endogenous ecdysteroids act in a similar fashion in facilitating the release of sheath proteins which have a stimulatory role in spermatogenesis.

Ultrastructural studies on the testicular sheaths of JHA treated pupae revealed hypertrophy of the cells, a large increase in endoplasmic reticulum and an

abundance of proteinaceous bodies. As mentioned earlier treatments of newly ecdysed pupae with JHA considerably increase the endogenous ecdysteroid titre (Mona, 2001). Increased ecdysteroid titre might be responsible for the large increase in protein synthesis in the testicular sheaths.

4.5. Summary

1. To elucidate the role of hormones and effects of hormone analogues/ agonists on spermatogenesis in *S. mauritia*, the classical approach of neck/ thorax-ligation to eliminate the endogenous hormone sources and the hormone replacement therapy was employed for the investigation.

2. In one group of sixth instar larvae, ligations were carried out between the head and prothorax, thus effectively separating the cephalic endocrine structures (brain, corpora cardiaca, corpora allata) from the rest of the body. Such sixth instar larvae which lack brain, corpora cardiaca-corpora allata complex, but possess prothoracic glands were designated as neck-ligated. In another set of experiments, ligations were also carried out between pro- and mesothorax. These larvae which lacked not only the brain, corpora cardiaca, corpora allata, but also the prothoracic glands were designated as thorax-ligated. Ligations were carried out on day 1 and day 4 larvae as eupyrene/ apyrene sperm differentiation take place during these days of larval-pupal transformation.

3. Neck/thorax-ligated day 1 larvae of *S. mauritia* showed the premetamorphic behaviour of gut purge and survived for 7 $\pm$ 1 days. Histological and ultrastructural studies were conducted on the testes of the ligated larvae on day 5. Histological studies show that differentiation of various types of spermatocysts was completely blocked and the testes contained only an undifferentiated mass of tissue.

4. Ultrastructural investigations revealed an undifferentiated mass of tissue with indistinguishable spermatogonial and spermatocyte cysts. Most of the cells were necrotic or degenerating with numerous autophagic vacuoles, pycnotic nuclei, multivesicular sac remnants, membraneous whorls, endoplasmic reticulum without ribosomes and mitochondria with less dense matrix. The demarcation of individual cysts was found to be totally absent. The dimorphic sperms, eupyrene and apyrene, present in normal spermiogenesis were absent in the ligated larvae. Possibly the inhibition of

spermatogenesis is due to the lack of cerebral factors and/or prothoracic gland secretions.

5. In the next set of experiments neck/thorax ligated sixth instar day 1 larvae were treated repeatedly with 5 μg juvenile hormone analogue (JHA) for 3 days. These larvae did not show the premetamorphic behaviour of gut purge. The testes lobes remained unfused and the volume of the testes decreased considerably. Histological preparations of testes showed the presence of large empty spaces in the testicular tissue and the germinal cells were necrotic and degenerating.

6. Ultrastructural studies of the testes of neck-ligated day 1 sixth instar larvae treated with JHA for showed a few germinal cells to be necrotic and degenerating, while majority of the cells had a normal appearance. The degenerating germinal cells had highly condensed nuclei without a prominent nuclear envelope and the cytoplasm contained elongated mitochondria without cristae and short stretches of endoplasmic reticulum with dispersed ribosomes. The normal development of germ cells might be due to the presence of prothoracic glands in neck-ligated larvae which are capable of spontaneous secretion of ecdysteroids. Further it is well known that cellular response to ecdysteroids is enhanced in the presence of JH. These studies suggest that ecdysteroids promote spermatogenesis.

7. Ultrastructural investigations were carried out on the testes of sixth instar day 1 thorax-ligated larvae treated with 5 μg JHA for 3 days. The ultrastructural features, revealed that most of the spermatocysts were indistinguishable and the germ cells possessed pycnotic nuclei, autophagic vacuoles, few elongated mitochondria and short stretches of rough endoplasmic reticulum. Degenerating interstitial cells with elongated nuclei and ruptured nuclear envelope were also observed. The testicular sheath revealed the presence of intercellular spaces. Elongated nucleus, mitochondrial aggregates, rough endoplasmic reticulum, glycogen particles, and lipid droplets were altogether absent. Internal and external tunics lacked electron-opacity and polarized distribution of organelles.

8. In another series of experiments sixth instar day 1 larvae were neck-ligated and treated with a single dose of 5 μg of ecdysone agonist RH 5992. These larvae showed the premetamorphic behaviour of gut purge, but failed to pupate. Histological and ultrastructural studies on the testes of treated larvae revealed the presence of well differentiated spermatogonial, spermatocytal and spermatid cysts. Spermatid cysts showed nuclear and acrosomal elongation. Eupyrene sperm bundles were present, but apyrene sperm

bundles were absent. These observations suggest that endogenous ecdysteroids or ecdysteroid mimics treated exogenously accelerate spermatogenesis.

9. To further analyse the involvement of hormonal factors and effects of hormone analogues on eupyrene/apyrene spermiogenesis, day 4 sixth instar larvae were ligated and treated with JHA or ecdysone agonist RH 5992. The effects of these treatments on metamorphosis and spermatogenesis were studied.

10. Neck-ligated day 4 sixth instar larvae neck-ligated treated with 5 µg JHA, transformed into headless pupae. Histological and ultrastructural studies conducted on the testes of headless pupae revealed that most of the early germinal cells to be necrotic and degenerating. Necrosis did not affect the eupyrene spermatozoa. In the testicular sheaths no clear distinction could be made between the internal and external tunics. Nuclei contained clumped chromatin along with the ruptured nuclear envelope. Vacuoles were predominant with numerous mitochondrial population and few evenly dispersed glycogen particles. Intercellular channels were absent.

11. Neck-ligated day 4 larvae treated with a single dose of 5 µg RH 5992 moulted into larval-pupal intermediates. The intermediates had larval thoracic legs and highly sclerotized pupal cuticle in the thoracic tergum and abdominal region. Testes lobes were found to be fused. Histological preparations showed the presence of few spermatogonial and spermatocyte cysts. Spermatid cysts were present in large numbers.

12. Ultrastructural studies on the testes of day 4 neck-ligated larvae treated with RH 5992 revealed the presence of spermatid cyst, consisting of nucleus, mitochondrial derivatives with axoneme and multivesicular body. Some of the spermatid cysts possessed few empty vacuoles and abnormal mitochondrial derivatives. These features suggest that treatment of RH 5992 might have resulted in hyperecdysonism. The cells of testicular sheath possessed nuclei with unevenly distributed chromatin. Extraordinary increase in glycogen deposit was seen. The sheath varied in thickness and displayed hypertrophic zones.

13. Newly ecdysed day 0 pupae were treated with a single dose of 5 µg JHA. High mortality was observed and the surviving pupae showed various abnormalities in pupal-adult development. Most of the treated pupae failed to emerge as adults. When the pupal cases of unemerged pupae were dissected they were found to contain adultoids. Histological and

ultrastructural studies on the testes of treated pupae revealed necrotic/degenerating cells and few malformed or abnormal eupyrene spermatozoa. Spermatocysts were indistinguishable. Degeneration occurred mostly in the spermatogonial cells which showed pycnotic nuclei and numerous myeloid structures. Mitochondria were relatively few in number.

14. The testicular sheath of JHA treated pupae varied considerably in thickness. Internal and external tunics were indistinguishable. The sheath lacked glycogen droplets and the nuclei were irregular in shape. Numerous mitochondrial population of varying shapes, short stretches of rough endoplasmic reticulum and large deposits of proteinaceous granules were observed. These ultrastructural features indicated hypertrophy of the cells.

15. In another set of experiments, newly ecdysed day 0 pupae were treated with a single dose of 10 μg RH 5992. High morality was observed towards the end of pupal period. Few pupae survived for 9±1 days, but failed to emerge as adults. Histological preparations of the testes revealed the presence of numerous sperm bundles but the number was less compared to control pupae.

REFERENCES

Aboim A.N. (1945). Dévelopment embryonnaire et post-embryonnaire des gonades normales et agamétiques de *Drosophila melanogaster*. *Rev. Suisse Zool.* 52, 53-154.

Afzelius B.A., Baccetti B. and Dallai R. (1976). The giant sperm of Notonecta. *J. Submicrosc. Cytol.* 8, 149-161.

Alrubeai H.F. and Gorell T.A. (1981). Hormonal control of testicular protein synthesis in developing *Tenebrio molitor*. *Insect Biochem.* 11, 337-342.

Ambika B. and Prabhu V.K.K. (1978). Endocrine influences on spermatogenesis in the red cotton bug, *Dysdercus cingulatus* (Pyrrhocoridae: Heteroptera). *Entomon.* 3, 165-175.

Ammann H. (1954). Die postembryonale Entwicklung der Weiblichen Geschlechtsorgane in der Raupe Von *Solenobia triquetrella* F.R (Lepid.) mit exgänzenden Bemerkungen über die Entwicklung des männlichen Geschlechts apparates. *Zool. Jahrb. Anat.* 73, 337-394.

Anderson J.M. (1950). A cytological and cytochemical study of the testicular cyst cells in the japanese beetle. *Physiol. Zool.* 23, 308-316.

Andre J. (1959). Etude du microscope électronique de l'évolution du chondriome pendant la spermatogénése du papillon du chou *Pieris brassicae*. *Ann. Sci. Nat. Zool.* 12, 283-308.

Andre J. (1961). Sur quelques détails nouvellement connus de l'ultrastructure des organites vibratiles. *J. Ultrastruct Res.* 5, 86-108.

Ashrafi S.H. and Roppel R.M. (1973). Radiation - induced partial sterility related to structurally abnormal sperms of *Plodia interpunctella* (Lepidoptera: Phycitidae). *Ann. Entomol. Soc. Am.* 66, 1309-1314.

Baccetti B. (1972). Insect Sperm cells. *Adv. Insect Physiol.* 9, 315-397.

Baccetti B. and Afzelius B.A. (1976). *The Biology of the Sperm Cell. Monographs in Developmental Biology* 10, S. Karger, Basel, pp.254.

Baccetti B. and Bairati A. (1965). Indagini Sull' Ultrastructtura delle cellule germinali maschili *Dacus oleae* ed in *Drosophila melanogaster*. *Redia* 49, 1-29.

Baccetti B., Dallai R. and Callaini (1981). The spermatozoon of Arthropoda, *Zootermopsis nevadensis* and isopteran sperm phylogeny. *Int. J. Invertebr. Rep.* 3, 87-99.

Baccetti B., Dallai R., Pallini V. and Afzelius B.A. (1977). Protein of insect sperm mitochondrial crystals. Crystallomitin. *J. Cell. Biol.* 73, 594-600.

Bairati A. (1967). Struttura ed Ultra struttura dess' apparato genitale maschile di *Drosophila melanogaster. Meig. Z. Zellforsch. mikrosk. Anat.* 76, 56-99.

Bairati A., Perotti M.E. and Gioria M.R. (1980). Ultrastructural study on the occurrence of DNA in mitochondria of spermatozoa. *J. Submicrosc. Cytol.* 12, 599-609.

Balamani E. and Nair V.S.K. (1989). Effects of antijuvenile hormone agents and a juvenile hormone analogue on neck-ligated post-feeding last instar larvae of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Insect Sci. Appl.* 10, 583-589.

Balamani E. and Nair V.S.K. (1991). On the morphogenetic role of juvenile hormone analogue in the prepupal stage of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Inverteb. Reprod. Dev.* 20, 175-179.

Balamani E. and Nair V.S.K. (1992). Inhibitory effect of a juvenile hormone analogue on prothoracic gland activity in the penultimate and last larval development of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Zool. Anz.* 228, 182-187.

Bao S.N., Quagio-Grassiotto I. and Dolder H. (1989). Acrosome formation in *Ceratitis capitata* (Diptera, Tephritidae). *Cytobios*, 58, 93-100.

Bawa S.R. (1964). Electron microscope study of spermiogenesis in a fire-brat insect, *Therombia domestica* Pack. 1. Mature spermatozoon. *J. Cell. Biol.* 23, 431-446.

Benny T.M. and Nair V.S.K. (1999). Involvement of ecdysteroids in the fusion of testes lobes of *Spodoptera mauritia* Boisd. *(Lepidoptera: Noctuidae). J. Entomol. Res.* 23(4), 343-345.

Bhakthan N., Nair K.K. and Borden J.H. (1969). Occurrence of a fat body layer around the testis of *Ips Confusum. Ann. Entomol. Soc. Am.* 62, 1495-1496.

Bigliardi E., Baccetti B., Burrini G. and Pallini V. (1970). The spermatozoon of Arthopoda XII. The distribution of some enzymes in the insect sperm tail. In *Comparative spermatology* (Ed. Baccetti B) pp.451-464. Academic, New York.

Blaine W.D. and Dixon S.E. (1976). Testicular development in the Cockroach, *Periplaneta americana* L. *Proc. ent. Soc. Ont.* 106, 60-65.

Bloch D.P. and Brack S.D. (1964). Evidence for the cytoplasmic synthesis of nuclear histone during spermiogenesis in the grasshopper *Chortophaga virdifasciata* (De Geer). *J. Cell Biol.* 22, 327-340.

Bollenbacher W.E., Vedeckis W.V., Gilbert L.I. and O'Connor J.D. (1975). Ecdysone titers and prothoracic gland activity during the larval-pupal development of *Manduca sexta*. *Dev. Biol.* 44, 46-53.

Bonhag F.P. and Wick J.R. (1953). The functional anatomy of the male and female reproductive systems of the milkweed bug, *Oncopeltus fasciatus* (Heteroptera: Lygaeidae). *J. Morphol.* 93, 177-283.

Buder J.E. (1917). Die Spermatogenese Von *Deiliphela euphorbiae* L. *Arch. Zellforsch.* 14, 26-78.

Cantacuzene A.M. (1967). Effects compares de l' allalectomie sur l' activite des glandes annexes males et le comportement sexual de z Acridiens, *Schistocerca gregaria* et *Locusta migratoria. C.R. Acad. Sci.* Paris D. 265, 224-227.

Cantacuzene A.M. (1970). 'L'annexe centriolaire du spermatozoide des insects. In *Comparative Spermatology*. (Ed. Baccetti, B) Academic Press, New York, pp.553-563.

Carpenter J.E. and Chandler L.D. (1994). Effects of sublethal doses of two insect growth regulators on *Helicoverpa zea* (Lepidoptera: Noctuidae) reproduction. *J. Entomol Sci.* 29, 429-435.

Chase J.A. and Gilliland F.R. Jr. (1972). Testicular development in the tobacco bud worm. *Ann. Entomol. Soc. Am.* 65, 901-906.

Chaudhury M.F.B. and Raun E.S. (1966). Spermatogenesis and testicular development of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyraustidae). *Ann. Entomol. Soc. Am.* 59, 1157-1159.

Cholodkovsky N. (1905). Ueber den Bau des Dipterenhodens. *Z. Wiss. Zool.* 82, 389-410.

Cooper K.W. (1950). Normal spermatogenesis in *Drosophila*. In: *Biology of Drosophila*, (Ed. Demerec, M.) pp.1-61. New York: Hafner.

Cymborowski B. and Stolarz G. (1979). The role of juvenile hormone during larval-pupal transformation of *Spodoptera littoralis*: Switchover in the sensitivity of the prothoracic gland to juvenile hormone. *J. Insect Physiol.* 25, 939-942.

Dai J.D. and Gilbert L.I. (1997). Programmed cell death of the prothoracic glands of *Manduca sexta* during pupal-adult metamorphosis. *Insect Biochem. Mol. Biol.* 27, 69-78.

Dai J.D. and Gilbert L.I. (1998). Juvenile hormone prevents the onset of programmed cell death in the prothoracic glands of *Manduca sexta*. *Gen. Comp. Endocr.* 109, 155-165.

Dallai R. (1970). The spermatozoon of Arthropoda XI. Further observations on Collembola. In: *Comparative Spermatology* (Ed. Baccetti, B.), Academic Press, New York, pp.275-279.

Dallai R. and Afzelius B.A. (1980). Characteristics of the sperm structure in Heteroptera (Hemiptera). *J. Morphol.* 164, 301-309.

Dallai R. and Afzelius B.A. (1990). Microtubular diversity in insect spermatozoa: Results obtained with a new fixative. *J. Struct. Biol.* 103, 164-179.

Dallai R. and Afzelius B.A. (1993). Development of the accessory tubules of insect sperm flagella. *J. Submicrosc. Cytol. Pathol.* 25, 499-504.

Dallai R. and Afzelius B.A. (1995). Sperm structure in Trichoptera. II. The aflagellate spermatozoa of *Hydroptila orthotrichia* and *Stactobia* (Hydroptilidae). *Int. J. Insect Morphol. Embryol.* 24, 161-170.

Dallai R., Lupetti P., Afzelius B.A. and Mamaev B.M. (1996). The sperm structure of the gall-midges *Anaretella* and *Lestremia*. *Tissue and Cell.* 28, 331-338.

Dallai R., Lupetti P., Frati F., Mamaev B.M. and Afzelius B.A. (1997). Characteristics of spermatozoa from five gall-midge species. *Acta. Zoologica* (Stockholm) 78, 33-37.

Danilova L.V. (1973a). Electron microscopic study of meiosis in diploid male silkworms. *Ontogenez*, 4, 40-48.

Danilova L.V. (1973b). Electron microscopic study of meiosis in oligopyrene spermatocytes of the silkworm. *Ontogenez*, 4, 281-287.

Danilova L.V. and Vereiskaya V.N. (1968a). Microtubules in the spermatids of the silkworm *Bombyx mori*. *Tsitologiya*, 10, 684-689.

Danilova L.V. and Vereiskaya V.N. (1968b). An electron microscope study of the spermatid heads in the silk worm. *Tsitologiya*, 10, 1361-1367.

Danilova L.V. and Vereiskaya V.N. (1969). Ultrastructure of the middle piece in spermatids in di-, tri- and tetraploids of silkworm *Bombyx mori* L. *Izvestia Academia Nauk SSSR. Seria Biologicheskaia.* 50, 50-61.

Danilova L.V. and Vereiskaya V.N. (1970). Lamellar bodies in spermatogenesis in the silkworm *Bombyx mori*. *Ontogenez* 1, 501-508.

Danilova L.V., Gaber E.S. and Shinyaeva L.I. (1984). Spermiogenesis and the ultra structure of mature sperm in *Eurygaster intergriceps. Gamete Research.* 9, 75-86.

Darvas B. and Varjas L. (1990). Insect growth regulators. In *Armoredscale Insects, Their Biology, Natural Enemies and Control* (Ed. by Rosen D.) Vol. B., pp.392-408, Elsevier Science Publishers, B.V. Amsterdam.

Dass C.M.S. and Ris H. (1958). Submicroscopic organisation of the nucleus during spermiogenesis in the grasshopper. *J. Biophys. Biochem. Cytol.* 4, 129-132.

Deb D.C. and Chakravorty (1981). Juvenoid - induced effects on the growth and differentiation of testis in the rice moth, *Corcyra cephalonica. J. Insect Physiol.* 27, 397-402.

Demant C. (1912). Der Geschlechtapparat von *Dytiscus marginalis. A. Wiss. Zool.* 103, 171-299.

Denlinger D.L. (1979). The physiology of pupal diapause in fleshflies. In "*Current Topics in Insect Endocrinology and Nutrition.*" (Ed. by Bhaskaran G., Friedman S. and Rodriguez J.G.) pp.131-160. Plenum Press, New York.

Dhadialla T,S., Carlson G.R. and Le Dat. P. (1998). New Insecticides with ecdysteroidal and juvenile hormone activity. *Ann. Rev. Entomol.* 43, 545-569.

Dumser J.B. (1980). The regulation of spermatogenesis in insects. *Ann. Rev. Entomol.* 25, 341-369.

Dumser J.B. and Davey K.G. (1974). Endocrinological and other factors influencing testis development in *Rhodnius prolixus*. *Can. J. Zool.* 52, 1011-1022.

Dumser J.B. and Davey K.G. (1975). The *Rhodnius* testis: hormones, differentiation of germ cells and duration of the moulting cycle. *Can. J. Zool.* 52, 1682 - 1689.

Edwards J.S. (1961). On the reproduction of *Prionoplus reticularis* (Coleoptera: Cerambycidae) with general remarks on reproduction in the Cerambycidae. *Q. J. microsc. Sci.* 102, 519-529.

Ephrussi B. and Beadle G.W. (1936). A technique of transplantation for *Drosophila*. *Amer. Nat.* 70, 218-225.

Erickson R.P. (1990). Post-meiotic gene expression. *Trends in Genetics*. 6, 264-268.

Etman A.A.M. and Hooper G.H.S. (1979). Developmental and reproductive biology of *Spodoptera litura* (Lepidoptera: Noctuidae). *J. Aust. Entomol. Soc.* 18.

Favard P. and Andre J. (1970). The mitochondria of spermatozoa. In. *Comparative spermatology* (Ed. Baccetti B.) pp.415-429; Academic Press, New York.

Fawcett D.W., Anderson W.A. and Phillips D.M. (1971). Morphogenetic factors influencing the shape of the sperm head. *Dev. Biol.* 26, 220-251.

Ferenz H.J. (1963). Juvenile hormone induced sexual maturity in males of *Pterostichus nigrita* F. *Experientia,* 29, 356.

Fernadez A.P. and Bao S.N. (1996). Ultrastructural study of the spermiogenesis and localization of tubulin in spermatid and spermatozoon of *Diabrotica speciosa*. *Cytobios*. 86(347), 231-241.

Fernades A.P., Curi G., Franca F.G.R., Bao S.N. (2001). Nuclear changes and acrosome formation during spermiogenesis in *Euchistus heros* (Hemiptera: Pentatomidae). *Tissue and Cell*, 33(3), 286-293.

Fox P.D., Hewitt G.M. and Hall D.J. (1974). DNA replication and RNA transcription of euchromatic and heterochromatic chromosome regions during grass hopper meiosis. *Chromosoma*. 45, 43-62.

Friedlander M. (1976). The role of transient perinuclear microtubules during spermiogenesis of the warehouse moth, *Ephestia cautella*. *J. Submicrosc. Cytol*. 8, 319-326.

Friedlander M. (1982). Juvenile hormone and regulation of dichotomous spermatogenesis during the larval diapause of the codling moth. *J. Insect Physiol*. 28, 1009-1012.

Friedlander M. (1983a). Phylogenetic branching of Trichoptera and Lepidoptera: an Ultrastructural analysis on comparative spermatology. *J. Ultra Struct. Res*. 83, 141-147.

Friedlander M. (1983b). A Comparative study on the spermatogenesis of Trichoptera and Lepidoptera. In *The Sperm Cell* (Ed. Andre, J.). Martinus Nijhoff, The Hague, pp.436-439.

Friedlander M. (1989). 20-Hydroxyecdysone induces glycogen accumulation within the testicular sheath during *in vitro* spermatogenesis renewal in diapausing codling moth (*Cydia Pomonella*) *J. Insect Physiol*. 35(1), 29-39.

Friedlander M. (1997). Control of the eupyrene - apyrene sperm dimorphism in Lepidoptera. *J. Insect Physiol*. 43(12), 1085-1092.

Friedlander M. and Benz G. (1981). The eupyrene-apyrene dichotomous spermatogenesis of Lepidoptera. Organ culture study of the timing of apyrene commitment in the codling moth. *Int. J. Invertbr. Rep*. 3, 113-120.

Friedlander M. and Benz G. (1982). Control of spermatogenesis resumption in post-diapausing larvae of the codling moth. *J. Insect Physiol*. 28, 349-355.

Friedlander M. and Brown J.J. (1995). Tebufenozide (Mimic R) a nonsteroidal ecdysone agonist, induces spermatogenesis reinitiation in isolated abdomens of diapausing codling mothlarvae (*Cydia pomonella*). *J. Insect Physiol*. 41, 403-411.

Friedlander M. and Gitay H. (1972). The fate of the normal - anucleated spermatozoa in inseminated females of the silkworm, *Bombyx mori. J. Morphol.* 138, 121-130.

Friedlander M. and Hauschteck-Jungen E. (1982). Differential basic nucleoprotein kinetics of the two kinds of Lepidoptera spermatids: nucleate (eupyrene) and anucleate (apyrene). *Chromosoma* 85, 387-398.

Friedlander M. and Miesels S. (1977). Spermatid anucleation during the normal atypical spermatogenesis of the warehouse moth, *Ephestia cautella. J. Submicrosc. Cytol.* 9, 173-185.

Friedlander M. and Reynolds S.E. (1988). Meiotic metaphases are induced by 20-hydroxyecdysone during spermatogenesis of the tobacco hornworm, *Manduca sexta. J. Insect Physiol.* 34, 1013-1019.

Friedlander M. and Reynolds S.E. (1992). Intratesticular ecdysteroid titres and the arrest of sperm production during pupal diapause in the tobacco horn worm, *Manduca sexta. J. Insect Physiol.* 38, 693-703.

Friedlander M. and Wahrman J. (1966). Giant centrioles in neuropteran meiosis. *J. Cell Sci.* 1, 129-144.

Friedlander M. and Wahrman J. (1971). The number of centrioles in insect sperm: a study in two kinds of differentiating silkworm spermatids. *J. Morphol.* 134, 383-397.

Friedlander M., Jans P. and Benz G. (1981). Precocious reprogramming of eupyrene-apyrene spermatogenesis commitment induced by allatectomyof the penultimate larval instar of the moth, *Actias selene J. Insect Physiol.* 27, 267-269.

Friedlander M., Jeshtadi A. and Reynolds S.E. (2001). The structural mechanism of trypsin-induced intrinsic motility in *Manduca sexta* spermatozoa *in vitro. J. Insect Physiol.* 47(3), 245-255.

Fujishita M., Ohnishi E. and Ishizaki H. (1982). The role of ecdysteroids in the determination of gut-purge timing in the saturniid *Samia cynthia ricini. J. Insect Physiol.* 28, 961-967.

Fukuda S. (1944). The hormonal mechanism of larval moulting and metamorphosis in the silkworm. *J. Fac. Sci. Univ. Tokyo. Sect. IV. Zool.* 6, 478-532.

Fukushima T. and Yagi S. (1975). Hormonal effect on cultivated insect tissues. III. Effects of α- and β-ecdysone or prothoracic glands on spermiogenesis in two noctuid insects *in vitro* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* 10, 220-225.

Fuller M.T. (1993). Spermatogenesis. In *The Development of Drosophila melanogaster*. (Eds. Bate M. and Arias. A.M.). Cold Spring Harbor Laboratory Press, New York. pp.71-147.

Garcia-Bellido A. (1964). Analyse de physiologischen bedingungen des vermehrynegswachstums momlicher Keimsellen von *Drosophila melanogaster. Wilhelm Ronx Arch. EntWi. Mech. Org.* 155, 611-631.

Garvey L.K., Gutierrez G.M. and Krider H.M. (2000). Ultrastructure and morphogenesis of the apyrene and eupyrene spermatozoa in the gypsy moth (Lepidoptera: Lymantriidae). *Ann. Entomol. Soc. Am.* 93(5): 1147-1155.

Gelbic I. and Metwally M.M. (1981). Changes in the development of male germinal cells in *Spodoptera littoralis* caused by the effects of juvenoids. (Lepidoptera: Noctuidae). *Acta Entomologica. Bohemoslovaca.* 78, 10-17.

Gelman D.B. and Borkovec A.B. (1986). Head critical periods for controlling apyrene and eupyrene spermiogenesis in the European corn borer *Ostrinia nubilalis*. In *Insect Neurochemistry and Neurophysiology*. (Ed. by Borkovec A.B. and Gelman D.B.) pp.371-374, Humana Press, Clifton, N.J.

Gelman D.B., Woods C.W. and Borkovec A.B. (1988). Effects of ecdysone and 20-hydroxyecdysone on apyrene spermiogenesis in the European corn borer, *Ostrinia nubilalis. J. Insect Physiol.* 34, 733-738.

Gelman D.B., Woods C.W. and Borkovec A.B. (1989). Ecdysteroid synthesis by testes of sixth instars and pupae of the European corn borer *Ostrinia nubilalis. J. Insect Physiol.* 34, 733-738.

Giebultowicz J.M., Loeb M.J. and Borkovec A.B. (1987). *In vitro* spermatogenesis in lepidopteran larvae. Role of the testis sheath. *Int. J. Invertbr. Reprod. Dev.* 11, 211-226.

Gilbert L.I., Bollenbacher W.E., Augui N., Granger N.A., Sedlak B.J., Gibbs D. and Buys C.M. (1981). The prothoracicotropes: Source of the prothoracicotropic hormone. *Amer. Zool.* 21, 641-653.

Gilbert L.I., Rybczynski R. and Tobes B. (1996). Endocrine cascade in metamorphosis. In "*Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect cells*" (Eds. Gilbert L.I., Tata J.R. and Atkinson B.G.) pp.59-107 Academic Press. San Diego.

Girardie A. and Vogel A. (1966). Etude du controle neuro humoral de l'activite sexuelle male de *Locusta migratoria* (L.) *C.R. Acad. Sci. Paris.* D. 543-546.

Godulla J. (1985). Quadriflagellar primary spermatocytes in the cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Cell and Tissue Res.* 242, 681-683.

Goldschmidt R. (1917). Ver suche zur spermatogenese *in vitro. Arch. Zellforsch.* 14, 421-450.

Gondos B. (1984) Germ cell differentiation and intercellular bridges. In *Ultrastructure of Reproduction* (Eds. van Blerkom J. and Motta P.M.) Martinus Nijhoff, Boston, The Hague, Dordrecht, Lancaster. pp. 31-45.

Grenier S. and Grenier A.M. (1993). Fenoxycarb, a fairly new insect growth regulator: a review of its effects on insects. *Ann. Appl. Biol.* 122, 369-403.

Gruetzmacher M.C., Gilbert L.I., Granger N.A., Goodman W. and Bollenbacher W.E. (1984). The effect of juvenile hormone on prothoracic gland function during the larval-pupal development of *Manduca sexta* as *in situ* and *in vitro* analysis. *J. Insect Physiol.* 30, 331-340.

Guraya S.S. (1998). In *Cellular and Molecular Biology of Gonadal Development and Maturation in Mammals. Fundamental and Biomedical Implications.* pp. 264. Narosa Publishing House, New Delhi.

Hannah - Alava A. (1965). The premeiotic stages of spermatogenesis. *Adv. Genet.* 13, 157-226.

Henning W. and Kremer H. (1990). Spermatogenesis of *Drosophila hydei. Int. Rev. Cytol.* 123, 129-175.

Herman W.S. and Peng P. (1976). Juvenile hormone stimulation of sperm activator production in male Monarch butterflies. *J. Insect Physiol.* 22, 579-581.

Hiruma K. (1980). Possible roles of juvenile hormone in the prepupal stage of *Mamestra brassicae. Gen. Comp. Endocr.* 41, 392-399.

Hiruma K., Shimada H. and Yagi S. (1978). Activation of prothoracic gland by juvenile hormone and prothoracicotropic hormone in *Mamestra brassicae*. *J. Insect. Physiol.* 24, 215-220.

Hoage T.R. and Kessel R.G. (1968). An electron microscope study of the process of differentiation during spermatogenesis in the drone honeybee (*Apis mellifera*) with special reference to centriole replication and elimination. *J. Ultrastruct. Res.* 24, 6-32.

Hoffmann K.H. and Behrens W. (1982). Free ecdysteroids in adult male crickets, *Gryllus bimaculatus*. *Physiol. Entromol.* 7, 269-279.

Holt G.G. and North D.T. (1970). Effect of gama irradiation on the mechanism of sperm transfer in *Trichoplusia ni*. *J. Insect Physiol.* 16, 2211-2222.

Iriki S. (1941). The two sperm types in the silkworm and their functions. *Zool. Mag.* (Tokyo). 53, 123-124.

Jagannadh V. and Nair V.S.K. (1992). Azadirachtin-induced effects on larval-pupal transformation of *Spodoptera mauritia*. *Physiol. Entomol.* 17, 56-61.

Jagannadh V. and Nair V.S.K. (1993). Effects of azadirachtin and juvenile hormone analogue on neck-ligated post-feeding larvae of *Spodoptera mauritia*. *Indian J. Exptl. Biol.* 31, 199-200.

Jamieson B.G.M. (1987). *The ultrastructure and phylogeny of insect spermatozoa*, Cambridge University Press, Cambridge, England, pp.320.

Jans P., Benz G. and Friedlander M. (1984). Apyrene spermatogenesis inducing factor is present in the haemolymph of male and female pupae of the codling moth. *J. Insect Physiol.* 30, 495-497.

Jarvis T.D. and Rees H.H. (1990). Studies on Ecdysteroid Biosynthesis, Investigation in Testes. Presented at IXth Ecdysone Work Shop, Paris. *Invert. Reprod. Devel.* 18, 1-2, 119.

Jones R.T. (1978). The blood/germ cell barrier in male *Schistocerca gregaria*: The time of its establishment and factors affecting its formation. *J. Cell Sci.* 31, 145-163.

Kambysellis M.P. and Williams C.M. (1971a). *In vitro* development of insect tissues II. The role of ecdysone in the spermatogenesis of silkworms. *Biol. Bull.* 141, 541-552.

Kambysellis M.P. and Williams C.M. (1971b). *In vitro* development of insect tissues. I. Macro molecular factor prerequisite for silkworm spermatogenesis. *Biol. Bull.* 141. 527-540.

Kambysellis M.P. and Williams C.M. (1972). Spermatogenesis in the cultured testes of the cynthia silk worm: effects of ecdysone and prothoracic glands. *Science.* 175, 769-770.

Kasuga H., Osanai M. and Aigaki T. (1985). Post testicular maturation of spermatozoa of the silkworm, *Bombyx mori. Zool. Sci.* 2, 953.

Katsuno S. (1977a). Studies on the eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). I. The intratesticular behaviour of the spermatozoa at various stages from the fifth instar to the adult. *Appl. Ent. Zool.* 12, 142-153.

Katsuno S. (1977b). Studies on eupyrene and apyrene spermatozoa in the silkworm *Bombyx mori* (Lepidoptera: Bombycidae). IV. The behaviour of the spermatozoa in the internal reproductive organs of female moths. *Appl. Entomol. Zool.* 12, 352-359.

Katsuno S. (1977c). Studies on eupyrene and apyrene spermatozoa in the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). V. The factor related to the separation of eupyrene sperm bundles. *Appl. Entomol. Zool.* 12, 370-371.

Katsuno S. (1978). Studies on eupyrene and apyrene spermatozoa in the silk worm, *Bombyx mori* (Lepidoptera: Bombycidae) L. VII. The motility of sperm bundles and spermatozoa in the reproductive organs of males and females. *Appl. Entomol. Zool.* 13, 91-96.

Katsuno S. and Tamazawa S. (1979). Studies in apyrene and eupyrene spermatozoa in the male reproductive organs of the tetraploid male of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Entomol. Zool.* 14, 438-444.

Kaur A., Kishen Rao B., Thakur S.S. and Sabita Raja (1987). Effect of juvenile hormone analogue hydroprene on the testes of the bug, *Leptocoris*

coimbatorensis Gross. (Hemiptera: Cordeidae). *J. Anim. Morphol. Physiol.* 34, 39-44.

Kawamura N., Yamashiki N., Saitoh H. and Sahara K. (2000). Peristaltic squeezing of sperm bundles at the late stage of spermatogenesis in the silkworm, *Bombyx mori. J. Morphol.* 246(2), 53-58.

Kaye J.S. (1962). Acrosome formation in the house cricket. *J. Cell. Biol.* 12, 411-431.

Kessel R.G. (1970). Spermiogenesis in the dragonfly with special reference to a consideration of the mechanisms involved in the development of cellular asymmetry. In *Comparative Spermatology* (Ed. Baccetti. B), Academic Press, New York, pp.531-554.

Kiguchi K. and Riddiford L.M. (1978). A role of juvenile hormone in pupal development of the tobacco horn worm, *Manduca sexta*. *J. Insect Physiol.* 24, 673-680.

Kiguchi K., Agui N., Kawasaki H. and Kobayashi K. (1985). Developmental time-table for the last larval and pharate pupal stages in the silkworm, *Bombyx mori*, with special reference to the correlation between the developmental events and hemolymph ecdysteroid levels. *Bull. Seric. Exp. Sta.* 30, 83-100.

Kim C.W., Kim W.K., Lee K.O and Kim C.S. (1982). A study on gonad in *Pieris rapae* L. II. Ultrastructural studies on the testis during larval - stages. *Kor. J. Ent.* 12, 7-13.

Kinefuchi H. (1978). Studies on eupyrene and apyrene spermatozoa of Lepidoptera. *Memoirs of the Faculty of Education of Niigata University.* 19, 21-31.

King R.C. and Akai H. (1971 a). Spermatogenesis in *Bombyx mori* I. The canal system joining sister spermatocytes. *J. Morphol.* 134, 47-56.

King R.C. and Akai H. (1971b). Spermatogenesis in *Bombyx mori* II. The Ultrastructure of synapsed bivalents. *J. Morphol.* 134, 181-194.

Koeppe J.K., Fuchs M., Chen T.T., Hunt L.M., Kovalick G.E. and Briers T. (1985). "The role of juvenile hormone in reproduction". In *Comprehensive Insect Physiology, Biochemistry and Pharmacology.* (Ed. By Kerkut G.A. and Gilbert L.I.) vol. 8, pp. 165-203, Pergamon Press, New York.

Kondo R., Matsuura E.T. and Chigusa S.I. (1991). Further observations of paternal transmission of *Drosophila* mitochondrial DNA by PCR selective amplification method. *Genetical Research*. 59, 81-84.

Koolman J., Scheller K. and Bodenstein D. (1979). Ecdysteroids in the adult male blowfly *Calliphora vicina*. *Experientia*. 35, 134-135.

Kramer S.J., Baker F.C., Miller C.A., Cerf D.C., Schooley D.A. and Menn J.J. (1983). Juvenile hormone antagonists. In *Pesticide Chemistry, Human Welfare and Environment.* (Ed. by Miyamoto J. and Kearney P.C.). Vol. 1, pp.177-182. Pergamon Press, New York.

Lachance L.E. and Olstad G. (1988). Spermiogenesis of *Heliothis virescens*: an ultrastructure study of eupyrene sperm in sterile backcross males. *Ann. Entomol. Soc. Am*. 81(2), 301-307.

Lai-Fook J. (1982). Testicular development and spermatogenesis in *Calpodes ethlius* Stall (Hesperiidae: Lepidoptera). *Can. J. Zool*. 60, 1161-1171.

Leclercq-Smekens M. (1978a). 'Reproduction d' *Euproctis chrysorrhea* L. (Lépidoptère: Lymantriidae) I. 'Etude de l'organogenèse des testicules et de la spermagogenèse'. *Bulletin de la classe des sciences*, 64, 304-317.

Leclercq-Smekens M. (1978b). 'Reproduction d' *Euproctis chrysorrhea* L. (Lépidoptère: Lymantriidae) II. Aspects cytologiques et r'le de la cellule apicale (Cellule de Verson) des testicules'. *Bulletin de la classe des sciences.* 64, 318-332.

Lee K.O., Kim C.W. and Kim W.K. (1982). A study on the gonad in *Pieris rapae* L. III. Ultrastructural study on the testis during the metamorphosis from prepupa to pupa. *Kor. Soc. Elect. Micros*. 12, 45-53.

Lee P.E. and Wilkes A. (1965). Polymorphic spermatozoa in the hymenopterous wasp, *Dahlbominus*. *Science*. 147, 1443-1446.

Lender T.H. and Duveav-Hagege J. (1963). La Survie et la differenciation en culture *in vitro* de gonades de larves de dernier age de *Galleria mellonella* (Lepidoptera: pyralidae). *Dev. Biol*. 6, 1-22.

Leviatan R. and Friedlander M. (1979). The eupyrene apyrene dichotomous spermatogenesis of Lepidoptera I. The relationship with postembryonic

development and the role of the decline in juvenile hormone titre towards pupation. *Dev. Biol.* 68, 515-524.

Loeb M.J. (1986). Ecdysteroids in testis sheaths of *Heliothis virescens* larvae: An immunocytological study. *Arch. Insect Biochem. Physiol.* 3, 173-180.

Loeb M.J. (1994). Characterization of genital tract growth factor like activity from testis sheath and fat body of *Heliothis virescens*. *Arch. Insect Biochem. Physiol.* 26, 263-277.

Loeb M.J. and Birnbaum M.J. (1981). The relationship of haemolymph osmotic pressure to spermatogenesis in the tobacco bud worm, *Heliothis virescens*. *Int. J. Invert Reprod.* 4, 67-79.

Loeb M.J., Brandt E.P. and Birnbaum M.J. (1984). Ecdysteroid production by the testes of Tobacco bud worm, *Heliothis virescens*, from last larval instar to adult. *J. Insect Physiol.* 30, 375-381.

Loeb M.J., Brandt E.P. and Masler E.P. (1985). Modulation of the rate of spermatogenesis by the central nervous system of the tobacco bud worm, *Heliothis virescens*. *J. Invert Reprod.* 8, 39-49.

Loeb M.J., Brandt E.P., Giebultowicz J.M. and Woods C.W. (1986). Endocrine regulation of the testes and of spermatogenesis in insects. In "*Advances in Invertebrate Reproduction.*" (Ed. by Andries J.C. and Dhainaut A.) Vol.4, pp.93-100, Elsevier, Amsterdam.

Loeb M.J., Brandt E.P., Woods C.W. and Bell R.A. (1988). Secretion of ecdysteroid by sheaths of testes of the Gypsy moth, *Lymantria dispar* and its regulation by testes ecdysiotropin. *J. Exp. Zool.* 248, 94-100.

Loeb M.J., Brandt E.P., Woods C.W. and Borkovec A.B. (1987). An ecdysiotropic factor from brain of *Heliothis virescens* induces testes to produce immunodetectable ecdysteroid *in vitro*. *J. Exp. Zool.* 243, 275-282.

Loeb M.J., De Loof A., Gelman D.B., Hakim R.S., Howard J., Kochansky J.P., Meola S.M., Schoofs L., Steel C., Vafopoulou X., Wagner R.M. and Woods C.W. (2001). Testis ecdysiotropin, an insect gonadotropin that induces synthesis of ecdysteroid. *Arch. Insect Biochem. Physiol.* 47(4), 181-188.

Loeb M.J., Woods C.W., Brandt E.P. and Borkovec A.B. (1982). Larval testes of tobacco bud worm. A new source of insect ecdysteroids. *Science*. 218, 896-898.

Lomen F. (1914). Der Hoden Von *Culex pipiens*. *Jena Z. Naturwiss*. 52, 567-628.

Lum P.T.M. (1977). High temperature inhibition of development of eypyrene sperm and of reproduction in *Plodia interpunctella* and *Ephestia cautella*. *J. Georgia. Entomol. Soc.* 12, 199-203.

Mancini K. and Dolder H. (2001). Ultrastructure of apyrene and eupyrene spermatozoa from the seminal vesicle of *Euptoleta hegesia* (Lepidoptera: Nymphalidae). *Tissue and Cell*. 33(3), 301-308.

Mazzini M. (1976). Giant spermatozoa in *Divales bipustulatus* F. (Coleoptera: Cleridae). *Int. J. Insect Morphol. Embryol*. 5, 107-115.

Medeiros M. and Silveira M. (1996). Ultrastructural study of apyrene spermatozoa of *Alabama arillacea* (Lepidoptera: Noctuidae) with tannic acid containing fixative. *J. Submicrosc. cytol. Pathol*. 28, 133-144.

Menon M. (1969). Structure of the apical cells of the testis of the tenebrionid beetles *Tenebrio molitor* and *Zophobas rugipes*. *J. Morphol*. 127,409-430.

Metwally M.M. and Gelbic I. (1974). Effects of juvenoids on spermatogenesis on the Egyptian cotton leaf worm (Lepidoptera: Noctuidae). *Symposium on regulation of Insect Reproduction*, Institute of Entomology, Czechoslovak Academy of Sciences, Prague (Abstract).

Meves F. (1907). Die Spermatozytenteilungen bei der Honigbiene (*Apis mellifica* L.), nebst einigen Bemerkungen über Chromatin reduktion. *Arch. mikrosk. Anat*. 70, 414-491.

Miyamoto J., Hirano M., Takimoto Y. and Hatakoshi M. (1993). Insect growth regulators for pest control, with emphasis on juvenile hormone analogs: Present and future prospects. In *Pest Control With Enhanced Environmental Safety* (Ed. Duke S.O., Menn J.J., Plimmer J.R.). Acs symp. Ser. 524, 144-168. Washington, D.C. Am.

Mona P.M. (2001). On the developmental profile of hormones in *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae) *Ph.D. Thesis*, University of Calicut.

Muckenthaler F.A. (1964). Autoradiographic study of nucleic acid synthesis during spermatogenesis in the grasshoppers, *Melanoplus differentialis*. *Exp. Cell. Res.* 35, 531-597.

Nagata H.,Ohtaki and Sakurai S. (1987). Role of low ecdysteriod titre in acquisition of competence for pupal transformation in *Bomby mori J. Insect Physiol.* 33, 657-663.

Nair V.S.K. (1981). Hormonal control of the mating behaviour in the females of *Spodoptera mauritia* (Lepidoptera: Noctuidae). *Curr. Sci.* 50, 690-691.

Nair V.S.K. (1993). Applied potential of insect hormone research: perspectives and prospects. In *Chemical Ecology of Phytophagous Insects.* (Ed. Ananthakrishnan T.N. and Raman A.) pp. 89-104, Oxford – IBH Publ. Co., New Delhi.

Nair V.S.K. and Rajalekshmi E. (1989). Effects of fluoromevalonate on penultimate and last instar larvae of *Spodoptera mauritia* Boisd. *Indian J. Exp. Biol.* 27, 170-173.

Nath V. (1956). Cytology of spermatogenesis. *Int. Rev. Cytol.* 5, 395-453.

Nishiitsutsuji-Uwo J. (1961). Further studies on the developmental features of the male germ cells in the prospective diapausing and non-diapausing swallow tails, *Papilio xuthus. Annotnes. Zool. Jap.* 34, 30-37.

Nowock J. (1973). Growth and metamorphosis in the testes of *Ephestia kuehniella in vitro. J. Insect Physiol.* 19, 941-949.

Numata H. and Hidaka T. (1980). Development of male sex cell in the swallow tail *Papilio xuthus* L. (Lepidoptera: Papilionidae) in relation to pupal diapause. *Appl. Entomol. Zool.* 15, 151-158.

Omura S. (1936). Studies of the reproductive system of the male of *Bombyx mori J. Fac. Agr. Hokkaido Univ.* 387, 153-230.

Osanai M., Ajgaki T., Kasuga H. and Yonezawa Y. (1986). Role of arginase transferred from the vesicula seminalis during mating and changes in amino acid pools of the spermatophore after ejaculation in the silkworm, *Bombyx mori. Insect. Biochem.* 16, 879-885.

Osanai M., Kasuga H. and Aigaki T. (1990). Physiology of sperm maturation in the spermatophore of silkworm, *Bombyx mori. In Advances in Invertebrate Reproduction* (Eds. Hoshi and O. Yamashita), Vol. 5, pp. 531-536.

Ohtaki T., Yamanaka F. and Sakurai (1986). Differential timing of pupal commitment in various tissues of the silkworm, *Bombyx mori. J. Insect Physiol.* 32, 635-642.

Palli S.R., Primavera M., Tomkins W., Lambert D. and Retnakaran A. (1995). Age specific effects of a nonsteroidal ecdysone agonist, RH-5992, on the spruce budworm *Choristoneura fumiferana* (Lepidoptera: Tortricidae). *Eur.J.Entomol.* 92, 325-332.

Personne P. and Anderson W.A. (1969). Compartimentage des activités enzymatiques dans le spermatozoide de certains mollusques. I. Localization des déhydrogenase. *J.Cell Sci.* 4, 693-707.

Phillips D.M. (1970). Insect sperm: their structure and morphogenesis. *J. Cell Biol.* 44, 243-277.

Phillips D.M. (1971). Morphogenesis of the lacinate appendages of lepidopteran spermatozoa. *J. Ultrastruct. Res.* 34, 567-585.

Phillips D.M. (1974). *'Spermiogenesis'*, Academic Press, New York and London, pp. 1-68.

Pitnick S., Spicer G.S. and Markow T.A. (1995). How long is a giant sperm? *Nature*. 375, 109-109.

Pratt S.A. (1962). An election microscope study of nebenkern formation and differentiation in spermatids of an hemipteran insect *Murgantia histrionica* (Hemiptera: Pentatomidae). *J. Morphol.* 126, 31-66.

Pradeep A.R. and Nair V.S.K. (1989). Morphogenetic effects of precocene II in the Brown plant hopper *Nilaparvata lugens* Stal (Homoptera: Delphacidae). *Proc. Indian Natn Sci. Acad.* 55, 381-386.

Rasmussen S.W. (1973). Ultrastructural studies of spermatogenesis in *Drosophila melanogaster* Meigen. *Z. Zellforsch. Mikrosk. Anat.* 140, 125-144.

Retnakaran A. (1970). The male reproductive system of the Spruce Bud worm *Choristoneura fumiferna* (Lepidoptera: Totricidae). I. Spermatogenesis. *Ann. Entomol. Soc. Am.* 63, 851-859.

Retnakaran A., Granett J. and Ennis T. (1985). Insect Growth Regulators. *In Comprehensive Insect Physiology, Biochemistry and Pharmacology.* (Ed.by the Kerkut G.A. and Gilbert L.I)Vol. 12, pp, 529-601. Pergamon Press, Oxford.

Richard R.D., Lachance L.E. and Proshold F.I. (1975). An ultrastructural study of sperm in sterile hybrids from crosses of *Heliothis virescens* and *Heliothis subflexa. Ann. Entomol. Soc. Am.* 68, 35-39.

Riddiford L.M. (1976). Juvenile hormone control of epidermal commitment *in vivo* and *in vitro.* In *"The Juvenile Hormones"* (Ed. by Gilbert L.I.) pp. 198-219. Plenum Press, New York.

Riddiford L.M. (1978). Ecdysone-induced change in cellular commitment of the epidermis of the tobacco hornworm, *Manduca sexta,* at the initiation of metamorphosis. *Gen. Comp. Endocr.* 34, 438-446.

Riddiford L.M. (1985). Hormone action at cellular level. In *"Comprehensive Insect Physiology, Biochemistry and Pharmacology".* (Ed. by Kerkut G.A. and Gilbert L.I) vol. 8, pp. 37-84. Pergamon Press, Oxford.

Riemann J.G. (1970) Metamorphosis of sperm of the cabbage looper, *Trichoplusia ni*, during passage from the testis to the female spermatheca. In *Comparative Spermatology.* (Ed. Baccetti B.) Academic Press, New York, pp. 321-331.

Riemann J.G. (1973). Ultrastructure of sperm in FI progeny of irradiated males of the Mediterranean flour moth, *Anagasta kuehniella. Ann. Entomol. Soc. Am.* 66, 147-153.

Riemann J.G. and Gassner G. (1973). Ultrastructure of lepidopteran sperm within spermathecae. *Ann. Entomol. Soc. Am.* 66, 154-159.

Riemann J.G. and Thorson B.J. (1971). Sperm maturation in the male and female genital tracts of *Anagasta kuehniella* (Lepidoptera: Pyralidae). *Int. J. Insect. Morphol. Embryol.* 1, 11-19.

Roosen-Runge E.C. (1977). In *The Process of Spermatogenesis in Animals.* Cambridge University Press, pp.214.

Rule H.D., Godwin P.A. and Waters W.E. (1965). Irradiation effects on spermatogenesis in the gypsy moth, *Prothetria dispar. J. Insect Physiol.* 11, 369-378.

Sado T. (1963). Spermatogenesis of the silk worm and its bearing on radiation induced sterility. *J. Fact. Agric. Kyushu Univ.* 12, 359-386.

Safranek L., Cymborowski B. and Williams C.M. (1980). Effects of juvenile hormone on ecdysone-dependent development in the tobacco hornworm, *Maduca sexta. Biol. Bull.* 158, 248-256.

Sakunthala C. and Nair V.S.K. (1995). Effects of ecdysone agonist RH 5849 on embryonic and postembryonic development of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Indian J. Exp. Biol.* 33, 690-693.

Sakurai S. (1983). Temporal organization of endocrine events underlying larval-larval ecdysis in the silkworm *Bombyx mori. J. Insect. Physiol.* 29, 919-932.

Sakurai S. (1990). Hormonal mechanism underlying the switch in the response of the prothoracic gland to juvenile hormone. *Advances in Invertebrate Reproduction,* V (Ed: Hoshi M. and Yamashita O.) pp. 435-440.

Sakurai S., Okuda M. and Ohtaki T. (1989). Juvenile hormone inhibits ecdysone secretion and responsiveness to prothoracicotropic hormone in prothoracic glands of *Bombyx mori. Gen. Comp. Endocr.* 75, 222-230.

Salama H.S. (1976). Spermatogenesis and testicular development in the gypsy moth *Porthetria dispar.* L. *Z. Angew. Ent.* 81, 102-110.

Sam Mathai and Nair V.S.K. (1983). Effects of precocene II on last instar larvae of *Spodoptera mauritia* (Lepidoptera: Noctuidae). *Curr. Sci.* 52, 376-377.

Sam Mathai and Nair V.S.K. (1984a). Treatment of larvae with repeated doses of precocene II induces precocious metamorphic change in *Spodoptera mauritia* (Lepidoptera: Noctuidae). *Arch. Insect. Biochem. Physiol.* 1, 199-203.

Sam Mathai and Nair V.S.K. (1984b). Effects of precocene II on ovarian follicle development and vitellogenesis in *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *abst. XVII. Internt. Cong. Entomol.* p.21.

Sam Mathai and Nair V.S.K. (1984c). Morphogenetic effects of precocene II on the larval stages of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). In proceedings of the fifth All India Symposium on Developmental Biology. (Ed. by Agerwal S.K. and Goel S.C.) pp.59-66.

Sam Mathai and Nair V.S.K. (1984d). Effects of a juvenile hormone analogue on acid and alkaline phosphatase activities in the pupae of *Spodoptera mauritia* (Lepidoptera: Noctuidae). *Comp. Physiol. Ecol.* 9, 253-256.

Santha P.C. and Nair V.S.K. (1986). Age-dependent responses of last instar larvae of *Spodoptera mauritia* to prococene II. *Physiol-Entomol.* 11, 335-341.

Santha P.C. and Nair V.S.K. (1987). Larval-pupal transformation of *Spodoptera mauritia* (Lepidoptera: Noctuidae). Effects of a juvenile hormone analogue. *Zool. Anz.* 218, 379-384.

Santha P.C. and Nair V.S.K. (1988). Precocene induced abnormalities in F1 generation of *Spodoptera mauritia. Zool. Anz.* 220, 104-110.

Santha P.C. and Nair V.S.K. (1991). Precocene-induced changes in the cerebral neurosecretory cells of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Zool. Anz.* 226, 163-173.

Santha P.C., Sankaranarayanan P. and Nair V.S.K. (1987). Precocene-induced effects on pupal-adult development of *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *Geobios New Reports.* 6, 35-38.

Saxena B.P. and Tikku. K. (1989). Ultrastructural studies of two tissues in *Dysdercus koenigii* Testis And their role in spermiogenesis. In *Regulation of Insect Reproduction* (Eds. Tonner M., Soldan T. and Bennettova B.) pp.141. Academia Praha.

Saxena B.P., Tikku K. and Koul D. (1977). A new insect chemosterilants isolated from *Acorus calamus* L. *Nature.* 270, 512-513.

Scheepens M.H.M. and Wysoki M. (1985). Testicular development, spermatogenesis and chromosome of *Boarmia selenaria* Schiff (Lepidoptera: Geometridae). *Int. J. Invertbr. Reprod.* 8, 337-348.

Schmidt E.L. and Williams C.M. (1953). Physiology of insect diapause. V. Assays of the growth and differentiation hormone of Lepidoptera by the method of tissue culture. *Biol. Bull. Mar. Biol. Lab. Woods Hole.* 105, 174-187.

Shepherd J.G. (1975). A polypeptide sperm activator from male saturniid moths. *J. Insect. Physiol.* 21, 9-22.

Shimizu T. and Yagi S. (1978). Hormonal effects on cultivated insect tissues. IV. The role of testis in spermiogenesis of the cabbage army worm, *Mamestra brassicae in vitro. Appl. Entomol. Zool.* 13, 278-282.

Shimizu T. and Yagi S. (1982). Hormonal effect on cultivated insect tissues. V. The role of testis protein in spermiogenesis of cabbage armyworm. *Mamestra brassicae in vitro.* (Lepidoptera: Noctuidae). *Appl. Ent. Zool.* 17, 385-392.

Shinyaeva L.I (1981). Effect of juvenile hormone analogues on spermatogenesis in *Eurygaster integriceps* during the period of prediapause and diapause formation. *Zool. Zh.* 60, 1198-1204.

Silberglied R.E., Shepherd J.G. and Dickinsons J.L. (1984). Enuchs: the role of apyrene sperms in Lepidoptera? *American Naturalist.* 123, 255-265.

Sjostrand F.S. and Afzelius B.A. (1958). Electron microscopy on grasshopper spermatids. In *Electron Microscopy, Proceedings of the stockholm Conference on Electron Microscopy 1956.* (Eds. Sjostrand F.S. and Rhodin J.) Almqvist and wiksell, Uppsala, pp. 164-167.

Smagghe G. and Degheele D. (1992). Effect of RH 5849, the first non steroidal ecdysteroid agonist, on larvae of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol.* 21, 119.

Smagghe G. and Degheele D. (1994). Action of a novel nonsteroidal ecdysteroid mimic, tebufenozide (RH 5992), on insects of different orders. *Pestic. Sci.* 42, 85-92.

Smagghe G. and Degheele D. (1997). Comparative toxicity and tolerance for the ecdysteroid mimic tebufenozide in a laboratory and field strain of cotton leaf worm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 90, 278-282.

Smith D.S. (1968). In *Insect cells.* Oliver and Boyd, Edinburgh, pp. 307-335.

Snodgrass R.E. (1935). *Principles of Insect Morphology.* New York: Mc Graw-Hill.

Sridevi R., Aparna Dutta-Gupta (Ray) and Ramamurty P.S. (1989). Spermatogenesis in *Spodoptera litura* (Lepidoptera: Noctuidae). *Entomon* 14, 1-9.

Stall G.B. (1982). Insect control with growth regulators interfering with the endocrine system. *Entomol. Exp. Appl.* 31, 15-23.

Sugai E. (1965). Formation of sperm dimorphism and glycogen of the testicular membrane in the silkworm *Bombyx mori. Zool. Mag.* 74, 276-282.

Svoboda J.A., Thompson M.J. and Robbins W.E. (1972). Azasteroids: potent inhibitors of insect molting and metamorphosis. *Lipids.* 7, 553-556.

Szollosi A. (1975). Electron microscope study of spermiogenesis in *Locusta migratoria* (Insect: Orthoptera). *J. Ultrastruct. Res.* 50, 322-346.

Szollosi A. and Marcaillou L. (1977). Electron microscope study of the blood testis barrier in an insect, *Locusta migratoria. J. Ultrastruct. Res.* 59, 158-172.

Szollosi A., Riemann J. and Marcaillou C. (1980) Localization of the blood testis barrier in the testis of the moth *Anagasta kuhniella. J. Ultrastruct Res.* 72, 189-199.

Takeda N. (1972). Effect of ecdysone on spermatogenesis in the diapausing slug moth pharate pupa *Monema flavescens. J. Insect Physiol.* 571-580.

Takeuchi S. (1969). Endocrinological studies of spermatogenesis in the silk worm *Bombyx mori. Dev. Growth. Differ.* 11, 8-28.

Talbot W.S., Swyrd E.A. and Hogness D.S. (1993). Drosophila tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell.* 73, 1323-1337.

Tokuyasu K.T. (1975). Dynamics of spermiogenesis in *Drosophila melanogaster.* VI. Adaptive significance of 'onion' nebenkern formation. *J. Ultra struct. Res.* 53, 93-112.

Trandaburu V., Beratlief C. and Trandaburu T. (1976). Fine structure of normal and gamma-irradiated testis of *Hyphantria cunea* Drury (Lepidoptera: Arctidae) *Zoologische Jahrbucher Abteilung fur Anatomie.* 96, 579-585.

Truman J.W. (1971). Physiology of insect ecdysis. I. The eclosion behaviour of saturniid moths and its hormone release. *J. Exp. Biol.* 54, 805.

Truman J.W. and Riddiford L.M. (1974). Physiology of insect rhythms. III. The temporal organization of the endocrine events underlying pupation of the tobacco horn worm. *J. Exp. Biol.* 60, 371-382.

Truman J.W., Riddiford L.M. and Safranek L. (1974). Temporal patterns of response to ecdysone and juvenile hormone in the epidermis of the tobacco horn worm *Manduca sexta. Dev. Biol.* 39, 247-262.

Unnithan G.C., Nair K.K. and Bowers W.S. (1977). Precocene-induced degeneration of the corpus allatum of adult females of the bug *Oncopeltus fasciatus. J. Insect. Physiol.* 23, 1081-1094.

Venugopalan A.K., Benny T.M. and Nair V.S.K. (1994). Testicular development and spermatogenesis in *Spodoptera mauritia* Boisd. (Lepidoptera: Noctuidae). *J. Anim. Morphol. Physiol.* 41, 125-134.

Verson E. (1889). Zur Spermatogenesis. *Zool. Anz.* 12, 100-103.

Vishwanath C.S. and Mittal P.K. (1974). Morphological and cyto chemical studies in the spermatogenesis of *Locusta migratoria* (L). *Research Bulletin* (N.S) *of Punjab University* Vol. 25, Parts, 1-11, pp. 9-15.

Watson R.D. and Bollenbacher W.E. (1988). Juvenile hormone regulates the sterioidogenic competence of *Manduca sexta* prothoracic glands., *Molec. Cell. Endocrinol.* 57, 251-259.

Wigglesworth V.B. (1936). The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). *Quart. J. Micr. Sci.* 79, 91-121.

Williams C.M. (1967). The juvenile hormone II. Its role in the endocrine control of molting, pupation and adult development in the cecropia silkworm. *Biol. Bull. woods Hole.* 121, 572-585.

Wing K.D. (1988). RH-5849, a nonsteroidal ecdysone agonist; Effects on a Drosophila cell line. *Science.* 241, 467-469.

Wing K.D., Slawecki R.A. and Carlson G.R. (1988). RH-5849, a non-steroidal ecdysone agonist: effects on larval Lepidoptera. *Science*. 241, 470-472.

Wolf K.W. (1990). Sheathed nuclear division in primary spermatocytes of *Orgyia antiqua.* (Lepidoptera, Insecta). *Bio systems.* 24 (1), 5-15.

Wolf K.W. (1993). Extracellular tubular elements within testes of *Phragmatobia fuliginosa L.* (Arctiidae: Lepidoptera). *Invert. Reprod. Dev.* 24 (2), 137-142.

Wolf K.W. (1996a). Cytology of Lepidoptera. VIII. Acetylation of alpha-tubulin in mitotic and meiotic spindles of two Lepidoptera species, *Ephestia kuehniella* (Pyralidae) and *Pieris brassicae* (Pieridae). *Protoplasma* 190, 88-98.

Wolf K.W. (1996b). Immunocytochemical evidences of alpha tubulin reserve at the tip of the Mediterranean meal moth, *Ephestia kuehniella* Z. (Pyralidae: Lepidoptera). *Acta Zoologica (Stockholm).* 77, 79-84.

Wolf K.W. and Kyburg J. (1989). The restructuring of the flagellar base and the flagellar necklase during spermatogenesis of *Ephestia kuehniella* Z. (Pyralidae: Lepidoptera). *Cell Tissue Res.* 256, 77-86.

Wolf K.W. and Mesa A. (1993). Synaptonemal polycomplexes in spermatids: a characteristic trait of Orthoptera. *Chromosome Research*, 1, 181-188.

Wyss C. (1976). Juvenile hormone analogue counteracts growth stimulation and inhibition by ecdysones in clonal *Drosophila* cell line. *Experientia.* 32, 1272-1274.

Yagi S. (1975). The role of juvenile hormone in diapause and phase variation in some lepidopterous insects. In *The Juvenile Hormones* (Ed. Gilbert L.I). pp. 288-300. Plenum Press, New York.

Yagi S. and Fukushima T. (1975). Hormonal effects on cultivated insect tissues II. Effects of juvenile hormone on spermiogenesis of the silkworm, *Bombyx mori.* L., *In vitro* (Lepidoptera: Bombycidae). *Appl. Ent. Zool.* 10, 77-83.

Yagi S. and Kuramochi K. (1976). The role of juvenile hormone in larval duration and spermiogenesis in relation to phase variation in the tobacco cut worm

Spodoptera litura (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* 11, 133-138.

Yagi S., Kondo E. and Fukaya M. (1969). Hormonal effect on cultivated insect tissues I. Effect of ecdysterone on cultivated testes of diapausing rice stem borer larvae. *Appl. Ent. Zool.* 4, 70-78.

Yamashiki N. and Kawamura N. (1997). Behaviours of nucleus, basal bodies and microtubules during eypyrene and apyrene spermiogenesis in the silkworm, *Bombyx mori. Dev. Growth Differ.* 39 (6), 715-722.

Yasuzumi G. (1974). Electron microscope studies on spermiogenesis in various animal species. *Int. Rev. cytol* 37, 53-120.

Yasuzumi G. and Oura C. (1964). Spermatogenesis in animals as revealed by electron microscopy XIII. Formation of a tubular structure and two bands in the developing spermatid of the silkworm, *Bombyx mori. Zeitschrift fur Zellforschung und Mikroskopische Anatomie,* 64, 210-226.

Zick K. (1911). Die postembryonale Entwicklung des mannlichen Geschlechtsapparats der schmetterlinge. *Z. Wiss. Zool.* 98, 430-447.

Zylberberg L. (1969). Contribution a l' 'etude dela double spermatogenèse chez un lépidoptère (Pieris brassicae L., Pieridae). *Annales des sciences Naturelles, Zoologie.* 11, 569-626.