CHAPTER 6

ULTRASTRUCTURAL STUDIES ON
REPRODUCTIVE SYSTEM
6.1 INTRODUCTION

The entire lives of insect depend on efficient reproductive capacities of the species, which in most insects are regulated by accessory reproductive glands among other factors (Gillott, 1998). Studies on the nature of the components of accessory glands and secretions in insects are rather scanty (Gillott, 1988; Kaulenas, 1992), in spite of the importance of these substances to insect reproduction. Accessory gland secretions of male insects may affect virtually all aspects of female reproductive activity and improve its reproductive efficiency (Gillott, 2003). Reproductive cells of insects have been studied through the use of electron microscopy by a few investigators (Dallai and Afzelius, 1980; 1982; Danilova et al., 1984; Jamieson et al., 1999; de Almeida and Cruz-Landim, 2000; Alves et al., 2006). The ultrastructure of the reproductive tract of male honey bee, Melipona bicolor, has been described by Dallacqua and Cruz-Landim (2003). There is little information available on the ultrastructure of the reproductive system of heteropterans (Dallai and Afzelius, 1980; 1982; Jamieson et al., 1999). The ultrastructure of the sperm of water strider (Tandler and Moriber, 1966; Turner 1972) and Gerris sperm (Tandler and Moriber, 1966) are extensively studied.

The studies on the ultrastructure of heteropteran testes, mesadenes and the germ cells of testes are scanty (Furieri, 1963; Rosati et al., 1976; Dallai and Afzelius, 1980; Itaya, 1980; Dorn et al., 1992; Motzko, 1992). To date no
information is available on the changes in the ultrastucture of testes and mesadenes of heteropterans due to carbaryl toxicity and hence this study.

6.2 MATERIALS AND METHODS

6.2.1 Tissue preparation for electron microscopy

6.2.1.1 Scanning electron microscopy

The scanning electron micrographs of spermatozoa in the vasa deferentia were examined in the present study. The vas deferens of male reproductive system was taken and tore with a fine needle to liberate the sperms and fixed the contents in 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) for about 24 hr. The tissue was dehydrated in alcohol series and finally dipped in isoamyl acetate. Dehydrated samples were transferred into Whatman No.1 filter paper, dried in a critical point drier with liquid carbon dioxide for half an hour. The sample was then put on adhesive pad, labelled and coated with gold and observed under scanning electron microscope (Hitachi S-2400).

6.2.1.2 Transmission electron microscopy

a) Ultrathin sectioning

The dehydration procedure of the tissue samples (testes and mesadenes) were described elsewhere (see, chapter 5). The block surface was
trimmed using a diamond knife (Diatome\textsuperscript{®}) to yield ultrathin sections of 50-70 nm. The sections were taken on to the shiny side of the copper grid.

**b) Staining for transmission electron microscopy**

The ultrathin sections were stained in uranyl acetate, by immersing the grids with the section side up, for 2 hrs. This was followed by washing the sections in methanol-water mixture (100\%, 80\% and 50\%). Thereafter the sections were stained for 10 min in lead citrate in 0.1 N NaOH (prepared in CO\textsubscript{2} free water). The grids were washed in four changes of distilled water and air dried. Stained ultrathin sections were observed under the electron microscope (Model Hitachi H-600) at an accelerating voltage of 75 KV and photographs (Kodak III ford film) were taken.

**6.3 RESULTS**

**6.3.1 Ultrastructural studies on mesadenes**

The ultrastructure of mesadenes of *I. limbata* are shown in figures 30-32. The ultrastructure of mesadenes of *I. limbata* show secretory epithelium with outer muscular sheath and are two layered around the cylindrical gland and the basement membrane surrounds the muscle layer. Numerous mitochondria are visible in the secretory cell (Fig. 30 A). Newly formed secretory vesicles in the mesadenes of *I. limbata* shows dense irregular plaques scattered at the surface of the vesicle (Fig. 30 B). The apical region of
the secretory cell shows tightly packed secretory granules are (Fig. 30 C). The tracheoles are enclosed in the cellular coat of mesadenes (Fig. 30 C, D and 31 A, B). There are vacuoles visible in the secretory cells of treated insects (Fig. 31 D). The nucleoplasm appear more dissociated from each other in the treated insects compared with the normal (Fig. 32 B). The chromatin granules are scattered with slight obliterations in treated mesadenes cell nucleus compared with control. The empty spaces in the cytoplasm and nucleoplasm of the secretory cells of mesadenes are more prominent in treated insects compared with the normal (Fig. 31 D and 32 B).

6.3.2 Ultrastructural studies on testes

The ultrastructure of testis wall is given in figure 33. The figure shows that the testes of *I. limbata* are externally surrounded by two membranes, tunica externa and the tunica interna. A number of mitochondria (presumptive mitochondria) are seen in the inner layer (Fig. 33 A). In the treated insect, the testis wall contains many lipid droplets and vacuoles compared to normal (Fig. 33 B).

6.3.3 Ultrastructural studies on spermatogenesis

The scanning electron micrographs of spermatozoa in the vasa deferentia of *I. limbata* are presented in figures 34-36. The vas deferens of *I. limbata* contains unbundled long spermatozoa. Both ends of the sperm are pointed with slightly prominent triangle shaped head region (see, arrow in
Fig. 35 A). The diameter of the sperm is below 1µ (Fig. 36). There is no observable morphological difference in the spermatozoa of treated insects in scanning electron micrographs.

The ultrastructure of different stages of spermatogenesis is presented in figures 37-40. The sex cells in the testes such as early and late spermatids are described in figure 37. The early spermatid of *I. limbata* showed a prominent proacrosomal vesicle (Fig. 37 A) which give rise to dense acosome cap in the late spermatid (Fig. 37 C). The late spermatid of *I. limbata* shows round large nucleus, centriole adjunct, and an axonemal and mitochondrial basis. During the development of the spermatids in the testis of *I. limbata* showed stretching and extensive elongation of the nucleus which transforms the sperm into a cylindrical shape (Fig. 37 E-F). The C.S. of the sperm head of *I. limbata* showed thick ring of tubular acrosome (Fig. 40 C). The anterior region of the sperm shows mitochondrial derivative and axoneme in the centre (Fig. 40). The mitochondrial derivative of *I. limbata* showed electron dense and electron transparent area (Fig. 38 E). The formation of 2nd axoneme in *I. limbata* is depicted in figure (see, arrow in Fig. 37 B and 38 F). There are two axonemes towards the posterior region of sperm tail (see, arrows in Fig. 39 C). The crystalline structure is clearly visible in the L.S. as fish bone (Fig. 38 C). The L.S. of the mid region of sperm tail showed mitochondrial derivatives and axoneme (Fig. 39). Towards the posterior region of the sperm tail of *I. limbata* the mitochondrial derivatives become thin and are less visible making
the two axoneme clearly visible (Fig. 39 C). The C. S. of the sperm showed cross bridge between mitochondrial derivative and axoneme it was prominent towards the tail region (Fig. 39 D). The developmental stages of sex cells in the testes show large number of vacuoles and lipid droplets in treated insects than normal (Fig. 37 D, 38 C, D, F and 40 B). The axoneme is flanked by two mitochondrial derivatives with no observable difference between normal and treated insects.

6.4 DISCUSSION

The wall of the paired accessory glands, mesadenes of *I. limbata* consists of single layer of epithelial cells. The basal surface of mesadenes bordering the hemocoel is covered with a thin basement membrane and muscle bands spin, below this lies the nuclei. The muscular layer showed tracheoles in between the outer and inner basement membrane. The apical region of the cell consists of abundant mitochondria. The apical region of mesadenia is filled with secretory epithelial cells packed with abundant secretory granules. The ultrastructure of mesadenia of *I. limbata* is comparable to the features described in *O. fasciatus* (Dorn et al., 1992).

There is some marked ultrastructural difference in the nucleus and in the basement region of mesadenes compared to normal. The corpora allata removed nymphs of cockroach showed partially developed accessory glands and tubule (decreased tracheation) formation (Dixon and Blaine, 1973). Thus,
insects with a defective nervous system neither juvenile hormone nor ecdysone is being produced. The ecdysone analogue, ecdysterone injected into the newly metamorphosed and allatectomized adults, observed increased tracheation (Dixon and Blaine, 1973). Changes in tracheation also affect the efficiency of cell respiration. The tracheolation observed in the carbaryl treated insects needs further studies and clarification. The changes in the accessory gland observed in the insects treated with carbaryl can be compared to that of corpora allata removed nymphs.

The chromatin granules are scattered with slight obliteration in treated mesadenia cell nucleus of *I. limbata* compared to normal insects. The empty spaces in the nucleoplasm of the secretory cells of mesadenes are more prominent in treated insects compared to the normal. The nuclei are pressed together in the case of BHC poisoning with scattered chromatin granules (Misra, 1981). The destruction of cytoplasmic and nuclear material, nuclear disarray and shrinkage, and consequent vacuolization of the cells are observed in fish, *C. punctatus* treated with organophosphate, malathion (Dubale and Shah, 1979). The nuclei are not apparently discernible due to their reduction in size with malathion and phosphamidon in the midgut tissues of adult *Hieroglyphus nigrorepletus* after 24 hrs of single lethal dose (Misra, 1981).

The wall of the testes of *I. limbata* showed the basic features as seen in other insects (Tembhare, 1997). The testes of *I. limbata* showed the
developmental stages of sperm. Insect spermatozoa are generally filamentous and consist of a tiny triangular head connected to a very long tail (Jamieson et al., 1999). The scanning electron micrographs of spermatozoa in the vasa deferentia of *I. limbata* showed triangular head with long tail as seen in *Palembus dermestoides* (de Almeida and Cruz-Landim, 2000).

The early spermatid of *I. limbata* contains a prominent proacrosomal vesicle that give rise to the acrosome in the late spermatid. The formation of spermatid has been described by Danilova et al. (1984) and Jamieson et al. (1999) in heteropteran insect. The round chromatin dense nucleus in the early stage of sperm formation (spermiogenesis) and thin, comma-shaped nucleus during elongation in *I. limbata* is comparable with acridae spermiogenesis (Alberti and Storch, 1976). During spermiogenesis the chromatin condenses and the nucleus strongly elongates. The acrosomal vacuole elongates during spermiogenesis, and finally possesses a cylindrical shape. The contents of the acrosome of *I. limbata* are uniformly dense in most of the areas. The acrosome is usually located in the apical end of the sperm head in hemipteran Nepomorpha, it lies along the sperm head (Lee and Lee, 1992). The apically placed acrosome of spermatid has a conical shaped electron dense region in *I. limbata*. The sperm head of *I. limbata* showed a thick ring of tubular acrosome as seen in the Nepomorpha bug (Lee and Lee, 1992). In some insects, however, centriole adjunct material is present early in spermiogenesis disappears before maturity. The function of the centriole adjunct has been
generally considered as a head to tail attachment structure (Gatenby and Tahmisian, 1959). The centriole adjunct observed in the early stage of the spermiogenesis of *I. limbata* is very thick and dense in appearance in. It usually surrounds the base of the mitochondrial derivatives and the axial filament complex where all of these structures attach to the posterior end of the nucleus.

The tail region of the spermatozoa of *I. limbata* consists of two mitochondrial derivatives which extend in parallel along the flagellum. During spermiogenesis it extends along the flagellum in most insect species. The length and size is enormous and occupy the largest part of the spermatozoon in some insects (Afzelius *et al.*, 1976; Mazzini, 1976; Pitnick *et al.*, 1995). The longitudinal accessory bodies are absent in *I. limbata* as like other heteropterans (Dallai and Afzelius, 1980). In *I. limbata*, mitochondrial derivative has crystalline material. The crystalline material in heteropteran mitochondrial derivatives has a "fish bone" pattern in longitudinal sections (Rosati *et al.*, 1976; Baccetti *et al.*, 1977). The crystalline material of mitochondrial derivative contains high percentage of proline (Baccetti *et al.*, 1977). A decline observed in the titer of proline in hemolymph treated insect (see, chapter 3) may lead to structural deformities in crystalline structure of the mitochondrial derivative. However the ultrastructural studies do not indicate any observable difference in the crystalline structure of normal and treated group of insects with limited resolution.
The present study showed biflagellarity of the spermatozoa of *I. limbata*. The heteropterans are characterized by two flagella towards the posterior region of tail (axial filament). The biflagellation can be seen only towards the posterior of tail (Klowden, 2002). In *I. limbata* towards the posterior of tail region two axoneme of the flagellum is clearly visible, one is smaller. Furieri (1963) claims that the spermatozoon of *P. apterus* has two flagella proximally, surrounded by the two mitochondrial derivative but one is shorter so that distally only one, still bordered by the mitochondria is seen.

The primary spermatocytes of *S. littoralis* (Godula, 1985) and *D. melanogaster* (Rasmussen, 1973) are reported to have four flagella. The insect *I. limbata* has two flagella. There are insect spermatozoa with no flagellum (Dallai et al., 1975; Dallai and Afzelius, 1994) and those with a hundred flagella (Baccetti and Dallai, 1978). He regards larger numbers of flagella observed is abnormal. Trandaburu and Trandaburu (1971) noted biflagellate condition as occasional variant in *Graphosoma italicum* (Heteroptera), 2 or 4 flagella with a pair of mitochondrial derivatives regarded as abnormal (Muramoto and Mizoguchi, 1980).

The axoneme is characterized with the short hand formula 9 + 9 + 2, i.e., nine accessory microtubules, nine peripheral doublets and two central microtubules as it was found in most insects (Jamieson et al., 1999). In *I. limbata* nine accessory microtubules, nine peripheral doublets and two central
microtubules are visible as described in lepidopteran (Alves et al., 2006) and in heteropterans (Rosati et al., 1976).

The two thirds of the axoneme of *I. limbata* are surrounded by the mitochondrial derivatives. In the sperms of the fire-bug, *P. apterus* (Pyrrhocoridae), the derivative-axonemal bridges have usually large end-feet which are curved and solid (Jamieson et al., 1999). In *Lygaeus equestris* (Lygaeidae) the derivatives are irregular in outline and contact each other at one point only. In the case of *I. limbata* the two mitochondrial derivatives are mirror images with no connection with each other. The mitochondrial derivative of heteropterans is large comma-shaped, medianly contiguous and almost completely embracing the axoneme (Werner, 1966; Lee and Lee, 1987, 1991). Rosati et al. (1976) have described the mitochondrial derivatives as remarkably uniform and partially crystallized in *Gonocerus insidator*.

The testes wall and the germ cells in the testes of treated insect show many vacuoles and lipid droplets in the outer coat which are limited in the normal insect. Each single spermatozoon is surrounded by a secretion sheath. Within the secretion matrix three different kinds of secretions are present. The first type of secretion consists of small droplets which are densely distributed and characterized by a bright center and a dark border. The second kind is a large electron-dense secretion droplet with a very irregular shape. These droplets often partially surround the spermatozoa. The third and rarest type of
secretion is a droplet which appears less electron-dense (Jamieson et al., 1999). In the treated insect there is an immense in the size of vacuoles and lipid droplets. Marked vacuolation in the testes, testes sperm, and the basal region of the mesadenes of *I. limbata* are observed in treated insects. The X-irradiated testes of *Dermestes frischii* (Coleoptera) showed increased vacuole formation and lipid droplets in the testes wall (Hodge, 1983). Similar abnormalities have been found in the epithelial cells of kidneys in rats and monkeys with carbaryl exposure (Serrone et al., 1966).

Inhibition of cholinesterase causes accumulation of acetylcholine in synapses, resulting in different malfunctions of the nervous system (Fukuto, 1990). Toxic injury to the reproductive system can results from chemical action of the central nervous system and or gonads to interfere with the complex hormonal regulation by modifying ovulatory functions and altering spermatogenesis. Also, some toxic agents can act on the development of gonads in the fetus and compromise reproductive function upon sexual maturity (Shank, 2004). The results of the present study depict histological deformities in the mesadenes, in the testes and spermatogenesis in testes of insects compared to normal groups. The results presented here also provide evidence for toxic effects of carbaryl to non-target organisms like *I. limbata* and therefore on environmental pollution.