EFFECTS OF CARBARYL ON THE MALE REPRODUCTIVE SYSTEM OF *IPHITA LIMBATA*

Thesis submitted to the University of Calicut for the Degree of Doctor of Philosophy in Zoology under the Faculty of Science

By

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CERTIFICATE

This is to certify that the thesis entitled "Effects of carbaryl on the male reproductive system of *Iphita limbata*", is an authentic record of research work carried out by Miss. Binitha, V.S., under my guidance and supervision, in partial fulfillment of the requirement of the Degree of Doctor of Philosophy under the Faculty of Science, University of Calicut. No part of this thesis has been presented previously for any other Degree.

Dr. K.V. Lazar
(Supervising teacher)

DECLARATION

I, Binitha, V.S., do hereby declare that the thesis entitled "Effects of carbaryl on the male reproductive system of *Iphita limbata*", is a research work carried out by me in the Department of Zoology, University of Calicut, under the guidance of Dr. K.V. Lazar. I further declare that no part of this thesis has been submitted previously for any other Degree.

Calicut University,
December 17, 2008.

Binitha, V. S.

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Dedicated to

My Beloved Parents

GENERAL INTRODUCTION

Food production and disease control are the most important challenges facing humanity in the 21st century that deserves special attention. These challenges are associated with the increasing global human population and with the control of arthropod pests. Arthropod pests are responsible for global harvest crop losses of approximately 20-50% of potential production and for transmitting a number of world's most important diseases (Thacker, 2002). Therefore controlling the arthropod pests is critical for the enhanced food production and disease control. The persistent use of organic pesticides has saved millions of lives by controlling human diseases and by greatly increasing the yields of agricultural crops.

Insect pest control, an essential component of crop protection and public health, has evolved over a recorded history of three millennia (Casida and Quistad, 1998; Ware, 2000). Plants were used as sources of insecticidal compounds by the Egyptians during the time of Pharaohs. Sulfur was first referred to by Homer in 1000 BC as a fumigant for pest control, and in California, it is still used in larger amounts than any other pesticide. Pliny, in 77 AD, makes reference to the use of arsenic, soda and olive oil. Chinese employed moderate amounts of mercury and arsenic for body louse control by the AD 100-200. Nicotine in the form of tobacco extracts were reported in 1690s as the first plant derived insecticide, followed by the pyrethrums and rotenone in the early 1800s. Synthetic organics in the 1940s to 1970s largely replaced with inorganic and botanicals with the introduction of

organophosphates, methylcarbmates, organochlorines and pyrethroids, and are still widely used today for insect control (Thacker, 2002; Tomizawa and Casida, 2005). The main target for these chemical compounds is the insect nervous system, at a very limited number of primary points: acetylcholinesterase for organophosphates and carbamates, sodium channels for DDT and pyrethroids, octapamine receptors for formamidine and GABA-gated chloride channels for cyclodienes and avermectins (Graf, 1993).

The number of pesticide application increased year after year. The major problem with these broad spectrum modern insecticides concerns their effect on both target and non-target beneficial species. Van den Bosch (1966) reported that the application of insecticide produced a greater loss of beneficial arthropods than any other agricultural practices. Another deplorable aspect of the utilization of broad spectrum toxins for insect control has been associated with their effect on the environment. Most of these pesticides are discharged into the soil and aquatic system and found to be highly toxic to many organisms in the ecosystem. Human can be exposed to pesticides by eating contaminated food or drinking contaminated water, by inhaling pesticide droplets from the atmosphere, by coming into contact with treated vegetation or through involvement in their manufacture or application.

In recent past, the carbamate compounds are preferred over other pesticides because of their reversible inhibitory effect on acetylcholinesterase

(Goldberg *et al*., 1963; Pradhan and Mhatre, 1970; Bhavan and Geraldine, 2002; Srinivasan, 2005). Among these carbamates, carbaryl was found to be the most commonly detected pesticide in the environment (Caroline, 1994) which is used to kill a range of chewing and sucking insects on over 120 agricultural crops over 100 species of insects (Caroline, 1994; Clive, 1994; WHO, 1994). Carbaryl persists in the plants (Antonovich, 1970; Molozhanova, 1970) and fruits after treatment (Antonovich, 1970; Yadav and Jaglan, 1982; Frank *et al*., 1989). Based on the anticipated coverage of plant protection measures, the requirements of technical grade pesticides have been estimated and approximately 80,000 metric tons in India during 1982 and the production of carbaryl used in the present study was estimated to be 7,500 metric tons during that year (Krishnamurthy and Dikshith, 1982).

As like most other pesticides carbaryl also pollute every component of the ecosystem and found to be highly toxic to many non-target organisms, like predators and parasites of the insect pest, earthworms, some of the predatory vertebrates etc. and can also concern their adverse effects upon the human population. The sub-lethal dose of carbaryl exposure to organisms resulted in abnormalities of sperm and damages in the testis of rats, mammals and some other animals (Meeker *et al*., 2004; Xia *et al*., 2005). There is some fair amount of direct but frequently contradictory experimental evidence showing the effect of sub-lethal dosage of insecticide carbaryl on female insect reproductive system of western corn root worm (Ball and Su, 1979).

Hitherto hundreds of contributions to the topographical anatomy of insects including the reproductive system have been made. However, the morphology of the heteropteran male reproductive system was studied in a few insects (Ambika, 1973; Sareen and Kaur, 1987; Dorn *et al*., 1992; Lemos *et al*., 2005). There have been only a few studies so far on spermatogenesis in hemipteran insects (Ambika, 1973; Sareen and Kaur, 1987). Further the effects of carbaryl on the reproductive system of hemipteran insects were not studied. In the present study a heteropteran bug, *Iphita limbata*, was taken as a model organism to study the effects of carbaryl on biochemical and histological aspects in the male reproductive system of non-target organisms. The structure of the male reproductive system of *I. limbata* was also studied in detail. The present study also focuses on the toxicity of carbaryl in general to non-target organisms and its importance in assessing the public and environmental health risks.

CHAPTER 1

REVIEW OF LITERATURE

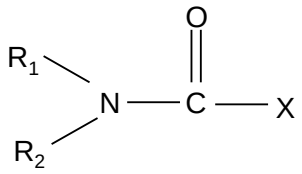
1.1 Introduction

The carbamates are the esters of carbamic acid and represent one of the three major groups of modern synthetic insecticides. Carbamates were first developed by the Geigy Corporation in 1951 but not made commercially available until 1956 (Baron, 1991). Research into carbamates began following a search for compounds other than organophosphates that had anticholinesterase activity. One of the known compound of this group was alkaloid physostigmine, found in the Calabar bean (*Physostigma venenosum*), which had been used for trial by ordeal in its native West Africa. The toxic constituent, physostigmine or eserine, was isolated one hundred years ago and identified in 1925 as the methylcarbamate ester of eseroline. Physostigmine is the only known naturally occurring carbamate ester. Successful development of carbamates as insecticides was initiated by the researches of Hans Gysin in Switzerland, and Robert Metcalf and co-workers in the United States (Hussel, 1990).

1.2 Carbamates

The carbamates are produced from carbamic acid and have a similar mode of action that of organophosphates as they also block the enzyme acetylcholinesterase (Thacker, 2002). This process of enzyme inhibition is called carbamylation (Oonnithan and Casida, 1968). The carbamylation process is relatively less stable, i.e., the enzyme is not blocked for so long.

Because of its action as a reversible cholinesterase inhibitor its use is preferred over other insecticides like organophosphates and chlorohydrocarbons, which are irreversible cholinesterase inhibitors. Cholinesterase depression is common to all the carbamate pesticide both in blood and tissues. The reversibility of acetylcholinesterase inhibition confers advantage to carbamates over organophosphates (Thacker, 2002). The general structure of carbamate is shown below



In which R_1 and R_2 are hydrogen, methyl, ethyl, propyl or short chain alkyls and X is phenol, naphthalene or other hydrocarbon rings.

The commonly used carbamates are given in table 1.

Table 1: The commonly used carbamates

Sl. No.	Common Name	Chemical Name	Trade Name
1.	Aldicarb	2-Methyl-2(methylthio) propionaldehyde-o-(methyl carbamoyl) oxime	Temik®
2.	Bendiocarb	2,2-Dimethyl-1,3-benzodiox-ol-4-yl N-methylcarbamate	Dycarb®, Tatto®, Seedox®
3.	Carbaryl	1-Naphthyl-N-methylcarbamate	Sevin®, Carbacide®

4.	Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranyl N-methylcarbamate	Furadan®, Brifur®
5.	Carbosulfan	2,3-Dihydro-2,2-dimethyl 7-benzofuranyl [(dibutylamino) thio] N-methylcarbamate	Advantage®, Marshal®
6.	Dioxycarb	2-(1,3-Dioxolan-2-yl) phenyl-N-methylcarbamate	Electron®, Famid®
7.	Formentanate HCl	3-Dimethylaminomethylene-amino phenyl N-methylcarbamate	Carzol®, Dicarzol®
8.	Mecarbam	S-(N-ethoxycarbonyl-N-methyl carbamoylmethyl)o,o-diethyl phosphorodithioate	Afos®, Murfotox®, Pestan®,
9.	Methiocarb	3,5-dimethyl-4-(methylthio)-phenyl N-methylcarbamate	Draza®, Mesurol®, Slug Guard®
10.	Methomyl	S-Methyl-N-[(methyl carbamoyl) oxy]theoacetimidate	Lannate®, Lanox®
11.	Mexacarbate	4-Dimethylamino-3,5-xylyl-N-methylcarbamate	Zectran®
12.	Oxamyl	N-N-dimethyl-2-methyl carbamoyl oxyimino-2-(methylthio) acetamide	Vydate®
13.	Pirimicarb	2-(Dimethylamino)-5,6,2-isopropoxyphenyl N, N-dimethylcarbamate	Abol®, Afox®
14.	Propoxur	2-Isopropoxyphenyl-N-methylcarbamate	Baygon®, Propagan®
15.	Thiodicarb	Dimethyl-N,N-{thiobis(methyl imino)carbonyloxy}-bis(ethanimidothioate)	Larvin®, Nirval®
16.	Trimethocarb	4:1 mixture of the 3,4,5- and 2,3,5-isomers of trimethyl phenyl N-methylcarbamate	Broot®, Landrin®

1.2.1 Carbaryl

The most important carbamates to have been developed include carbaryl, carbofuran, methiocarb, pirimicarb, propoxur and aldicarb (Thacker, 2002). The first carbamate to be used was carbaryl. This was first synthesized in 1953 and introduced for use in crop protection in 1956 (EPA, 2003). In India carbaryl was introduced by UNION CARBIDE in the early 1960s (whose pesticide interests were taken over by RHONE-POULENC after Bhopal gas diaster). Currently, over 300 products containing carbaryl are actively registered with the EPA (PBPPD, 2001). Its low mammalian toxicity has meant that it has been widely used around the home as well as in agricultural production.

Carbaryl controls insect pests of field crops, forage, vegetables, fruits, nuts, shade trees, ornamentals, forests, lawns, turf and rangeland, as well as pests of domestic animals (Caroline, 1994; WHO, 1994). It is mostly used against caterpillar pests on apples, pests on citrus fruit, mangoes, bananas, strawberries, nuts, vines, olives, okra, cucurbits, peanuts, soyabeans, cotton, rice, tobacco, cereals, beet, maize, sorghum, alfalfa, potatoes, ornamentals and forestry. Carbaryl is used to control a variety of pests, including moths, beetles, cockroaches, ants, ticks and mosquitoes (Tomlin, 2000). Carbaryl is used against ectoparasites of humans and animals, including against head louse on children (Whitehead, 1995). Carbaryl formulations include baits,

dusts, wettable powders, granules, and oil, molasses, and aqueous dispersions and suspensions (EPA, 2003).

The chemical name of carbaryl is 1-napthyl N-methylcarbamate (EPA, 2003). Its structure is shown in figure 1.

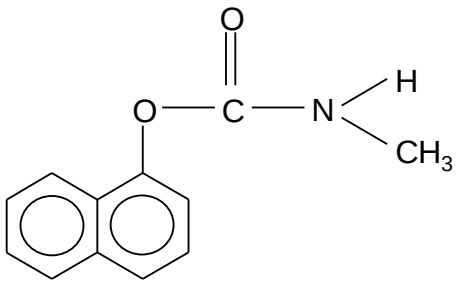


Figure 1: Chemical structure of carbaryl.

The common name carbaryl is in general use except in Eastern Europe, where aryl alum is used, and in the USSR, where the trade name Sevin® is used as a common name. Other trade names have included Atoxan®, Caprolin®, Carbacide®, Carbamine®, Carpolin®, Cekubaryl®, Denapon®, Denopton®, Devicarb®, Dicarbam®, Gamonil®, Hexavin®, Karbaspray®, Karbatox®, Karbosep®, Mervin®, NAC®, Panam®, Rayvon®, Septene®, Sevinox®, Sevidol®, Tercyl® and Tricarmam®. The code designations are ENT 23969, UC 7744 and OMS 29. The CAS (chemical abstract service) registry number is 63-25-2 (Baron, 1991).

1.2.1.1 Physical and chemical properties

Carbaryl is a white to light tan solid with a mild phenolic odour (HSDB, 1997). It has the empirical formula, $C_{12}H_{12}NO_2$ and a molecular weight of 201.20. The melting point of carbaryl is 142 ^{o}C and a vapour pressure of less than 4×10^{-5} mm Hg at 26 ^{o}C (Kidd and James, 1991). Carbaryl has low volatility and low air-water partition coefficient. Thus, only limited evaporation can be anticipated after treatment (Baron, 1991). The dimensionless air-water partition constant for carbaryl (Henry's law constant) was found to be 5.3×10^{-6} (Schemburg *et al*., 1991). Lee *et al*. (1990) calculated that, 50 days after treatment, 0.63% of the carbaryl applied to soil could have been volatilized and 78.84% degraded.

Carbaryl disrupts the normal functioning of the insect nervous system and cause toxicity by contact or ingestion (Tomlin, 2000). It also disrupts nervous system by adding a carbaryl moiety to the active site of the acetylcholinesterase enzyme, which prevents it from interacting with acetylcholine (Klassen *et al*., 1996). The chemical neurotransmitter acetylcholine is used to relay nervous system signals across the nerve synapse. Acetylcholinesterase is the enzyme responsible for the breakdown of acetylcholine once it is released into the synapse. When the enzyme is inhibited, surplus acetylcholine builds up, resulting in nervous overstimulation. The carbaryl group is released from the active site of

acetycholinesterase by spontaneous hydrolysis and restoring nerve function (Gray, 1996).

1.2.1.2 Absorption and distribution

The toxicity of carbaryl is greatly influenced by the vehicle and route of exposure and the importance of these factors vary among the insecticidal carbamates (Baron, 1991). Carbamates are readily absorbed during passage through the gastrointestinal tract, and absorption is so partly related to the vehicle in which they are administered (IPCS, 1994). The most important human exposure route is dermal, and those occupationally exposed, such as insecticide formulators and applicators and farm workers (Baron, 1991). The greatest risk to these individuals would be from working with carbamates under conditions of high temperature. Low-level exposure to residues in foods may occur wherever carbamates are used on edible commodities and where tolerances have been granted for such uses. Once absorbed, carbaryl was rapidly distributed to the tissues and organs. Metabolism and eliminations are relatively rapid, no evidence has been found for bioaccumulation of carbamates (Baron, 1991).

Penetration of carbaryl through rat skin depended on the solvent, being greater in acetone than in benzene or corn oil, rapid early penetration was by the parent compound (O'Brien and Dannelley, 1965). In a percutaneous absorption study with rats, about 57% of a continuously applied dose of [^{14}C]

carbaryl in acetone penetrated the shaved skin in 168 hr. The absorption rate was 0.18 μg/cm^2/hr; $t_{1/2}$ was 1.26 hr for absorption and 67 hr for elimination (Knaak *et al*., 1984). In mice, $t_{1/2}$ for acute dermal penetration of [^{14}C] carbaryl in acetone was 12.8 min, and the label was detected in the blood, tissues and excreta within 5-15 min after application. By 8 hr after application, 73.3% of the dose had appeared in the excreta, while 4.9% remained in the intestine and 2.6% in the liver; levels in other tissues and organs ranged from less than 0.1% to 0.6% (Shah *et al*., 1981). In rats, carbaryl was absorbed more rapidly from the intestine than from the stomach (Cambon *et al*., 1981). In mice, about 69% of an intubated dose of carbaryl was absorbed within 60 min. The $t_{1/2}$ for absorption was 17 min. Within an hour, 16.9% of the dose appeared in the urine and 8.6% in exhaled CO_2 (Ahdaya *et al*., 1981).

The gastric intubation of rats with [^{14}C] carbaryl, the percentage of the dose per gram of tissue ranged from less than 0.1% to nearly 0.4% after 1 hr; levels had significantly declined in liver and fat (Tanaka *et al*., 1980). The acute oral exposures of rats to carbaryl at 450-1500 mg/kg, residues were detected in tissues at 48 hr after dosing. In rats that died, minimum residue levels were 11.7 ppm in the liver, 5 ppm in the brain and 3.6 ppm in the heart (Mount *et al*., 1981).

1.2.1.3 General metabolism

As with other carbamates the principal metabolic pathways are hydroxylation, hydrolysis, and epoxidation, resulting in numerous metabolites subjected to conjugation, forming water-soluble sulfates, glucuronides, and mercapturates (Carpenter and Livestone, 1961; Dorough *et al*., 1963; Dorough and Casida, 1964; Knaak *et al*., 1965; Menzie, 1969; Bend *et al*., 1971). Hydrolysis of carbaryl results in the formation of 1-napthol, carbon dioxide, and methylamine (Carpenter and Livestone, 1961; Sakai and Matsumura, 1971). Hodgson and Casida (1961) have reported the first evidence for carbaryl hydroxylation. Carbaryl is metabolized by a rat liver microsome system, requiring $NADPH_2$ and oxygen, to form formaldehyde yielding derivative.

Animals metabolize carbaryl both by hydrolytic (hydrolysis and hydroxylation) and non-hydrolytic pathways. The principal metabolic pathways such as hydrolysis and ring hydroxylation metabolize carbaryl to 1-naphthol and hydroxylated naphthylmethylcarbamate, which form water-soluble sulfates, glucuronides and mercapturates. Carbaryl rapidly metabolized in mammals to 1-napthol, CO_2 and methylamine by hydrolysis (Baron, 1991). The hydroxylation resulted in 4-hydroxycarbaryl, 5-hydroxycarbaryl, N-hydroxymethylcarbaryl, 5,6-dihydro-5,6-dihydroxycarbaryl and 1,4-naphthalendiol. Carbaryl is soluble under normal

storage conditions but is hydrolyzed rapidly at pH 10 or above (PIP, 1996). Biotransformation of carbaryl is basically similar in humans, rats, guinea pigs, monkeys and sheep; the major difference being the extent to which carbaryl was hydrolyzed to yield 1-naphthol. Much less hydrolysis occurs in monkeys or pigs than in humans (Knaak *et al*., 1968; Sullivan *et al*., 1972; Lin *et al*., 1975).

Carbaryl has been shown to be metabolized *in vitro* by cells from both animal and plant sources and the main product was 1-naphthol (Chin *et al*., 1979). Partial metabolism of carbaryl to CO_2 was demonstrated *in vitro* (Palut *et al*., 1970). Most mammals given napthyl-labelled carbaryl excreted 68-74% of the dose in the urine and 2-11% in the feces within 24 hrs of administration (Knaak *et al*., 1968; Sullivan *et al*., 1972). The metabolism of up to 85% of carbaryl occurs within 24 hrs after administration (EPA, 1987).

The hydrolysis of carbaryl to 1-naphthol was probably the critical step in the house flies (Eldefrawi and Hoskin, 1961). The housefly metabolites of 1-naphthol and naphthalene have been characterized (Terriera *et al*., 1961). The administration of ^{14}C-labelled carbaryl to house flies yielded ^{14}C dioxide (Dorough *et al*., 1963). Flies appeared to metabolize carbaryl through an initial hydroxylation and form an unstable intermediate from *p*-nitrophenyl dimethylcarbamate which may be the N-methyl derivative of the compound (Hodgson and Casida, 1960, 1961). In no case the complete metabolic

pathway of a carbamate insecticide in an insect has been elucidated (Baron, 1991).

1.2.1.4 Biochemical effects of carbamates

Bordy *et al*. (1983) found that at 24 hr post dosing with methmoyl, a carbamate, at 40 mg/kg/day by oral intubation, caused significant increase in serum alkaline phosphatase, glutamate oxaloacetae transaminase, serum triglycerides, phospholipids, free fatty acids and cholesterol in rats. A single oral 500 mg/kg dose of carbaryl or seven doses of 71 mg/kg/day increased the activities of acid phosphatase, aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) in the liver and kidney but did not affect the activities of alkaline phosphatase, lactate dehydrogenase or succinate dehydrogenase (Kiran *et al*., 1985).

Acid and alkaline phosphatases activities increased significantly after pyrethrum treatment in both brain and ventral nerve cord with ganglia whereas acetylcholine esterase decreased rapidly in newly emerged *Schizodactylus monstrous* (Banerjee *et al*., 1984). Activity levels of proteases were significantly elevated due to sub-lethal dose of carbaryl in bliser beetle, *Mylabris pustulata* (Bharathi and Govindappa, 1985 a). In beetles exposed with carbaryl, there was depletion in the levels of all the four nitrogenous end products in the malpighian tubules such as free ammonia, glutamine, uric acid and urea, indicating that the tubules were actively extracting the excretory

products from blood into the pellets (Bharathi and Govindappa, 1985 b). The activities of ALAT and ASAT were markedly elevated in the hemolymph. These changes were similar to those under stress conditions (Bharathi and Govindappa, 1985 c). After exposure all digestive enzymes were inhibited in the foregut but activated in the midgut after the exposure. The effects of short term exposure to the pesticide were reversible (Bharathi and Govindappa, 1986).

Excessive utilization of lipids occurs under toxic impact of insecticides like carbaryl and lindane in the scorpion, *Heterometrus fulvipus* (Rajyalakshmi and Reddy, 1991). Blister beetles exhibited lipid oriented metabolic pattern during pesticide exposure. There was a significant depletion of total lipid occurred both during short-term and prolonged exposure to sub-lethal dose of carbaryl (Bharathi and Govindappa, 1985 d). An elevation in the levels of ASAT and ALAT was observed in maternal and embryonic tissues of *H. fulvipus*, with carbaryl and lindane treatment after 48 hrs. Reports are available regarding the toxic effects of pesticides such as organophosphates, organochlorine and carbamate groups on the physiology and biochemistry of some fresh water fishes (Webb and Brett, 1973; Arunachalam *et al*., 1980; 1985; Palanichamy *et al*., 1986; Vasanthi and Ramaswamy, 1987).

1.2.1.5 Toxicity of carbaryl

1.2.1.5.1 Acute toxicity of carbaryl

Exposure to a single high concentration is likely to elicit an immediate (acute) response that is qualitatively different from repeated exposure (chronic) to the same chemical at much lower concentrations, for example, a single exposure to 1 mg of carbaryl to a rat will result in the killing of large number of liver cells and death due to liver failure within 5-6 days; exposure to a few nanograms of carbaryl for several months does not kill liver cells but does result in liver cancer (Shank, 2004).

The acute toxicity, expressed as the LD_{50} varies considerably according to species, formulations and vehicles. The oral LD_{50} of carbaryl to rat ranges from 200 to 850 mg/kg and from 100 mg/kg to 650 mg/kg in mice. Cats are sensitive to carbaryl with an LD_{50} of 150 mg/kg, whilst pigs and monkeys are less susceptible having an LD_{50} greater than 1000 mg/kg. Based on the LC_{50} values assessed in the laboratory studies to banana rhizome weevil showed that carbaryl was found to be least toxic than aldicarb, carbofuran and phorate in increasing order (Visalakshi *et al*., 1986). The acute toxicity of carbaryl to rat of both sexes was > 500 mg/kg (LD_{50}). The symptoms of acute intoxication are typical of acetylcholinesterase inhibition. The acute oral, dermal and inhalation exposure of rats and rabbits to carbaryl at doses ranging from 450 to 1500 mg/kg resulted in transient

acetylcholinesterase inhibition in the brain, plasma and erythrocytes ranging from 30% to greater than 65%. At the higher doses, other blood parameters were also affected (Mount *et al*., 1981; Kossakowski and Lysek, 1982).

Bostanian *et al*. (2000) used carbaryl to manage aphids, maggots and leaf cutting caterpillars in the apple orchard. Ahmad *et al.* (2002) revealed that carbaryl has much intrinsic toxicity against the oblique-banded leaf roller, *Christoneura rosaceana* than the eighteen other insecticides used and exhibited a very low level of resistance against it. The experiments on insecticide susceptibilities of cat fleas showed that, of the eleven strains tested only two field strains developed tolerance against carbaryl. Sean *et al.* (1982) showed that technical grade carbaryl was toxic to clerids only at high doses (LD_{50}=287 μg/g of body weight and LD_{90}=2242.1 μg/g of body weight) by topical application. Carbaryl at 2 pounds of toxicant per acre gave evidence of a lasting residual control of cotton leaf predator (*Bucculatric thurberiella*), salt marsh caterpillar (*Estigmene acrea*), and cotton bollworm larvae (Shorey *et al*., 1962). A 5% sevin provided effective control of the corn earworm, *Heliothis zea* (Shorey *et al*., 1962).

Ball and Su (1979) studied the toxicity of carbaryl to female *Diabrotica virgifera* by topical application. Lawrence *et al*. (1973) reported the topical treatment of carbaryl on *Chrysopa rufilabris* on duration of larval, pupal and adult survival and 50% WP of carbaryl gave the lowest percent of

survival and had unfavourable effects on *C. rufilabris.* Visalakshi *et al*. (1986) showed that carbaryl controls rice swarming caterpillar, *Spodoptera mauritia*. The insecticide toxicity on the diamond black moth described a significantly high larval mortality with carbaryl after 72 hrs of treatment (Hill and Foster, 2000). Effect of carbaryl on eggs, larvae and adults of the green lace-wing, *Chrysopa scelester*, was studied by Krishnamoorthy (1985) and found that at 0.10% caused 100% adult mortality. He has also observed that carbaryl is highly toxic to first instar larvae and adults but low to medium toxic to second and third instar larvae of the green lace-wing. Lecrone and Simlonitz (1980) studied the toxicity of carbaryl to green peach aphid, *Myzus persicae, and Coleomegilla maculata* and *Chrysopa oculata*. Toxicity of carbaryl insecticide to *Amblyscius fallacis* and *Typhlodromus pyri* was studied by Watve and Leink (1976) and found that *A. fallacis* was extremely susceptible to carbaryl while *T. pyri* was highly tolerant. The recommended field rate/100 gal water of carbaryl is 2.016 kg and the LC_{50} value for *A. fallacis* is 0.2 kg. Residual toxicity of carbaryl was observed in first instar larvae of spotted bollworm and was found to be most persistent and effective which gave 37.93% mortality on the 15^{th} day after treatment (Patil and Pokharkar, 1977). Carbaryl provides extensive protection of *Ponderosa* pine trees against western pine beetle attack (Smith *et al.* 1977). Tsai and You (1962) obtained excellent control of spotted bollworm with 0.1% carbaryl. Unequal response of Douglass fire tussock moth, *Orgyia pseudotsugata* was found among four

populations to the pesticide carbaryl and the range of LD_{50} value was 14.1 to 172.0 μg/g body weight (Stock, 1979).

1.2.1.5.2 Chronic toxicity of carbaryl

The toxicity of carbaryl to common prawn (*Palaemon serratus*) was studied for 29 days in the adults and the induction thresholds for inhibitory effects of acetylcholinesterase were determined (Bocquene *et al*., 1991). Carbaryl with sub-lethal dose showed some effects on protein metabolism of fresh water fish (Rao *et al*., 1987; Reddy and Bashamohideen, 1987). Rath and Mishra (1980) have reported a reduction in protein content in *Tilapia mossambica* with chronic exposure to pesticide media. The protein content was reduced in *Oreochromis mossambicus*, *Mystus vittatus* and *Channa striatus* which were reared at carbofuran media for different duration of exposure (Palanichamy *et al.,* 1989). The acid and alkaline protease activities were increased in muscle, liver gill and intestine of *M. vittatus* (Palanichamy *et al*., 1989). There was significant inhibition of acetylcholinesterase activity observed in the selected tissues of *Metapenaeus monoceros* after chronic exposure (Reddy *et al*., 1990). The residual toxicity studies of some of the commonly used insecticides in the first instar larvae of spotted bollworm revealed that out of the insecticides tested, carbaryl was observed to be the most persistent and effective that gave 37.93% mortality on the 15^{th} day after treatment (Patil and Pokharkar, 1977). In female western corn rootworm, sub-

lethal dosage of carbaryl stimulated oviposition and extended longevity (Ball and Su, 1979).

1.2.1.6 Effects of carbaryl on non-target organisms

Carbaryl is classified by the World Health Organisation as moderately hazardous (WHO, 1992). The toxicity varies considerably according to species and formulations. The mice, rats and cats were very sensitive with a low range of carbaryl. The pigs and monkeys were less susceptible to carbaryl. Toxic effect of carbamide groups of pesticides on the fresh water fishes were reviewed (Webb and Brett, 1973; Arunachalam *et al*., 1985; Palanichamy *et al*., 1986). The toxicity of carbaryl in adult prawn, *P. serratus*, was studied in the adults and it inhibits acetylcholinesterase (Bocquene *et al*., 1991). Carbaryl was toxic to *Macrobrachium malcolmsonii* (Bhavan and Geraldine, 2002). Toxic effect of carbaryl in the respiratory movements of an air breathing fish, *C. striatus* exposed to sevin was also studied (Anbu and Ramaswamy, 1991). Carbaryl is very highly toxic to shrimp, crab and oysters (EPA, 2002).

According to EPA report carbaryl can range from highly to slightly toxic to freshwater fish on an acute basis and is moderately toxic to ocean and estuary fish. Salmon, trout, and perch are the most sensitive species and are killed by concentrations between 250 and 970 ppb (EPA, 2002). Carbaryl is used in grasshopper baits, might cause harm to the small mammals who share

grasshopper habitat and carbaryl exposed mice ran more slowly and were more apt to cannibalize their offspring than unexposed mice (Punzo, 2003). Relyea and Mills (2001) studied the interactions between carbaryl predatory salamanders and tree frog tadpoles and showed 60% mortality of tadpole exposed to 50 ppb and adding a second stress to the tadpoles, carbaryl induced mortality to 97%. The toxic concentration of carbaryl at 50 ppb harmed the survival of tadpoles (Rohr *et al*., 2003).

Carbaryl fits into integrated pest management programs, because it is relatively non toxic to coleopterous predators *Enoclerus lecontei* and *Enoclerus sphegeus* using residual film and topical application method but highly toxic to western pine beetle *Dendrotonus brevicornis* (Sean *et al*., 1982; Robertson and Gillette, 1978; Greene, 1983). The toxicity of chemical insecticides to parasitoids and predators at reduced dosages in increasing order of toxicity was malathion < carbaryl < toxaphene < methyl parathion (Wilkinson *et al*., 1975). The estimated LC_{50} (AI g/Acre (0.405 hectar) of carbaryl for 8 species of parasitoids and predators are 18.2, 286.0, 54.5, 18.2, 376.8, 13.6, 68.1, 1362.0 for *Compoletis sonorensis, Chelonus blackburni, Brachymeria intermedia, Meteorus leviventris, Varia ruralis, Hippodamia convergens, Chrysopa carnea* and *C. carnea* larvae respectively (Wilkinson *et al*., 1975).

The broad spectrum of effectiveness of carbaryl against many agricultural pests has been recognized for several years and has been confirmed by the results of Shorey *et al*. (1962). Carbaryl is also lethal to many non-target insects, including bees and beneficial insects (Kidd and James, 1991). EPA's databases show that numerous bee kill incidents also have been reported in several states (EPA, 2003). With regard to beneficial insects, the insecticide used reduced populations of parasitic Hymenoptera. Carbaryl was known to be highly toxic for honey bees. When ingested, LD_{50} of carbaryl was found to be 0.18 µg/ bee (Alvarez *et al*., 1970) and the contact LD_{50} for carbaryl in adult honey bee was 1.3 µg/ bee (Stevenson, 1978).

1.2.1.7 Biochemical effects of carbaryl

1.2.1.7.1 Effects on protein

The acute oral administration of carabaryl to rats at doses ranging from 50 to about 500 mg/kg affected the levels of a variety of enzymes, amino acids, neurotransmitters of other substances in the blood and brain (Baron, 1991). Effects reported include a decrease in serum protein levels, blood free amino acids and brain acetylcholinesterase concentrations and changes in free amino acids metabolism in the liver and brain. Rath and Mishra (1980) have reported a reduction in protein content in *T. mossambica* when exposed to pesticide media. Effects of carbaryl on protein metabolism in some freshwater

fishes have been reported (Rao *et al*., 1987; Reddy and Bashamohideen, 1987; Palanichamy *et al.*, 1989)

A decrease in cellular protein in the Hela cells was noted (Blevins and Dunn, 1975). Carbaryl showed considerable protein-binding ability in cultured human embryonic lung cells (Murakami and Fukami, 1983). Miller *et al*. (1979) demonstrated that carbon derived from carbaryl binds to microsomal proteins. Human serum albumin reacted *in vitro* with the ester group of carbaryl and catalyzed the hydrolysis and liberation of 1-naphthol. This reaction is similar to an "esterase type" action (Casida and Augustinson, 1959) called carbamylation. Enzyme mediated binding of carbaryl to rat hepatic microsomal protein occurred *in vitro* in the presence of NADPH and oxygen (Neskovic *et al*., 1978). In a protein binding study with rats, carbaryl in the serum was bound primarily to albumin and partly to globulin and lipoprotein in the cytosol fraction of the intestinal mucosa (Tanaka *et al*., 1981). Carbaryl inhibited the synthesis of DNA, RNA and proteins in cultured rat and human embryonic lung cells (Lockard *et al*., 1982; Murakami and Fukami, 1983). The level of proteins showed an initial increase followed by a drastic decline in late larval stages, of the midgut tissue of *S. mauritia* with carbaryl administration (Nair, 1995).

1.2.1.7.2 Effects on amino acids

The sub-chronic oral administration of carbaryl to rats at 95 mg/kg /day for 30 days slightly decreased erythrocyte alanine levels (Jeleniewicz and Szczepaniak, 1980). Effect of carbaryl on tryptophan metabolism in rats was studied by Ashraf *et al.* (1990). Carbaryl administered to rats for 3 months at a daily intragastric dose of 60 mg/kg/day decreased the levels of tryptophan in the blood and decrease in amino acid concentration in the brain tissue and liver which later became normalized in both the tissues (Podolak-Majczak and Tyburezyk, 1984).

The radiolabelled metabolic products of carbaryl were covalently bound to amino acid residues of microsomal proteins that accounts to 99.2-99.7% of the bound radioactivity (Miller *et al*., 1979). Carbaryl binds to free amino acids of the blood (IPCS, 1994). Boyd and Boulanger (1968) reported an increased susceptibility to carbaryl toxicity in Albino rats fed a protein-deficient diet. An increase in the ratio of amino acid nitrogen to creatinine in the urine after carbaryl treatment may represent a decrease in the ability of the proximal convoluted tubule to reabsorb the amino acids (Knaak *et al*., 1968). Carbaryl inhibited the incorporation of 3H-uridine and 14C-labelled aminoacids into RNA and proteins in cultures of Hela cells and the effect was dose dependent (Myhr, 1973). Human serum albumin reacted *in vitro* with the

ester group of carbaryl. There was a temporary reduction in the ability to reabsorb amino acids at the highest dose (Wills *et al*., 1968).

1.2.1.7.3 Effects on glucose

Disturbances have been reported in the carbohydrate metabolism and protein synthesis and detoxification function of the liver in mammals. Carbaryl is a weak inducer of hepatic microsomal drug-metabolizing activity (WHO, 1994). A single application of carbaryl at 30 mg/rat produced transient hypoglycemia at 20 hr followed by hyperglycemia at 44 hr and carbaryl inhibited lactate gluconeogenesis, and to some extent, gluconeogenesis from fructose pyruvate and alanine. Glycerol glucogenesis was unaffected (Orzel and Weiss, 1966). Intraperitonial doses of carbaryl as low as 5 mg/kg produced a hyperglycemic response in intact or hypophysectomized rats. Hyperglycemic responses have also been reported in rabbits and dogs administered with carbaryl (Weiss *et al*., 1964; 1965). Orzel and Weiss (1966) found a rise in blood glucose correlated with the onset and duration of tremors and the degree of brain ChE inhibition in rats that were treated intrapertitoneal with 5 and 25 mg carbaryl/kg. The authors suggested the hyperglycemic effect was due to increased secretion of epinephrine. Hyperglycemia is thought to result from cholinergic stimulations as it is found in acute intoxications with organophosphorous compounds (Kaloyanova, 1963). In isolated rat hepatocytes, carbaryl was reported to inhibit

gluconeogensis, reduce lactate dehydrogenase and asparate aminotransferase activities, and enhance glucose-6-PO_4 activity (Parafita and Otero, 1983, 1984 a, b). The carbohydrates of midgut tissue of *S. mauritia* showed an initial increase in the first few days of carbaryl administration followed by a drastic decline in late larval stages (Nair, 1995).

1.2.1.8 Effects of carbaryl on organ systems

Carbaryl in the adrenals of rat induced the histopathological alternations (Baronia *et al*., 1992). Carbaryl has been established to be a neurotoxicant and effective poisonous chemical when added into mammalian body (Padilla and Hooper, 1992; Takahashi *et al*., 1994). Administration of the maximum tolerated dosage of carbaryl to mice for about 18 months did not increase the incidence of tumors (Innes *et al*., 1969). Carbaryl in the diet of rats at 400 ppm for 2 years did not affect the incidence of tumors (Carpenter and Livestone, 1961). Triolo *et al*. (1982) reported that carbaryl at 1000 ppm in the diet of mice for 20 weeks did not cause tumors. The dietary exposure of 20 ppm carbaryl to chicken for three months suppresses the immunity (Singh *et al*., 2007).

Marked vacuolation of the epithelium of the proximal tubules of kidney of rats and monkeys receiving very large doses of carbaryl were reported by Serrone *et al*. (1966). A 30-day exposure of rats to carbaryl at 10 ppm in their drinking water produced treatment related liver histopathology

and slight decrease in platelet count and activity of clotting factor VII (Lox, 1984).

The effects of carbaryl on the nervous system are primarily related to cholinesterase inhibition and are usually transitory. The effects on the central nervous system were studied in rats and monkeys. In a small study on pigs, carbaryl was reported to produce a number of neuromuscular effects (150 mg/kg body weight in the diet for 72-82 days). No evidence of demyelination was observed in the brain, sciatic nerve, or in spinal cord sections examined microscopically. Carbaryl has been reported to effect coagulation. There have been no reports of confirmed induction of mitotic recombination and gene conversion in prokaryotes and eukaryotes *in vitro*. Negative results were obtained in tests for gene mutations and chromosome damages. Chromosomal damage with high dosage of carbaryl has been reported *in vitro* in human, rat and hamster cells. Carbaryl has been shown to induce disturbance in the spindle fibre mechanism in mammalian cells *in vitro* (IPCS, 1994). Delescluse *et al.* (2001) suggested that carbaryl provoked strong DNA-damaging activity in the human lymphoblastoid cell line. Carbaryl has special toxicity to somatic and germ cells in animals (Siboulet *et al*., 1984; Pant *et al*., 1995; 1996), however, others reported the contrary results (Osterloh *et al*., 1983; Bigot-Lasserre *et al*., 2003).

Effects of carbaryl on endocrine system

It is known that pesticides can cause certain type of cancers, birth defects, sterility problems, genetic mutations and behavioral changes. In recent years researchers have also begun to investigate the effects of pesticides on the human endocrine system because of the evidence that some pesticides may be responsible for altering the gender of species. Hormones are chemical messengers that regulate all biological processes in animals. These processes include blood sugar levels, growth and function of the reproductive system, and the development of the brain and nervous system (EPA, 2005). Hormones are mostly produced by endocrine glands like the ovaries, testes, pituitary gland and thyroid gland. Carbaryl's ability to disrupt hormones and the endocrine system was first demonstrated only a decade after its marketing began when researchers from the USSR Academy of Medical Sciences described its effect on the endocrine glands of rats (Shtenberg and Rybakova, 1968). Researchers from Tulane University showed that carbaryl inhibited the activity of two sex hormones, estradiol and progesterone, in human cells (Klotz *et al*., 1997). Carbaryl was identified as an endocrine disruptor (EPA, 2005). Chemicals that interfere with the hypothalamic-pituitary-testicularaxis have dramatic impact on male reproduction (Shank, 2004). Inhibitors of testosterone synthesis and inducers of phase I metabolism to accelerate the removal of circulating testosterone (e.g., several organochlorine compounds) decrease male fertility. Genotoxic

compounds interfere with normal meiosis by damaging DNA and chromosomes and inhibiting nucleic acid synthesis; mutations in the DNA and abnormal chromosomes can generate incompetent sperm or no sperm at all. Reproductive functions in both the male and female are under hypothalamic-pituitary control (Klassen, 2001).

The effect of carbaryl on the neuroendocrine system was studied in rats. Spermatozoon motility was reduced progressively with the duration of the exposure of carbaryl with 70 mg/kg/day. The histochemical studies of hypophysis showed changes indicative of an increase in the activity of the cells producing a luteinizing gonadotrophy, i.e., an increase in the size of the cells, loss of granules, and hyalinization of the cytoplasm. Histological examination of the adrenal glands revealed an increase in the size of mitotic activity of cells in the zone glomerulosa. Cells with large nucleus or two nuclei were present in the fascicular zone. It is likely that the effects of carbaryl on the reproductive organs are mediated by the endocrine glands (Rybakova, 1966; Shtenberg and Rybakova, 1968; Shtenberg *et al*., 1970).

1.2.1.9 Effects on reproduction

The toxic effects of carbaryl related to reproductive toxicology (Schrag and Dixon, 1985; Baranski, 1993; Savitz *et al*., 1997; Juhler *et al*., 1999; Xia *et al*., 2005) and genetic toxicology (Grover *et al*., 1989; Renglin *et al*., 1999; Delescluse *et al*., 2001) have also been extensively investigated. With the

exception of a small number of studies (Ball and Su, 1979; Martin, 1982; Osterloh *et al*., 1983), all adverse reproductive and developmental effects were noted. Kazarnovskaya and Vasilos (1977) had shown that carbaryl suppressed mitosis, changed the rate of the mitotic phase, and significantly increased the number of pathological forms of mitosis in a human embryonic fibroblast culture and exerted a pronounced chromosome breaking effect, at a concentration of 100 μmol/litre, 26 hr and 50 hr after treatment. The cytogenetic effects induced included mostly metaphase and anaphase fragments and anpahase bridges are time-dependent (Wuu and Grant, 1966).

In a study with 101 non-exposed men with 49 men currently or previously employed in carbaryl production, no relationship was found between the intensity or duration of exposure and either sperm count (Whorton *et al*., 1979). On reexamining the sperm samples from this study and comparing them against a new control group of 34 non-exposed workers in the same plant, Wyrobek *et al*. (1981) reported morphological abnormalities in the sperm from the exposed workers, not related to estimated exposure levels. Another evaluation of these same sperm samples showed no differences in sperm count or morphology between exposed and control groups (Mac-Leod, 1982). Reviews of the reproductive effects of carbaryl (Weil *et al*., 1972; Kuhr and Dorough, 1976; Crammer, 1986) note frequent reports of reproductive injury by even small doses of carbaryl. Many of these

effects have not been verified in other laboratories. In these studies, rats were exposed to carbaryl by daily pre oral intubation at dosages ranging from 2-30 mg/kg/day and for periods ranging from 1 to 12 months; one study assessed reproductive effects over 5 generations. Effects reported at dosages as low as 2 mg/kg/day were functional and focal histological changes in the testes (including reduced motility and survival time of sperms), and increased hypophyseal secretion of gonadotropic hormones. Reproductive effects reported for dosages as low as 2 mg/kg/day were decreased fertility in both sexes, increases in still births and pup mortality, and delayed pup development (Rybakova, 1967, 1968; Vashakidze, 1967, 1975). Trifonova (1984) reported reduced ovarian function at carbaryl dosages of 40-80 mg/kg/day but not at 20 mg/kg/day. Vashakidze (1965) reported teratogenicity and decreased reproduction at sub-chronic intubated dosages of 100 mg/kg/day and higher; but not at 50 mg/kg/day.

The oral administration of carbaryl to male rats at 200 mg/kg/day on 3 days a week for 90 days showed no clinical signs, effects on fertility, or histopathological changes in the testes, liver, kidney or brain (Dikshith *et al*., 1976). Kitagawa *et al*. (1977) reported reduced sperm count with carbaryl per week orally for 1 year in rats. Carbaryl given to mice at 34 mg/kg/day for 5 days did not affect either the weight of the testes and sex glands or the ability of the prostate to assimilate and metabolize testosterone (Dieringer and Thomas, 1974; Thomas *et al*., 1974). In three-generation reproduction studies

with rats, carbaryl in the diet at 10,000 ppm (about 500 mg/kg/day, maternally toxic dosage) reduced fertility (Baron, 1991).

1.3 Studies on the hemolymph of insects

One of the interesting features of insect hemolymph is that it contains very large amounts of free amino acids, much more than in the body fluids of other animals (Chen, 1962). These amino acids contribute to the osmotic value of hemolymph and account for a substantial portion of the cations and anions of hemolymph (Tembhare, 1997; Nation, 2002). The subject has been reviewed by Auclair (1953); Buck (1953); Bheemeswar (1959); Wyatt (1961); Chen (1962, 1966); Chefurka (1965); Jeuniaux (1971) and Florkin and Jeuniaux (1974). About 35-65% of non-protein nitrogen of hemolymph represents the amino acids. The free amino acid content of the blood of insects is strikingly high in comparison with that of other animals. All the known amino acids have been demonstrated in hemolymph of various insect-species (Srivastava and Auclair, 1975; Febvay *et al*, 1995; Sasaki and Ishikawa, 1995; Sanstorm and Moran, 2001; Calatayud *et al*., 2002). About 35-60% of non-protein nitrogen of hemolymph represents the amino acids (Tembhare, 1997).

The various aspects of the hemolymph proteins in insects have been studied by many investigators (Price, 1973; Chen, 1978; Wyatt and Pan, 1978; Gope and Prasad, 1980). It has established that the protein

concentration of insect hemolymph is generally higher than that of the internal fluids of other invertebrates and is almost similar to that of the blood of man (Florkin and Jeuniaux, 1974). The total hemolymph proteins occur within the range of 1 to 20 mg/100 ml (Tembhare, 1997). The presence of an open circulatory system in insects means that hemolymph perocolates around and through the fat body lobes and the adipocytes that are arranged for maximum exposure to the circulatory fluid. The 10 amino acids considered essential for insects in general: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Dadd, 1985). Quite a number of amino acids in the hemolymph such as alanine, aspartic acid, glutamine and tyrosine play an important role in the synthesis of chitin, polyphenols and other important constituents of the cuticle (Dhillon and Sidhu, 1977).

1.4 Studies on phosphatases

Acid phosphatases (ACPH) are considered as marker enzymes of lysosomal activity and have great significance in biochemical studies. Acid phosphatases have gained importance as clinical diagnostical tools in the detection of gynaecological conditions, metastasizing prostate cancer, bone conditions including rheumatic osteoblastoma, bone cancer, osteogenesis imperfecta, liver diseases such as Goucher's disease, hyperparathyroidism and chronic renal failure (Nakasato *et al*., 1999; Macejewski *et al*., 2001).

During massive destruction of tissues, lysosomal activity is an important factor (Van Prett-Verkuil, 1978). The histolysis represents a programmed cell death, a hormonally induced and neurologically activated cytolytic mechanism. These phosphatases catalyse the hydrolysis of phosphoric acid esters. Of the phosphoric acid esters, phosphomonoesters and phosphodiesters are of importance as constituents of cells. Day (1949) studied distribution of alkaline phosphatase histochemically in different regions of digestive tract of a number of insects. Functional significance of alkaline phosphatase was studied first by Moog (1946). Its localization in plasma membrane perhaps played an important role in transport of phosphate through cellular membranes. Hardonk and Koudstaal (1976) reported that this activity is facilitated by phosphatase or phosphotransferase action. Pearse (1961) and Srivastava (1966) studied that high activity of alkaline phosphatase indicated increased phosphate transfer from one alcohol to the other. Ide and Fischman (1969) and Farquhar *et al*., (1974) suggested that the lysosomal enzymes undergo metabolic transformation *in vivo*, resulting in change of substrate specificity. Hiromu (1969) reported that this enzyme helps in metabolism and transphosphorylation. The pivotal studies of de Duve and colleagues led to the characterization of lysosomes as the membrane-limited sub-cellular organelles which contain acid hydrolases (de Duve and Wattiaux, 1966; de Duve, 1970; Bainton, 1981).

Beel and Feir (1977) studied the changes in acid phosphatase activity in the testes and hemolymph of the 5th instar male *Oncopeltus fasciatus* at various time intervals and found that the activity of ACPH was higher in the hemolymph. In *Drosophila melanogaster*, the ACPH is present in higher levels during embryogenesis (Yasbin *et al*., 1978). Tissues having energy requirements need a readily available source of phosphate which is provided by acid phosphatase (Blum, 1970). Under physiologic conditions the enzyme acid phosphatase may be involved specifically in the dephosphorylation of naturally and physiologically occurring phosphate esters. Acid phosphatase plays an important role during stress condition. It releases inorganic phosphorus to the system and helps to maintain the metabolic activity. The lysosomes function *in situ* as scavenging organelles and help in degradation of macromolecules of cellular origin and from invading microorganisms (Bainton, 1981).

1.5 Histomorphology of the male reproductive system of insects

The reproductive system of the male insect has been studied in varying degrees of detail in many orders (Phillips, 1970; Roosen-Runge, 1977; Muse, 2002; Alves, 2006) including heteropteran (Ambika, 1973; Dorn *et al*., 1992; Lemos *et al*., 2005). The reproductive system of the male insect consists of testes, vas deferens, accessory glands and an ejaculatory duct (Tembhare, 1997; Klowden, 2002). All parts of the system may produce secretions that

aid the transfer of sperm to the female (Happ, 1992). Each testis generally consists of a number of tubes or follicles in which spermatozoa are matured (Nation, 2002). The follicles bound together by a peritoneal coat. Each tubule opens via a vas efferens into the mesodermal sperm duct or vas deferens, and the paired vasa deferentia unite to connect with the ectodermally derived ejaculatory duct which terminates in the gonopore. The vas deferens is usually expanded over part of its length to form a seminal vesicle or sperm storage organ. The testis consists of series of parallel tubes or follicles that empty into a vas deferens (Roosen-Runge, 1977). The zones of maturation stages of sperm exist along the length of a typical follicle. In the 'Growth Zone' (Zone I), the spermatogonia divide by mitosis into many diploid spermatocytes within a sac or cyst which generally arise from the same spermatogonial cell and their development is synchronized. The spermatocytes may undergo more mitotic divisions; there are five to eight divisions in Acrididae and seven in *Melanoplus*; but eventually in the 'Zone of Maturation' (Zone II), meiosis and haploid spermatids are produced (Klowden, 2002). Normally, four sperm are produced from each spermatocyte. In Zone III, the 'Region of Transformation', the mature sperm develop. Usually, the mature sperm remain bundled together in Zone III (Jamieson *et al*., 1999; Nation, 2002). The testis follicles of sexually mature males are full of cysts, the spermatocysts, within which spermatogenesis proceeds. A cyst is a group of germ cells surrounded by an epithelium. The proximally situated cysts contain

spermatogonia or spermatozoa. The late gonial and meiotic divisions are incomplete and the daughter cells remain connected by cytoplasmic bridges. The cells of a cyst hence form a functional syncytium. They divide in synchrony and develop as clones; the number of spermatids per cyst will thus generally be equal to an integral power of 2, for instance 2^5, 2^6, 2^7, or 2^8, depending on the species (Jamieson *et al*., 1999). Rasmussen (1973) found some exception to such a synchrony. The interconnected spermatids within a cyst remain aligned side by side throughout spermatogenesis, so that a transverse section through the cyst cuts all cells at the same level (Phillips, 1974). Apical cells are known to be larval structures, gradually disappearing in older animals (Carson, 1945; Menon, 1969). Numerous spermatogonia were already well packed in the terminal portion and they grew and differentiated into spermatocytes and spermatids as early as the late 4^{th} instar. The number of all these members increases with the growth of the animal. In most insects spermatogonia and spermatocytes were developed in the pupal and nymphal stages, and the testes of the imago contain only spermatids and spermatozoa (Roosen-Runge, 1977). In some water beetles, the two testes are long and coiled single tubes (Jamieson *et al*., 1999). In Diptera, the testes consist of a simple, elongated and undivided sac (French and Hoopingarner, 1965). Apical cell complex has been observed in the heteropteran *Oncopeltus* (Bonhag and Wick, 1953; Economopoulos and Gordon, 1971). A remarkable, perhaps unique, feature of insect spermatogenesis is the association of

primary spermatogonia with a large cell or cellular complex at the apex of the testis or of each follicle, the so-called apical cell (Roosen-Runge, 1977). It is presumably derived from the primordial germ cell, but doesnot give rise to spermatogonia. The early spermatogonia have cytoplasmic extensions connecting them to the apical cell, as if receiving some kind of signal substance from that cell. The apical cell of this type is not found in Protura (Berlese, 1910), which is of interest as this animal group may not be closely related to the true insects (Roosen-Runge, 1977). The processes of spermatogenesis and spermiogenesis provide typical examples of the profound morphological changes that occur during terminal differentiation of specialized cells (Phillips, 1974). Deb *et al*. (1983) recorded the follicle of testes contains a succession of zones, in *Chrysocris stollii* (Pentatomidae). Bhalerao *et al*. (1991) showed that first zone of testis contained spermatogonia which have distinct nuclei. The second zone has spermatocytes. In the last zone spermatozoa are present in bundles. Sperms of 6.64 mm long (Yanders and Perras, 1960) and more than 10 mm long (Joly and Bressac, 1994) have been reported for *Drosophila hydei*.

In many cases, accessory glands are formed as diverticula from the vas deferens (Tembhare, 1997). In other insects the mesodermally derived ducts are themselves glandular (Jamieson *et al*., 1999). The male accessory genital glands of insects may be ectodermal or mesodermal in origin, known as ectadenia and mesadenia, respectively (Tembhare, 1997). The number and

arrangement of the accessory glands varies considerably between the different groups of insects (Nation, 2002). Each accessory gland consists of a single layer of epithelial cells, the fine structure of which depends on their stage of development and the nature of the secretion produced (Chapman, 1998).

The morphological and histochemical studies on the spermatogenesis in the bug, *Halys parvus* (pentatomidae: Homoptera) were carried out in detail (Sareen and Kaur, 1987). Histomorphological observations on the spermatogenesis in the normal and the transplanted testis of the *Dysdercus cingulatus* (Heteroptera) were also studied (Ambika, 1973). Srivastava *et al.* (1985) studied the histopathological effects of X-irradiation on the testes of the *Dysdercus koenigii*. The testicular degeneration in *D. Koenigii* after microwave exposure has been studied (Bhalerao *et al*., 1991). Rajendra *et al*. (2001) reported the male sterility associated with the over expression of noncoding of *hsrω* gene in cyst cells of testes of *D. melanogaster*.

1.5.1 Ultrastructure of testes

The wall of the testes of insect has three layers. The outermost is the peritoneal coat, followed by a middle muscle fibre supporting the inner most coat of epithelial cells. A number of mitochondria, endoplasmic reticulum and some ribosomes are seen in the peritoneal cells (Tembhare, 1997; Klowden, 2002). The muscle fibers are usually single cell in thickness but at some

places even double cells are seen. There are a few mitochondrial bodies in them.

1.5.2 Ultrastructure of sperm

Insect spermatozoa have been described at ultrastructrual level (Baccetti, 1972). Diversity among insect spermatozoa is seemingly endless. The length of the spermatozoon can vary from 1.7 μm as in the termite *Reticulitermes lucifugus* (Baccetti *et al*., 1981), to 58,000 μm as in *Drosophila bifurcata* (approximately 20 times the length of the male producing it) (Pitnick *et al*., 1995). A great diversity of shapes exists in insect sperms and any of the four main constituents (nucleus, acrosome, mitochondria and flagellum) may be lacking or be the dominant one (Jamieson *et al*., 1999).

Heteropteran sperm

The investigation of spermatozoal ultrastructure in eight families in four of the seven infra orders of the Heteroptera (classification of Stys and Kerzhner, 1975), Dallai and Afzelius (1982) considered the four characters for heteropteran sperm such as the presence of two or three crystals within each of the two mitochondrial derivatives, (rather than a single one), presence of bridges between the two mitochondrial derivatives and axonemal microtubules 1 and 5, the absence of longitudinal accessory bodies and a

prominent Zipper-line along the sperm tail to be unique synapomorphies (autapomorphies) for the Heteroptera, within a framework of many variations, and have confirmed the taxon-specific nature of sperm morphology. The crystalline material in heteropteran mitochondrial derivatives has a "fish bone" pattern in longitudinal sections; the patterns have been elucidated by Rosati *et al*. (1976) and Baccetti *et al*. (1977).

The spermiogenesis in most insect means an elongation of the entire cell and not least of the nucleus. This elongation is accompanied by, perhaps caused by, the appearance of microtubules encircling the acrosome and the nucleus and collectively named the manchette (Kessel, 1966, 1970). It is found not only in insects but in most animal spermatozoa. Fawcett *et al*. (1971) are of the opinion that the shape of the sperm head is probably not the consequence of external modelling by pressures applied to the condensing spermatid nucleus by microtubules in the perikaryal cytoplasm but may be largely determined from within by a specific genetically controlled pattern of aggregation of the molecular subunits of DNA and protein during condensation of the chromatin.

The sperm tails are usually free and beat in a coordinated fashion. Large and small spermatozoa are also seen in pentatomid bugs, such as *Murgantia histrionica* and *Rhaphigaster* sp. (Bowen, 1920). In the sperm of the fire-bug, *Pyrrhocoris 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. Rosati *et al*. (1976) have described the mitochondrial derivatives of *G. lineatum italicum* and *Nezara viridula smarogdula*. They have remarkably uniform derivatives, regarded as partially crystallized. Their position is, however, different in *G. lineatum* where the derivatives arise close together in the centriole region whilst in *N. smarogdula* they arise one behind the other from a small cavity in the nucleus. The inner region is filled with structural material visible in and between the coils. It appears like a series of parallel lines, evidently corresponding with 30 A^{o} thick longitudinally arranged globular filaments that in some regions become elements of a honeycomb network (Jamieson *et al*., 1999). The classical form of the insectan axoneme was established by Phillips (1966), chiefly for the scavenger fly *Sepsis*. Baccetti (1972) found that all ptergote insects have 9+9+2 axoneme except for Ephemeroptera. The paracrystalline material often has a herringbone pattern in longitudinal section as in the Diptera, Homoptera, Odonata, Dermoptera, Psocoptera, Hemiptera, Neuroptera, Coleoptera and Hymenoptera (Phillips, 1970). In the typical insectan sperm tail the axoneme is flanked not only by two mitochondrial derivatives but also by two elongate bodies, or less commonly one body, which may show a paracrystalline structure (Jamieson *et al*., 1999).

a) Acrosome formation

The early spermatid contains a prominent Golgi body that will give rise to the acrosome. It is sometimes called the acroblast. It produces a secretion vesicle on its concave side (sometimes called maturing or secretory side), which migrate to the nucleus. In some insects the acrosomal vesicle is the only component of the acrosome region (Jamieson *et al*., 1999). In some insects, such as the heteropteran *Gerris, Notonecta* and *Hydrometra*, the acrosome has an intricate geometry of tightly packed tubules (Tandler and Moriber, 1966; Dallai and Afzelius, 1980) or a regular meshwork with a honeycomb pattern (Afzelius *et al*., 1976; Werner *et al*., 1988). The acrosome is lacking in many species, but is more than 2.5 mm long in the heteropteran, water strider, *Gerris* (Tandler and Moriber, 1966). The acrosome of most insect species has a conical or rod like shape, although in some group more complicated types are seen such as arrow heads in Tettigonidae (Baccetti *et al*., 1970 b; Guerra *et al*., 1990), a wine-glass shape in several Neuroptera (Afzelius and Dallai, 1979) and a large flattened disc in some saldid bugs (Afzelius *et al*., 1985).

The acrosome of insect sperm is formed from the Golgi apparatus (Clayton *et al.*, 1958; Cruz-Landim and Ferreira, 1971; Cruz-Landim, 1979 a; Kaye, 1962; Shay and Biesele, 1968) which is usually termed the acroblast and which lies distal to the nucleus. The outer laminated part of the acroblast,

the externum, is horse-shoe shaped whereas an inner area, the internum, is relatively structureless. The acrosome typically forms in the internum within the concavity of the laminae of *Acheta domestica* (Clayton *et al.*, 1958; Kaye, 1962), *Centhophilus secretus (*Shay and Biesele, 1968), *Myogryllus s*p. (Cruz-Landim, 1979 b).

b) Mitochondrial transformation

Mitochondria in insect spermiogenesis generally undergo a remarkable series of transformations, first to form what is called a 'Nebenkern' (a German term meaning alongside the nucleus) and later to form the so-called mitochondrial derivatives. Pratt (1968) has described several steps in Nebenkern formation in the hemipteran *M. histrionica*. She found that many filamentous mitochondria aggregate in late telophase of the second meiotic division and occupy the space between the cleavage furrow and the chromosome plate. Gradually the mitochondria fuse to form longer units and approach each other closely. They also anastomose with their neighbours. A network is then formed that consists of two unconnected and interlocked network of rings. In a cross section the Nebenkern will look like a jigsaw puzzle with the many mitochondrial profiles that form two halves, each consisting of several concentric layers (Tokuyasu, 1975). Finally two halves of the Nebenkern extend to become the two elongated mitochondria which extend in parallel along the flagellum. The two mitochondrial derivatives are

derived from a spherical mass (Nebenkern) formed by the fusion of all the mitochondria of spermatid. With the growth of flagellum of the spermatid, these mitochondria become realigned to give the two longitudinal mitochondrial derivatives of the mature spermatozoon (Payne, 1966; Meyer, 1968; Pratt, 1968; Szöllösi, 1975; Cruz-Landim, 1979 a).

Pratt (1968) assumed that the process of Nebenkern formation may be one that will divide the mitochondrial material equally in two parts to maintain symmetry and that it is one that will organize the mitochondrial material for its specific role in the mature spermatozoon. The two mitochondrial derivatives in many species have unequal diameters. It is a process where the mitochondrial DNA complements from all mitochondria of a single spermatid are given the opportunity to meet, perhaps for a proof-reading and correction of the genetic information (Tokuyasu, 1975).

Evidence for origin of the mitochondria of the spermatid in association with the nuclear envelope and from, or at least under the control of nuage material emanating from the nucleus is presented by Cruz-Landim (1979 c) for *Myogryllus* sp. The two mitochondria formed during spermiogenesis extend along the flagellum in most insect species and differ from their equivalents in somatic cells in three respects: (1) their length and size is relatively enormous and in some species they occupy 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 oriented perpendicularly to the longitudinal axis (Phillips, 1970, 1974) and (3) the mitochondrial matrix contains a conspicuous crystalline material, which may occupy most of the mitochondrial space or part of it; in later case it is close to the mitochondrial membrane at the side bordering of flagellar axoneme. The mitochondrial crystalline protein, crystallomitin, present in the sperm of most insects (largely from a study of the heteropteran *Notonecta*) has been defined by Baccetti *et al*. (1977).

c) Flagellar growth

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). The flagellum has a central core of nine microtubular doublets surrounding two central singlet microtubules, the well-known 9+1 organelle. The wall of the two central microtubules contains 13 protofilaments as does the dynein arm carrying A-tubule of the doublets (Jamieson *et al*., 1999). The wall of B-tubule contains 10 protofilaments and a smaller filament. The lumen of these various tubules appears clear or electron-dense, depending on the species (Dallai and Afzelius, 1990). The newly formed flagellum has a simple 9+2 axonemal structure, but eventually accessory tubules (also called peripheral singlets) develop. In most insect orders, the axoneme is

characterized with the short hand formula 9+9+2, i.e., nine accessory tubules, nine doublets and two central microtubules (Alves, 2006).

The early insectan spermatid contains a single centriole (Friedländer and Wahrman, 1966, 1971), whereas other animals generally have a diplosome. In most animals, the centrioles replicate after the first meiotic division, which gives the first spermatocyte with four centrioles; no such replication occurs in insects according to Friedländer and Wahrman (1971). In this connection it is of interest to note that the primary spermatocytes of lepidopteran insect *Spodoptera littoralis* (Godula, 1985) and dipteran insect, *D. melanogster* (Rasmussen, 1973) are reported to have four flagella.

d) Sperm surface

Freeze-fracture replicas of the heteropteran spermatozoa have shown that the sperm tail has a triple row of intramembranous particles running along the spermatid (Dallai and Afzelius, 1982). They are seen mainly in the P-face of the membrane. This structure was called the Zipper-line in analogy with the better-known Zipper in mamalian spermatozoa (Friend and Fawcett, 1974). Contrary to the mammalian zipper, the hemipteran sperm tail has an asymmetric location, as it is located between the axoneme and one of the two mitochondrial derivatives. Zipper-lines are also seen in the freeze-fracture replicas of fruit fly (Baccetti *et al*., 1971) and mosquito spermatozoa (Báo and de Souza, 1992) and might be common in insect spermatozoa.

e) Centriole adjunct

The centriole of the mature spermatozoon is surrounded by a material termed centriolar adjunct. In most insects it has a rather homogeneous or finely granular appearance (Dallai *et al*., 1996). The centriolar adjunct consists mainly of proteins, although the presence of RNA has also been demonstrated (Baccetti, *et al*., 1970 a; Cantacuzene, 1970). Species of at least 15 orders of the Insecta have been reported to contain a centriole adjunct (Lindsey and Biesele, 1974). Although the adjunct varies morphologically in the species studied, it usually surrounds, asymmetrically, the bases of the mitochondrial derivatives and the axial filament complex where all of these structures attach to the posterior end of the nucleus. In some insects, however, centriole adjunct material present early in spermiogenesis disappears before maturity. The function of the centriole adjunct has not been established but it is generally considered as a head to tail attachment structure (Gatenby and Tahmisian, 1959), and a nidus for the attachment of the upper ends of the mitochondrial derivatives, needed for cushioning the robust flagellar movement (Lindey and Biesele, 1974).

f) Nucleus

The nucleus is usually an elongated, anteriorly tapered cylinder, and truncated posteriorly. It is sometimes helically coiled and may even coil around the flagellum (Jamieson *et al*., 1999).

g) Accessory bodies

In the typical insectan sperm tail, the axoneme is flanked not only by two mitochondrial derivatives but also by two elongate bodies, or less commonly one body, which may show a paracrystalline structure. These are the deltoid or accessory bodies. In heteropterans longitudinal accessory bodies are absent (Dallai and Afzelius, 1980).

h) Centriole

The studies of Phillips (1970) in 195 species of 15 orders of insects concluded that, in all sperms both centrioles disappear during spermiogenesis and absence of a centriole was directly demonstrated for Homoptera and Heteroptera.

1.5.3 Ultrastructure of accessory glands

The accessory glands differ in size, shape, number, anatomical placement and embryological origin (Adiyodi and Adiyodi, 1975; Grassé, 1977). The muscular layer outside the epithelium constituted of a variable number of sheets of muscular fibers. These muscles are generally innervated (Kimura *et al*., 1987). Most accessory glands consist of a secretory epithelium surrounded by a muscular sheath, for most glands there are 2 or more layers of muscle cells which spiral obliquely to the right or to the left, around the generally cylindrical gland (Happ, 1992). The thickness of the muscle coat

seems to be correlated with the consistency of the product and is surrounded by two or three layers of muscle cells. Basement membranes surround the muscle layer and also coat the basal surface of the secretory cells. Tracheoles, enclosed in their cellular coats, run between the muscle cells and deep into the secretory epithelium (Happ, 1992). The materials produced by the accessory glands are transferred to the female during copulation, and they frequently have a long term effect on her reproductive behaviour and physiology (Chen, 1984; Happ, 1992; Schooneveld *et al*., 1997; Wolfner, 1997; Gillott, 1998). The accessory reproductive gland components exerts their effects at all phases of the reproductive biology of the mated females, from the moment sperm is deposited in the reproductive tract to egg deposition. Components with sperm-related functions seen likely to act mainly within the female reproductive tract, whereas those molecules that influence female behaviour and physiology typically appear to pass through the wall of the tract into the hemocoel to reach their site of action (Eberhard, 1996; Gillott, 1988), female insects are far from being "silent partners". Thus, it may be anticipated that females strongly influence the manner and extend to which male accessory reproductive gland (ARG) materials function. The past two decades have seen the publication of two comprehensive review of male ARG (Chen, 1984, Gillott, 1988), as well as several that deal with specific aspects, namely structure and development of ARG (Happ, 1984, 1992), endocrine regulation of ARG secretory activity (Gillott and Gaines, 1992; Gillott, 1996), and the

role of ARG secretions in egg production (Vahed, 1998; Gwyne, 2001; Gillott, 2002) and protection (Blum and Hilker, 2002; Eisner *et al*, 2002; Gillott, 2002). For males which mate repeatedly, the glands must go through recurrent secretory cycles to produce the charge of semen and paraseminal material during copulation. The maturation of accessory glands is regulated by endocrine and neuroendocrine factors in almost all groups (Happ, 1992).

Biochemistry of accessory gland secretions

As like mammals, insect semen and its accompaniments are an aggregation of biochemical constituents derived from morphologically diverse and complex glands. The heterogeneous products of these glands include both the seminal fluids and paraseminal fluid mixtures which promote sperm maturation, facilitate transfer of sperm, provide nourishment for stored sperm, contribute nutrients for investment into egg yolk and modulate the behaviour or the physiology of the female (Leopold, 1976). The mesadenia, simplex and duplex are just a few of the terms which describe the organs that produce seminal or paraseminal secretions (Happ, 1992). Some of these glandular products are proteins. Smaller molecules including sugars and lipids are reported in the semen (Gilbert, 1982). Secreted proteins appear to be manufactured *de novo* within the epithelium. Delivery of sperm to the female requires a vehicle which is produced by the accessory glands. Secretions of male accessory glands contain a variety of bioactive molecules. When

transferred during mating, these molecules exert wide-ranging effects on female reproductive activity. The accessory gland secretions may affect virtually all aspects of the female's reproductive activity. Male-derived accessory gland proteins that are transferred to female during mating have profound effect on female reproductive physiology including mating inhibition, and effect on sperm utilization and storage (Gillott, 2003).

Although proteins and carbohydrates were regarded as ubiquitous components of the accessory glands of insects (Leopold, 1976) more work has been done on the analysis of protein components of the accessory glands of several species of insects (Chen, 1984). The accessory secretions may mingle with the sperm, may precede the sperm mass, may enclose it, or may follow along afterward. It is accepted that the glycogen serves as source of energy for various activities of insect (Steele, 1982). Blum *et al*. (1962) demonstrated presence of fructose, glucose and trehalose in the ARGs of bee, *Apis mellifera*. Inositol is supposed to be the main carbohydrate present in the ARG complex of *Periplaneta americana* (Leopold, 1976). An ovipositor stimulant in the male accessory gland extract of *S. littura* has been studied by Sridevi *et al*. (1987). Baumann (1974) reported the presence of glucose and xylose as the important components that stimulate egg maturation and oviposition in the female insect. A complex mixture of protein in the accessory gland of *A. domestica* was reported by Kaulenas (1976). Lange and Loughton (1984) demonstrated the presence of a variety of proteins with a

wide range of molecular weights in the accessory glands of *Locusta migratoria*. There are about 80 secreted accessory gland proteins (Wolfner *et al*., 1997; Swanson *et al*., 2001; Mueller *et al*., 2004) in *D. melanogaster*. The genes of male reproductive tract that encode proteins and peptides have strong effect on male and female fitness (Chapman, 2001; Wolfner, 2002). Davies and Chapman (2006) identified cysteine-rich secretory protein genes in the accessory gland of *D. melanogaster*. Several studies have shown that the proteins from the accessory reproductive glands are transported during copulation (Leopold *et al*., 1971; Terranova *et al*., 1972; Kaulenas, 1976; Chen, 1984).

CHAPTER 2

ACUTE EFFECTS OF CARBARYL ON THE INSECT

2.1 INTRODUCTION

The carbamates are known for its reversible inhibitory effect on acetylcholinesterase (Srinivasan, 2005). Cholinesterase inhibition is common to all the carbamate pesticides both in blood and tissues (Fukuto, 1990). In the fish, *Labeo rohita*, carbaryl affects several biochemical changes such as depletion of metabolic reserves with increased levels of free sugars and high protein turn over under lethal stress (Rajamannar and Manohar, 2000). Carbaryl also disturbs several other physiological processes in insects which are under the control of neuroendocrine system (Singh, 1986). The utilization of major biochemical constituents due to acute toxicity of carbaryl is also reported in fresh water prawn (Bhavan and Geraldine, 2002). Only little information is available on the acute toxicity of carbaryl, expressed at LD_{50} (Stevenson, 1978, Sean *et al*., 1982; Tomizawa and Casida, 2005). The present chapter reports the changes in the levels of total glucose, total protein and total free amino acids in plant bug, *I. limbata*, on the acute toxicity of carbaryl at LD_{50} concentration. The toxicity of carbaryl is also noted with the recovery time to its normal condition as the time is an important factor in dose-response relationship.

2.2 MATERIALS AND METHODS

2.2.1 Rearing of insect

The plant bug, *Iphita limbata*, comes under the family Pyrrhocoridae of the order Heteroptera, are collected locally. The insects are reared under the laboratory conditions at 28 ^{o}C ± 2 and natural photoperiod, and fed with sprouted green gram seeds. *I. limbata*, is a hemimetabolous, polyphagous bug but not a harmful pest. They are oval shaped with bright red coloured with black markings. The males are comparatively smaller than the females about 1.6 ± 0.1 cm in length and distinguished by their external genitalia.

2.2.2 Carbaryl treatment

The adult male insects with more or less same size and age were used for experimental purposes. The experimental insects taken from the stock culture were kept in a separate cage for about 7 days prior to use. A stock culture of adult male *I. limbata* maintained in the laboratory was used for treatment. The following concentrations of carbaryl (50% wettable powder, Aventis Crop Science limited, Mumbai), i.e., 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 µg per 2 µl were prepared in acetone and the same was applied topically on the bugs as described by Ball (1977) and Kodrik *et al*. (2002). Similarly an equal volume of acetone was applied and used as control. Each experiment was replicated 5 times, each with 10 male insects. The acetone

treated insects were defined as 'control' and the carbaryl treated insects as 'treated'. The mortality of insect was noted at 48 hr and the mean value of mortality was taken for probit analysis. The 48 hr LD_{50} value was calculated using unweighed regression method of probit analysis (SPSS software).

The LD_{50} of carbaryl was topically applied to 75 male insects, to study its acute effect on the bug. The survived insects after the topical application of LD_{50} of carbaryl were used for biochemical estimations. The biochemical studies were conducted at 1 day intervals up to 4^{th} day (1-4 days) and 2 day intervals up to 8^{th} day. Equal number of replicates was done for both treated and control groups. The weighed insects were homogenized in distilled water and the homogenate was precipitated with 80% aqueous ethanol. It was centrifuged (Rota 4R-V/FA) at 4 ºC at 2000 rpm for 10 min and the supernatant was used for total glucose and total amino acid estimation and the precipitate was processed for the estimation of protein.

2.2.3 Estimation of total protein, total free amino acids and total glucose

The precipitate obtained after centrifuging the homogenate was successively extracted in ethanol-chloroform (2:1) ethanol-ether (2:1) and ether at room temperature. The final residue was extracted with 0.5 N perchloroacetic acid at 90 ºC for 15 min. The residue left over the hot acid extraction was dissolved in 0.5 N NaOH. The total protein in the sample was estimated according to Lowry *et al*. (1951) using bovine serum albumin

(Fraction V, Sigma) as standard. The blue colour developed was measured at 660 nm (or red filter) against a reagent blank in a spectrophotometer (UVmini-1240, UV-visible spectrophotometer, Shimadzu).

The supernatant obtained after centrifugation was used for the estimation of total free amino acids. The aliquots of 0.5 ml each were taken (duplicate) and made the volume to 1 ml with distilled water and was estimated according to the method of Lee and Takahashi (1966). The intensity of the colour against a reagent blank at 570 nm was measured using a UV spectrophotometer.

The supernatant obtained after centrifugation was used for estimation of total glucose. The aliquots of 0.2 ml each were taken (triplicate) and made the volume to 1 ml with distilled water and the total glucose was estimated according to Nelson (1944) and Somogyi (1952). The colour developed was measured in a UV-spectrophotometer at 620 nm against a reagent blank.

2.3 RESULTS

2.3.1 Probit analysis

The effective lethal dose of carbaryl to the insect *I. limbata* at different concentrations was recorded and probit analysis was done to estimate the LD_{50} value. The LD_{50} of carbaryl and percentage of survival of insects at different concentrations were shown in figure 2. It was found that 4.8, 3.4, 2.5

and 1.6 µg in 2 µl of acetone per insect gave 100, 75, 50 and 25% of mortality i.e., 0, 25, 50 and 75% of survival respectively, within 48 hr of single exposure of carbaryl.

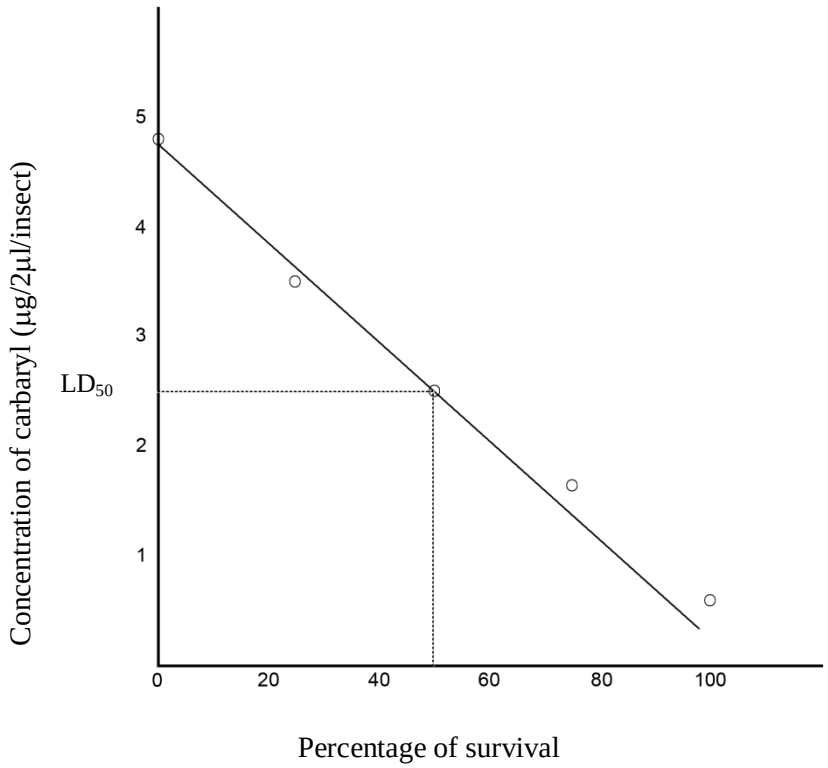


Figure 2: Effect of different doses of carbaryl on the survival of *I. limbata.* Dotted line indicates the LD_{50}.

Immediately after treatment with carbaryl, the bugs exhibited hyperactivity, tremors and prostration. At a concentration of 5 µg, insects showed hyperactivity within 10-20 min after treatment and did not recover from poisoning. While 2.5 µg concentration insects appeared normal for a few hours then exhibited hyperactivity and 50% of the insects were survived. The control groups were showed no mortality within 48 hr.

2.3.2 Acute effects of carbaryl on insect

2.3.2.1 Effect on total protein

The acute effect of carbaryl on total protein of *I. limbata* was estimated. The results were recorded in table 2 and figure 3. The amount of total protein was decreased in the carbaryl treated insects when compared with the control. In general, there was a significant decrease in total protein up to 4 days in treated insects compared to the control. The total protein in carbaryl treated insect was significantly decreased to 10% on the first day ($p < 0.05$), 14% on 2nd day ($p < 0.05$), 12% on 3rd day ($p < 0.001$) and 17% on 4th day ($p < 0.05$). There was no significant difference in total protein on the 6th and 8th days after treatment of carbaryl.

Table 2: The amount of total protein in control and treated insects

Total protein (mg/g tissue)		
Days	Control	Treated
1	54.71 ± 4.77	49.49 ± 2.18*
2	53.42 ± 5.84	46.16 ± 3.49*
3	51.07 ± 5.32	45.64 ± 2.66**
4	54.31 ± 4.18	45.01 ± 2.94*
6	53.17 ± 2.67	48.16 ± 3.15
8	53.53 ± 4.08	49.87 ± 4.32

Value are means ± SDs (n=5).
Values are significant at * $p < 0.05$; ** $p < 0.01$ level.

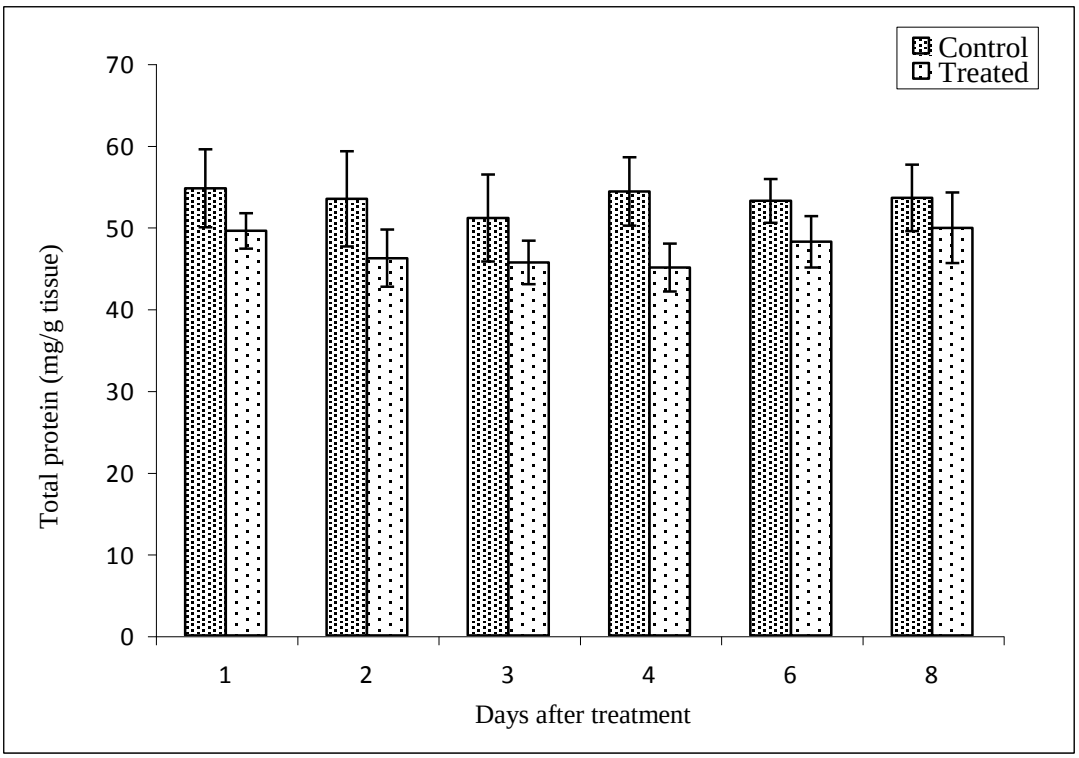


Figure 3: The amount of total protein in control and treated insects. The vertical bars represent SD.

2.3.2.2 Effect on total free amino acids

The total free amino acids in the treated insects were estimated and the results were presented in table 3 and figure 4. The data showed significant increase in total free amino acids in the initial period (1-2 days) after the application of carbaryl compared to the control insects. The level of total free amino acids in treated insects showed significant elevation of 13% on 1st day ($p < 0.05$) and 3% on 2nd day ($p < 0.01$) and a significant decrease of 6% on 3rd day ($p < 0.01$) over the control insects. There was no significant difference in total free amino acids on 3rd day after carbaryl administration.

Table 3: The amount of total free amino acids in control and treated insects

Total free amino acids (mg/g tissue)		
Days	Control	Treated
1	21.02 ± 1.94	23.78 ± 1.88*
2	21.68 ± 2.43	22.38 ± 2.13**
3	19.44 ± 2.82	18.29 ± 2.45**
4	21.72 ± 1.75	20.80 ± 2.74
6	20.15 ± 2.27	21.64 ± 2.96
8	21.04 ± 3.43	21.22 ± 2.37

Value are means ± SDs (n=5).
Values are significant at * $p < 0.05$; ** $p < 0.01$ level.

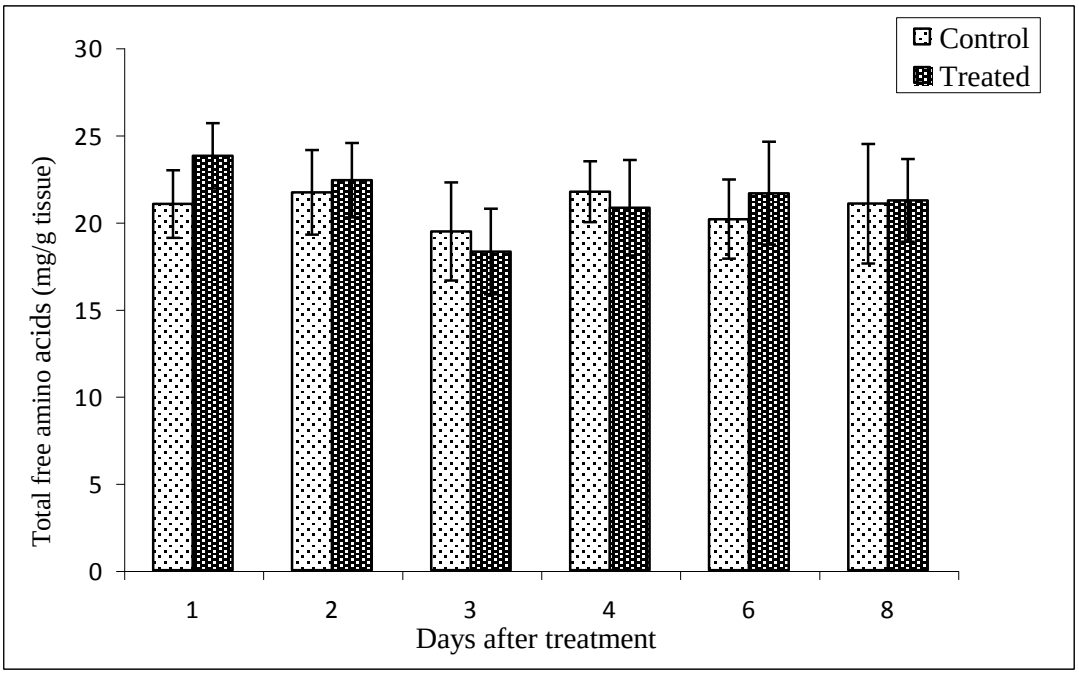


Figure 4: The amount of total free amino acids in control and treated insects. Vertical bars represent SD.

2.3.2.3 Effect on total glucose

The effect of carbaryl on total glucose in *I. limbata* was estimated, the results are presented in table 4 and figure 5. The results showed an increase in total glucose in the initial period (1-2 days) which was followed by a decline after 3rd day onwards in treated insects. The amount of total glucose was significantly elevated to 25% on 1st day ($p < 0.01$) and to 3% on 2nd day ($p < 0.05$). There was 10% of significant decrease in total glucose on 3rd day ($p < 0.001$), 21% on 4th day ($p < 0.05$) and 19% on 6th day ($p < 0.05$). The decrease in the amount of total glucose on 8th day after single application of carbaryl showed no significant difference in treated insects compared to control. The insects more or less regained its normal glucose level on 8th day after carbaryl treatment.

Table 4: The amount of total glucose in control and treated insects

Total glucose (mmol/g tissue)		
Days	Control	Treated
1	18.88 ± 2.25	23.58 ± 2.65**
2	19.45 ± 2.94	20.13 ± 2.82*
3	19.20 ± 1.71	17.45 ± 1.67***
4	18.36 ± 2.77	16.52 ± 2.82*
6	21.26 ± 1.91	16.09 ± 1.45*
8	20.74 ± 2.58	19.97 ± 2.14

Values are means ± SDs (n=5).
Values are significant at * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ level.

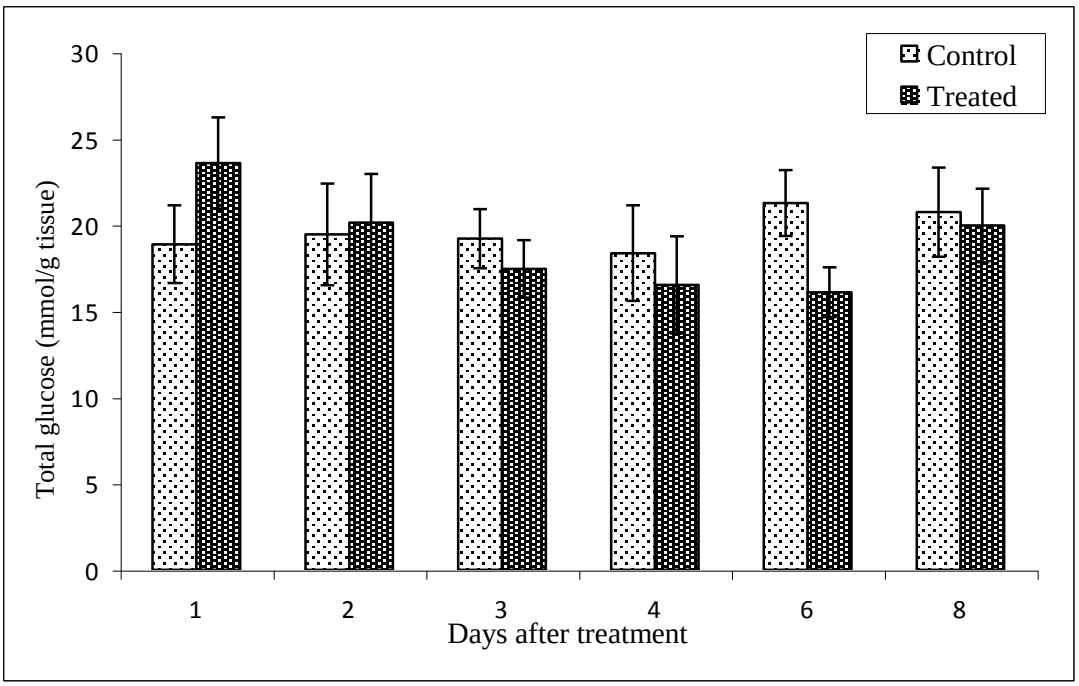


Figure 5: The amount of total glucose in control and treated insects. Vertical bars represent SD.

Percentage difference in the amount of total protein, total free amino acids and total glucose

The percentage changes in the amount of total protein, total free amino acids and total glucose in treated over control insects are presented in the table 5 and figure 6. The percentage change in total protein in the treated over control insects (E/C) was more or less same throughout the period of exposure. The percentage changes in the amount of total free amino acids showed an increase up to 2 days after application of carbaryl. The ratio of total free amino acids showed a dip on the 3rd day after application compared to control which remained more or less same up to 8th day. The pattern of changes in the ratio of total glucose was same as total free amino acids.

Table 5: Percentage changes in the amount of total protein, total free amino acids and total glucose in treated over control insects

Days	Total protein (E/C %)	Total free amino acids (E/C %)	Total glucose (E/C %)
1	85 ± 2.41	114 ± 2.96	123 ± 4.75
2	90 ± 3.89	110 ± 4.64	116 ± 3.49
3	90 ± 4.57	86 ± 7.46	80 ± 5.12
4	88 ± 8.11	89 ± 5.64	84 ± 7.06
6	91 ± 5.80	84 ± 8.19	81 ± 3.11
8	96 ± 3.74	97 ± 4.23	92 ± 4.79

Value are means ± SDs (n=5).

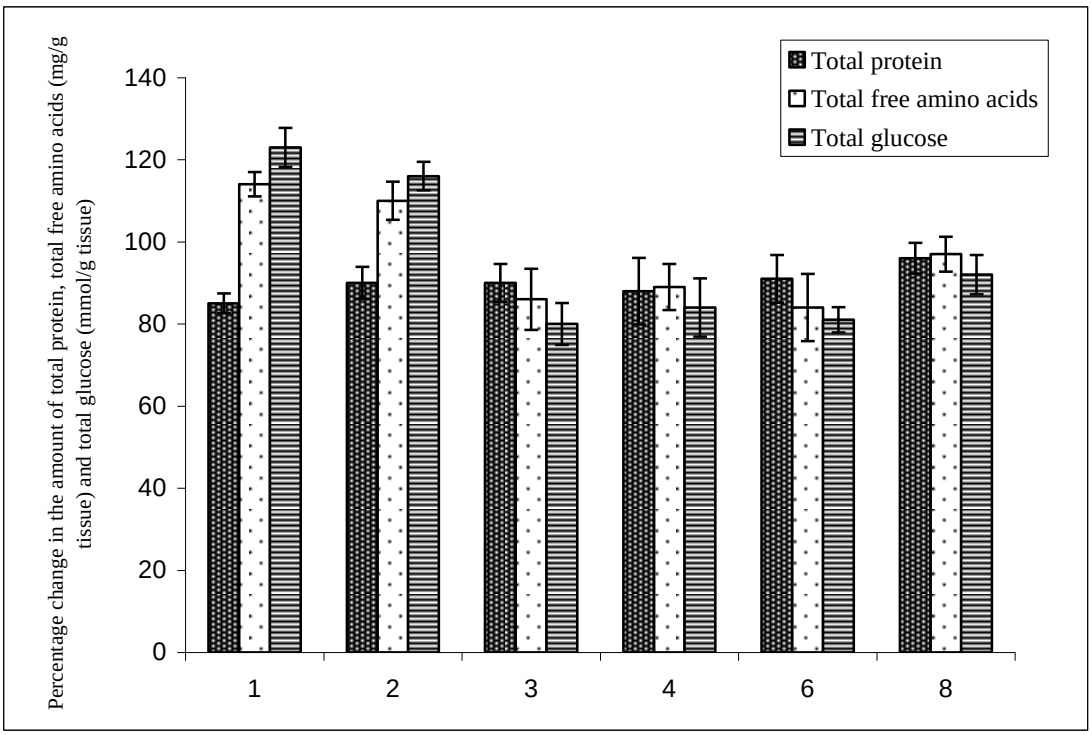


Figure 6: The ratio of total protein, total free amino acids and total glucose in treated over control insects. Vertical bars represent SD.

2.4 DISCUSSION

The behaviour of insects and all animals is governed by interactions among neurons within their nervous systems. Insecticides have been selected and sometimes designed for their remarkable ability to kill insects. Most of them attack specific sites within the insect nervous system (Narachashi, 1976). Therefore it is not surprising that insecticides at levels that do not lead to mortality can influence behaviour. The tremors and prostration observed in the bug after treatment of carbaryl explains the inhibition of acetylcholinesterase activity of the compound. The recovery of the insect after 48 hr of carbaryl treatment demonstrates the reversible nature of cholinesterase inhibitor. The behavioral change observed in the bugs also demonstrates the cholinergic stimulations due to the acute effect on the nervous system.

Carbaryl at 4.8 μg/2 μl and higher doses had an acute effect on adult male *I. limbata*, 2.5 μg/2 μl was the median lethal dose at 48 hr of exposure. The contact LD_{50} observed in *I. limbata* in the present study is comparable to the value reported in honeybees, i.e., 1.3 μg/honeybee (Stevenson, 1978).

The changes in the amount of total protein, total free amino acids and total glucose observed in the bug when exposed at LD_{50} concentration of carbaryl demonstrate a mechanism similar to that of stress. Similar results were presented by Orzel and Weiss (1966) in rats with a dose of LD_{50}

produced hyperglycemia at 44 hr. The application of permethrin resulted in an increased titre of two hyperglycemic neuropeptides in the central nervous system (CNS) and the hemolymph of the fire bug, *P. apterus* (Kodrik and Socha, 2005). It has been reported that insecticides induce the release of hyperglycemic neurohormones within 48 hrs of treatment, due to the direct action of these chemicals upon neurosecretory cells (Singh and Orchard, 1982). This leads to the production of glucose through glycogenolysis and gluconeogenesis (Rajamannar and Manohar, 2000). The carbaryl treated prawns showed a reduction in glycogen and an elevation in total sugar level (Bhavan and Geraldine, 2002). Inhibition of protein synthesis is a common action of toxicants (Shank, 2004). Bisazir is an effective chemosterilant for insects, of its cause significant reductions in protein and an enhancement in total free amino acids activity have been observed (Srivastava and Kumar, 1984). The intensive proteolysis and the elevation of total free amino acids are suggested as an alternative mechanism for the production of energy rich compounds under pesticide induced stress (Rajamannar and Manohar, 2000). They have also reported an elevation of total free amino acids, a dip in tissue proteins, total carbohydrates and glycogen reserves in fishes as a result of carbaryl treatment (Rajamannar and Manohar, 1998).

A remarkable drop in the level of the total carbohydrates in fish exposed to pesticides, particularly to carbaryl exposure suggesting its mobilization to meet the higher demands under pesticide stress. In

consonance with the decreased tissue glycogen levels the blood glucose level increased concomitantly indicating the mobilization of glycogen as glucose from blood to the tissues (Sahib, 1984; Rajamannar and Manohar, 1998). The results of the present study indicate extensive mobilization and utilization of energy resources of the animal for survival during stress. Most of the changes with respect to total protein, total free amino acids and total glucose encountered in the initial period of exposure of carbaryl get restored within a week demonstrating that the short-term residual property of carbaryl.

CHAPTER 3

EFFECTS OF CARBARYL ON HEMOLYMPH

3.1 INTRODUCTION

The hemolymph, in most insects, is a greenish yellowish fluid containing a large number of cells or hemocytes and various organic and inorganic constituents. About 90% of hemolymph is water, which determines its total volume (Tembhare, 1997). The insect hemolymph serves as a bathing medium for various tissues and organs as they lack an epithelial lining of a true coelom. Therefore, the hemolymph forms the meeting place of both the raw materials required and the products of various physiological activities of the insect body. Since the hemolymph is not directly connected with the external environment any change in it can be taken as a measure of the physiological state of the internal environment of the intact animal. Analysis of the hemolymph, therefore, may provide one of the most reliable data which can be used as an index of the physiological activity (Buck, 1953; Wyatt, 1961). The insect hemolyph is noted for its high titre of free amino acids (Chen, 1962). All of the amino acids that occur in proteins (total amino acids) also are found in free form (free amino acids) in the blood, extracellular fluid and within cells. It is depleted by the utilization of amino acids for the synthesis of proteins and for the production of other nitrogenous constituents of the body. Any excess of supply over utilization is oxidized, providing high-energy phosphate (McGilvery, 1970). Carbaryl administration adversely affected the hemolymphatic organic constituents and was responsible for the mortality even at sub-lethal doses (Bharathi and Govindappa, 1987). Any

effective change in the hemolymph may also affect the reproductive system. According to Loeb and Birnbaum (1981), changing osmotic pressure of the hemolymph contributes to the control of development and degeneration of sperm in *Heliothis virescens*. A change in the amino acid pool and enzyme phosphatases will directly influence the protein turn over, i.e., anabolism and catabolism of proteins and will thus reflect the physiological state of the insect. Studies on the effect of carbaryl on the hemolymph in the titres of protein, phosphatases (acid and alkaline phosphatases) and amino acids in heteropteran insects are also not reported.

3.2 MATERIALS AND METHODS

3.2.1 Treatment of carbaryl at LD_{50} level

The hemolymph was collected from insects before (0 hr) and after (24 hr) the application of LD_{50} of carbaryl by amputating one of the antennae. Acetone treated insects were used as control. The pooled hemolymph samples were collected from 20 bugs (5 µl of hemolymph was collected from each bug with a calibrated capillary tube and transferred to eppendorf tubes placed on crushed ice), centrifuged for 2 min at 2000 rpm. The supernatant of hemolymph 10 µl for each was used to estimate the titres of total protein, acid phosphatase and alkaline phosphatase.

3.2.1.1 Estimation of hemolymph total protein

The hemolymph was precipitated with 80% ethanol and the supernatant was removed by centrifugation at 3500 rpm for 10 min. The precipitate was then successively extracted with ethanol: chloroform, ethanol: ether and finally ether at room temperature. The final residue was extracted with 3% perchloric acid at 90 ºC for 15 min. The residue left over the hot acid extraction was dissolved in 0.1 N NaOH. Total protein was estimated according to the method of Lowry *et al*. (1951).

3.2.1.2 Estimation of acid phosphatase (ACP) and alkaline phosphatase (ALKP) activities

The estimation of acid phosphatase was carried out according to the method described by Sadasivam and Manikam (1992). The assay system for acid phosphatase activity consisted of hemolymph, 0.1M citrate buffer pH 5.0 and substrate *p*-Nitrophenyl phosphate (PNPP) in a final volume of 1 ml (triplicate). Heat denatured hemolymph was used for control in both acetone treated and carbaryl treated insects. Incubation was done at 37 ºC for 30 min in a water bath. The *p*-Nitrophenol released by enzymatic hydrolysis was measured at 405 nm with UV-spectrophotometer against the reagent blank.

The estimations of alkaline phosphatase activity (triplicate) were done at pH 10.0 using 0.1 M sodium glycinate buffer in the assay system. One unit of enzyme activity (acid phosphatase and alkaline phosphatase) was defined

as one µmol of *p*-Nitrophenol formed per min per mg crude protein as the assay conditions described above.

3.2.1.3 IR spectrum of hemolymph free amino acids

The LD_{50} of carbaryl was topically applied on *I. limbata* and after 1 hr, hemolymph was taken as described elsewhere (see, chapter 3). The hemolymph was precipitated with 5% TCA in an eppendorf tube, and was centrifuged at 2000 rpm for 5 min. The supernatant was dried over silica gel. Similarly the hemolymph of acetone treated insects was used for comparison. The spectrum of the material in a potassium bromide disc was taken as described by Silverstein and Webster (2002) with IR spectrometer ((FT/IR-4100 type A).

3.2.2 Treatment of carbaryl at sub-lethal dose level

The male insects were administered topically with a sub-lethal dose of carbaryl (0.6 µg/2 µl/insect) every 5 days for a period of 60 days on the dorsal anterior portion of the abdomen under the wings using a micropipette. Solvent (acetone) treated control was run along for comparison. Estimations were done after a period of 60 days.

3.2.2.1 Sample preparation for the assay of total amino acids in the hemolymph

The hemolymph was collected from insects as described elsewhere. Each sample (both control and treated) was mixed with 1 ml of 6 N HCl to

break the peptide bonds and the test tubes were then filled with nitrogen and sealed. It was then kept at 121 ºC for 24 hr for hydrolysis. The hydrolyzed sample was filtered and flash evaporated repeatedly adding distilled water till the traces of chlorine removed. The residue obtained was made up to 2 ml with 0.05 M HCl.

3.2.2.2 Sample preparation for the assay of individual free amino acids in the hemolymph

The hemolymph was collected from insects as described elsewhere. Each sample (both control and treated) was deproteinized with 1 ml of 5% TCA and the precipitate was removed by centrifugation at 3000 rpm for 10 min. The supernatant obtained was flash evaporated and the residue was made up to 0.5 ml with 0.05 M HCl.

The total amino acids and free amino acids present in the hemolymph were determined as per the method described by Ishida *et al*. (1981).

3.2.2.3 Analysis of amino acids in the hemolymph with amino acid analyzer (HPLC-LC10AS)

The sample was filtered through a membrane filter of 0.45 μm and 20 μl of the filtrate was injected to an amino acid analyzer (HPLC-LC 10AS) equipped with cation column packed with a strongly acidic cation exchange resin, styrene divinyl benzene copolymer with sulphonic group. The column was sodium type, ISC-07/S 1504 Nam, with 19 cm length and 5 mm diameter.

The instrument was equipped with Shimadzu FL 6A fluorescence detector and Shimadzu CR 6A chrompac recorder. The mobile phase of the system consists of two buffers, buffer A (trisodium citrate, methanol and perchloric acid in double distilled water, pH adjusted to 3.2 by perchloric acid) and buffer B (trisodium citrate and boric acid in double distilled water, pH adjusted to 10 by 4 N NaOH). Gradient system was followed for effective separation of amino acids. The flow rate of the mobile phase was programmed in order to achieve maximum separation. The total run was programmed for 60 min. The oven temperature was maintained at 60 ^{o}C. The amino acids were eluted from the column by step wise elution, i.e., acidic amino acids first followed by neutral and then basic amino acids.

The amino acid analysis was done with non-switching flow method and fluorescence detection after post column derivatization with O-phthaldehyde (OPA). The $–NH_2$ functional group reacted with OPA (OPA buffer-sodium carbonate, boric acid and potassium sulphate in double distilled water), producing fluorescent substance in the presence of 2 mercapto-ethanol. In the case of proline and hydroxy proline, imino group was converted to amino group with sodium hypochlorite reagent (prepared in OPA buffer).

Amino acid standard (Sigma Chemical Co., St. Louis, USA) was also run to calculate the concentration of amino acids in the sample. The elution

time of each amino acid standard was determined and their positions were noted in the chromatogram. The quantification of amino acids was done by comparing the respective peak areas in the chromatogram of the sample with the standard. The amino acid content was calculated and expressed as μmol/ml hemolymph.

3.2.2.4 Estimation of tryptophan

The hemolymph was hydrolyzed in 2 ml of 2 N NaOH at 110 ^{o}C for 24 hr in a sealed tube with pure nitrogen. The hydrolyzed sample was neutralized to pH 7.0 with 6 N HCl using phenolphthalein indicator. The solution was then filtered through Whatman No.1 filter paper and filtrate was used for estimation. The H_2SO_4 of 50%, 25% sucrose and 0.6% thioglycolic acid were added to a test tube. Tubes were then kept for 5 min in water bath at 45-50 ^{o}C and cooled. The sample was then added to the test tubes. Five independant trials were made for both control and treated. A standard tryptophan (10 μg/ml) was run in a similar way. The samples were made up to 5 ml with 0.1 N HCl and allowed to stand for 5 min to develop colour. The absorbance was measured against reagent blank at 500 nm in a spectrophotometer. The amount of tryptophan in the hemolymph sample was expressed as μmol/ml hemolymph.

3.3 RESULTS

3.3.1 Acute effects of carbaryl in the hemolymph

3.3.1.1 Total protein in the hemolymph

The total protein in the hemolymph of treated and control insects are presented in table 6 and figure 7. The results indicate a significant ($p < 0.01$) decrease (14%) in total protein in the hemolymph of insects at 24 hr after carbaryl administration. The control group showed slight increase in the hemolymph total protein but it is statistically insignificant.

Table 6: Total protein in the hemolymph of control and treated insects

	Total protein (mg/ml)	
	0 hr	24 hr
Control	20.09 $\pm$ 1.3	20.34 $\pm$ 0.77
Treated	21.75 $\pm$ 0.34	19.04 $\pm$ 0.69**

Values are means ± SDs (n=6).
Values are significant at ** $p < 0.01$ level.

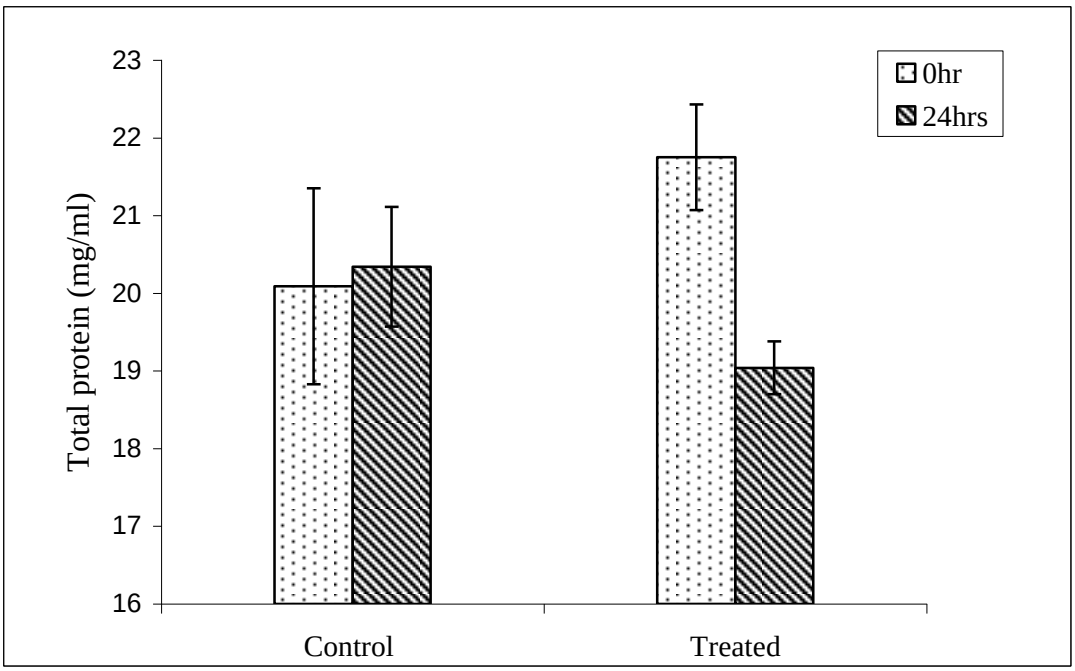


Figure 7: Total protein in the hemolymph of control and treated insects. Vertical bars represent SD.

3.3.1.2 Acid phosphatase activity in the hemolymph

The results of the assay of acid phosphatase activities are presented in table 7 and figure 8. There is significant ($p < 0.01$) increase (55%) in acid phosphatase activity in the carbaryl treated insects at 24 hr compared to control.

T**able 7:** Acid phosphatase activity in the hemolymph of control and treated insects

	Acid phosphatase activity (µmol/min/ml)	
	0 hr	24 hr
Control	17 ± 4.3	18 ± 6.3
Treated	20 ± 6.0	31 ± 8.0**

Values are means ± SDs (n=6).
Values are significant at $^{**}p < 0.01$ level.

Specific activity of acid phosphatase

The specific activity of acid phosphatase in the hemolymph of *I. limbata* is presented in the table 8 and figure 9. The results showed significant ($p < 0.05$) increase in carbaryl treated insects at 24 hr.

Table 8: The specific activity of acid phosphatase in the hemolymph of control and treated insects

	Acid phosphatase activity units	
	0 hr	24hr
Control	0.846 ± 0.21	0.884 ± 0.30
Treated	0.919 ± 0.27	1.628 ± 0.45*

Values are means ± SDs (n=6).
Values are significant at * $p < 0.05$ level.

3.3.1.3 Alkaline phosphatase activity in the hemolymph

The alkaline phosphatase activity in the hemolymph of *I. limbata* is given in table 9 and figure 8. The results showed significant ($p < 0.01$) increase (46%) in alkaline phosphatase activity in the carbaryl treated insect at 24 hr compared to control.

Table 9: Activity of alkaline phosphatase in the hemolymph of control and treated insects

	Activity of alkaline phosphatase (µmol/ml)	
	0 hr	24 hr
Control	6.6 ± 1.6	7.6 ± 2.0
Treated	7.3 ± 2.2	10.7 ± 1.3**

Values are means ± SDs (n=6).
Values are significant at ** $p < 0.01$ level.

Specific activity of alkaline phosphatase

The specific activity of alkaline phosphatase in the hemolymph of treated insect is presented in the table 10 and figure 9. The results showed significant ($p < 0.01$) increase in unit activity of alkaline phosphatase at 24 hr after treatment compared to control.

Table 10: The specific activity of alkaline phosphatase in the hemolymph of control and treated insects

	Alkaline phosphatase activity units	
	0 hr	24 hr
Control	0.326 ± 0.079	0.373 ± 0.98
Treated	0.335 ± 0.101	0.561 ± 0.68**

Values are the means ± SDs.
Values are significant at ** $p < 0.01$ level.

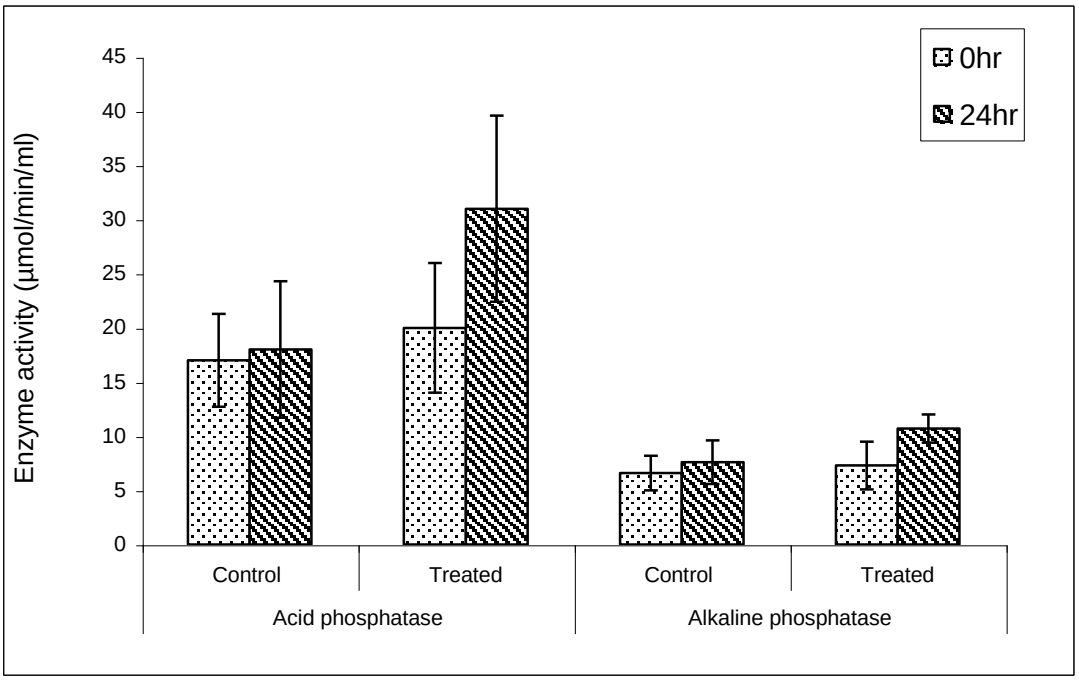


Figure 8: Activity of acid phosphatase and alkaline phosphatase in the hemolymph of control and treated insects. Vertical bars represent SD.

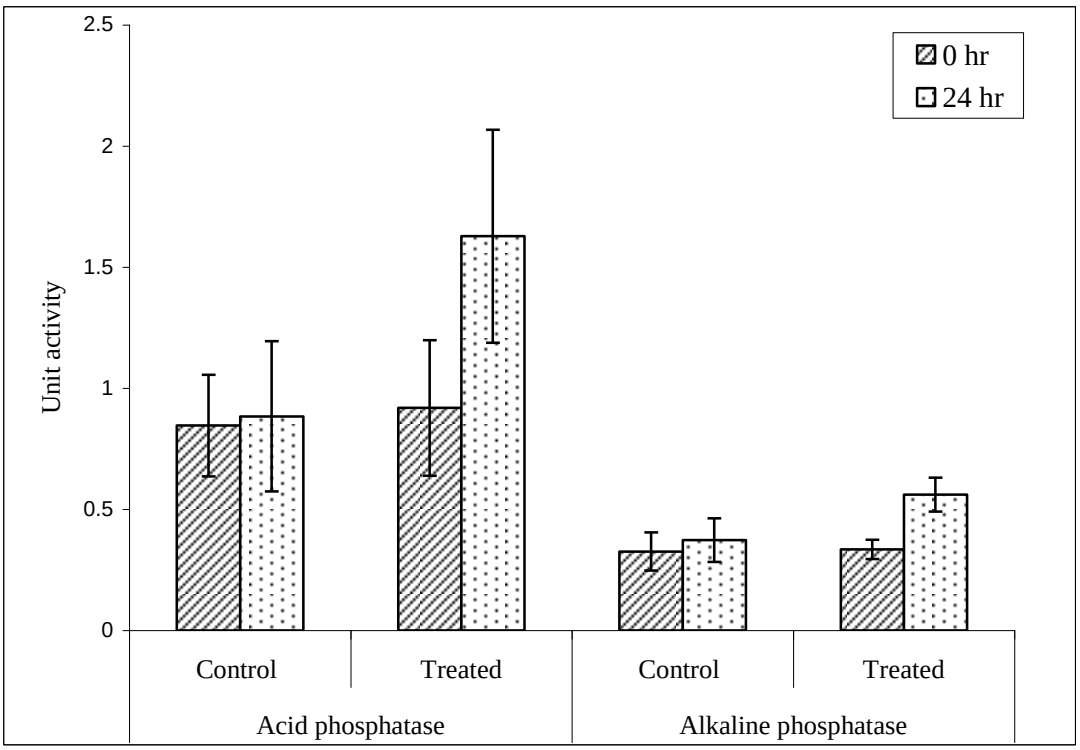


Figure 9: The specific activity of acid phosphatase and alkaline phosphatase in control and treated insects. Vertical bars represent SD.

3.3.1.4 IR spectrum of hemolymph free amino acids

The IR spectrum of the hemolymph free amino acids of *I. limbata* at 1 hr after carbaryl application is shown in figure 10. The IR spectrum of carbaryl treated insect showed intensification of the band formed at ~ 1637 cm^{-1} compared to control at 1 hr after treatment. The peak at ~ 3433 cm^{-1} showed a broadening in carbaryl treated samples. The frequency of band at ~ 3464 cm^{-1} is shifted to ~ 3433 cm^{-1} in the treated samples compared to control.

3.3.2 Chronic effects of carbaryl in the hemolymph amino acids

3.3.2.1 Total amino acids in the hemolymph

The total amino acid profile of hemolymph of *I. limbata* was analysed using HPLC separation and the results are presented in the table 11 and figure 11. The analysis detected 18 and 17 amino acids in control and treated insects respectively. The following amino acids are detected in the hemolymph: aspartic acid (A), threonine (T), serine (S), glutamic acid (E), proline (P), glycine (G), alanine (A), cysteine (C), valine (V), methionine (M), isoleucine (I), leucine (L), tyrosine (Y), phenylalanine (F), histidine (H), lysine (L) and arginine (R). Among this cysteine is below the level of detection in the carbaryl treated insects after chronic exposure. In general, the levels of total amino acids showed a decrease in the treated insects. The results showed 14% reduction in total amino acids in treated insects after chronic exposure to carbaryl.

The relative abundance of total amino acids in control insect is as follows:

Glutamic acid > aspartic acid > alanine > leucine > glycine > proline > arginine > valine > serine > isoleucine > threonine > tyrosine > phenylalanine > histidine > tryptophan > lysine > methionine > cysteine.

The relative abundance of total amino acid profile in carbaryl treated insect is as follows:

Glutamic acid > aspartic acid > alanine > glycine > leucine > proline > arginine > serine > valine > isoleucine > threonine > tyrosine > phenylalanine > histidine > lysine > tryptophan > methionine.

Table 11: Total amino acids in the hemolymph of control and treated insects after chronic exposure

Sl. No.	Amino acids	Control (μmol/ml)	Treated (μmol/ml)
1	Aspartic acid (D)	45.18	42.70
2	Threonine (T)	12.03	10.94
3	Serine (S)	17.27	15.82
4	Glutamic acid (E)	58.37	53.42
5	Proline (P)	24.51	19.37
6	Glycine (G)	27.95	24.61
7	Alanine (A)	40.43	34.63
8	Cysteine (C)	0.24	B.L.D.
9	Valine (V)	18.61	15.33
10	Methionine (M)	3.20	1.13
11	Isoleucine (I)	15.88	11.06
12	Leucine (L)	28.37	23.98
13	Tyrosine (Y)	11.60	9.14
14	Phenylalanine (F)	11.55	9.09
15	Histidine (H)	8.24	6.24
16	Lysine (K)	4.15	3.90
17	Arginine (R)	19.53	17.61
18	Tryptophan (W)	4.5	3.47
	Total	351.6	302.44

B.L.D-Below the level of detection.

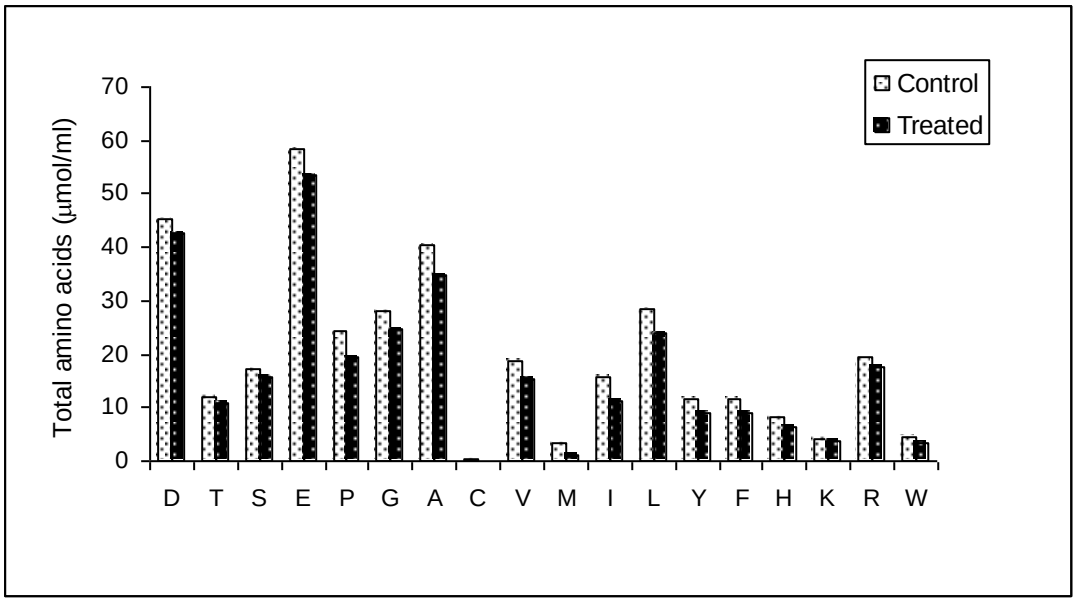


Figure 11: Total amino acid profile of hemolymph in control and treated insects after chronic exposure.

3.3.2.2 Individual free amino acids in the hemolymph

Analysis of hemolymph free amino acids using HPLC separation revealed 18 individual free amino acids. The results of the estimation are presented in table 12 and figures 12 and 13. The results showed 46% of reduction in the amount of total free amino acids in the hemolymph of carbaryl treated insects. The difference between control and treated (C-E) insects showed decrease in individual free amino acids except valine, isoleucine, leucine and arginine in treated insects.

The relative abundance of individual free amino acids in control insect is as follows:

Taurine > alanine > proline > aspartic acid > glycine > serine > glutamic acid > threonine > histidine > arginine > methionine > valine > tyrosine > leucine > lysine > phenylalanine, cysteine > isoleucine.

The relative abundance of individual free amino acids in carbaryl treated insects is as follows:

Alanine > proline > aspartic acid > glycine > taurine > glutamic acid > histidine > arginine > valine > serine > methionine > leucine > threonine > tyrosine > lysine > isoleucine > phenylalanine > cysteine.

Table 12: Individual free amino acids in the hemolymph of control and treated insects after chronic exposure

Sl. No.	Amino acids	Control(C) (μmol/ml)	Treated (T) (μmol/ml)	C–T (μmol/ml)
1	Aspartic acid (D)	2.13	1.77	0.36
2	Threonine (T)	0.41	0.15	0.26
3	Serine (S)	0.63	0.22	0.40
4	Glutamic acid (E)	0.56	0.41	0.15
5	Proline (P)	2.78	1.94	0.84
6	Glycine (G)	0.83	0.78	0.05
7	Alanine (A)	3.19	2.80	0.39
8	Cysteine (C)	0.08	0.04	0.04
9	Valine (V)	0.20	0.22	-0.02
10	Methionine (M)	0.23	0.20	0.03
11	Isoleucine (I)	0.056	0.08	-0.024
12	Leucine (L)	0.16	0.18	-0.002

13	Tyrosine (Y)	0.19	0.14	0.05
14	Phenyl alanine (F)	0.08	0.06	0.02
15	Histidine (H)	0.36	0.35	0.01
16	Lysine (K)	0.15	0.11	0.04
17	Arginine (R)	0.24	0.29	-0.05
18	Taurine	6.78	0.63	6.12
	Total	18.85	10.31	8.66

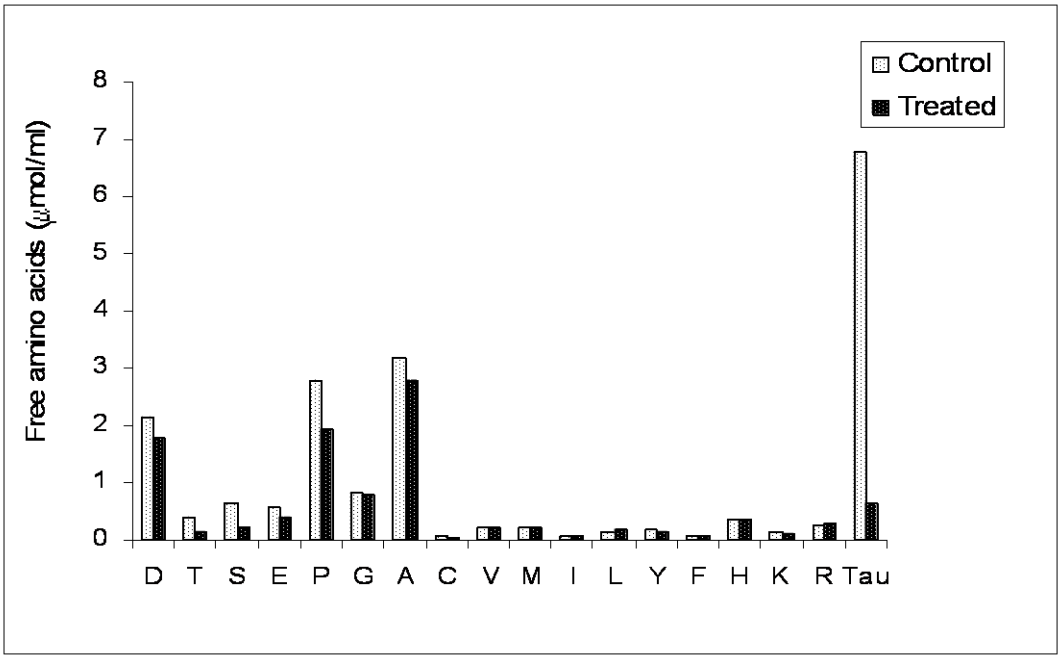


Figure 12: The individual free amino acid profile of hemolymph in control and treated insects.

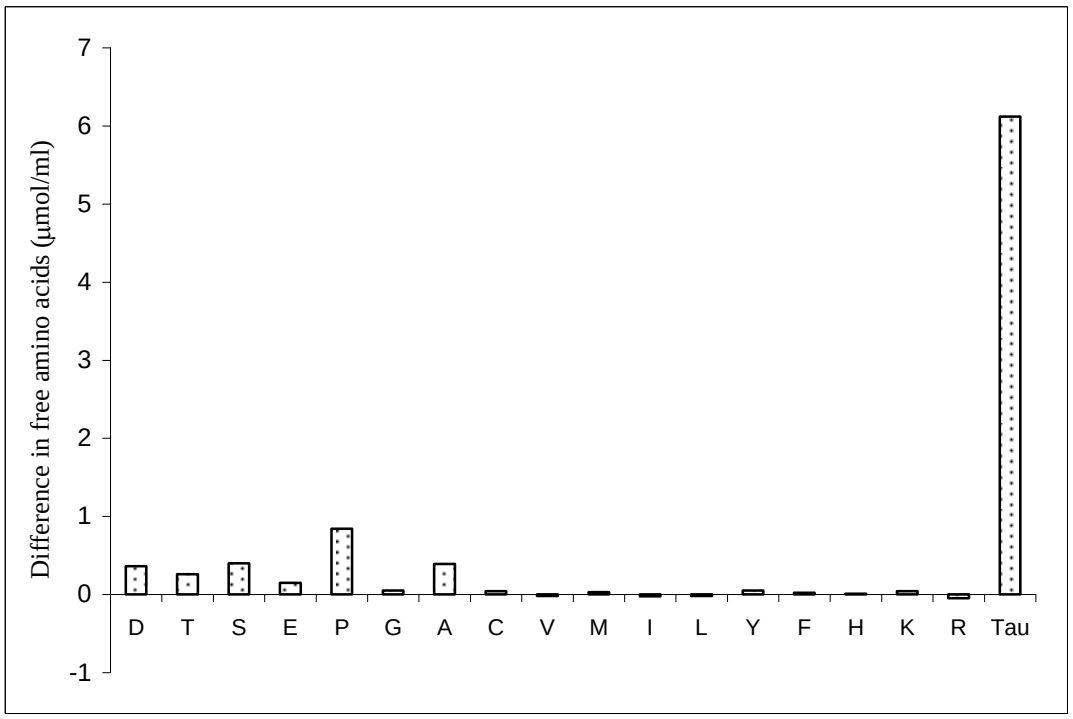


Figure 13: The difference in the amount of individual free amino acids between control and treated insects.

3.4 DISCUSSION

3.4.1 Acute effects of carbaryl in the hemolymph

3.4.1.1 Effect on total protein

The amount of hemolymph total protein in *I. limbata* showed a significant decrease in carbaryl treated insects. A decrease in protein content was also reported in carbaryl exposed *M. vittatus* (Palanichamy *et al.*, 1989). Rath and Mishra (1980) have also reported a reduction in protein content in *T. mossambica* exposed to pesticide media. Rao *et al*. (1987) have found that benthiocarb, a carbamate, decreased the soluble and insoluble protein content

in different tissues of *Sarotherodon mossambicus*. The observed decrease in the levels of proteins is probably to counteract the pesticide toxic impact.

3.4.1.2 Effects on phosphatases

Disease, starvation, aging, stress and environmental factors are known to influence physiological and biochemical state of animals, by exhibiting marked changes in the activities of several enzymes (Knox and Greengard, 1965; Mathur, 1976). A change in enzymes of carbohydrate metabolism under pesticidal impact is well documented (Eller, 1971; Bhatia *et al*., 1972; Hendrickson and Bowden, 1976; Dragomirescu *et al*., 1979; Sastry and Siddiqui, 1983; Mehler, 1988).

The elevated levels of acid and alkaline phosphatases in the hemolymph of *I. limbata* in response to carbaryl treatment indicate a mechanism similar to necrosis induced reactions. When the tissues exposed to toxins necrosis will occur. Once a cell dies, the lysosomes within it release phosphatases, nucleases, proteases and other enzymes digesting the cell by the process of autolytic necrosis (Shank, 2004). It was found that there was an increase in the release of phosphatases with carbaryl exposure. Acid phosphatase, predominently regarded as the marker enzyme has been found in Golgi cisternae and lysosomes. The quantity of alkaline phosphatase is directly proportional to the amount of transfer across cell boundaries and damage to plasma membrane assembly. Increased activity of acid phosphatase

indicates a breakdown of lysosomes causing the release of lysosomal enzymes which have proteolytic action (Bell *et al*., 1970; Maczon, 1976).

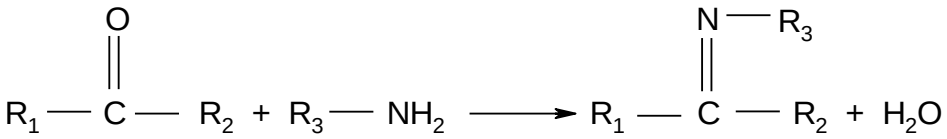
Alkaline phosphatase has been shown to be intimately associated with protein synthesis (Pilo *et al*., 1972) and spermatogenesis (Pavlikova and Repas, 1975). The increased alkaline phosphatase activity in the hemolymph of *I. limbata* is to compensate the protein reduction in treated insects. There is significant reductions in alkaline phosphatase activity and an enhancement in acid phophatase activity have been observed after bizasir treatment in *Earias fabia* (Srivastava and Kumar, 1984). In *S. monstrous* acid and alkaline phosphatase activities increased significantly in both brain and ventral nerve cord as a result of pyrethrin adminitration (Banerjee *et al*., 1984).

Acid and alkaline phosphatases activities were increased in nervous tissue and decreased in haemolymph of female adult cockroach, *P. americana*, after the application of bendiocarb, sumicidin, quinalphos and monocrotophos (Rejender, 1986). He suggested that the difference in enzyme activities may be due to unequal liberation of hydrolytic enzymes by lysosomes of damaged cells due to insecticide treatment. The BHC also decreased both phosphatase activities in larvae of *Leucopholis* (Govindwar and Bhawane, 1989). The activity of acid phosphatase was significantly enhanced, whereas alkaline phosphatase activity was significantly inhibited

after the treatment with mexacarbate insecticides in ear cutting caterpillar, *Mythimna separate* (Pandey and Sharma, 1995).

3.4.1.3 Acute effect on free amino acids

The IR spectrum of hemolymph shows that there is imine formation at 1 hr after LD_{50} of carbaryl administration. The nitrogen analogs of ketones and aldehydes are called imines, azomethines or Schiff bases. These compounds are formed by the condensation of primary amines with ketones or aldehydes. The general structure of an imine is given below.



In the presence of O_2 and H_2O, such imines will quite readily hydrolyze. However, protonation of the Schiff base which is stabilized by hydrogen bonding to the oxygen increasing the acidity of Cα proton. The carbanion formed by the loss of Cα proton is stabilized by the electron delocalization, towards the positively charged nitrogen atom (Nelson and Cox, 2005). IR Spectrum of hemolymph free amino acids of treated insects at 1 hr after carbaryl treatment depicts a band formed at ~1637 cm^{-1} which was found to be intensified compared to control. The strong absorption at ~1637 cm^{-1} is attributed by the superimposed >C=N and >C=O stretching

(Silverstein and Webster, 2002). The possible chemical reaction of imine formation and intra molecular weak bonding are depicted in figure 14.

Due to the imine formation there is an increased possibility of intramolecular hydrogen bonding between oxygen of carboxylic group and H of NH of carbaryl, which is clearly indicated by the broadening of peak at ~ 3433 cm^{-1} in the samples taken from carbaryl treated insects. The shifting of –OH bond to lower frequency region (~ 3433 cm^{-1} in treated insects to ~ 3464 cm^{-1} in control insects) is also attributed to intra molecular hydrogen bonding as in figure 14, i.e., on hydrogen bonding the strength of –OH bond decreases due to stretching vibration of –OH occur in lower frequency region. The –OH stretching bands move to lower frequencies usually with increased intensity and band widening show the extend of hydrogen bonding (Silverstein and Webster, 2002).

The changes in the peak at ~ 1637 cm^{-1} observed in the hemolymph of *I. limbata* at 1 hr after carbaryl topical application, between control and treated insects and the broadening of the peak at ~ 3433 cm^{-1} in the treated condition indicates imine formation in the insect hemolymph with the treatment of carbaryl. This resulted in reduced availability of free amino acids in the metabolic pool with consequent changes in metabolism. Moosmann *et al*. (2001) have reported that imines are formed during toxic exposure to detoxify the substance. They have also suggested that different imine

compounds have neuroprotective antioxidant function and possess excellent cytoprotective potential in diverse paradigms of oxidative neuronal cell death.

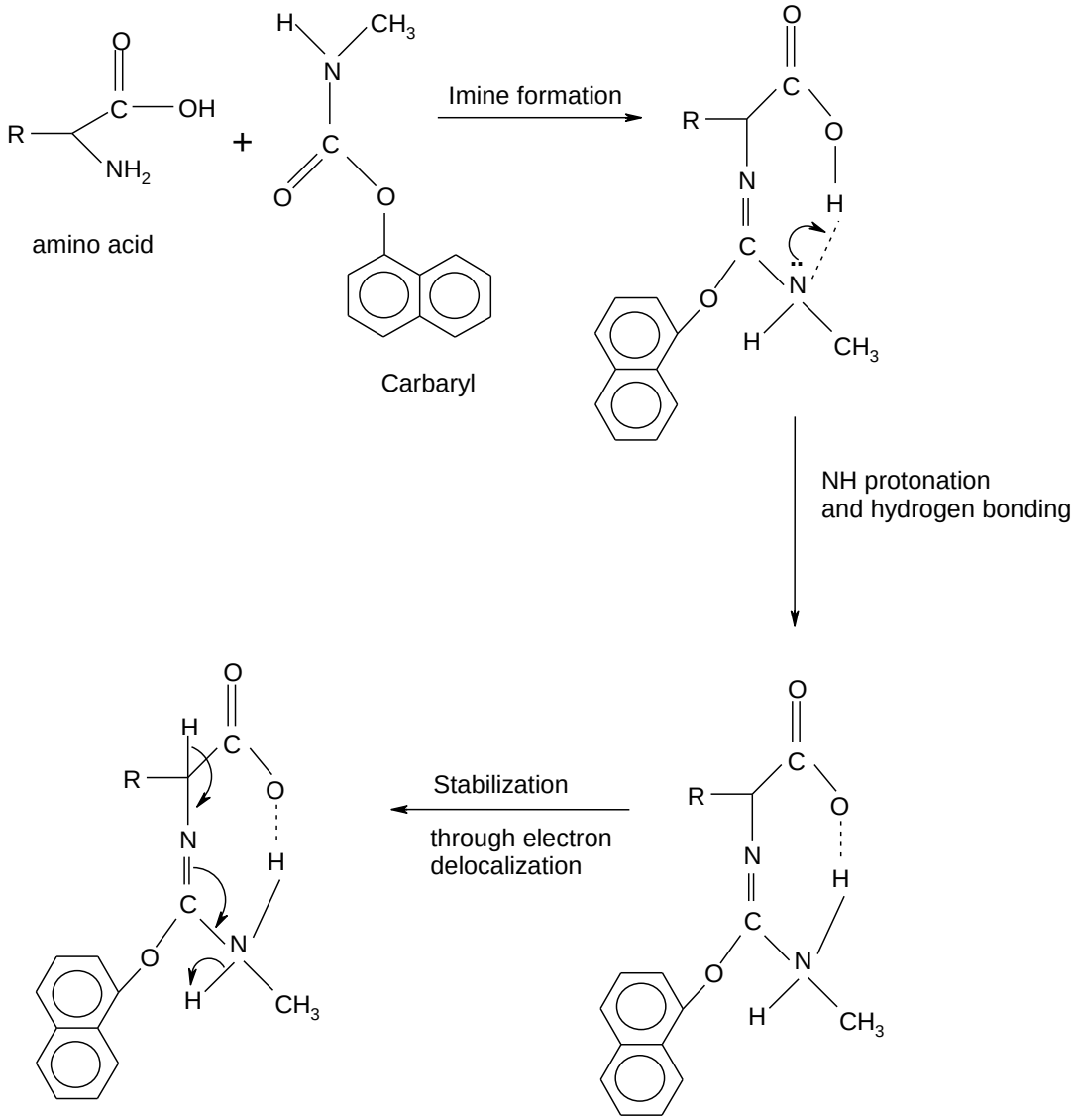


Figure 14: Formation of imine and intramolecular weak bonding of hydrogen in treated insects.

3.4.2 Chronic effects of carbaryl on hemolymph amino acids

3.4.2.1 Effect on total amino acids

There is considerable reduction in the total amino acid profile in the hemolymph of carbaryl treated insects. The changes observed in the hemolymph amino acids of *I. limbata* after chronic exposure with carbaryl reflects the synthesis and degradation of protein with a higher utilisation against less synthesis when compared with control group. As the general metabolism is impaired by the carbaryl, the homeostatic mechanism must also be affected. The reduction in the amino acid in the treated insect when compared to the control observed in the present study can be attributed to the reduction in protein degradation. Other physiological functions of free amino acids in the hemolymph such as buffering capacity of the hemolymph and the maintenance of acid base balance become disturbed due to carbaryl exposure.

A persual of the literature reveals that in general there is no difference in the metabolism of amino acids in insects and other animals except that there is a change in the magnitude of the metabolism of individual amino acids or in their level. Nutritional studies proved that the ten essential amino acids in rat (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine) are also essential for insect (Chen, 1962).

3.4.2.2 Effect on individual free amino acids

The effects of carbaryl on individual free amino acids are discussed below. The amino acids which are listed follow the classification of Umbarger (1978).

1) Glutamate family

Glutamic acid

The topical application of carbaryl affects the metabolism of glutamic acid as its concentration decreased per unit volume of hemolymph compared to control individuals. Glutamine is also formed from glutamic acid in insects. Glutamine is used for the synthesis of uric acid which is a characteristic compound of insect hemolymph.

Proline

The proline is synthesized from arginine. The proline synthesis from arginine results in the production of ornithine and urea. Arginine to proline pathway via ornithine is predominant in insects. The proline is extensively oxidized to yield energy in insects. Thus proline metabolism has multiple function i.e., as an energy source (Bursell, 1963, 1966; Bursell *et al.*, 1974; Sactor, 1975) and a precursor for the synthesis of cuticular structure. Thus the reduction in proline, one of the major energy sources, will directly affect the energy generating potential of the organism.

The amount of proline showed a decrease in treated insects per unit volume of hemolymph when compared with control. The cuticular protein (resilin, fibroin and elastin) of insects shows a predominance of proline residue. Proline forms the important amino acid in the synthesis of integumentary structures and their decrease in treated insects suggests that it may affect the integumentary structure of insect.

Arginine

The arginine in the hemolymph of *I. limbata* showed a dip in the carbaryl treated insect. The reduction in the concentration of arginine may affect the arginine to proline pathway and the energy resources of the insect.

Lysine

The levels of lysine showed a dip after chronic exposure of carbaryl compared to the control.

2) Serine family

Serine

Serine is a common constituent of dietary proteins, but it can be also synthesized from glucose. The turnover of serine in the amino acid pool is almost as rapid as that of glutamate and aspartate. The continual formation of the amino acid insures a supply of one-carbon units independent of the immediate dietary supply. Specific transaminase, which uses alanine rather

than glutamate, converts hydroxypyruvate to serine. After chronic exposure of carbaryl to insects showed decrease in the hemolymph serine per unit volume in the treated insects.

Glycine

The amount of glycine in treated insect is less compared to controls. One of the important functions of glycine is its participation in the synthesis of structural proteins of animals (Seifter and Gallop, 1966). The analysis of the amino acid residues of structural proteins in insects revealed that they are unique in the high content of glycine (Lucas *et al*., 1960; Bailey and Weis-Fogh, 1961; Srivastava, 1971). The structural proteins are important during the maturation of sperms; the lower concentration of glycine in treated insects indicates its reduced availability for the synthesis of structural proteins. Detoxification of certain metabolites can be affected through their interaction with amino acids.

Taurine

Taurine is ubiquitous among a variety of animal species by its abundance in the free amino acid pool (Soupart, 1962). The information on taurine metabolism in insect is very limited though insect hemolymph contains an appreciable amount of the amino acid. The concentration of taurine was found to be reduced to about 1/10 in the carbaryl treated insects. In *Drosophila* and *Phormia* the level of taurine in the hemolymph is high

(Chen and Hanimann, 1965; Levenbook and Dinamarca, 1966). Taurine is considered to be metabolically inactive and virtually the total material synthesized in the nymph is carried over to the adult and most of adult taurine is present in the thorax suggesting its association with the flight muscle (Chen, 1985). The taurine is not merely a metabolically inert end-product of methionine catabolism. Rather, taurine may function in biological systems as a general detoxifier, eliminating excessive cholates and scavenging chlorine oxidants. Taurine may thereby specifically protect cells from self-destruction during process that generates oxidants (Wright *et al*., 1986). Free taurine, is found in millimolar concentrations, especially in the tissues that are excitable, rich in membranes, and that generate oxidants (Wright *et al*., 1985). Taurine possesses a more acidic acid function as well as a more acidic ammonium function than other amino acids (Wright *et al*., 1986). The capacity of amino acid dipole ions to form a metal complex is an important feature of their biological activity. Despite the significant biological effect of taurine on calcium and zinc binding in living systems, a contribution by direct interaction between taurine and metals appears to be minimal (Wright *et al*., 1986). Taurine, as a nutrient is to protect cell membranes by attenuating toxic compounds.

The chlorpromazine, a phenothiazine derivative, is a tranquilizer and antimemetic, inhibits taurine uptake by a synaptosomal preparation from rat cerebral cortex (Schmidt and Schwankl, 1975). The milk of many species

contains taurine. The carbaryl causes depletion of taurine and may affect the infants taurine metabolism (or lactating mammals). Taurine is rapidly transferred from the rat to the pup via the milk. In the human milk, some amount of carbaryl was detected (WHO, 1994).

There are a number of possible synthetic routes by which taurine could be derived from precursor cysteine (Oja and Kontro, 1983). During stress condition, cysteine concentration becomes minimal. In carbaryl treated insects the peak of cysteine is below the level of detection. So as a precursor, the reduction of cysteine is one of the reasons for a decline in the amount of taurine in treated insects. The sharp decline in taurine of insect hemolymph on the exposure of carbaryl may also result in degenerative changes in other tissues that are seen in other animals (Wright *et al*., 1986).

3) Aspartate family

Aspartic acid

The concentration of aspartic acid in the treated insects showed a decline when compared with the control. Aspartic acid is an important precursor in the synthesis of purines, pyrimidines and structural proteins. Aspartate also plays an important role in transamination reactions and thus functioning as a connecting link between the metabolism of carbohydrates and proteins (Katunuma *et al*., 1968). The reduced amount of aspartic acid in

insects may affect the transamination reaction, purine and pyramidine metabolism and gluconeogenesis.

Methionine

Methionine in the hemolymph of adult *I. limbata*, showed a decrease in the treated insect compared to control. Methionine is used for the formation of succinyl CoA which participate in the oxidation of certain fatty acids (Stryer, 1988). So a reduction in its concentration may also affect fatty acid oxidation and protein synthesis. When the methyl group of methionine is utilized for the various purposes, the primary sources for methyl group used in the regeneration of methionine are choline. The inhibition of acetylcholine esterase by carbaryl can result in the reduction of free choline and subsequently affect the regeneration of methionine.

Threonine

The concentration of threonine declined to about 1/3 in the treated insect compared to control. Threonine can be converted to glycine and then to serine. Serine can be catabolised for liberating energy. Thus metabolism of threonine is involved in the formation of proteins and energy (Mehler, 1988). The reduction in threonine due to carbaryl may cause abnormalities in the metabolism of this amino acid and the related ones.

4) Pyruvate family

Alanine

The alanine is declined by about 12% in the treated insect compared to control. It follows that the synthesis of structural proteins may be hindered with carbaryl treatment as alanine is an important fraction in the structural proteins (Lazar and Mohamed, 1988). The reduced levels of alanine may also affect gluconeogenesis (Harris, 1988).

Valine, leucine and isoleucine

These are similar amino acids and they form homologous proteins in various organisms in which these branched chain amino acids replace each other in certain positions without greatly altering the functional properties of the proteins. In their degradation all of them are initially transminated to corresponding α-keto acids, which are then oxidatively decarboxylated to yield a derivatives of CoA.

In carbaryl treated insects these three amino acids showed a unique increase against a decline in all other free amino acids except arginine in its hemolymph. As these three amino acids are involved in energy metabolism the increased concentration in treated insects indicate the increased need for energy during carbaryl induced stress.

5) Aromatic family

Phenylalanine

The nitrogen of phenylalanine was transferred to α-ketoglutarate, producing glutamate. The aromatic amino acids have a metabolism as complex as that of the sulfur amino acids. Phenylalanine is oxidized to form tyrosine, and tyrosine is further metabolized, with the ultimate formation of fumarate and acetoacetate. The phenylalanine in the hemolymph of *I. limbata*, showed a decrease in carbaryl treated groups. Phenylalanine is converted to tyrosine. So the reduction in its concentration due to carbaryl will also affect the tyrosine metabolism.

Tyrosine

The concentration of tyrosine in the hemolymph of *I. limbata,* was affected due to carbaryl treatment. The level of tyrosine was reduced by 25% in the treated group of insects. The tyrosine in the epidermis is converted to ortho-diphenyl N-acetyldopamine which is secreted into the cuticle (Hackman, 1974) and in the epicuticle it meets a diphenol oxidase, which oxidises in its corresponding quinone. The quinone diffuses into the bulk of the cuticle, where it reacts spontaneously with amino group. When quinone reacts with an amino group the nitrogen atom is linked in the aromatic ring by a covalent bond and the quinone is reduced to a catechol residue. In another view the tanning agent, N-acetyl dopamine is not always oxidised to a

quinone but that the side chain can be activated to give a reactive intermediate which would link the cuticular proteins together (Anderson and Barrett, 1971; Anderson, 1979; Vincent and Hillerton, 1979). Tyrosine plays a key role in the sclerotization and melanization of cuticle. The reduction of tyrosine in the hemolymph of treated insects points to the impairment of the tanning and sclerotization process.

Histidine

There was only a slight difference observed in the concentration of histidine in the treated insect compared to control. Histidine can be catebolised to glutamate. Its decarboxylation produces histamine (Stryer, 1988).

6. Tryptophan

The essential amino acid tryptophan is usually the least abundant of the amino acid in the diet, and is not a major substrate for the generation of high-energy phosphate. However, the unusual indole ring that it contains is used as a precursor for a variety of cellular components. The balance of the carbon skeleton is metabolized to CO_2 by way of alanine made from the side chain and crotonyl CoA formed from the ring, which can be converted to glucose and acetoacetate, respectively. The sharp decline observed in the amount of tryptophan in the treated insect is indicative of metabolic imbalance due to carbaryl toxicity.

CHAPTER 4

BIOCHEMICAL EFFECTS OF CARBARYL ON REPRODUCTIVE SYSTEM

4.1 INTRODUCTION

To date, very few data is available on the composition of total proteins, total free amino acids and total glucose in the reproductive system of insects. Data on the changes in the composition of total protein, total free amino acids and total glucose in the reproductive system on the administration of various pesticides on insects are also not available. Blum *et al*. (1962) demonstrated the presence of fructose, glucose and trehalose in the accessory gland of *A. mellifera.* Carbohydrates are necessary for the normal functioning of the reproductive system as well the development of the embryo (Chippendale, 1978). There is some evidence to support the hypothesis that reduction in carbohydrate metabolism is associated with the killing action of some insecticides (Clark and Butz, 1961). The major components of ARG secretions, in both quantity and importance as modulators of female reproductive activity, are proteins. Pesticides also affect the protein metabolism (Rao *et al*., 1987; Reddy and Bashamohideen, 1987). The present study was undertaken to analyze the total protein, total free amino acids and total glucose in the male reproductive system of *I. limbata* on the administration of sub-lethal dose of carbaryl over a period of 60 days.

4.2 MATERIALS AND METHODS

4.2.1 Topical application of sub-lethal dose of carbaryl

Topical application of sub-lethal dose of carbaryl on *I. limbata* is described elsewhere (see, chapter 3). The saline treated normal insects were

also maintained for comparison. The biochemical estimations were made at every 15 days in the treatment period up to 60 days.

4.2.2 Estimation of total proteins, total free amino acids and total glucose

The reproductive system of male insect was carefully dissected out using a fine needle and watchmakers forceps from experimental insects with the aid of binocular stereozoom dissection microscope (Zeiss D_4) to a modified insect Ringer solution (7.5 gm NaCl, 0.135 gm KCl, 0.135 gm $CaCl_2$ and 0.120 gm $NaHCO_3$ in 1 litre of distilled water) prepared according to Lum (1961). Ten male insects were used for each experiment and seven replicates were done. The reproductive system was dissected out, weighed in an electronic balance (APCO, India) and homogenized in distilled water. The homogenate was precipitated with 80% aqueous ethanol the supernatant was removed by centrifugation at 3500 rpm for 10 min. The precipitate was then successively extracted with ethanol: chloroform, ethanol: ether and finally ether at room temperature. The final residue was extracted with 0.5 N perchloric acid at 90 ºC for 15 min. The residue left over the hot acid extraction was dissolved in 0.1 N NaOH and total protein was estimated according to the method of Lowry *et al*. (1951).

The supernatant obtained after centrifugation of the precipitated homogenate was used for the estimation of free amino acids according to the method of Lee and Takahashi (1966). Total glucose in the supernatant was estimated according to Nelson (1944) and Somogyi (1952).

4.3 RESULTS

4.3.1 Changes in the amount of total protein

Carbaryl administered with sub-lethal dose for a period of 60 days showed a gradual decrease in the amount of total protein content in the reproductive system of *I. limbata*. The results are presented in the table 13 and figure 15. There is no significant change in total protein level up to 15 days, thereafter the levels of total protein declined up to 60 days.

The significant changes in total protein levels of reproductive system were determined by analyzing the data with ANOVA. The ANOVA of the data was presented in table 14. There was no significant change in total protein up to 30^{th} day between normal, control and treated groups. Estimations made on 45^{th} and 60^{th} days showed a significant decrease in total protein in carbaryl treated insects in the treatment period.

Table 13: The amount of total protein in the male reproductive system of normal control and treated insects

Total protein (mg/g tissue)			
Days	Normal	Control	Treated
1	34.69 ± 4.53	33.23 ± 3.07	33.50 ± 3.50
15	35.03 ± 3.84	34.84 ± 3.45	33.85 ± 4.70
30	33.45 ± 2.81	34.33 ± 4.52	30.07 ± 4.27
45	34.44 ± 6.29	32.76 ± 4.47	26.78 ± 2.44*
60	33.82 ± 4.10	31.62 ± 2.92	24.39 ± 2.50***

Values are means ± SDs (n=7).
Values are significant at * $p < 0.05$; *** $p < 0.001$ level.

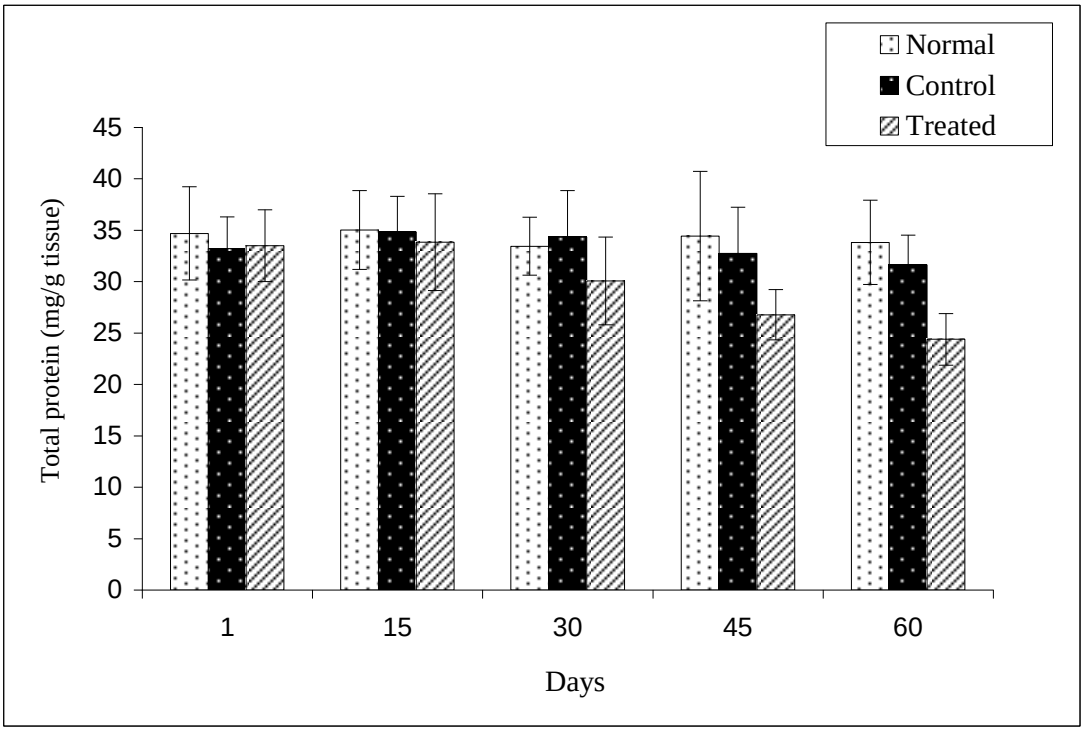


Figure 15: The amount of total protein in the male reproductive system of normal, control and treated insects.

Table 14: ANOVA of the data on the amount of total protein in the reproductive system of normal, control and treated insects

Days	Variance	Sum of squares	df	Mean square	F	Sig.
1	Between groups	9.415	2	4.707	0.308	0.739
	Within groups	274.883	18	15.271		
	Total	284.298	20			
15	Between groups	5.675	2	2.838	0.175	0.841
	Within groups	292.472	18	16.248		
	Total	298.147	20			
30	Between groups	72.313	2	36.156	1.975	0.168
	Within groups	329.516	18	18.306		
	Total	401.829	20			
45	Between Groups	212.752	2	106.376	4.618	0.024
	Within Groups	414.653	18	23.036		
	Total	627.406	20			
60	Between Groups	325.594	2	162.797	14.601	0.000
	Within Groups	200.693	18	11.150		
	Total	526.287	20			

4.3.2 Changes in the amount of total free amino acids

The changes in total free amino acids levels in the male reproductive system of *I. limbata* are presented in the table 15 and figure 16. The amount of total free amino acids in the male reproductive system of *I. limbata* is more or less the same up to 15^{th} day of the treatment period in normal, control and treated insects. Thereafter the levels of total free amino acids declined up to 60 days.

The significant changes in the amount of total free amino acids in the reproductive system were determined by analyzing the data with ANOVA. The ANOVA of the data are presented in table 16. The data showed the difference in the amount of total free amino acids up to 30^{th} day is not significant in carbaryl treated insects. Estimations made on 45^{th} and 60^{th} days showed that the levels of free amino acids in the reproductive system are significantly ($p < 0.001$) low in treated insects and the magnitude of dip is sharp in the latter.

Table 15: The amount of total free amino acids in the male reproductive system of normal, control and treated insects

	Total free amino acids (mg/g tissue)		
Days	Normal	Control	Treated
1	17.28 ± 2.15	18.11 ± 2.69	17.62 ± 3.44
15	16.50 ± 2.48	17.74 ± 2.02	15.57 ± 2.17
30	17.06 ± 2.85	16.07 ± 2.46	13.58 ± 3.14
45	15.94 ± 2.90	15.88 ± 3.34	9.56 ± 1.27***
60	16.28 ± 2.21	14.23 ± 2.04	8.86 ± 1.38***

Values are means ± SDs (n=7).
Values are significant at *** $p < 0.001$ level.

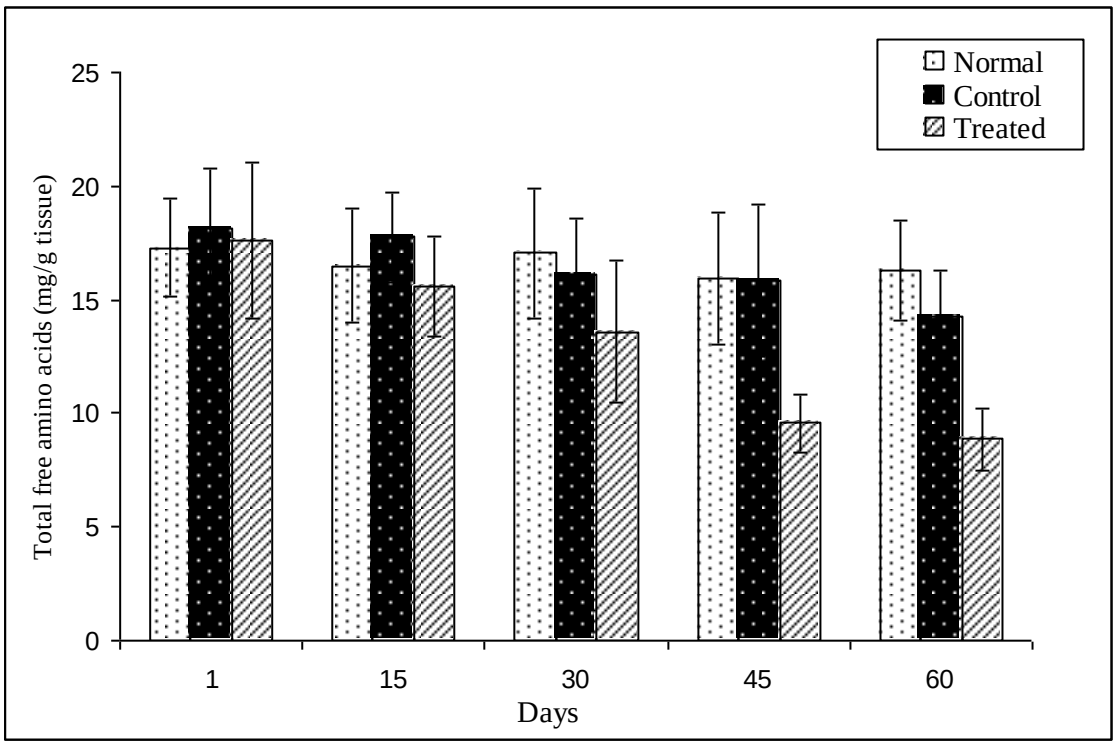


Figure 16: The amount of total free amino acids in the male reproductive system of normal, control and treated insects.

Table 16: ANOVA of data in the levels of total free amino acids in the reproductive system of normal, control and treated insects

Days	Variance	Sum of squares	df	Mean square	F	Sig.
1	Between groups	6.430	2	3.215	0.394	0.680
	Within groups	146.686	18	8.149		
	Total	153.116	20			
15	Between groups	2.458	2	1.229	0.247	0.784
	Within groups	89.624	18	4.979		
	Total	92.083	20			
30	Between groups	20.140	2	10.070	1.214	0.320
	Within groups	149.274	18	8.293		
	Total	169.414	20			
45	Between Groups	226.998	2	113.499	16.073	0.000
	Within Groups	127.105	18	7.061		
	Total	354.104	20			
60	Between Groups	163.418	2	81.709	20.695	0.000
	Within Groups	71.069	18	3.948		
	Total	234.487	20			

4.3.3 Changes in the amount of total glucose

The changes in the amount of total glucose in the reproductive system of *I. limbata* on carbaryl administration are presented in table 17 and figure 17. The results showed a decrease in total glucose in the reproductive system after chronic exposure with carbaryl. The reduction in total glucose level is more prominent on 45^{th} and 60^{th} day in carbaryl treated insects.

The significant changes in total glucose levels of reproductive system were determined by analyzing the data with ANOVA. The results of the analysis were given in table 18. As indicated in the data the levels of total glucose in the reproductive system of normal, control and treated insects was more or less the same up to 30th day of carbaryl treatment and the difference was found to be not significant. There was significant ($p < 0.001$) depletion in the level of total glucose on 45th and 60th day in treated insects.

Table 17: The amount of total glucose in the male reproductive system of normal, control and treated insects

	Total glucose (mmol/g tissue)		
Days	Normal	Control	Treated
1	14.21 ± 1.64	13.12 ± 1.42	12.91 ± 1.20
15	13.40 ± 2.52	13.44 ± 1.99	12.81 ± 2.29
30	13.81 ± 1.10	14.08 ± 1.75	11.88 ± 2.03
45	14.02 ± 1.90	13.84 ± 1.04	8.48 ± 1.51***
60	13.34 ± 1.63	12.92 ± 2.29	7.99 ± 1.42***

Values are means ± SDs.
Values are significant at *** $p < 0.001$ level.

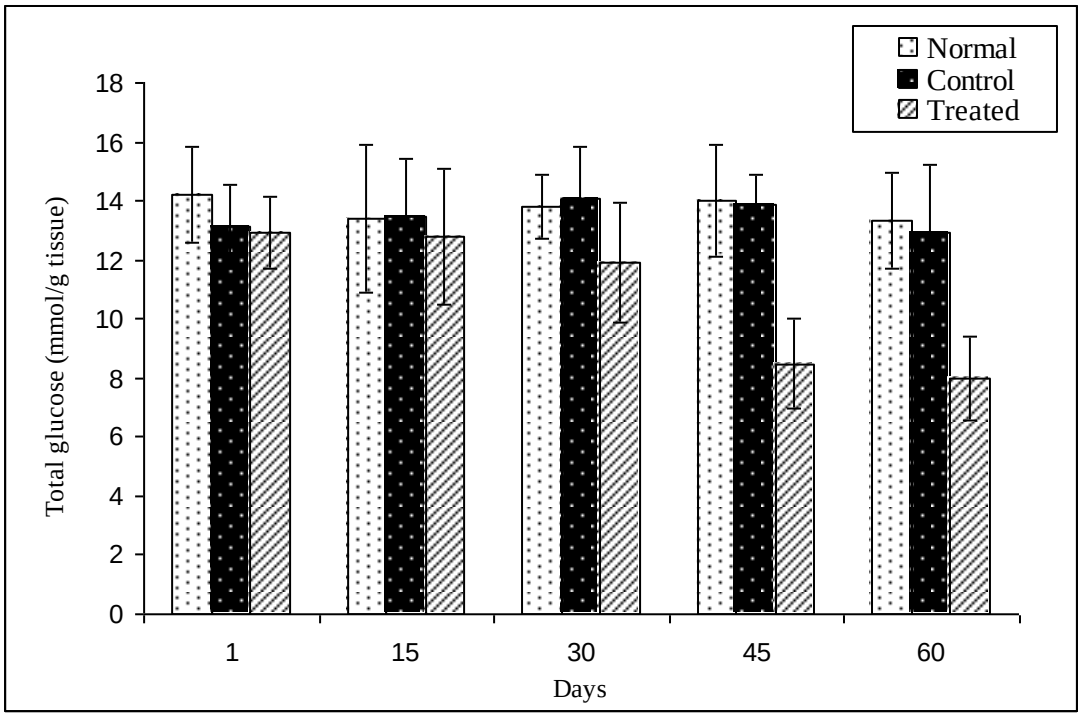


Figure 17: The amount of total glucose in the male reproductive system of normal, control and treated insects**.**

Table 18: ANOVA of the data on the amount of total glucose in the reproductive system of normal, control and treated insects

Days	Variance	Sum of squares	df	Mean square	F	Sig.
1	Between groups	0.845	2	0.423	0.205	0.817
	Within groups	37.135	18	2.063		
	Total	37.980	20			
15	Between groups	6.955	2	3.478	0.247	0.784
	Within groups	93.363	18	5.187		
	Total	100.319	20			
30	Between groups	5.550	2	2.775	0.683	0.517
	Within groups	73.080	18	4.060		
	Total	78.630	20			
45	Between Groups	125.487	2	62.744	26.903	0.000
	Within Groups	41.979	18	2.332		
	Total	167.467	20			
60	Between Groups	123.832	2	61.916	18.713	0.000
	Within Groups	59.556	18	3.309		
	Total	183.338	20			

4.4 DISCUSSION

It is well known that the accessory glands of the male reproductive system of insects mainly produce proteins and function as a vehicle for sperm transfer to the females (Wigglesworth, 1936). The reproductive system of the bug and the secretions of the accessory glands as a whole play an important role in the regulation of the physiology of the female reproductive system and

it contain several stimulatory substances, most of them are proteins (Bhosale *et al*., 1987). Retnakaran (1971) has demonstrated protein synthesis in all the differentiating cells of the testes of *Choristoneura fumiferana* during spermatogenesis. The results of the present study revealed that there is a dip in the levels of total protein in the male reproductive system. The sub-lethal effect of carbaryl on protein metabolism in the freshwater catfish, *M. vittatus*, showed a decrease in protein content of muscle, liver, gill and intestine (Palanichamy *et al.* 1989).

Protein plays a vital role in the development and maturity of reproductive organs and any defect may result in the reproductive failure of the insect. Salma (1964) has reported that, the corpora allata of *P. apterus* regulate the utilization of protein by ovaries and note the synthesis of protein. Hill (1962) and McCaffery (1971) have shown that corpora allata was responsible for the uptake of hemolymph protein into the oocytes. The insecticide carbaryl mainly affects this coordination by the inhibition of acetylcholiesterase. Carbaryl may also directly affect the adrenergic nerve endings, reported by Hassan (1971) while studying the effects of carbaryl on the synthesis and degradation of catecholamine in rat at 100 or 700 mg/kg for 7 months.

A male with fully depleted accessory glands does not inseminate under normal conditions (Ramalingam, 1974; Hausermann and Nijhout, 1975). The

decreased production of protein in the accessory gland after allatectomy was due to a lack of juvenile hormone and when corpora allata were implanted; its production was restored to normal (Blaine and Dixon, 1973). Electrophoresis also showed that allatectomy caused a change in the concentration of a great many proteins rather than just one, which also suggest that it is affecting protein metabolism generally. Gillott and Friedel (1976) have found that corpus allatum prevents normal increase in protein content of the ARGs of adult male *Melanoplus sanguinipes*. Gillott and Friedel (1977) reported that a fecundity-enhancing and receptivity-inhibiting substance in the accessory glands of *M. sanguinipes* is a proteinacious substance. These ARG substance increases fecundity in mated females and prevent them from undergoing a second mating. So in the case of carbaryl treated insects the levels of total proteins showed a decrease was may be due to the affect on the nervous system which leads to reduction in the release of hormones such as ecdysone, juvenile hormone and so on. It will directly affect the secretory activity of the accessory gland of the male reproductive system of *I. limbata*.

The male *Cecropia* moth transfers juvenile hormones (JH 1 and JH 2) to the female during copulation which is stored in the female ARGs (Shirk *et al*., 1980). The fate of JH in the female is not known since it has been shown that vitellogenin synthesis in this moth is not influenced by JH. The male silk moth injected with ^{14}C leucine when allowed to mate with the female, the accessory reproductive structures of the female showed accumulation of

labeled protein. It is suggested that the proteins from the male are transferred during copulation. The reduction in the biochemical component such as total protein in the male insect reproductive system with carbaryl exposure for a long run may also directly influence the female reproductive success.

Prolonged treatment of carbaryl causes a reduction in total protein and total free amino acids in the male reproductive system of *I. limbata*. Similar reduction of protein and amino acids in the testis was observed in the bisazir treated male spotted bollworm, *E. fabia* (Srivastava and Kumar, 1984). Exposure of fish to sub-lethal concentrations of carbaryl and phenthoate and their combination showed variations in the levels of sucrose soluble proteins in muscle, gill and liver tissues. This suggests high protein hydrolysis to occur during pesticide impact. The impact of carbaryl and phenthoate and their combination on the fish tissues obviously suggest the dearrangement of the structural integrity of tissues which may be lethal, if the fish were exposed for prolonged periods. To support this trend, there are many reports confirming tissue damage to occur under pesticide toxic impact (Chakrabarthy and Konar, 1974; Dikshith *et al*., 1979; Pawar and Katdare, 1983). Based on the changes observed in tissue protein levels, tissue free amino acid content can also be considered to have been influenced in insects exposed to pesticides. Testicular homogenate and hemolymph of *Poecilocerus pictus* showed 19 free amino acids in the hemolymph and 15 were noted in the testes, and 10 were common to both (Dikshith *et al*, 1968). The level of hemolymph amino

acids showed reduction in carbaryl treated insects (see, chapter 3) resulting an imbalance in the uptake and utilization of proteins.

The largest stores of carbohydrate for energy metabolism are glycogen and trehalose, with glucose usually playing a more minor role. Glycolysis is the major pathway by which insects degrade carbohydrates for energy release in the form of glucose. The main substrates for gluconeogenesis in insects are those amino acids which on degradation yield pyruvate or an intermediate of the TCA cycle. On carbaryl exposure the total free amino acids in the reproductive system of *I. limbata* showed depletion throughout the time of exposure period. Similarly total glucose showed a dip in the reproductive system of *I. limbata* with chronic exposure. George and Ambrose (1999) observed a reduction in organic constituents such as carbohydrates and proteins in the alimentary canal as well as in the entire animal of *Rhynocoris kumari* on the treatment with monotophos, dimethoate, methylparathion, quinalphos and endosulfan. The depletion in the levels of total protein, total free amino acids and total glucose with carbaryl treatment may be due to the effect on nervous system. Pesticides like carbaryl have deleterious effects on several non-target animals resulting in a change in the well balanced dynamics of the natural ecosystem.

CHAPTER 5

HISTOMORPHOLOGY AND HISTOPATHOLOGY OF REPRODUCTIVE SYSTEM

5.1 INTRODUCTION

As to the male reproductive organs of insects matters have remained as obscure as ever in morphology as well as in physiology. The complex series of behavioral and physiological events of reproduction are coordinated by the nervous and hormonal systems. The insecticides can upset this coordination and result in diminished reproductive success (Fukuto, 1990; Shank, 2004). Most often only the end results of sub-lethal poisoning is associated with physiological events such as spermatogenesis, sperm motility, oogenesis and fertilization of eggs. The various bioactive compounds of the environment are capable of crossing the blood-testis barrier and affecting male reproductive mechanisms (Okumura *et al*., 1975). The effects of carbaryl on the reproductive system of animals have been studied (Vashakidze, 1965; Dikshith *et al*., 1976; Ball and Su, 1979; Wyrobek *et al*., 1981; Mac-Leod, 1982; Martin, 1982; Osterloh *et al*., 1983). A number of agricultural chemicals, including carbaryl affect the reproductive system, resulting in adverse outcomes such as abortion, still birth, birth defects, and infertility (Schrag and Dixon, 1985; Baranski, 1993). The studies of chemical exposure in relation with poor semen quality and genotoxic effects on spermatozoa showed adverse effect on reproduction in carbaryl exposed workers (Oliva *et al*., 2001).

The internal organs of reproduction of the male insect are deceptively simple in their basic plan and consist of testes, vasa deferentia, accessory glands and an ejaculatory duct (Dorn *et al*., 1992; Lemos *et al*., 2005). Here the histomorphology of the male reproductive system of *I. limbata* is studied with special emphasis on the effect of carbaryl on testes, mesadenes (paired accessory glands) and spermatozoa in the vasa deferentia.

5.2 MATERIALS AND METHODS

The administration of carbaryl to experimental insects and dissection of male reproductive system were described elsewhere (see, chapter 4).

5.2.1 Tissue preparation for light and phase contrast microscopy

The dissected tissues of male reproductive system of control and treated insects were measured with calibrated ocular micrometer. Histological sections were prepared according to the method of Humason (1966). The tissue was fixed in aqueous Bouin's fluid for 12-18 hrs. The Bouin's fluid was removed from the tissue by washing with running water and then with distilled water. It was then dehydrated through a series of ethyl alcohol dilutions and cleared in methyl benzoate, and finally a dip in benzene. The dehydrated tissue was infiltered with molten paraffin and finally embedded in paraffin.

The testes of *I. limbata* were dissected out into a clean slide, squashed with a drop of modified insect Ringer solution and different stages of spermatogenesis were observed under phase contrast microscope. A smear of testes were prepared to examine the sex cells in a small drop of modified insect Ringer. The testes and vasa deferentia of the male reproductive system of *I. limbata* were transferred separately into a clean slide and tore with a fine needle while immersed in a modified Ringer solution to observe the germ cells. The sperm bundles in the testes and spermatozoa in the vasa deferentia were counted. The spermtozoa in the vasa deferentia were mixed with 1 ml of modified Ringer solution in an eppendorf tube and 1 µl suspension was counted with the aid of inverted microscope (TCM 400, Digi 3, Labomed, USA). Fifteen replicates with 15 observations (5 from bottom, 5 from top and 5 from the middle of sample 1 ml) were made as described by LaChance *et al*. (1972). The sperm count was also taken from the control group of insects. The morphology and histology of the whole reproductive system and histopathology of the testes and mesadenes were mainly studied.

Serial sections of 6-7 µ thick were made by using a rotary microtome, (Leitz 1512 model) with attached microtome knife. Ribbons obtained from sectioning were adhered onto thoroughly cleaned slides with Mayer's egg albumin and spreaded. The slides were dried on hot plate, then dewaxed, hydrated and stained with Harris haematoxylin (Frank, 1944) and

counterstained with eosin Y (Davenport, 1964). The tissue sections on the slides were again dehydrated in ethanol dilutions and cleared in xylene before mounting. In order to compare the changes reproductive system of the carbaryl treated insects were also examined immediately after dissection without fixing or staining. The histological sections were observed under trinocular research microscope with bright field/ phase (Axioskop 2 Plus, Zeiss, Germany) and with inverted microscope under different magnifications and photographed with camera mounted on microscope.

5.2.2 Semi-thin sectioning

a) Fixation

The tissue samples (testes and mesadenes) of 1 mm^3 size were fixed in 3% gluteraldehyde in phosphate buffer (pH 7.4) for a minimum of 24 hrs at 4 ^oC. The samples were washed with phosphate buffer at 4 ^oC for four changes of 10 min each and were post-fixed in 1% O_sO_4 for 2 hrs at 4 ^oC. The samples were again washed in phosphate buffer for four changes of 15 min each and finally rinsed in distilled water for 5 min.

b) Dehydration

Dehydration of samples were carried out in the ascending grades of acetone in the following order: 50% acetone for 10 min × 2 in 4 ^oC; 70% cold acetone 10 min in cold, 70% acetone at RT for 10 min; 90% acetone for 10

min × 2 and 100% acetone at RT for 10 min × 2 and with dry acetone at RT for 10 min × 2. After dehydration samples were cleared in propylene oxide for 10 min.

c) Infiltration

Infiltration of the samples with polybed 812 (Epoxy resin-Polysciences Inc., USA) were carried out as follows. Propylene oxide: resin in 3:1 ratio for 1½ hours, propylene oxide: resin in 1:1 ratio for 1½ hours, propylene oxide: resin in 1:3 ratio kept overnight in vacuum for 12 hrs and finally the samples were infiltrated in pure resin for 1-2 hrs under vacuum.

d) Embedding

Samples were embedded in moulds containing resin (Polybed 812 mixed with Dodecenyl Succinic Anhydride, DDSA- Hardener), Nadic Methyl Anhydride (NMA-Hardener), Dimethylaminomethyl Phenol (DMP-accelerator) in appropriate ratios as per the manufacturer's instructions (Polysciences Inc., USA) and placed in an oven at 60 ^{o}C for 3 days for polymerization of the resin blocks.

The blocks were sectioned on a microtome (Lieca Ultra-cut Model UCT) using glass knife to obtain sections of 1 μm thick. Semi-thin plastic sections were transferred into a drop of water on a glass slide and stained with 1% toludene blue. The glass slide was then heated on a hot plate for few

seconds and thereafter the sections were washed with distilled water, air dried and mounted with DPX mountant. The sections were viewed with a trinocular microscope. Images were captured using a digital camera.

5.3 RESULTS

5.3.1 Morphology of the male reproductive system

Analysis under microscope revealed that the male reproductive system of *I. limbata* lies below the alimentary canal and is surrounded by fat body. The position of the reproductive system in the abdominal cavity is presented in figure 18 B. The male reproductive system of *I. limbata* is presented in figure 18 and 19. Diagram of the dorsal view (Fig. 19) of the male genital apparatus of *I. limbata*, showed paired testes (T) with 7 follicles (TF) join to a paired vasa deferentia (VD) with seminal vesicle (SV). The vasa deferentia in the distal region shows adhering paired accessory glands (PAG), mesadenes with lobules filled with secretions. The vasa deferentia empty in to the sperm duct (SD). The wall of the sperm duct consists of cuticle and is surrounded by erection fluid (EF). The erection fluid reservoir (EFR) covered the unpaired accessory gland (UAG).

The structure of male reproductive system showing testes, vasa deferentia, accessory glands and an ejaculatory duct are presented in figure 20.

Testes

The figure (18 B) showed that each testis of *I. limbata* was laid ventral to the alimentary canal of the 3rd and 4th abdominal segment. They were found to be externally covered with the fat body. Each testis of a pair was free from each other, oval in shape 1.57 ± 0.42 mm in length. The width varied considerably at different regions and it was maximum at the middle region and 0.75 ± 0.36 mm in width (Fig. 20 A).

Follicles

The testes of *I. limbata* are composed of 7 follicles each measured 0.2 ± 0.06 mm in width. Each follicle bears a distinct epithelial wall and the internal space is filled with the male germ cells undergoing development. The unstained photomicrographs of sex cells in different stages of development in the testes follicle are presented in the figure 21. The germ cells are arranged systematically in the follicle (Fig. 21 A).

Vasa deferentia

The follicles of *I. limbata* are connected to the vasa deferentia through the short fine vasa efferentia at the distal end of the testes. The vasa deferentia of *I. limbata* are 2.1 ± 0.25 mm in length, which receive the sperms from the testes and facilitate the transport of sperms to the ejaculatory duct. The width of vasa deferentia of *I. limbata* is 0.29 ± 0.09 mm. The seminal vesicles are

dilations of vasa deferentia 0.34 ± 0.07 mm in width (Fig. 20 A). The spermatozoa in the vasa deferentia are presented in figure 22 D

Accessory glands

The paired accessory glands (mesadenes) of *I. limbata* are vesicular in appearence. The mesadenes of *I. limbata* are 0.63 ± 0.23 mm in length and 0.51± 0.14 mm in width. The vasa deferentia with adhering mesadenes open into the ejaculatory duct. There is an unpaired bean shaped accessory gland and it opens into the cuticular ejaculatory duct which is presented in figure 20 D.

Ejaculatory duct

The ejaculatory duct of *I. limbata* is covered with erection fluid reservoir and cuticular intima. The ejaculatory duct of *I. limbata* is 0.46 ± 0.18 mm in length and 0.81 ± 0.23 mm in width. The diameter of the ejaculatory duct or sperm duct alone is 0.13 ± 0.032 mm.

The effect of topical application of sub-lethal dose of carbaryl to the reproductive system of adult insect was studied and the micrometric measurements were presented in table 19. The results showed no significant reduction in the size of the testes, vasa deferentia, accessory glands and ejaculatory duct of *I. limbata* after 60 days of carbaryl treatment at a sub-

lethal dose administration at 5 day intervals. The observed variations in the measurement of treated insects were not significant ($p > 0.05$).

Table 19: The micrometric measurement of each part of the male reproductive system of control and treated insects

Regions of reproductive system	Control		Treated	
	Length (mm)	Width (mm)	Length (mm)	Width (mm)
Testis	1.57 ± 0.42	0.75 ± 0.36	1.58 ± 0.40	0.70 ± 0.35
Vas deferens	2.1 ± 0.25	0.31 ± 0.09	2.0 ± 0.26	0.28 ± 0.02
Mesadenia	0.63 ± 0.23	0.51 ± 0.14	0.59 ± 0.17	0.54 ± 0.14
Unpaired accessory gland	0.46 ± 0.18	0.87 ± 0.23	0.41 ± 0.10	0.86 ± 0.21
Ejaculatory duct	2.8 ± 0.49	0.24 ± 0.04	2.5 ± 0.39	0.24 ± 0.03

Values are means ± SDs (n=50).
Values are significant at $p > 0.05$ level.

5.3.2 Histology of the male reproductive system

The histology of the male reproductive system of *I. limbata* is presented in figure 23-25. The histological studies of the reproductive system of *I. limbata* showed that the testes with follicles are bounded on the outside by a compact fibrous connective tissue layer forming the basement membrane which supports the germinal cells (Fig. 23 A and 24). Each follicle is divided by a thin membrane into apical germarium and a series of cysts containing developing germ cells (Fig. 23 A and 24).

The vas deferens of *I. limbata* showed sperms in C.S. (Fig. 23 B). The T.S. of vas deferens showed outer epithelial cells and spermatozoa arranged internally (Fig. 23 C). There are two types of accessory glands in *I. limbata*, paired accessory glands or mesadenes and an unpaired accessory gland (Fig. 23 D). The glandular pockets of mesadenes are filled with secretions (Fig. 25 B). The C.S. of unpaired accessory gland of *I. limbata* is covered with erection fluid reservoir and internally with secretory epithelial cells with secretions (Fig. 25 A and C). The erection fluid reservoir has no access to the genital ducts. The secretions of the unpaired gland are released into the glandular lumen (Fig. 25 A) which opens in to the sperm duct (Fig. 25 C). The C.S. of the sperm duct showed covering with erection fluid reservoir and spermatozoa internally (Fig. 25 D). The semi-thin sections of mesadenes are presented in figure 26. The figure shows lobules of mesadenia filled with secretions with outer epithelial cells. The lumen of vas deferens is clearly visible in the semi-thin section of mesadenia (Fig. 26 C).

On the basis of the histological observations, the testes follicle of *I. limbata* can be divided into three zones: such as growth zone (Zone I), maturation zone (Zone II) and transformation zone (Zone III), which together represent the entire process of spermatogenesis (Fig. 21 A and 24). The different stages of spermatogenesis in the testes are presented in the figures 27-29. The spermatogonia of *I. limbata* are enclosed within a cyst in the growth zone which undergoes mitosis (Fig. 28) and spermatocytes are formed

within a sac or cyst showing synchronized development (Fig. 27 A-D). The number of cells in the cyst of spermatogonia is limited compared to spermatocytes. The spermatocytes undergo meiotic division (Fig. 27 C-D). In the zone of maturation, spermatids are formed and occur in cysts. The elongation of tiny tail and prominent nucleus is clearly visible in spermatids (Fig. 29 A-B). The immature spermatozoa formed from spermatids measure 362 $\pm$ 42 μm in length and exhibit a beaded appearance (Fig. 22). The mean bundle count for testes sperm is 80 $\pm$ 17. Mature sperms are developed in zone III and the transformed spermatozoa are transferred to the vas deferens through vas efferens. The basal region of the testes follicles of *I. limbata* is filled with ripe spermatozoa with prominent head region and long tail (Fig. 29 C). The spermatozoa in the vas efferens is unbundled (Fig. 30). The unbundled mature insect spermatozoa in the vas deferens of *I. limbata* are found to be filamentous and are 559 $\pm$ 62 μm long (Fig. 22).

5.3.3 Histopathology

5.3.3.1 Effects on mesadenes

The semi-thin sections of mesadenes of treated insect are presented in figure 26. The figure showed large number of vacuoles and enlarged nuclei in the epithelial wall of mesadenes indicating degradation of nuclei in carbaryl treated insects (Fig. 26 F). In the lobules of both control and treated have secretory pockets filled with secretory vacuoles (Fig. 26 A-D).

5.3.3.2 Effects on testes

The histological studies on the effect of topical application of carbaryl on insect show little histological changes on testes and are presented in figures 27- 28. As indicated in the figure, the cytoplasm of spermatocyte cysts show small vacuoles (Fig. 27 B) and spermatozoa are clumped together in the treated insect testes (Fig. 27 F). There is no observable change in spermatocyte division. The treated insects show loose arrangement of spermatids in the testes compared to normal (Fig. 28 B). The nuclei of the outer wall of the testes show vacuole formation and degenerating nuclei and their cytoplasm contracts (Fig. 28 F). The nuclei in the outer wall of the testes are deeply stained and not clearly visible when compared with normal.

5.3.3.3 Effect of carbaryl on sperm

The vasa deferentia of *I. limbata* possess only matured filamentous sperms. The count of the sperm in the treated insects show a significant ($p < 0.05$) decrease after 60 days of chronic exposure and the results are presented in the table 20. There is no much observable morphological difference in the sperms of control and treated insects with light microscopic observation.

Table 20: The sperm count in the vas deferens of control and treated insects after chronic exposure

Sperm count/insect	
Control	Treated
419,300 ± 44,250	351,344 ± 52,584*

Values are means ± SDs (n=18).
Values are significant at *$p < 0.05$ against control.

5.4 DISCUSSION

The male reproductive tract of heteropteran insect typically consists of a pair of testes and a pair of vasa deferentia that end in a median ejaculatory duct (Davey, 1985; Happ, 1992; Nijhout, 1994; Chapman, 1998). The basic morphological features of the male reproductive system and testicular development in *I. limbata* consist of same pattern reported for other heteropterans. In general, each testis is composed of a number of testis tubes or follicles (Nijhout, 1994; Chapman, 1998). Each follicle has its own germarium, analogous to the ovarioles of females, except that the testicular follicles are generally encased in a common sheath, making the testis a fairly compact body (Nijhout, 1994). In *I. limbata* the number of follicles in the testes are found to be 7 and it is comparable to the results in *P. nigrispinus* (Heteroptera: pentatomidae) which has 6 follicles in the testes (Lemos *et al*., 2005). The number of follicles per testes, suggesting that the number of follicles is species-specific, as are the number of ovarioles per ovary in Heteroptera (Biining, 1994). The testes follicles of *I. limbata* have a

germarium in the apical region; testicular follicles are lined by a layer of cells and it opens into the vasa deferentia as seen in other insects (Roosen-Runge, 1977).

The vasa deferentia of *I. limbata* are usually expanded over part of its length to form seminal vesicle or sperm storage organ. Accessory glands, which open into the vasa deferentia or the ejaculatory duct, are present in most insects (Happ, 1992; Chapman, 1998). In other insects the mesodermally derived ducts are themselves glandular called mesadenes (Davey, 1985). In *I. limbata* the paired accessory glands, (mesadenes) are found to be a glandular structure of vasa deferentia, open to the median ejaculatory duct. The paired gland mesadenes considered as the proper male accessory gland (Bonhag and Wick, 1992). The unpaired accessory gland of *I. limbata* showed an internal layer of epithelium is lined with cuticle shows the feature of ectedenia type (Tembhare, 1997). In *I. limabta*, both mesadenia and ectadenia may occured together (Tembhare, 1997). The bean shaped unpaired accessory gland is covered with erection fluid reservoir as found in heteropteran *O. fasciatus* (Dorn *et al*., 1992). The spermatozoa of *I. limbata* are kept together in bundles in the proximal region of testes and sperms are found unbundled in the vasa deferentia. Large and small spermatozoa are seen also in pentatomid bugs, such as *M. histrionica* (Bowen, 1920). In *I. limbata* small spermatozoa with beaded like structure are found but the mature spermatozoa in vas deferens are long and not beaded. The sperms of *I. limbata* remain free from

the bundle when it reaches the vasa deferentia as seen in other insects (Klowden, 2002).

The follicles of testes of *I. limbata* contain a succession of zones, as reported in *C. stollii* (Pentatomidae), in which sex cells in different stages of development are found (Deb *et al*., 1983). As in other heteropterans the first zone of *I. limbata* testes follicle contain spermatogonia which have distinct nuclei, the spermatocytes within a sac or cyst that generally arise from the same spermatogonial cell and their development is synchronized in *I. limbata*. The mature spermatozoa are developed in *I. limbata* in the region of transformation. In the testes of adult *Gerris,* each bundle of approximately 128 late spermatids or mature spermatozoa are enveloped by a cellular sheath derived from a cyst cell (Pollister, 1930). The spermatozooa of *I. limbata* is very long (556 $\pm$ µm) and is comparable with that of *R. prolixus* which measures 300 µm (Jamieson *et al*., 1991).

Effects of carbaryl on male reproductive system

Despite the importance of Heteroptera as biological control agents, few reports exist on the internal morphology and the effect of carbamates on the reproductive tract of insect group especially with reference to male reproductive system. The results of the present study showed no morphological abnormalities in the reproductive system of the bug after carbaryl exposure. There are some histological deformities observed in the

accessory gland of *I. limbata* of treated insect male reproductive system which may lead to the effective secretory activity of the cells. The histological effect of carbaryl on the accessory gland will lead to reproductive failure in both male and female. The endocrine system has central role in regulating the release of sperm from the testes (Riemann *et al*., 1974).

The results of the present study show that the vacuoles in cytoplasm of spermatocytes in the cyst of treated insect testes compared with normal. The testis of *I. limabta* showed compact arrangement of the germ cells which are supported by the wall of the testis. The spermatids show loose arrangement in bundles in the testes of treated insects. The sperm heads are clumped and deeply stained in carbaryl treated insects of *I. limbata*. The cytoplasm of the germ cells shows degeneration in treated insects. The vacuolization in the testes wall are prominent with degenerated nuclei. The HCH (hexachlorocyclohexane) treatment in male *P. pictus* showed more or less similar results (Ahi, 1988). In the *P. pictus,* tissue hyperactivity and cellular deformations were noticed in the testes after HCH treatment (Ahi, 1988) and apholate on the reproductive organs of *Locusta* (Viswanath *et al*., 1978). It can be concluded that carbaryl makes the tissue hyperactive and there is a stress which may brings about cellular deformations. The hypertrophied sperms reflected disturbed physiological state of activity observed by Viswanath *et al*., (1976). Dikshith *et al*. (1976) reported no histopathological change in the testes of male rats after carbaryl treatment.

There is reduction in the count of sperms in the present study as reported by Kitagawa *et al*. (1977). The reduction in sperm count observed in the carbaryl exposed insects may be due to the delay in the division of germ cells of the spermatogonia and spermatocytes. There is significant reduction in sperm count observed in *A. kuhniella* with marked effects of decapitation and exposure to continuous light, but lack of any effect of severing the abdominal nerve cord (Riemann *et al*., 1974). The release of mature spermatozoa from the cysts in the testes displays a circadian rhythmicity that are initially inhibited by 20-hydroxyecdysone. The decline of 20-hydroxyecdysone is thus necessary in order for sperm to be released. According to Jones (1978), meiosis in *Schistocerca gregaria* males depend on the presence of ecdysteroids. Juvenile hormone also controls the development and secretion of the male accessory glands (Klowden, 2002). The carbaryl affects the nervous system which may lead to the functional imbalance in the production of hormones such as juvenile hormone and ecdysterone by corpora allata and corpora cardiaca respectively. So the carbaryl exposures may affect the central nervous system (endocrine system) resulting a decrease in sperm count. Men exposed to genotoxic agents showed elevated frequencies of spermatozoa with chromosomal abberration (Robbins *et al*., 1997; Harkonen *et al*., 1999; Sram *et al*., 1999; Naccarati *et al*., 2003; Xu *et al*., 2003). Aneuploidy in germ cells is the major cause of infertility, abortion, and congenital diseases (Nishikawa *et al*., 2000).

Xia *et al*. (2005) evaluated that there is possible association between carbaryl exposure and numerical chromosomal aberrations. Carbaryl suppressed mitosis, changed the rate of the mitotic phase, and significantly increased the number of pathological forms of mitosis in a human embryonic fibroblast culture (Kazarnovskaya and Vasilos, 1977). When compared with the control insects the division of spermatocytes shows no visible difference in treated insects after 60 days of carbaryl treatment.

In mice injected intraperitonially with 0.4 mg of carbaryl either once or daily for a week, the incidence of sperm abnormalities was reportedly increased, but no degenerative changes in the testes were seen (Degraeve *et al*., 1976). Carbaryl administered to mice intraperitonially for 5 day for up to 68 days did not affect testis weight or histology, sperm count, or frequency of sperm abnormalities (Martin, 1982; Osterloh *et al*., 1983). In the present study significant difference in sperm count is seen in carbaryl treated insects but no effect on the morphology of testes and revealed the harmful effect of carbaryl to the histology of male reproductive system.

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®) 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_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 Ill ford film) were taken.

6.3 RESULTS

6.3.1 Ultrastructural studies on mesadenes

The ultrstructure 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 ultrastucture 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 acrosome 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 in to 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 2^{nd} 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 in to 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. melanogster* (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 hisological 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.

SUMMARY

1) Topical application of single dose of carbaryl at 2.5 µg/2 µl/insect caused 50% of mortality in the heteropteran plant bug, *I. limbata.*

2) There was a significant depletion in the levels of total protein of the insect immediately after treatment with LD_{50} of carbaryl but regained to its normal level within a week.

3) Total free amino acids and glucose levels showed an elevation in the bug on carbaryl administration which came down to the normal level within a week.

4) The total protein in the hemolymph showed significant reduction at 24 hr after carbaryl administration.

5) There was an elevation in acid and alkaline phosphatase activities in the hemolymph at 24 hr after carbaryl administration.

6) The IR spectrum of hemolymph free amino acids showed imine formation in carbaryl exposed insects. Imine formation was suggested to be a mechanism to detoxify the compound.

7) Topical application of carbaryl at 0.6 µg/2 µl / insect at 5 day intervals for a period of 60 days resulted in a reduction in the titres of total amino acids (14%) and total free amino acids (46%) of its hemolymph.

8) Except arginine and the branched chain amino acids, i.e., valine, leucine and isoleucine, all other free amino acids showed a reduction in the hemolymph of carbaryl treated insects.

9) The relative abundance of individual free amino acids in control insect is as follows:

Taurine > alanine > proline > aspartic acid > glycine > serine > glutamic acid > threonine > histidine > arginine > methionine > valine > tyrosine > leucine > lysine > phenylalanine, cysteine > isoleucine.

10) The relative abundance of individual free amino acids in carbaryl treated insects is as follows:

Alanine > proline > aspartic acid > glycine > taurine > glutamic acid > histidine > arginine > valine > serine > methionine > leucine > threonine > tyrosine > lysine > isoleucine > phenylalanine > cysteine.

11) Chronic exposure of carbaryl for a period of 60 days resulted in a decline in the levels of total protein, total free amino acids and glucose in the male reproductive system of *I. limbata.*

12) The morphological features of the reproductive system of *I. limbata* are described. Its reproductive system consists of a paired testes, paired vasa deferentia, paired accessory glands (mesadenes), unpaired

accessory gland which is covered with erection fluid reservoir and an ejaculatory duct or sperm duct with cuticular intima.

13) The micrometric measurements of the reproductive system showed no significant difference between control and carbaryl treated insects.

14) The follicle of testis bears a distinct follicular layer with its internal space filled with the male germ cells such as spermatogonia, spermatocytes, spermatids and spermatozoa which are undergoing development.

15) The histology of testes, vasa deferentia, mesadenes, an unpaired accessory gland and an ejaculatory duct of the male reproductive system of *I. limbata* are described.

16) The mature spermatozoa present in the vasa deferentia of *I. limbata* are filamentous and 559 $\pm$ 62 μm long.

17) The number of sperms in the vas deferens showed a significant reduction in the carbaryl administered insects.

18) The mesadenes, the paired accessory glands of the male reproductive system of *I. limbata*, showed glandular pockets that are filled with secretions. The semi-thin sections of the accessory glands showed an increase in the size of the nucleus and number of vacuoles in carbaryl treated insects compared with control.

19) The spermatids are loosely arranged in the testes of the treated insects compared to the normal.

20) The sperm heads appeared clumped in the testes of carbaryl administered insects whereas in the control it was evenly dispersed.

21) The cytoplasm of spermatocyte cysts showed small vacuoles in treated insects.

22) Treatment of carbaryl resulted in the appearance of vacuoles and degeneration of nuclei in the testis wall.

23) The scanning electron micrograph of spermatozoa of *I. limbata* depicts its triangle shaped head and long tail. There was no difference between the morphological features of the sperms of control and treated insects.

24) The accessory glands of the reproductive system of *I. limbata* contain a muscle layer with tracheoles, basement membranes, prominent nuclei and mitochondria.

25) The basal region of the secretory cells of mesadenes of carbaryl administered insects showed large vacuoles.

26) The chromatin granules were scattered with slight obliterations in nucleus of mesadenes of carbaryl administered insects.

27) The ultrastructure of the testes showed different stages of spermatids: early spermatid with large spherical nucleus and prominent proacrosomal vesicle, late spermatid with acrosome cap and elongated spermatid with fibrillar type of nuclear condensation.

28) The late spermatid of *I. limbata* depicts nucleus and an acrosome in the head region, thick centriole adjunct in the neck region and mitochondrial derivative and axoneme in the tail region.

29) The sperms of *I. limbata* showed (important characteristics of heteropteran sperms) such as absence of accessory bodies, large comma shaped mitochondrial derivatives and axoneme, and derivative-axoneme cross bridge.

30) The spermatozoa of *I. limbata* showed biflagellarity towards the posterior region of its tail with 9 + 9 + 2 axoneme organization.

31) There was no observable difference in the structure of mitochondrial derivative and axoneme in the carbaryl treated insects compared to normal.

32) The number of vacuoles and lipid droplets in the outermost matrices of the spermatozoa of testes were more prominent in the treated insects compared to the normal.

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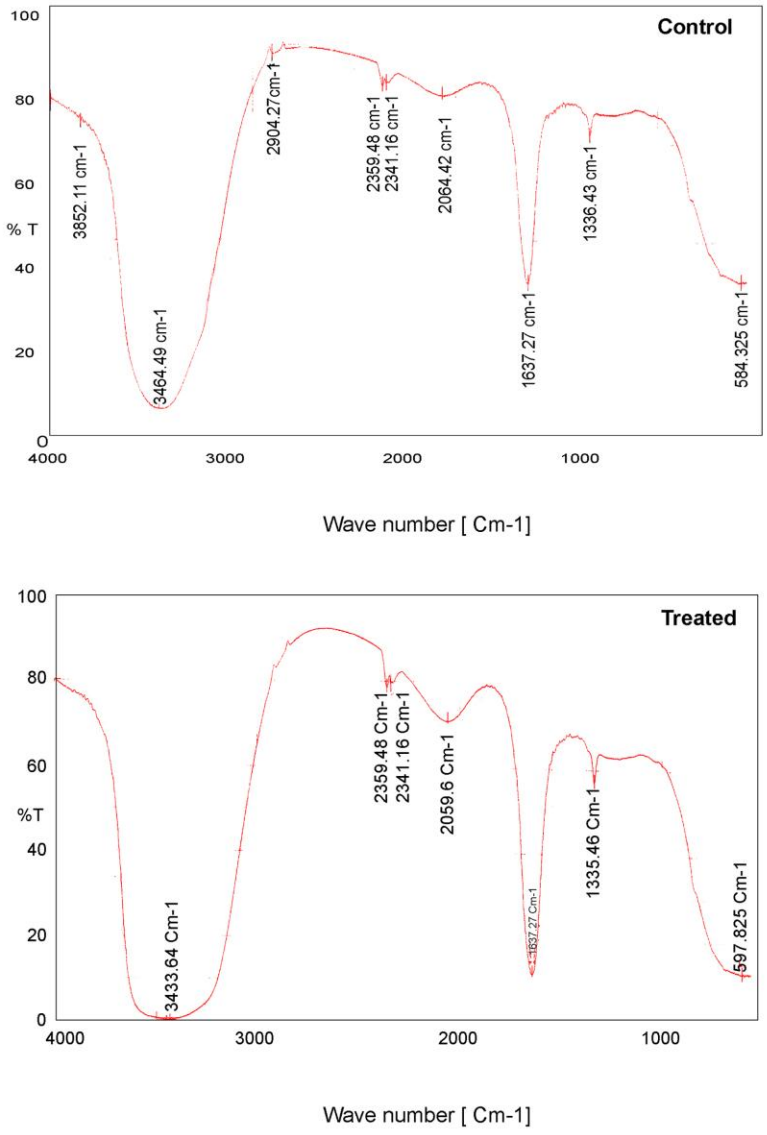

Figure 10: Infrared spectrum of hemolymph free amino acids in control and treated insects.

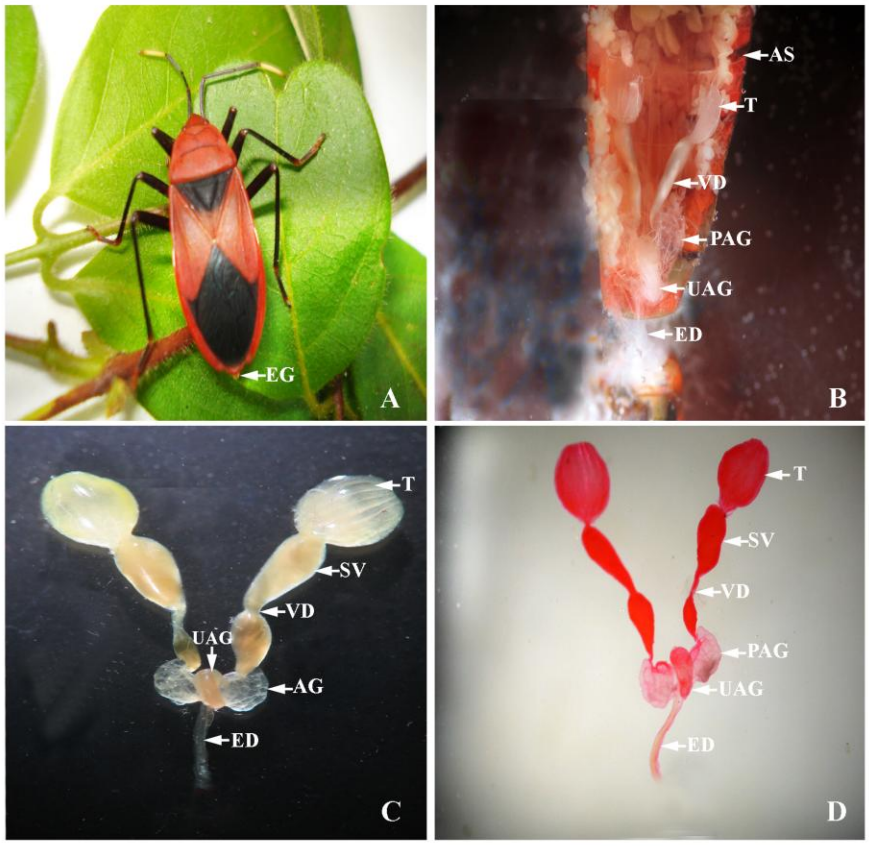

Figure 18: A. Photograph of male *I. limbata*, with external genitalia (EG). **B.** Light micrograph showing the position of reproductive system in the abdominal cavity and the abdominal segment (AS) of insect. **C.** The whole reproductive system of *I. limbata* with testes (T), seminal vesicles (SV), vasa deferentia (VD), paired accessory glands (PAG), unpaired accessory gland (UAG) and an ejaculatory duct (ED) (before fixing and staining). **D.** Reproductive system stained with eosin.

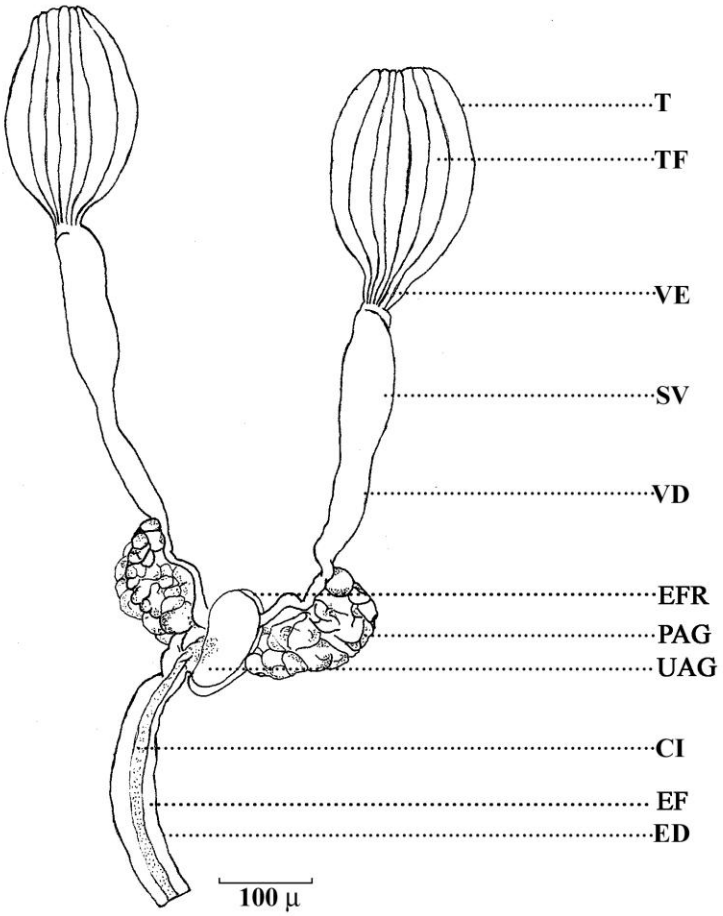

Figure 19: Diagram of the male genital apparatus of *I. limbata*, dorsal view. The paired testes (T) with 7 testes follicles (TF) joined to paired vasa deferentia (VD) with seminal vesicles (SV). The vasa deferentia in the distal region shows adhering paired accessory glands (PAG), the mesadenes, with lobules filled with secretions. The vasa deferentia empty in to the sperm duct (SD). The wall of the sperm duct consists of cuticlar intima and is surrounded by erection fluid (EF) which has no access to the genital ducts. The erection fluid reservoir (EFR) lies above the unpaired accessory gland (UAG). Scale bar = 100 µm).

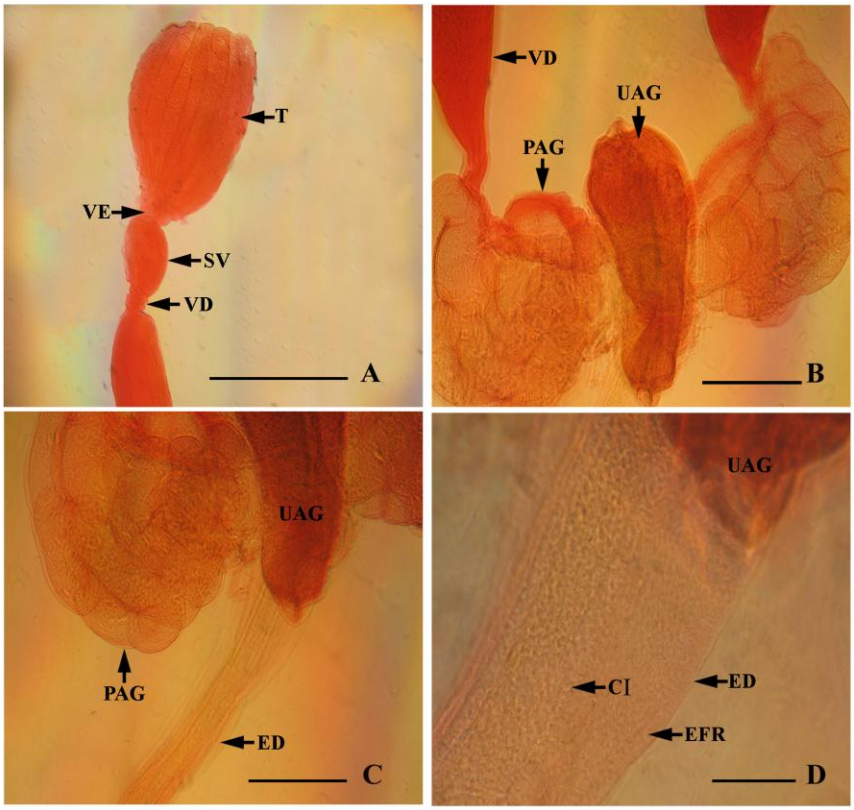

Figure 21: Enlarged view of the portions of male reproductive system of *I. limbata*. **A.** Testis (T), seminal vesicle (SV) and vas deferens (VD). **B.** Vas deferens with vesicular paired accessory glands (PAG) and an unpaired accessory gland (UAG). **C.** The unpaired accessory gland which continues in to the sperm duct or ejaculatory duct (ED). **D.** The wall of the ejaculatory duct consists of cuticular intima (CI) and externally erection fluid reservior (EFR). Scale bars: A-C = 30 µm; D = 20 µm.

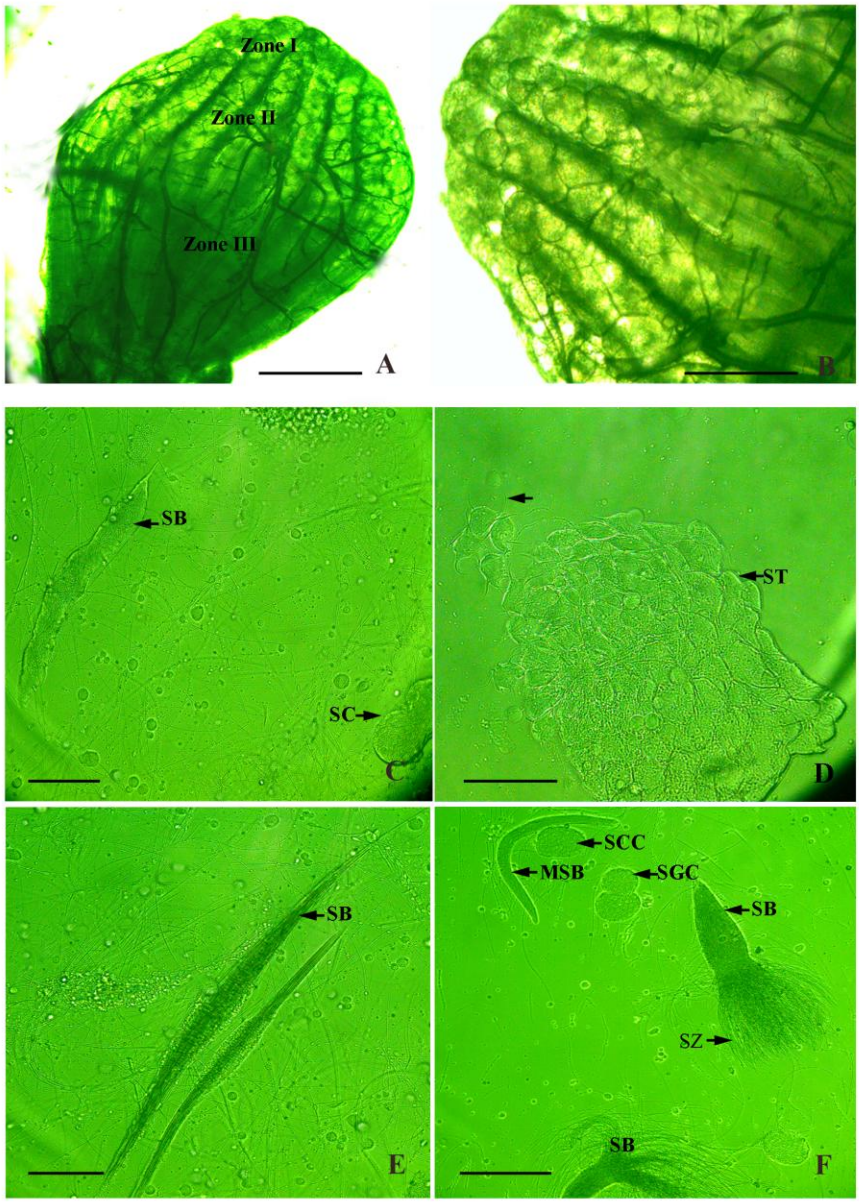

Figure 21: Unstained phase contrast photomicrographs of sex cells in different stages of development in the testes. **A-B.** Testis showing zones of sex cell differentiation. **C.** Elongating spermatocytes (SC) and immature sperm bundle. **D.** The late stage of spermatids (ST) showing tail region (arrow). **E.** Spindle-shaped cyst with broad central region. **F.** Spermatogonial (SGC) and spermatocyte cysts (SCC), and mature sperm bundle (MSB). Moving spermatozoa which were partially separated from the bundle (SB). Scale bars = 10 μm.

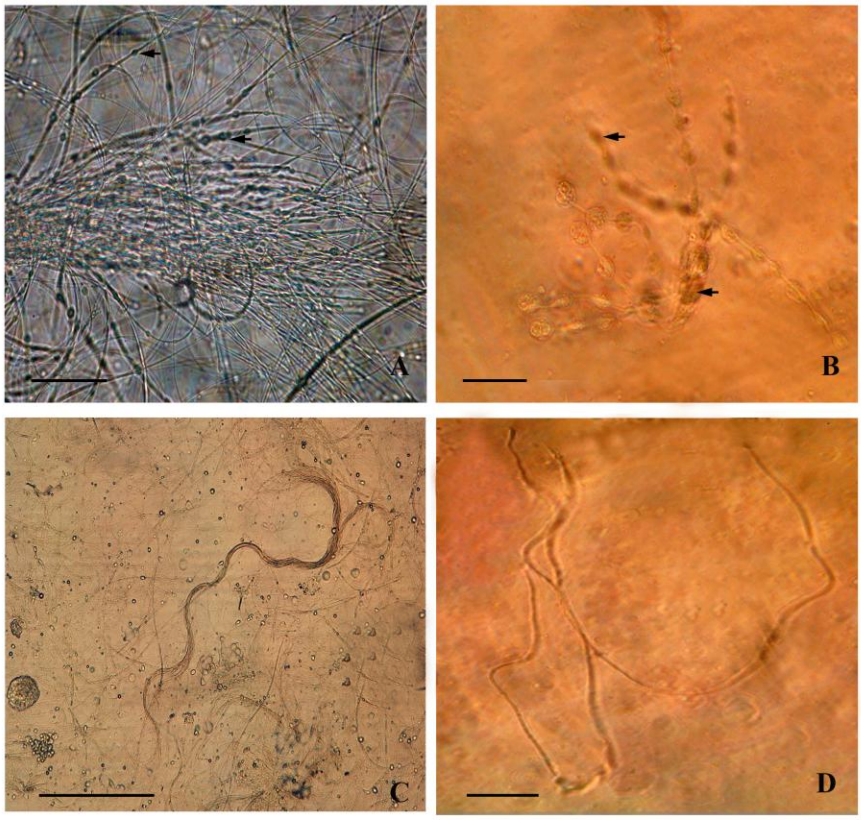

Figure 22: Unstained phase contrast photomicrographs of spermatozoa. **A-B.** Immature short spermatozoa in the testes with beaded appearance (arrows). **C.** Long mature spermatozoa in the testes. **D.** Mature spermatozoa of vasa deferentia. Scale bars =10 μm.

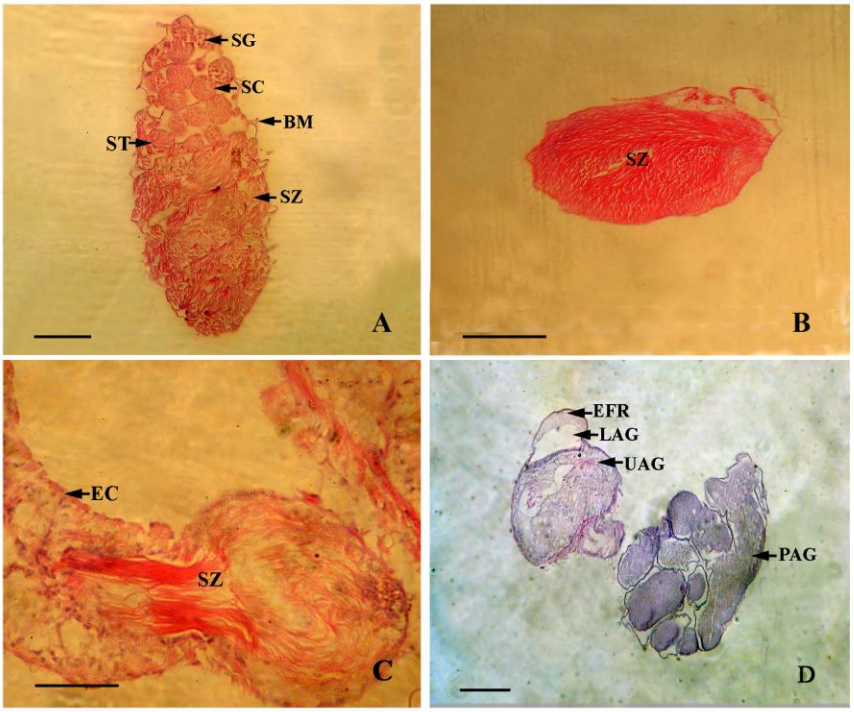

Figure 23: The light micrographs of different regions of male reproductive system. **A.** The L.S of testis filled with germ cells in anterior region with spermatogonia (SG), spermatocytes (SC), spermatids (ST) and spermatozoa (SZ) towards the posterior region. **B.** C.S of vasa deferentia filled with spermatozoa. **C.** T.S of the vas deferentia showing epithelial cells (EC) and internally filled with spermatozoa. **D.** The C.S of paired (PAG) and unpaired (UAG) accessory gland. Sections were stained with eosin/hematoxylene. Scale bars = 30 μm; C = 20 μm.

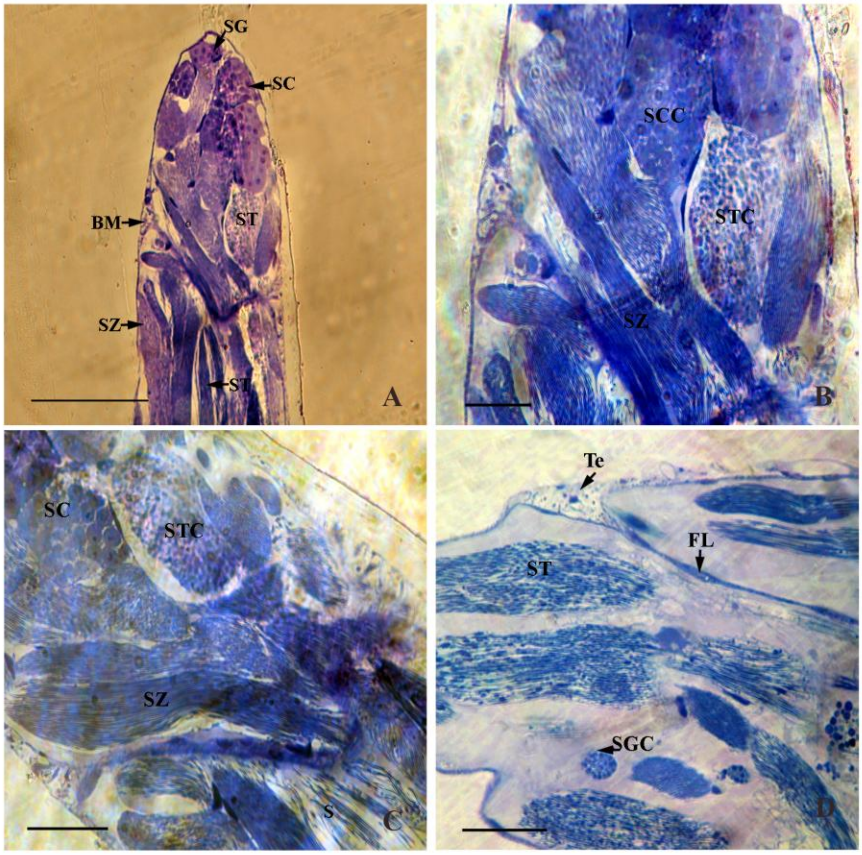

Figure 24: Semi-thin sections of testes. **A-B.** T.S. of testis showing spermatogonial cyst (SGC), spermatocyte cyst (SCC), spermatid cyst (STC) and spermatozoa (SZ), which is covered by basement membrane (BM). **C.** T.S. of the testis of treated insects showing no visible change. **D.** L.S. of the testis showing follicular epithelium (FL) separates the germ cells and an outer covering of tunica externa (Te). Sections were stained with toludene blue. Scale bars = 10 μm.

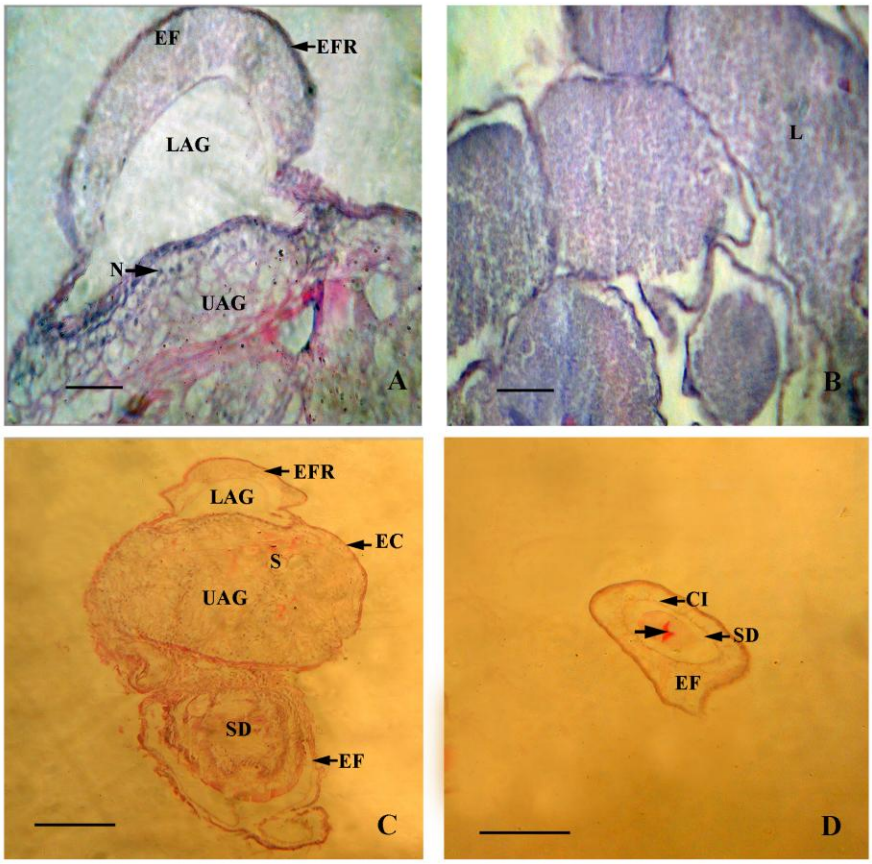

Figure 25: A. The C.S. of unpaired accessory gland (UAG). The erection fluid reservoir (EFR) lies above the unpaired accessory gland. The secretion of the unpaired accessory gland is released in to the glandular lumen (LAG). The epithelial cells showing nucleus (N). **B.** The lobules (L) of paired accessory gland (PAG) showing dense secretions. **C.** The T.S. of unpaired accessory gland showing secretions in the centre. The secretion (S) of the unpaired accessory gland is released in to the glandular lumen which continues into the sperm duct or ejaculatory duct (SD). **D.** The C.S. of the sperm duct (SD) with spermatozoa (arrow) showing cuticular intima and is surrounded by erection fluid (EF). Sections were stained with eosin/hematoxylene. Scale bars: A-B = 20 μm; C-D = 30 μm.

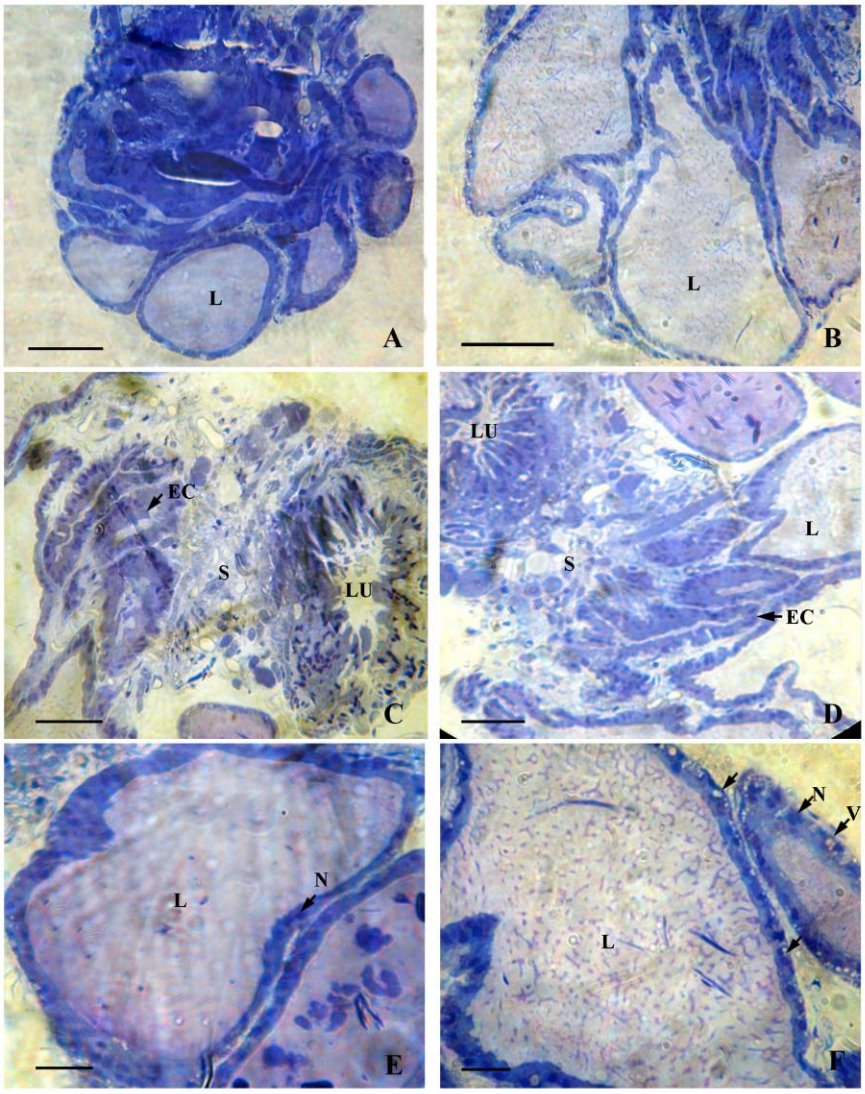

Figure 26: Semi-thin sections of mesadenes. **A-B.** Semi-thin section of mesadenes showing lobules filled wirh secretions with outer epithelial cell (EC) with nucleus (N). **C.** section of mesadenes showing secretions (S) in the lobules and the lumen (Lu) of the vas deference (VD). **D.** The section of mesadenes of treated insect showing no visible change. **E.** Lobule of mesadenes secretions and outer secretory epithelial cells. **F.** Lobule of the treated insect mesadenes showing large number of vacuoles (V) in the outer secretory epithelial cells and enlarged nucleus. Sections were stained with toludene blue. Scale bars = 10 µm.

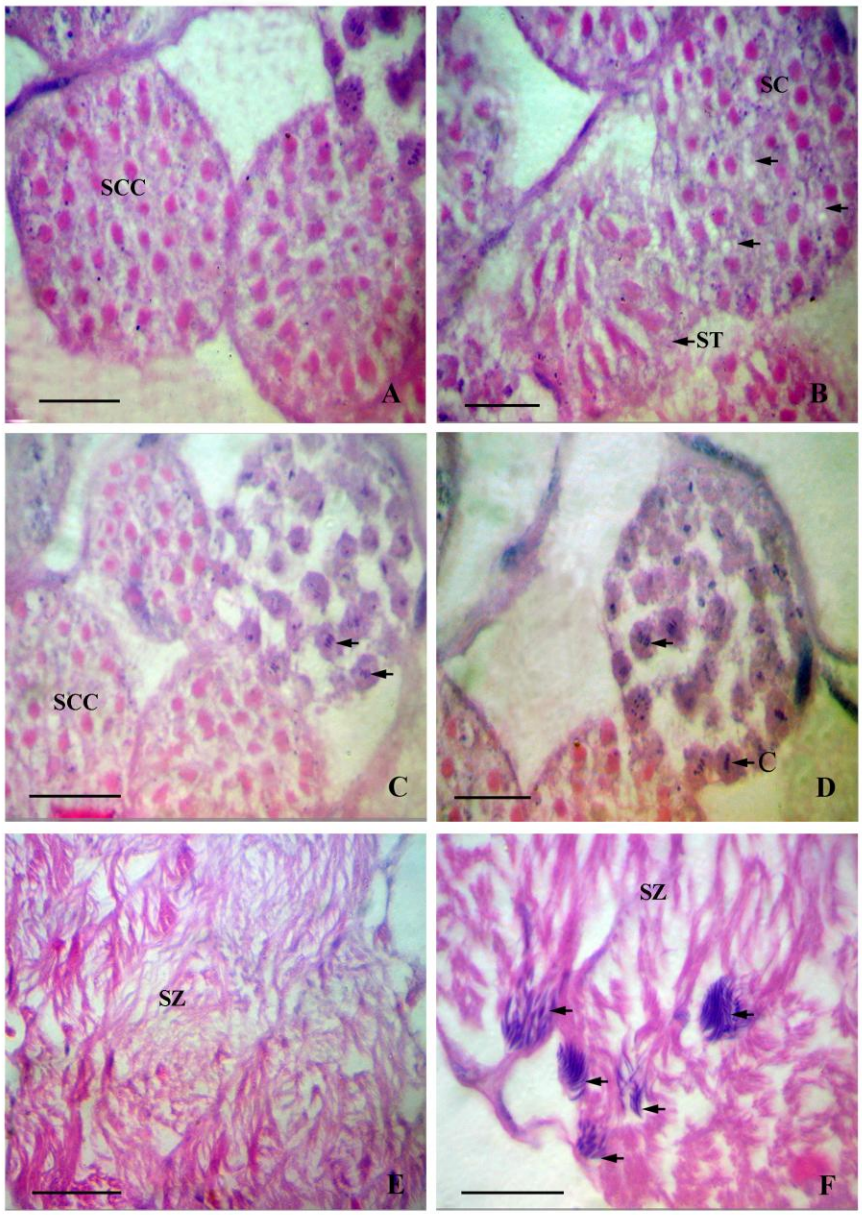

Figure 27: The germ cells in the testes. **A.** section of the testis showing spermatocyte cyst (SCC) with spermatocytes connected with cytoplasmic bridges. **B.** Treated insect testis showing spermatocyte cyst with small cytoplasmic vacuoles (arrows) and early spermatid (ST). **C.** Meiotic division of spermatogonia showing stages of cell division, cells in metaphase, alignment of chromosomes (C) in the same plane (arrows). **D.** Meiotic division of spermatgonia of treated insect with no observable difference. E. Spermatozoa (SZ) of testis in the distal region. **F.** Spermtozoa of testis showing clumped sperm heads (arrows) in the treated insect. Sections were stained with eosin/hematoxylin. Scale bars = 20 µm.

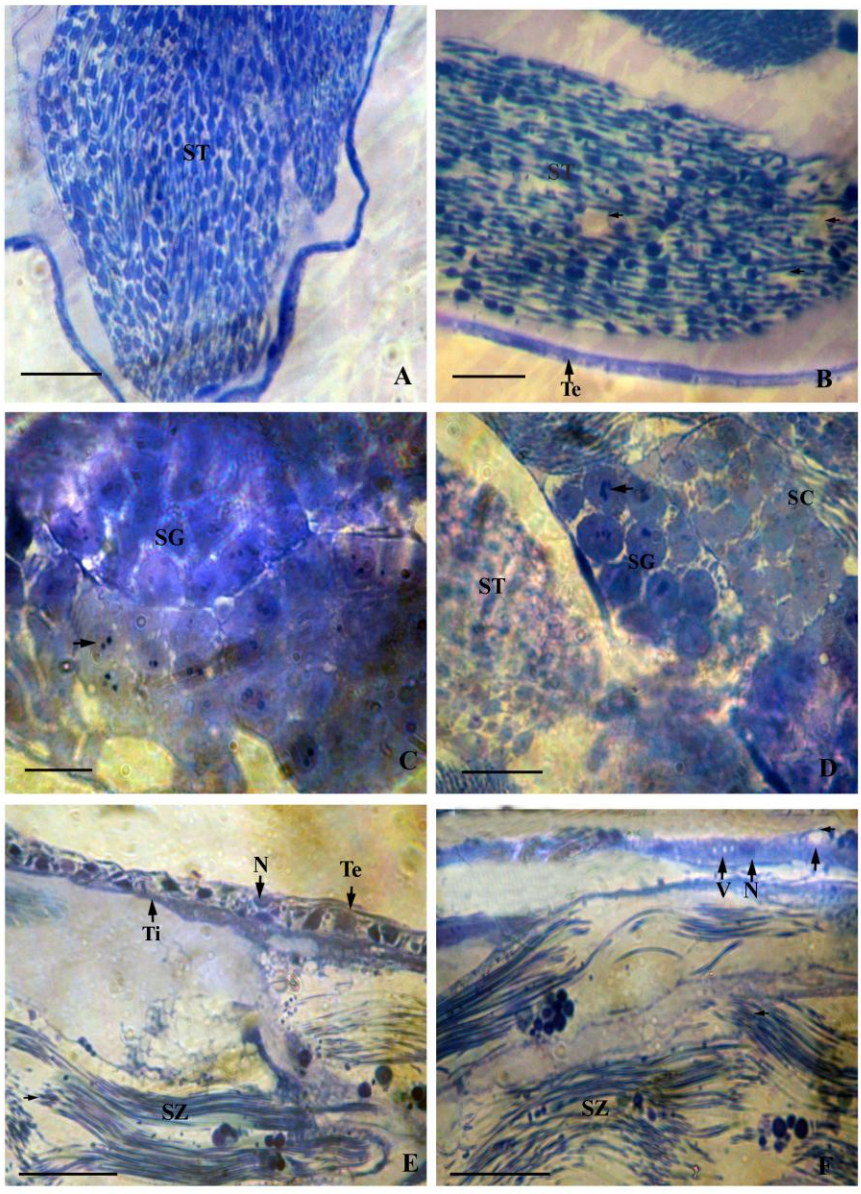

Figure 28: Semi-thin section of testis. **A.** Early spermatid cyst with compact arrangement. **B.** The treated insect showing loose arrangement of spermatid in the testis. **C.** Mitotic prophase of spermtogonial cell with well formed polygonal nuclei (arrows). **D.** Prophase spermatogonial cells, ring formation and broken ring formation (C-shaped) (arrows). **E.** The testis wall with tunica externa (Te), tunica interna (Ti) and clearly visible nuclei (N) and long spermatozoa (SZ) with conical shaped head. **F.** Wall of testis of treated insect showing vacoules and become reduced in size, and the nucleus not clearly visible. Sections were stained with toludene blue. Scale bars = 10 μm.

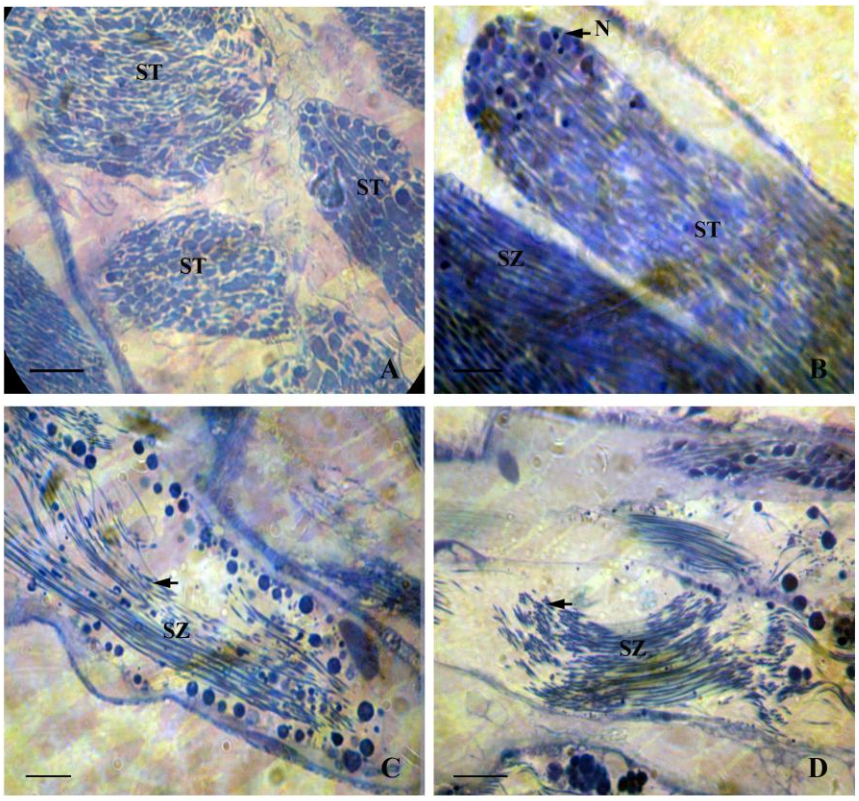

Figure 29: Semi-thin sections of testes. **A.** Differentiation of spermatocytes in to early spermatids (small cyst) and late spermatids (ST) with large number of cells. **B.** Elongated spermatid cyst showing spherical nuclei. **C.** The vas efferens showing unbundled spermatozoa with slightly prominent head region (arrow) and long tail region. **D.** Treated insect showing clumped spermatozoa in the vas efferens. Sections were stained with toludene blue. Scale bars = 10 μm.

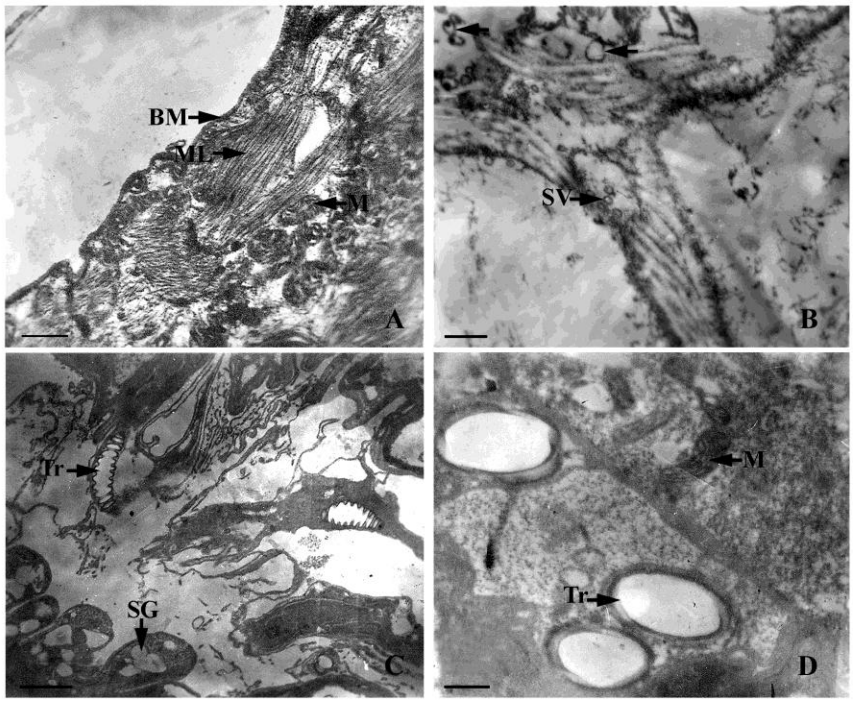

Figure 30: Ultrastructure of mesadenes. **A**. Muscle layer (ML) and basement membrane (BM) surround the secretory epithelium of the paired accessory gland. The secretory cells contain scattered mitochondria (M). **B**. Newly formed secretory vesicles (SV) of secretory cell with dense irregular plaques are scattered at the surface of the vesicle. **C**. The apical edge of the secretory cells of AG showing tracheoles (Tr). The secretory granules (SG) are packed tightly in the apical region. **D**. The tracheoles with cuticle in the secretory cell. Scale bars = 0.5 μm.

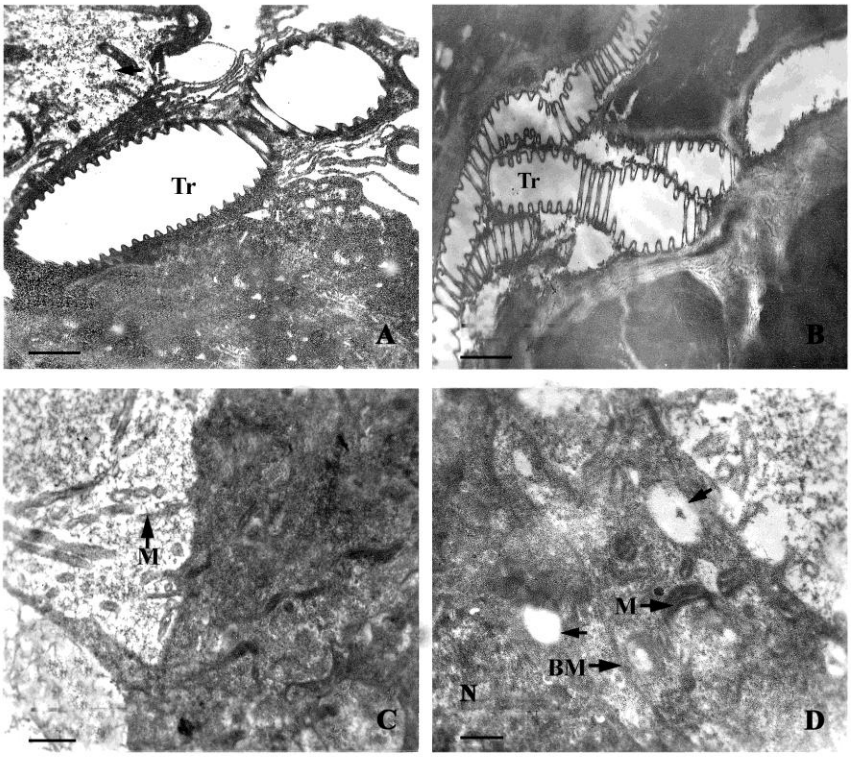

Figure 31: A. The tracheoles (Tr) in the mesadenes of normal insect. **B.** Tracheoles in the mesadenes of treated insect. **C.** The basal region of the mesadenes showing abundant mitochondria (M) in normal insect. **D.** The basal region of the secretory cells showing vacuoles (arrows) in the treated insect. Scale bars = 0.5 µm.

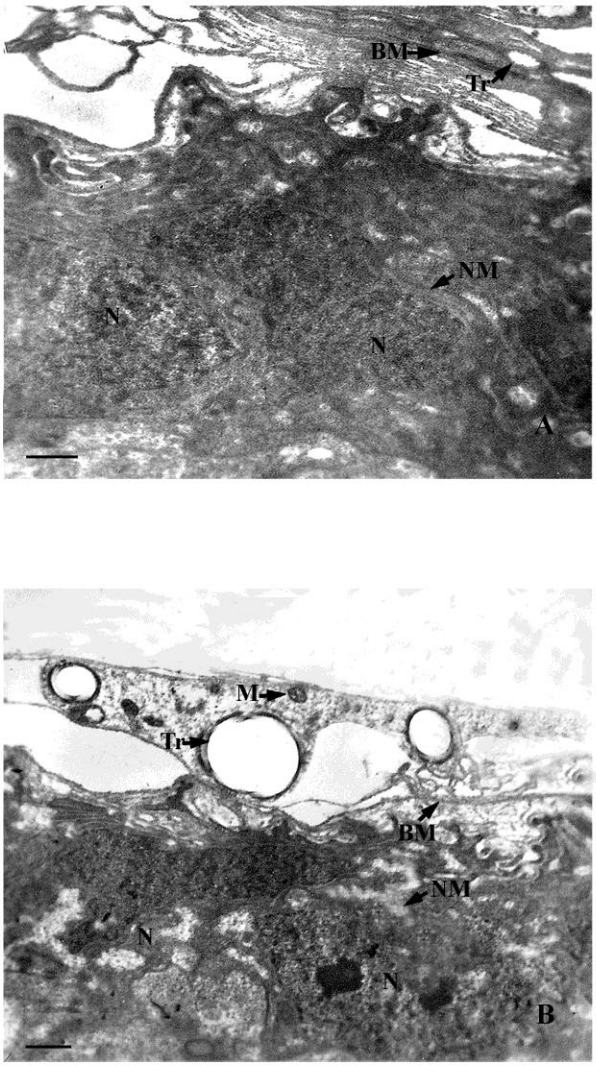

Figure 32: A. The secretory cells of mesadenes showing dense nucleus (N) with distinct nuclear membrane (NM) in the normal insect. **B.** The nucleoplasm appear more dissociated or condensed with slight obliterations in treated mesadenes cell nucleus. Scale bars = 0.5 μm.

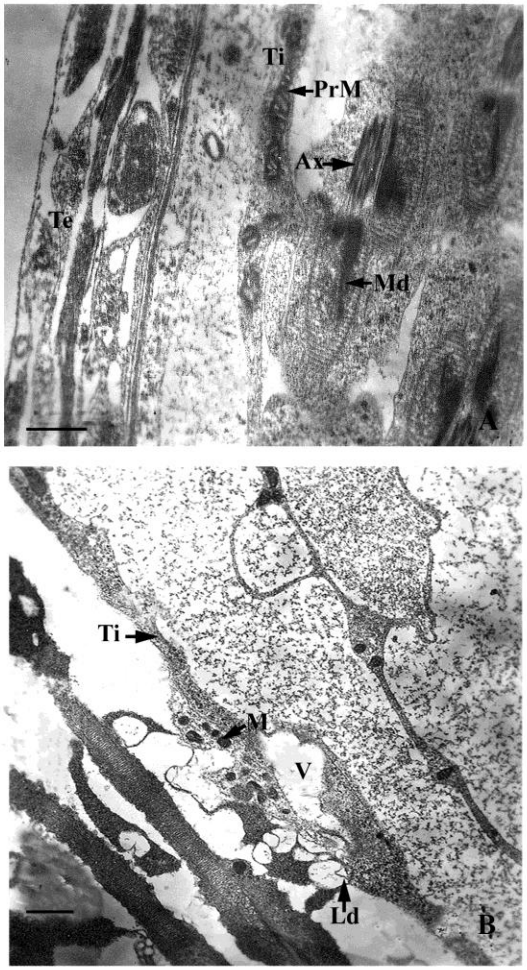

Figure 33: Transverse section of testes wall. **A.** Testis wall of normal insect showing 2 layers of testis, Te-Tunica externa, structureless envelope and Ti-Tunica interna with presumptive mitochondria (PrM). The testis is internally filled with spermatozoa showing axoneme (Ax) and mitochondrial derivative (Md). **B.** Transverse section of testis wall of treated insect showing degradation of tunica interna with large vacuoles (V) and lipid droplets (Ld). Scale bars = 0.5 μm.

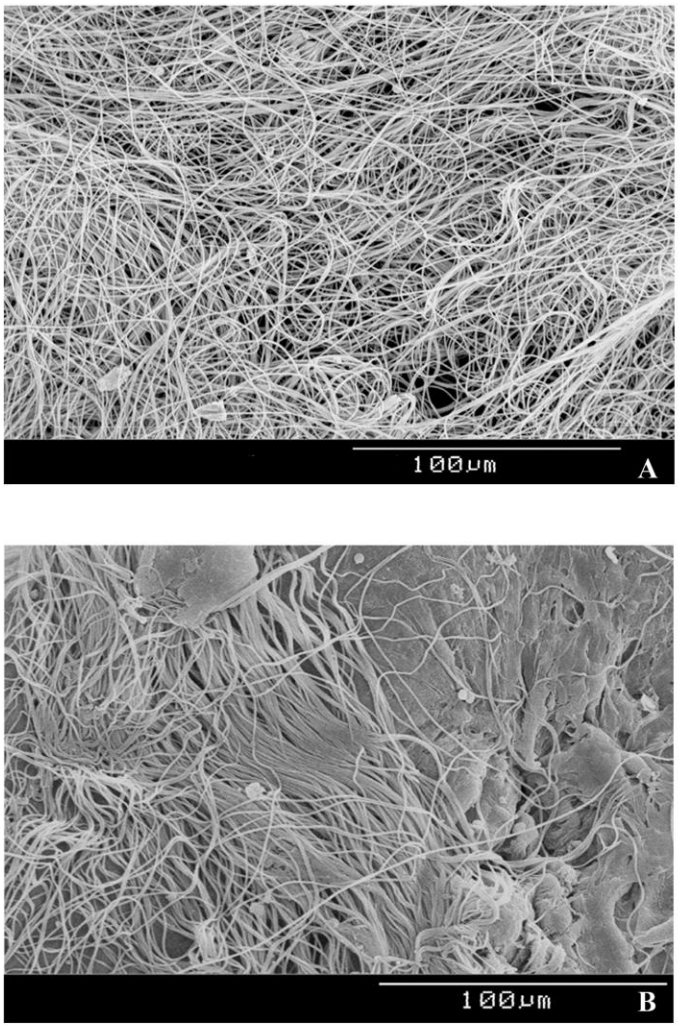

Figure 34: Scanning electron micrographs of spermatozoa in the vasa deferentia. **A.** spermatozoa of normal insect. **B.** spermtozoa of treated insect.

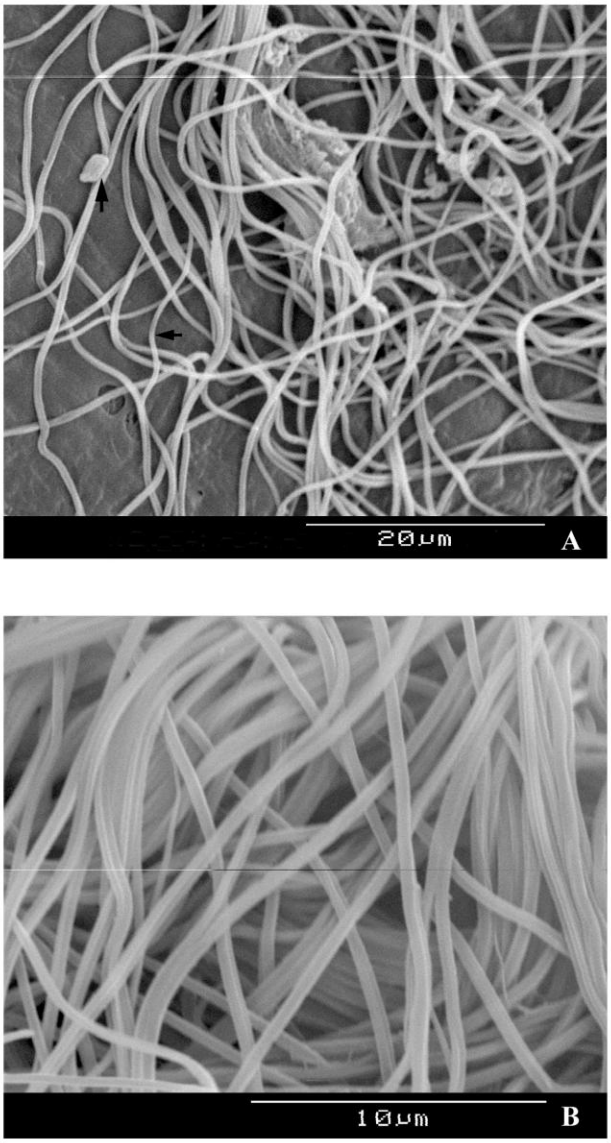

Figure 35: A. Scanning electron micrograph of spermatozoa in the vasa deferentia of normal insect showing triangle shaped head and long tail (arrows). **B.** Spermatozoa of treated insect with no visible difference.

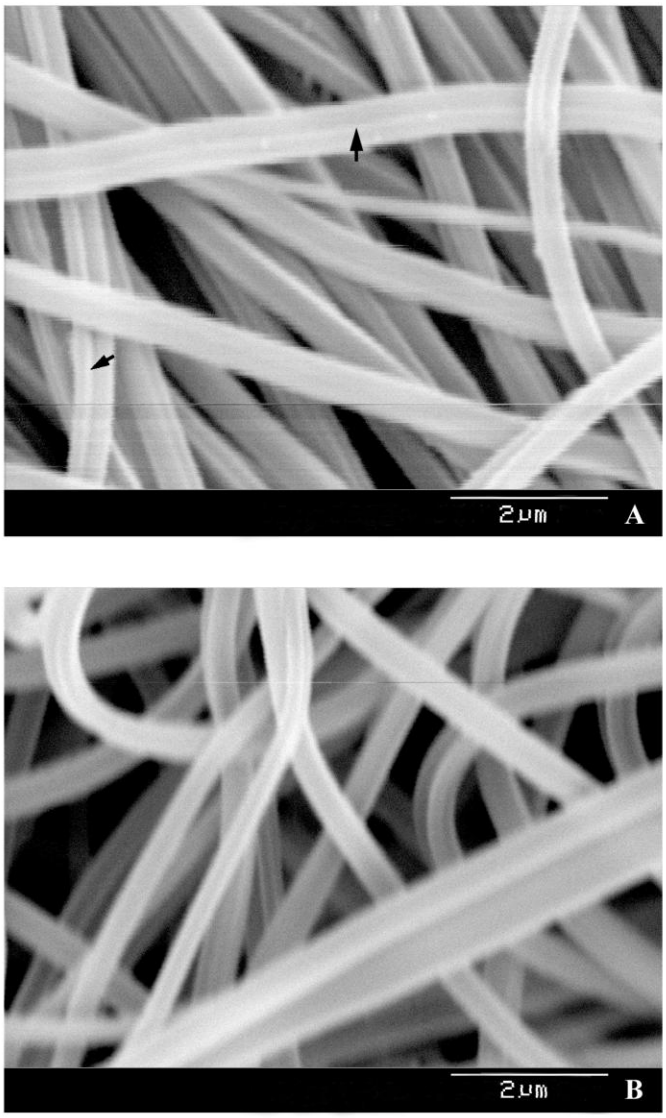

Figure 36: A. Scanning electron micrograph of spermatozoa in the vasa deferentia showing biflagellarity (arrow). **B.** Spermatozoa of treated insect with no visible difference.

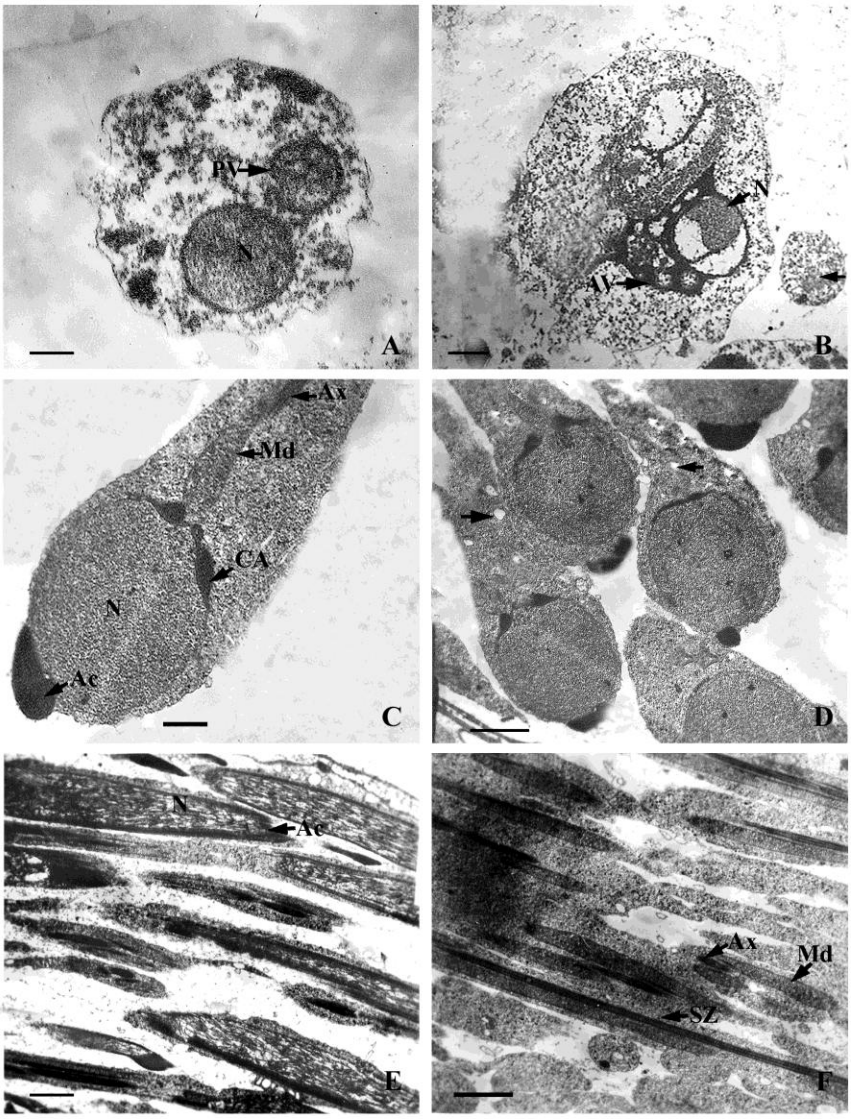

Figure 37: Different stages of spermatogenesis. **A.** Early stages of spermatogenesis with a large spherical nucleus (N) and provesicle (PV) **B.** Acrosomal vacuole (AV) on anterior pole of bean shaped nucleus. of spermatid. Two axonemes (arrow) are visible in the C.S. of spermatid. **C.** Horse shoe shaped acrosome cap in the posterior region of late spermatid, large round nucleus, centriole adjunct (CA), an axonemal (Ax) and mitochondrial basis (Md), which migrates into an indentation at the posterior pole of the nucleus and the nuclear surface with unique arrangements. **D.** Treated insect with vacuoles towards the posterior region (arrows). **E.** Elongation of spermatid during spermiogenesis, chromatin condensation, the elongation of nucleus and acrosomal vacuole during spermiogenesis. **F.** Elongated spermatozoa (SZ) with cylindrical shape. Scale bars = 0.5 µm.

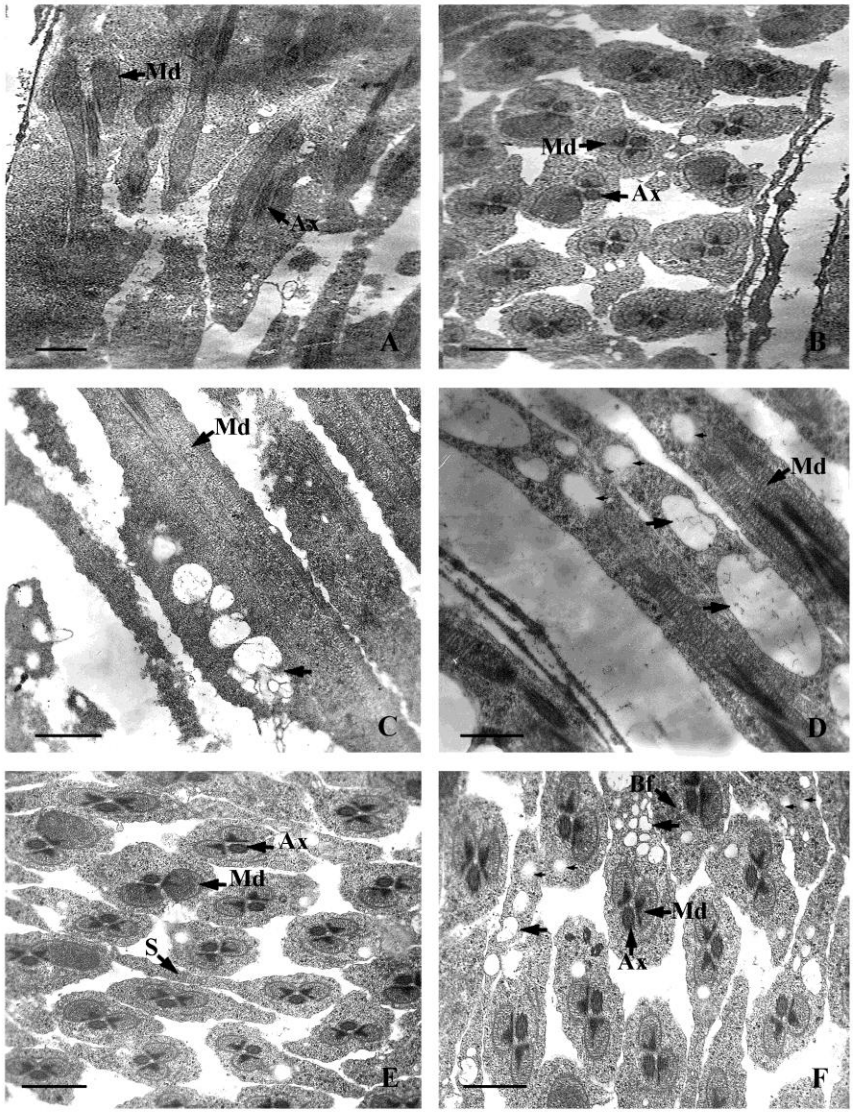

Figure 38: A. Longitudinal section of the spermatid in the testis. **B.** C.S. of the sperm showing mitochondrial derivative (Md) and axoneme (Ax). **C-D.** L.S of the treated insect spermatozoa with large number of vacuoles (small arrows) and lipid droplets (large arrows), the fish bone llike pattern of mitochondrial (Md) crystalline material is visible. **E.** Presence of crescent shaped mitochondrial derivative with axoneme in the centre portion of normal insect sperm. **F.** The C.S of the sperm showing large number of vacuoles (small arrows) and lipid droplets (large arrows) in the outer most matrix of treated insect. The two axonemes (biflagellarity, BF) are clearly visible. Scale bars = 1.0 μm.

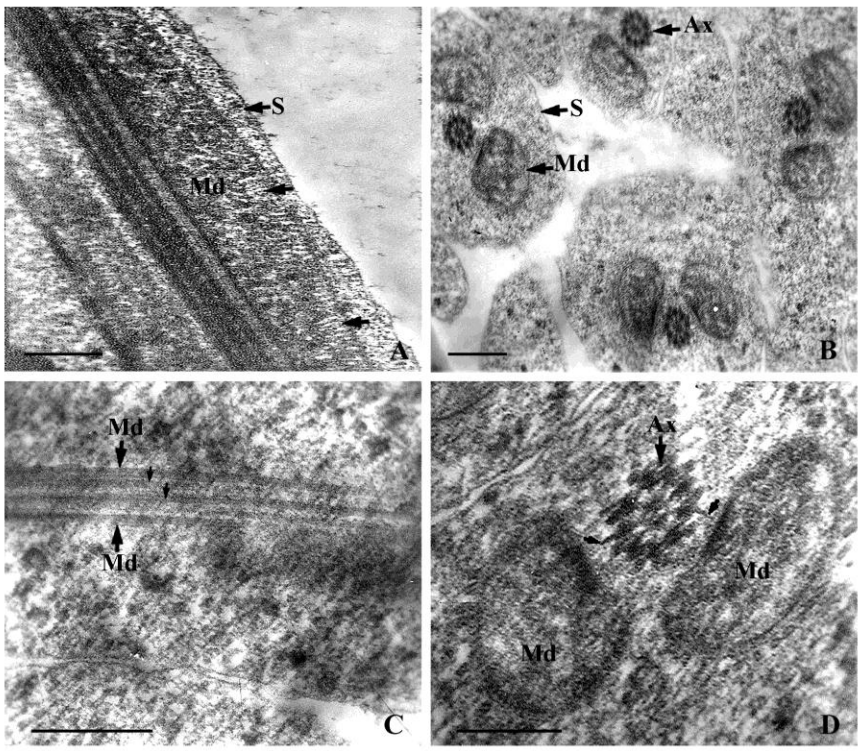

Figure 39: A. L.S. of the posterior region of spermatozoa with mitochondrial derivative (Md) with crystallistion (arrow), axoneme (Ax) and sperm surface (S). **B.** C.S. of sperm showing crescent shaped mitochondrial derivative (mirror image) and axoneme of 9+9+2 organisation. **C.** L.S. of tail region towards the posterior of the sperm showing two axoneme (small arrows). **D.** C.S. of the sperm showing cross bridge between axoneme and mitohondrial derivatives. Notice the cross bridge (arrow). Scale bars = 0.25 μm.

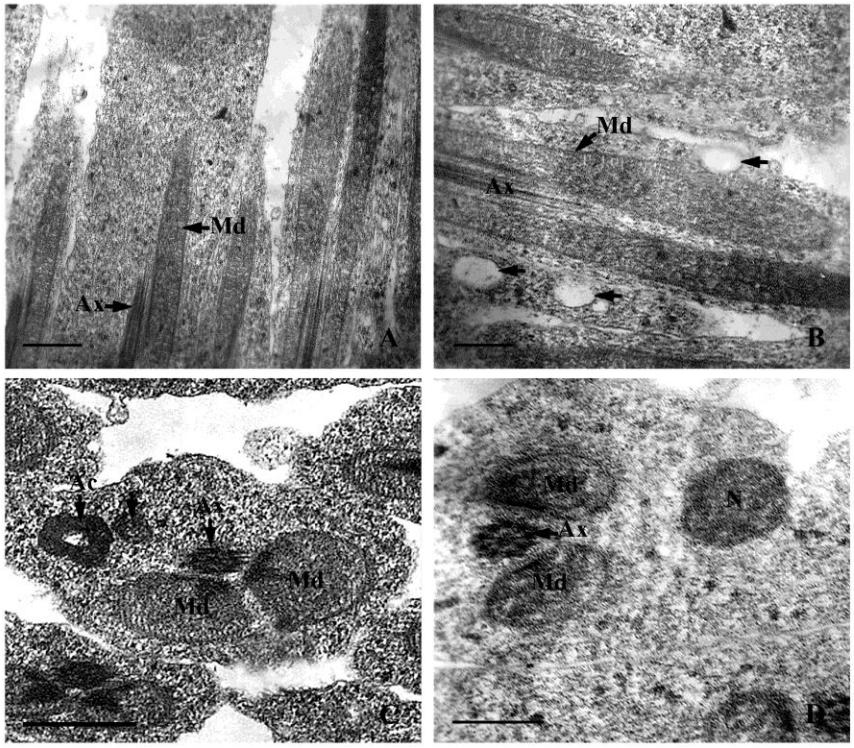

Figure 40: Ultrastructure of head region of spermatozoa . **A.** L.S. of sperm showing mitochondrial derivative (Md) and axoneme (Ax). **B.** L.S. of spermatozoa of treated insect with vacuoles in the outer surface (arrows). **C.** C.S. of the sperm head showing tubular thick ring shaped acrosome vacuole (AC), round nucleus (N), axoneme and two mitochondrial derivative in the normal insect **D.** Round nucleus, mitochondrial derivatives and clearly visible axoneme of spermatozoa in the normal insect. Scale bars = 1.0 μm.