STUDIES ON OXIDATIVE STRESS DURING THE LIFE CYCLE OF *IPHITA LIMBATA*

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

By

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CERTIFICATE

This is to certify that this thesis entitled "STUDIES ON OXIDATIVE STRSS DURING THE LIFE CYCLE OF *IPHITA LIMBATA*" is a bonafide research work done by Gracy Thomas from December 2000 to January 2005 in the laboratory of Insect Physiology and Biochemistry of the department under my supervision and guidance, in partial fulfillment of the requirement of the Degree of Doctor of Philosophy under the Faculty of Science, University of Calicut. I also certify that no part of this thesis has been presented before for any other degree.

Dr. K.V. Lazar

DECLARATION

I, Gracy Thomas do hereby declare that this thesis entitled "STUDIES ON OXIDATIVE STRSS DURING THE LIFE CYCLE OF *IPHITA LIMBATA*" submitted by me to the University of Calicut for the award of the degree of Doctor of Philosohy under the Faculty of Science is the result of the bonafide research work carried out by me under the guidance of Dr. K.V. Lazar, Lecturer, Department of Zoology, University of Calicut. I further declare that the results presented in this thesis have not been submitted previously for any degree.

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Gracy Thomas

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Introduction

INTRODUCTION

Although oxygen is vital to life, it may also contribute to aging and illness in organisms. When oxygen is metabolized, cells form by-products called "free radicals." Free radicals interact with the components of the cell, disrupting the structure of other molecules and resulting in cellular damage. Such damage is believed to contribute to aging and various health problems.

Oxygen-derived radicals or reactive oxygen species (ROS) are produced in abundance in all animal cells and tissues as part of the normal metabolic processes. However, organisms have numerous natural defenses either to prevent their formation or to neutralize them after they are formed. In spite of the natural defenses, it has been estimated that each human cell undergoes 10,000 "hits" by free radicals each day. Although most free radicals are potentially harmful, others are essential to many intracellular metabolic reactions. For example, they are deployed by phagocytic cells to kill ingested microorganisms.

Low levels of free radicals are necessary for a number of important physiological functions including the inflammatory response, cell division, and white blood cell action against bacterial infection. Free radical formation is controlled by a complex network of beneficial compounds known as antioxidants. Antioxidants are capable of stabilizing or deactivating free radicals before they attack the cells. But providing proper antioxidant protection is a challenge the animals face. Thus it is important to maintain a system of checks and balances between antioxidants and free radicals and their compounds.

In the present study the precise physiological and biochemical changes that the plant bug, *Iphita limbata* Stal (Heteroptera: Pyrrhocoridae) underwent on the administration of tryptophan, an antioxidant, along with the control food, honey, were followed. This included the separation and assay of tissue components and enzymes using standard biochemical techniques. On estimation of these components, an analysis of the data was made so as to elucidate the overall effects of oxidative stress leading to the morphological and physiological changes to the insect. The present work also attempts to elucidate the biochemical changes in the plant bug, *Iphita limbata,* during its Pactive and diapause stages.

The primary aim of the study was to investigate whether oxidative stress has consequences during the life span of the adult plant bug, *Iphita limbata.* The specific objective of the present work was also to study the free radical turnover rates in the adult *I. limbata.* The H_2O_2 generation in the insect was evaluated in context of the insect metabolism and the effect of a reducing amino acid, tryptophan, on the free radical turnover rates and metabolism during the adult stage of the insect was analysed. The results are interpreted in the context of the current understanding of the molecules that cause oxidative stress at the cellular level, with the antioxidant systems that function at the cellular and organism level, and with the role of dietary materials in oxidative stress.

REVIEW OF LITERATURE

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Review of Literature

REVIEW OF LITERATURE

Oxidative stress

Oxygen is the primary oxidant in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. Oxidative stress results from the metabolic reactions that use oxygen, and it has been defined as a disturbance in the equilibrium status of pro-oxidant/antioxidant systems in intact cells. When additional oxidative events occur, the pro-oxidant systems outbalance the antioxidant, potentially producing oxidative damage to lipids, proteins, carbohydrates, and nucleic acids, ultimately leading to cell death in severe oxidative stress (Halliwell and Gutteridge, 1989). Animals have multiple protective mechanisms against oxidative stress and succeed in preventing cell damage to the extent that these protective mechanisms are effective. Many dietary constituents are important sources of protective agents that range from antioxidant vitamins and minerals to food additives that might enhance the action of natural antioxidants (Packer, 1994).

Free radicals

Free radicals are oxygen atoms (or oxygen containing molecules) that are produced normally as intermediaries in cellular processes and respiration, and in the degradation of fatty acids. Free radicals are also generated by phagocytes (a type of immune cell) in the destruction of bacteria and virally infected cells, which leads some to speculate that increased oxidative stress occurs during inflammatory immune responses.

Oxygen species

Radicals of oxygen (superoxide anion, hydroxyl radical, and peroxy radicals), reactive non-radical oxygen species such as hydrogen peroxide and singlet oxygen, as well as carbon, nitrogen, and sulphur radicals comprise the variety of reactive molecules that can constitute an oxidative stress to cells. It has been estimated that a maximum of 5 % of the total oxygen metabolism of liver tissue results in the production of partially reduced oxygen species. This represents a significant stress under normal conditions, and there is evidence that some cellular damage occurs under these conditions (Sies, 1991; Packer, 1994; Halliwell, 1989).

Effects of oxidants on carbohydrates

Hydroxyl radicals react with carbohydrates by randomly abstracting a hydrogen atom from one of the carbon atoms, producing a carbon- centered radical. This leads to chain breaks in important molecules like hyaluronic acid in a process involving intermediates such as peroxyl radicals. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals. This phenomenon apparently accounts for a significant decrease in the synovial fluid of affected joints (James, 1999; Weir, 1996).

Cellular antioxidants

Although oxygen is vital to life, scientists are also finding that this essential element may contribute to aging and illness. When oxygen is metabolized, cells form by-products called "free radicals." Free radicals interact with the components of the

cell, disrupting the structure of other molecules and resulting in cellular damage. Such damage is believed to contribute to aging and various health problems.

Oxygen-derived free radicals (reactive oxygen species – ROS) are produced in abundance in all animal cells and tissues as part of the normal metabolic processes. However, organisms have numerous natural defenses either to prevent their formation or to neutralize them after they are formed. In spite of the natural defenses, it has been estimated that each human cell undergoes 10,000 "hits" by free radicals each day (Weir *et al.*, 1996; James, 1999). Although most free radicals are potentially harmful, others are essential to many intracellular metabolic reactions. For example, they are deployed by phagocytes to kill ingested microorganisms.

In 1956, D. Harman proposed a free radical theory of aging, hypothesizing that reactive oxygen species (ROS) are generated throughout all cells and tissues, resulting in progressive, random damage to enzymes, proteins, unsaturated lipids, cell membranes, and to the genes. The end result is cell senescence. Since then, abundant experimental and observational evidences support the idea that aging is the sum of all free radical reactions throughout all cells and tissues, or at least they are major the contributors to it.

Antioxidants are compounds with a chemical affinity for free radicals. They exist in abundance and bond with free radicals before they can cause damage. Antioxidants are of five classes: enzymes, such s catalases, peroxidases, and superoxide dismustase (SOD); peptides, such as glutathione; phenolic compounds like vitamin E and plant flavonoids; nitrogen compounds which includes various amino acids; and

carotenoids, most notably beta-carotene.

Other agents may have antioxidant effects through replenishing mechanisms - vitamin C, for instance, helps to recycle vitamin E, and NAC (N-acetyl cysteine) provides an important component of glutathione.

The most effective antioxidant in oxidative stress depends on the specific molecules causing the stress, i.e., superoxide anion, lipid peroxides, iron-generated hydroxyl radical, etc., and the cellular or extra cellular location of the source of these molecules. As an example, damage to a cell membrane occurs from both internally and externally generated oxidative stress. This damage is most effectively prevented by vitamin E which reacts with peroxyl and hydroxyl radicals, carotenoids which react with singlet oxygen, and possibly by membrane bound proteins. The chain-breaking antioxidant function of vitamin E in membranes results from its close association with polyunsaturated components of the membrane (Sies, 1991; James, 1999).

Antioxidants protect key cell components from damage by neutralizing the free radicals. Antioxidants that occur naturally in the body or are consumed through the diet may block damage to cells. However, over time, damaged cells can accumulate and lead to age-related diseases. In an effort to combat free-radical activity, scientists are studying the effects of increasing individuals' antioxidant levels through the diet and dietary supplements.

Oxygen is a required component of normal animal metabolism. Like any process that generates heat, safeguards are necessary to prevent damage to the cell from high levels of oxygen turnover. These safeguards exist in the form of both enzymatic

and non-enzymatic reactions. The enzymatic reactions require cofactors such as selenium and zinc, while the non-enzymatic systems rely on the presence of antioxidant molecules such as ascorbic acid or beta-carotene. Stress in a variety of forms can overwhelm these safeguards, resulting in the production of highly reactive forms of oxygen called free radicals. A large number of scientific publications over the last three years have implicated free radical reactions in the pathology of more than 50 human diseases. In fact, the oxidative stress resulting from the large-scale production of these free radicals is now believed to be a feature of most, if not all, human chronic diseases, including AIDS, cancer, cardiovascular diseases, arthritis, chronic fatigue syndrome, psoriasis and asthma. Furthermore, drugs used to treat these diseases may themselves contribute to increased oxidative stress. Radiation and cancer chemotherapy damage normal cells and produce reactive oxygen. (James, 1999)

Free radical damage to cells occurs when the antioxidant defenses of the organisms are overwhelmed and has been implicated in the pathogenesis of most acute and chronic debilitating diseases and also in the normal aging process. It is believed that disease progression may be retarded by supplementing the natural antioxidant defenses. And in addition, several of the dietary antioxidants have immune stimulating properties. Strong support of the immune system is critical in the treatment of any infectious disease and diseases such as cancer, as well as defending yourself against colds and flu (Diplock, 1991).

There is evidence showing that antioxidants can potentially delay the aging process, and also protect against the development of age-related diseases. Antioxidant properties of honey were effective against deteriorative oxidation reactions in foods.

The antioxidant capacity of the honey appeared to be primarily due to their phenolic composition as opposed to enzymatic antioxidants and ascorbic acid.

Antioxidant enzymes in cells

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is a very important enzyme that functions as a cellular antioxidant. It is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion. It catalyses the dismutation of superoxide anion in the following manner.

$$2\,O_2^{\cdot-} + 2\,H^+ \rightarrow O_2 + H_2O_2$$

The absence of this enzyme is lethal. The amount of superoxide dismutase is controlled by specific redox-sensitive genes in cells. There is also an extracellular form of superoxide dismutase in plasma, lymph, and synovial fluid that is different from the intracellular forms of the enzyme (Packer, 1994). The extracellular enzyme may function at cell surfaces.

Catalase

Catalase is a heme protein that catalyzes the reaction shown below in which hydrogen peroxide is detoxified. It is usually found in peroxisomes except in cells like erythrocytes that do not contain these organelles. In that case catalase is a cytoplasmic enzyme. Catalase provides a protective role that is similar to that of glutathione peroxidase because both are important means of removing hydrogen peroxide. Both

catalase and glutathione peroxidase are important in hydrogen peroxide detoxification (Packer 1994).

$$2\,H_2O_2 \rightarrow O_2 + 2\,H_2O$$

Glutathione peroxidase

Glutathione peroxidase is a cytoplasmic and mitochondrial enzyme that is important for detoxifying H_2O_2 in most cells. This protein is a seleno protein, i.e., it contains a selenocysteine amino acid at the active site instead of a normal cysteine. The selenium that replaces the normal sulfur in this amino acid has enhanced nucleophillic properties and ionizes more readily to release a proton. It is a much more effective catalyst in the reaction catalyzed by this enzyme.

Biochemical composition of insect tissues

Haemolymph

The haemolymph of insects serves as a bathing medium of its various tissues and organs as they lack an epithelial lining of true coelom. Therefore, the haemolymph forms the meeting place of both the raw materials required and the products of various physiological activity of the insect body. Since the haemolymph is not directly connected with the external environment in the case of alimentary canal, 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 most reliable data, which can be used as an index of the physiological activity (Buck, 1953; Wyatt, 1961).

It is found that during the diapause no nitrogenous materials are eliminated from the animal as a result of the cessation of excretory activity. This will result in an accumulation of nitrogenous metabolites in the internal milieu. Therefore, the analysis of haemolymph during the period will give clues to the various mechanisms by which the animal is adapted to deal with the nitrogenous end-products, so as not to develop toxicity.

Haemolymph proteins in insects

Proteins have many reactive sites that can be damaged during oxidative stress, but interest has centered on three measurable events. First, aggressive radicals such as hydroxyl radical can fragment proteins in plasma, and the fragmented products of specific proteins, if known, can be detected. This fragmenting is associated with reactions at specific amino acids such as proline and histidine. Second, proteins may contain metal binding sites that are especially susceptible to oxidative events through interaction with the metals. These reactions usually produce irreversible modifications in amino acids that might be involved in metal ion binding, e.g., histidine. These modifications may produce signal sequences that are recognized by specific cellular proteases that degrade such proteins. Finally, many intracellular proteins have "reactive" sulphydryls groups on specific cysteine residues that can be modified (oxidized) to specific forms (disulphides) that can be reduced again by metabolic processes. Similarly, some proteins have a "reactive" methionine that can undergo a reversible modification to methionine sulphoxide. The disulphide and sulphoxide forms of these two amino acids may actually serve a protective role, since the metabolic reversibility of the protein modification effectually detoxifies the oxidative species that

caused the modification. The reversible nature of the modifications of cysteine and methionine also suggests that oxidative modifications of this type may have a role in regulating metabolic events in the cells under oxidative stress (James, 1999).

Protein is an extremely common dietary constituent of insects and it is readily digested by the gut. The various aspects of the haemolymph proteins in insects have been studied by many investigators (Buck, 1953; Wyatt, 1961; Chen, 1966, 1978; Jeuniaux, 1971; Price, 1973; Wyatt and Pan, 1978). It has been established that the proteins are synthesized in the fat body and releases into the haemolymph, which are subsequently sequestered into the fat body and stored there depending upon the physiological condition of the animal. But the necessity for the occurrence of such a process has not been well explained (Dortland, 1978), though Martin *et al.*, (1971) suggested the possibility of source. Conformational change in the structure of the proteins during the temporary retention in the haemolymph is necessary before being stored in the fat body.

The protein concentration of insect haemolymph 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 general observation is that the concentration of proteins increases during the larval stages and decreases at the pupal and adult stages (Eagle and Woods, 1960; Wyatt, 1961; Chen, 1966, 1971; Jeuniaux, 1971; Florkin and Jeuniaux, 1974).

Insects are known for its capacity to regulate the osmotic pressure of the haemolymph in spite of the variation in its volume. (Florkin, 1966). During dehydration

the haemolymph volume decreases but the osmotic pressure remains constant due to a concomitant lowering of the concentration of the solutes. The reverse phenomenon is observed during re-hydration (Djajakusumah and Miles, 1966). Therefore, the various ingredients of the haemolymph may contribute to the maintenance of its osmotic pressure by changing the free amino acid concentrations of the haemolymph. A survey of the haemolymph proteins of insects (Wyatt and Pan, 1978) revealed that many low molecular weight proteins are present in it.

Haemolymph total free amino acids

Of the many important characteristic features of insect haemolymph, the high titre of total free amino acids stands outstanding. The subject has been reviewed by Florkin and Morgulis, 1949; Auclair, 1953; Buck, 1953; Duchateau and Florkin, 1958; Bheemerwar, 1959; Florkin, 1939,1966; Gilmour, 1961,1965; Wyatt, 1961; Chen, 1962, 1966, 1971; Chefurka, 1965; Corrigan, 1970; Jeuniaux, 1971 and Florkin and Jeuniaux, 1974. In general, the previous investigation revealed that the amount of the total free amino acids in insect is about 5 to 20 times higher than other invertebrates and represents about 0.5 to 2%.(wt/v) of the haemolymph. Attempts have been made to compare the concentration of total free amino acids in different phases of life cycle of insects. It has been generally observed that the total free amino acids are much higher in the larva than pupa or adult. Many insects, particularly plant-sucking species consume a liquid diet which is high in amino nitrogen concentration. The amino acids and amides present in such diet far exceed the normal amounts required for life processes. Akao, (1943) has suggested that when the total free amino acid pool of the body is in excess of the actual demand for total free amino acids in the haemolymph,

the excess accumulation proves fatal to the organism and their removal from the body is necessary.

According to many investigators, there exists a relationship among haemolymph total free amino acids and excretory total free amino acids (Mall and Pall, 1982; Lazar, 1983). Lazar, (1983) showed that during the development of *Spodoptera mauritia* larva, there occurs a gradual increase in both haemolymph and excretory total free amino acids from phase 1 larva to phase IV larva. Nakayama, (1991) reported the changes in haemolymph total free amino acids concentration in accordance with development. Changes in the concentration of total free amino acids in haemolymph with regard to the physiological state of insects are also visualized by Maddrell and Gardiner; (1980), Osanai and Yonezawa, (1986) and Whitton *et al.* (1995).

Fat body proteins

The fat body proteins have been investigated extensively in insects (Kilby, 1963; Karnavar and Nayar, 1973; Price, 1973; Wyatt, 1975; Chen, 1978; Wyatt and Pan, 1978 pg.23; Lazar, 1983). A few studies have been made in relation to physiological changes in the larva of insects (Wiggles-Worth, 1942; Coles, 1965a; Loughter and West, 1965; Chippendale and Beck, 1967; Nair *et al.*, 1967; Hill and Goldsworthy, 1968; Chippandale and Kilby, 1969; Collins, 1969, 1974, 1975; Terra *et al.*, 1973).

Fat body total free amino acids

The interest in the investigation of free amino acids in the fat body is due to the fact that the tissue is an active site of the intermediary metabolism of amino acids (Kilby, 1963). Investigation on the content of free amino acids in the haemolymph of the animal revealed that there is a conspicuous change in the amount of the material during the active and diapause phase of the animal.

Fat body urea

The accumulation of urea during the periods of water stress has been demonstrated in semi terrestrial and terrestrial animals (Balinsky *et al.*, 1967, 1971; Gorden *et al.*, 1961; Scheer and Markal, 1962; Tereafs sand Schoffeniels, 1962; Janssens, 1964, Janssens and Cohen, 1968; De Jorge and Peterson, 1970; Jungreis, 1971; Campbell *et al.*, 1972; Tramell and Campbell,1972. Another possible function of urea is its effect in the reduction of evaporative water loss (De Jorge and Peterson, 1970; Horne, 1971, 1973, 1977 a, b; Campbell *et al.*, 1972; Newman and Thomas, 1975; Jungreis, 1976). During dormancy the metabolism in general is expected to be at a low level (Campbell, 1973; Cohen, 1976) in order to conserve the energy resources of the animal. The occurrence of urea has been demonstrated in the haemolymph of a few insects (Chefurka, 1965; Cochran, 1975).

Fat body creatinine

Phosphocreatinine, the phosphogen characteristic of vertebrate muscle was thought to be subtotaled by phospshoarginine in invertebrates and this forms the basis of invertebrate phosphogen theory (Baldwin, 1967). The concentration of creatine and

creatinine in fat body shows an inverse correlation depicting that creatinine is a metabolite of creatine as in vertebrates (Block and Schoenheimer, 1939; Borsook and Dubnoff, 1947 a, b).

Insect diapause

Diapause refers to the state of arrested growth or reproduction that is typical of many hibernating or aestivating arthropods (Lees, 1956). One must distinguish diapause from quiescence. Some borderline cases do occur, but certain physiological mechanisms can be recognized in the diapausing insect which are absent in the quiescent (Tauber and Tauber, 1976). Harvey, (1962) stated that diapause is a state of developmental arrest which persists even when environmental conditions are favorable for growth. In some insects the arrest is facultative: environmental stimuli direct the organism either to continue or to terminate development. In other insects the arrest is obligatory. In both facultative and obligatory diapause, control over development is exercised by the endocrine system (Beck, 1968).

Organisms living in temperate regions face seasonal challenges, such as absence of food, harsh winter conditions, and the need to synchronize reproduction with suitable conditions for breeding. Insect adaptations to these challenges include migration,

dormancy, and seasonal phenotypic variation; various combinations of these traits constitute a diapause syndrome (Andrewartha, 1952; Tauber *et al.*, 1986; Danks, 1987; Leather *et al.*, 1993). Diapause is a state of arrested development, characterized by low metabolic activity, reduced motor activity and increased resistance to environmental extremes, and is usually hormonally controlled in insects (Nijhout, 1994). Diapause can occur during any insect life stage; reproductive diapause refers to delayed reproductive development in the adult stage.

The course of diapause includes changes in the insect's physiology and sensitivity to environmental stimuli against a backdrop of seasonal changes. Diapause induction occurs along a spectrum ranging from complete control by external factors to complete control by genetic factors (Tauber *et al.*, 1986). External stimuli that induce diapause almost always precede adverse conditions, and include both abiotic and biotic factors (reviews by Tauber *et al.*, 1986; Leather *et al.*, 1993). After the onset of diapause, there is typically a period of intensification followed by maintenance and eventual termination. These steps are referred to as diapause development (Tauber *et al.*, 1986). Diapause development may end via multiple pathways, following a predetermined course not influenced by external cues or terminating in response to external stimuli. Hodek, (1983) refers to these pathways as "horotelic" (Greek: hora = right time, telos = fulfilment) and "tachytelic" (Greek: tachys = quick) respectively. These pathways may operate singly or together. The rate of diapause development is often driven by temperature, but other stimuli include photoperiod, food, moisture, parasitoid / host interactions and mating (reviewed in Tauber *et al.*, 1986; Danks, 1987).

Temperature effects on diapause are variable (Saunders, 1967, 1968) and temperature may also affect the induction of diapause through photoperiodic influences (Sullivan and Wallace, 1967). High temperatures tend to avert diapause in long-day species, although low temperatures may avert diapause in some cases also. Apparently temperatures are important in determining whether or not photoperiod can act. Temperature and photoperiod act differently on different developmental stages to cause diapause (Eskafi and Legner, 1974).

The principal stimulus for the onset of diapause is photoperiod, although temperature, water and diet may be involved. Diapause may terminate abruptly when the brain regains its full function. All insect stages may enter diapause. In the larva and pupa diapause is an arrest in molting controlled by the brain-thoracic gland system. In adult insects diapause is characterized by an inhibition in the maturation of eggs associated with corpus allatum failure (de Wilde and Boer, 1961). Diapause in the early embryo of *Melanoplus differentialis* involves an interruption in embryogenesis.

Each of the following endocrine organs of insects is associated with some form of diapause: (1) brain-thoracic gland, (2) corpus allatum and (3) subesophagael ganglion. It is recognized that insect diapause is an endocrine deficiency syndrome of the prothoracic glands (or the corpora allata). There is little doubt that diapause found in the growing stages is due to a temporary absence of neurosecretory activity in the brain. In the case of adult diapause there may be active inhibition of the corpora allata (de Wilde, 1962).

Seasons exert their influence on diapause according to their particular form. In *Hippelates* eye gnats the egg enters diapause following a period of desiccation (Legner, Olton and Eskafi, 1966). Larvae of the navel orangeworm, *Amyelois transitella,* enter diapause following a period of drought (Legner, 1983). The causes of diapause in parasitic Hymenoptera are not simple. In many species the individuals may enter a state of diapause at a time when the environment is favorable to continuous development and increase of the species (Flanders, 1944, 1972; Simmonds, 1948).

It is now recognized that insect diapause is an endocrine deficiency syndrome of the prothoracic glands (or the corpora allata). There is little doubt that diapause found in the growing stages is due to a temporary absence of neurosecretory activity in the brain. In the case of adult diapause, there may be active inhibition of the corpora allata (de Wilde, 1982). The neurohormonal control of eastern North American monarch diapause has been elucidated (Barker and Herman, 1973; Herman, 1975, 1981, 1985), and photoperiod and temperature have been shown to influence reproductive development in post-eclosion monarchs (Barker and Herman, 1976). The duration of diapause is extremely variable among species. Nine days to 200 days and even 12 years (e.g., *Sitodiaplosis* sp. midge) are known (Etzel and Legner, 1999). A general requisite for breaking diapause is the taking up of water from the environment, which is probably related to the increasing metabolic activity of awakening insects.

MATERIALS AND METHODS

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Materials and methods

MATERIALS AND METHODS

The experimental insect

Iphita limbata Stal (Heteroptera: Pyrrhocoridae) are moderately sized, brightly coloured insects. They exhibit strongly contrasting red and black colouration. They are polyphagous and omnivorous. Cannibalism is also observed. Mouthparts are exclusively adapted for piercing and sucking fluids from plants and animals. The mandibles and maxillae are modified to form slender bristle like stylets, which rest on the grooved labium. Both pairs of stylets are hollow seta like structures, capable of limited protrusion and retraction by means of muscular action.

The head is rather small with well-developed antennae, which are thick and four segmented. The last segment of the antennae is white in colour. Ocelli are absent.

The prothorax and scutellum are brownish black. The three pairs of legs are adapted for walking. Both pairs of wings are normally developed and are folded flat over the dorsum with the apical portions overlapping. Though they possess well-developed wings they do not fly.

Body is large, robust with red colour. Sexual dimorphism is seen. The females are larger with broader abdomen. The cerci are absent. The ovipositor is well developed. The insects are diurnal in habit.

Diapause of *I. limbata*

I. limbata exhibits diapause when they are undisturbed from late June to early February which varies with environmental stimuli such as the onset of monsoon, temperature and humidity. The nature of diapause has been observed for three consecutive years in the laboratory from March 2001 to June 2004.

Rearing and bioassay

Adult bugs were collected locally from the field by hand picking. The insects are sexually dimorphic. The males are comparatively smaller with their external genitalia projecting at the posterior side. For the study of the biology of the animals, males and females were collected at random and bred in the laboratory. Only males, more or less of the same age and size were used for the biochemical analysis. The male insects were segregated into two groups in separate insect rearing boxes and maintained in the culture room at normal temperature and humidity. They were fed on 15 % (v/v) honey soaked in cotton buds. The buds were renewed daily. The dead insects were removed from the boxes immediately to keep the hygienic condition. The insect's boxes were protected from ants by water barriers. Adult insects, acclimatized to the laboratory conditions for two weeks were used for the experiments.

Treatment of tryptophan

The experimental insects were fed with 15 % (v/v) honey mixed with 5 % (w/v) tryptophan soaked in cotton buds. The control sets of insects were fed on 15 % (v/v) honey. The insects fed with honey supplemented with tryptophan are called treated

insects and those fed with honey alone are called normal insects. The buds were renewed daily. Experimental studies on the effects of tryptophan on the insect were initiated 24 hours after feeding. The experiment was conducted on alternate days for four weeks. The control insects were also tested for the same parameters.

Determination of the weight of insects

The fresh weights of the insects were noted at forty-eight hour intervals in the case of both normal and treated insects. They were separately killed with the ethyl acetate and dried in an oven at 100°C for one hour and then at 60°C to a constant weight. The variation in the total fresh and dry weight of the insect was determined.

To determine the differential weight of normal insects for the year, the fresh weight of insects were noted every month. Then the insects were killed with the ethyl acetate and dried in an oven as mentioned above. The differences in the water content of normal and treated insects were determined by finding out differences between the fresh and dry weight.

Determination of the volume of total haemolymph

The measurement of the volume of haemolymph was made directly using a fine calibrated capillary tube. For extracting the haemolymph, the insect was anaesthetized slowly with diethyl ether as described by Mohamed (1974). The insect antennae were amputated with a sharp scissors and the haemolymph that oozed out was immediately drawn into a calibrated capillary tube and its volume found out. To ensure complete

extraction of haemolymph, the body of the insect was pressed gently until no more haemolymph was oozing out of the wound.

Determination of weight of the total fat body

The fat body of appropriate number of treated and the normal insects were dissected out carefully in ice-cold insect ringer on separate clean sides. Water adhering to the fat body lobes were wiped off carefully with a filter paper and weighed immediately. Variations in the fresh weight of fat body in the treated and normal insects were determined.

Biochemical analyses of fat body of insects under experimental dietary regimen

For the estimation of various biochemical analyses the pooled fat body of normal and treated insects was homogenized separately in 0.1M phosphate buffer of pH 7.0. This extract was then used to estimate the total proteins, total free amino acids, glucose, urea, creatinine, hydrogen peroxide, the enzyme activity of catalase and the activities of alanine aminotrasferase (AlAT) and aspartate aminotrasferase (AsAT). The biochemical analyses were carried on every alternative day for four weeks for the treated and normal insects.

Eight insects each were collected for the normal and the treated groups and dissected out in ice-cold insect ringer. The pooled fat body samples were isolated from the insects of each set. The extract was stored at -20° C until the estimations were carried out.

Estimation of total protein

Total protein was estimated following the method of Lowry *et al.*, (1951) using crystalline bovine serum albumin (fraction V, Sigma) as standard. The proteins were precipitated with trichloroacetic acid (TCA). 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° C for fifteen minutes. The residue left over the hot extraction was dissolved in 1 N sodium hydroxide. The blue colour developed was measured against a reagent blank at 540 nm in a Shimadzu UV 250 spectrophotometer.

Estimation of total free amino acids

The total free amino acid content in the tissues was estimated by the method of Lee and Takahashi (1966). The homogenized tissues was precipitated with 10 % sodium tungstate and 2/3 N H_2SO_4 and centrifuged at 2000 rpm for 20 minutes. The resultant supernatant was used for the amino acid estimation. The colour developed was read at 540 nm against a reagent blank in a spectrophotometer.

Estimation of glucose

Glucose was estimated according to Morgan (1975). Homogenates of the tissues were deproteinised by 0.3 N barium hydroxide and 5% zinc sulphate and filtered. The filtrate was then used for the estimation and the blue colour developed was read at 540 nm against a reagent blank in spectrophotometer.

Estimation of urea

Urea was estimated in the tissues using Fearon reaction, modified by Beale and Croft (1961). The homogenates were deproteinised with 0.3 N barium hydroxide and 5% zinc sulphate and filtered. The filtrate was treated with diacetyl monoxime-phenyl anthranilic acid and then with activated acid phosphate reagent and heated for eleven minutes. The colour developed was read after cooling at 535 nm in a spectrophotometer.

Estimation of creatinine

Creatinine content in the tissue was estimated based on Jaffe reaction by Me Fate, *et al.*, (1954). The homogenates were precipitated with 10 % sodium tungstate and 2/3 N H_2SO_4 and centrifuged at 2000 rpm for 20 minutes. The filtrate was used for the estimation and the yellow orange colour developed was read at 520 nm in a spectrophotometer.

Estimation of H_2O_2 and catalase activity

Both estimations were done based on the formation of soluble coloured peroxotitanium complex by the reaction of H_2O_2 with potassium titanium oxalate which was measured at 410 nm.

The determination of catalase activity was based on estimating the amount of residual hydrogen peroxide in the assay mixture after incubation of a known amount of H_2O_2 with the enzyme extract for a fixed time interval.

The assay system consisted of 0.5 ml of the homogenized tissue extract in 0.1 M phosphate buffer, pH 7.0 and 0.88 M hydrogen peroxide in a total volume of 1ml. The mixture was incubated at 37° C for 30 minutes. The reaction was terminated by adding 0.8 ml of 20 % TCA. The assays for the controls were prepared by adding TCA before the incubation. The test and control assay mixtures were centrifuged at 3000 rpm for ten minutes and the supernatant was used for the estimation as described above. The differences in the values of the control and test give the amount of H_2O_2 oxidized in thirty minutes.

Estimation of alanine aminotransparase (AlAT) and aspartate aminotransparase (AsAT) activity

The activity of AlAT and AsAT was estimated following the method of Reitman and Frankel (1957) using pyruvic acid as standard. The homogenates of the tissues were centrifuged and the clear supernatant was directly taken for the enzyme assay. In the case of AlAT the substrate mixture was made of α - oxoglutarate and L-alanine in phosphate buffer of pH 7.4. This was incubated with the enzyme source for one hour. The colour developed was read after ten minutes at 520 nm in a spectrophotometer. In the case of AsAT the substrate mixture of α - oxoglutaric acid and L-aspartic acid in a phosphate buffer of pH 7.4 was incubated with the enzyme source for one hour. The colour developed was read after ten minutes at 520 nm in a spectrophotometer. One unit enzyme activity corresponds to the formation of one mole of keto acid/ minute at 37° C under the experimental conditions.

Biochemical changes during diapause

The biochemical analysis of the fat body and haemolyph were followed for the insects collected from the field under active and diapause stages.

Biochemical analyses of haemolymph

For the biochemical analyses in haemolymph, pooled haemolymph samples were extracted from appropriate number of insects. Haemolymph samples were extracted from insects of pre-diapause, diapause and post-diapause periods. The samples were immediately processed before melanization and clotting takes place.

The collected haemolymph was deproteinised with 80% ethyl alcohol (v/v) and centrifuged at 5,000 rpm for 15 minutes. The supernatant was collected and the residue was again treated with 80% ethanol, centrifuged and the supernatant added to the original supernatant. This was repeated for three times and the combined supernatant was evaporated to dryness in a hot air oven at 60°C, re-dissolved in a known volume of 1% HCl. This extract was then used to estimate the total proteins, total free amino acids, glucose, urea and creatinine using the procedures cited earlier.

Determination of individual free amino acids in haemolymph using HPLC

The individual amino acids were determined using HPLC according to Ishida *et al.*, (1981). The sample in the vials were carefully washed with 6N HCl and transferred into the test tubes. The volume was made up to 5 ml and the tubes were heat-sealed after filling pure nitrogen gas. Hydrolysis was carried out in a hot air oven at 110°C for 24 hours. After hydrolysis, the contents were removed and filtered into round bottom

flasks through Whattman filter paper No.42. The flasks were flash-evaporated to remove traces of HCl and the process was repeated for three times with distilled water. The residue was made up to I ml with 0.05M HCl. The samples thus prepared were filtered again through a membrane filter of 0.45μm and 20μl was injected in to Shimadzu HPLC-LC 10AS having a column packed with a strongly acidic cation exchange resin *i.e.* styrene divinyl benzene copolymer with sulfinic group. The column is of sodium type [ISC-07/s 1504 Na-Shimadzu]. The mobile phase consists of two buffers, Buffer A (sodium citrate, absolute alcohol, perchloric acid, pH 3.2) and buffer B (sodium citrate, boric acid, 4N NaOH, pH 10). The oven temperature was maintained at 60°C, the amino acids were eluted from the column by step wise elution *i.e.* acidic amino acids first, followed by neutral and then finally basic amino acids. The eluted amino acids were detected by using a fluorescence detector after post column derivitization with O-phthalaldehyde. In the case of proline and hydroxyproline, imino group was converted to amino group with sodium hypochlorite.

Biochemical analyses of fat body

Eight insects each were collected during active and diapause seasons and dissected out in ice-cold insect ringer. The pooled fat body samples were isolated from the insects of each set. The extract was stored at –20° C until the estimations were carried out. For the estimation of various biochemical analyses the pooled fat body of active and diapause insects was homogenized separately in 0.1M phosphate buffer of pH 7.0. This extract was then used to estimate the total proteins, total free amino acids, glucose, urea, creatinine, hydrogen peroxide, the enzyme activity of catalase and the

activities of alanine aminotrasferase (AlAT) and aspartate aminotrasferase (AsAT). The biochemical analyses were carried on every month for one year.

The physiological significance of the variations in the titre of each material was discussed. An attempt has been made to elucidate the homeostatic mechanism with which the animal undergoes its active and diapause periods.

RESULTS

Gracy Thomas "Studies on oxidative strss during the life cycle of iphita limbata" Thesis. Department of Zoology, University of Calicut, 2005

Results

RESULTS

Development and growth of the insect

Internal fertilization takes place in *Iphita limbata*. The eggs (plate: 1) are laid in clusters of about 100. They are ovoid, creamy white with an average diameter of 1 mm. It takes eight to ten days for the development of the egg. The day before hatching, the egg becomes strongly pigmented and exhibits movements beneath the outer covering.

The nymphs emerge from the egg in a form which differs from the adult only in the undeveloped state of the reproductive organ and external genitalia and in morphologically unimportant details of shape and size. The newly hatched nymphs are pale red in colour with about 0.01g average weight. The young resembles the adults in general form and mode of life. Post embryonic development is consequently one of gradual growth, unaccompanied by any striking morphological change. The distinctive features of growth are the acquisition of wings and genitalia. The young bugs are bright red with black legs and antennae.

The wing rudiments are not discernable in the first instar but later become visible externally as wing pads which gradually increase in size. The insect during its growth undergoes four moults and there are five instars in *I. limbata*. The final instar is the fully mature form which is known as the adult or imago. The growth from the nymph to the imago is a simple one and is unaccompanied by conspicuous morphological changes though the changes at the last nymphal moult are usually greater than at proceeding ones.

Plate: 1. The eggs of *Iphita limbata*

Plate: 2a. The adult *Iphita limbata* (dorsal view)

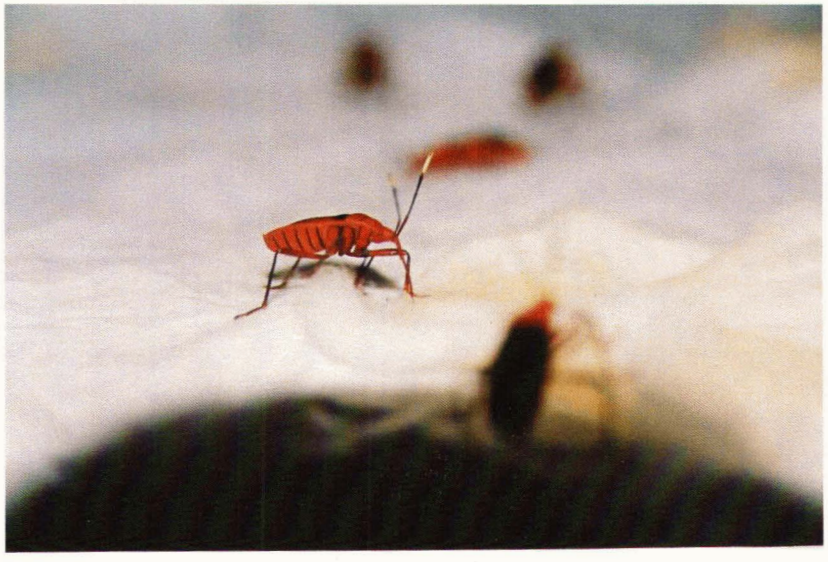


Plate: 2b. The adult *Iphita limbata* (side view)

The adult insects are red in colour with black patches on their wings (plate: 2). They exhibit sexual dimorphism.

These insects enter diapause regularly every year from the end of June to February. During diapause they are seen in groups on the ventral surface of some leaves, which may be fresh or dry (plate: 3). During this period they are inactive as they stop feeding, breeding and moving except that of its antennae. The mortality rate of those insects in diapause is found to be very low or almost nil. During diapause development is suspended and cannot be resumed unless diapause is first broken by an appropriate environmental change.

Biological effects of tryptophan

Weight of insects during experimental dietary regimen

The fresh weight and the dry weight of the adult insects were determined on alternate days beginning from day two. The findings are recorded in table 1 and figure 1:1 to 1:4.

Table 1

Figure 1:1 to 1:4

There was gradual increase in the weight of insects up to the fourteenth day and the weight suddenly decreased in both normal and treated ones. The decrease in weight in normal was found to be 36.75% and in treated it was 36.28 %.

Plate: 3a. Diapause on *Enterolobium saman*

Plate: 3b. Diapause on *Piper nigrum*

Table: 1. Fresh and dry weight of insect during dietary regimen

Dietary regimen in days	Weight in mg			
	Fresh weight/insect		Dry weight/insect	
	Normal	Treated	Normal	Treated
2	198 ±21.02	223 ± 23.69	76 ± 10.62	91 ± 11.25
4	196 ± 19.24	186 ± 21.62	84 ± 12.03	100 ± 12.47
6	203 ± 21.51	196 ± 20.96	82 ± 11.08	92 ± 13.01
8	205 ± 20.13	186 ± 22.87	100 ± 13.03	93 ± 11.47
10	207 ±20.89	180 ± 19.85	105 ± 11.96	98 ± 12.05
12	208 ± 21.87	178 ± 21.45	104 ± 12.04	104 ± 13.06
14	209 ± 22.36	163 ± 20.87	107 ± 14.87	98 ± 12.12
16	191 ± 20.95	160 ± 18.96	96 ± 10.97	100 ± 11.04
18	168 ± 17.68	158 ± 20.99	92 ± 11.84	101 ± 12.45
20	165 ± 20.45	157 ± 18.34	90 ± 12.25	102 ± 20.98
22	164 ± 23.10	155 ± 22.85	85 ± 11.81	102 ± 11.41
24	148 ± 12.43	144 ± 14.57	82 ± 10.96	85 ± 12.14

The values are the means of five determinations with standard deviation.

Water content in the normal and treated insects

There was gradual decrease in the amount of water content in both normal and treated ones. But the decrease was more conspicuous in the treated. The results are recorded in table 2 and figure 2.

Table: 2

Figure: 2

Table: 2. Water content in the normal and treated insects

Dietary regimen in days	Weight of insect in mg	
	Normal	Treated
2	122	132
4	112	86
6	121	104
8	105	93
10	102	82
12	104	74
14	102	65
16	95	60
18	72	57
20	75	55
22	79	53
24	66	59

The values are derived from the mean values of fresh and dry weight of insects given in table 1.

Weight of fat body in the normal and treated insects

The fat body of *I. limbata* is white in colour and consists of loosely aggregated irregular and diffuse tissue which is freely suspended in the haemocoel so that they are intimately bathed by the blood.

The weight of fat body during dietary regimen is recorded in table 3 and figure 3.

Table 3

Figure 3

There was a steady increase in the amount of fat body during the experiment. The weight of fat body in the normal insects was found to be more than the treated ones up to the end of third week and it gradually decreased. In the case of treated ones there was no decrease in the weight of fat body even after the third week. But the weight became more or less half by the end of the first week. Then it started increasing steadily.

Table: 3. Weight of fat body in the normal and treated insects

Dietary regimen in days	Weight of fat body in mg	
	Normal	Treated
2	30.7 ± 4.21	33.1 ± 4.91
4	32.6 ± 5.41	31.1 ± 3.25
6	36.9 ± 3.18	18.1 ± 3.61
8	39.2 ± 4.15	17.1 ± 2.87
10	47.1 ± 6.84	28.4 ± 3.54
12	46.1 ± 5.71	38.1 ± 4.45
14	46.7 ± 6.62	40.3 ± 4.87
16	48.1 ± 5.21	42.1 ± 5.96
18	52.3 ± 6.81	43.2 ± 4.29
20	54.4 ± 6.13	44.2 ± 6.42
22	61.8 ± 7.54	45.4 ± 5.15
24	53.4 ± 7.26	47.2 ± 6.77
26	47.6 ± 5.02	49.3 ± 5.86
28	46.2 ± 6.16	54.3 ± 5.94

The values are the means of five determinations with standard deviation.

BIOCHEMICAL EFFECTS OF TRYPTOPHAN

Total protein content of the fat body in the normal and treated insects

The content of total protein in the fat body of normal and treated insects are given in table 4 and figure 4:1 to 4:2.

Table 4; Figure 4:1 to 4:2

There was a steady increase of protein content in the fat body for three weeks and then it declined slowly in both normal and treated insects. The protein levels of fat body per insect showed an initial increase for a week and then it maintained a constant level around the mean for the rest of the experimental period in normal insects. But in the case of treated insects, there was an increase in protein content up to the 22nd day. Then it slowly decreased to come close to the mean.

Total free amino acids in fat body in the normal and treated insects

The total free amino acid content of the fat body of normal and treated insects during the experiment is recorded in table 5 and figure 5:1 to 5:2.

Table 5

Figure 5:1 to 5:2

The total free amino acids in the normal insects showed an initial increase, which then maintained more or less a steady rate after four days. In the case of treated insects the initial increase in the total free amino acids was much higher (more than 100%) and then decreased to a very low amount that is about 80% below the mean. Then it maintained more or less a steady rate during the rest of the experimental days.

Table: 4. Total protein content in fat body in the normal and treated insects

Dietary regimen in days	Total protein			
	mg/g fat body		mg/insect	
	Normal	Treated	Normal	Treated
2	25.77 ± 3.20	25.09 ± 4.87	0.66 ± 2.03	0.83 ± 0.09
4	52.96 ± 5.35	57.60 ± 6.03	1.75 ± 0.24	2.09 ± 0.36
6	69.73 ± 7.04	68.85 ± 7.78	2.73 ± 0.37	2.67 ± 0.21
8	76.80 ± 8.35	86.11 ± 8.41	2.76 ± 0.38	3.76 ± 0.48
10	88.33 ± 9.98	88.46 ± 9.58	3.22 ± 0.33	3.65 ± 0.47
12	96.74 ± 9.65	91.24 ± 9.84	3.50 ± 0.44	3.25 ± 0.38
14	105.74 ± 13.01	96.12 ± 9.79	3.41 ± 0.21	2.86 ± 0.38
16	112.56 ± 11.57	101.65 ± 11.84	3.70 ± 0.54	4.69 ± 0.13
18	115.22 ± 11.45	106.26 ± 10.57	3.39 ± 0.55	5.31 ± 0.24
20	121.37± 12.98	112.35 ± 12.25	3.61 ± 0.36	5.87 ± 0.58
22	124.69 ± 13.68	114.08 ± 12.34	3.99 ± 0.36	6.37 ± 0.65
24	121.63 ± 12.76	114.65 ± 12.09	3.88 ± 0.22	5.96 ± 0.85
26	117.86 ± 12.03	118.96 ± 11.85	3.57 ± 0.75	5.44 ± 0.31
28	114.08 ± 13.14	112.83 ± 11.64	3.02 ± 0.24	5.09 ± 0.25
30	101.52 ± 11.84	108.57 ± 21.07	2.80 ± 0.34	4.71 ± 0.28
32	99.33 ± 12.03	105.25 ± 12.61	2.78 ± 0.56	4.43 ± 0.15
34	92.88 ± 11.87	103.25 ± 11.75	2.76 ± 0.25	4.22 ± 0.38
36	83.13 ± 12.05	98.65 ± 11.94	2.88 ± 0.21	3.85 ± 0.30
38	75.71 ± 10.89	95.54 ± 10.43	2.89 ± 0.21	3.63 ± 0.15

The values are the means of five determinations with standard deviation.

Table: 5. Total free amino acids in fat body in the normal and treated insects

Dietary regimen in days	Total free amino acids			
	mg/g fat body		mg/insect	
	Normal	Treated	Normal	Treated
2	3.35 ± 0.45	4.96 ± 0.44	0.086 ± 0.019	0.165 ± 0.019
4	2.95 ± 0.25	4.52 ± 0.41	0.231 ± 0.020	0.175 ± 0.006
6	2.14 ± 0.06	1.55 ± 0.05	0.026 ± 2.004	0.056 ± 0.003
8	1.96 ± 0.08	0.48 ± 0.05	0.078 ± 1.005	0.017 ± 0.001
10	2.10 ± 0.01	0.86 ± 0.30	0.075 ± 2.010	0.028 ± 0.002
12	2.15 ± 0.60	1.56 ± 0.45	0.073 ± 0.008	0.056 ± 0.007
14	2.20 ± 0.23	1.98 ± 0.30	0.071 ± 0.013	0.060 ± 0.001
16	2.28 ± 0.05	1.74 ± 0.04	0.075 ± 0.005	0.064 ± 0.004
18	2.34 ± 0.07	1.62 ± 0.02	0.078 ± 0.008	0.068 ± 0.003
20	2.51 ± 0.04	1.47 ± 0.06	0.082 ± 0.004	0.073 ± 0.005
22	2.58 ± 0.03	1.32 ± 0.02	0.083 ± 0.006	0.074 ± 0.007
24	2.30 ± 0.03	1.71 ± 0.01	0.085 ± 0.003	0.083 ± 0.005
26	2.21 ± 0.07	2.34 ± 0.04	0.085 ± 0.005	0.094 ± 0.006
28	2.05 ± 0.04	2.78 ± 0.02	0.086 ± 0.006	0.100 ± 0.008
30	2.12 ± 0.04	2.51 ± 0.03	0.087 ± 0.012	0.096 ± 0.006
32	2.26 ± 0.08	2.49 ± 0.04	0.092 ± 0.015	0.0 92 ± 0.007
34	2.38 ± 0.05	2.32 ± 0.03	0.095 ± 0.012	0.088 ± 0.021
36	2.49 ± 0.05	2.21 ± 0.02	0.098 ± 0.015	0.085 ± 0.014
38	2.64 ± 0.04	2.13 ± 0.04	0.101 ± 0.015	0.081 ± 0.009

The values are the means of five determinations with standard deviation.

Glucose levels in fat body in the normal and treated insects

The glucose level per mg and per insect in the fat body of the normal and treated insects during the experiment is given in table 6 and figure 6:1 to 6:2.

Table 6

Figure 6:1 to 6:2

The glucose content in the fat body per unit weight showed an initial increase till the tenth day. The glucose content in the treated insects showed a double increase on the second day and then suddenly decreased till the sixth day of the experiment. Then it maintained a consistent level till the end of third week. By the end of fourth week the glucose level per unit weight was found to be very low and had more or less the same amount in both normal and treated insects.

The glucose content per insect showed the same pattern as that of unit weight on the second day. But the glucose content had a gradual decrease in both normal and treated insects until the third week. Then it started slowly rising again.

Urea levels in fat body during dietary regimen

The changes in the urea levels in fat body per unit weight as well as per insect of both normal and experimental insects are given in table 7 and figure 7:1 and 7:2.

Table 7

Figure 7:1 and 7:2

Table: 6. Glucose levels in fat body during dietary regimen

Dietary regimen in days	Glucose			
	mg/g fat body		Mg/insect Treated	
	Normal	Treated	Normal	Treated
2	3.15 ± 0.41	5.90 ± 0.41	0.11 ± 0.02	0.20 ± 0.03
4	3.21 ± 0.43	5.04 ± 0.31	0.10 ± 0.02	0.18 ± 0.02
6	3.24 ± 0.34	3.67 ± 0.37	0.08 ± 0.01	0.13 ± 0.03
8	3.48 ± 0.33	3.58 ± 0.38	0.07 ± 0.01	0.11 ± 0.02
10	3.94 ± 0.37	3.23 ± 0.24	0.06 ± 0.01	0.10 ± 0.01
12	3.24 ± 0.42	3.21 ± 0.41	0.07 ± 0.01	0.10 ± 0.02
14	2.55 ± 0.23	3.16 ± 0.38	0.08 ± 0.02	0.07 ± 0.01
16	2.47 ± 0.31	3.12 ± 0.33	0.07 ± 0.02	0.07 ± 0.02
18	2.26 ± 0.37	3.07 ± 0.31	0.06 ± 0.01	0.06 ± 0.01
20	2.18 ± 0.35	3.06 ± 0.35	0.05 ± 0.02	0.05 ± 0.01
22	1.87 ± 0.31	2.90 ± 0.30	0.04 ± 0.01	0.04 ± 0.01
24	1.67 ± 0.22	1.82 ± 0.23	0.01 ± 0.01	0.02 ± 0.01
26	1.65 ± 0.24	1.77 ± 0.16	0.04 ± 0.01	0.06 ± 0.02
28	1.63 ± 0.23	1.68 ± 0.12	0.05 ± 0.01	0.05 ± 0.01
30	1.44± 0.24	1.55 ± 0.24	0.06 ± 0.01	0.07± 0.01

The values are the means of five determinations with standard deviation.

Table: 7. Urea levels in fat body in the normal and treated insects

Dietary regimen in days	Urea			
	mg/g fat body		mg/insect	
	Normal	Treated	Normal	Treated
2	0.44 ± 0.03	0.59 ± 0.06	0.013 ± 0.002	0.019 ± 0.001
4	0.90 ± 0.11	0.62 ± 0.05	0.038 ± 0.001	0.019 ± 0.002
6	1.59 ± 0.05	1.03 ± 0.03	0.059 ± 0.004	0.019 ± 0.002
8	0.94 ± 0.17	0.88 ± 0.09	0.037 ± 0.003	0.022 ± 0.001
10	0.66 ± 0.13	0.64 ± 0.04	0.030 ± 0.002	0.018 ± 0.002
12	0.45 ± 0.07	0.58 ± 0.01	0.021 ± 0.002	0.015 ± 0.002
14	0.38 ± 0.04	0.20 ± 0.04	0.018 ± 0.003	0.008 ± 0.003
16	0.44 ± 0.03	0.23 ± 0.03	0.022 ± 0.002	0.010 ± 0.001
18	0.50 ± 0.08	0.25 ± 0.01	0.027 ± 0.003	0.010 ± 0.002
20	0.56 ± 0.07	0.28 ± 0.03	0.032 ± 0.002	0.014 ± 0.002
22	0.62 ± 0.06	0.30 ± 0.03	0.038 ± 0.003	0.016 ± 0.001
24	0.74 ± 0.01	0.35 ± 0.04	0.040 ± 0.004	0.017 ± 0.001
26	0.87 ± 0.05	0.39 ± 0.04	0.044 ± 0.005	0.018 ± 0.002
28	0.99 ± 0.09	0.43 ± 0.05	0.045 ± 0.003	0.019 ± 0.001

The values are the means of five determinations with standard deviation.

The normal insects showed much higher urea content than the treated ones in unit weight of fat body as well as per insect. The values per unit weight of fat body showed an increase in urea levels from day one to six. The peak amount was shown on day six in both the cases but the increase in normal ones was much higher than the treated. The difference between the normal and treated on the sixth day was three times. After the sixth day there was decline in the urea levels up to the fourteenth day. Then it steadily increased.

The treated insects showed much lower urea content than the normal ones through out the experimental days.

Creatinine levels in the fat body in the normal and treated insects

The creatinine levels in the fat body of normal as well as treated insects are given in table 8 and figure 8:1 and 8:2.

Table 8

Figure 8:1 and 8:2

The creatinine levels per unit weight as well as per insect in normal and treated ones showed more or less the same pattern. Initially there was a sudden shooting of creatinine at 24 hours followed by a decline to the normal level at 72 hours which remained the same through out the experiment in both normal as well as treated insects.

Hydrogen peroxide levels in fat body in the normal and treated insects

The hydrogen peroxide levels per unit weight of fat body and per insect of both normal and treated insects are given in table 9 and figure 9:1 and 9:2.

Table 9; Figure 9:1 and 9:2

Table: 8. Creatinine in fat body in the normal and treated insects

Dietary regimen in days	Creatinine			
	mg/g fat body		mg/insect	
	Normal (x 10^{-2})	Treated (x10^{-2})	Normal(x10^{-2})	Treated (x10^{-2})
2	52.39 ±7.24	101.59 ±10.15	2.23 ±0.45	3.36± 0.38
4	50.81 ± 8.65	57.54 ±6.13	1.56 ±0.16	1.78 ±0.27
6	39.14 ±3.98	24.99 ±1.65	1.45 ±0.19	0.28 ±0.03
8	20.87 ±1.68	21.03 ±2.21	0.82 ±0.03	0.25 ±0.01
10	16.02 ±1.25	16.97 ±2.01	0.75 ±0.04	0.38 ±0.09
12	12.41 ±2.08	15.64 ±1.95	0.66 ±0.05	0.59 ±0.03
14	12.31 ±1.21	14.70 ±1.87	0.57 ±0.11	0.59 ±0.14
16	10.16 ±1.01	13.87 ±2.23	0.51 ±0.08	0.61 ±0.05
18	9.83 ±1.28	13.62 ±1.01	0.48 ±0.03	0.62 ±0.08
20	9.62 ±1.95	13.47 ±1.45	0.46 ±0.04	0.63 ±0.05
22	9.42 ±1.10	12.26 ±1.58	0.44 ±0.02	0.60 ±0.06
24	8.85 ±1.95	12.19 ±1.65	0.41 ± 0.03	0.61 ±0.08
26	7.31 ±1.20	13.26 ±1.25	0.40 ±0.01	0.66 ±0.06
28	6.61 ±0.60	12.92 ±1.01	0.41 ± 0.03	0.69 ±0.07
30	6.74 ±1.95	11.86 ±1.96	0.45 ±0.03	0.69 ±0.05
32	6.77 ±1.03	10.73 ±1.78	0.48 ±0.05	0.69 ±0.07
34	9.98 ±2.13	11.34 ±1.13	0.52± 0.04	0.71 ±0.09
36	11.83 ±1.97	11.79 ±2.03	0.59 ±0.04	0.74 ±0.06
38	13.569 ±2.25	12.16 ±1.75	0.6.3 ±0.08	0.79 ±0.08

The values are the means of five determinations with standard deviation.

Table: 9. Hydrogen peroxide levels in fat body in the normal and treated insects

Dietary regimen in days	Hydrogen peroxide			
	mg/g fat body		mg/insect	
	Normal	Treated	Normal	Treated
2	1.031 ± 0.02	2.279 ± 0.03	0.032 ± 0.002	0.075 ± 0.008
4	3.665 ± 0.04	5.438 ± 0.04	0.156 ± 0.006	0.168 ± 0.012
6	1.182 ± 0.02	3.487 ± 0.04	0.044 ± 0.008	0.063 ± 0.007
8	1.322 ± 0.02	2.759 ± 0.02	0.052 ± 0.009	0.051 ± 0.006
10	0.756 ± 0.01	1.339 ± 0.01	0.036 ± 0.005	0.038 ± 0.004
12	0.812 ± 0.01	0.597 ± 0.01	0.040 ± 0.009	0.023 ± 0.004
14	0.762 ± 0.01	1.065 ± 0.02	0.036 ± 0.002	0.025 ± 0.003
16	0.695 ± 0.01	0.858 ± 0.02	0.038 ± 0.005	0.022 ± 0.005
18	0.634 ± 0.01	0.543 ± 0.02	0.035 ± 0.005	0.020 ± 0.004
20	0.563 ± 0.01	0.392 ± 0.01	0.035 ± 0.007	0.018 ± 0.002
22	0.543 ± 0.02	0.525 ± 0.02	0.034 ± 0.008	0.024 ± 0.003
24	0.597 ± 0.01	0.595 ± 0.01	0.034 ± 0.004	0.026 ± 0.002
26	0.624 ± 0.02	0.621 ± 0.01	0.033 ± 0.005	0.032 ± 0.003
28	0.699 ± 0.02	0.671 ± 0.01	0.032 ± 0.002	0.036 ± 0.002

The values are the means of five determinations with standard deviation.

On the basis of per insect weight of fat body of normal insects, the level of hydrogen peroxide increased from day one and reached its peak on day four followed by a sudden decline on day six. When the hydrogen peroxide levels per insect was considered it showed the same pattern in the case of normal as well as treated insects. The hydrogen peroxide levels in the treated insects per unit weight and per insect showed the same results, but in the case of unit weight of fat body showed two to three fold increase initially than the normal insects.

Catalase activity in fat body in the normal and treated insects

The activity of enzyme catalase per unit fresh tissue and per insect were estimated in the fat body of both normal and treated insects and are displayed in table 10 and figure 10:1 and 10:2.

Table 10

Figure 10:1 and 10:2

The enzyme activity was very high from day one to four. The peak activity per insect was on day four in both normal and treated insects. There was a two fold increase in the enzyme activity two times on day four when we consider the whole insect in both normal as well as treated ones. But in the case of unit weight of fat body the enzyme activity was at its peak in the treated insects on day six and it was double than that of the normal insects. The enzyme activity became consistent from sixth day.

Table: 10. Catalase activity in fat body in the normal and treated insects

Dietary regimen in days	Catalase activity			
	mg/min/g fat body		mg/min/insect	
	Normal	Treated	Normal	Treated
2	14.91 ± 1.21	11.85 ± 1.24	0.45 ± 0.03	0.39 ± 0.04
4	19.25 ± 2.02	25.21 ± 2.08	0.82 ± 1.51	0.78 ± 0.05
6	11.04 ± 1.05	28.19 ± 3.87	0.40 ± 0.03	0.51 ± 0.07
8	10.73 ± 1.04	23.35 ± 2.98	0.42 ± 0.02	0.39 ± 0.08
10	9.97 ± 0.21	14.13 ± 1.03	0.42 ± 0.05	0.40 ± 0.03
12	9.19 ± 1.05	10.98 ± 1.41	0.42 ± 0.09	0.42 ± 0.04
14	8.79 + 1.04	10.41 ± 1.65	0.41 ± 0..05	0.42 ± 0.04
16	7.68 ± 1.04	10.23 ± 1.07	0.41 ± 0.05	0.42 ± 0.04
20	6.98 ± 0.93	9.85 ± 1.69	0.39 ± 0.04	0.42 ± 0.09
22	6.25 ± 0.89	9.12 ± 1.51	0.39 ± 0.08	0.42 ± 0.03
24	6.16 ± 0.76	7.71 ± 0.92	0.39 ± 0.03	0.41 ± 0.06
26	5.89 ± 0.89	7.22 ± 0.81	0.32 ± 0.04	0.36 ± 0.03
28	5.93 ± 0.65	6.37 ± 0.72	0.29 ± 0.05	0.31 ± 0.05
30	5.67 ± 1.23	4.97 ± 0.58	0.26 ± 0.02	0.27 ± 0.02

The values are the means of five determinations with standard deviation.

Alanine aminotrasferase activity in fat body in the normal and treated insects

The of alanine aminotransferase (AIAT) activity in fresh tissue of fat body per unit weight and per insect of both normal and treated insects are presented in table 11 and figure 11:1 and 11:2.

Table 11

Figure 11:1 and 11:2.

The alanine aminotransferase activity showed a concave curve. On the second day of the experiment the enzyme activity in fat body was four to five times higher than the mean. Then it suddenly declined to the end of second week to maintain a consistent level till the end of the experiment. The initial enzyme activity in the treated insect showed a much higher rate than the normal ones.

Aspartate aminotrasferase activity in fat body in the normal and treated insects

Aspartate aminotrasferase (AsAT) activity in the fat body per unit weight and per insect is given in table 12 and figure 12:1 and 12:2.

Table 12

Figure 12:1 and 12:2

The aspartate aminotrasferase activity was found to be around the mean in the case of normal insects when the weight per insectwas considered. But in the case of treated insects the enzyme activity showed a gradual and steady increase after the first week of the experiment. The increase in the activity in the treated insects at the end of the experiment was found to be about six times higher than the normal ones.

Table: 11. Alanine aminotranferase activity in fat body in the normal and treated insects

Dietary regimen In days	Alanine aminotransferase activity			
	mg/g fat body		mg/insect	
	Normal ($x10^{-3}$)	Treated($x10^{-3}$)	Normal ($x10^{-3}$)	Treated ($x10^{-3}$)
2	117.71 ± 12.02	168.85 ± 12.04	3.006 ± 0.031	5.619 ± 0.061
4	113.52 ± 13.06	77.81 ± 7.02	3.755 ± 0.041	2.775 ± 0.032
6	112.77 ± 11.03	59.69 ± 6.05	2.623 ± 0.032	2.369 ± 0.021
8	73.77 ± 8.0 1	42.45 ± 4.01	2.582 ± 0.022	1.983 ± 0.014
10	65.14 ± 7.01	27.18 ± 3.04	1.048 ± 0.023	0.950 ± 0.078
12	40.69 ± 5.87	19.62 ± 2.35	0.858 ± 0.016	0.809 ± 0.008
14	32.68 ± 4.43	19.58 ± 3.20	0.836 ± 0.021	0.713 ± 0.065
16	16.34 ± 2.16	15.98 ± 2.96	0.812 ± 0.018	0.092 ± 0.009
18	15.43 ± 2.76	18.77 ± 4.23	0.784 ± 0.008	0.071 ± 0.008
20	15.89 ± 2.02	17.97 ± 1.96	0.643 ± 0.145	0.103 ± 0.021
22	15.93 ± 2.42	18.69 ± 2.03	0.565 ± 0.064	0.319 ± 0.061
24	17.83 ± 3.65	18.03 ± 3.05	0.558 ± 0.058	0.554 ± 0.061
26	18.44 ± 2.02	17.98 ± 2.68	0.531 ± 0.048	0.564 ± 0.045
28	20.49 ± 3.65	17.95 ± 2.14	0.516 ± 0.067	0.576 ± 0.062
30	19.88 ± 2.21	17.89 ± 3.03	0.514 ± 0.104	0.596 ± 0.101
32	18.73 ± 2.07	17.81 ± 2.05	0.511 ± 0.071	0.614 ± 0.062
34	17.64 ± 2.12	17.59 ± 2.21	0.521 ± 0.078	0.631 ± 0.071
36	16.37 ± 2.04	17.32 ± 3.08	0.523 ± 0.051	0.646 ± 0.087
38	15.69 ± 2.987	17.77 ± 3.23	0.524 ± 0.042	0.676 ± 0.051

The values are the means of five determinations with standard deviation.

Table: 12. Aspartate aminotransferase activity in fat body in the normal and treated insects

Dietary regimen In days	Aspartate aminotransferase activity			
	mg/g fat body		mg/insect	
	Normal ($x10^{-6}$)	Treated ($x10^{-6}$)	Normal ($x10^{-6}$)	Treated ($x10^{-6}$)
2	149.6 ± 15.96	250.0 ± 30.61	4.59 ± 0.30	8.26 ± 1.20
4	113.0 ± 12.23	321.6 ± 42.03	4.81 ± 0.92	12.24 ± 1.65
6	221.5 ± 30.90	159.6 ± 21.23	8.18 ± 0.80	2.88 ± 0.21
8	172.0 ± 20.01	56.3 ± 60.13	6.73 ± 1.01	0.96 ± 0.16
10	61.3 ± 8.21	67.1 ± 71.56	2.88 ± 0.36	15.70 ± 1.25
12	732.6 ± 70.10	380.3 ± 41.01	3.37 ± 0.32	14.43 ± 1.03
14	613.2 ± 68.13	430.4 ± 50.04	3.36 ± 0.461	17.32 ± 2.65
16	531.4 ± 51.23	571.8 ± 67.21	3.31 ± 0.432	25.31 ± 3.31
18	421.2 ± 50.32	724.3 ± 83.31	3.36 ± 0.324	31.78 ± 4.23
20	211.4 ± 31.03	871.5 ± 90.24	3.37 ± 1.241	39.14 ± 4.91
22	62.3 ± 8.23	1038.4 ± 81.05	3.84 ± 0.063	47.15 ± 5.31
24	113.6 ± 11.04	1071.1 ± 98.20	5.23 ± 0.710	51.63 ± 7.01
26	167.8 ± 20.36	1103.7 ± 91.04	7.89 ± 0.213	56.11 ± 6.10
28	214.4 ± 24.20	1135.1 ± 89.25	9.91 ± 0.95	65.92 ± 5.05

The values are the means of five determinations with standard deviation.

BIOCHEMICAL ANALYSES OF *IPHITA LIMBATA* DURING ACTIVE AND DIAPAUSE STAGES

Weight of insects and their fat body

The fresh weight of the insects and their fat body for a period of one year is recorded in table 13 and figure 13a, 13b and 13c.

Table 13

Figure 13a, 13b and 13c

Table: 13. Weight of insect and its fat body during the active and diapause stages

	Weight in mg.	
Month	Weight/insect	Fat body/insect
June	209.74 ± 25.38	38.12 ± 2.82
July	198.52 ± 26.91	37.36 ± 3.51
August	194.35 ± 10.34	32.52 ± 4.03
September	189.63 ± 18.92	33.48 ± 3.18
October	188.28 ± 19.15	30.64 ± 4.52
November	188.92 ± 21.87	27.53 ± 2.97
December	189.65 ± 19.98	19.32 ± 1.43
January	187.76 ± 10.51	12.66 ± 1.17
February	185.37 ± 23.79	07.25 ± 0.82
March	190.25 ± 31.82	11.47 ± 2.56
April	196.51 ± 20.62	19.73 ± 3.41
May	204.45 ± 19.85	28.23 ± 3.32
June	209.34 ± 18.32	36.54 ± 3.11

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of February.

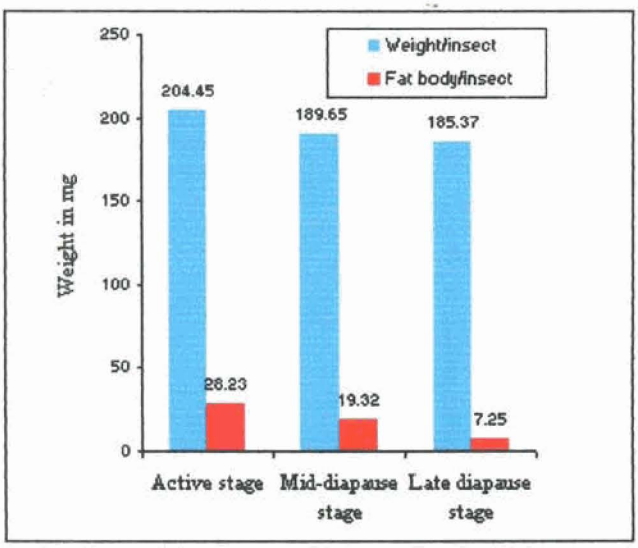


Figure: 13c. Weight of insect and its fat body during the active and diapause stages

The fresh weight of the insect was maximum during the active months and it gradually declined during diapause. The weight was least in February and maximum in June. The reduction in weight was 11.48 %.

The weight of fat body was found to be maximum in June and there was gradual decrease during the months of diapause. By December there was 50 % decrease of fat body and in February it was found to be least with a decrease of 81.58%. During the post diapause period the amount of fat body increased steadily.

Total protein levels during the active and diapause stages

The amount of total protein in the haemolymph of insects during active and mid-diapause stages is given in figure 14a.

Figure 14a

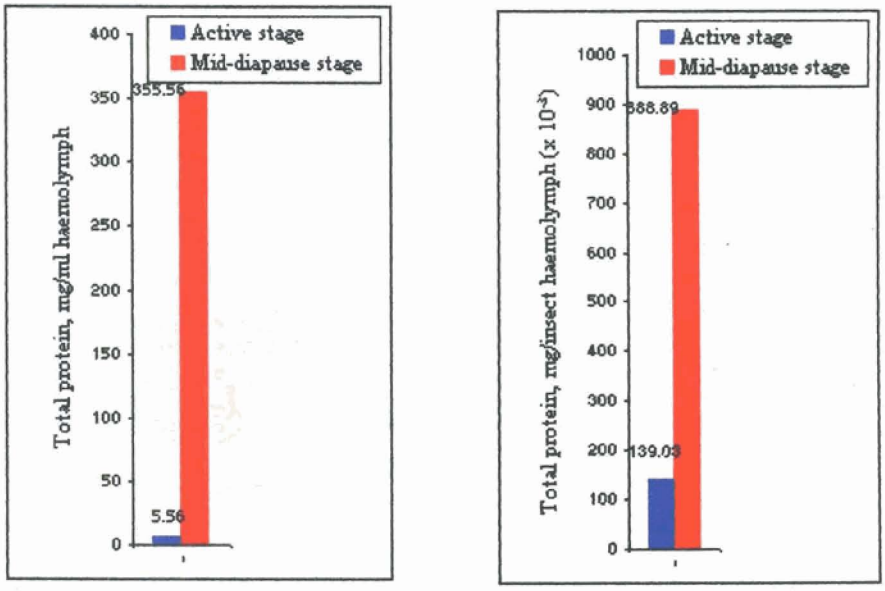


Figure: 14a. Total protein in haemolymph during active and diapause stages.

The amount of total protein determined in fat body during active and diapause stages of insects are given in table 14 and figure 14b and 14c:1 and 14c:2.

Table 14

Figure 14b

Figure 14c:1 and 14c:2

The levels of protein in the fat body and haemolymph were high during the active period and it was reduced to half during diapause period.

Table: 14. Total protein content of fat body during active and diapause stages

Month	Total protein	
	mg/g fat body	mg/ insect
April	47.66 ± 5.32	2.07 ± 0.39
May	69.44 ± 8.11	1.95 ± 0.26
September	54.56 ± 7.64	1.8 ± 0.35
October	37.12 ± 5.75	1.06 ± 0.22
November	19.14 ± 3.66	0.45 ± 0.063
December	25.88 ± 4.28	0.78 ± 0.094
January	35.68 ± 6.05	0.72 ± 0.087
February	72.07 ± 9.57	0.67 ± 0.091

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of February.

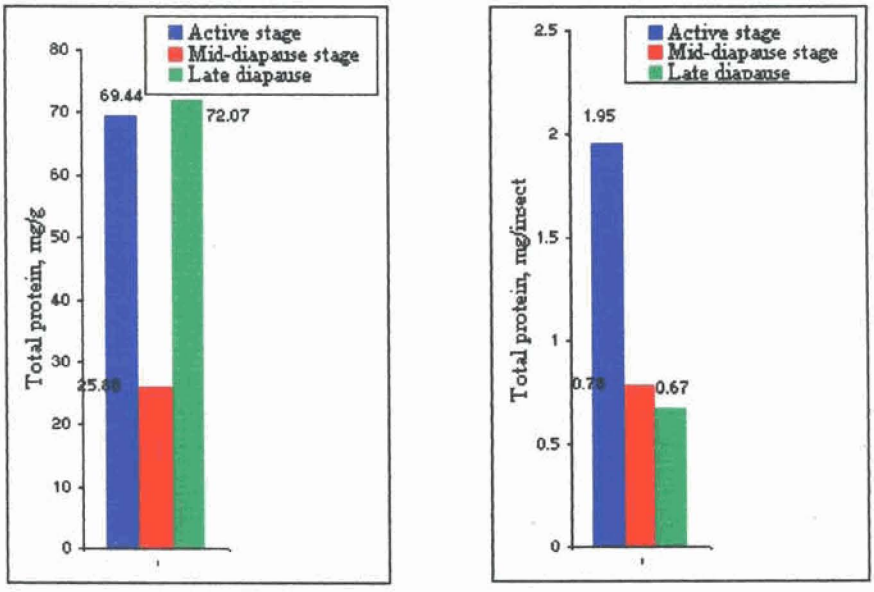


Figure: 14b. Total protein in fat body during active and diapause stages.

Total free amino acids in the haemolymph of *I. limbata* during the active and diapause stages

The amount of total free amino acids in the haemolymph of *I. limbata* during the active and diapause stages are given in figure 15a.

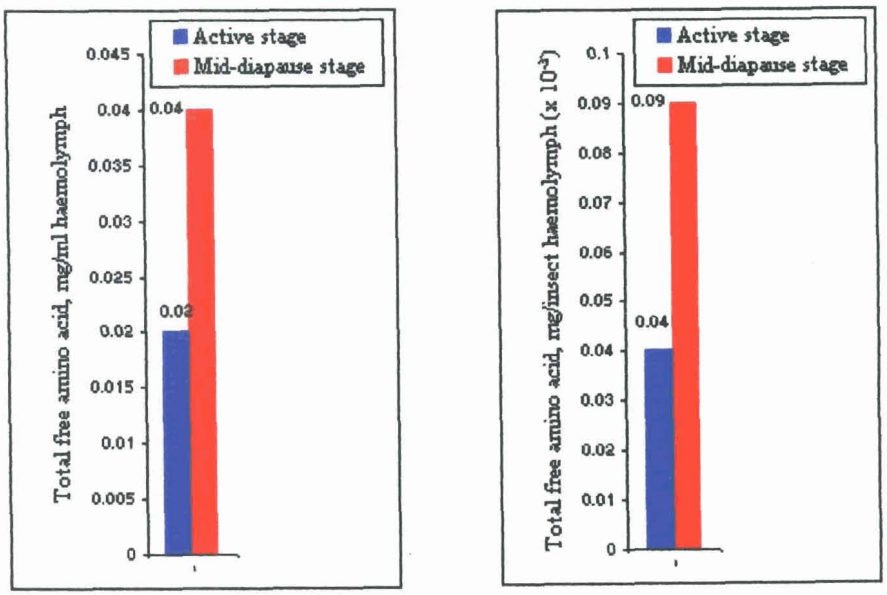


Figure: 15a. Total free amino acids in haemolymph during active and diapause stages.

The amount of total free amino acids during the active stage was found to be 16.15µg/ml and 0.04µg/insect. During diapause the total free amino acids in the haemolymph was almost doubled recording 35.42 µg/ml and 0.09µg/insect.

Total free amino acids in fat body during the active and diapause stages

The total free amino acids in fat body during the active and diapause periods were determined. The values are given in table 15 and figure 15b:1; 15b:2 and 15c.

Table 15

Figure 15b:1 and 15b:2

Figure 15c

Table: 15. Total free amino acids in fat body during active and diapause stages

Month	Total free amino acid	
	mg/g fat body	mg/insect
May	5.66 ± 0.61	0.15 + 0.026
September	46.48 ± 6.42	1.53 ± 0.191
October	24.79 ± 2.97	0.84 ± 0.095
November	10.64 ± 1.86	0.25 ± 0.037
December	8.43 ± 0.94	0.25 ± 0.042
January	10.29 ± 1.53	0.21 ± 0.031
February	12.15 ± 1.87	0.11 ± 0.024

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of February.

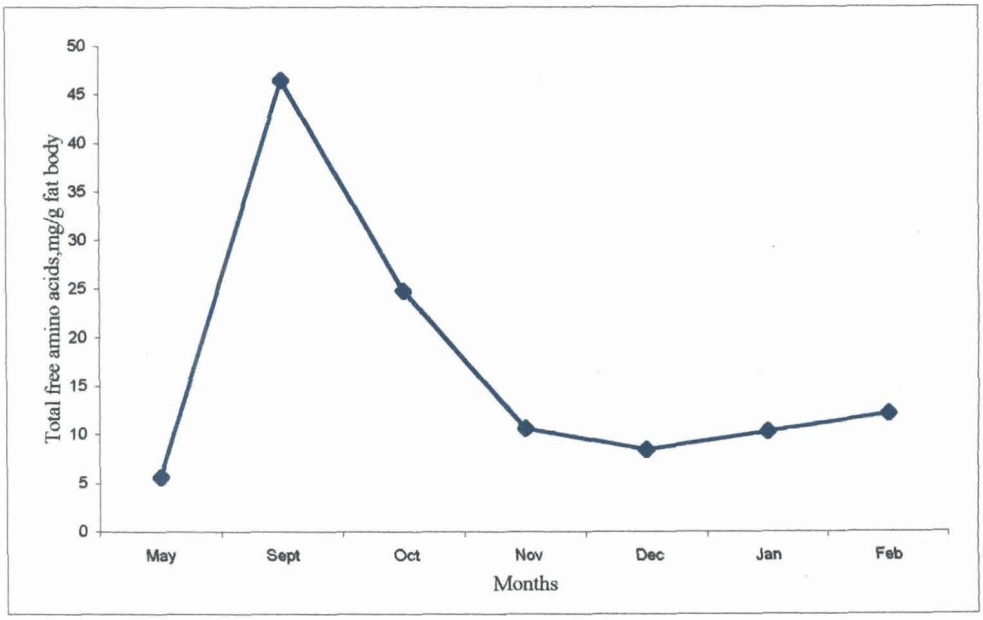


Figure 15b:1. Total free amino acids in fat body during active and diapause stages

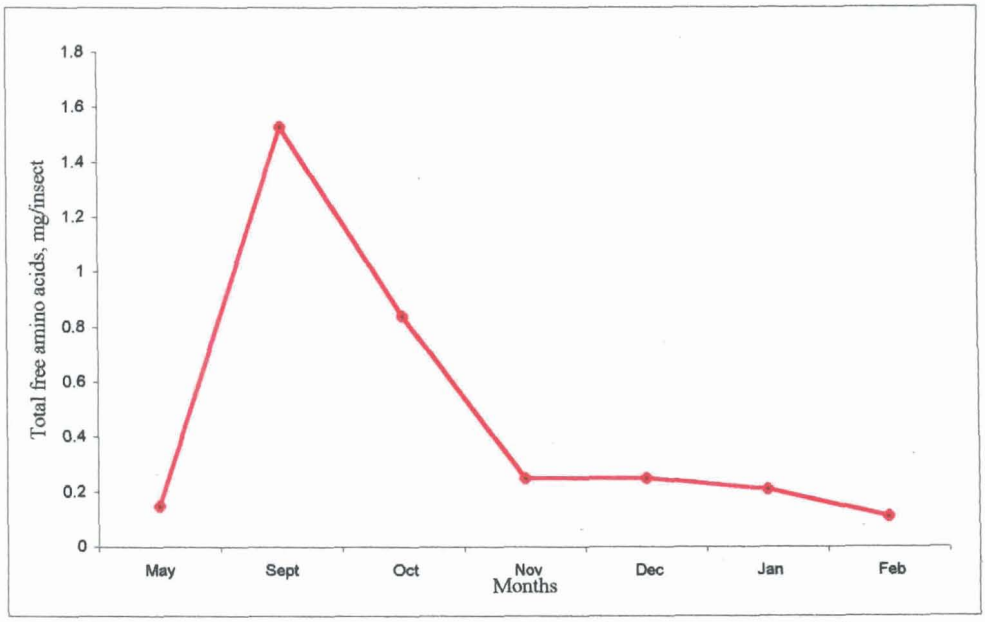


Figure 15b:2. Total free amino acids in fat body during active and diapause stages

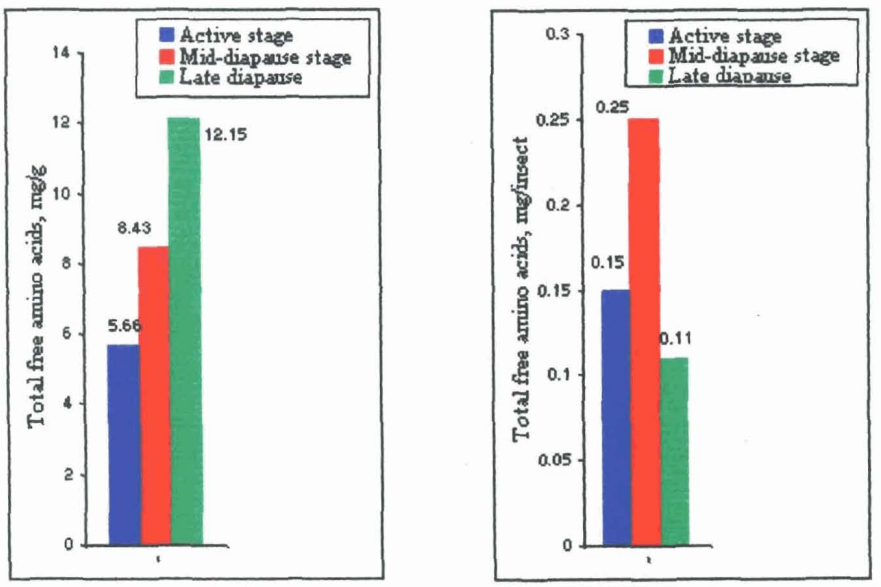


Figure: 15c. Total free amino acids in fat body during active and diapause stages.

The titre of total free amino acids during the active stage was much low when compared to diapuase stage on the basis of unit weight and total tissue. As per insect during the early diapause stage the amount was nearly nine times higher than the active stage. Then it declined gradually by the end of diapause. But as per unit weight it always maintained a higher level than the active stage.

Individual free amino acids in the haemolymph of *I. limbata* during the active and diapause stages

The composition of each amino acid as percent of total free amino acids in the haemolymph of *I. limbata* during diapause stage and active stage is given in table 16 and figure 16a, 16b and 16c:1 to 16:16.

Table 16

Figure 16a, 16b and 16c:1 to 16c:16

Table: 16. Composition of each amino acid as percent of total amino acids in the haemolymph of *I. limbata*

	Active	Mid-diapause	Late diapause
Amino acid	May	December	February
Glutamic acid	27.51	14.55	20.47
Alanine	18.68	8.90	12.92
Valine	8.41	7.61	6.58
Glycine	7.56	7.01	6.98
Serine	5.53	12.47	9.02
Leucine	3.68	7.78	6.54
Arginine	3.30	3.8	0.82
Proline	3.27	1.42	3.27
Aspartic acid	2.92	8.12	6.33
Isoleucine	2.02	5.23	3.50
Threonine	1.53	4.83	3.78
Tyrosine	1.35	1.46	1.37
Phenylalanine	1.09	4.38	4.46
Methionine	1.03	0.85	0.57
Lysine	1.02	3.83	3.06
Histidine	0.21	0.15	0.42
Total	89.11	92.39	90.55

The experiments were conducted from May 2002 to February 2003. Diapause starts at the end of June and ends by the end of February.

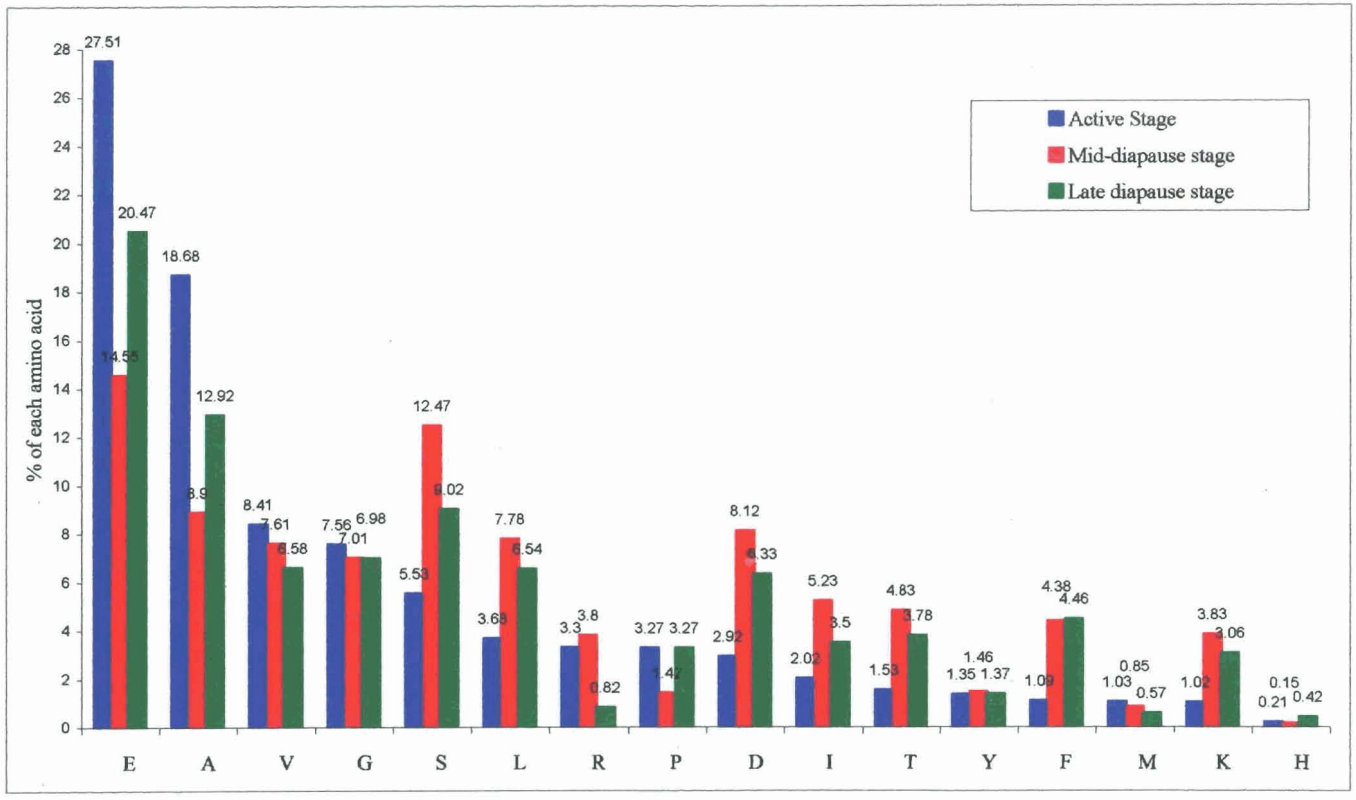


Figure: 16a. Composition of each amino acid as percent of total amino acids in the haemolymph of *I. limbata.*

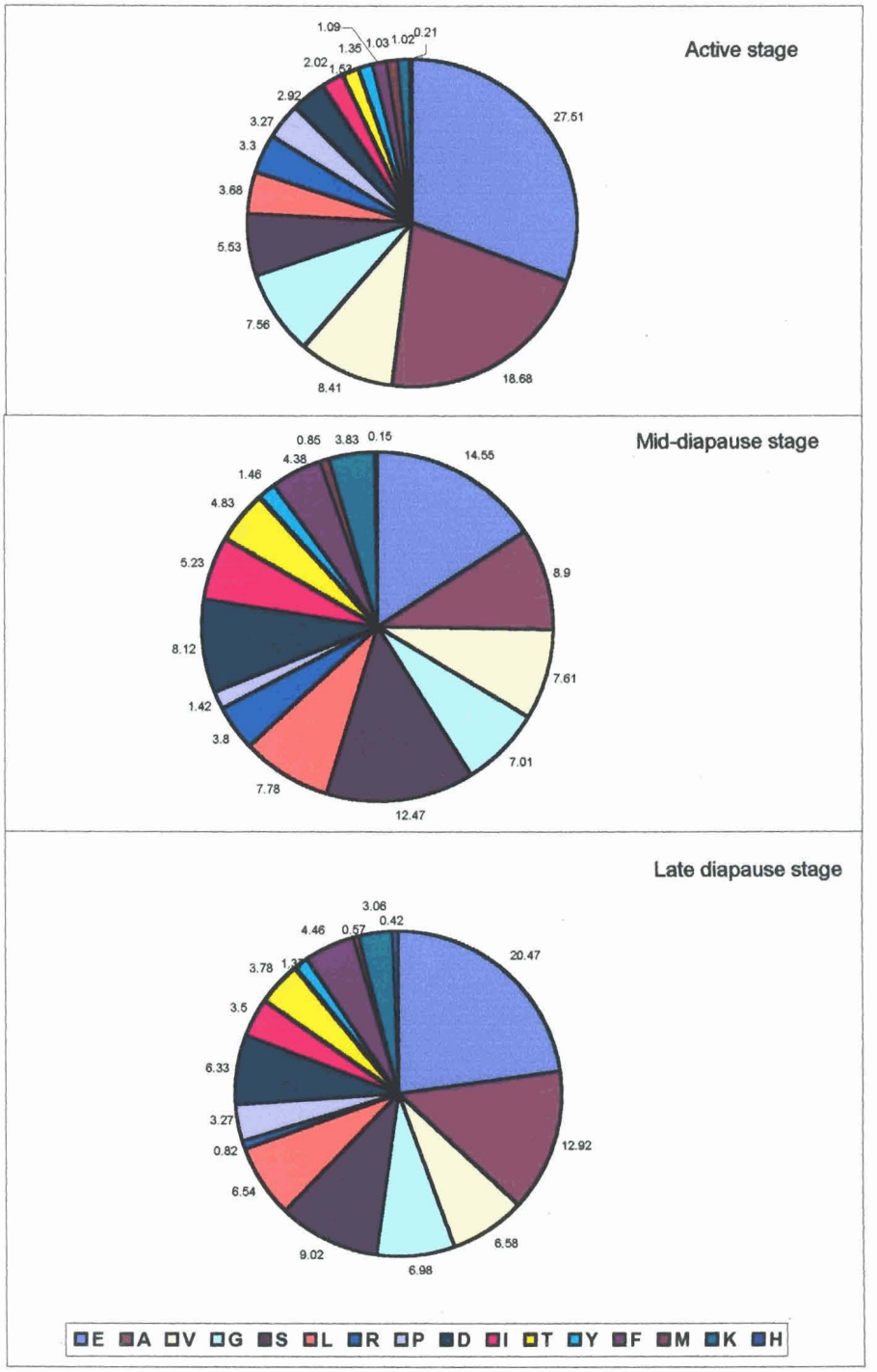
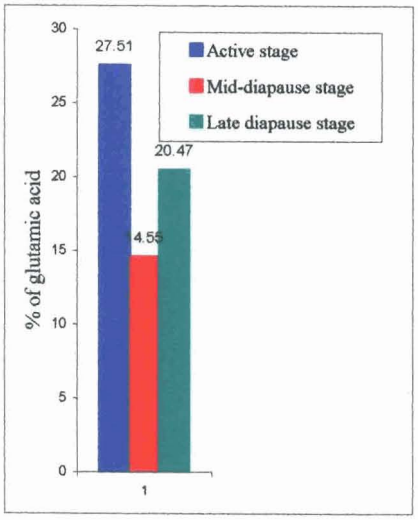
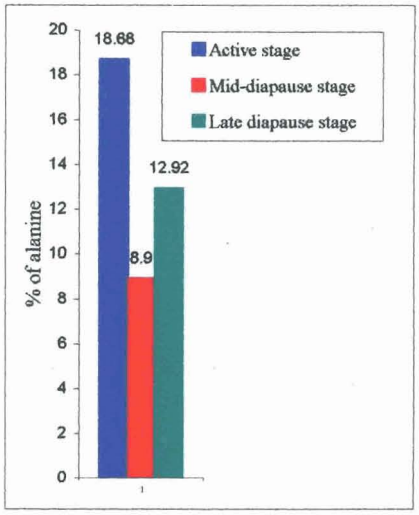


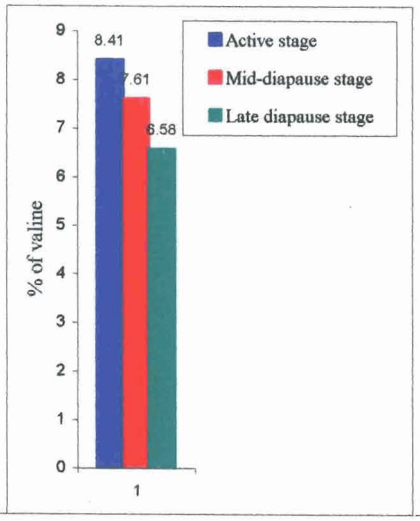
Figure 16b. Composition of each amino acid as percent of total free amino acids in the haemolymph of *I. limbata* during active, mid-diapause and late diapause stages.



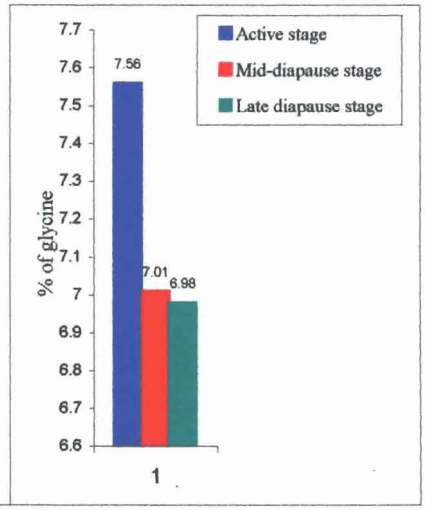
1. Glutamic acid



2. Alanine

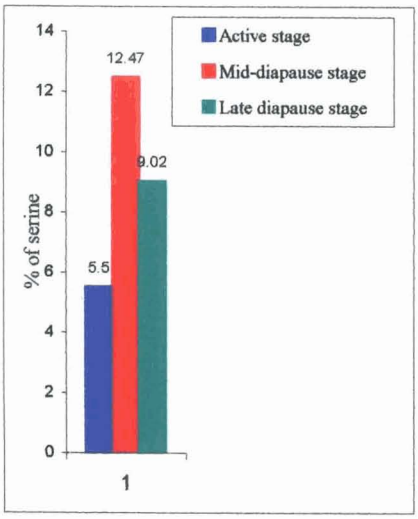


3. Valine

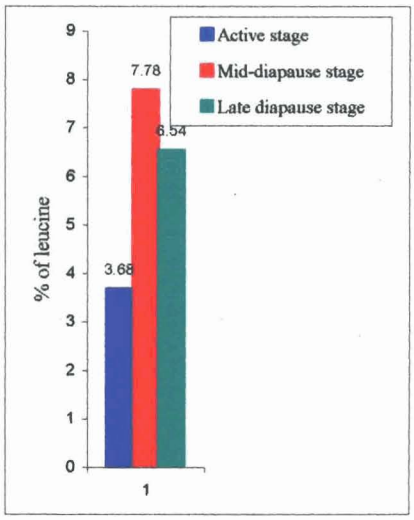


4. Glycine

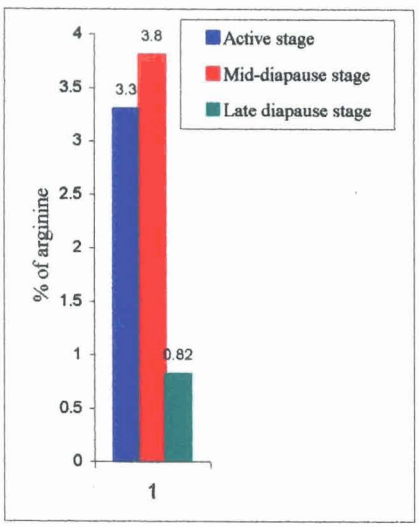
Figure 16c: 1-4. Percentage of individual amino acids in the haemolymph of *I. limbata.*



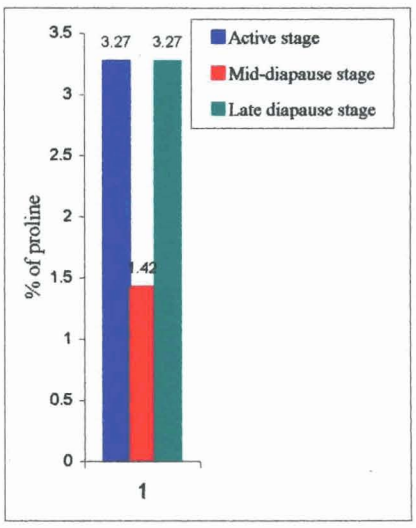
5. Serine



6. Leucine

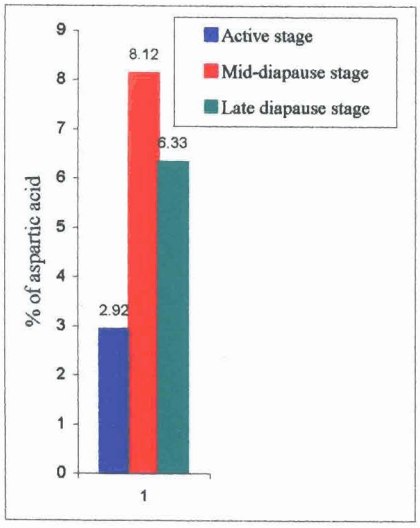


7. Arginine

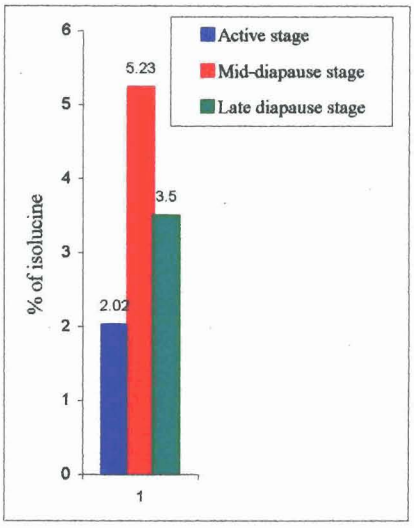


8. Proline

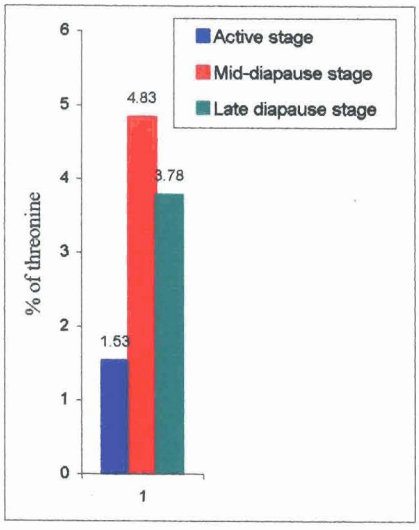
Figure 16c: 5-8. Percentage of individual amino acids in the haemolymph of *I. limbata.*



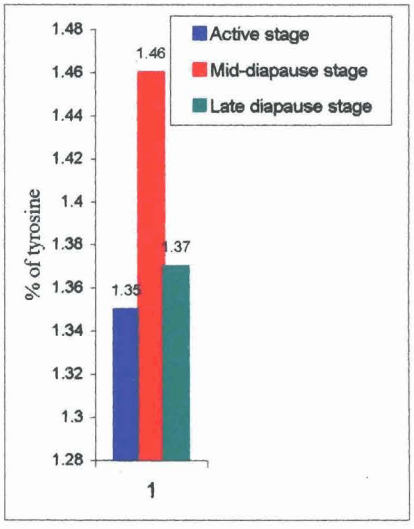
9. Aspartic acid



10. Isoleucine

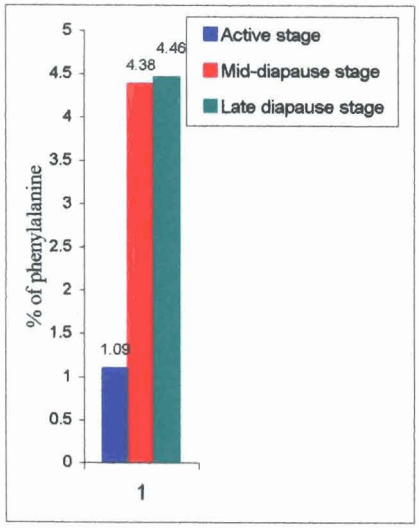


11. Threonine

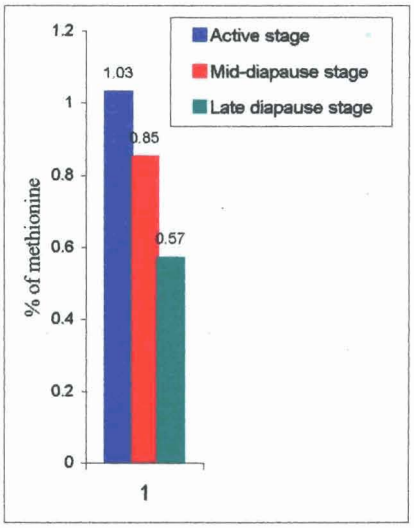


12. Tyrosine

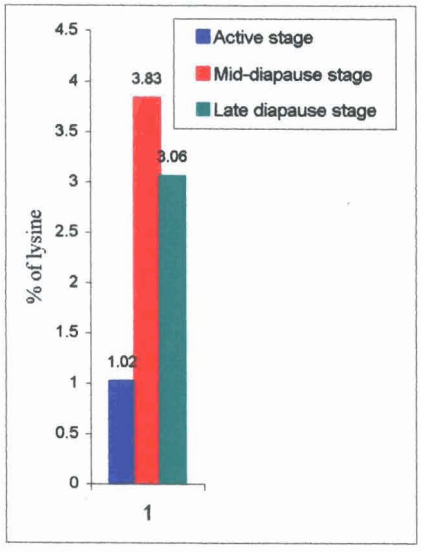
Figure 16c:9-12. Percentage of individual amino acids in the haemolymph of *I. limbata.*



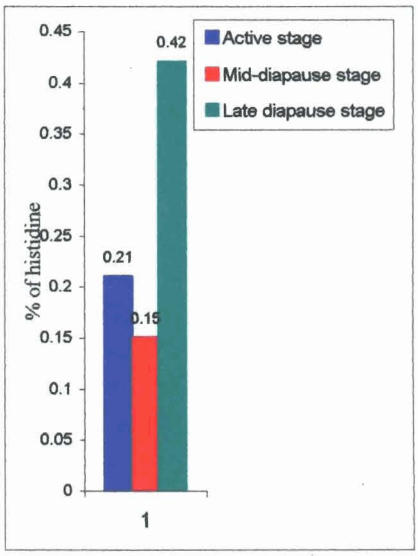
13. Phenylalanine



14. Methionine



15. Lysine



16. Histidine

Figure 16c:13-16. Percentage of individual amino acids in the haemolymph of *I. limbata*

A total of sixteen free amino acids were separated from the haemolymph and the composition of each of them was determined. The following are the amino acids identified – glutamic acid, alanine, valine, glycine, serine, leucine, arginine, proline, aspartic acid, isoleucine, threonine, tyrosine, phenylalanine, methionine, lysine and histidine. The above list is given in their decreasing order in the case of active insects.

Glutamic acid is found to be the most abundant with 27.51% of the total free amino acids. This is followed by alanine with 18.68%. The percent of histidine is noted as the least. The composition of free amino acids in the haemolymph during the diapause stage is found to be much different. The percent of glutamic acid, alanine and proline are reduced to more or less half the amount during mid-diapause. But an increase was noted in the case of proline, glutamic acid and alanine by the end of diapause. There is much increase in the case of serine, leucine, aspartic acid, isoleucine, threonine, phenylalanine, histidine and lysine during diapause. The phenylalanine content during diapause stage is four times higher than that of active stage and lysine content is more than three times higher than that of the active ones. There is not much variation in the amount of valine, glycine, tyrosine and methionine between the active and diapause stages. Arginine which remained more or less constant till mid-diapause was reduced to about one fourth by the end of diapause.

Glucose levels in haemolymph during active and diapause stages

The variations in the amount of glucose in haemolymph during the active and diapause stages are given in figure 17a.

Figure 17a

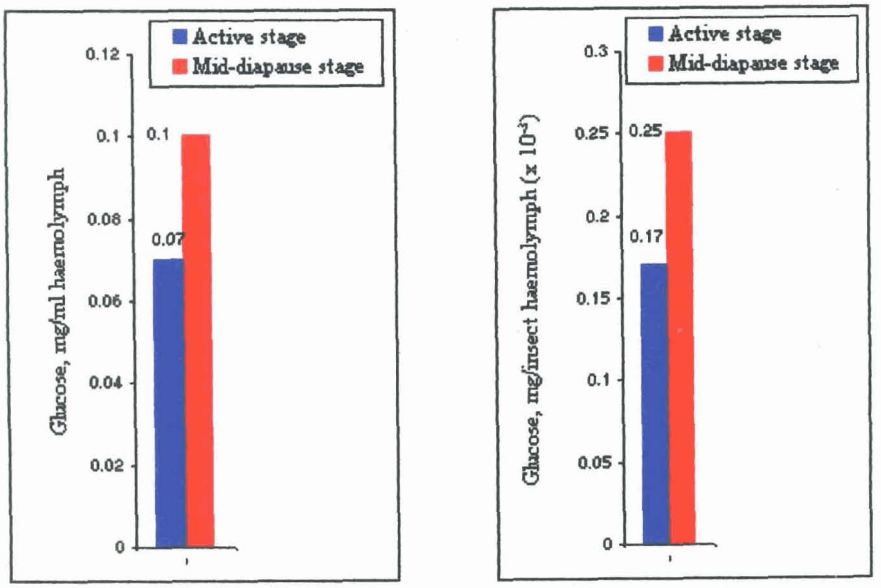


Figure: 17a. Glucose in haemolymph during active and diapause stages.

In the case of haemolymph the titre value showed low glucose level during active stage and a higher amount during diapause stage.

Glucose levels in fat body during active and diapause stages

Glucose levels in fat body during active and diapause stages are recorded in table 17, figure 17b and 17c:1 and 17c:2.

Table 17

Figure 17b

Figure 17c:1 and 17c:2

Table: 17. Glucose content in fat body during the active and diapause stages

Month	Glucose	
	mg/g fat body	mg/insect
May	2.53 ± 0.28	0.071 ± 0.014
September	1.18 ± 0.15	0.028 ± 0.003
October	0.87 ± 0.11	0.017 ± 0.001
November	0.55 ± 0.07	0.013 ± 0.002
December	0.61± 0.09	0.018 ± 0.003
January	0.67 ± 0.08	0.014 ± 0.002
February	1.01 ± 0.12	0.009 ± 0.001

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of February.

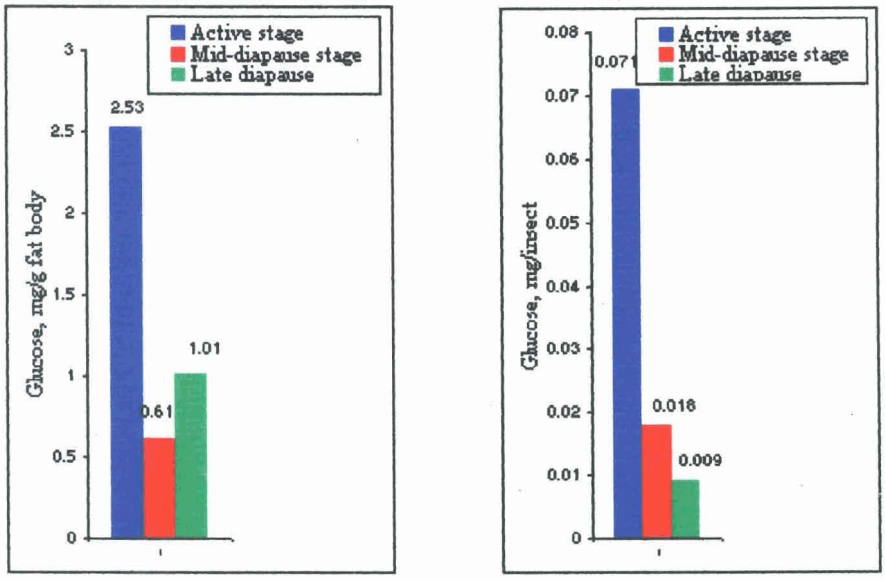


Figure: 17b. Glucose in fat body during active and diapause stages.

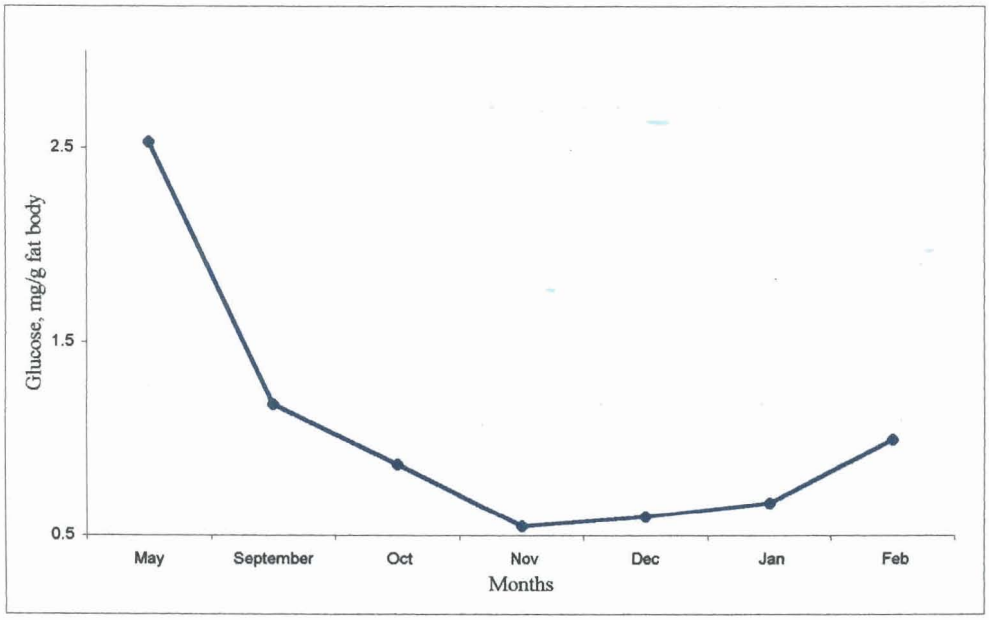


Figure 17c:1.Glucose content of fat body during active and diapause stages

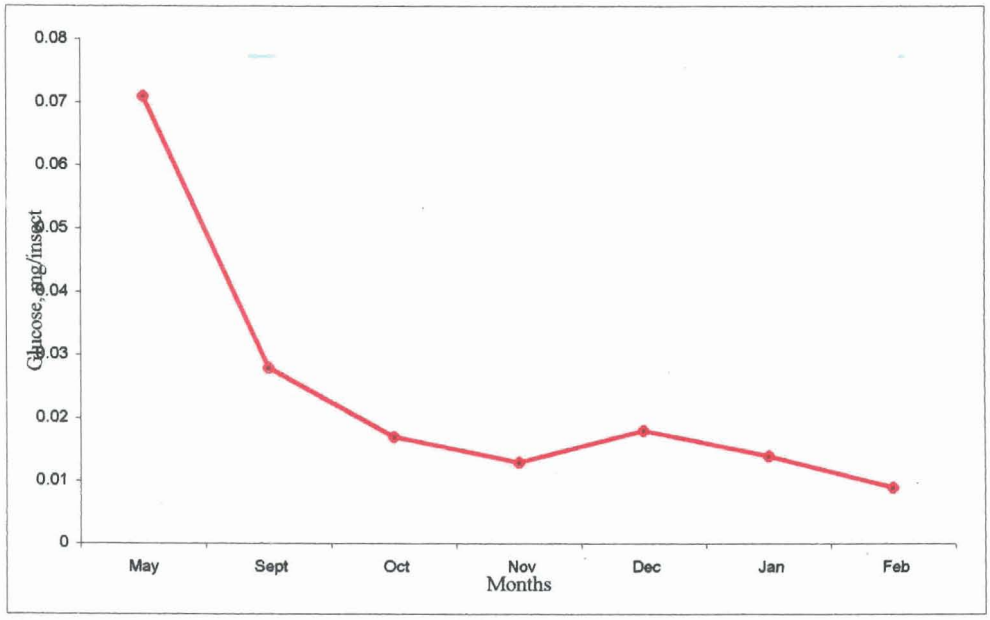


Figure 17c.2. Glucose content in fat body during active and diapause stages

The amount of glucose in fat body during diapause stage is found to be negligible. But the active stage had considerable amount of glucose.

Urea levels in haemolymph during active and diapause stages

The changes in the urea levels in haemolymph during active and diapause stages are recorded in figure 18a.

Figure 18a

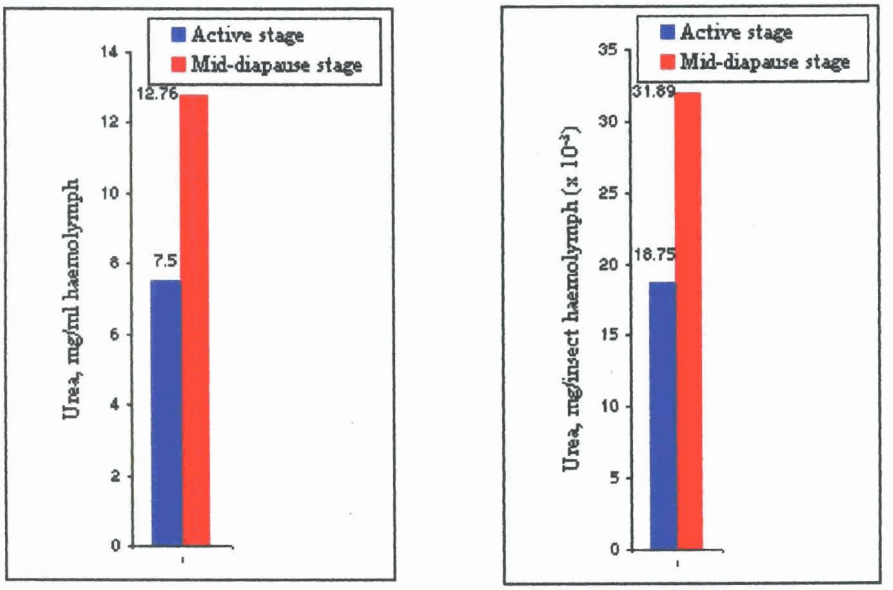


Figure:18a. Urea in haemolymph during active and diapause stages.

In the case of haemolymph the diapause stage had a two third increase of urea than the active stage.

Urea levels in fat body during active and diapause stages

The changes in the urea levels in fat body during active and diapause stages are given in table 18, figure 18b and 18c.

Table 18

Figure 18b:1 and 18b:2

Figure 18c

Table: 18. Urea levels in fat body during the active and diapause stages

Month	Urea	
	mg/g fat body	mg/insect
May	0.834 ± 0.094	0.036 ± 0.005
September	0.909 ± 0.102	0.033 ± 0.004
October	0.854 ± 0.088	0.026 ± 0.003
November	0.801 ± 0.091	0.019 ± 0.002
December	0.392 ± 0.045	0.012 ± 0.002
January	0.416 ± 0.049	0.009 ± 0.001
February	0.523 ± 0.062	0.009 ± 0.001

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

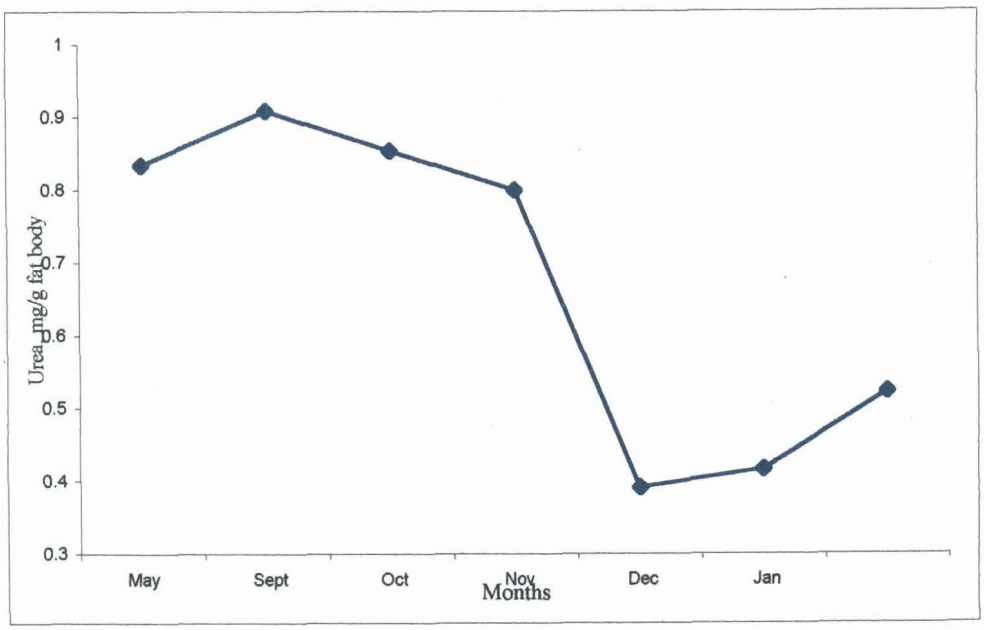


Figure 18b:1. Urea content in in fat body during active and diapause stages

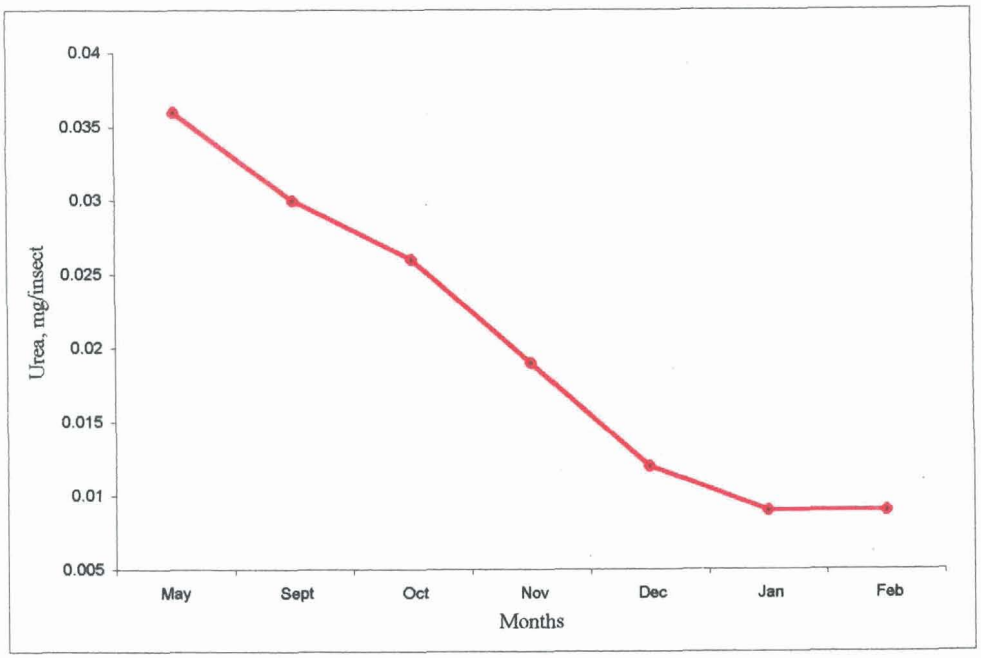


Figure 18b:2. Urea content in in fat body during active and diapause stages

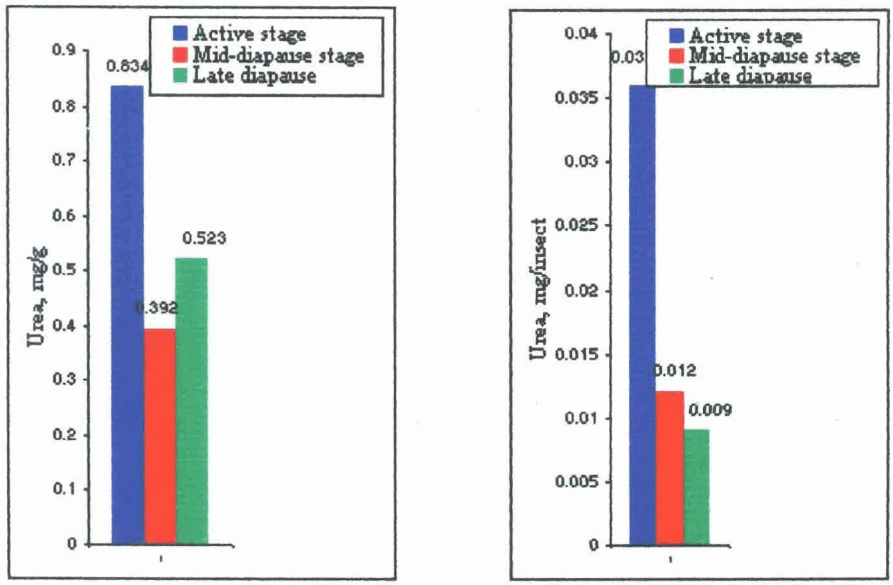


Figure:18c. Urea in fat body during active and diapause stages.

The titre values of urea in fat body depicted a gradual decrease from a higher active stage to a lower diapause stage. During the active stage the urea levels in fat body were double than that of diapause stage.

Craetinine levels in haemolymph during the active and diapause stages

The changes in the creatinine content in haemolymph during active and diapause stages are given in figure19a.

Figure19a

The haemolymph during diapause stage had a considerable higher amount of craetinine.

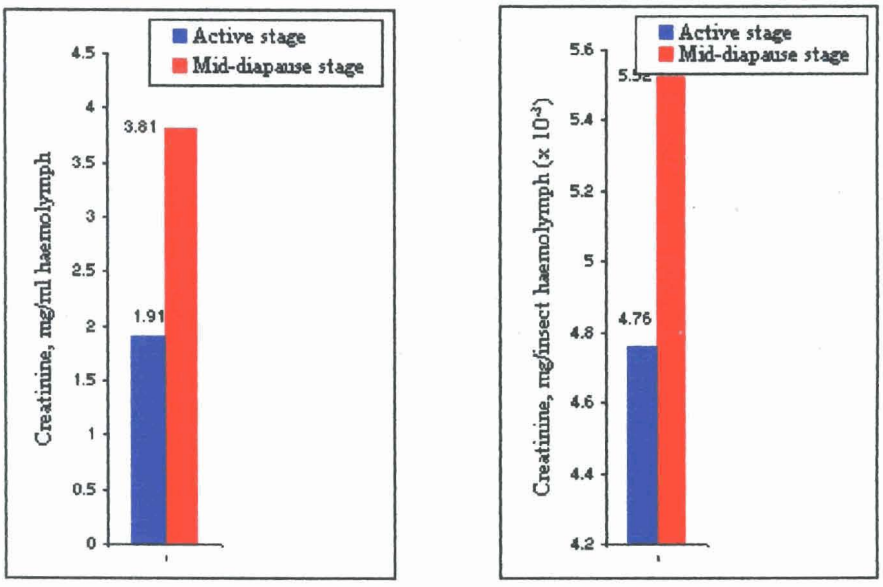


Figure:19a. Creatinine in haemolymph during active and diapause stages.

Craetinine levels in fat body during the active and diapause insects

The changes in the creatinine content in fat body during active and diapause stages are given in table 19, figure 19b and 19c.

Table 19

Figure 19b

Figure 19c:1 and 19c:2

The amount of creatinine in fat body during the diapause stage is found to be only one tenth of the active stage.

Table: 19. Creatinine content in fat body during the active and diapause stages

	Creatinine	
Month	mg/g fat body (x 10^{-2})	mg/insect (x 10^{-2})
May	143.8 ± 11.2	4.23 ± 0.052
September	94.8 ± 10.3	3.11 ± 0.037
October	54.7 ± 6.7	1.94 ± 0.025
November	17.5 ± 1.8	0.34 ± 0.038
December	16.7 ± 1.9	0.33 ± 0.042
January	22.9 ± 2.6	0.52 ± 0.064
February	38.6 ± 4.2	0.43 ± 0.051

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

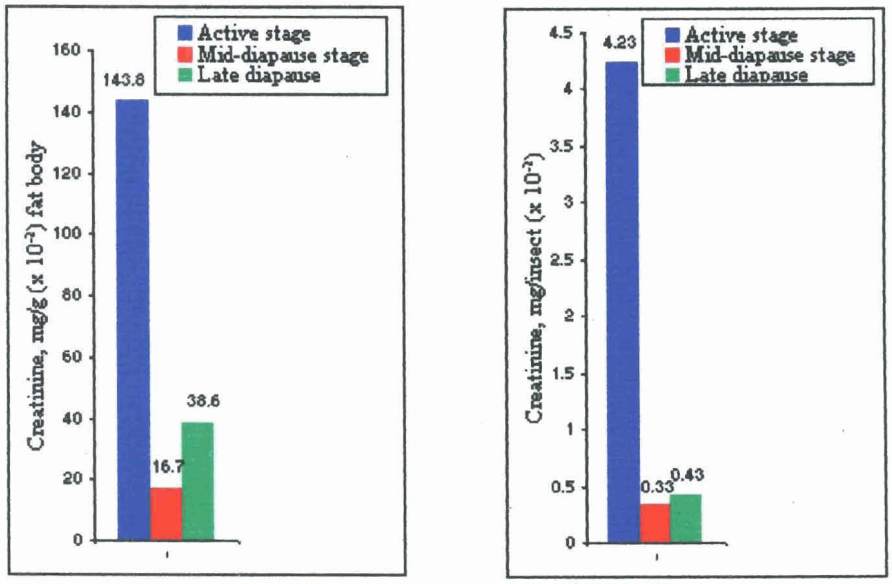


Figure: 19b. Creatinine in fat body during active and diapause stages.

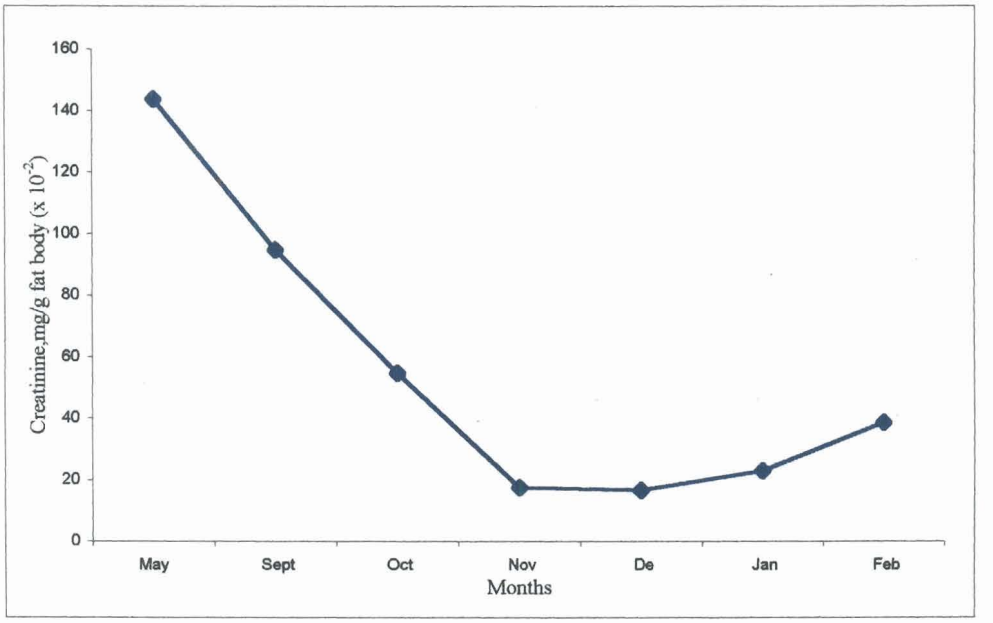


Figure 19c:1. Creatinine content in fat body during active and diapause stages

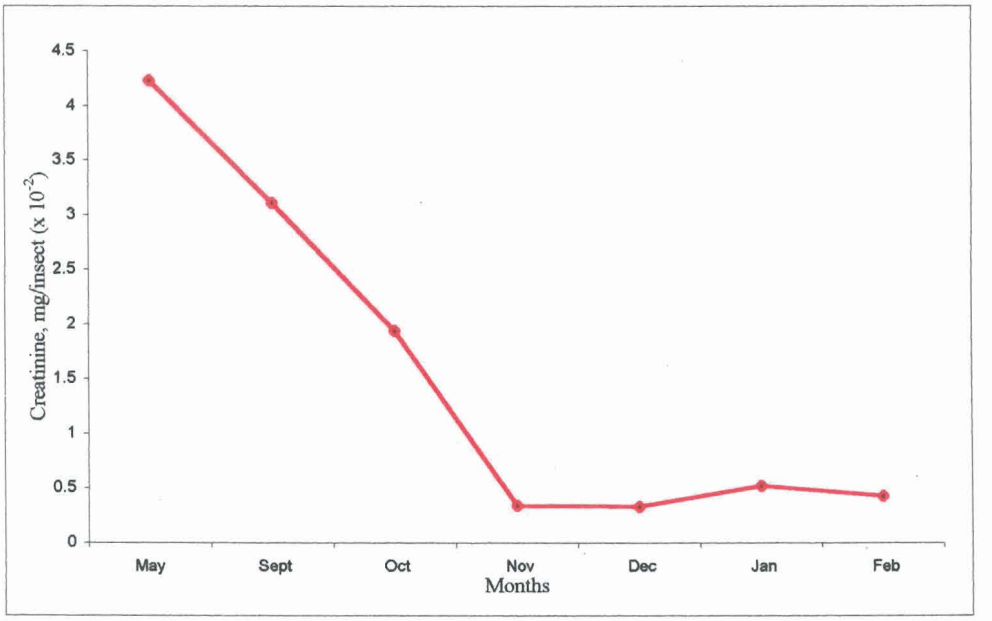


Figure 19c:2. Creatinine content in in fat body during active and diapause stages

Hydrogen peroxide levels in fat body during active and diapause stages

The content of Hydrogen peroxide of fat body during active and diapause stages are given in table 20, figure 20a and 20b.

Table 20

Figure 20a:1 and 20a:2

Figure 20b

Table: 20. Hydrogen Peroxide levels in fat body during the active and diapause stages

	Hydrogen peroxide	
Month	mg/g fat body	mg/insect
May	1.765 ± 0.182	0.077 ± 0.011
September	2.442 ± 0.213	0.081 ± 0.009
October	1.739 ± 0.147	0.052 ± 0.006
November	1.044 ± 0.102	0.025 ± 0.003
December	0.241 ± 0.036	0.007 ± 0.001
January	0.978 ± 0.114	0.021 ± 0.003
February	3.589 ± 0.427	0.033 ± 0.004

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

Hydrogen peroxide in fat body was found to be very low during mid-diapause stage but increased by the end of diapause stage.

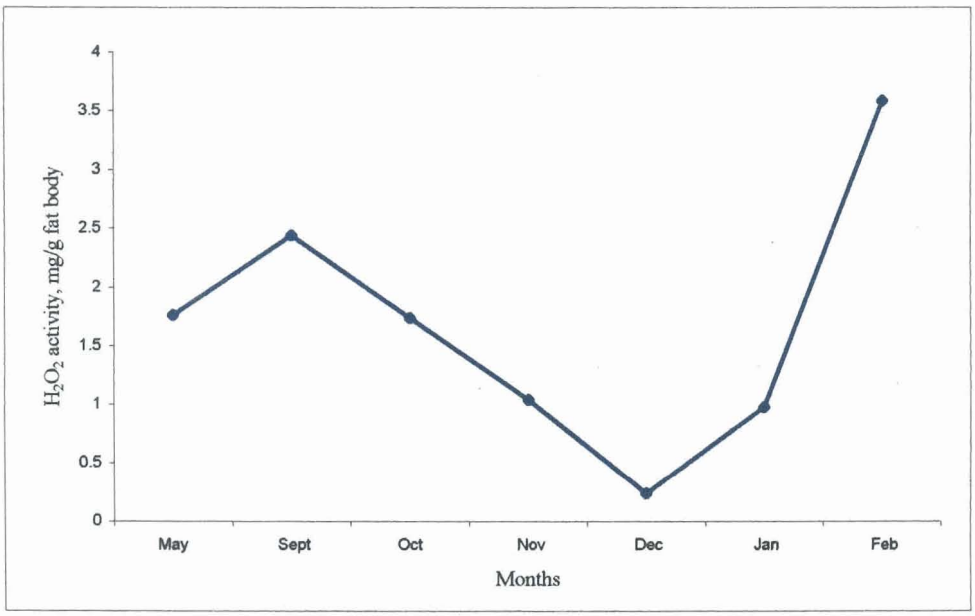


Figure 20a:1. Hhydrogen peroxide in fat body during active and diapause stages

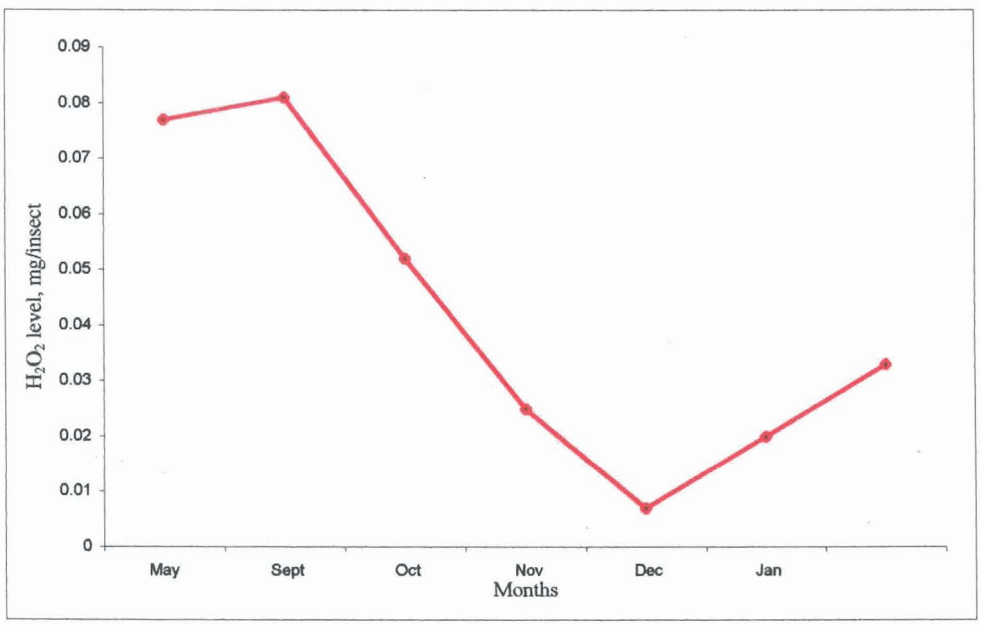


Figure 20a:2. Hydrogen peroxide in fat body during active and diapause stages

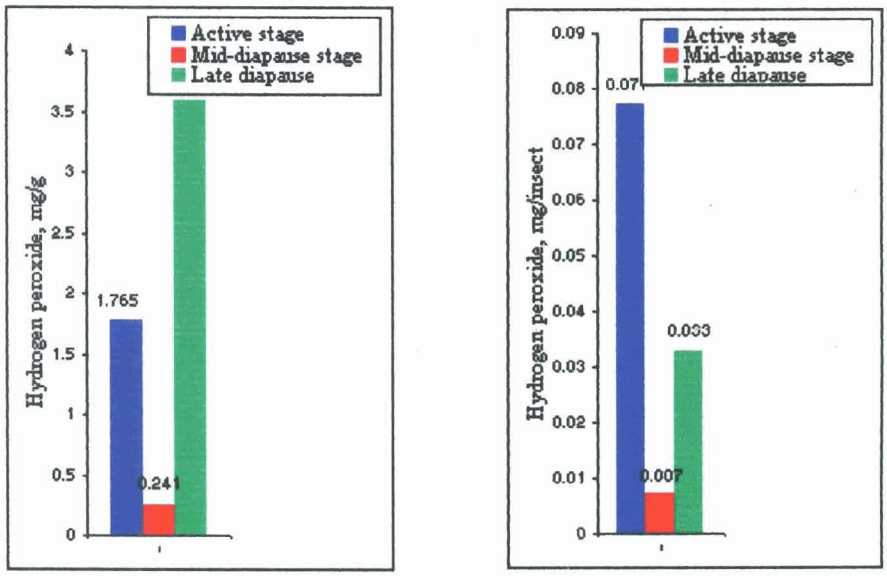


Figure:20b. Hydrogen peroxide in fat body during active and diapause stages.

The catalase activity in fat body during the active and diapause stages

The catalase activity in fat body during active and diapause stages is given in table 21, figure 21a and 21b.

Table 21

Figure 21a and 21b

The catalase activity in fat body had nearly 60% decrease during the mid-diapause stage as per unit weight and in the whole insect during mid-diapause and late diapause. But the catalase activity during late diapause as per unit weight was found to be more or less equal to that of active stage.

Table: 21. Catalase activity in fat body during active and diapause stages

	Catalase activity	
Month	mg/min/g fat body	mg/min/insect
May	14.91± 1.56	0.452 ± 0.051
September	9.14 ± 1.03	0.301 ± 0.035
October	6.94 ± 0.93	0.237 ± 0.031
November	4.25 ± 0.38	0.178 ± 0.024
December	3.86 ± 0.39	0.116 ± 0.012
January	3.56 ± 0.38	0.091 ± 0.011
February	12.72 ± 1.36	0.118 ± 0.016

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

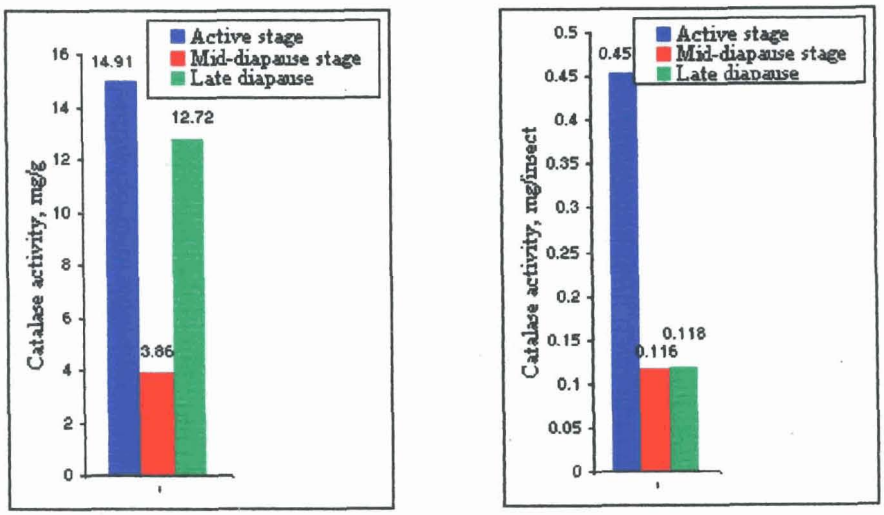


Figure: 21a. Catalase activity in fat body during active and diapause stages.

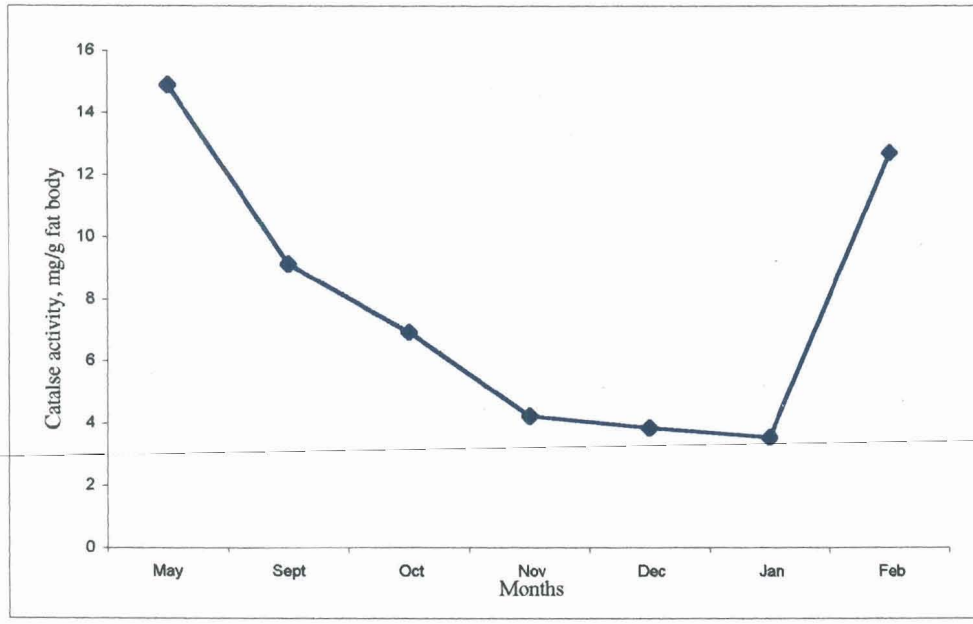


Figure 21b:1. The catalase activity in fat body during active and diapause stages

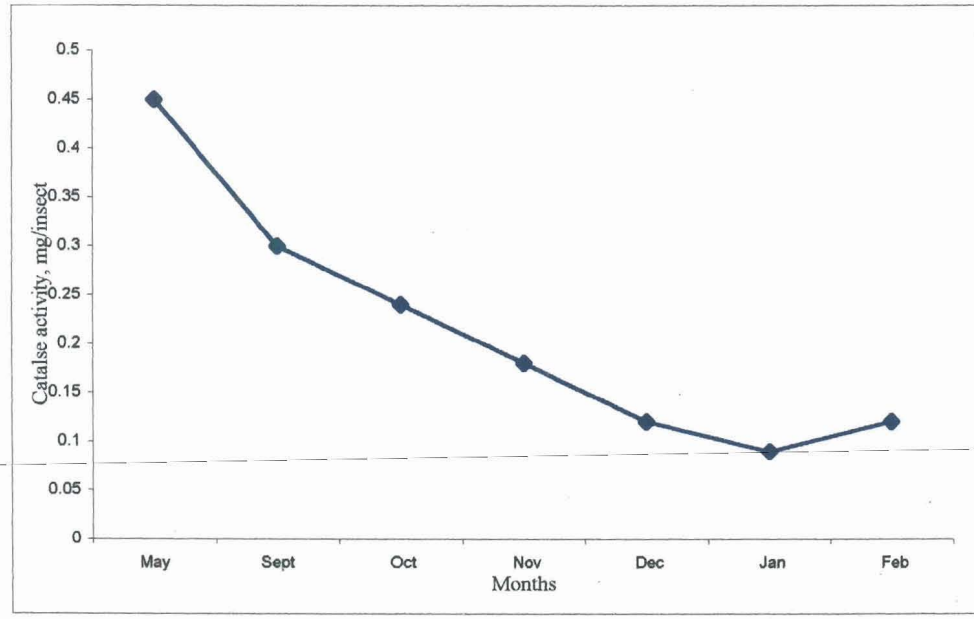


Figure 21b:2. The catalase activity in fat body during active and diapause stages

Alanine aminotrasferase (AIAT) activity in fat body during active and diapause stages

The enzymatic activity of alanine aminotransferase in fat body is given in table 22, figure 22a and 22b.

Table 22

Figure 22a:1 and 22a:2

Figure 22b.

Table: 22. Alanine aminotransferase activity in fat body during the active and diapause stages

	Alanine aminotransferase activity	
Month	mg/g fat body($x10^{-3}$)	mg/insect ($x\ 10^{-3}$)
May	117.97 ± 12.26	3.725 ± 0.426
September	13.42 ± 1.94	0.442 ± 0.053
October	12.82 ± 1.67	0.352 ± 0.042
November	12.18 ± 1.78	0.289 ± 0.036
December	8.01 ± 0.98	0.143 ± 0.018
January	8.28 ± 1.07	0.172 ± 0.015
February	12.39 ± 1.54	0.115 ± 0.012

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

The titre value of alanine aminotransferase during the diapause stage was found to be very low and almost negligible compared to the active stage.

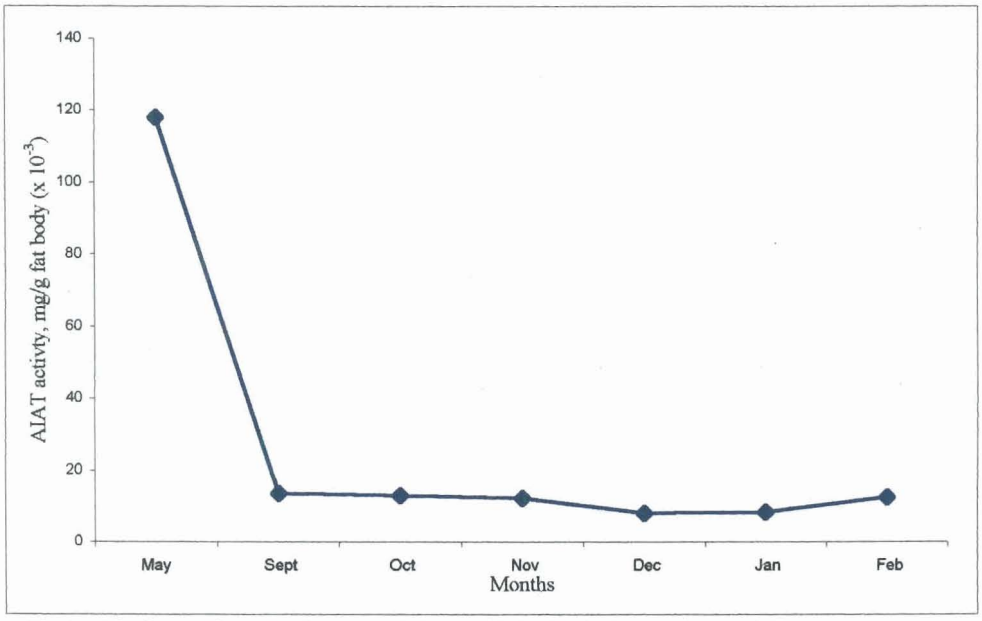


Figure 22a:1. Alanine aminotransferase activity in fat body during active and diapause stages

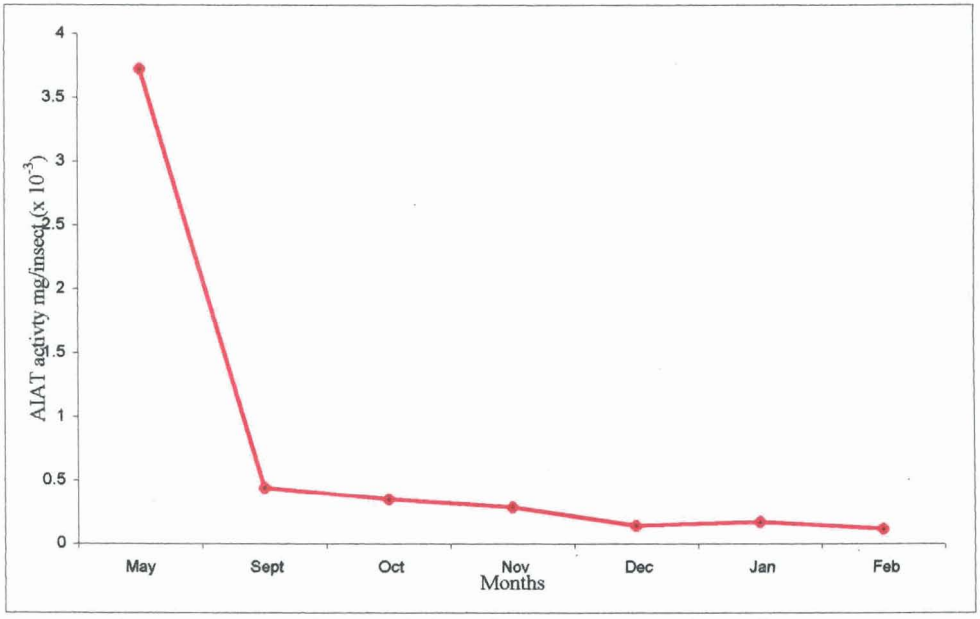


Figure 22a:2. Alanine aminotransferase activity in fat body during active and diapause stages

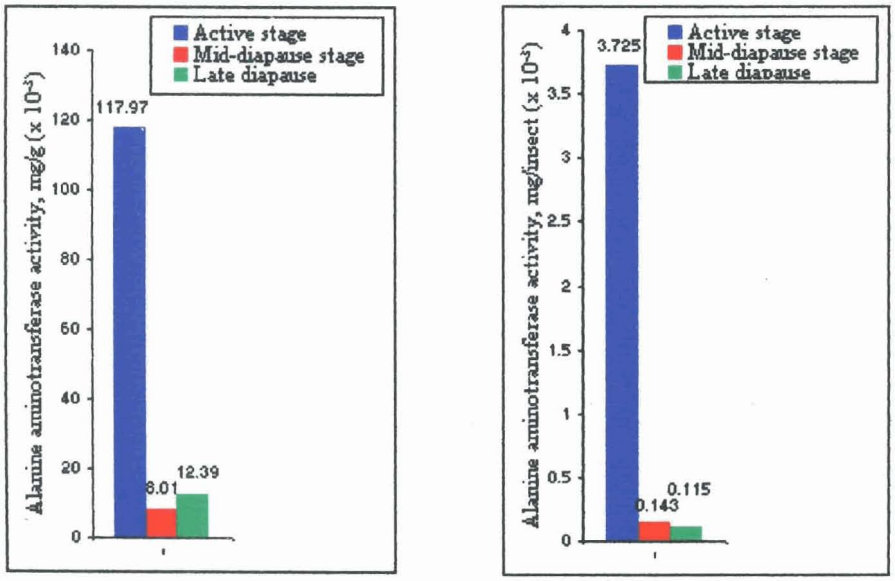


Figure:22b. Alanine aminotransferase activity in fat body during active and diapause stages.

Aspartate aminotrasferase (AsAT) activity in fat body during active and diapause stages

The enzymatic activity of aspartate aminotransferase in fat body is given in table 23, figure 23a and 23b.

Table 23

Figure 23a

Figure 23b

The titre value of aspartate aminotransferase during the diapause stage was found to be very low and almost negligible compared to the active stage.

Table: 23. Aspartate aminotransferase activity in fat body during the active and diapause stages

	Aspartate aminotransferase activity	
Month	mg/g fat body ($x10^{-6}$)	mg/insect ($x\ 10^{-6}$)
May	195.36 ± 21.32	5.487 ± 1.012
September	7.18 ± 0.95	0.237 ± 0.035
October	8.45 ± 1.05	0.222 ± 0.031
November	9.22 ± 1.65	0.219 ± 0.036
December	3.77 ± 0.52	0.113 ± 0.019
January	4.31 ± 0.63	0.089 ± 0.018
February	6.01 ± 0.86	0.056 ± 0.014

The values are the means of five determinations with standard deviation. The experiments were conducted from June 2002 to June 2003. The diapause starts from late June and ends by the end of late February.

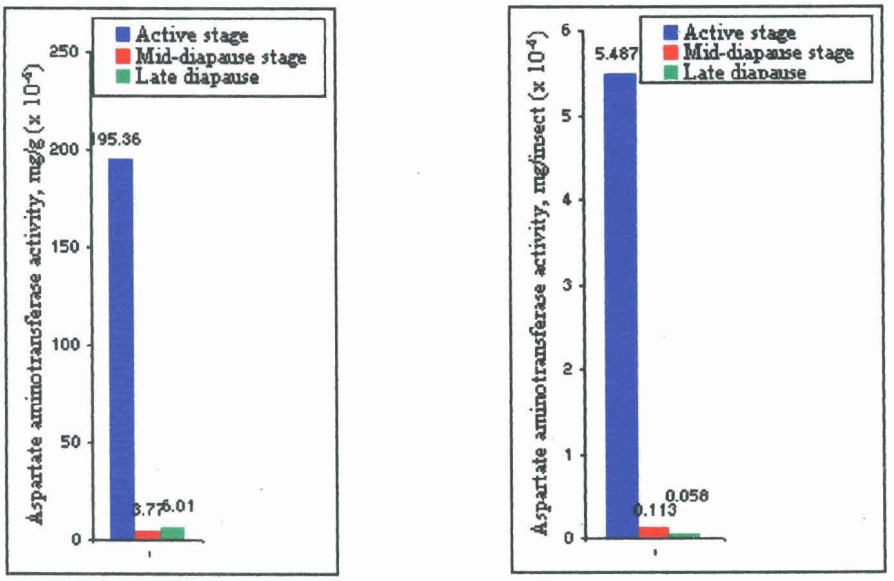


Figure: 23a. Aspartate aminotransferase activity in fat body during active and diapause stages.

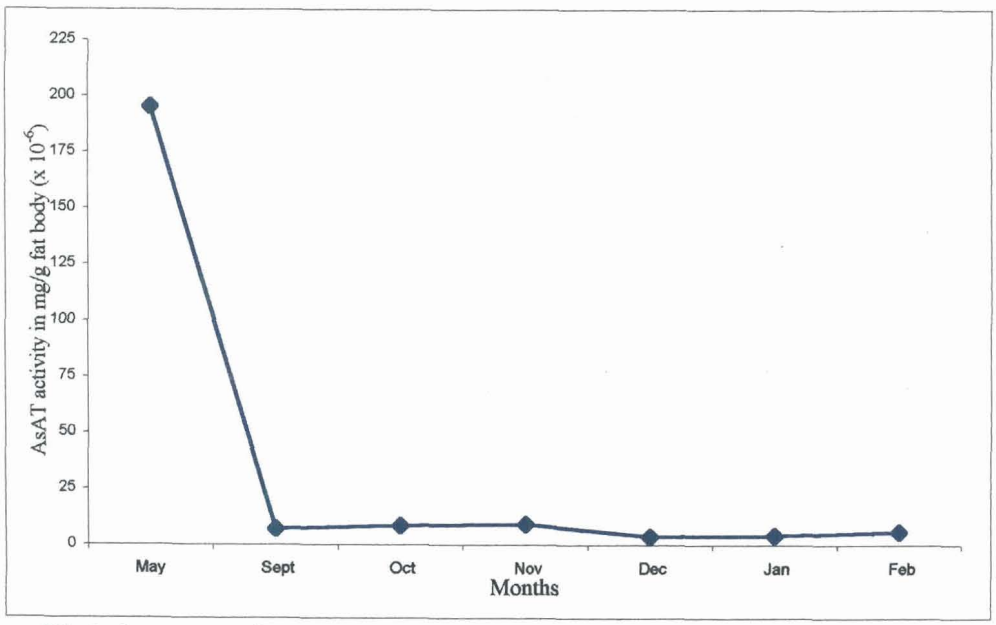


Figure 23b:1. Aspartate aminotranferase activity in fat body during active and diapause stages

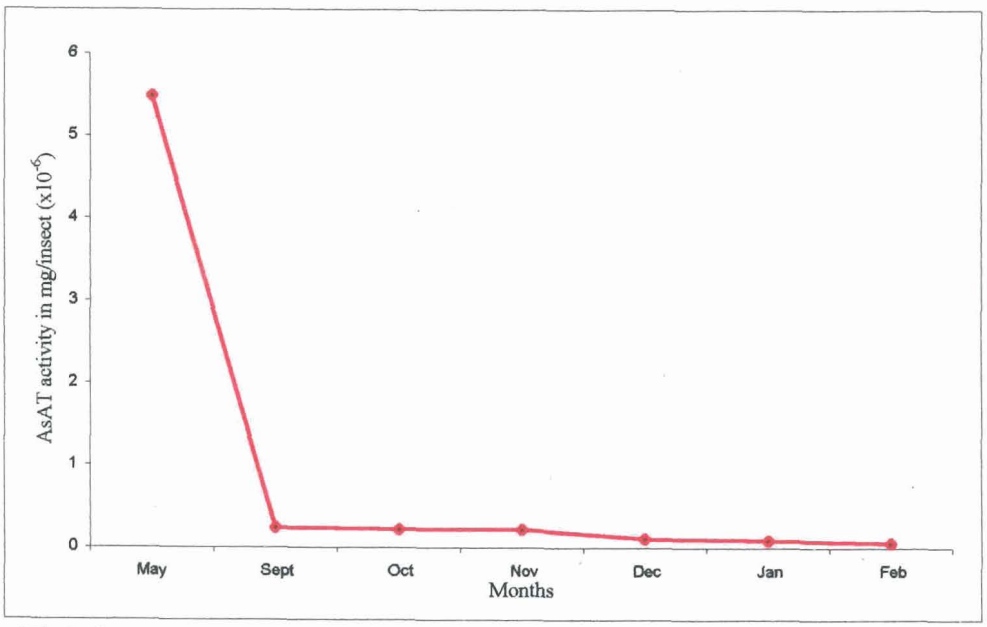


Figure 23b:2. Aspartate aminotransferase activity in fat body during active and diapause stages

The consolidated data of biochemical analysis of haemolymph and fat body of active and diapause stages

The consolidated data of biochemical analysis of haemolymph and fat body of active and diapause stages are given in tables 24, 25 and figures 24a, 24b, 25a and 25b.

Tables 24 and 25

Figures 24a and 24b

Figures 25a and 25b

When the biochemical constituents of haemolymph are considered the amount of total protein, total free amino acids glucose, urea, and creatinine showed a higher concentration during diapause than during the active stage. The variation in total protein was much higher than the rest.

The biochemical constituents of fat body also had considerable variations between the active and diapause stages. The amount of total free amino acids alone increased during diapause while total protein, glucose, urea, creatinine, hydrogen peroxide, catalase activity, and activity of the transferases decreased sharply during diapause.

Table: 24.Biochemical constituents of haemolymph during active and mid-diapause stages

	mg/ml haemolymph		mg/ insect (x 10^{-3})	
	Active	mid-diapause	Active	mid-diapause
Total proteins	5.56	355.56	139.03	888.89
Total free amino acids	0.02	0.04	0.04	0.09
Glucose	0.07	0.10	0.17	0.25
Urea	7.5	12.76	18.75	31.89
Creatinine	1.91	3.81	4.76	5.52

The data represents the results of the determinations made which corresponds to active and mid-diapause stages respectively.

Table: 25. Biochemical constituents of fat body during active and middiapause stages

	Per unit weight		Per insect	
	Active	Mid-diapause	Active	Mid-diapause
Total proteins, mg/g	69.44	25.88	1.95	0.78
Total free amino acids, mg/g	5.66	8.43	0.15	0.25
Glucose, mg/g	2.53	0.61	0.071	0.018
Urea, mg/g	0.834	0.392	0.036	0.012
Creatinine, mg/g (x 10^{-2})	143.8	16.70	4.23	0.33
H_2O_2, mg/g	1.765	0.241	0.077	0.007
Catalase, mg/min/g	14.91	3.86	0.452	0.116
AlAT, mg/g (x 10^{-3})	117.97	8.013	3.725	0.143
AsAT, mg/g (x 10^{-6})	195.36	3.765	5.486	0.113

The data represents the results of the determinations made which corresponds to the active and mid-diapause stages respectively.

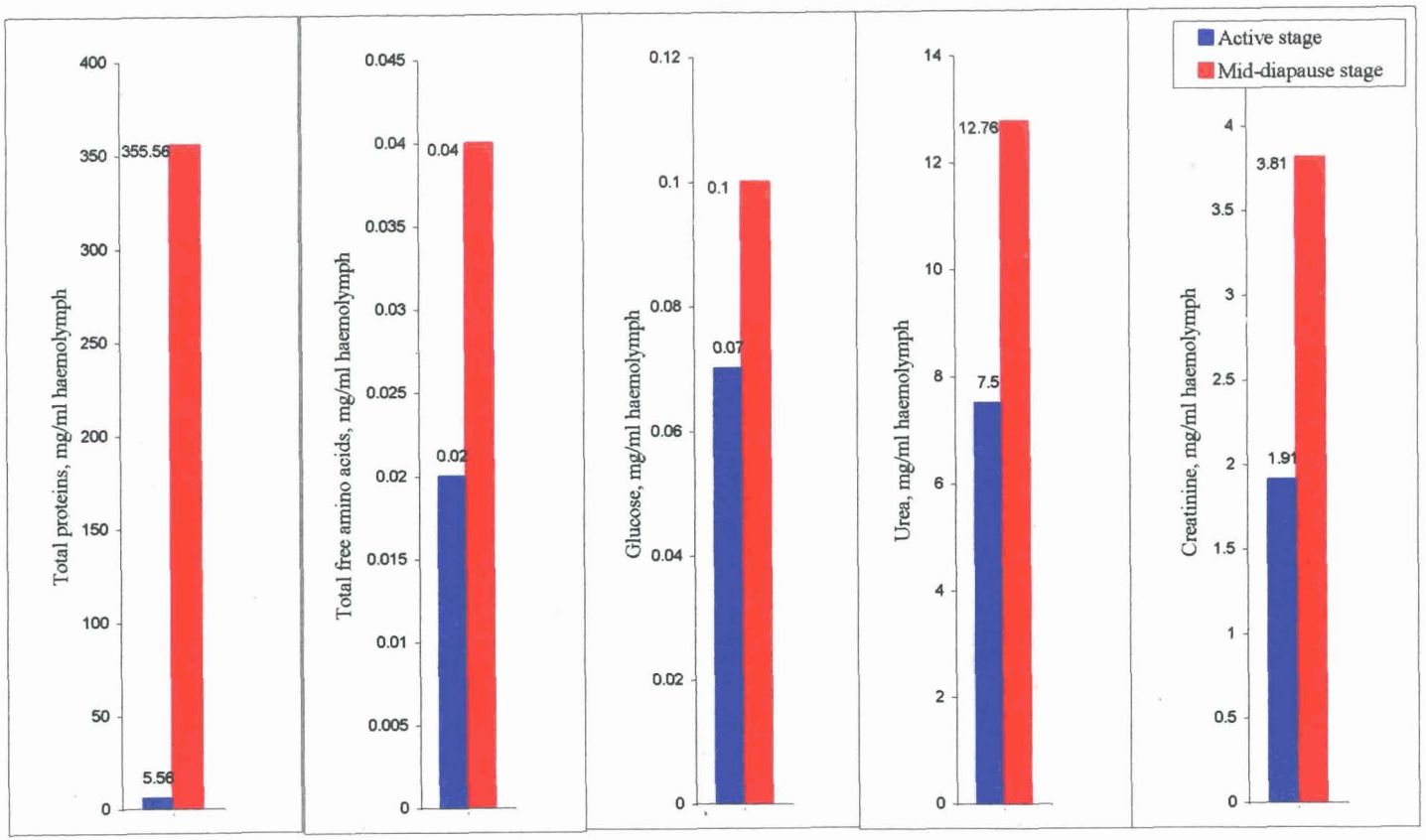


Figure 24a. Biochemical constituents of haenolymph, mg/ml, during active and mid-diapause stages

DISCUSSION

Gracy Thomas "Studies on oxidative strss during the life cycle of iphita limbata" Thesis. Department of Zoology, University of Calicut, 2005

Discussion

DISCUSSION

Biology of *Iphita limbata*

Biological changes due to the administration of tryptophan

In the normal honey fed and honey with tryptophan fed insects the fresh weight of insects showed a decline. However in the latter case the water content of the bugs was less when compared with those of normal insects. It can be seen that the bulk of the fresh weight of the body was due to its water content and that the insect lost most of its water content as the experiment proceeded (fig.2). The result clearly indicates that tryptophan has diuretic effect on the bug.

Biochemical effects on the administration of tryptophan

Biochemical analyses were carried out for a period of one month during which the experimental insects were fed with honey supplemented with tryptophan and its effects were evaluated.

Total proteins

It is established that there is an extensive storage of total proteins in the fat body of insects during the larval- pupal transformation and pupal diapause (Legner, 1983). In the present study, there was an accumulation of protein in the fat body on the treatment of honey and honey supplemented with tryptophan and the changes were more or less same in both cases.

Proteins are the most studied nitrogenous compounds in the insect fat body. A variation in the content of fat body proteins is observed in the normal and treated insects during experiment. There was a six fold increase in the level of protein during the experimental period. The variation was more striking when the amount was calculated for total insect fat body where there was a six fold increase during experiment. From the above observation it is obvious that there is an accumulation of proteins in the fat body during the course of the experiment. Accumulation of protein in the final instar larval fat body of insects has been demonstrated in many insects (Kilby, 1963; Karnavar and Nayar, 1973; Price, 1973; Wyatt, 1975; Chen, 1978).

The storage of protein is of universal occurrence in insects. In insects the fat body is the chief storage tissue, which stores nutrients like carbohydrates, proteins, lipids and other metabolites like uric acid. The present observation of the elevated levels of proteins in the fat body of *I. limbata* on the administration of honey and honey with tryptophan can be correlated with its storage. The magnitude of the storage of proteins during the experimental period indicates that the storage steadily increased during the experiment about six times than the initial amount (fig.4). It seems that honey and tryptophan induces the accumulation of protein in fat body, much similar to that of the pupal stages of holometabolus insects.

Total free amino acids

The occurrence of a high titre of amino acid in the haemolymph and fat body is a universal phenomenon in insects (Tobe and Laughton, 1969). Administration of honey and

tryptophan had an inducing effect of accumulation of free amino acids in the fat body (fig.5). The titre of amino acid in the fat body can be used as a pointer in the evaluation of the dynamic metabolism of proteins and of the organism in total. A change in the amino acid pool will directly influence the protein turnover; *i.e.*, the synthesis and degradation of proteins which obviously reflect the physiological state of the organism.

Glucose

Initially the tryptophan treated insect showed an increase in glucose levels followed by a gradual decrease till the end of the experiment (fig.6). The decrease may be due to its transportation to blood and also due to low rate of gluconeogenesis. This suggests a reduction in the stored carbohydrate and in gluconeogenesis. The elevated level of glucose during the experimental dietary regimen is linked with the elevated levels of total free amino acids and transaminases observed during the same period.

Urea

Urea is a protein-unfolding agent that can accumulate locally to high concentrations in tissues of many organisms (David *et al.*, 1999). A considerable amount of urea was found in the fat body of the insect (fig.7). There was a sudden increase in the amount of urea during the first week of the experiment which was reduced to normal level within a short period showing the active nature of excretion by the animal. The levels of urea on the administration of honey and tryptophan are in tune with the levels of proteins observed during the same period. In the treated insects there was a sharp reduction in the water content of the bug. This results in a situation similar to that of diuretic. The accumulation

of urea in the tissues of insects is connected with osmoregulatory aspects of the internal environment of the animal (Baldwin, 1967).

Creatinine

The turnover of creatinine has been considered as a pointer in the evaluation of the active protoplasmic mass in animals. The analysis of the fat body during dietary regimen revealed that creatinine is present initially in significant quantity and was reduced to almost one third by the fourth day and maintained a very low level throughout the experiment (fig.8). The reduced amount of creatinine in the fat body of the animal indicates a reduced turnover of creatinine as product of metabolism. The results are also in tune with the accumulation of proteins in the fat body during the same period.

Hydrogen peroxide and catalase activity

Catalase is known to catalyse the decomposition of hydrogen peroxide to water and oxygen and thus protects their cell from oxidative damage by H_2O_2 and OH^- (Bandopadhyay *et al.* 1999). Since insects lack glutathione peroxide activity, catalase activity provides the sole enzymatic mechanism for the removal of H_2O_2 (Orr, *et al.*, 1992).

The pattern of catalase enzyme activity in the fat body of *I. limbata* may be due to the changes in the physical activity and feeding habits of the animal as recorded by Sohal *et al.*, (1986).

Sohal *et al.*, (1985) studied the mechanism of aging in the housefly and the efficiency of the mechanism of protective against the intermediates of oxygen metabolism.

Catalase activity as well as the concentration of inorganic peroxide seems to steadily decline with the age. The constitutive levels of the enzymes SOD, catalase, glutathione, transferase and its peroxidase activity and glutathione reductase correlate well with natural feeding habits of the lepidopterus larva of the cabbage looper (*Trichoplusia ni*), southern armyworm (*Spodoptera eridania*) and black swallowtail (*Papilio polyxenses*) and their relative susceptibility to peroxidents plant allelochemicals, quercetin (a flavonoid) and xanthotoxin (a photoactive furanocoumarin) (Ahmad and Pardini, 1990).

The catalase activity of both honey fed and honey with tryptophan fed insects were found to follow the same pattern (fig.10). They had an initial increase in the enzyme activity and then maintained a low steady state. Restriction of caloric intake lowers steady-state levels of oxidative stress and damage, retards age-associated changes in mammals (Sohal and Weindruch, 1996). The measurement of the peroxide level in the fat body of normal and treated insects gave a clear picture of the enzyme activity as both followed the same pattern. The results indicate that honey and tryptophan has a transient effect on elevating the levels of hydrogen peroxide and catalase activity in the bug, which did not sustain on prolonged administration.

Alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) activity

Aminotransferases are of fundamental importance in the metabolism of proteins and amino acids. A balanced amino acid pool is a primary requirement for protein synthesis and transamination as this is one of the chief mechanisms which functions as a regulator of this pool (Reddy *et al.*, 1991). So the activity of AlAT and AsAT was studied

(fig.11 and 12). Increased activity of transaminase means enhanced mobilization of free amino acids into transamination activities. The AlAT forms a general index of amino acid break down.

Biological changes during diapause

The adults of *I. limbata* undergo diapause as indicated by its active and dormant phases during the twelve months of the year. The prediapause and the postdiapause are active stages of the bug. During the diapause phase they cease to feed, excreate and reproduce and virtually show no movements except its antennae. This lasts about seven to eight months. In this stage they are also seen in groups resting under some leaves which may be fresh or dry (plate: 3).

The changes in the total fresh weight of insects during active and diapause stages showed considerable variation (fig.13a). After the insect entered into diapause the weight gradually decreases probably due to water loss and also due to the decrease in the amount of fat body (fig.13b and 13c). In insects the greater part of the fat body is made up of cells called trophocytes which are vacuolated and distended by stores of glycogen, fat or proteins. The trophocytes are very much like some blood cells and there may be a close relationship between the two. The trophocytes accumulate reserves of food of which fat is the most usual. The stored food in variable forms depends on the diet. Carbohydrate in fat body is usually in the form of glycogen. Protein may also be present. Protein is not usually stored in large quantity in adult insects. These food reserves are of particular importance in insects during non feeding periods. They also enable the insects to survive during the

period of diapause and usually, as preliminary to diapause, extensive reserves are accumulated (Legner, 1983). In the present study, at the end of diapause the total weight of fat body was reduced to about 1/5 of the active stage. It may be noted that the fresh weight of insect was reduced to only by 10% at the same stage. The decrease in the weight of fat body during diapause was noted to be less than 25% of the weight of fat body during the active period.

One of the most significant features of the diapause stage of the insect is that they apparently show no excretion of nitrogenous end products. Since the nitrogenous end products accumulated should not disturb the equilibrium of the internal milieu, a change in the metabolism of the animal and thus a change in the nature of the nitrogenous end products should take place.

Similar cases of diapause have been reported in insects (Legner, 1979, 1983; Saunders, 1981). In the case of monarch butterflies diapause is correlated with temperature (Brower, 1985). In *I. limbata* the dipause is correlated with monsoon and temperature. The active period end by June and the insect enter diapause with the beginning of monsoon. The insects leave the diapause stage as the temperature rises slowly after the winter.

Biochemical changes during diapause

Levels of total proteins during diapause

There is inverse correlation in the levels of total proteins in the haemolymph and fat body; during diapause fat body proteins are depleted with a corresponding increase in

the haemolymph (fig.14a; 14b and 14c). Mobilization of fat body reserves is necessary for their utilization during diapause. The results indicate that the fat body reserves are in a dynamic state; the uptake and sequestration are related with the physiological condition of the insect. The changes in the levels of free amino acids in the tissues further give evidence to the above.

Levels of total free amino acids during diapause

Investigation on the content of free amino acids in the haemolymph and in the fat body of the animal revealed that there was a conspicuous increase in the amount of the material during the active and diapause phases of the animal. The changes observed in the total free amino acids in the insect in haemolymph and in fat body (fig. 15a, 15band 15c) points to the dynamic protein turnover taking place during the active and the diapause periods. The increase in the amino acid content during the prediapause period is attributed to the fact that this period is a very active stage in the animal and the accumulation of the materials for its future maintenance takes place during this stage. The amino acids derived from the food also are retained to the maximum in the body after satisfying the amino acid requirement for its protein synthesis during the growth period. It may be possible for the insect to spare a considerable amount of free amino acids as a result of vigorous feeding activity during active stage.

Any depletion or repletion of amino acids will indicate a shift in the equilibrium between synthesis and degradation of body proteins. The true picture of these dynamic changes could be obtained by evaluating the changes in the total content of free amino

acids in the haemolymph and in fat body. At the beginning of diapause there was a sharp increase in total free amino acids in the fat body which declined gradually during the diapause (fig. 15a, 15b and 15c). The results indicate that the proteolytic activity was maximum during the initial period of diapause.

Levels of individual free amino acids in the haemolymph during diapause

Glutamic acid

Glutamic acid is the most abundant individual free amino acid which accounts to 27.51% of the total free amino acids during the active phase (fig. 16c:1). Glutamic acid is an important amino acid in the synthesis of puriens, pyramidines and NAD and in the transmission reaction, which form the connecting link between the metabolism of carbohydrates and proteins. It is already mentioned that the high concentration of uric acid is a characteristic feature of insect haemolymph and therefore their synthesis invariably requires large amount of glutamine. Further the amines have an important role in the metabolism of ammonia as a major nitrogenous excretory product.

Proline

The amount of proline constitutes about 3.27% of the total free amino acids of the haemolymph of *I. limbata* (fig.16c:8). Proline has a multiple function in the animal, as an energy source (Bursell, 1963, 1966; Bursell *et al.*, 1974; Sactor, 1975), and as a precursor in the synthesis of cuticular structures. It has been suggested that arginine to proline pathway is predominant in insects (Sactor, 1975). The conversion of arginine to proline

results in the production of urea. Therefore the metabolism of arginine results in the subsequent increase in the amount of proline and also in the production of urea and ornithine.

Arginine

It can be observed that arginine at the active and mid-diapause stage (fig. 16c:7) is more or less equal (3.3% and 3.8%) showing the absence of arginine metabolism and the non production of urea as the insects are in the non excretory period. But by the end of diapause that is in February the amount was found to be very low that is 0.82%. In correspondence with this the praline level by the end of diapause was found to be high, indicating its return to the active excretory period.

Lysine

Lysine occurs consistently in the haemolymph of the insect (Chen, 1962). The concentration in the active and diapause stages show much variation. During the active stage the animal has 1.02% of Lysine while during diapause stage it is increased to 3.83% (16c:15).

Glycine

The amount of glycine during the active and the diapause stages of the haemolymph are more or less constant (7.56% and 7.01%) (fig. 16c:4). 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

protein in insects revealed that they are unique in the high content of glycine (Lucas *et al.*, 1960; Bailey and Weis-Fogh, 1961; Srivastava, 1971).

Serine

Serine is found in the haemolymph of *I. limbata* (fig. 16c:5). In the diapause insects its concentration increased more than double than that of active stage. Serine has been reported to be a significant fraction in Lepidopteron haemolymph, but its origin and metabolism are unknown (Chefurke, 1965). It has been suggested that serine arises from fatty acids.

Aspartic acid

The concentration of aspartic acid increased nearly four times during diapause stage (fig. 16c:9). Aspartic acid is an important precursor in the synthesis of puriens, pyramidins, and structural proteins. Aspartate also plays an important role in transmission reactions and thus functioning as a connecting link between the metabolism of carbohydrates and proteins (Katunuma *et al.*, 1968).

Methionine

The amount of methionine per total tissue in the haemolymph during active stage decreased from 1.03% to 0.85% (fig.16c:14). Methionine is used for the formation of succinyl CoA which participates in the oxidation of certain fatty acids (Stryer, 1988). So the reduction in its concentration may also affect fatty acid oxidation and protein synthesis.

Threonine

The concentration of threonine gradually decreased during the feeding period and increased during the non-feeding period. It increased from 1.53% to 4.83% (fig. 16c:11). Threonine can be converted to glycine and then to serine. Serine can be catabolised for liberating energy. Thus threonine involves in the formation of proteins and energy metabolism (Mehler, 1988). The sharp rise in the concentration of threonine shows its utilization by insects in diapause.

Alanine

Alanine is one of the predominant amino acids of the *I. limbata*, which constitutes 18.68 % of the total free amino acids (fig. 16c:2). During diapause the concentration of alanine decreases conspicuously (8.9%). One of the important functions of the alanine is its participation in the synthesis of structural proteins (Lazar, 1983).

Valine, leucine, isoleucine

These are similar amino acids which form homologous proteins in various organisms in which these branched chain amino acids replaced each other in certain positions without greatly altering the functional properties of the proteins. During the active stage valine showed 8.41% (fig. 16c:3) of the total free amino acids and leucine with 3.68% (fig. 16c:6) and isoleucine with 2.02% (fig. 16c:10). But during non-feeding diapause stage the amount of valine decreased to 7.61% while that of the other two

increased to 7.78% and 5.23% respectively. These three amino acids are involved in energy metabolism and protein synthesis.

Phenylalanine

Phenylalanine is found to be in low concentration (1.09%) during the active feeding stage and it has a sharp increase to 4.38% during the non-feeding diapause stage (fig. 16c:13). Phenylalanine is converted to tyrosine (Stryer, 1988).

Tyrosine

The concentration of tyrosine in the haemolymph during the active and the diapause stages remain more or less constant (fig. 16c:12).

Histidine

Histidine can be catabolised to glutamate. Its decarboxylation produces histamine (Stryer, 1988). The concentration of histidine was doubled by the end of diapause stage (fig. 16c: 16).

The account given above indicates that the amino acids necessary for the synthesis of structural proteins play a prominent role in *I. limbata.* It is particularly significant that certain amino acids like glutamic acid, alanine, arginine, proline, methionine, serine, leucine, aspartic acid, isoleucine, threonine, metheonine, lysine, and histidine are obviously affected by the diapause (fig. 16a). The concentrations of glutamic acid, alanine, arginine, proline and methionine decreased during diapause stage. The concentrations of

the rest of the amino acids increased during diapause stage. Valine, glycine and tyrosine had no significant difference in their concentrations. Increase of amino acids in the haemolymph especially in the non-feeding period might be due to the inability of the insect to incorporate them in the metabolic processes.

The relative proportion of free amino acids during active, mid-diapause, and late diapause stages are given below.

Active period

Glutamic acid > alanine > valine > glycine > serine > leucine > arginine > proline > aspartic acid > isolucine > threonine > tyrosine > phenyl alanine > methionine > lysine > histidine.

Mid-diapause period

Glutamic acid > serine > alanine > aspartic acid > leucine > valine > glycine > isolucine > threonine > phenyl alanine > lysine > arginine > tyrosine > proline > phenyl alanine > methionine > histidine.

Late diapause period

Glutamic acid > alanine > serine > glycine > valine > leucine > aspartic acid > phenyl alanine > threonine > isolucine > proline > lysine > tyrosine > arginine > methionine > histidine.

In general there is a decline in the glutamate family of amino acids during diapause but their levels show a recovery at the end of diapause indicating their utilization during active life. It may be noted that the glutamate family of amino acids are important in gluconeogenesis and its subsequent utilization for energy.

Levels of glucose during diapause

The glucose content in fat body showed a four fold decrease during diapause (fig. 17a, 17b and 17c). This corresponds with the decrease in the percentage of alanine in haemolymph which is almost half the amount that is found in the active ones. The amount of glucose in haemolymph is inversely proportional to the levels of glucose in fat body.

Levels of urea during diapause

I. limbata exhibits an excretory active period and an inactive no excretory diapause period. So the chances of an accumulation of metabolites in the diapause phase of the insects are quite evident. The present investigation of the variation in the concentration of urea in the haemolymph and fat body were helpful to elucidate the physiological significance involved in the maintenance of urea level in the haemolymph and in fat body and its role in the control of osmoregulation. The occurance of urea has been demonstrated in the haemolymph of a few insects (Buck, 1953; Chefurka, 1965; Cochran, 1975). Sumida *et al.* (1990, 1995) have shown that urea concentration tend to decline toward larval-pupal transformation in the haemolymph of *Bombyx mori*.

The variation of urea in haemolymph and in the fat body found during the different periods was very conspicuous (fig. 18a, 18b and 18c). The amount of urea in haemolymph was almost double during diapause. But in fat body it is reduced to about 40%.

The amount of urea in the fat body has an important role in detoxication mechanism, osmoregulation and in the intermediary metabolism. The total turnover of urea in the fat body of *I. limbata* revealed that it maintains high content of urea during the active phase, *i.e.* nearly double the amount during active phase and a low content of urea in the diapause phase. The maximum retention of urea occurs at the active metabolic phase. Irrespective of the high content of urea in the fat body the total content in the animal is comparatively low during the diapause phase. During diapause the ammonia produced as a result of the catabolism of protein is to be detoxified immediately as the animal is unable to eliminate it by way of excretion. Sumida, *et al.*, (1990, 1995) have shown that urea concentration tend to decline toward larval-pupal transformation in the haemolymph of *Bombyx mori.*

It has been suggested that the accumulation of urea during periods of water stress was due to the enhanced synthesis of the material or the increased availability of the urea cycle intermediates, which in turn is due to the enhanced catabolic activity and in the failure of its excretion. On the whole, it can be seen that the tissue level of the material is a reflection of the homeostatic adaptation of the animal to maintain its internal environment. The accumulation of urea in the tissues of *I. limbata* is obviously connected with osmoregulatory aspects of the internal environment of the animal in order to survive during periods of water shortage. In the marine elasmobranches, the build up of urea in the tissues

(Baldwin, 1967) is indispensable for its survival in the hyper-osmotic (external) environment where they inhabit. But in the lungfishes or in the aestivating frogs the above situation does not exist (as they are not in contact with a hyper osmotic external environment). Instead they have to maintain a hyper-osmotic internal environment during water stress.

Levels of creatinine during diapause

The results of the analysis of creatinine in haemolymph and fat body of active and diapause insects were in contrast to each other (fig. 19a, 19b and 19c). The amount of creatinine in haemolymph almost doubled during diapause while it reduced to nearly one-ninth in fat body. It may be noted that the conversion of creatine to creatinine results in the accumulation of the latter in the haemolymph when the excretory activity stops. It may also be noted that the accumulation of creatinine in haemolymph is marked by its subsequent decline in the fat body there by indicating its sequestration from the latter during diapause. The result also indicates that the origin of creatinine is partly of dietary and partly of metabolic. Its higher concentration in fat body during the active metabolic phase suggests that creatinine is the product of protein metabolism and indicate its relationship with energy metabolism.

Creatinine has been reported in the final instar larvae of *Orthaga exvinacea* and *Spodoptera mauritia* (Lazar and Mohammed, 1991; Kuzhivelil and Mohammed, 1997). Its presence was also reported by Mangalalaxmy, (2002) in the haemolymph and fat body of the final instar larva of *Bombyx mori*. The variation found in creatinine was very

conspicuous during the active and diapause periods. The steep fall in concentration of creatinine in fat body between active and diapause periods indicates its effective removal from the fat body. The retention of the material in the active and diapuse period further points to their osmoregulatory function.

The turnover rate of creatinine has long been considered as a pointer in the evaluation of the protoplasmic mass in animals. Folin, (1905) identified that there are an endogenous and exogenous phases of protein metabolism, the former is relatively constant and is independent on the availability of protein from external source. This has been confirmed by many investigators (Albanese and Wangerin, 1944; Brody, 1945; Miller and Blyth, 1952; Kumar *et al.*, 1959; Platt *et al.*, 1964; Chin, 1966).

Levels of hydrogen peroxide and catalase activity during diapause

There is an almost 90% reduction in the amount of H_2O_2 in fat body and the catalase activity was reduced to less than half during diapause (fig. 20a, 20b, 21a and 21b). The death rate of the insect was found to be extremely low and was almost nil during the long period of diapause (7-8 months). The aging of the insects also plays a role in determining the enzyme activity. Physical activity is a determining factor of the enzyme activity (Sohal *et al.*, 1986). The results are in tune with the hypothesis of free radical reduction and prolonged life span in the organisms (Harman, 1956).

Alanine aminotransferase (AlAT) and aspartate aminotransferase (AsAT) activity during diapause

I. limbata has active feeding phase and a non-feeding diapause phase. In the case of *Spodoptera mauritia* an increase in the level of enzyme activity is seen only in the feeding stages, and the activity declines with the cessation of feeding until pupation. However the larva maintains a relatively higher level of enzyme activity during larval pupal transformation. This can be interpreted in terms of histolysis and histogenesis occurring in the larva prior to pupation. An increase in aminotransferase activity noticed in the larvae of *Drosophila melanogaster* and mammals during post-natal development (Miller, 1969) is explained as inevitable for keeping pace with increasing demand of keto acid for gluconeogenesis during starvation or post-natal development. The high level of aminotransferase observed in the normal insects during active stage is in tune with the anabolic phase of the insect (fig. 22a, 22b, 23a and 23b).

High aminotranferase activity in mammalian liver can be induced by starvation (Katunuma *et al.*, 1968; Snell, 1979). Similarly young mammals feeding on milk with low carbohydrate content and rich protein have a higher titre of aminotransferases (Dymsza *et al.*, 1964). These conditions are linked to gluconeogenesis for substituting carbohydrate. The important physiological functions of alanine aminotransferases are for the maintenance of the amino acid pool at proper level for protein synthesis (Meister, 1965), the supply of metabolites for energy metabolism (Sacktor, 1974) and as catalyzes of interactions between protein and carbohydrate metabolism (Katunuma *et al.*, 1968). A

sharp reduction in AlAT and AsAT activity during diapause in the fat body can be correlated to the non-feeding and low metabolic rate of the animal.

SUMMARY

Gracy Thomas "Studies on oxidative strss during the life cycle of iphita limbata" Thesis. Department of Zoology, University of Calicut, 2005

Summary

SUMMARY

Tryptophan has a diuretic effect on *Iphita limbata.*

Both honey and tryptophan induces accumulation of protein and total free amino acids in fat body, much similar to that of the pupal stages of holometabolus insects.

The adult stage of the insect is divided into an active feeding period and an inactive non-feeding diapause period. In the feeding period, normal activities like feeding, excretion, movements and reproduction take place whereas they are totally inactive during the diapause stage except the movement of their antennae. These observations lead to the suggestion that there will be a change in the metabolism and physiology of the internal environment of the animal.

The active and diapause stages of the insects are characterized by the drastic changes in its fresh weight, dry weight and in the fresh weight of the fat body. The changes in the water content of the animal during a period of twelve months and the changes in the weight of the tissue in the normal and treated insects during the experimental dietary regimen indicate that the analytical data must be evaluated on the basis of unit tissue and total tissue.

The physiological significance of the temporary retention of proteins in the fat body and in the haemolymph during active and diapause stages has been suggested to be a mechanism to maintain the osmotic pressure of the medium. It has been argued that the

retention of proteins in the haemolymph and their sequestration into the fat body are in a dynamic equilibrium synchronized with the gross physiological state of the organism.

The total free amino acids and individual free amino acids in the haemolymph vary conspicuously during the active and diapause stages of the insect.

It has been suggested that the titre of free amino acids in the haemolymph is related to the protein turn over in the animal.

The levels of urea on the administration of honey and tryptophan are in tune with the levels of proteins observed in the same period.

The reduced amount of creatinine in the fat body of the animal indicates a reduced turnover of creatinine as product of metabolism. The results are also in tune with the accumulation of proteins in the fat body during the same period.

By the end of diapause the total weight of fat body was reduced to about 1/5 of the active stage.

Honey and tryptophan has a transient effect on elevating the levels of hydrogen peroxide and catalase in the bug, which did not sustain on prolonged administration.

A sharp reduction in AlAT and AsAT activity during diapause in the fat body can be correlated to the non-feeding and low metabolic rate of the animal.

The changes in the amount of urea in the haemolymph and fat body exhibit an inverse realationship. It has been suggested that the removal of urea from the fat body and

its subsequent storage in the haemolymph during diapause is causatively linked to the osmoregulatory mechanism of the tissues and is an adaptation to maintain the internal environment of the organism. It has also been concluded that the development of ureotelism in the insects is not essentially directed towards the problem relating the hyperosmotic stress, but partly towards the homeostatic mechanisms operating in the organism in response to the changes in their internal and external environment.

The amount of urea in fat body during the active phase is reduced to one-fourth in the diapause phase of the insect. It has been suggested that the synthesis of urea in the animal may be associated with the osmoregulation of the animal.

The amount of creatinine in the fat body and haemolymph is very high when compared to that of the human blood. The pattern of content of creatinine in the haemolymph indicates that its metabolism in the insect resembles to that of the vertebrate pattern and thus contradicts the classical concept on invertebrate phosphogens.

Creatinine constitutes a small proportion of the total non-protein nitrogen of the fat body. The amount of creatinine in haemolymph is low in the active feeding phase and high in the non-feeding diapause phase. It is just the opposite in the case of fat body.

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