NEUROHORMONAL REGULATION OF METABOLISM IN THE PADDY PEST *SPODOPTERA MAURITIA* BOISD.

(NOCTUIDAE : LEPIDOPTERA)

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By

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Dedicated

to

My Parents

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CERTIFICATE

This is to certify that this thesis is an authentic record of work carried out by **P. Shylaja Kumari** from July 1993 to April 1999 under my supervision and guidance in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** under the Faculty of Science of the University of Calicut. No part of this thesis has been presented before for any other degree. I also certify that P. Shylaja Kumari has passed the Ph.D. qualifying examination of the University of Calicut held in December 1995.

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DECLARATION

I hereby declare that this thesis has not previously formed the basis for the award of any other degree/diploma.

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GENERAL INTRODUCTION

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter I

GENERAL INTRODUCTION

Insects are the most successful group of animals on earth and one of the reasons for their success is their ability to utilize lipids effectively as substrates for processes such as flight, reproduction, embryogenesis, metamorphosis and growth. In general, lipid metabolism in insects resembles that in mammals and it includes the biosynthesis of various lipids and their oxidation. Carbohydrates, proteins and lipids form the principal classes of organic compounds that are found in insects and other organisms. The rates of metabolic reactions in insect tissues vary considerably under different conditions and respond to physiological stresses that may arise during development, during environmental changes, or during flight. Within the last half-century, but more especially in recent years, numerous hormones has been shown to regulate a myriad of events in the life cycle of insects.

The endocrine system coordinates the body's internal physiology, regulates its development throughout life and helps it to adapt to nutritional and other external environmental changes. Moulting, cuticle synthesis, diapause, reproduction, water balance and salivary gland secretions are only a few of the mechanisms that are known to be regulated by the endocrine system. It has become increasingly evident that many hormones express their effects through control of metabolic activity. Certain hormones act directly on metabolic pathways. Lipid and carbohydrate metabolism are regulated by

adipokinetic hormones and hyperglycaemic hormones respectively, produced and released from the neurosecretory cells of the corpora cardiaca of many insects.

The present study is an attempt to test the presence of active components in the corpora cardiaca/allata-brain complex of the paddy armyworm moth *Spodoptera mauritia* that are involved in the regulation of the metabolism of lipids and carbohydrates. The cross-reactivity of these components on two other insects, *Iphita limbata* (lipid release, *in vivo* and *in vitro*) and *Periplaneta americana* (sugar release, *in vivo* and *in vitro*) were also tested. The activities were also tested with syn Lom-AKH-I for comparison.

REVIEW OF LITERATURE

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter II

REVIEW OF LITERATURE

Insects in the course of their evolution have developed both neural and hormonal systems to enable them to cope with the complexities of their environment providing for homeostasis, integration, differentiation and growth. Some of these systems are truly unique and are found only in insect species while others are also found in other groups of organisms. Hormones are used by insects to regulate many phases of their life processes. Growth and development are regulated primarily by the ecdysteroids and juvenile hormones whereas the neurosecretory hormones regulate various homeostatic mechanisms as well as other relatively rapid physiological, developmental and behavioural processes.

The centre of the endocrine system of the insect consists of neurosecretory cells in the brain. The axons from these cells carry neurosecretory granules to the corpus cardiacum (CC) from where the hormones are liberated into the blood. The CC also secretes a number of its own hormones. One of these hormone, prothoracicotropic hormone (PTTH), activates a second endocrine organ, the thoracic gland, and causes this to secrete the moulting hormone (ecdysone) which acts directly upon the growing tissues. A third endocrine organ, lying just behind the corpora cardiaca, is the corpora allata (CA) which is active throughout the young stages of the insect. The CA secretes the juvenile hormone (JH) or neotinine.

II.1. Hormones in insects

An endocrine system functions as an important link between the environment and various physiological and developmental events in the organism. The endocrine organs produce hormones which are carried by the blood, to various organs of the body, coordinating their long term activities. The hormones of insects are many and varies in their effects and even hormones from a single organ may have a variety of effects.

Insect hormones are of three chemical types. The first is the lipid hormones and include juvenile hormone and ecdysone. The second group is the peptide hormones which are synthesized by the neurosecretory cells and secreted into the haemolymph via neurohaemal organs and the third group is the biogenic amines such as octopamine, which are released from nerve endings.

II.1.1. Lipid hormones

Lipid hormones comprises ecdysone and juvenile hormone (JH) which are involved in the growth and metamorphosis in insects. In most insect species, reproduction and development are dependent on the secretion of JH by the CA. The JH control several important physiological processes including metamorphosis, reproduction, diapause, polymorphism and pheromone

production. Pfeiffer (1945) demonstrated that the CA were essential for egg development and maturation. The CA remain inactive during the early adult stage of feeding and somatic growth when reserves accumulate (Johnson and Hill, 1975) but later trigger a metabolic switch to the stage of lipovitellin synthesis for oogenesis. The absence of JH from CA prevents vitellogenesis and the consequent lipid transfer from fat body to ovaries in association with the diacylglycerol carrying lipoprotein, lipophorin (Chino *et al.*, 1977, 1981). Larval moulting is initiated by an ecdysteroid moulting hormone, in the presence of JH, while pupation occurs in the absence of JH (De Kort and Granger, 1981). In adult fat body, the induction of vitellogenin synthesis by JH has been intensively investigated in the cockroach, *Leucophaea maderae* (Engelmann, 1984) and the locust, *Locusta migratoria* (Wyatt, 1988; Braun and Wyatt, 1992; Zhang *et al.*, 1993; Wyatt *et al.*, 1994). Insect development, growth and the formation of larval structures are largely regulated by JH (Riddiford, 1986, 1994).

The work of Plagge (1938) and Fukuda (1940) led to the identification of a thoracic centre for moulting, which were later identified as the prothoracic glands, which in turn, were stimulated by the brain hormone or PTTH to secrete ecdysone. Ecdysone is responsible for the growth and development. Substrate transport and metabolism is important during growth

and development of insects and ecdysones must therefore be involved, directly or indirectly, in the regulation of these processes. Arnold and Regnier (1975) reported that ecdysteroids have some effects on lipid flux. In *Aedes aegypti* a blood meal may stimulate the ovaries to release α-ecdysone (Hagedorn *et al.*, 1975) and it is ecdysone that stimulates vitellogenin synthesis in the fat body. However, JH is required prior to these events, otherwise the fat body is not competent to respond to the ecdysone (Flanagan and Hagedorn, 1977). Ecdysone-controlled specific vitellogenin synthesis was reported in *Musca domestica* (Adams *et al.*, 1985).

II.1.2. Peptide hormones

Insect peptide hormones are involved in the control of a wide range of physiological, biochemical and developmental functions, including water balance, lipid and carbohydrate metabolism, muscle contraction, reproduction, growth and metamorphosis. Peptide hormones are chemical messengers in the insect nervous system and some of them act as endocrine regulators. A large number of biologically active insect neuropeptides have been sequenced and some of them synthesized. An understanding of the physiological roles of these peptides can reveal their intricate regulatory mechanisms. The peptides are now recognised to act not only as neurohormones but also as neurotransmitters and neuromodulators. These hormones are either released

from the nervous system into the haemolymph as circulating neurohormones or are released at specific sites as neurotransmitters.

Proctolin, the first insect neuropeptide identified (Brown, 1967) and isolated from the proctodeum of the cockroach, *Periplaneta americana* (Brown and Starratt, 1975) is a pentapeptide, widely distributed among insects (Brown, 1967). This peptide was later sequenced (Arg-Tyr-Leu-Pro-Thr; Starratt and Brown, 1975). Proctolin exhibited potent contractile effect on the hind gut or proctodaeal muscles. Originally described as an excitatory neuromuscular transmitter in the hindgut of the cockroach, proctolin exhibits potent activity in a variety of muscle preparations including hindgut, heart, extensor tibiae and oviduct. Proctolin or proctolin like peptides have been found to be widely distributed throughout the arthropods including several insects (O'Shea and Adams, 1981; Lange *et al.*, 1986; 1988; Baines and Downer, 1991; Puiroux *et al.*, 1992) and crustaceans (Bishop *et al.*, 1984; Siwicki *et al.*, 1985).

Many other peptides, with similarity in their amino acid sequences to each other and biological activity, have been identified mainly from CC of different insects. Corpora cardiaca are the main neurosecretory organs of insects resembling the hypothalamo-pituitary complex in mammals. They store and release neurohormones synthesized by the neurosecretory cells in

the brain. In addition, CC contain intrinsic glandular cells which forms a distinct lobe in some insects. Neurohormones produced by the brain cells are confined to the storage lobe.

The first hormone to be reported among these was the hyperglycaemic (HGH) factor from the CC of the cockroach, *P. americana* (Steele, 1961). The major source of HGH in all species is the CC, although smaller quantities of active factor may also be found in the CA (Steele, 1969). A detailed account of HGH is given elsewhere.

There has been increasing evidence for a hypoglycaemic hormone activity in concert with HGH. The first report of such a factor was that of Dixit and Patel (1964) from the honeybee. Selective removal of CA and/or CC proved that the CC-CA complex was the site of release of the hypotrehalosaemic factor (Chen and Friedman, 1977). A hypoglycaemic factor is also known to exist in the head region of some dipteran species (Duve, 1978). Kramer *et al.* (1982) isolated an insulin like material from the honeybee, *Apis mellifera* and from *Manduca sexta* and the material was called 'insect insulin'. They also reported that the hypoglycaemic factor and the insulin like peptide was one and the same.

Similarly, a hypolipaemic factor has been found to be localized in the lateral protocerebrum, storage lobe of the CC and in the midgut of locust

(Loughton, 1987). Orchard and Loughton (1980) reported that the hypolipaemic hormone is stored and released from the storage lobes of the CC. Downer and Steele (1972) demonstrated a reduction in haemolymph lipid with an increase in fat body lipid in *P. americana* following an injection of CC-extract.

Diuretic and antidiuretic hormones control water balance, waste removal, and ion balance. These homeostatic factors are responsible for maintaining a stable internal environment under constantly changing conditions. Diuretic activity has been found throughout the insect neuroendocrine system; within the brain, ventral nerve gaglia, corpora cardiaca and corpora allata. One of the neuropeptide that stimulates diuresis in *Locusta* has been isolated (Schooley *et al.*, 1987) from extracts of locust thoracic and suboesophageal ganglia and structurally characterized (Proux *et al.*, 1987). Locust diuretic hormone stimulates water transport by increasing the second messenger cyclic adenosine 3',5'-monophosphate (cAMP), (Proux and Herault, 1988). Kataoka *et al.* (1989) have isolated and characterized a diuretic hormone from extracts of pharate adult heads of *M. sexta*. An antidiuretic hormone of the house cricket, *Acheta domesticus* exerts an antidiuretic effect by inhibiting fluid secretion by the Malpighian tubules (Spring *et al.*, 1988).

The eclosion hormones are large (> 6 kd) molecules synthesized in the brain and released from the CC at precise intervals just before ecdysis. Eclosion hormones stimulate larval, pupal and adult ecdysis, along with other developmental changes (For a review see Truman, 1985). The eclosion hormones are isolated from pharate adult brain tissue of the silkworm *Bombyx mori* (Kono *et al.*, 1987) and the tobacco hornworm, *M. sexta* (Kataoka *et al.*, 1987; Marti *et al.*, 1987). Allatotropins are factors that stimulate the secretion of JH by the CA. Kataoka *et al.* (1989) have isolated and structurally characterized an allatotropin from head extracts of pharate adult *M. sexta*.

Another group of peptide hormones identified in the insect CC is adipokinetic hormones (AKHs) which regulate the mobilization of lipids from the fat body, its transport and oxidation in flight muscles. The AKHs are the most extensively studied insect neurohormones. A detailed account of these peptide hormones is given elsewhere.

Scarborough *et al.* (1984) isolated two cardioacceleratory peptides from *P. americana*, which they designated as CC-I and CC-II, which also exhibited strong hyperglycaemic activity when injected into cockroaches. The factors M-I and M-II described by O'Shea *et al.* (1984) have been found to be same as CC-I and CC-II.

Recently several other neurohormones are identified in insects using bioassay and radioimmunological and immunohistochemical techniques (Kramer, 1985). FMRF-amide (Phe-Met-Arg-PheNH_2) is one of the most studied invertebrate peptide. FMRF-amide immunoreativity is widespread in the animal kingdom and several peptides containing a C-terminal sequence identical or similar to FMRF-amide have been isolated and sequenced (Greenberg *et al.*, 1988). FMRF-amide related peptides (FaRPs) have potent effects on contraction of heart muscle (Cuthbert and Evans, 1989; Duve *et al.*, 1993), oviduct (Peeff *et al.*, 1993), hindgut (Holman *et al.*, 1986) and skeletal muscles of the legs (Evans and Mayers, 1986; Elia and Orchard, 1995). FaRPs are released from neurohaemal areas into the haemolymph from where they circulate as neurohormones to a distant target site (Robb and Evans, 1990; Elia *et al.*, 1993). The release of FaRPs from the CC have been demonstrated in *M. sexta* (Carroll *et al.*, 1986), *Calliphora vomitoria* (Duve *et al.*, 1992) and *Schistocerca gregaria* (Robb and Evans, 1990). In the blood sucking insect, *Rhodnius prolixus*, it was shown that FaRPs were released into the haemolymph at specific times following a blood meal (Elia *et al.*, 1993). In locust (Walther *et al.*, 1991)and crey fish (Mercier *et al.*, 1993), FaRPs have been shown to directly affect neuromuscular transmission.

Besides the various peptides described above, there has been lot of evidence to indicate the presence of a number of vertebrate hormones in insects. Mammalian gastrin/ cholecystokinin-like peptides were reported from *B. mori* (Yui *et al.*, 1980), *Calliphora erythrocephala* (Duve and Thorpe, 1981) and *Eristalis aeneus* (El-Salhy *et al.*, 1980). Blowfly brain contains factors that are similar to the C-terminal fragments of vertebrate gastrin and cholecystokinin (Dockray *et al.*, 1981). The two cockroach myotropins, leuosulfakinin I and leucosulfakinin II were the first invertebrate neuropeptides structurally characterized containing sulfated tyrosine residue (Nachman *et al.*, 1986). Both peptides exhibit strong sequence homology with the C-terminal sequences of the human brain-gut peptides cholecystokinin and gastrin, which also contain a sulfated tyrosine residue. Leucopyrokinin was the most abundant of the *L. maderae* myotropic neuropeptides (Holman *et al.*, 1986). Other vertebrate hormones detected in insects include somatostatin, vasopressin, neurophysin, enkephalin, endorphin, substance P, pancreatic polypeptide and prostaglandins (For a review, see Kramer, 1985). The presence of vertebrate hormones in insects suggests that they are phylogenetically ancient substances and that pharmacological agents which affect hormone action in vertebrates may function in insects also.

II.1.3. Biogenic amines

Biogenic amines are found in the haemolymph and nervous system of many insect species where they may function as neurotransmitters, neuromodulators or neurohormones (For a review, see Orr *et al.*, 1985; Pannabecker and Orchard, 1988; Macfarlane *et al.*, 1990; Orchard *et al.*, 1993; Blau *et al.*, 1994). Insect nervous system contain high levels of octopamine, dopamine and serotonin or 5-hydroxytryptamine (Evans, 1986). Octopamine has been the most widely studied because of its relationship to insect "fight or flight" response. It regulates lipid and carbohydrate mobilization (Orchard *et al.*, 1981, 1982, 1983; Downer *et al.*, 1984; Pannabecker and Orchard, 1986 a). It serves as a neuromodulator of neuromuscular transmission and muscle contraction in insect skeletal muscle (Evans and O'Shea, 1978; Evans, 1981) and controls insect visceral muscles in the gut and ovaries (Orchard and Lange, 1987). Octopamine also regulates flight muscle metabolism (Candy, 1978). It initiates light emission from the firefly light organ (Nathanson, 1979), and mediates some insect behaviours (Brookhart *et al.*, 1988).

Numerous examples of amines controlling peptide neurosecretion exist in both invertebrates and vertebrates. Octopamine regulates release of AKHs from intrinsic neurosecretory cells of the locust glandular lobe (Orchard and Loughton, 1981; Pannabecker and Orchard, 1986). In *Periplaneta* octopamine

appears to act on neurosecretory cells within the CC to elicit release of HGH (Downer *et al.*, 1984). Flanagan and Berlind (1984) demonstrated that 5-HT facilitate diuretic hormone release from the neurosecretory cells of the abdominal neurohaemal organ of *R. prolixus*. Another aminergic link with insect neurosecretion appears in *Rhodnius* where amines are involved, perhaps at interneurons, in enhancing release of ovulation hormone (Orchard *et al.*, 1983).

II.2. Adipokinetic hormones

Peptides of the AKH family are wide spread among insects, and are probably the best studied insect peptides. Adipokinetic hormone is one of the most extensively investigated insect neurohormone of recent years (Jaffe *et al.*, 1986; Goldsworthy and Mordue, 1989; Ziegler *et al.*, 1990; Van Marrewijk *et al.*, 1992, 1996; Gaede, 1996). The existence of an AKH in the CC of locusts which regulated lipid utilization during prolonged flight was first suggested by Mayer and Candy (1969) in *S. gregaria* and Beenakkers (1969 a) in *L. migratoria* respectively. The locust AKH, was found to have cross-reactivity with the red pigment concentrating hormone (RPCH) of the shrimp, *Pandalus borealis* (Fernlund, 1974). When amino acid analysis and sequencing were carried out, it was found that these peptides had similar amino acid sequence and they were thus included in the adipokinetic hormone/red pigment

concentrating hormone (AKH/RPCH) family. Meanwhile a large number of peptides were identified and added to this group (Table II.1).

The AKH was released into the haemolymph from the CC during flight, and was responsible for the characteristic increase in haemolymph lipid (Beenakkers, 1965). Besides lipid mobilization from the fat body during flight (Spencer, 1975; Goldsworthy *et al.*, 1986, 1992), AKHs control the oxidation of fatty acids in flight muscle (Robinson and Goldsworthy, 1977), inhibit lipid synthesis by the fat body (Gokuldas *et al.*, 1988; Gokuldas, 1989; Lee and Goldsworthy, 1995), regulate the conversion of lipoprotein in haemolymph (Wheeler and Goldsworthy, 1985), inhibit synthesis of proteins (Carlisle and Loughton, 1986; Moshitzky and Applebaum, 1990; Cusinato *et al.* 1991), accelerate heart contraction (Scarborough *et al.*, 1984), muscle contraction (O'Shea *et al.*, 1984), and activate fat body phosphorylase (Steele, 1963; Siegert and Mordue, 1994). Recently inhibition of RNA synthesis by AKHs has been reported (Kodrik and Goldsworthy, 1994).

Peptides that characteristically elevate haemolymph lipids or carbohydrates from stores in the fat body have been structurally identified in a number of major insect orders (Gaede, 1990 a). At present there are at least 31 identified members of this group of peptide hormones (AKH/RPCH) from the orders Odonata, Orthoptera, Dictyoptera, Lepidoptera, Coleoptera and

Table II.1. Primary structures of peptides of the AKH/RPCH family

Sl. No.	Name	Amino acid sequence	Reference
1	*Locusta* AKH-I	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Stone *et al.* (1976)
2	*Phymateus* AKH	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-SerNH_2	Gaede *et al.* (1996)
3	*Decapotoma* CC	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-Trp-Gly-AsnNH_2	Gaede (1995)
4	*Carausius* HrTH-I	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gaede *et al.* (1992)
5	*Carausius* HrTH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gaede and Rinehart (1987 a)
6	*Phymateus* CC	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-SerNH_2	Gaede and Kellner (1995)
7	*Tabanus* HOTH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-Trp-Gly-TyrNH_2	Jaffe *et al.* (1989)
8	*Heliothis* HrTH	pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-AsnNH_2	Jaffe *et al.* (1988)
9	*Romalea* CC	pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2	Gaede *et al.* (1988)
10	*Blaberus* HrTH	pGlu-Val-Asn-Phe-Ser-Pro-Gly-Trp-Gly-ThrNH_2	Hayes *et al.* (1986)
11	*Platypleura* HrTH I & II	pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-AsnNH_2	Gaede and Janssens (1994)
12	*Manduca* AKH	pGlu-Leu-Thr -Phe-Thr-Ser-Ser- Trp-GlyNH_2	Ziegler *et al.* (1985)
13	*Pseudagrion* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Gly-TrpNH_2	Janssens *et al.* (1994)
14	*Libellula* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Ser-TrpNH_2	Gaede (1990 c)
15	*Empusa* AKH	pGlu-Val-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Gaede (1991 a)
16	*Anax* AKH	pGlu-Val-Asn-Phe-Ser-Pro-Ser-TrpNH_2	Gaede *et al.* (1994)
17	*Periplaneta* CAH-I	pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH_2	Witten *et al.* (1984)

18	*Gryllus* AKH	pGlu-Val-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Gaede and Rinehart (1987 b)
19	*Tenebrio* HrTH	pGlu-Leu-Asn-Phe-Ser-Pro-Asn-TrpNH_2	Gaede and Rosinski (1990)
20	*Pandalus* RPCH	pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2	Fernlund and Josefsson (1972)
21	*Locusta* AKH-II	pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH_2	Siegert *et al.* (1985)
22	*Schistocerca* AKH-II	pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Siegert *et al.* (1985)
23	*Melolontha* CC	pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-TrpNH_2	Gaede (1991 b)
24	*Onitis* CC-I	pGlu-Tyr-Asn-Phe-Ser-Thr-Gly-TrpNH_2	Gaede (1997)
25	*Onitis* CC-II	pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-TrpNH_2	Gaede (1997)
26	*Locusta* AKH-III	pGlu-Leu-Asn-Phe-Thr--Pro-Trp-TrpNH_2	Oudejans *et al.* (1991)
27	*Microhodotermes* CC	pGlu-Ile-Asn-Phe-Thr-Pro-Asn-TrpNH_2	Liebrich *et al.* (1995)
28	*Polyphaga* HrTH	pGlu-Ile-Thr-Phe-Thr-Pro-Asn-TrpNH_2	Gaede and Kellner (1992)
29	*Periplaneta* CAH-II	pGlu-Leu-Thr-Phe-Thr-Pro-Asn-TrpNH_2	Witten *et al.* (1984)
30	*Tabanus* AKH	pGlu-Leu-Thr-Phe-Thr-Pro-Gly-TrpNH_2	Jaffe *et al.* (1989)
31	*Phormia* HrTH	pGlu-Leu-Thr-Phe-Ser-Pro-Asp-TrpNH_2	Gaede *et al.* (1990)

Diptera (Gaede, 1990 a, 1992 a, 1996, 1997; Keeley *et al.*, 1991; Gaede *et al.*, 1994). Such a hormone has never been described for any Hymenopteran species (Woodring *et al.*, 1994).

II.2.1. Source of AKH

The insect corpora cardiaca (CC) are the major neurohaemal organs that store and release neurohormones synthesised by neurosecretory cells in the brain (Orchard and Loughton, 1985). The AKHs are produced in the intrinsic secretory cells of CC. In locusts, these intrinsic cells form a separate lobe called the glandular lobe. The majority of the adipokinetic activity in locusts is located in the glandular lobe (Goldsworthy *et al.*, 1972; Hekimi and O'Shea, 1985). Stone and Mordue (1979) revealed that adipokinetic activity of the glandular lobe resides in the electron-dense granules of 200-600 nm diameter detected by Rademakers and Beenakkers (1977) and Krogh and Normann (1977). Schooneveld *et al.* (1983) demonstrated immunochemically that the glandular lobes of the CC where AKH-I is stored in secretory granules, respond with high intensity to antiserum raised against Tyr-AKH-I.

Adipokinetic hormones have been reported in several insects. These hormones are released during flight in the monarch butterfly, *Danaus plexippus* and the butterfly *Vanessa cardui* (Dallmann *et al.*, 1981), the tobacco hornworm moth *M. sexta* (Ziegler and Schulz, 1986), the horsefly *Tabanus*

atratus and corn earworm moth *H. zea* (Jaffe *et al.*, 1988). These neuropeptides have also been detected in crude CC-extracts from a variety of insects (For reviews see, Goldsworthy and Gaede, 1983; Gaede, 1988, 1990 c). In different insects, AKHs vary only slightly in their chain length and amino acid sequence.

II.2.2. Isolation and structural characterization

The CC of insects contain intrinsic glandular cells producing a variety of bioactive factors affecting metabolic processes. The grasshopper neuropeptide adipokinetc hormones (AKH-I and II) were among the first of a growing family of structurally similar arthropod hormones and neuroregulators to be isolated and sequenced. These peptides have been isolated and purified using reversed phase high performance liquid chromatography (RP-HPLC, Gaede *et al.*, 1984) and the blocked peptides sequenced using fast atom bombardment mass spectrometry-tandem mass spectrometry (Ziegler *et al.*, 1985). Adipokinetic hormone I was isolated from the glandular lobes of *L. migratoria* and *S. gregaria* by Stone *et al.* (1976) and presented a complete structural elucidation of AKH-I using about 650 nmol of the hormone. It was shown to be an uncharged decapeptide having a molecular weight of 1158 and with the following amino acid sequence.

pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH_2

Adipokinetic hormones are N- and C- terminally blocked; pyroglutamic acid (PCA) as the blocked NH_2 terminal residue and amide on the threonine as the COOH-terminal residue. The primary sequence was confirmed later by synthesis of the hormone (Broomfield and Hardy, 1977; Yamashiro *et al.*, 1981). This peptide is now denoted as Lom-AKH-I (*Locusta migratoria* adipokinetic hormone- I (Raina and Gaede, 1988).

A second adipokinetic hormone was isolated from CC of the locust, *S. gregaria* (Carlson *et al.*, 1979) and *L. migratoria* (Gaede, 1984) and designated as AKH-II-S and AKH-II-L respectively. They are octapeptides with blocked N- and C- termini as in AKH-I (Siegert *et al.*, 1985). A third new adipokinetic hormone (Lom-AKH-III) was isolated from the glandular lobes of the CC of *L. migratoria* (Oudejans *et al.*, 1991) with similarly blocked N- and C- termini.

The amino acid sequence of AKH-II-S, AKH-II-L and AKH-III-L are

AKH-II-S **pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH$_2$**

AKH-II-L **pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH$_2$**

AKH-III-L **pGlu-Leu-Asn-Phe-Thr-Pro-Trp-TrpNH$_2$**

Adipokinetic and hypertrehalosaemic peptides have been found in the neuroendocrine organs of several insect orders. All these peptides are octa-nona-decapeptides and are included in one family, the AKH-RPCH family

because they are similar in structure and function. The common structural features are a pyroglutamyl residue at the N-terminus and an amide at C-terminus. Furthermore, residues 4 and 8 respectively are always phenylalanine and tryptophan (Wheeler *et al.*, 1988; Gaede, 1990 a). In four of them (AKH-I, AKH-II-S, AKH-II-L and RPCH), the first four amino acids from the N-terminus are identical, while in others conservative changes are observed; at position 2, leucine to valine, at position 5, threonine to serine, at position 3, an aspargine to threonine replacements are found. At position 7, replacements of aspargine by glycine and serine and at position 10, a change from threonine to tyrosine and aspargine are noticed. Substitution at position 6 can be a proline, serine, alanine or threonine residue.

II.2.3. Synthesis and release of AKH

The corpora cardiaca of the locust *S. gregaria*, provide a model system to study neuropeptide biosynthesis and processing. Hekimi and O'Shea (1985, 1987) using an *in vitro* organ culture, studied the synthesis of AKHs in *S. gregaria*. Moshitzky *et al.* (1987 a) found that AKHs are synthesized in the brain of locusts. Noyes and Schaffer (1990) reported that AKH-I and AKH-II are synthesized from very small mRNAs of 550 bases in *S. nitans*. O'Shea and Rayne (1992) demonstrated that the biosynthesis of AKH-I and II involves the enzymatic processing of larger, precursor polypeptides. This bigger peptide

molecule is called Pro-AKH. Two other small polypeptides (P_1 and P_2) which showed immunoreactivity to anti-AKH and contained [^{3}H] tryptophan were proposed to be the precursors of AKHs. Prior to processing the AKH prohormones form dimers in the endoplasmic reticulum which are the direct precursors of AKHs (Hekimi *et al.*, 1989). The glandular lobes of the CC contain approximately 6000-10000 intrinsic neurosecretory cells which are devoted to the biosynthesis of the AKHs (Hekimi *et al.*, 1989; Aellen *et al.*, 1989). Oudejans *et al.* (1990) reported that in *L. migratoria* total time necessary for biosynthesis and processing of the bioactive AKHs is only 75 minutes.

Flight is the primary stimulus for the release of AKHs in adult locusts. Release of AKH was found to be under the control of secretomotor centers in the lateral areas of the protocerebrum (Rademakers, 1977) and this control is probably by octopaminergic neurons (Orchard and Loughton, 1981). Jutsum and Goldsworthy (1976) have suggested that the concentrations of metabolic fuels, such as trehalose and diacylglycerols in the haemolymph control the release of AKH. Houben and Beenakkers (1973) have demonstrated that the release of hormones is signalled by a decrease in haemolymph trehalose which becomes depleted after a few minutes of flight. However, sucrose injections also cause a delay in lipid mobilization (Cheeseman *et al.*, 1976) indicating

that the drop in trehalose level during the first few minutes of flight cannot be the primary stimulus for AKH release (for a review, see Goldsworthy, 1983). Elevated levels of intracellular Ca^{2+} are found to be one of the critical factors which initiate the process of AKH release. Pannabecker and Orchard (1987) reported that an influx of extracellular Ca^{2+} into the neurosecretory cells provide an essential trigger for initiating AKH release. Pannabecker *et al.* (1987) showed that electrical stimulation of the nerve supplying the CC (nervi corpori cardiaci, NCC-II) results in hormone release. Octopamine mimicked this activity accompanied by an elevation of cAMP and requires extracellular Ca^{2+}. The decline of octopamine levels after 15-20 min of flight is accompanied by a decline in haemolymph trehalose levels, which in turn may contribute to the continued release of AKHs.

Although originally described as adult hormones, AKHs have been shown to serve functions in larval insects. The presence of AKHs have been reported in the larval stages of the *L. migratoria* and *S. gregaria* (Goldsworthy *et al.*, 1973; Gaede *and* Beenakkers, 1977; Hekimi and O'Shea, 1985; Siegert and Mordue, 1986). However, larval AKH is less active than adults (Mwangi and Goldsworthy, 1977) either due to insufficient cAMP level to activate the lipase system (Gaede and Beenakkers, 1977) or due to lack of C-proteins needed in the lipoprotein conversion (Mwangi and Goldsworthy, 1977).

Material with adipokinetic activity is released *in vitro* from neurosecretory cells of the CC in larval locusts (Pannabecker *et al.*, 1987).

II.2.4. Metabolism of AKH

Despite the great advances made in the study of insect peptide hormones and transmitters, there is relatively very little known about the mechanisms of inactivation of insect neuropeptides. Not much work has been done to examine the fate of AKH after they affect the target tissues. The half-life of AKH in the circulation of locusts has been estimated to be approximately 24 minutes (Cheeseman *et al.*, 1976; Cheeseman and Goldsworthy, 1979). Mordue and Stone (1978) reported that AKH-I activity 'disappears' from the bathing medium of semi-isolated locust Malpighian tubule (MT) preparations and concluded that breakdown of AKH-I takes place within the cells of the MTs. Siegert and Mordue (1987) have studied the degradation of syn AKH-I using homogenates of MTs from *S. gregaria* and reported that the breakdown products did not exhibit lipid mobilizing activity.. Though AKH is readily destroyed by many potent proteases, but such enzymes are not reported in insect haemolymph. Inactivation of AKH may be achieved *in vivo* by extracellular inactivating enzymes, either by circulating peptidases or by peptidases on surfaces of tissues in contact with the haemolymph. Matsas *et al.* (1983) reported that peptide inactivating enzymes are limited in number

but relatively broad in their peptide specificity. The inactivation of many neuropeptides is accomplished by a family of related enzymes in both vertebrates and invertebrates. Cell surface peptidases have an important role in the metabolism and inactivation of neuropeptide transmitters and hormones (Mckelvy and Blumberg, 1986; Turner *et al.*, 1985; Erdos and Skidgel, 1989). These proteins are anchored to the plasma membrane and with their active sites facing the extracellular space, they are well placed to hydrolyse peptide messengers (Kenney *et al.*, 1987).

Rayne and O'Shea (1992) reported that AKH is very stable in *S. gregaria* haemolymph, but not in MTs, fat body or flight muscles. The mechanism proposed that AKH-degrading enzymes located on the surface of cells are responsible for AKH metabolism. The fat body is the major target tissue of AKH and binds these peptides through its receptors which may be followed by internalization and breakdown of the peptide. The breakdown products are AKH I-1, AKH I-2 and AKH I-3 (Siegert and Mordue, 1992 a,b).

There is some evidence to indicate a possible role of endopeptidase in inactivating peptide hormones in peripheral tissues of insects. The breakdown was initiated by cleavage of the Asn-Phe bond of both AKH-I and II and this hydrolysis was sensitive to inhibition by phosphoramidon and EDTA (Rayne and O'Shea 1992). The AKH-inactivating endopeptidase is effective *in vitro*

between pH 6.5 and pH 8.0, that it requires divalent cations, and is sensitive to phosphoramidon, a specific inhibitor of mammalian endopeptidase. Lamango and Issac (1993) have identified a phosphoramidon sensitive endopeptidase activity from the head membranes of *M. domestica*. The phosphoramidon was able to protect AKH-I from degradation by head membranes.

II.2.5. Quantitation of AKH

Though the mode of action of AKHs have been studied in detail (see Mordue and Stone, 1981; Beenakkers *et al.*, 1981a), very little information is available on the hormone titre in the CC. Stone *et al.* (1976) estimated the AKH content of CC to be between 400-700 pmole in *S. gregaria* and 200-500 pmole in *L. migratoria* on the basis of amino acid analysis. Siegert and Mordue (1986) reported that in 6 weeks old adults contain an amount of 900 pmole AKH-I in *S. gregaria* and more than 540 pmole in *L. migratoria*. They also noticed that in both insects, AKH-I and II are synthesized for more than ten days after the adult moult. The CC of *S. gregaria* contained more of the peptides than *L. migratoria*. In most stages, females contained more hormones than males. In *S. gregaria* AKH-I increases from 190 pmole (early 5th instar) to 920 pmole (in adult males, 6 weeks old) and to 1200 pmole (in females, 6 weeks old). Likewise, AKH-II increased from about 50 pmole to 180 pmole in

males and 260 pmole in females. The maximum amount of AKH-I found in adult *L. migratoria* was approximately 550 pmole in males and about 750 pmole in females. Adipokinetic hormone-II was around 100 pmole and 125 pmole in males and females respectively. At any given time during the period studied, the CC contained more AKH-I than AKH-II: the molar ratio, AKH-I : AKH-II ranged from 2:1 to 6:1. Moshitzky *et al.* (1987 b), using radioimmunoassay quantified AKH-I from the locust, *L. migratoria* CC and reported a value between 175 and 440 pmole per CC which corresponds to the values determined using bioassay techniques (Stone *et al.*, 1976). Quantification with RP-HPLC also indicates that the total content of both AKH-I and II changes between stages.

Ziegler *et al.* (1984) suggested that the *Manduca* CC contained 10-20 times less adipokinetic peptide than that in the locust. The content of AKH in the CC of *M. sexta* was estimated by radioimmunoassay to be 33 pmole per CC pair in adults, and 3.5 pmole per CC pair in larvae (Fox and Reynolds, 1990). The smaller quantity of hormone in adult *Manduca* compared with the quantity of AKH-I in the adult locust reflects difference between the two species in the time spent in sustained flight as well as the greater longevity of the adult locust (several weeks) compared with the adult moth (one week). A similarity with the locust is that a considerable increase in the peptide content

of the CC occurs during metamorphosis. Evidently the demands of flight in the adult stage require a larger store of hormone. Orchard *et al.* (1991) reported that the CC of *Pseudaletia unipuncta* males contain approximately 17.6 pmole of *Manduca*-AKH equivalents. This is almost similar to the amounts observed in other Lepidoptera (Ziegler *et al.*, 1985; Ziegler, 1990) but considerably lower than the 500-1000 pmole reported for the locusts (Orchard, 1987).

II.2.6. Structure - activity relationships

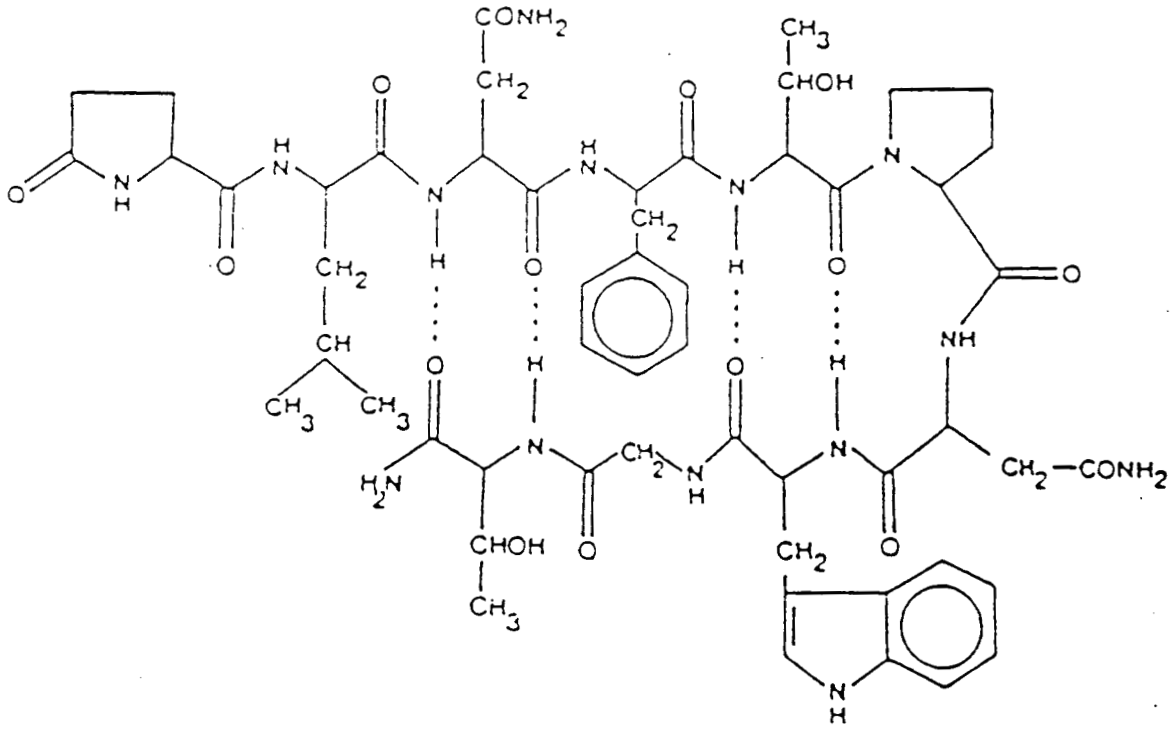
Generally, binding of neuropeptide molecules to specific membrane bound receptor proteins in target cells is a prerequisite for exerting hormonal activity. The structure of the peptides of the AKH/RPCH family have been found to be remarkably stable with only exchanges by a few selective amino acids because of the interdependency of the hormone and the receptor. However, different biological functions can be achieved by differentiation of the specific receptor or receptor binding sites during evolution. Different binding properties have been detected for the receptors in the fat body of the locusts, *L. migratoria* and *S. gregaria* (Stone *et al.*, 1978); the receptors in *S. gregaria* are more specific in response than those of *L. migratoria* (Gaede, 1989). The biological information is encoded within the structure of the different peptides. The structure-activity relationship elucidate the special

structural features of the peptides which are important for receptor recognition. Extensive structure-activity relationship studies for the actions of AKHs on lipid mobilization have been carried out (Stone *et al.*, 1978; Mordue, 1980; Hardy and Sheppard, 1983; Orchard, 1987; Wheeler *et al.*, 1988; Keeley *et al.*, 1991).

Peptides with a chain length of at least 8 amino acid residues were found to be eliciting a biological response. Furthermore, the N-terminal pyroglutamate and the amidated C-terminal are essential and at the C-terminus, the natural sequence (..... Trp-Gly-ThrNH_2) is necessary for maximum activity, whereas the sequence (..... Trp-Thr-GlyNH_2) has reduced potency. Analogue of AKH with 3,4-dehydroproline at position 6 exhibits a reduction of activity to 20% (Hardy and Sheppard, 1983). Studies on secondary structure of AKH showed the existence of a β-bend among residues 5-8 (Stone *et al.*, 1978); the structure is further strengthened by hydrogen bonding between residues 5 and 8 and 3 and 10 (Fig. II.1). Replacements of 5-10 residues resulted in reduced activity (Hardy and Sheppard, 1983; Mordue and Morgan, 1985).

Gaede (1990 a,b) proposed that different binding requirements exists for the AKH/RPCH peptides with respect to their receptors in shrimps, locusts and cockroaches. In cockroach HTH the blocked termini are important and

Fig. II.1. Adipokinetic hormone - secondary structure demonstrating the postulated β-bend involving residues 5-8.



[Redrawn from Mordue and Morgan (1985)]

the amino acids, Phe, Pro and Trp at position 4, 5 and 8 are essential for receptor binding. Using natural and synthetic analogues of the peptides Hayes *et al.* (1986), Ford *et al.* (1988) and Hayes and Keeley (1990) reported that pGlu, Phe and and Trp are essential for the initial recognition of the receptor and maximal response. They also proposed that the N-terminal octapeptide region is more important in recognizing the receptor than the C-terminal dipeptide.

In *P. americana*, a Pro residue at position 6 is not essential for maximum response. Gaede (1990 b) proposed that the two endogenous peptides of *P. americana* with hypertrehalosaemic activity exhibited identical dose response curves. The tenebrionid peptide (Tem-HrTH) has an identical structure to that of *Periplaneta* peptide-I, but in position 2 it is Leu instead of Val. In *Mantid* peptide at position 5 it is a Thr instead of Ser. Gaede (1990 b) stated that an Asn or Gly residue at position 7, but no Ser are important for receptor binding. The fact that Phe at position 4 is more important for receptor interaction, is adipokinetic in locusts and *M. sexta* and hypertrehalosaemic in *P. americana* and *Blaberus discoidalis* suggested that Phe is conserved in all AKH/RPCH peptides so far investigated (Gaede, 1990 b; Hayes and Keeley, 1990; Fox and Reynolds, 1991).

Hayes and Keeley (1990) suggested that in *B. discoidalis* HTH, a

hydrogen bond between Ser and Trp amide gives maximum intrapeptide interaction. In AKH-I of *L. migratoria* a hydrophobic cluster of Leu, Phe and Trp are important for receptor interaction. The endogenous peptides were found to be the most potent molecules to achieve optimal binding in *Blaberus* (Hayes and Keeley, 1990) and *Manduca* (Fox and Reynolds, 1991). Goldsworthy *et al.*, (1986) and Gaede (1990 b) reported that the *Melolontha* peptide which is exactly the same as the *Tenebrio* peptide, but contains the Tyr at position 4 shows a much reduced potency. The other octapeptide which showed a largely reduced potency, was the *Polyphaga* hypertrehalosaemic peptide. It differs from the quite active *Periplaneta* hypertrehalosaemic peptide II (Goldsworthy *et al.*, 1986; Gaede, 1990 b). In the cockroach *B. discoidalis* the Tyr analogue of the endogenous HTH is almost as active as the parent molecule (Hayes and Keeley, 1990). Gaede (1993) proposed that the *Tabanus* hypotrehalosaemic peptide was even less active than the *Tabanus* adipokinetic peptide. It seemed therefore, that the hydrophobic Tyr at position 10 instead of neutral Thr in the AKH-I is responsible for the reduced potency. The *Heliothis* hypertrehalosaemic peptide show a reduced activity because of lack of a Pro residue at position 6, which is necessary to keep the molecule in a folded position (Stone *et al.*, 1978; Goldsworthy *et al.*, 1986; Wheeler *et al.*, 1990; Gaede, 1992 b).

II.2.7. Physiological actions of AKH

A primary function of the neuropeptides belonging to the AKH/RPCH family in insects is believed to be the rapid mobilization of substrates required for fuelling high energy activities such as flight. The actions of the adipokinetic family peptides are quite diverse and certainly broader than their name implies. In the locust, *L. migratoria*, which utilizes carbohydrate initially and changes to lipid during prolonged flight, the role of AKH-I and II in stimulating the release of diacylglycerol from the fat body has been well documented (Mayer and Candy, 1969; Goldsworthy, 1983; Gaede, 1990 a). The hormone was later found to control other functions like transport and unloading of lipids as specific lipoprotein complex; flight muscle metabolism, myotropic activity, fat body protein synthesis, synthesis of fatty acids, synthesis of cytochrome hemes during fat body mitochondriogenesis and JH-mediated protein synthesis (Robinson and Goldsworthy, 1977; Asher *et al.*, 1984; Goldsworthy, 1983; Goldsworthy and Wheeler, 1984; Goldsworthy and Mordue, 1989; Gaede, 1990 a, 1992, b; Keeley *et al.*, 1991).

II.2.7.1. Lipid mobilization

During flight and other activities of energy demand in locusts, triacylglycerol (TAG) reserves in the fat body are mobilized, released into the

haemolymph as diacylglycerol (DAG) and transported by lipophorin to flight muscles or other sites of utilization (Chino and Downer, 1982). Lipophorin and Ca^{2+} are required for DAG release from fat body. Cyclic AMP has been proposed as a specific second messenger in mediating the action of AKH (Spencer and Candy, 1976; Gaede and Beenakkers, 1977). Pines *et al.* (1981) proposed that the lipid mobilization in locust fat body proceeds in a similar manner to the mobilization of lipid in vertebrate tissue in which the production of cAMP is followed by activation of protein kinase and phosphorylation of lipase.

Lipids stored as TAGs in the fat body provide the major source of energy for flight in many insects. The AKH stimulate the fat body to degrade metabolic stores for the synthesis and release of circulating metabolites. These metabolites serve as the major energy sources for peripheral tissues, such as muscles and are usually lipids (as in locusts, Mayer and Candy, 1969) or carbohydrates (as in cockroaches, Steele, 1961). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in larvae and lipids in adults (Zielger, 1984; Ziegler *et al.*, 1984). Commencement of flight in adults of species which rely on lipid as a flight fuel initiates the mobilization of lipid stores from the fat body. In *Tenebrio molitor, P. americana, Carausius morosus* and *Gryllus bimaculatus*, there were no enhanced lipid release after flight or

injection of the hormone extract from their CC. When CC-extract was injected into locusts, these extracts had adipokinetic effect (see reviews, Goldsworthy, 1983; Beenakkers *et al.*, 1985; Gaede, 1990 a, b; Cusinato *et al.*, 1991).

The importance of cAMP in hormone action was first recognized by Sutherland and Rall (1958). Adipokinetic hormones appear to exert their effects on locust fat body through cAMP as the second messenger. In adult *L. migratoria* injection of CC-extracts or synthetic AKH results in a 3-5 fold increase in fat body cAMP (Gaede and Holwerda,1976; Gaede and Beenakkers, 1977; Gaede, 1979; Orchard *et al.*, 1982; Asher *et al.*, 1984; Goldsworthy *et al.*, 1986; Chino *et al.*, 1989). A similar response was observed in *S. gregaria* (Spencer and Candy, 1976). Pines and Applebaum (1978) have reported a cAMP and cGMP stimulated protein kinase activity in locust fat body suggesting mobilization of DAG by cAMP or cGMP dependent protein kinases. The role of cAMP in the mobilization of lipid was substantiated by the finding that theophylline, a phosphodiesterase inhibitor, stimulates DAG output and potentiates the action of AKH in the fat body of *S. gregaria* (Spencer and Candy, 1976). They also showed that dibutyryl cAMP or theophylline mimicked the action of the CC-extract and theophylline with CC-extract increased the incorporation of [^{3}H] adenine into cAMP. Wang *et al.* (1990) have detected a positive relationship between cAMP and DAG.

Adenylate cyclase and phosphodiesterase together determine the concentration of cAMP, the activity of both enzymes being regulated by Ca^{2+} (Morishima, 1979). Spencer and Candy (1976), Wang *et al.* (1990) and Van Marrewijk *et al.* (1991) have demonstrated a dependence on extracellular calcium for AKH-I stimulated release of DAG in locusts. Hydrolysis of phosphoinositides is responsible for receptor activated calcium mobilization from intracellular stores followed by entry of extracellular calcium across the plasma membrane (Putney, 1987; Putney *et al.*, 1981, 1989).

II.2.7.2. Lipid transport

Haemolymph lipid transport has been the subject of considerable research interest since the transport vehicle, lipophorin was discovered in the 1960s and various aspects have been reviewed by several authors (Gilbert and Chino, 1974; Chino, 1985; Beenakkers *et al.*, 1986; Shapiro *et al.*, 1988; Ryan, 1990; Kanost *et al.*, 1990; Van der Horst, 1990; Law *et al.*, 1992; Weers *et al.*, 1993; Ziegler *et al.*, 1995; Engler *et al.*, 1996). Lipids represent the main source of energy in starved or long-term flying insects (Weis-Fogh, 1952; Beenakkers *et al.*, 1985). The lipid composition of the haemolymph of a large number of insects has been compiled. (Bailey, 1975; Downer and Mathews, 1976) and it has been found that neutral lipids constitute the major part of hemolymph lipids. Lipids are insoluble in water and hence animals transport

them in the form of lipoproteins in their aqueous body fluids. In general, DAGs represent the main lipid component. In insects, the main haemolymph lipoprotein is lipophorin (Chino *et al.*, 1981; Beenakkers *et al.*, 1988). Insect lipophorin probably exists in all insect species and serves as a reusable shuttle to transport various lipids including DAG, cholesterol, hydrocarbons and carotenoids between the tissues (Chino, 1985). Ryan (1990) reported that lipophorin is produced in the fat body. Lipophorins are involved in the transport of DAG from the sites of absorption to the sites of storage (Weintraub and Tietz, 1973; Chino and Downer, 1979; Tsuchida and Wells, 1988). Role of lipophorin in the transport of cholestrol from midgut (Chino and Downer, 1979) and in the transport of hydrocarbons from the oenocytes (Katase and Chino, 1984) have been established. The specific lipoproteins with which DAG is associated in the haemolymph was first isolated and purified from the pupal haemolymph of *Hyalophora cecropia* (Thomas and Gilbert, 1968) and *Philosamia cynthia* (Chino *et al.*, 1969). Thomas and Gilbert (1968) called it high-density lipoproteins. Other names have been proposed for this protein; such as diacylglycerol- carrying lipoprotein-1 (DGLP-1) (Chino *et al.*, 1969), lipoprotein-1 (Gilbert and Chino, 1974) and diglyceride-transporting lipoprotien (Mwangi and Goldsworthy, 1977). A female specific lipoprotein, vitellogenin has been isolated and purified from the haemolymph

of *H. cecropia* (Thomas and Gilbert, 1968). Vitellogenin is involved in lipid transfer from the fat body to ovaries in association with lipophorin (Chino *et al.*, 1977). In view of the functional multiplicity of lipophorin as a reusable shuttle, insect lipophorin differs from mammalian plasma lipoproteins. In mammals, the main lipid components of transport system are TAG and unestrified fatty acids which combine with plasma proteins.

The ability of lipophorin to be used as a reusable shuttle of lipid was hypothesized (Chino and Kitazawa, 1981; Chino, 1985). Subsequently numerous studies have provided support for the shuttle hypothesis (Van Heusden *et al.*, 1987, 1991, 1997; Surholt *et al.*, 1991; Gondim *et al.*, 1992). Van Heusden *et al.*(1991) hypothesized that in order for lipophorin to act as a shuttle of lipid, a lipid- depleted lipophorin must be capable of reloading with lipid. Lipophorin also appears to be important in the transport of free fatty acid in haemolymph (Soulages and Wells, 1994 a). Similarly lipophorins have been shown to transport steroids such as cholesterol which is important in steroid hormone production (Chino, 1985). The lipophorin of the south western corn borer, *Diatraea grandiosella* (Bergman and Chippendale, 1992) among other species (De Kort and Koopmanschap, 1987) carries carotenoids non-specifically, presumably in the core of the particle, and retains the pigment during diapause. The sesquiterpene JH, a key regulator of

development, is transported in the haemolymph by a number of binding proteins. One of these has been identified in *Leptinotarsa decemlineata* (De Kort and Koopmanschap, 1989), and *Diploptera punctata* (King and Tobe, 1992). The main organs of lipid loading of the lipophorin shuttle are the midgut during absorption of dietary lipids (Chino and Downer, 1979; Turunen, 1979) and the fat body during mobilization of stored lipids (Beenakkers *et al.*, 1985). Lipid unloading has been shown to occur at the fat body cells during lipid deposition (Tsuchida and Wells, 1988) and at flight muscle cells that utilize fatty acids as fuel (Van der Horst, 1990). It also occurs at other lipid storing cells such as the epithelial lipocytes of the MTs of *Aeshna* (Kukel and Komnick, 1989).

The dynamics of loading of DAG- carrying haemolymph lipoproteins during flight have been studied in locusts. In the locust AKH stimulates lipid mobilization induced by flight activity, or injection of AKH-1 into resting locusts, resulted in significant shifts in the haemolymph protein pattern involving lipoprotein- protein interactions (Beenakkers *et al.*, 1985; Van der Horst, 1983; Goldsworthy and Wheeler, 1984). In locusts, haemolymph lipoproteins are qualitatively different during and after flight from those at rest (Goldsworthy, 1983; Chino, 1985). In resting locusts, DAG is associated principally with a single lipoprotein termed A_{yellow}, but during flight this is

transformed to a higher molecular weight lipoprotein A^+ by the combination of A_{yellow} , non-lipid containing protein C_L and DAG. Injection of AKHs also results in the appearance of A^+ (activated lipophorin) which carries 10-15 times more lipid (Mwangi and Goldsworthy, 1977; Wheeler and Goldsworthy, 1985). It has been suggested that under the influence of AKH, low molecular weight protein- apoprotein-III (apLp-III) associate reversibly with existing high density lipophorin, A_{yellow} (HDLP) in the haemolymph and DAG released from the fat body to produce a new low density lipophorin (LDLP) + A^+ lipoprotein. This new lipophrorin is involved in the transport of lipids to the flight muscles (Mwangi and Goldsworthy, 1977; Van der Horst *et al.*, 1979; Wheeler and Goldsworthy, 1985). Studies show that the formation of lipoprotein A^+ is a gradual process and that intermediate forms arise between A_{yellow} and A^+ in the hemolymph (Goldsworthy *et al.*, 1985). Van Heusden and Law (1989) have investigated AKH-induced lipid mobilization from the fat body of *M. sexta*. Lipid transfer particle which transfer DAG to lipophorin have been reported from *L. migratoria* and *M. sexta* and has been purified from the haemolymph of the locust *L. migratoria* (Hirayama and Chino, 1990), the American cockroach, *P. americana* (Takeuchi and Chino, 1993) and housefly, *M. domestica* (Capurro and de Bianchi, 1990).

II.2.7.3. Lipid utilization

Adipokinetic hormones, which mobilize fat body lipids to make available fuel for primarily flight activities, also play a role in stimulating oxidation of lipids. The evolutionary success of the class Insecta has been facilitated by their ability to store large quantities of fat and use this substrate as a source of metabolic water to supplement strategies of water conservation. It is well established that locusts *L. migratoria* and *S. gregaria* can use both carbohydrates and fatty acids for flight muscle metabolism (Beenakkers, 1969 a, b; Candy, 1970). Haemolymph trehalose concentration falls rapidly during the first 10 minutes of flight and is stabilized at around 50% of the initial concentration after 30 minutes (Houben and Beenakkers, 1973; Jutsum and Goldsworthy, 1976). Concomitant with the decrease in trehalose level, the DAG content of the haemolymph is raised at the expense of fat body lipids under the action of the AKH (Beenakkers, 1969 b; Mayer and Candy, 1969).

Trehalose is the main substrate during the first 20-30 min of flight; however, during flights of longer duration, trehalose still appears to be consumed, although at a much lower rate than before (Jutsum and Goldsworthy, 1976; Van der Horst *et al.*, 1978), the main substrate then being fatty acids derived from haemolymph DAG. Worm and Beenakkers (1980) reported that in flight muscle of *L. migratoria* a change from carbohydrate to

lipid utilization is not due to absence of carbohydrate but the result of regulatory process. Mwangi and Goldsworthy (1981) demonstrated that the lipoprotein A^+ in the presence of AKH is able to provide lipids to the flight muscles during long-term flight or contraction of the flight muscles *in vitro* (Robinson and Goldsworthy, 1977). The switching over of fuel utilization requires a switch over in the metabolic pathways in the flight muscle. Robinson and Goldsworthy (1977) have shown that AKHs which mobilize fat body lipids to make available fuel for primary flight activities, also stimulates lipid oxidation in the working flight muscle of the locust. They suggested that AKHs appear to have a direct effect on the rate of lipid oxidation by increasing the activity of mitochondrial carnitine acyltransferase, thus providing more substrate for the tricarboxylic acid cycle. The increase in concentration of acyl carnitine appears to inhibit glycolysis (Storey, 1980; Goldsworthy and Wheeler, 1989). Candy (1978) has shown that octopamine stimulates the oxidation of a variety of substrates in a working perfused locust flight muscle. Large molecules of DAG carried by the haemolymph lipoprotein are hydrolysed before entry into the muscle cells, catalysed by a specific membrane bound lipoprotein lipase (Wheeler *et al.*, 1986) in the flight muscle. This lipoprotein lipase hydrolyses lipids carried as part of lipoprotien A^+ at much higher rates than those carried by A_{yellow}. Lipase appears to be regulated

by C_L-proteins in the haemolymph. In resting insects, lipase is inhibited due to high concentration of free C_L-proteins; but when lipoprotein A^+ is formed due to the action of AKH, concentration of C_L-proteins in the haemolymph decreases and lipase becomes activated (Wheeler *et al.*, 1986). Fatty acids from haemolymph acylglycerols are made available for oxidation in flight muscle mitochondria by the hydrolytic action of lipases (Beenakkers *et al.*, 1981 a, b, 1984, 1985; Candy, 1985; Downer, 1985; Beenakkers, 1991).

II.2.7.4. Inhibition of protein synthesis

Carlisle and Loughton (1979) demonstrated that injection of CC-extract or synthetic AKH into 5 day old 5th instar larva and adult of *L. migratoria* suppressed the incorporation of labelled amino acid (^{3}H-leucine) into haemolymph protein to 24%. This effect was demonstrated by Asher *et al.* (1984) in *Locusta* by using a preparation of dispersed fat body cells *in vitro*. Inhibition of protein synthesis in the locust system occurs at doses of AKH too low to bring about lipid release (Carlisle and Loughton, 1979). Higher doses of hormone had no effect on protein synthesis. Carlisle and Loughton (1986) also reported that protein synthesis in heart and midgut tissue were also inhibited by AKH. They have proposed that this inhibitory activity may function in the locust's overall adaptation to flight and it may also account for the presence of AKH both in larval locusts and other species which do not fly

for prolonged periods. They further assumed that AKH-I operates as a translational regulator of protein synthesis, since its inhibitory effect on fat body protein synthesis is very rapid. Moshitzky and Applebaum (1990) stated that in *Locusta*, AKH-I plays a major role in shutting of vitellogenin synthesis towards the end of maturation. Cusinato *et al.* (1991) have detected an octapeptide in the CC of *A. domesticus* and was found to inhibit the incorporation of (^{3}H) leucine into haemolymph proteins. The control of RNA synthesis by extracts of brain or peptides has been described in insect fat body (Osborne *et al.*, 1968; Lee and Keeley, 1994), prothoracic glands (Aizono *et al.*, 1986) and silk glands (Kodrik and Sehnal, 1991).

II.2.7.5. Inhibition of lipid synthesis

Aqueous extracts of central nervous system and CC of tsetsefly, *Glossina morsitans* contain a peptide factor which has potent inhibitory effect on lipid synthesis in the fat body (Pimley and Langley, 1981). The lipid synthesis inhibiting factor is believed to be synthesised in the median neurosecretory cells in the brain and stored in the CC. Gokuldas *et al.* (1988) and Gokuldas (1989) demonstrated that AKH play a dual role in the control of lipid synthesis in *S. gregaria*. It inhibit incorporation of [^{14}C] acetate into fatty acids and at the same time stimulate conversion of [^{14}C] glucose into glycerol component of glycerides.

II.2.7.6. Regulation of carbohydrate metabolism

Adipokinetic hormones have also been found to have hyperglycaemic activity on fat body. Hence they regulate the hyperglycaemic factor was reported in the CC of the cockroach, *P. americana* (Steele, 1961). These hormones activate fat body glycogen phosphorylase (Goldsworthy, 1970) thereby causing an elevation of haemolymph trehalose. Activation of glycogen phosphorylase by AKH was noticed in the locust fat body (Goldsworthy *et al.*, 1986). The purified peptides induce hyperglycaemia in *P. americana in vitro* (Scarborough *et al.*, 1984; Siegert, *et al.* 1986). Van Marrewijk *et al.* (1991) reported that AKH is dependent on Ca^{2+} for the rapid activation of glycogen phosphorylase.

II.3. Lipids in insects

Lipids have assumed considerable functional significance during the evolution of the class Insecta (Lubzens *et al.*, 1981; Downer, 1985; Turunen and Chippendale, 1989; Zera *et al.*, 1994). They form the essential structural components of the cell membrane and cuticle (Beament, 1961; Hadley *et al.*, 1968; Raison, 1980; Srivastava *et al.*, 1995), provide a rich source of metabolic energy for periods of sustained energy demand (Samuelson *et al.*, 1988; Ranganathan and Padmanabhan, 1994; Van Marrewijk *et al.*, 1996),

facilitate water conservation both by formation of an impermeable cuticular barrier and by yielding metabolic water upon oxidation. They are the primary energy source during periods of non feeding stages such as diapause (Downer and Matthews, 1976), long migratory flights (Beenakkers *et al.*, 1985) and during non feeding stages of development (Gilbert, 1967; Martin, 1969; Downer, 1985). Lipids serves as the essential components in the formation of the cuticle (Blomquist and Dillwith, 1985). Since the relative importance of each of these functions varies throughout development, the rate of lipid biosynthesis and the synthesis of specific lipid classes also would be expected to fluctuate according to physiological need.

As in other higher animals, lipids constitute the major energy reserve in Insects (Weintraub and Tietz, 1973; Pagani *et al.*, 1980; Kapur *et al.*, 1983; Jacome *et al.*, 1995). The amount of lipids in insects varies considerably depending on the stage of development, nutritional state, sex, environment, physiological stress and diapause. Most of the lipids in insects are stored in the fat body which is the major storage organ. Fat body is the main metabolic centre of insects thus making it comparable to the combined adipose tissue and liver in mammals (Haunerland and Shirk, 1995). The stored lipids consists mainly of neutral lipids. The content of TAG, the major component, in fat body and other tissues changes during the life of insects. Fat body also

contains other fractions such as phospholipids and sterols in smaller quantities.

The principal energy reserves in insects are the neutral lipids. The advantages of lipids over the other common metabolic reserve, glycogen, include a higher calorific content/unit weight, the liberation upon oxidation of two times more metabolic water than carbohydrate and the capacity to be stored in anhydrous form. Lipids are of great biochemical importance because of the role as the chief storage form of energy and because of their role in cellular structures. The lipids have variety of molecular shapes and size and their structure is very complex. Physiological studies indicate that fats may play a very important role in the energy metabolism of insects particularly during flight (Orchard and Lange, 1983 a, b; Wheeler and Goldsworthy, 1985; Zeigler and Schulz, 1986; Candy, 1989; Wang *et al.*, 1990; Gaede, 1992 a; Van Marrewijk *et al.*, 1996). Lipids play a structural role in cellular and intracellular membranes, and a metabolic role in most cellular activities such as energy production and storage, protein synthesis and cellular respiration.

II.3.1. Lipid metabolism in insects

Although, lipid metabolism in insects fundamentally resembles that in vertebrates, insects have developed specific systems for its mobilization,

transport and utilization during their evolution (Van der Horst and Beenakkers, 1980; Wheeler *et al.*, 1984; Wells and Law, 1988; Woodring *et al.*, 1989; Van Heusden and Law, 1989; Singh and Ryan, 1991; Surholt *et al.*, 1992; Oudegans *et al.*, 1996). In general, lipid metabolism in insects resembles that in mammals and it includes the biosynthesis of various lipids and their oxidation. Fat is stored and transported principally in the form of tri-and diacylglycerol and ester hydrolysis is a necessary prelude to oxidation of the fatty acid.

II.3.1.1. Biosynthesis of fatty acids

Fat body is the major site of fatty acid synthesis in insects and also serves as a storage organ for nutrient reserves. A major portion of the insect lipids is usually found in the fat body, with over 90% of total fat body lipid being present as TAG (Chino and Gilbert, 1965 b; Gilbert, 1967; Sacktor, 1970; Beenakkers *et al.*, 1981 a, b; Downer, 1985). The TAG is the main source of metabolic energy in insects which undergo prolonged periods of metabolic activity without feeding during diapause, migratory flight and non-feeding developmental stages of embryogenesis and pupation (Downer, 1985). These lipid stores are derived from absorbed dietary lipids transported to the fat body, through haemolymph. Insects also synthesize lipids from non lipid

precursors such as proteins, carbohydrates and amino acids (Halarnkar *et al.*, 1989).

Fatty acid biosynthesis in insect fat body occurs by a pathway similar to that occurring in mammals and other animal groups. The incorporation of labelled precursors such as [^{14}C] acetate, [^{14}C] glucose etc. have been used to trace the synthesis of fatty acids. Clements (1959) has succeeded in tracing the incorporation of labelled metabolites such as acetate, glucose and some amino acids into fatty acids in the isolated fat body tissue of *S. gregaria*. The major fatty acid produced was palmitic acid. Walker and Bailey (1970 a, b) have demonstrated the conversion of carbohydrate to lipid in the fat body of *S. gregaria*. Glucose is converted to pyruvate via the glycolytic and pentose phosphate pathways. The pyruvate is transported into mitochondria and oxidised to acetyl Co-A, which is converted to citrate. The citrate is transported into the cytosol and acetyl Co-A for fatty acid synthesis is regenerated by the action of citrate lyase. Storey and Bailey (1978 a) have demonstrated a similar pathway in the fat body of *P. americana*. The primary products of *de novo* fatty acid synthesis are C16: 0, C18: 0 and C18:1 (Robins *et al.*, 1960; Sridhara and Bhat, 1965 a). The synthesis of palmitic acid (C16:0) from acetyl Co-A involves the following reactions.

$$\text{Acetyl CoA} + 7 \text{ malonyl CoA} + 14 \text{ NADPH} + 14 \text{ H}^+ \rightarrow 1 \text{ Palmitic acid}$$

$$+ 7 \text{ CO}_2 + 8 \text{ CoA} + 14 \text{ NADP}^+ + 6 \text{ H}_2\text{O}$$

The two enzyme systems, acetyl-CoA carboxylase and fatty acid synthetase, responsible for effecting the fatty acid synthesis, have been reported in many insects. Acetyl-CoA carboxylase is involved in the conversion of acetyl-CoA to malonyl-CoA and fatty acid synthase complex converts malonyl-CoA to long chain fatty acids (Storey and Bailey, 1978 a; Lizarbe *et al.*, 1980; Munday and Hardie, 1984).

Conversion of malonyl-CoA to long chain fatty acids is catalysed by the fatty acid synthetase complex. Fatty acid synthetase complex in insect systems that have been studied the final chain length of fatty acids varies between C 14:0 (Takaya and Miura, 1968; Ryan *et al.*, 1982), C 16: 0 (Municio *et al.*, 1977) and C 18:0 (Thompson *et al.*, 1975). Fatty acid synthetase has been separated from *Lucilia Sericata* (Thompson *et al.*, 1975), *Galleria mellonella* (Thompson and Barlow, 1976) and *Ceritits capitata* (Municio *et al.*, 1977; Lizarbe *et al.*, 1977). Gavilanes *et al.*, (1979, 1981) described the enzyme complex in *C. capitata* as a lipoprotein with TAG as the most abundant lipid class. Metabolic studies using radiolabelled precursors have confirmed that most insects are unable to synthesize long chain polyunsaturated fatty acids (Fast, 1964; Dadd, 1981).

II.3.1.2. Biosynthesis of acylglycerols

Fatty acids produced by lipolysis from dietary lipids are stored in the fat body mainly in esterified form as TAG. Studies on the mechanism of acylglycerol formation in insects indicate that the synthetic pathway is similar to those described for other animal groups (Kennedy, 1961; Johnston, 1963). The incorporation of fatty acids into fat body acylglycerols has been demonstrated in a number of insect species both *in vivo* and *in vitro* (Bailey, 1975; Municio *et al.*, 1975).

The two pathways for the synthesis of TAG in mammals i.e., the α-glycerolphosphate and monoacylglycerol pathways are found also. The monoacylglycerol pathway is concerned primarily with DAG synthesis during demand and the glycerol-3-phosphate is involved in the synthesis of storage TAG. Tietz (1969) showed that in the locust fat body glycerolphosphate and diacylglycerol acyltransferase activities are associated with the microsomal fraction whereas the supernatant obtained from centrifugation at 140,000 x g contains phosphatidate phosphohydrolase, catalysing the conversion of phosphatidate to DAG and TAG. The mitochondrial fraction contains glycerolkinase enabling the conversion of glycerol to glycerol-3-phosphate which in turn is utilized by the fat body homogenates for acylglycerol synthesis.

II.3.1.3. Biosynthesis of phospholipids

As in vertebrates, the principal biosynthetic route of phospholipids in insects is the cytidine pathway (Kennedy and Weiss, 1956). The major phospholipids in insects are phosphatidyl choline and phosphatidyl ethanolamine. The cytidine pathway involves the phosphorylation of the appropriate base in the presence of phosphokinase, which is then 'activated' to yield cytidine diphosphoryl derivative. The cytidine phosphoryl derivative is finally cleaved and the phosphorylated base is transferred to a 1,2-diacylglycerol.

In insects, the major site of phosphoacylglycerol synthesis is fat body (Crone *et al.*, 1966; Shelly and Hodgson, 1970; Kulkarni *et al.*, 1971; Atella *et al.*, 1991) and small amounts are synthesized in other tissues such as nervous tissues, flight muscle and gut (Lenartowicz *et al.*, 1964; Bridges, 1972; Fournier *et al.*, 1995). In addition to cytidine pathway phosphatidyl choline syntheis may result from the methylation of ethanolamine (Hirata and Axelrod, 1980).

Total lipid biosynthesis and the synthesis of the major lipid classes fluctuate greatly during development of many insects. Because lipid is the main energy storage form for most insects, lipid biosynthesis, primarily of

TAG, should occur during periods of heavy feeding. Developmental changes in the synthesis of total lipids and individual lipid classes have been studied systematically in the Mediterranean fruitfly, *C. capitata* (Municio *et al.*, 1974), the cabbage looper, *Trichoplusia ni* (De Renobales and Blomquist, 1983; Dwyer *et al.*, 1986) and in the desert locust, *S. gregaria* (Walker and Bailey, 1970 b). The oxidative pentose shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are major sources of NADPH for lipid synthesis. It is known that dietary conditions affect the rate of fatty acid synthesis in animals (Hill *et al.*, 1958). The rate of fatty acid synthesis is the function of the nutritional state of the animal.

II.3.1.4. Oxidation of fatty acids

Most of the lipids in insects is stored in the fat body but is oxidised at other sites (Stone *et al.*, 1976; Pines *et al.*, 1981; Beenakkers *et al.*, 1985; Ziegler and Schulz, 1986; Gies *et al.*, 1988; Wheeler, 1989; Gaede, 1992 a, b; Soulages and Wells, 1994 a, b; Gaede *et al.*, 1996). In many insects lipids provide a major fuel for long term flight (Beenakkers, 1991). In locusts, lipid is stored in the fat body as TAG and during flight this is converted to DAG for transport to the flight muscles (Bailey, 1975). Locust flight muscle is able to hydrolyze DAG by a lipase (Crabtree and Newsholme, 1972) and the fatty acids produced are then oxidized by the muscle.

The energy contained in the fatty acids is released through the sequential removal of 2C-units in the form of acetyl-S-CoA. The units of acetyl-S-CoA undergo condensation with oxaloacetate to form citrate which enters the tricarboxylic acid cycle. (Beenakkers *et al.*, 1984, 1985; Candy, 1985; Downer, 1985). Oxidation of lipids takes place primarily in the flight muscles of insects. The initial activation of fatty acid is catalysed by the enzyme fatty acyl-S-CoA synthetase which has been demonstrated in several insect species (Zebe and Mc Shan, 1959; Domroese and Gilbert, 1964; Beenakkers and Henderson, 1967). The enzymes of β-oxidative pathway, β-ketoacyl-CoA thiolase and β-hydroxy-acyl-CoA dehydrogenase have been reported in flight muscle mitochondria of several insect species (Beenakkers, 1969; Storey and Bailey, 1978 a; Storey and Storey, 1981). Other requirements of β-oxidation such as Mg^{2+}, ATP, CoA and TCA cycle intermediates have been reported in insects (see, Downer, 1985). Under certain conditions, accumulation of ketone bodies, acetoacetate and D-3-hydroxy butyrate occur during fatty acid oxidation. β-Hydroxy butyrate dehydrogenase has been reported in locust flight muscle and fat body (Hill *et al.*, 1972) and acetoacetate CoA transferase has been detected in the flight muscles of several species (Beis *et al.*, 1980) and in the cerebral ganglia of locusts (Hill *et al.*, 1972).

II.3.2. Regulation of lipid metabolism

The lipid content of insects fluctuates during the animals life history. The changes reflect variations in the metabolic balance between lipid synthesis and lipid utilization, and result from direct or indirect action of hormones and/or neurohormones. Thus such processes as development, migration, diapause, reproduction and flight, all of which are under endocrine control, influence lipid metabolism. Various aspects of endocrine regulation of lipid metabolism have been reviewed by Gilbert and Chino (1974), Goldsworthy and Mordue (1974), Bailey (1975), Steele (1976), Goldsworthy (1983), Jaffe *et al.* (1986), Goldsworthy and Mordue (1989), Ziegler *et al.* (1990), Van Marrewijk *et al.* (1992). The metabolism of lipids in insects is regulated by a number of neuroendocrinological, physiological and environmental factors.

II.3.2.1. Hormonal control of lipid metabolism

The rates of metabolic reactions in insect tissues vary considerably under different conditions and respond to physiological stresses that may arise during development, during environmental changes or during flight. Lipid synthesis, release and transport from storage sites are also regulated in accordance with changing physiological requirements and part of this regulation is effected by hormones (Shapiro *et al.*, 1988; Ziegler, 1991; Soulages and Wells, 1994 a, b).

That hormones secreted from CA regulates lipid metabolism was reported in *P. americana*, (Bodenstein, 1953), *S. gregaria* (Odhiambo, 1966; Walker and Bailey, 1971) and *L. migratoria* (Strong, 1968). The corpus allatum synthesises and releases hormones that play important roles in developmental and reproductive regulation (Gilbert and King, 1973). It has long been recognized that allatectomy results in the accumulation of lipid content in the fat body and the hypertrophy can be reversed by implantation of active CA into the allatectomized individuals. The oxidation of [$U^{14}C$]-palmitate by *S. gregaria* fat body is unaffected by allatectomy (Walker and Bailey, 1971). The effect of JH is not sex specific, since the lipogenic effect of allatectomized male, *S.gregaria* is also reduced by implanting CA (Odhiambo, 1966). The above results support the view that the lipogenic effect of allatectomy is due to an increase in the rate of lipid synthesis and the JH is an inhibitor of lipid synthesis. In contrast to the aforementioned studies Butterworth and Bodenstein (1969) suggested that the implantation of female CA into male *Drosophila melanogaster* causes hypertrophy of fat cells and accumulation of lipids; an effect also produced by synthetic JH. Sroka and Barth (1976) have shown that JH has no mobilizing effect on fat body lipid in *Eublaberus posticus* in which rise in total fat body lipid following allatectomy

includes a large increase in the DAG component. But normally the concentration of DAG in the fat body is very low.

Corpus allatum hormone induces vitellogenin synthesis in several species of insects. Alletectomy prevents vitellogenin synthesis in *Leucophaea* (Engelmann, 1969)and *Periplaneta* (Bell, 1969). The absence of JH from CA inhibits vitellogenesis and the consequent lipid transfor from the fat body to ovaries in association with the DAG carrying lipopotein- ipophorin (Chino *et al.*, 1977, 1981).

A relationship of lipid accumulation with the synthesis of protein and lipid has been proposed by Steele (1976). In the absence of JH, the apoprotein moieties that normally facilitate the release and transport of lipid from the fat body are not produced which results in an accumulation of lipid and it also seems likely that the availability of additional non-lipid precursors in allatectomised insects contributes to the increased levels of fat body lipids. A physiological role for the action of JH in suppressing lipid accumulation in fat body was suggested by observations from the locust. The CA remain inactive during the early adult stage of somatic growth and lipid accumulation (Johnson and Hill, 1973, 1975), but later when they become active, the accumulation of lipid ceases and vitellogenesis begins (Hill and Izatt, 1974). This metabolic switch from the synthesis of lipid for migratory purposes to the

synthesis of lipovitellin for oogenesis appears to be triggered by JH (Hill and Izatt, 1974).

The insect moulting hormone, ecdysone, influence the synthesis of lipids in insects. The hormone is released as α-ecdysone from the prothoracic glands in response to a tropic brain hormone. Arnold and Regnier (1975) demonstrated the stimulatory effect of 20-hydroxy-ecdysone on hydrocarbon biosynthesis from [^{14}C] acetate in *Sarcophaga bullata*. Ecdysteroids though primarily known for controlling moulting in young insects, have been found to have effect on the lipid flux. Ecdysone-controlled specific vitellogenin synthesis was reported in *Lucilia Sericata* and *M. domestica* (Adams *et al.*, 1985). In *A. aegypti* the stimulus of a blood meal brings about release of an egg-development neurosecretory hormone from the brain (Hagedorn, 1974). This hormone causes the resting ovary to release α-ecdysone, which induces vitellogenin synthesis in the fat body.

Other hormones shown to influence the synthesis of lipid on the fat body include a factor of unknown composition in the medial neurosecretory cells in the mosquito *A. taeniorhynchus*. The removal of these cells prevents the conversion of sugar to lipid (Van Handel and Lea, 1965). The results suggest that the factor suppress glycogen synthesis, favouring the synthesis of lipid. Aqueous extracts of the central nervous system and CC of *G. morsitans* contain

a peptide factor which has a potent inhibitory effect on lipid synthesis in fat body (Pimley and Langley, 1981). This lipid synthesis inhibiting factor (LSIF) is believed to be synthesised in the medial neurosecretory cells of the brain and stored in the CC. The AKHs have been reported to inhibit lipid synthesis in the fat body of *S. gregaria* (Gokuldas *et al.*, 1988; Gokuldas, 1989).

II.3.2.2. Physiological and environmental factors influencing lipid metabolism

Besides the various neurohormones, neurotransmitters and neuromodulators that influence the metabolism of lipids in insects (Stone *et al.*, 1976; Beenakkers *et al.*, 1978, 1981 a; Ziegler and Schulz, 1986; Keeley *et* al., 1991; Gaede, 1996), certain environmental factors and dietary regimes also regulate the metabolism of lipids.

A physiological variable influencing lipid metabolism is starvation. An increase in ketone bodies acetoactate and 3-hydroxy butyrate due to lipid oxidation has been noticed in several tissues of locusts (Bailey *et al.*, 1971; Hill *et al.*, 1972) and cockroaches (Shah and Bailey, 1976). In *L. migratoria* starvation elevates haemolymph lipids four folds, although the effect is apparently not normally induced (Jutsum *et al.*, 1975; Mwangi and Goldsworthy, 1977; Cheeseman and Goldsworthy, 1979). Locusts from which

the glandular lobes of the CC have been removed, retain their capacity to mobilize lipid (Jutsum and Goldsworthy 1975). It was found that an increased level of lipid exerted a feed back inhibition of AKH release during flight. Orchard *et al.* (1982) suggested that the hyperlipaemic condition of starved locusts is due to octopamine. Starvation of adult cockroaches results in sequential mobilization of carbohydrates and lipids (Downer, 1985). In *Romalea microptera* starvation blocks the *Romalea* CC-stimulated hyperlipaemia (Spring and Gaede, 1987). Gokuldas (1989) has reported that in *S. gregaria,* starvation reduced the capacity of the fat body to synthesize lipid from acetate and refeeding reversed the condition. The excitation caused by handling result hyperlipaemia in *L. migratoria* (Orchard *et al.*, 1981) and in *A. domesticus* (Woodring *et al.*, 1989). The initial stimulation for the release of AKH involves receptors associated with wing or air movement, tarsal contact or metabolite levels (Goldworthy, 1983) and is mediated by neural cells, the cell bodies of which are located in the leteral regions of protocerebram. Excitation caused by handling stress in *Locusta* (Orchard *et al.*, 1981) and *A. domestiscus* (Woodring *et al.*, 1989) and heat and chemical stress in *Schistocerca* (Davenport and Evans, 1984 a, b) have been found to cause hyperlipaemia.

II.4. Carbohydrates in insects

Carbohydrates along with proteins and lipids, form the principal classes of organic compounds that are found in insects and other organisms. Carbohydrates contribute to the structure and functions of all insect tissues, and can be found in the nuclei, cytoplasm and membranes of cells, as well as in the extracellular haemolymph and supporting tissues. Carbohydrates are necessary for normal growth, development, reproduction and survival of individual species (Chippendale, 1978; Hayakawa and Chino, 1981; Ivanovic *et al.*, 1983; Shimada *et al.*, 1984; Hoshikawa, 1987; Ohtsu *et al.*, 1992; Kimura *et al.*, 1992). The requirements of carbohydrates in insects vary according to age, sex and metamorphic stage. Some carbohydrates have been shown to be nutritionally ineffective because they act as feeding deterrents or because they are incompletely hydrolysed or absorbed. The carbohydrates are necessary to meet the insect's energy demands for flight, reproduction and longevity (Wilps and Collatz, 1986; TsuTsui *et al.*, 1988; Ziegler and Schulz, 1986; Gaede, 1992 b; Zera *et al.*, 1994). Adults most commonly obtain their dietary carbohydrates from plant nectars and grain kernels and therefore consume mainly sucrose and starch. Besides having dietary requirement for sugars and starches, insects have also been found to require a dietary source of

some conjugated or derived carbohydrates in the form of vitamins and related compounds.

II.4.1. Carbohydrate metabolism in insects

In most insects carbohydrate reserves are present as glycogen and trehalose which can be readily converted into glucose (Crompton and Birt, 1967; Pant and Kumar, 1979; Islam and Roy, 1981). In addition, glycoproteins may be present, especially in the haemolymph. The synthesis of glucose may take place either by gluconeogenesis or by the process of interconversion from other sugars.

II.4.1.1. Glycogen synthesis

Glycogen is the major reserve polysaccharide of insects (Dezwann and Zandee, 1972; Pant and Kumar, 1981; Chhibber *et al.*, 1986; Mandal and Chaudhuri, 1992; Zera *et al.*, 1994). The synthesis of glycogen from glucose in insects is similar to those found in vertebrates. Glycogen reserves are most abundant in insect fat body, flight muscles and intestinal tissues (Wyatt, 1967; Pant and Morris, 1972; Pant and Kumar, 1979). Studies conducted on glycogen synthesis in the larval fat body of the silk moth, *H. cecropia* and the honeybee *A. mellifera* have shown that glycogen synthase is bound to glycogen particles in the cytosol. The glycogen in the flight muscles of the black fly,

Simulium vittatum, is present as β-particles. Insect glycogen may also be covalently linked to proteins or polypeptides. Substantial amounts of glycogen can also be found in other tissues and haemolymph. Glycogen deposits appear to provide an important energy source for the central nervous system. The extent of glycogen deposits varies between species and depends further on the developmental stage and activity level of the insect (Locke, 1964; Crompton and Birt, 1967; Mwangi and Goldsworthy, 1977; McClure and Steele, 1981; Ziegler and Schulz, 1986; Siegert and Mordue, 1992 c, 1994).

II.4.1.2. Trehalose synthesis

Trehalose functions as an important reserve carbohydrate in insects (Wyatt and Kalf, 1957; Chippendale, 1978; Hayakawa and Chino, 1981; Bloemen *et al.*, 1987; Gaede, 1991 b; Lee and Keeley, 1994; Siegert, 1995). Wyatt (1967) had demonstrated that trehalose, the non reducing disaccharide of glucose occur in many other insect species. Trehalose synthesis is an energy requiring process involving the incorporation of glucose donated from UDP-glucose. D-glucose is transferred from UDP-glucose to glucose 6-phosphate to form the 6-phosphate of α-α--trehalose and UDP in a reaction catalysed by α,α-trehalose phosphate synthase. Free trehalose is liberated from trehalose-6-phosphate by the action of phosphatase (Friedman, 1967; Steele and Hall,

1985; Steele and Paul, 1985; Steele *et al.*, 1988; Khan and Steele, 1993; Lee and Keeley, 1994; Keeley *et al.*, 1995; Thompson *et al.*, 1996).

Candy and Kilby (1961) demonstrated the presence of the biosynthetic pathway of trehalose in the fat body of *S. gregaria* and *L. migratoria*. The trehalose synthesis occur in the cytosol of the fat body cells. Bailey (1975) reported that in some species muscle and intestinal tissues may synthesise lesser amounts of trehalose. Trehalose is an important reserve disaccharide because it is readily hydrolysed to glucose, which is oxidised to provide energy, especially for insect flight. Glycogen and trehalose reserves are mobilized by enzymatically controlled reactions which generate glucose for the support of all life processes.

II.4.1.3. Glycolysis and pentose phosphate pathway

In insects the process of glycolysis whereby sugars are metabolized through their phosphates to pyruvic acid, is similar to that of other organisms (Crabtree and Newsholme, 1975). Pyruvic acid is then converted to acetyl-CoA, which is oxidised to CO_2 and water through the TCA cycle and terminal oxidation.

Although glycolysis provide the main pathway for the anaerobic conversion of glucose to pyruvate, the pentose phosphate pathway or hexose

monophosphate shunt also operates in insects (Chefurka, 1965; Friedman, 1970). This pathway is not important for energy generation in insects, but it can result in the complete oxidation of glucose-6-phosphate to CO_2 and H_2O without the involvement of glycolysis and the TCA cycle. In insects, the main functions of the pentose phosphate pathway are to provide NADPH for fatty acid synthesis, pentose phophate for nucleic acid synthesis and triose phosphate for glycogen synthesis.

The biosynthetic pathways for glycogen and trehalose have been described in a number of reviews (Chefurka, 1965; Wyatt, 1967; Bailey, 1975; Chippendale, 1978). Trehalose occurs in fat body as a readily available source of metabolic fuel that is produced and released during periods of metabolic demand, whereas glycogen represents a more permanent storage form of carbohydrate. Conversion of certain amino acids and glycerol into carbohydrate by reversal of glycolysis has been described in several insect species (Bailey, 1975). Gluconeogenesis occur in the fat body of *P. americana* (Storey and Bailey, 1978 b). Gluconeogenesis may represent a route of carbohydrate synthesis for supply of the nervous system during periods of prolonged starvation. The glycogen content is extremely variable and depends on the nutritional state of the insect. Locust flight muscle utilizes both carbohydrates and lipids during flight (Weis-Fough, 1952; Beenakkers, 1969).

The changes in the carbohydrate content of the fat body and in the amounts of carbohydrate consumed during growth (Walker *et al.*, 1970) suggest that carbohydrate may be converted to lipid during development.

II.4.2. Regulation of carbohydrate metabolism

Hormonal control of carbohydrate metabolism is studied in a large number of insects (Goldsworthy, 1971; Gaede and Lohr, 1982; Siegert and Ziegler, 1983; Ziegler and Schulz, 1986; Gies *et al.*, 1988; Wilps and Gaede, 1990; Ziegler, 1991; Keeley *et al.*, 1994, 1996; Raina *et al.*, 1995). Allatectomy resulted in accumulation of glycogen in the fat body of *Melanoplus, Drosophila, Calliphora, Periplaneta* and *Phormia* (Odhiambo, 1966). Corpora cardiaca extract was found to elevate the level of glycogen phosphorylase in *P. americana* (Steele, 1963). In mosquito, removal of neurosecretory cells greatly increases the glycogen content. Implantation of median neurosecretory cells reduces glycogen synthesis in operated females compared to that in unoperated females (Lea and Handel, 1970). The extract of corpus cardiacum stimulates the release of trehalose when added to isolated fat body (Wyatt, 1967). Weins and Gilbert (1965, 1967) found that CC-extract, when added to fat body *in vitro* increased the rate of respiration at the expense of lipids rather than carbohydrates. In diapausing and allatectomized *Pyrrhocoris*, the glycogen content of the fat body was significantly elevated

(Martin, 1969). Injury to the pupal integument of *Cecropia* silkworm stimulates the incorporation of glucose into blood trehalose and fat body glycogen (Wyatt, 1967).

In *P. americana* the additional trehalose that enters the haemolymph following injection of CC-extract results in the loss of glycogen from the tissues (Steele, 1963; Bowers and Friedman, 1963; Hanaoka and Takahashi, 1976). However, in the case of *L. migratoria* injection of CC-extract brings about the loss of glycogen with an increase in phosphorylase activity (Goldsworthy, 1970) but without any change in the concentration of haemolymph trehalose (Chalaye, 1969). In the last instar larvae of *M. sexta* CC-extract has a potent activating effect on phosphorylase (Ziegler, 1979). Siegert and Ziegler (1983) have demonstrated that the CC-extract of *M. sexta* larvae have been shown to release a factor during starvation that stimulates glycogenolysis. The CC-extract does not cause hypertrehalosaemia and no glycogenolysis in starved *Phormia regina* (Friedman, 1967). Steele (1980) reported that a polypeptide hormone trehalogon, convert phosphorylase b to a in the fat body. Goldsworthy (1970) demonstrated a decrease in locust fat body phosphorylase activity following cardiactectomy. Activation of phosphorylase by CC factor is associated with an increase in the concentration of cAMP in *P. americana* and *L. migratoria* (Gaede and Holwerda, 1976; Hanaoka and Takahashi, 1977;

Gaede, 1979). The octopamine stimulates cAMP synthesis in cockroach fat body (Gole and Downer, 1979). Adenylate cyclase, an enzyme responsible for the activation of phosphorylase, is present in the fat body of various species of insects including *B. mori* (Morishima, 1978, 1979). *H. cecropia* (Filburn and Wyatt, 1976) and *P. americana* (Hanaoka and Takahashi, 1977). The increase in phosphorylase a after treatment of the fat body with exogeneous cAMP (Steele, 1964; McClure and Steele, 1981) implicates the cyclic nucleotide in the activation of phosphorylase even in fat bodies which do not normally increase trehalose efflux when treated with CC-extracts.

Injection of CC-extract cause an elevation in the level of haemolymph carbohydrates in *P. americana* (Goldsworthy *et al.*, 1972). Cockroach CC contain a factor(s) which on injection into cockroaches, elevates haemolymph carbohydrate levels (Steele, 1961, 1963) and also contain a factor which can elevate haemolymph lipid levels in locusts (Goldsworthy *et al.*, 1972). The pure AKH can also induce hyperglycaemia in cockroaches (Holwerda *et al.*, 1977; Jones *et al.*, 1977). Dutrieu and Gourdoux (1967) had reported an elevation of haemolymph trehalose levels in *C. morosus*, after injection of extracts of CC. In *L. migratoria*, starvation depletes the carbohydrate reserves of the fat body and haemolymph (Goldsworthy, 1969). The head extracts of *A.*

mellifera cause hypertrehalosaemia in *P. americana* (Van Norstrand *et al.*, 1980).

The glandular lobes of the locust CC possess a glycogen phosphorylase activating factor (Goldsworthy, 1970) and it has been demonstrated that removal of these lobes results in lowered concentrations of haemolymph carbohydrate (Jutsum and Goldsworthy, 1975). They also reported that nerve sectioning, however, does not depress blood sugar levels. During sustained flight of the migratory locust, *L. migratoria*, when haemolymph trehalose levels are much lower and lipid has become the main fuel for flight, trehalose is still used as an additional energy source and is mostly derived from glycogen stores in the fat body (Beenakkers *et al.*, 1981 a, b). Flight activity of the locust induces an increase of phosphorylase activity in the fat body (Van Marrewijk *et al.*, 1980), which suggests that AKH, in addition to its hyperlipaemic effect, may also be involved in phosphorylase activation. The enzyme could be activated by injection of synthetic AKH (Gaede 1981 a). Jones *et al.* (1977) observed that an increase of haemolymph trehalose concentration in *Periplaneta* upon injection of AKH extracted from locust CC. Activation of glycogen phosphorylase and stimulation of glycogenolysis by the action of octopamine has also been reported for cockroach nerve cord (Robertson and Steele, 1972). Spencer and Candy (1976) suggested that

Ca^{2+} plays a pivotal role in hormonal activation of phosphorylase. McClure and Steele (1981) observed that the hypertrehalosaemic factor interact with adenylate cyclase to produce cAMP which then activates a protein kinase and results in increased calcium influx. Feeding insects generally contain low percentages of active phosphorylase (Ziegler and Gaede, 1984; Siegert *et al.*, 1986; Siegert, 1987 b); this level exceeded during metabolic stress, e.g., flight (Van Marrewijk *et al.*, 1980) or starvation (Siegert and Ziegler, 1983). The CC-extract induce hyperglycaemia in larvae and imagines and stimulate carbohydrate release *in vitro* from larval fat body and accelerate heart beat in adults of *T. molitor* (Rosinski and Gaede, 1988). The fat body of adult *M. sexta* contains low levels of active phosphorylase; this has also been observed in adult *L. migratoria* (Van Marrewijk *et al.*, 1991). Siegert and Mordue (1992 c) demonstrated that in *M. sexta* the active phosphorylase should steadily increase when fat body is incubated *in vitro* over a long period of time without glucose.

In the cockroach *P. americana*, flight caused an increase in haemolymph sugar (King *et al.*, 1986), but under certain stressful conditions, e.g., exposure to chlorinated insecticides, a rapid and dramatic decrease in haemolymph carbohydrate was observed (Orr and Downer, 1982). Hyperlipaemic and hyperglycaemic responses have been reported for a single peptide in *Heliothis*

zea (Jaffe *et al.*, 1988), and *Tabanus* species (Woodring and Leprince, 1992). The hypertrehalosaemic hormone stimulates the fat body to synthesise juvenile hormone regulated export proteins during the vitellogenic cycle in adult female *B. discoidalis* (Keeley *et al.*, 1991). Chilling activates phosphorylase in *M. sexta* with differential effects in larvae (Siegert, 1992) and adults (Siegert and Mordue, 1992 c).

MATERIALS AND METHODS

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter III

MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Insects

Adult *Spodoptera mauritia* (Noctuidae: Lepidoptera) males and females were collected locally during night when they come to light, reared in the laboratory and fed with diluted (50%) honey. Mated females laid eggs within 2 days and these eggs hatched into larvae. Larvae were fed with the grass *Ischaemum aristatum*. A colony was thus maintained in the laboratory. From this stock culture, required stages were drawn for the study. Sixth instar larvae (96 h), prepupae, pupae and adults were used for experiments.

Iphita limbata (Pyrrhocoridae: Heteroptera), a phytophagous bug, were collected locally from the fields and a stock culture of the insect was temporarily maintained in the laboratory and fed with green gram seedlings. Adult insects, after keeping at least for 3 days in the laboratory at normal temperature and humidity, were used for experiments.

Adult American cockroaches, *Periplaneta americana* were collected locally. These insects were kept in wooden cages with glass sides, (30 cm x 30 cm x 30 cm) and were provided with small cardboard boxes for them to hide during day time. The insects were maintained in the laboratory for at least 48 h before they were used for the experiments. Insects were fed on fresh potato

pieces which were changed daily and they had free access to water. Adult insects only were used for experiments.

III.1.2. Equipments

Binocular microscope

Colorimeter

Deep freezer (Kelvinator)

Electronic balance (Sartorius)

Hamilton syringes

High performance liquid chromatograph (HPLC)

Liquid scintillation counter (LSC) (LKB Pharmacia, ECIL)

Magnetic stirrer

Microfuge (Beckman)

Micropipettes (Finpipettes)

Oven

pH meter (Systronics)

Shaker water bath (Toshniwal)

III.1.3. Chemicals

Acetic acid

Acetonitrile (HPLC grade, 40%)

Ammonium acetate

Anthrone

Bovine serum albumin (BSA)

Calcium chloride

Chloroform

Disodium hydrogen phosphate

Glacial acetic acid

Glucose

Glycerol trioleate

HEPES (N-2-Hydroxyethyl piperazine-N-2 ethane sulphonic acid)

Magnesium chloride

Magnesium sulphate

Methanol

Orthophosphoric acid

POPOP (1-4-Bis[5 phenyl oxazol, 2yl] benzene)

PPO (2,5-diphenyl oxazole)

Potassium chloride

Sodium acetate

[1^{14}C] - Sodium acetate

Sodium chloride

Sucrose

Sulphuric acid

Synthetic locust adipokinetic hormone-1 (syn Lom-AKH-I)

Toluene

Trehalose

Synthetic locust adipokinetic hormone-I (syn Lom-AKH-I) was obtained from M/s Peninsula Laboratories, Inc., California, USA. All other laboratory chemicals or organic solvents were of analytical grade obtained through local suppliers. Glass distilled water was used for the preparation of all aqueous solutions and reagents.

III.1.4. Buffers and media

a) HEPES buffer

Fat body incubations for lipid release studies were done in a buffer having the following composition: NaCl, 10 mM; KCl, 12 mM; $MgSO_4$, 2 mM; Na_2HPO_4, I mM; $CaCl_2$, I mM; HEPES, 30 mM; Glucose 10 mM; Sucrose, 50 mM and BSA, 2% (w/v). The ingredients were dissolved in distilled water in a beaker, kept on a magnetic stirrer. The pH was adjusted to 7.2 using a digital pH meter.

b) Ammonium acetate buffer

Ammonium acetate buffer 0.05 M (pH 5.6) was prepared by mixing appropriate volumes of a solution of ammonium acetate and acetic acid.

c) Physiological saline

This saline solution, used for the incubation of fat body preparations in sugar release studies contained NaCl, 154 mM; KCl, 8.0 mM; $CaCl_2$ 1.8 mM and HEPES, 30 mM; pH 7.2.

III.1.5. Other solutions and reagents

a) Preparation of synthetic Lom-AKH-I solution

A stock solution of 200 pmoles/µl of the hormone was prepared from syn Lom-AKH-I. From this stock solution different concentrations ranging from 0.02 µM/10 µl to 1 µM/10 µl (0.02, 0.05, 0.075, 0.10, 0.125, 0.25, 0.50, 1.00 µM) were prepared by dilution with distilled water. These solutions were kept in a deep freezer at about -10°C in ependorf tubes and brought to room temperature just before the experiments.

b) Phosphovanillin reagent

The phosphovanillin reagent used for the colorimetric analysis of total lipid was prepared by mixing concentrated orthophosphoric acid and 0.525% (w/v) aqueous vanillin in the ratio 3:2 (v/v).

c) Lipid standard solution

Chromatographically pure glycerol trioleate was used to prepare lipid standard solution. Standard stock solution was prepared by dissolving 1 g of glycerol trioleate in 100 ml chloroform. Solutions containing different quantities of glycerol trioleate (50 μg to 500 μg) were prepared by diluting the stock solution with chloroform.

d) Anthrone reagent

Anthrone reagent, used for the colorimetric analysis of total sugar was prepared by dissolving 200 mg anthrone in 100 ml of concentrated sulphuric acid.

e) Trehalose standard solution

A standard stock solution was prepared by dissolving 100 mg trehalose in 10 ml of distilled water. From this stock solution different solutions having sugar ranging from 10 μg to 200 μg were prepared by diluting with distilled water. The solutions were kept frozen in a deep freezer.

f) [1^{14}C)]-Sodium acetate solution

The stock solution contained 0.1 mCi [1^{14}C] - Sodium acetate (specific activity 51.88 mCi/mole). The stock solution was diluted with 10 μM sodium acetate solution to get a 0.5 μCi/ml solution.

g) Scintillation cocktail

A toluene based scintillation cocktail was used in all isotopic experiments. This solution contained PPO and POPOP. They were taken in a ratio 5 g : 0.1 g per 1000 ml.

III.2. METHODS

III.2.1. Preparation of *Spodoptera* Hormone (SH)-extract

The brain with corpora cardiaca and corpora allata of adult *S. mauritia* were removed using jeweller's forceps under a binocular microscope. The brain CC/CA complex from many insects were put in a known volume of distilled water (5 μl per gland complex) taken in an ependorf tube (1.5 ml capacity) and the hormone extracted by heating the tube in a steam bath for 5 min. After hormone extraction, the tissue was separated by centrifuging at 5000 x g in a microfuge. The clear supernatant was used as the *Spodoptera* hormone extract (abbreviated as **SH-extract** hereafter). Lower concentrations of the extract were prepared by dilution with distilled water. Concentration is expressed as gland pair equivalent (abbreviated as **gpe** hereafter). The extracts were stored in a deep freezer till use.

III.2.2. RP-HPLC of hormone extract

Reversed phase - high performance liquid chromatography was used to determine the identity of the active components of SH-extract (both larval and adult). Samples of 20 µl were injected onto an ODS (Octadecylsilane) connected with a guard column, in an HPLC system using a Hamilton micro syringe. The extract was eluted in an isocratic run with 40% acetonitrile in (HPLC grade) (in 0.05 M ammonium acetate buffer, pH 5.6) as mobile phase for 40 min. The column effluents were monitored, at a wavelength of 280 nm. Synthetic AKH-1 equivalent to 8 nmole (400 pmole/µl) was used for another run in similar operation conditions in order to get a pattern to be compared to the earlier runs with the samples of SH-extract.

III.2.3. Preparation of fat body for *in vitro* incubation

Fat body from appropriate stage of the insects were removed and washed in ringer solution and blotted dry on filter paper. The fat body was chopped gently on a polyvinyl disc with a sharp razor blade. The chopped fat body was mixed using stainless steel needle and divided approximately into two equal halves. One half served as experimental and the other half as control. These halves were then put into preweighed incubation vials containing fixed volumes (200 µl) of incubation buffer and fat body weight

determined. The whole procedure was completed as quickly as possible (approx.5-6 min).

III.2.4. *In vitro* incubation

Flat bottomed glass vials (5 ml capacity) with bakelite screw caps were used for incubation. The incubation mixture contained 200 µl of the standard HEPES buffer and 10 µl of either the appropriate concentration of hormone solution (experimental) or 10 µl distilled water (control). The incubations were carried out for 30 min in a shaker water bath preset at 37°C.

III.2.5. *In vivo* incubation

III.2.5.1. Injection of hormones (SH-extract and AKH-I)

For *in vivo* experiments the hormone extract of different concentrations in 10 µl volume were injected into the haemocoel through the pleural membrane of the abdominal segments by a Hamilton microlitre syringe. Haemolymph samples (5 µl each) were collected from the insects by calibrated capillary tubes before and after 30 min of injecting the hormone. Similarly haemolymph samples were collected from insects injected with distilled water and were used as controls.

III.2.6. Extraction of lipids

After *in vitro* incubation, lipids released into the incubation medium was extracted by a modified Bligh and Dyer (1959) procedure. Using a micropipette, a known volume of the incubation medium was drawn out from the incubation vials taking care not to draw any fat body pieces. The drawn out samples were deproteinised with 1 ml of chloroform-methanol (1:2 v/v) mixture and were allowed to stand for at least 15 min. To the samples sodium chloride (1.0 M, 1.0 ml) and chloroform (1.0 ml) were added. The mixtures were shaken well and either kept for at least 15 min or centrifuged for 3 min at 1000 rpm to separate the organic and aqueous phases. The lower chloroform layer contained the extracted lipids. Samples (known volume) were drawn from the lower organic phase using micropipette and transferred to test tubes for quantitation of lipids.

III.2.7. Quantitation of lipids

From the lipid samples mentioned above, chloroform were evaporated off by keeping them at room temperature. The amount of lipids in the samples (extracted lipids from *in vitro* experiments and haemolymph samples (5 µl) from *in vivo* experiments) were measured as total phospovanillin positive materials (Frings *et al.*, 1972). For this, samples were mixed with

concentrated sulphuric acid (100 µl) and heated in a boiling water bath for 10 min to oxidise lipids into ketones. The test tubes were cooled to room temperature and phosphovanillin reagent (5 ml each) were added. The ketones develop a pink colour with phosphoric acid and vanillin. The content were mixed well and incubated at 37°C for 15 min. The absorbance were read against a reagent blank at 540 nm using Klett-Summerson photoelectric colorimeter. From these values, absorbance per mg of fat body tissue was calculated using the absorbance value of standard lipid solution (glycerol trioleate) or using a standard curve prepared from different concentrations of the standard lipid solution. From the values thus obtained from controls and experimentals, change in the pattern of lipid release due to the hormone action has been estimated.

III.2.8. Effect of SH-extract and synthetic AKH-1 on lipid synthesis

Experiments were carried out to investigate the effect of SH-extract and syn AKH on lipid synthesis by fat body from various stages of *S. mauritia*. Labelled sodium [1^{14}C]-acetate (0.05 µCi) in 50 µl Sodium acetate was included in the incubation medium of both controls (without hormone) and experimentals (with hormone). After 30 min of incubation at 37°C as before, activity was stopped by the addition of 1.0 ml of chloroform-methanol (1:2 v/v) into the incubation vials. Lipid extraction was completed using the

procedure described earlier. Acetate incorporated into lipids was determined by counting samples in a liquid scintillation counter. Samples (1.0 ml) drawn from the lower phase were transfered to scintillation vials and were dried and scintillation cocktail were added (5.0 ml) to the vials and mixed well before feeding into the counters.

III.2.9. Measurement of hyperglycaemic activity from the fat body of *S. mauritia* and *P. americana* by colorimetric method

The amount of sugar released from the fat body into the incubation medium in the presence or absence of SH-extract and syn AKH (both *in vivo* and *in vitro*) were measured colorimetrically using the anthrone method (Mokrasch, 1954). For *in vitro* experiments, fat bodies for incubation were prepared as described earlier. The incubation mixture contained 200 µl physiological saline and 10 µl of either the hormone extract or syn AKH of appropriate concentration (experimental) or 10 µl distilled water (control). The incubations were carried for 30 min in shaking water bath at 37°C.

Haemolymph samples were collected from *P. americana* before and after 30 min of injection of the appropriate concentration of the hormone. After *in vivo* incubation, the samples were diluted with 1.0 ml distilled water. The amount of sugar in the samples (extracted sugar from *in vitro* experiments and

haemolymph samples from *in vivo* experiments) were measured by modified anthrone method (Mokrasch, 1954) with trehalose as standard.

III.2.10. Analysis of lipids during growth and development by gravimetry

For the gravimetric analysis of total lipid content, sixth instar larvae, pupae and adults of *S. mauritia* were used. Live insects were separated from the colony and weighed. All life stages were dried at 40°C in an oven to constant weight. The total lipids were extracted from the dry matter with chloroform: methanol (1:2 v/v) and quantitated gravimetrically. The constant dry weight of the lipid free carcasses were determined by drying them at 40°C and this weight was subtracted from the original dry weight of insects. The difference (= lipid content) was expressed as percentage of dry body weight. This weight was cross checked by determining the lipid extracted into the chloroform-methanol mixture (gravimetrically).

Values from 10 independent determinations were made (from individual insects) and average were taken for each stage and were expressed as mean $\pm$ SEMs.

III.2.11 Analysis of data

The statistical analysis is broadly classified into two.

1) Parametric

2) Non-parametric

In parametric analysis we use an appropriate mathematical model whereas in non-parametric analysis, there is no such model. The validity of the model may be verified using descriptive statistics like mean, variance, skewness etc. If the verification rejects the validity of the model, the non-parametric method is appropriate.

In our data the variables consist of

1) Experiments (lipid release, sugar release, lipid synthesis etc.).

20 Controls (fat body difference)

3) The difference (E-C)

It is known that the variable (lipid release, sugar release etc.) is affected by the controllable factors (known factors), stage of insects and concentration of hormone (or difference in fat bodies in the case of control) and possibly the joint effect of these two (interaction). In this case the model can be taken as

variable = sum of effect of all known factors + random error

The known factors in the case of experimental data are the following.

1) Effect of stages
2) Effect of concentrations
3) Joint effect of stage and concentration (interaction)

The known factors in the case of control data are the following:

1) Effect of stages
2) Effect of fat body
3) Joint effect of stage and fat bodies

The known factors in the case of difference data are the following:

1) Effect of stages
2) Effect of concentration
3) Joint effect of stage and concentration

The random error occurs because of several unknown factors and this mainly includes experimental error. For the comparison of stages, concentrations and interactions, we make use of ANOVA technique in which we assume that the random error is statistically distributed according to normal (Gaussian) distribution with equal variances for all trials. A well known property of normal distribution is its symmetrycity. The symmetricity

can be varified using a measure called skewness. If the distribution is symmetric the skewness value will be very small i.e., near to zero. When we computed skewness in all cases we found that it is near to zero (see, Table III.1). Therefore the claim of normal distribution for the data is granted. To use usual ANOVA, all the variances must be equal. But in our case variances are significantly different (ref: Table III.1). Therefore generalised analysis of variance is appropriate. In generalised analysis of variance we use an appropriate transformation to make all the variances equal. The usual ANOVA technique is then applied in the transformed variable.

For the details about the Generalised ANOVA one can refer to Rao (1965). For a detailed discussion of designed experiments and ANOVA refer to Das and Giri (1986).

In ANOVA we have 3 sources of variations

1) due to stage
2) due to concentration or fat body
3) Interaction

The ANOVA tables, thus computed in each case are presented in the Results section. In all cases except *S. mauritia* lipid release (using SH-extract), the sample size in all catagories are the same. The sum/s of squares is

Table III.1. Quantity of lipids released (mg/g fb) from various stages of *S. mauritia* in the experimentals (with AKH), showing mean, variance and skewness of each set

Stages	Hormone concentration (μM)	Lipid release		
		Mean (mg/g fb)	Variance	Skewness
96 h	0.05	80.140	537.2566	0.459449
96 h	0.075	101.55	1176	1.088073
96 h	0.1	95.579	683.7099	0.43635
96 h	0.125	89.565	2587.775	0.725669
96 h	0.25	53.814	468.9358	-0.25873
96 h	0.5	58.965	923.8315	0.301467
96 h	1	89.549	787.1488	1.116346
Prepupa	0.05	111.13	2420.094	0.701327
Prepupa	0.075	74.501	251.7699	0.281428
Prepupa	0.1	111.10	2892.561	0.186933
Prepupa	0.125	56.559	232.5498	-0.45652
Prepupa	0.25	75.236	1010.023	0.800605
Prepupa	0.5	94.317	1148.481	-0.67905
Prepupa	1	118.31	1199.795	1.075028
Pupa	0.05	70.483	160.3918	-0.67343
Pupa	0.075	59.668	798.7897	1.511711
Pupa	0.1	68.441	341.7727	-0.629 24
Pupa	0.125	71.590	1205.267	0.39065
Pupa	0.25	55.066	134.6917	0.285251
Pupa	0.5	60.467	373.738	0.656807
Pupa	1	84.995	1272.196	0.84916
Adult	0.05	141.33	7898.089	2.04271
Adult	0.075	108.75	2390.938	0.4077
Adult	0.1	93.284	3061.357	0.653624
Adult	0.125	178.39	3160.766	-0.43503
Adult	0.25	99.841	649.1214	0.737104
Adult	0.5	83.562	1618.555	1.448074
Adult	1	97.422	679.8963	-0.14428

calculated using standard technique. But in other two cases, the sample sizes are not equal, and hence the adjusted sums of squares are used which are computed using the analysis of covariance technique.

If there is interaction, the effect of concentration differs on different stages. When we make a graph of stages versus variable (lipid release, sugar release, etc.) the plot of different concentrations will not be parallel. The line diagram or bar diagram is used to visualize the effect of stages on different concentrations and also the effect of concentrations on different stages.

In ANOVA table SS denotes the sum of squares, MSS denotes the mean sum of squares which is SS/df, df is degrees of freedom which is equal to number of cases of the factor minus one (n-1) for the individual effect and product of the individual degrees of freedom in the case of interaction. The F statistic is calculated by dividing due to the MSS the factor by MSS due to error.

IDENTITY OF ACTIVE COMPONENTS IN THE EXTRACT OF NEURONAL TISSUES FROM S.MAURITIA

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter IV

IDENTITY OF ACTIVE COMPONENTS IN THE EXTRACT OF NEURONAL TISSUES FROM *S. MAURITIA*

IV.I. Introduction

Neuropeptides play many diverse functional roles in insects as chemical messengers, controlling many aspects of growth, development and reproduction, as well as many important physiological and metabolic processes. They are either released from the nervous system into the haemolymph as circulating neurohormones or more specifically released as neurotransmitters at specific target sites. It has been possible to identify and characterize some of these neuropeptides. Much attention has recently been paid to investigate the chemical structure and structure-function relationship of these neuropeptides in insects (for reviews, see Stone *et al.*, 1978; Mordue and Morgan, 1985; Gaede, 1989; Goldsworthy and Wheeler, 1989; Keeley, *et al.*,1991). The adipokinetic hormones of locusts are the first and the best studied insect neuropeptides. They have been isolated, characterized and synthesized (Stone *et al.*, 1976; Broomfield and Hardy, 1977; Siegert *et al.*, 1985; Oudegans *et al.*, 1991) and their various activities have been established (O'Shea *et al.*, 1984 Scarborough *et al.*, 1984; Carlisle and Loughton, 1986; Gokuldas *et al.*, 1988; Kodrik and Goldsworthy, 1994). Following this a number of other peptide hormones have been characterized.

One of the major reasons for the slow pace in characterizing insect neuropeptides has been the difficulty in isolating enough material for analysis. This is because of the minute quantity of the active component present in the neurosecretory organs, which demands a large number of insects. Another reason was the instability of some of the peptide molecules extracted from the tissues. During the last one decade or so, most of these difficulties have been successfully overcome. This has been made possible by the advancement of biochemical techniques enabling very small quantities of peptides to be fractionated and detected from tissues. Such methods include chromatographic techniques- thin layer chromatography, gel filtration chromatography, and high performance liquid chromatography, which make use of various properties of the peptide molecules such as size, charge, adsorptive properties etc. Ultraviolet spectrophotometry, mass spectroscopy, fast atom bombardment mass spectrometry and various methods for amino acid analysis and sequencing have also been very useful tools.

High performance liquid chromatography on a reversed phase column (RP-HPLC) has become an essential tool in analytical biochemistry, enabling separation of a wide range of materials with less effort and time and with

more efficiency and reproducibility than other methods. Early attempts to apply this technique to peptide separation were not always successful because of poor resolution and recovery but the development of porous silica-based column packing possessing chemically bonded alkyl side chains have proved particularly successful. Such reversed phase columns have resulted in high and rapid resolution and recovery.

Insect endocrine research have achieved a fast pace with the help of this technique within the last few years. Consequently, a large number of biologically active compounds have been characterized and synthesized. Most of them are peptidic in nature. Witten *et al.* (1984) used 10 μ C_{18} bonded silica (Waters, μ-Bondapak) columns to compare synthetic and natural myoactive peptides (M I and M II) described by O'Shea *et al.* (1984), combined with fast atom bombardment mass spectroscopy. For HPLC, they used 1 mM ammonium acetate buffer (pH 4.5) and acetonitrile gradient (25% - 50%, 1 ml/min) as the mobile phase. Scarborough *et al.* (1984) purified two cardioacceleratory peptides, CC-1 and CC-2 from the CC-extract of *P. americana* using 9% 1-propanol in 0.1% trifluroacetic acid/water for CC-1 and 12% 1-propanol in 0.1% trifluroacetic acid for CC-2 in an aquapore C_{18} column. Siegert *et al.* (1985) used size exclusion

chromatography (SEC) and HPLC on a reversed phase followed by gas phase sequencing to elucidate the primary structures of locust AKH- II. Siegert and Mordue (1986) found that an aquapore RP-300 column with a solvent system of 0.1% trifluroacetic acid and a gradient of acetonitrile was efficient enough to quantitate the AKH in a single gland pair of CC from *S. gregaria* and *L. migratoria*. Spring and Gaede (1987) isolated a peptide which brought about hyperlipaemia in locusts and hyperglycaemia in cockroaches, from the CC of a non-flying grasshopper, *R. microptera* using C_{18} column (Nucleosil, 7 μm particle size). Gaede *et al.* (1992) used 5 μm C_8 column (Nucleosil-300) for the separation of peptides from many Ctenoid beetles. C_{18} columns were used for the separation of neuropeptides from tenebrionid and onitoid beetles (Gaede, 1994, 1997). Morgan *et al.* (1987) used a C_{18} reverse phase column to isolate a diuretic peptide from the locust CC. Hekimi and O'Shea (1987) in their studies with the precursors of AKH-1 used a C_{18} reverse column (Waters, μ-Bondapak) to separate the ^{3}H-tryptophan labelled AKH-1 and AKH-II synthesized by the CC *in vitro*. Jaffe *et al.* (1989) used a C_8 column (Du Pont) to isolate two peptides having adipokinetic and hypotrehalosaemic activities from CC of horse flies, *T. atratus*. Gaede *et al.* (1990) isolated a peptide from the CC of blowfly, *Phormia terraenovae* having hypertrehalosaemic activity using a reversed

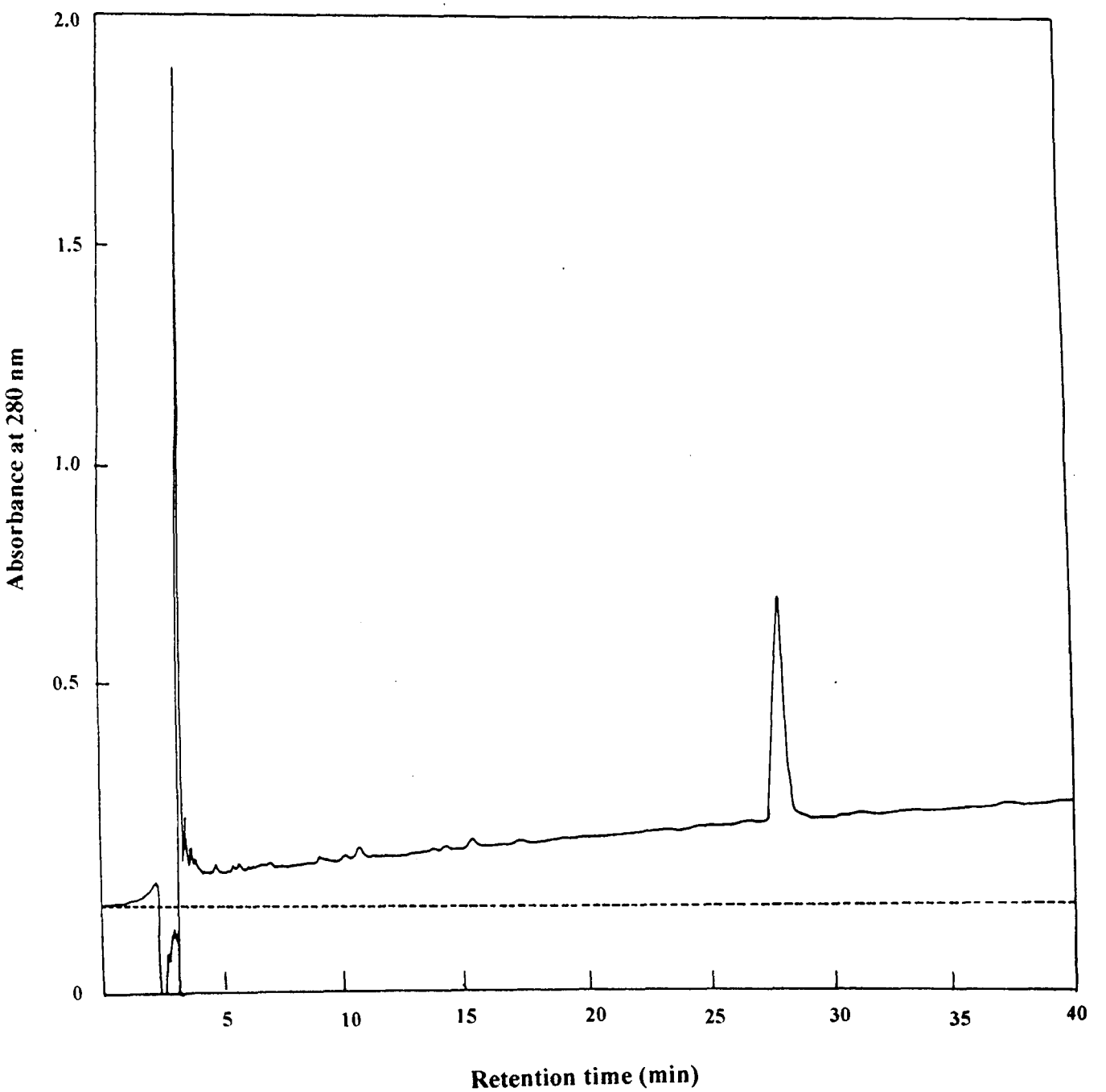
phase column (Spherisorb, 3 μ particle size). Oudejans *et al.* (1991) isolated and characterized the third adipokinetic hormone from the glandular lobes of CC of *L. migratoria* by reverse phase HPLC (Nucleosil, 5 μm particle size). Raina *et al.* (1995) isolated a neuropeptide from the CC of Cicadas having adipokinetic and hypertrehalosaemic activities by HPLC with a C_{18} column (supelco).

IV.2. Results

Isocratic separation of SH-extract

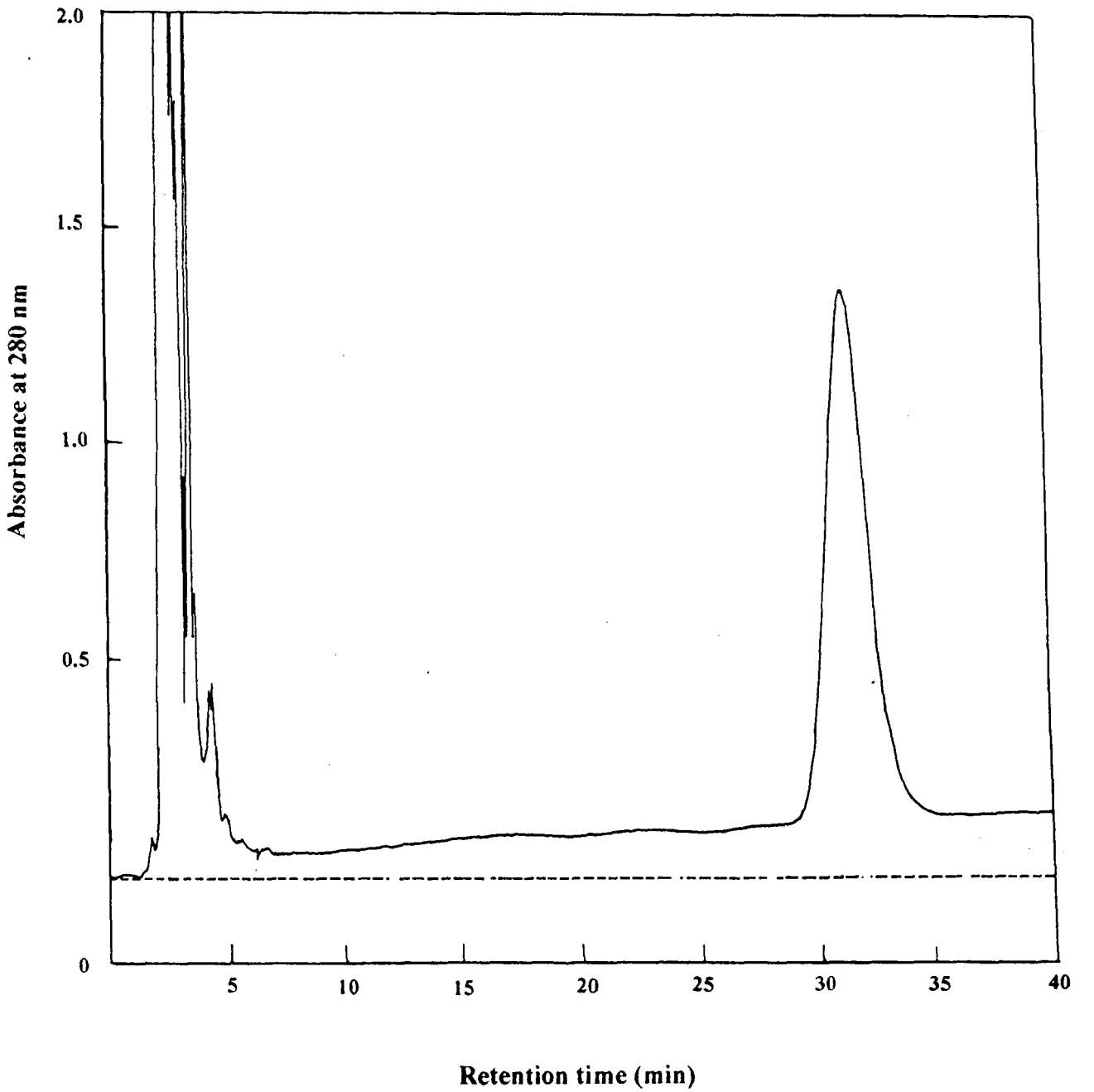
Figure IV (1-3) show the HPLC elution profile of syn AKH and SH-extract of adults and larvae respectively. During the first 4 or 5 min a large number of clustered peaks elute which represent materials that get eluted in acetonitrile having concentrations below 40%. The peaks appeared during this period may also comprise those due to the solvents as some of these peaks also appeared in the case of AKH. At a retention time of about 29 min, the peak corresponding to AKH appeared (Fig.IV.1). The adult SH-extract also gave a similar peak after a similar retention, which was a reasonably good peak (Fig.IV.2) whereas in the case of larval extract, the peak was not observed (Fig.IV.3). Comparing the peaks for the extracts to

Fig. 4.1. High performance liquid chromatogram of syn AKH



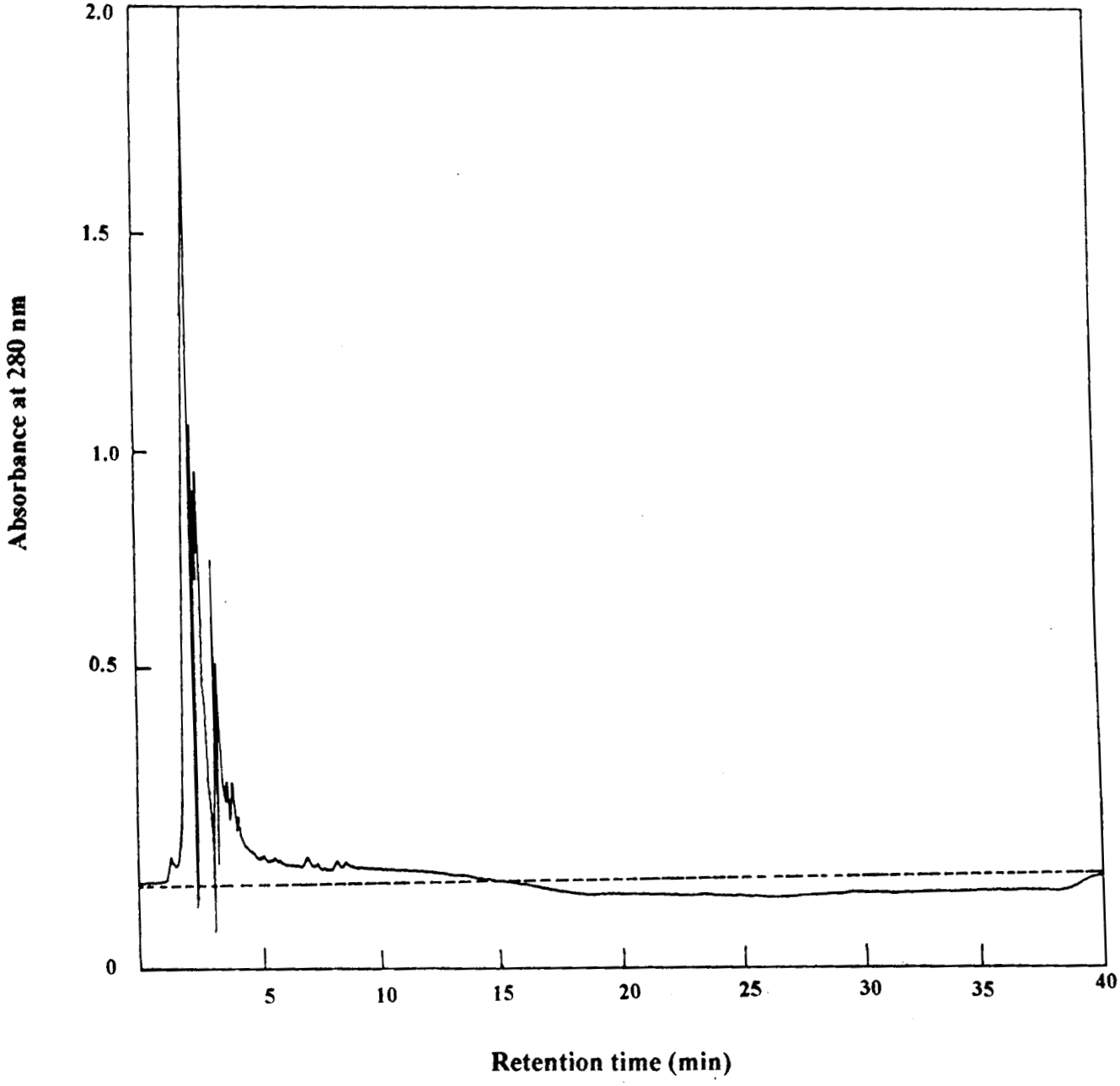
A solution of syn AKH containing 8 nmoles was run with 40% acetonitrile in a C_{18} reversed phase column.A flow rate of 1 ml/min was maintained and the column effluents were monitored at 280 nm.

Fig.4.2. High performance liquid chromatogram of SH-extract prepared from adults



An extract of 10 gland pair equivalent prepared from adults of *S.mauritia* was run with 40% acetonitrile in a C_{18} reversed phase column. A flow rate of 1 ml/min was maintained and the column effluents were monitored at 280 nm.

Fig. 4. 3. High performance liquid chromatogram of SH-extract prepared from larvae



An extract of 5 gland pair equivalent prepared from larvae of *S.mauritia* was run with 40% acetonitrile in a C_{18} reversed phase column. A flow rate of 1 ml/min was maintained and the column effluents were monitored at 280 nm.

that of the AKH, it appears that the extracts had compounds that have similar chemical characteristics to that of syn AKH.

On the basis of the area of the peak corresponding to 8 nmoles of syn AKH, calculation was made on the approximate quantity of active compound present in the peak appeared with the SH-extract (adult) injected (i.e.,10 gpe). It was found that each gland pair contained almost 7.35 nmoles of the material eluted after a retention time same as that of AKH. In the case of larval tissue extract, the peak corresponding to the peaks obtained for adult tissue and AKH, was not shown, whereas the peaks appearing during the initial 4-5 min were clearly shown. The absence of peak at 23 min suggests that the quantity of material present in larval tissues is very small which is not detectable at the sensitivity selected (2.0 FS).

IV.3. Discussion

The HPLC carried out with SH-extract from adult and larvae and syn AKH showed that the adult *Spodoptera* contained (an) active compound/s that have almost the same retention time as that of AKH. This peak was insignificant or absent in the extract made from larval tissue. The materials eluted during the first 4-5 min represented by the cluster of peaks might be due to the mobile phase (40% acetonitrile and ammonium acetate) used for

the isocratic run. Materials having mobility in acetonitrile of less than 40% concentration might also have got washed down the column at the beginning itself. The retention time of the main peak material (29-35 min) for the SH-extract (adult) comes near to that of AKH 27-29 min suggesting a similarity between the two. However, both these retention times seems to be more than the time reported earlier (Gokuldas *et al.*, 1988) in a similar experiment with AKH, which was about 23 min. This increased time of retention may be due to the difference in the elution procedure adopted, i.e., isocratic instead of a gradient system. The concentration of acetonitrile selected for the present run (40% v/v) (isocratic) is from the elution pattern obtained from the earlier gradient run mentioned above where the retention time for AKH-I was found be 23 min at a concentration of 40% of acetonitrile (Gokuldas *et al.*,1988). The pattern of elution obtained here only suggests that there is/are components with similar retention time as AKH but whether they are the only active components is not sure as the extract was not fractionated and tested individually.

STUDIES ON LIPID RELEASE FROM THE FAT BODY

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter V

STUDIES ON LIPID RELEASE FROM THE FAT BODY

V.1. Introduction

The role of neurohormones on lipid release in insects has been described in many insects. The report of AKHs from the locusts (Mayer and Candy, 1969; Beenakkers, 1969 a) was followed by a plethora of such reports from many insects (Goldsworthy *et al.*, 1972; Cheeseman *et al.*, 1976; Goldsworthy, 1983; Ziegler and Schulz, 1986; Keeley *et al.*, 1991; Gaede, 1992 a,b; Gaede, 1997). However, AKH-I of locust (Lom-AKH-I) is still the best studied among all of them. Now designated as AKH/RPCH family of peptides, they have similar structures (although with conservative changes of amino acids in their sequence) and functions but to various extents. Due to the similarity in structure many of these hormones exhibit cross-reactivity. For example, the locust AKH elicits pigment concentration in crustacea and the crustacean hormone brings about hyperlipaemia in locusts (Fernlund, 1974). Moreover, AKH exhibited hyperglycaemia in cockroaches whereas the cockroach hyperglycaemic hormone (HGH) elicits hyperlipaemia in locusts (Jones *et al.*, 1977).

The AKHs regulate energy metabolism during flight by mobilizing lipids from the fat body. In the case of locusts, AKH is also involved in the regulation and utilization of lipids by the flight muscles (Goldsworthy, 1983;

Goldsworthy and Wheeler, 1989; Orchard *et al.*, 1991). Ziegler *et al.* (1990) reported that AKHs are involved in the control of lipid metabolism in the larvae of *M. sexta.* Both endogenous natural adipokinetic peptides exhibit adipokinetic and phosphorylase activating activity (Gaede, 1981 a). Many Lepidoptera use lipids as the main fuel for flight (Zebe, 1954), but carbohydrates may also be used (Beenakkers *et al.*, 1981 a, b). Haemolymph lipid levels increase during tethered flight in the monarch butterfly, *D. plexippus,* and this may be controlled by a factor from the head (Dallmann and Herman, 1978). In the CC of *M. sexta* there is a peptide which has adipokinetic activity when injected into adults of *M. sexta* (Beenakkers *et al.*, 1978; Ziegler and Gaede, 1984). Injection of CC-extracts from *L. migratoria* into *M. sexta* increases haemolymph lipid levels (Ziegler and Gaede, 1984) and the injection of a very high dose (200 pmole) of locust AKH-I, results in loading of the lipid transport protein, lipophorin, in adult *M. sexta* (Shapiro and Law, 1983). Gaede and Spring (1987) reported that the flightless lubber grasshopper, *R. microptera* showed a very small adipokinetic response to its own hormones compared to its activity in locusts. Another flightless grasshopper, *Barytettix psolus* showed slight adipokinetic response when injected with extracts of its own CC or with locust AKH (Ziegler *et al.*, 1988). Injection of trehalose lowered phosphorylase activity as well as haemolymph

lipid concentration in starved adult *M. sexta* (Ziegler, 1991). Ziegler *et al.* (1995) reported that M-AKH cause lipid mobilization in both larvae and adult of *M. sexta*.

In the experiments described in the present chapter, fat body from the Lepidopteran insect *S. mauritia* was tested for its adipokinetic response to AKH (Locust) and extracts of its own neuronal tissues *in vitro*. Cross-reactivity of adipokinetic factors of *S. mauritia* were also tested both *in vivo* and *in vitro* on the fat body of *I. limbata*.

V.2. Results

V.2.1. Lipid release from the fat body of various stages of *S. mauritia* incubated with different concentrations of SH-extract *in vitro*

Incubations of fat body from various stages (viz., 96 h larva, prepupa, pupa and adult) of *S. mauritia* were carried out for 30 min with different concentrations of SH-extract and quantitative changes in total lipids released over control (containing distilled water instead of SH-extract) were measured as described. The results obtained were subjected to ANOVA and the data are provided in Table V.1. The analysis of the data shows that the effect is significant. The F-value obtained (125.45) when compared to the Table F-value (1.88) shows that there is a significant difference between different

Table V.1. ANOVA of data for lipid release from the fat body of various stages of *S. mauritia* at different concentrations of SH-extract *in vitro*

Experiment

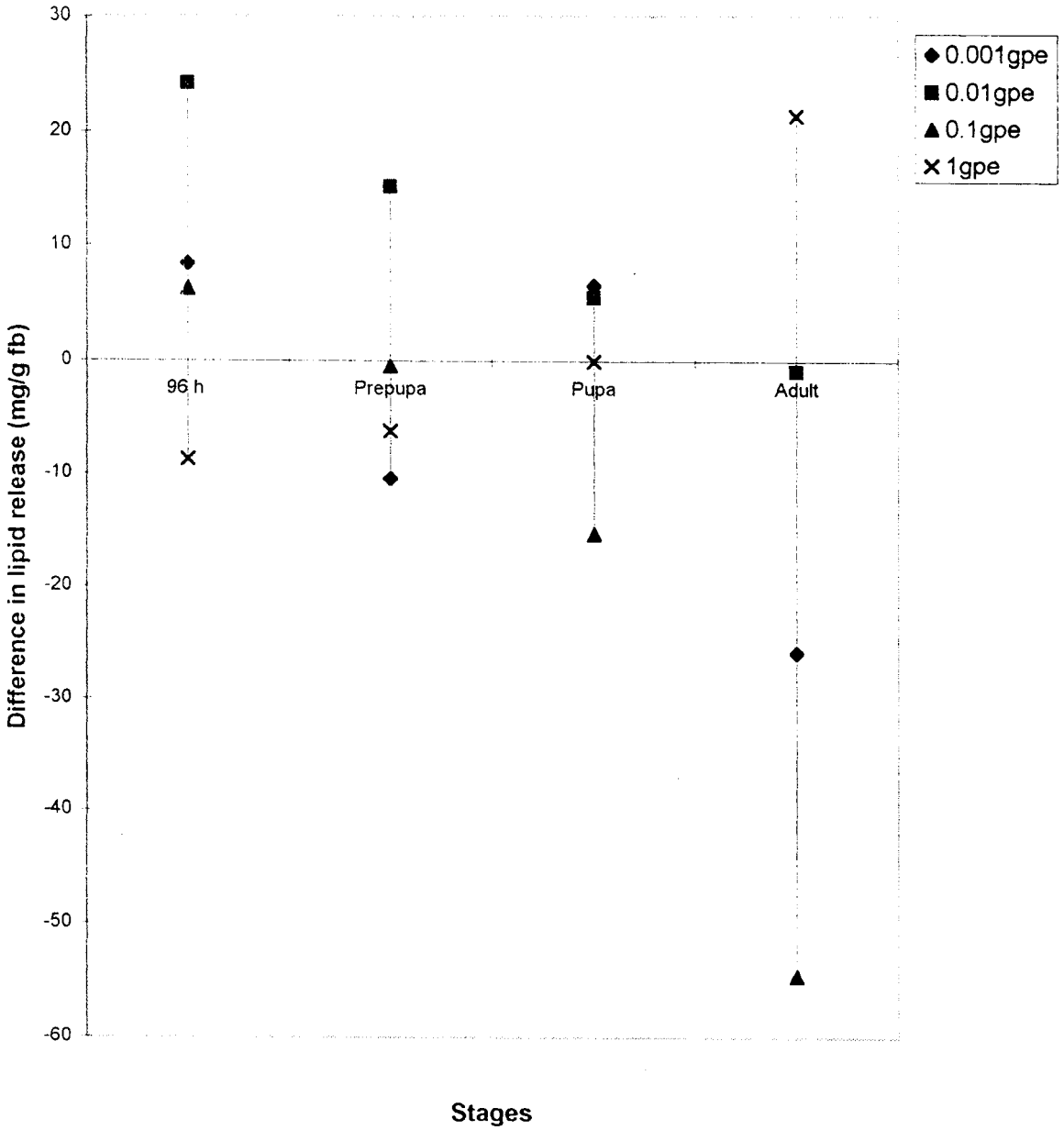
Source	df	SS	MSS	F	Tab. F
S x C x T	9	2833094	314788.2	125.45	1.88
Error	218	547036.3	2509.341		

The data used for ANOVA were obtained from analysis of lipid release using various stages of *S. mauritia* at different concentrations of SH-extract (Total cases considered are 125). 'T' indicate treatments.

stages of *S. mauritia* and various concentrations of SH-extract with respect to lipid release from the fat body.

Figure 5.1 presents the effect of various concentrations of SH-extract on fat body lipid release from *S. mauritia*. It is seen that the SH-extract have both stimulatory as well as inhibitory effects on fat body lipid release. Significant positive effects were obtained from all the stages tested. In the case of 96 h larva, 0.001, 0.01 and 0.1 gpe solutions of the hormone showed positive effect and the maximum release of lipid occurred was about 24 mg/g fb (with 0.01 gpe). However, 1.0 gpe concentration showed inhibitory effect by about 9.0 mg/g fb. The prepupal fat body responded positively, only to a hormone concentration of 0.01 gpe (15 mg/g fb). All the other concentrations of the hormone showed either no effect (0.1 gpe) or negative response. Lipid release was reduced by about 6-10 mg/g fb (0.001, 0.1 and 1.0 gpe). In pupal fat body, the maximum stimulation of lipid released was only 7 mg/g fb and this occurred at a concentration of 0.001 gpe; followed by 0.01 gpe (6 mg/g fb). At higher concentration (1.0 gpe) there was only inhibitory effect on the fat body. Adult fat body lipid release was stimulated only with a hormone concentration of 1.0 gpe. Maximum stimulation was about 21 mg/g fb. All the other concentrations showed negative effect on lipid release. The maximum inhibition was shown by a concentration of 0.1 gpe (by about 54

Fig. 5.1. Effect of different concentrations of SH- extract on lipid release *in vitro* from the fat body of various stages of *S.mauritia*

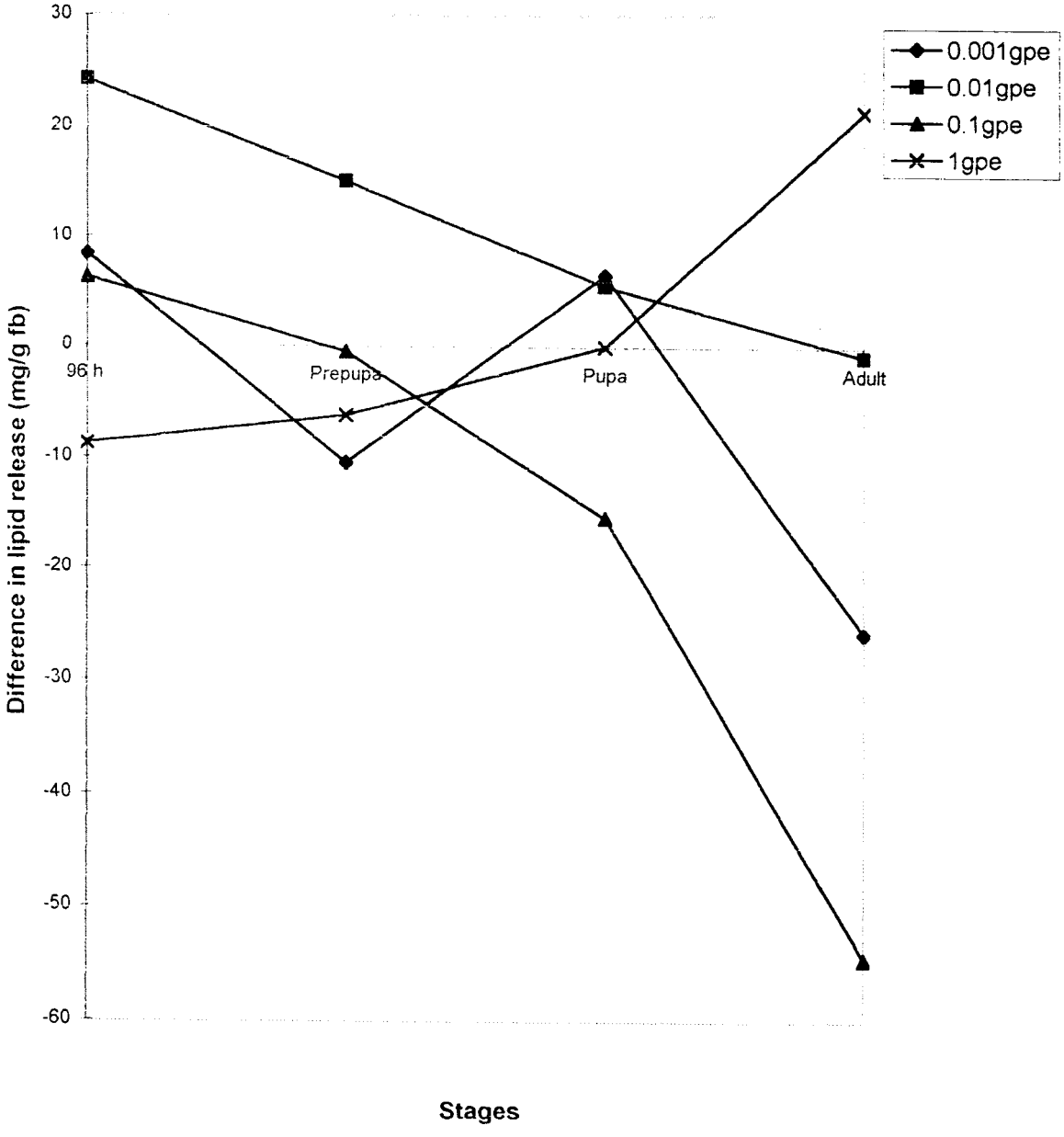


mg/g fb) followed by 0.001 gpe (by about 26 mg/g fb). At 0.01 gpe concentration, the hormone could not elicit any response on adult fat body. Thus in brief, the response of the various concentrations of the hormone had different pattern of activity on lipid release from fat body from different stages of the insect although these effects were significant.

Figure 5.2 shows the interaction between various stages of *S. mauritia* with different hormone concentrations on fat body lipid release. The graph clearly indicates that the response of the hormones on lipid release from different stages are significantly different. The activity is different at different stages. It is seen that different concentrations of SH-extract shows stimulatory as well as inhibitory effects on lipid release from the fat body from various stages of *S. mauritia.*

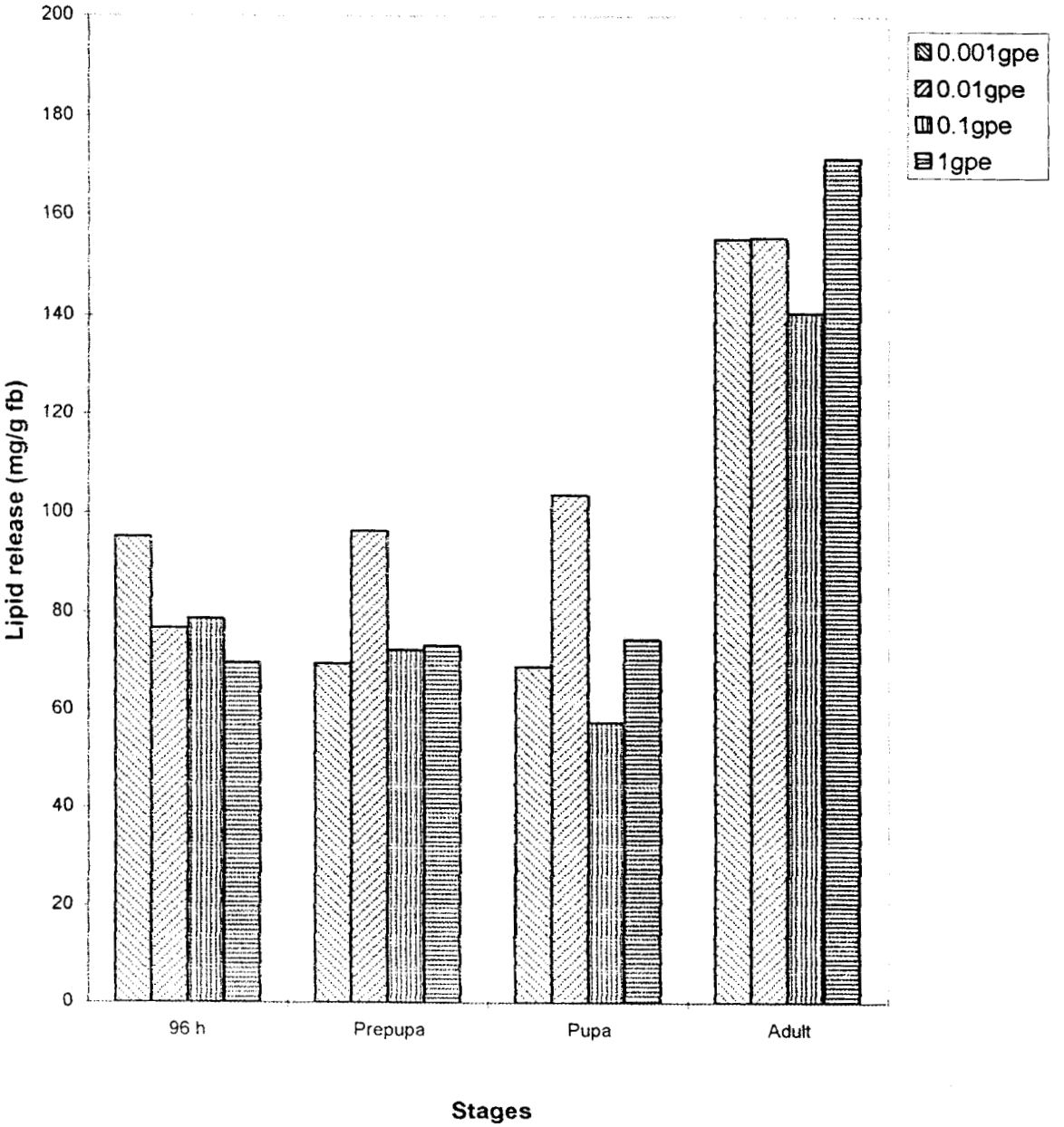
Figure 5.3 presents the average amount of lipids released in experimental observations using different hormone concentrations in various stages. The bar diagram presents a visual comparison of the lipid released from the fat body cells. Among all the stages, maximum lipid release was found to occur from the adult fat body. A hormone concentration of 1.0 gpe was the most effective one (172 mg/g fb). In adult, all the concentrations showed better response than any of the other stages tested. All the other stages showed almost similar range of lipid release, i.e., around 100 mg/g fb.

Fig. 5.2.Graph showing the interaction between various stages of *S.mauritia* and different concentrations of SH- extract



178

Fig. 53. Average lipid release from the fat body of *S.mauritia* incubated with different concentrations of SH-extract in various stages (n=8)



In prepupal and pupal fat body, however, the lipid release was higher (by about 96 mg/g fb and 104 mg/g fb respectively) at a hormone concentration of 0.01 gpe than other concentrations. In 96 h larval fat body, a concentration of 0.001 gpe seemed to have better lipid release activity than others. Figure 5.4 presents the amount of lipid released from the fat body of various stages of *S. mauritia* at different hormone concentrations. From the graph it is clear that the amount of lipid released from the fat body of the adult is always far better than the other stages with all the concentrations tried. The higher hormone concentration (1.0 gpe) shows a less prominent effect on fat body lipid release from all stages except adult.

V.2.2. Lipid release from the fat body of various stages of *S. mauritia* incubated with different concentrations of syn AKH *in vitro*

Fat body from various stages viz., 96 h larva, prepupa, pupa and adult of *S. mauritia* were incubated for 30 min without and with different concentrations of syn. AKH and total lipids released into the medium were extracted and measured as described in materials and methods section. The results obtained were subjected to ANOVA as described and the data are provided in Table V.2. The Table V.2.1 provides data obtained for the experimentals where the incubation mixture contained hormone at various concentrations. The first row of values are for the activity for different stages.

Fig. 5.4. Average lipid release from the fat body of various stages of *S.mauritia* at different concentrations of SH-extract (n=8)

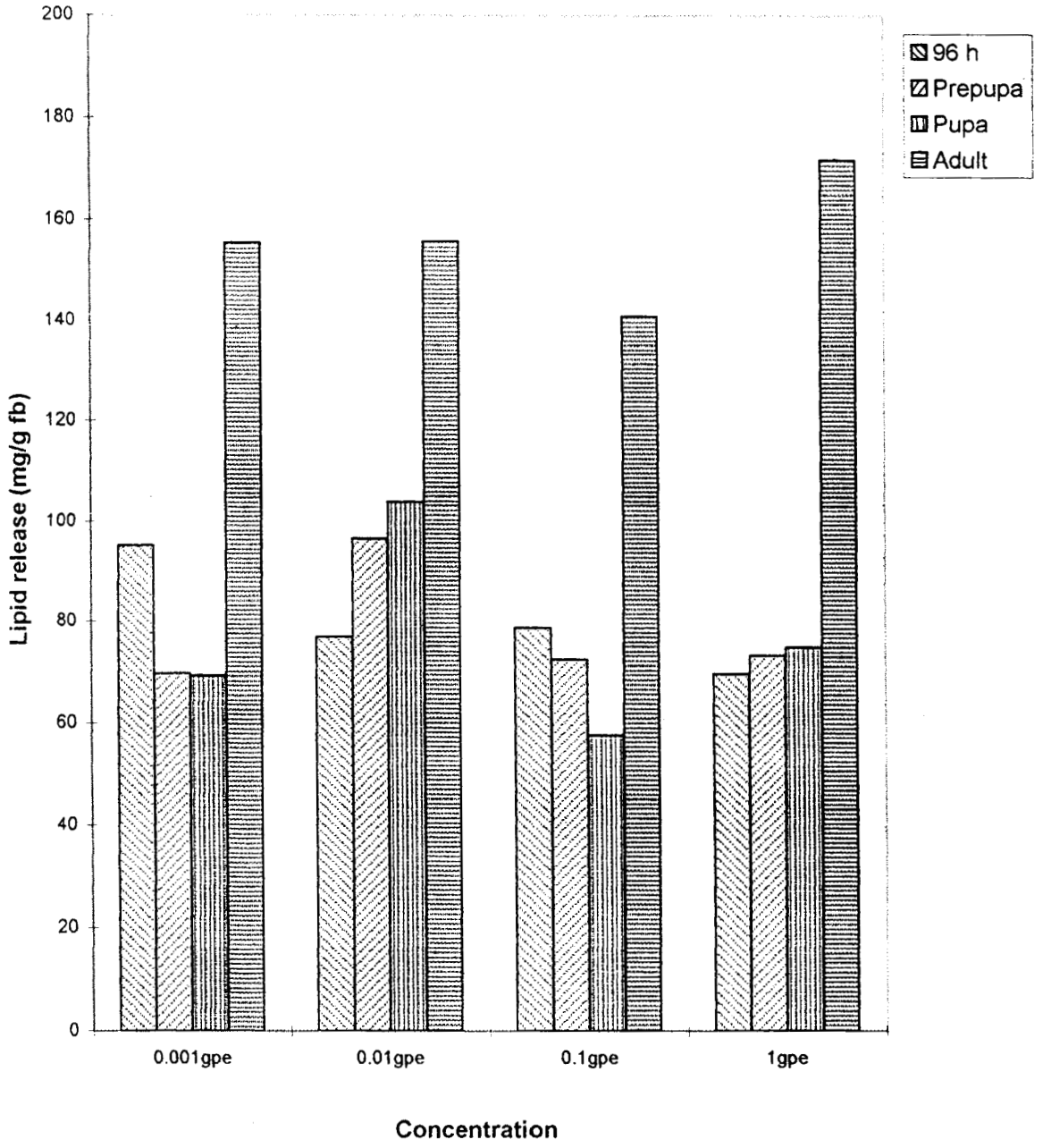


Table V.2. ANOVA of data for lipid release from the fat body of various stages of *S. mauritia* at different concentrations of syn AKH *in vitro*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	18.46082	6.153605	5.38	2.61
Conc. (C)	6	20.32324	3.387207	2.96	2.10
S x C	18	183.1473	10.17485	8.90	1.57
Error	196	224.0005	1.14286		

2. Control

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	103.6689	34.55632	30.24	2.61
Fat body (Fb)	6	206.6045	34.43408	30.13	2.10
S x Fb	18	703.0679	39.05933	34.18	1.57
Error	196	224	1.142857		

3. Difference

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	23.42301	7.807671	6.83	2.61
Conc. (C)	6	22.77499	3.812499	3.34	2.10
S x C	18	37.60709	2.089283	1.83	1.57
Error	196	224	1.142857		

The data used for ANOVA were obtained from analysis of lipid release using various stages of *S. mauritia* at different concentrations of syn AKH (Total cases considered are 224).

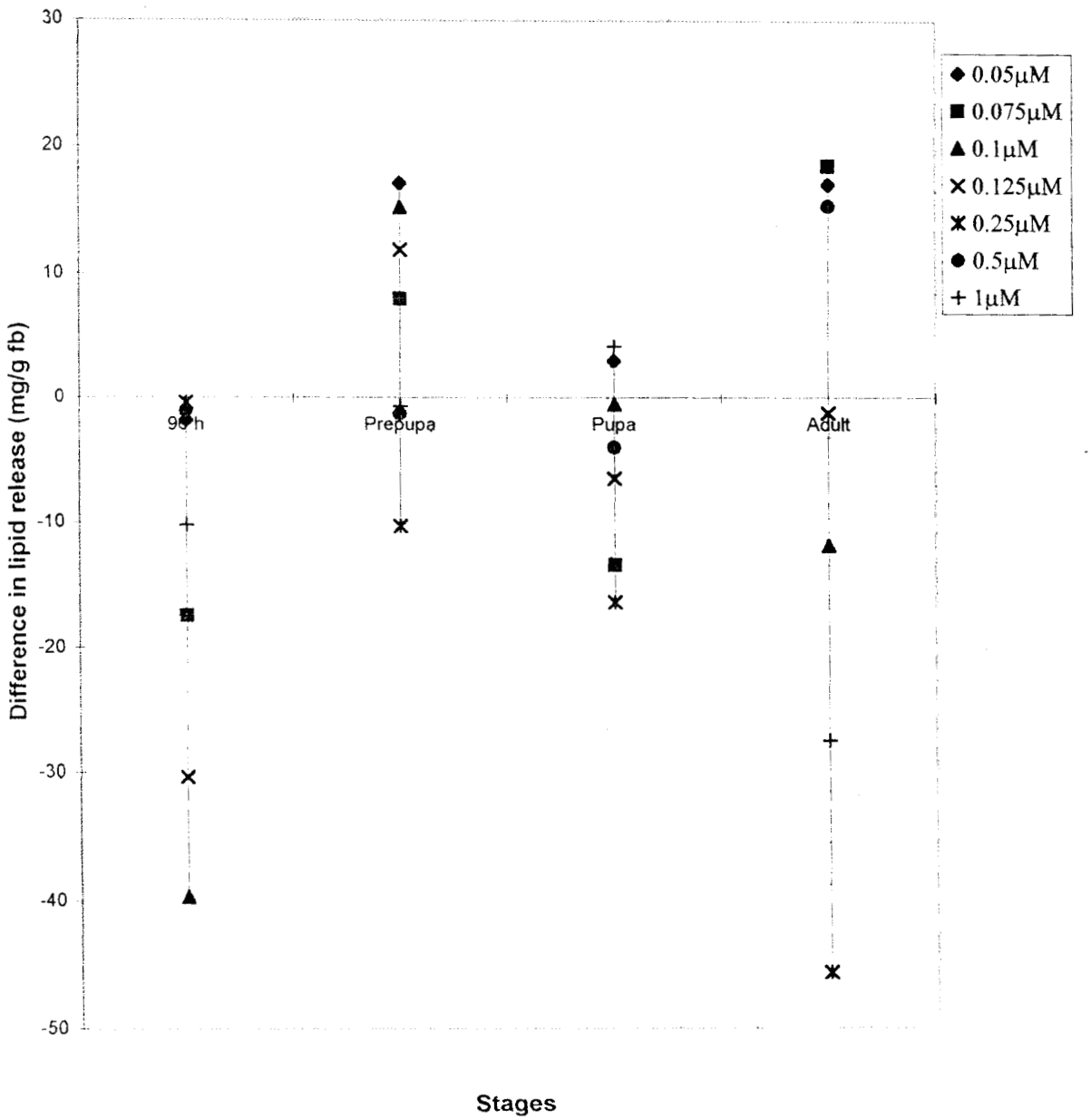
The F-value obtained (5.38) when compared to the Table F-value (2.61) shows that there is significant difference between the different stages with respect to lipid release. Likewise, the second row of values, obtained for various concentrations, shows that the calculated F-value is higher (2.96) than the Table F-value (2.10) and thus shows that different concentrations have significantly different actions on fat body with respect to lipid release. Third row of values which represent the analysis of the combined effect of stages and concentrations also shows high significance (F-value calculated 8.90 and tabulated 1.57).

Table V.2.2 likewise, presents the analysis of data for fat body incubations without the hormone (controls). Here the release of lipids is shown to be dependent on the stage, i.e., they are highly significantly different in different stages (F-value 30.24 compared to Table F-value of 2.61). Since the controls contained only distilled water in place of the hormones, the significant difference observed here can only be due to the difference brought about by different fat bodies, the inherent physiological difference between the fat body from different individuals (F-value 30.13 compared to Table F-value of 2.10). Third row of values which represent the analysis of the combined effect of stages and concentrations, shows the significant effect (F-value calculated 34.18 and tabulated 1.57).

Table V.2.3 provides the analysis of data for the difference between experiments and controls. The data shows that the difference between experimental and control observations is significant. The first row of values shows the significant difference between different stages of the insect (F-value is 6.83 compared to the Table F-value 2.61). The second row of values shows the data for the difference between experiments and controls with different concentrations (F-value calculated 3.34 and table F-value 2.10). The third row of values represent the difference of the values for the combined effect of stages and concentrations. This value also shows significant difference (F-value 1.83 compared to table F-value 1.57).

Figure 5.5 presents the effect of AKH on lipid release from the fat body from various stages. It appears that in the case of *S. mauritia* the synthetic hormone has more of inhibitory effect on lipid release when compared to its stimulatory effect. The only stimulatory effect on lipid release were shown by fat body from prepupa and adult. In the case of prepupa 0.05, 0.075, 0.1 and 0.125 μM solutions of the hormone showed positive effect and the maximum release of lipid occurred was about 17.0 mg/g fb (with 0.05 μM). Adult fat body responded positively to 0.05, 0.075 and 0.50 μM hormone solutions and they all had almost the same potency and released about 15-17 mg lipid/g fb. Other concentrations showed negative results to various degrees. Maximum

Fig. 5.5. Effect of different concentrations of syn AKH on lipid release *in vitro* from the fat body of various stages of *S.mauritia*



inhibitory effect (by about 45 mg/g fb) was given by 0.25 μM followed by 1.0 μM (by about 28 mg/g fb) and 0.1 μM (12 mg/g fb). A concentration of 0.125 μM was almost ineffective. Pupal fat body showed slight positive response but not to any appreciable levels, with 0.05 and 1.0 μM hormone. All the other concentrations showed negative response. Inhibitory effect of the hormone was the most prominent in the 96 h larval fat body, where hormone at all the concentrations tested gave inihibitory effects. Maximum inhibitory effect (by about 40 mg/g fb) was shown by a concentration of 0.1 μM followed by 0.125 μM (by about 30 mg/g fb). Concentrations of 1.0 μM and 0.075 μM gave inhibitions between 10 and 17 mg/g fb. Other concentrations (0.05, 0.25 and 0.50 μM) were more or less ineffective. Prepupal and pupal fat body in spite of having some positive response with some concentrations of the hormone also showed negative response. However, the extent of inhibition, just like their stimulatory effect was less prominent. Maximum inhibition in prepupal fat body was effected by 0.25 μM (by 10 mg/g fb) and in the case of pupal fat body it was about 13-16 mg/g fb (by 0.075 and 0.25 μM). Thus in brief, the activity of the hormone at various concentrations on lipid release from the fat body of various stages showed varying effects and these effects were significant.

Figure 5.6 shows the interaction of the stages and hormone concentrations with lipid release. The nature of the curve clearly suggests that the activity of the hormone on different stages is significantly different. The pattern of activity for different stages are markedly different. It is evident from the graph that the effect of most of the hormones in majority of the cases (stages as well as concentrations) show inhibition rather than stimulation of lipid release.

Figure 5.7 presents the amount of lipids released from the fat body from various stages in the experimental incubations with various concetrations of the synthetic hormone. The bar diagram provides a visual comparison of the lipid released. Maximum lipid release was stimulated in the adult with 0.125 μM (178 mg/g fb). In the adults, all the concentrations show better result than any of the other stages. Next better results were given by fat body from prepupal stage. The activity was still lower in 96 h larval fat body and the pupal fat body had the lowest activity. Figure 5.8 represents the same data as that of Fig. 5.7, but presented in a different way, i.e., activity of the concentrations in all the stages. There seems to have no common pattern for lipid release from the fat body when the data were represented in this fashion.

Fig. 5.6: Graph showing the interaction between various stages of *S.mauritia* and different concentrations of syn AKH

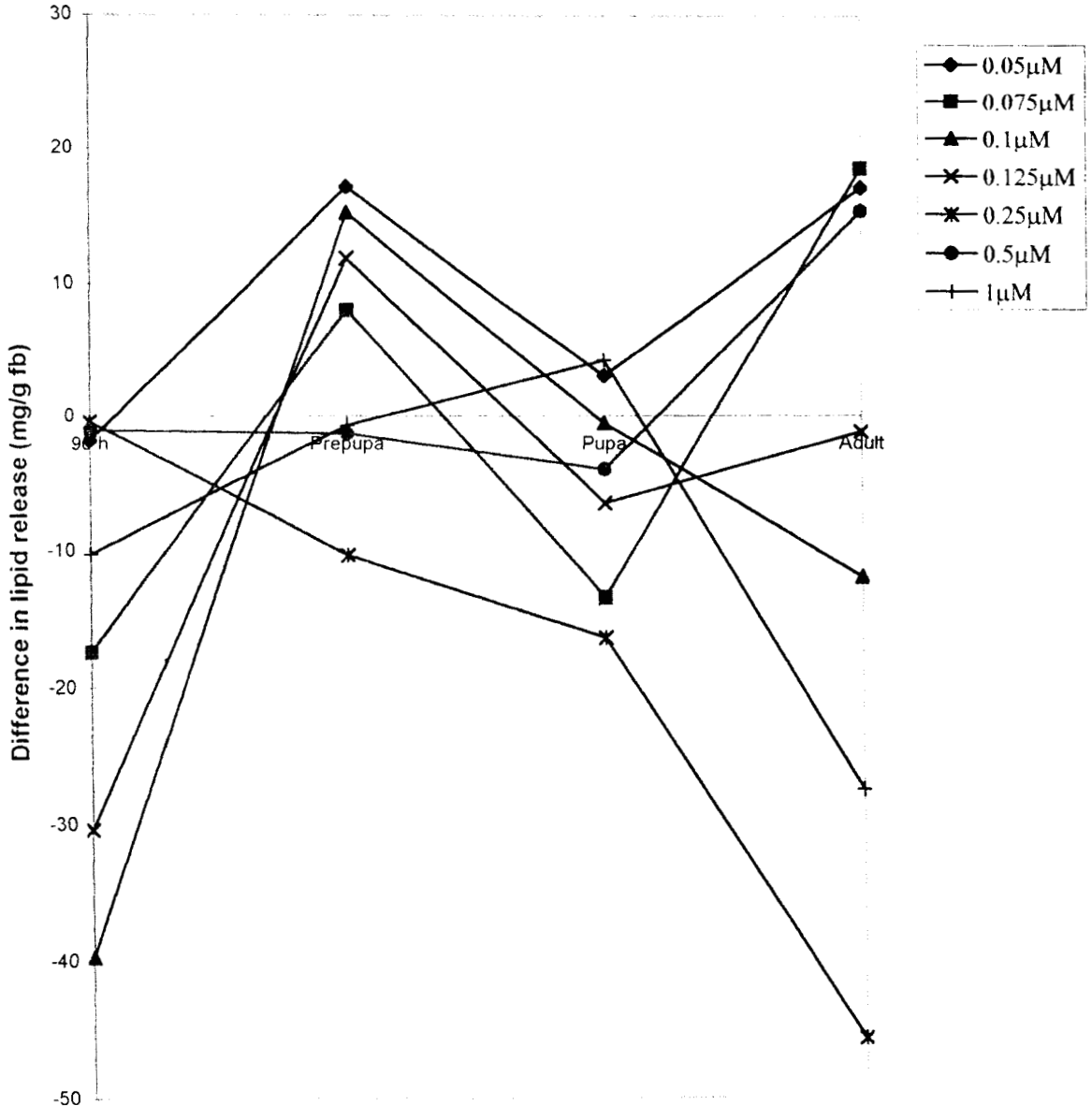


Fig. 5.7. Average lipid release from the fat body of *S.mauritia* incubated with different concentrations of syn AKH in various stages (n=8)

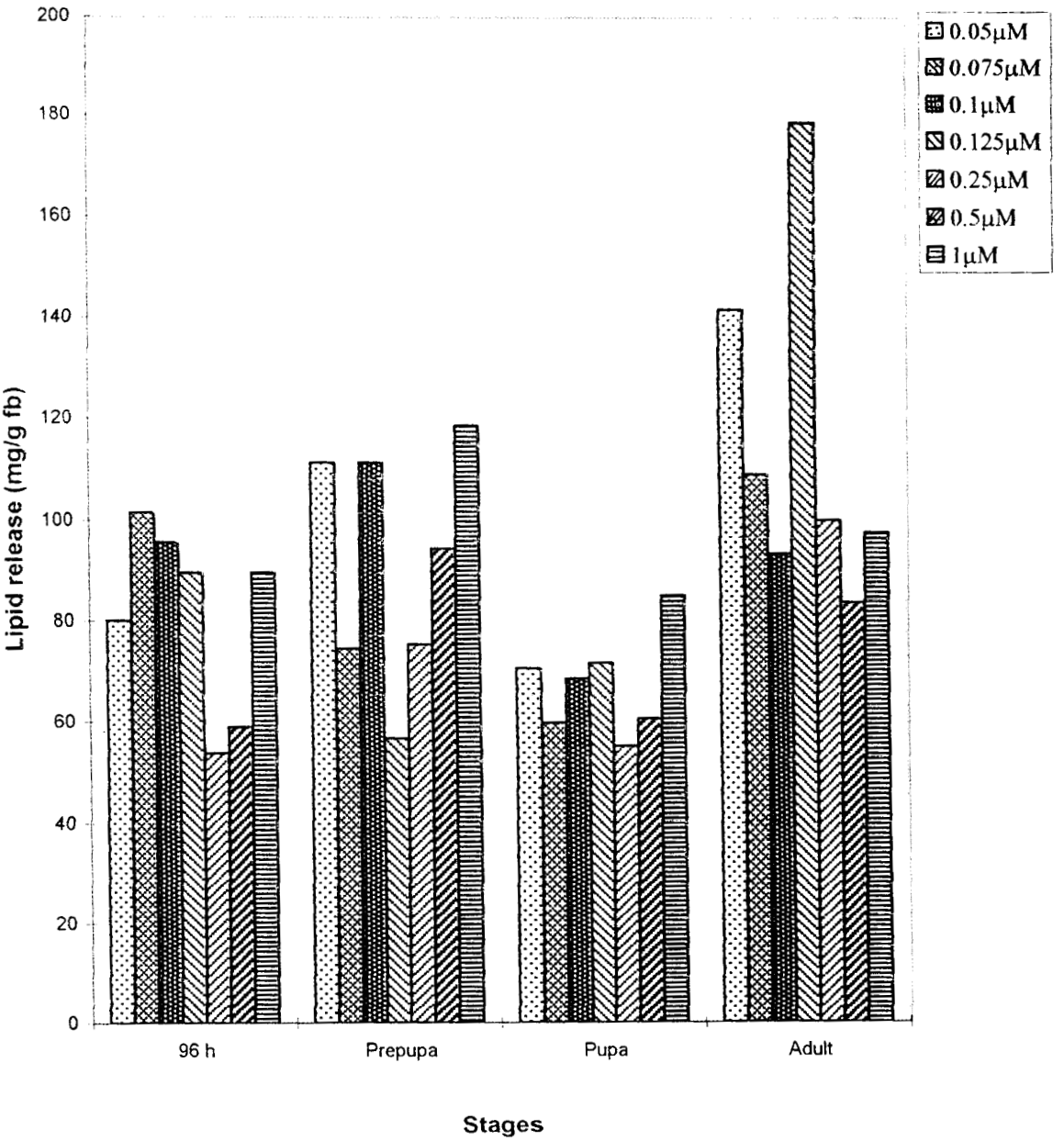
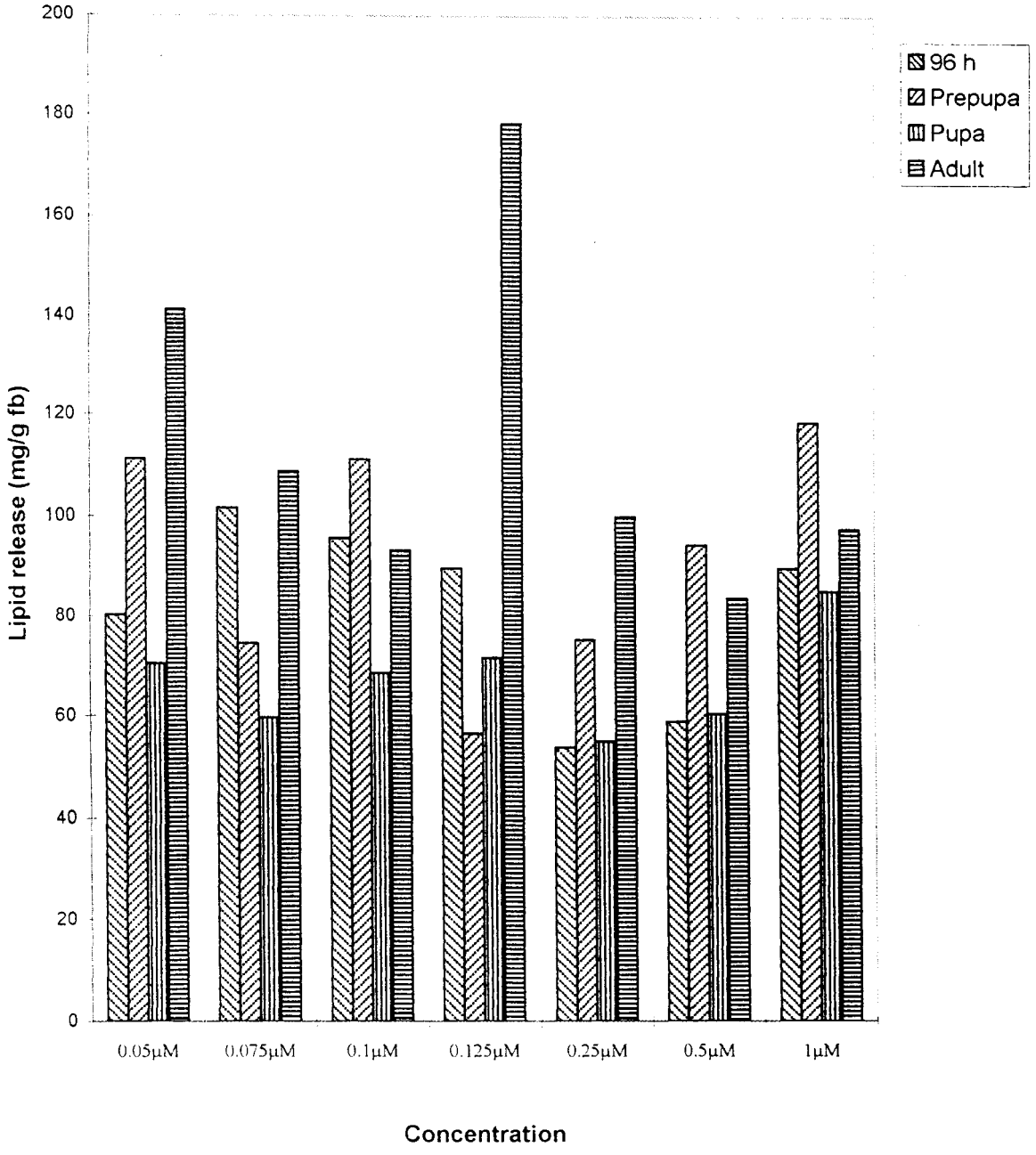


Fig. 5.8. Average lipid release from the fat body of various stages of *S.mauritia* at different concentrations of syn AKH (n=8)



V.2.3. Lipid release from the fat body of *I. limbata* incubated with different concentrations of SH-extract *in vitro*

Various concentrations of SH-extract were tested for their effects on lipid release from the fat body of *I. limbata*. The fat body was incubated for 30 min and total lipids released were measured in controls and experimentals as described in materials and methods section. The results obtained were subjected to ANOVA as described and the data are presented in Table V.3. Table V.3.1 analyses the data obtained for the experimentals where the incubation mixtures contained the hormone at different concentrations. The hormone showed significant difference in its effects on fat body lipid release with different concentrations (F-value 5.45 compared to Table F-value of 2.45). Table V.3.2 similarly, analyses the data for control observations where the incubation mixture contained distilled water instead of hormone. Here also the F-value is higher (2.95) than the Table F-value (2.45). The significant difference observed here can only be due to the difference in the activity of the fat body taken for the experiments. Table V.3.3 shows the difference between the experimental and control values subjected to ANOVA. The effects of different concentrations of the hormone on lipid release from the fat body are found to be significant. The F-value is 3.20 compared to the Table F-value of 2.45.

Table V.3. ANOVA of data for lipid release from the fat body of *I. limbata* incubated with various concentrations of SH-extract *in vitro*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Conc. (C)	4	26.13797	6.534492	5.45	2.45
Error	25	30	1.2		

2. Control

Source	df	SS	MSS	F	Tab. F
Conc. (C)	4	14.1633	3.540825	2.95	2.45
Error	25	30	1.2		

3. Difference

Source	df	SS	MSS	F	Tab. F
Conc. (C)	4	15.37539	3.843847	3.20	2.45
Error	25	30	1.2		

The data used for ANOVA were obtained from measurement of lipid released from fat body of adult *I. limbata* at different concentrations of SH-extract (Total cases considered are 30).

Figure 5.9 presents the extent of the effect of different concentrations of SH-extract on fat body lipid release from *I. limbata*. It appears that the maximum amount of lipids is released with a hormone concentration of 1.0 gpe (by 6.25 mg/g fb). Lower concentrations (0.001, 0.01 and 0.1 gpe) also showed positive effects (4.14, 0.58 and 4.92 mg/g fb), among which 0.01 gpe showed only very slight activity. Figure 5.10 provides the actual amount of lipids released in the experimental observations. The bar diagram provides a visual comparison of the lipid released with various hormone concentrations. Maximum lipid release was found in the incubations with a concentration of 1.0 gpe (by about 46 mg/g fb) followed by 0.001 gpe (45 mg/g fb). Lipid release in other cases were 30 mg/g fb (0.1 gpe), 21 mg/g fb (2.0 gpe) and 20 mg/g fb (0.01 gpe).

V.2.4. Lipid release from the fat body of *I. limbata* injected with different concentrations of SH-extract *in vivo*

Different concentrations of SH-extract were tested *in vivo* for their effects on lipid release from the fat body of *I. limbata*. The results obtained were subjected to ANOVA and is presented in Table V.4. It appears that the different concentrations of the hormone showed significant effect with respect to fat body lipid release *in vivo*. The F-value is 2.73 compared to Table F-value of 2.45.

Fig. 5.9. Effect of different concentrations of SH- extract on lipid release *in vitro* from the fat body of *I.limbata*

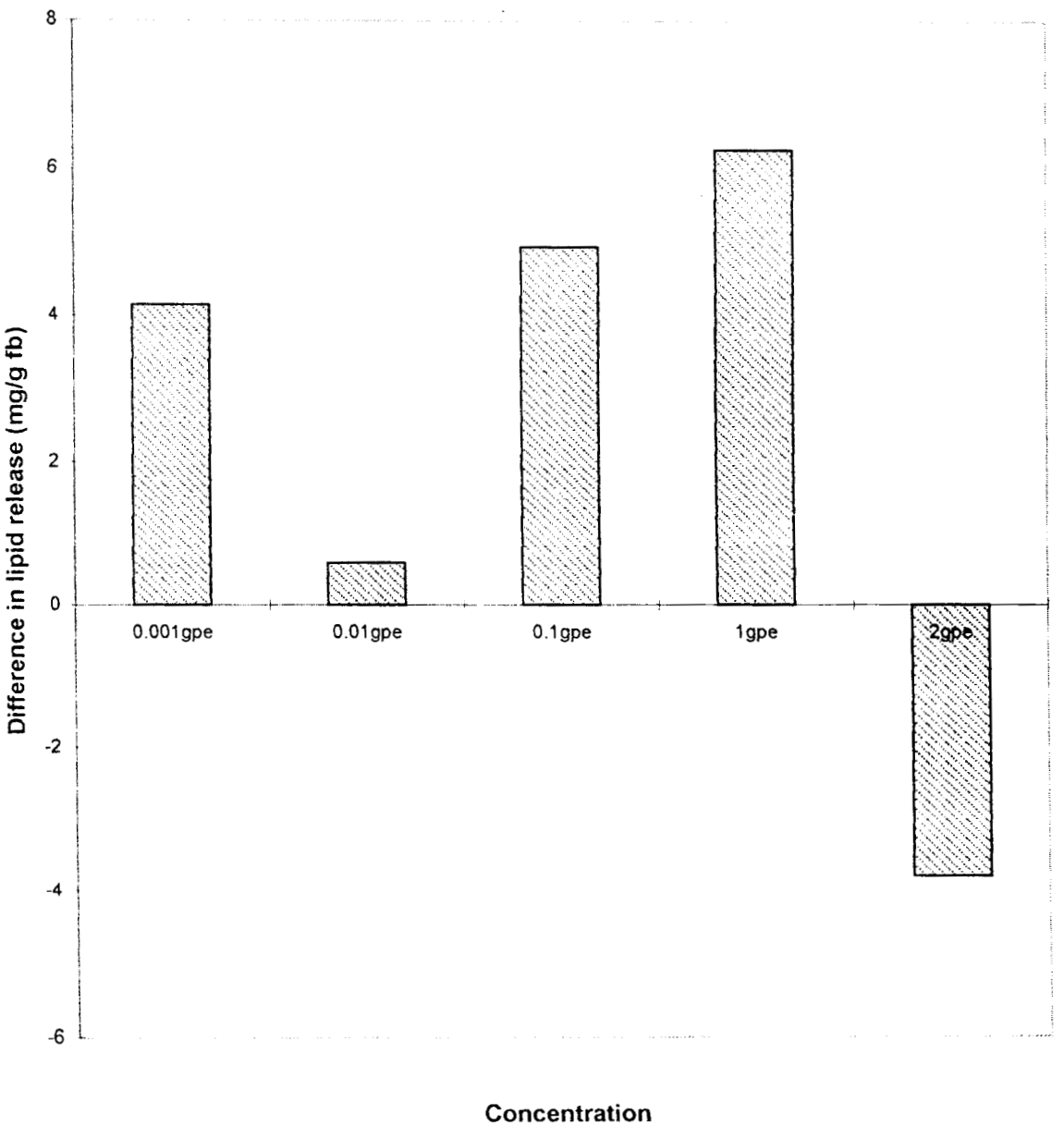
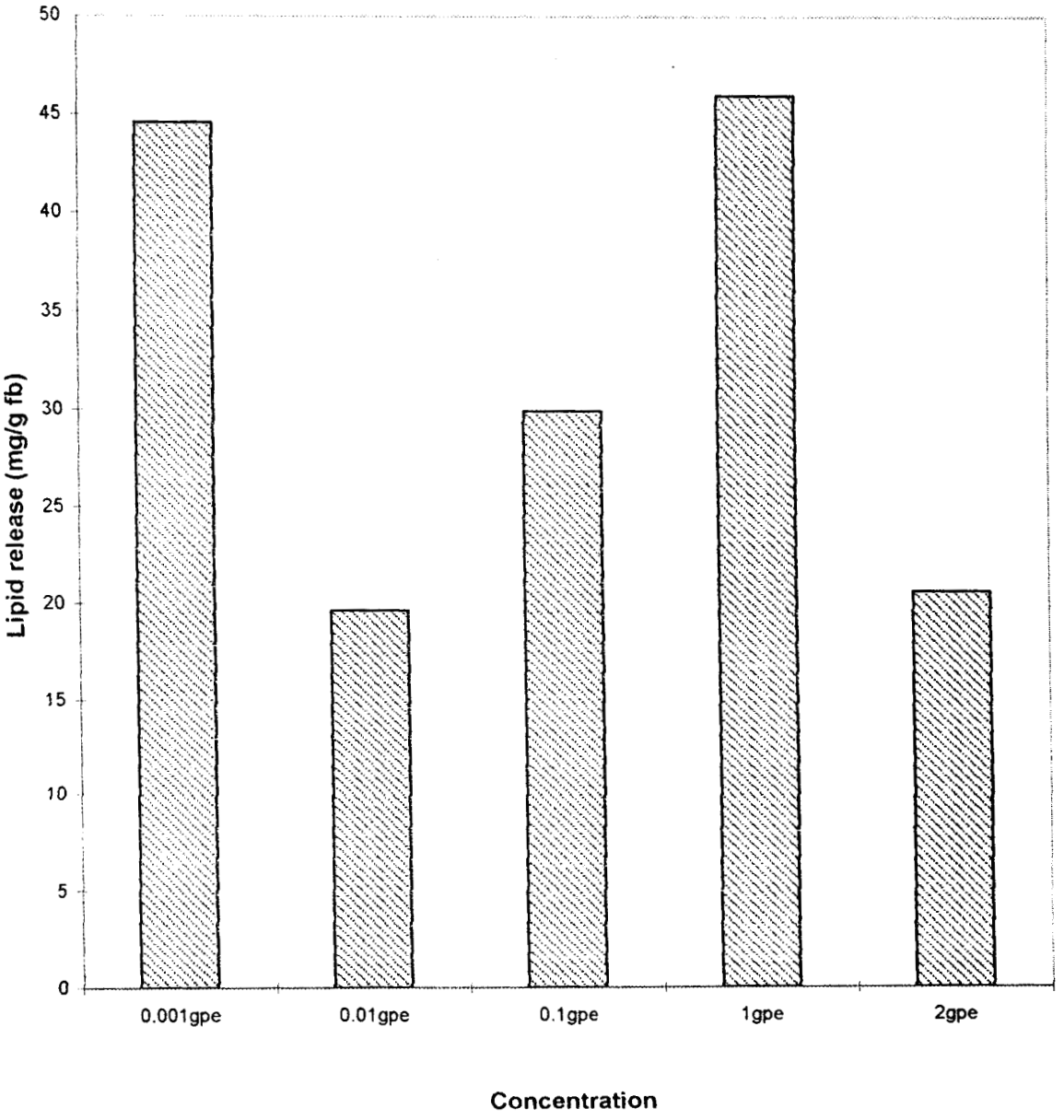


Fig. 5.10. Average lipid release from the fat body of *I.limbata* incubated with different concentrations of SH- extract *in vitro* (n=6)



04C

Table V.4. ANOVA of data for lipid release from the fat body of *I. limbata* at different concentrations of SH-extract *in vivo*

Experiement

Source	df	SS	MSS	F	Tab. F
Conc. (C)	5	139604.5	27920.9	2.73	2.45
Error	41	419855.5	10240.38		

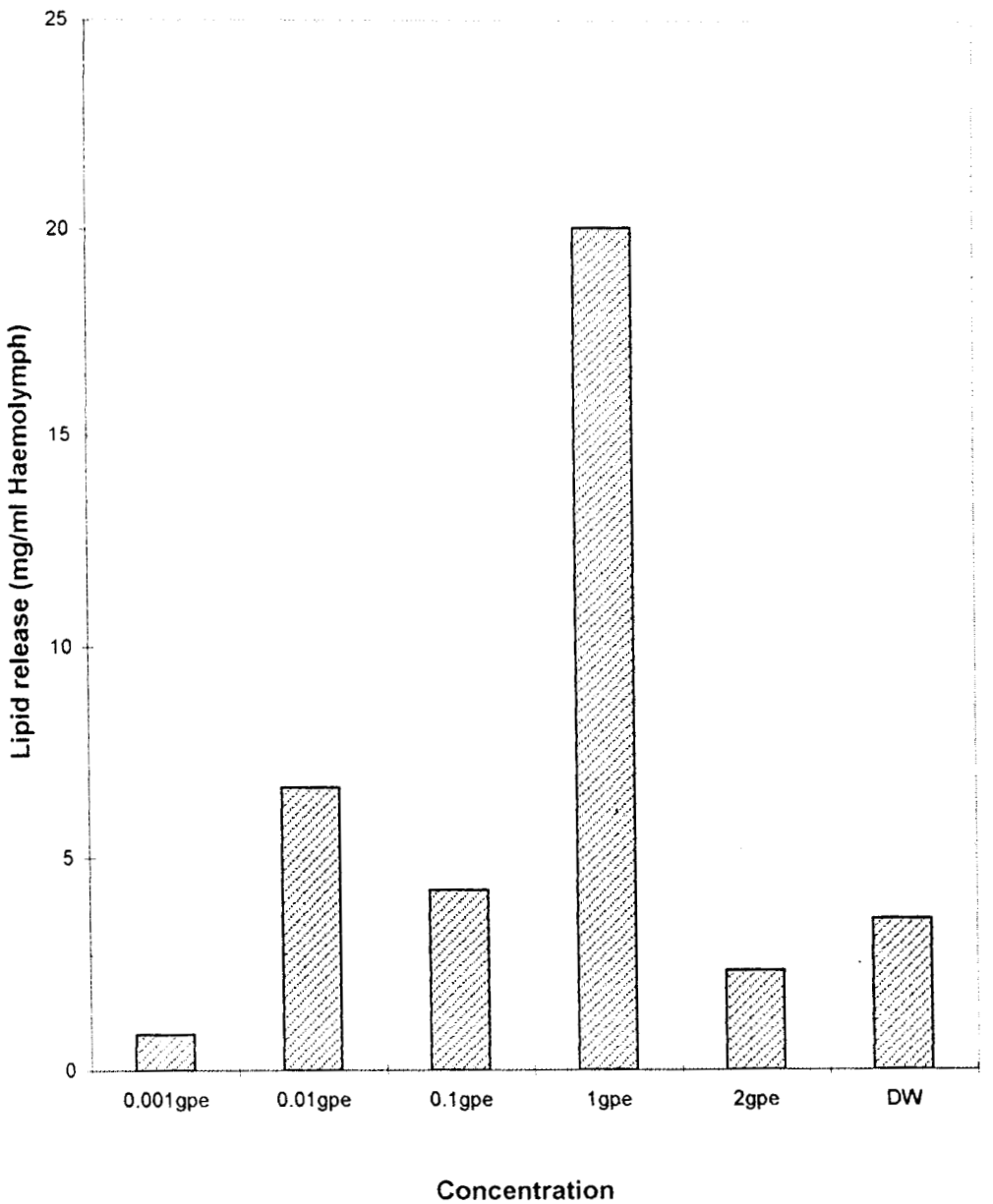
The data used for ANOVA were obtained from analysis of lipid release using *I. limbata* at different concentrations of SH-extract (Total cases considered are 39).

Figure 5.11 provides visual comparison of lipid released from the fat body by different concentrations of hormone extract injected. The maximum release of lipid occured was about 20.2 mg/g fb with a hormone concentration of 1.0 gpe. Some concentrations (0.001 and 2.0 gpe) showed lower values than the distilled water injected, only of about 1.3 mg/g fb and 2.2 mg/g fb respectively.

V.3. Discussion

Experiments carried out to study the effect of hormones (SH-extract and syn AKH) on lipid release from the fat body of the insects *S. mauritia* and *I. limbata* have shown interesting results. The SH-extract though active in *I. limbata* both *in vivo* and *in vitro*, and elicited a dose response *in vitro*, was not that much effective in *S. mauritia*. Most of the concentrations tried showed a reduction or reversal in their activity as the insect developed from larval to adult stage. In adults although there was a high rate of release with all the concentrations (Fig.5.3), the effect of the hormone was prominent only with a concentration of 1.0 gpe (Fig.5.2). Other three concentrations showed negative effects. The inhibitory effect of 0.1 gpe concentration was more (54 mg/g fb) than the stimulation obtained with 1.0 gpe (Fig.5.1). The gradual loss of response to the lowest concentration (0.001 gpe) of SH-extract, observed during larva-adult transformation (Figs.5.1 and 5.2) suggests that

Fig. 5.11. Effect of different concentrations of SH- extract on lipid release *in vivo* from the fat body of *I.limbata*



the larval fat body cells are sensitive to very low concentration of the hormone prevalent in larval stages. This helps the insect to release lipids to meet any energy requirements during emergencies such as starvation. The younger stages of insects have been found to have only smaller titre of hormones (Van Marrewijk *et al.*, 1983; Siegert and Mordue, 1986). This sensitivity is gradually lost through prepupal to pupal stage. This loss of sensitivity and reversal of the activity is also evident in the case of a concentration of 0.1 gpe. Here, the very low stimulatory effect get reversed to a very high inhibitory effect from larval to adult stage gradually through prepupal and pupal stages (Fig.5.2).

The loss of sensitivity to the hormone through prepupal to pupal stage is suggestive of the gradual loss of the adipokinetic response in the less active stages where less energy is required. In the pupal stage since the fat body has been completely histolysed the hormone may not have had the necessary cell membrane complements required for the proper response. It is also likely that due to the histolysis of the fat body most of the lipids must have been liberated into the haemolymph, resulting in a reduction of total lipid release (Fig.5.3). The inhibitory effect demonstrated with 0.1 gpe is difficult to explain in this context. In the case of a concentration of 1.0 gpe, there was a reversal of

activity from an inhibitory effect to a stimulatory effect during development from larval to adult stage.

The effect of syn AKH on lipid release from *S. mauritia* fat body was found to be almost similar to the effect of SH-extract except the case of 96 h larva. The synthetic hormone did not show any adipokinetic activity on the larval fat body. All the concentrations of the hormone tested showed inhibitory effects to various extent. Maximum inhibition was shown by 0.1 μM solution. The observed inhibition may be due to the non-functioning of the lipid release mechanism in the experimentals, as a result of the hormone present in the *in vitro* medium. In the controls on the other hand no such regulation is posed and there could be a natural release of lipids. The inhibition in the experimentals can also be due to the action of the hormone on the metabolic pathways leading to lipid synthesis inhibition resulting in a lowered level of released lipids. Such lipid synthesis inhibiting factors have been reported in *G. morsitans* (Pimley and Langley, 1981). Lipid synthesis inhibition by adipokinetic hormone have also been reported in *S. gregaria* (Gokuldas *et al.*, 1988).

In the prepupa lower concentrations were hyperlipaemic (0.25, 0.5 and 1.0 μM) and higher concentrations were hypolipaemic. However, both these activities were not appreciable. Maximum lipid release obtained was 17 mg/g

fb with a concentration of 0.05 μM. Slight inhibitory effect was shown only by 0.25 μM (by 10 mg/g fb). The stimulatory effect shown by this stage suggests that the fat body is still capable of responding to hormone to allow lipid release for utilisation during the prepupal stage where formation of chitinous tissue and other structures required for the formation of pupa take place (Sridhara and Bhat, 1965 b; Henson *et al.*, 1972). In the pupal stage, there was no stimulation of lipid release except some insignificant effect by 1.0 and 0.05 μM. All the other concentrations showed slight inhibitory activities. Being the most inactive stage, the pupa is expected to consume less energy for activities such as locomotion and therefore there is no necessity to have lipid mobilisation and the associated cellular machinery required for it. Moreover on the 7th day on which the insects were used for the experiments, most of the adult structures including the adult fat body must have been formed which is seen to favour more sugar release as a response to hormone treatment rather than lipids.

Adult fat body showed a mixed response to the adipokinetic hormone. There was more inhibition than stimulation. Inhibitory activity showed wide variation among the concentrations of 0.1, 0.125, 0.25 and 1.0 μM. Highest activity was shown by 0.25 μM (45 mg/g fb). Stimulatory effects were shown by 0.05, 0.075 and 0.5 μM concentrations, all of which showed almost similar

level of stimulation (15-17 mg/g fb). Although it is difficult to explain why both stimulatory as well as inhibitory effects are shown by the hormones on the adult fat body, it could be assumed that the adult fat body still have the ability to mobilise and release lipids in response to adipokinetic factors.

The SH-extract when tested on the fat body of *I. limbata*, an insect whose fat body has been found to respond positively to adipokinetic factors (syn AKH, Vrinda, 1997, unpublished) both *in vivo* and *in vitro*, was found to elicit hyperlipaemia both *in vivo* and *in vitro*. The results give additional support for the presence of compounds similar to adipokinetic hormone in the SH-extract. The loss of activity with the highest concentration (2.0gpe) may be due to the presence of increased amount of some inhibitory factors which could be there in the SH-extract. Such hypolipaemic factors have been reported in the locust (Orchard and Loughton, 1980; Loughton,1987). Hypolipaemic factors, have been presumably acting as regulatory factors on hyperlipaemia (Orchard and Loughton,1980; Loughton,1987). The factors believed to be having some common molecular features with vertebrate insulin have been proposed to have similar function as that of insulin. The pattern of hyperlipaemic activity and loss of activity at higher concentration were similar both *in vitro* and *in vivo* except that the hyperlipaemia was much effective with 1.0 gpe in *in vivo* (20.2 mg/ml hl) than the corresponding value

in vitro (6.25mg/g fb). The better result in the *in vivo* conditions is suggestive of the requirement of lipoproteins and other necessary complements for maximum lipid release (Chino, 1985; Shapiro *et al.*, 1988; Law *et al.*, 1992; Ziegler *et al.*, 1995; Engler *et al.*, 1996).

STUDIES ON LIPID SYNTHESIS IN THE FAT BODY OF S.MAURITIA

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter VI

STUDIES ON LIPID SYNTHESIS IN THE FAT BODY OF *S. MAURITIA*

VI.I. Introduction

In animals, lipids serve both as fuel reserves and as structural components of cells. In insects, during somatic growth, structural lipids, such as phospholipids, sterols and cuticular lipids, are synthesized in larger quantities whereas synthesis of storage lipids (di-or triacylglycerols and free fatty acids) predominate during other times. Storage lipids are utilized during non-feeding periods (such as starvation, diapause, moulting etc.) or during intense physical and physiological activities (for example, flight, oogenesis etc.). Thus it is clear that in the life cycle of insects, there is great fluctuation in the synthesis and utilization of lipids.

Variation in lipid synthesis during development has been studied in many insects. Walker and Bailey (1970 b) reported that in male desert locust the dietary carbohydrates are converted to lipid and the major period of lipogenesis was between the fifth and the eighth day after adult emergence. It has also been found that the patterns of changes of enzymes associated with the conversion of carbohydrates to acetyl- CoA for lipogenesis correlate well with the observed increase in the fat body lipid content (Walker and Bailey, 1970 a).

Studies on lipid metabolism in insects have been carried out by

following the incorporation of different labelled substrates into total and various subclasses of lipids and different fatty acids. Incorporation of labelled precursors such as acetate, glucose, trehalose, amino acids and fatty acids has been studied in many insects (Clements, 1959; Zebe and McShan, 1959; Walker and Bailey, 1970 a; Chang and Friedman, 1971). Walker and Bailey (1970 b) followed the incorporation and distribution of label from acetate, glucose, trehalose and citrate into different lipid classes in the adult desert locust. Label from acetate was mainly incorporated into triacylglycerols (more than 70%). The free fatty acid fraction contained less than 2% of the total incorporation. Municio *et al.* (1973) made a comparative study on the *in vitro* and *in vivo* incorporation of [^{14}C]- acetate into different lipid classes by homogenates of different stages of the insect *C. capitata*. They found that the pattern of lipid labelling *in vitro* depended on the time of incubation and stage of development. Larval and pharate adult homogenates incorporated [^{14}C]-acetate rapidly during the first 60 min, mainly into phospholipids. In the *in vivo* experiments triacylglycerols accounted for the highest percentages of incorporation in both stages of development whereas free fatty acids and phospholipids show low incorporation. Stephen and Gilbert (1969) using gas chromatographic and mass spectrographic analysis demonstrated that the major fatty acids present in the silkmoth *H. cecropia* are palmitic, palmitoleic,

stearic, oleic, linoleic and linolenic acids. Their studies using labelled acetate, palmitate and linoleate showed that the insect can synthesize only straight-chain saturated and mono-unsaturated fatty acids from acetate.

It is well established that lipid synthesis in animals is regulated by hormones. This has been studied in detail in mammalian tissues such as adipose tissue and liver which helped to draw the complete picture of regulatory mechanism in lipid synthetic pathways. It has been found that in these tissues, lipogenesis is subject to short-term and long-term control mechanisms. Glucagon, insulin and presumably cyclic AMP appear to be involved in adaptive changes in the quantity of the lipogenic enzymes (long-term control) (Gibson *et al.*, 1972). Cyclic AMP and glucagon have been found to inhibit lipogenesis (Hamis and Yount, 1975). Cyclic AMP has been found to exert its inhibitory action at various levels such as aerobic glycolysis (possibly phosphofructokinase, and pyruvate kinase), pyruvate dehydrogenase system, citrate transport, acetyl-CoA carboxylase, fatty acid synthetase system and fatty acid esterification (for reviews, see Saggerson, 1985; Harris *et al.*, 1979).

Insect CC have been shown to secrete a number of hormones controlling various aspects of lipid metabolism. Corpora cardiaca extract have been

shown to increase the synthesis of neutral glycerides in the fat body of *P. americana* at the expense of that in the haemolymph (Downer and Steele, 1972). It is possible that the hypolipaemic factor described from the storage lobe of CC in *Locusta* by Orchard and Loughton (1980) could be a similar factor. The lipid synthesis inhibiting factor (LSIF) reported by Pimley and Langley (1981) has been found to inhibit lipid synthesis from labelled amino acids. Gokuldas *et al.* (1988) and Gokuldas (1989) have reported that locust AKH also inhibit lipid synthesis in fat body of younger stages of the locust *S. gregaria* from their ^{14}C-acetate incorporation studies. Gokuldas (1989) has also found that the hormones inhibits incorporation of ^{14}C-acetate into fatty acids by younger locusts and stimulated ^{14}C-glucose incorporation into lipids.

Bahjou *et al.* (1990) reported that lipogenesis occured in the absence of added carbohydrate in the medium, but it was stimulated by the addition of glucose, especially trehalose. They also reported that intestinal insulin like peptide (ILP) also stimulated *in vitro* lipogenesis in a dose-dependent fashion. The amount of lipid synthesized *in vitro* in *A. aegypti* by the ovaries was extremely small, less than 1/1000 of the amount of lipid that accumulates in the oocytes (Ziegler, 1997).

The experiment described in this section were conducted to investigate any possible role of the hormone extracted from the neuronal tissues of *S. mauritia* and that of syn AKH, on the lipid synthesis in the fat body of *S. mauritia*.

VI.2. Results

Effect of SH-extract and syn AKH on lipid synthesis in the fat body of *S. mauritia*

Lipid synthesis by the fat body from various stages of *S. mauritia* and the effect of SH-extract and syn AKH on lipid synthesis were studied by using radiolabelled acetate as the precursor. Fat body, prepared as described elsewhere, were incubated with SH-extract (1.0 gpe) and AKH (100 pmole). The results were subjected to ANOVA and is presented in Table VI. Table VI.1 provides the data obtained for the experimentals where the incubation mixture contained the hormones. The first row of values shows the activity with different stages. The F-value is lower (1.41) when compared to the table F-value 2.61 showing that there is no significant difference in the lipid synthesis between fat body from different stages. The second row of values derived from the effect of the hormones (SH-extract and AKH) shows that F-value is less than 0.01 compared to the Table F-value of 2.10. This indicates that the effect of both hormones (AKH and SH extract) on lipid synthesis by fat body

Table VI. ANOVA of data for the effect of SH-extract and syn AKH on lipid synthesis by the fat body from various stages of *S. mauritia*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	5.0895	1.6965	1.41	2.61
SH & AKH	1	8.087158E-04	8.087158E-04	0.00	2.10
S x H	3	5.303909	1.76797	1.47	1.57
Error	40	48	1.2		

2. Control

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	4.237747	1.412582	1.18	2.61
Fat body (Fb)	1	6.208672	6.208672	5.17	2.10
S x Fb	3	6.673065	2.224355	1.85	1.57
Error	40	48.00001	1.2		

3. Difference

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	11.65876	3.886254	3.24	2.61
SH & AKH	1	4.105449E-03	4.105449E-03	0.00	2.10
S x H	3	4.9042	1.634733	1.36	1.57
Error	40	48	1.2		

The data used for ANOVA were obtained from analysis of lipid synthesis using different concentrations of SH-extract and syn AKH (Total cases considered are 48). 'H' indicates the hormones.

are not significant. The third row of values which shows the combined effect of stages and hormones with lipid synthesis shows that the F-value is (1.47) smaller compared to the Table F-value of 1.57 indicating that the interaction of stages and concentration also does not bring out any significant change in the lipid synthesis in the fat body.

Table VI.2 presents data for controls. This shows the level of significance among fat bodies of different stages. The first row of values shows the activity of different stages in control experiments. The F-value is 1.18 compared to the Table F-value of 2.61 showing that the difference between stages is not significant. The second row of values show the difference among fat bodies as there was no hormone added in the incubation mixture. The F-value is 5.17 compared to Table F-value of 2.10, showing that the difference is significant. The third row of values show the interaction of stages and fat bodies with lipid synthesis. The effect is significant as the F-value is greater (1.85), compared to table F-value of 1.57. Table VI.3 provides the data on the difference between experiments and controls from different stages and with the two hormones (SH-extract and AKH). The table shows that there is a significant difference between stages as far as the stimulation of lipid synthesis was concerned. The F-value is 3.24 compared to Table F-value of 2.61. There was no significant difference between the effects of SH-extract

and AKH (F-value is less than 0.01 compared to Table F-value of 2.10). When stages and concentrations were considered together (stages x concentration), the F-value was 1.36 and Table value was 1.57, indicating again that the activity is not significant.

Figure 6.1 shows the effect of SH-extract and AKH on lipid synthesis by the fat body of various stages of *S. mauritia*. It appears that the hormones react differently with various stages viz., 96 h, prepupa, pupa and adult. Stimulatory effects on lipid synthesis were shown by fat body from prepupa and adult. In the case of prepupa, maximum stimulation of label incorporation was about 84 DPM/mg fb by SH-extract and 6 DPM/mg fb by AKH. In adult fat body the maximum stimulation occurred with SH-extract was about 82 DPM/mg fb and with AKH, about 61 DPM/mg fb. On pupal fat body both of these hormones showed inhibitory activity. The 96 h larval fat body reacted differently to two hormones. In this stage, AKH has a stimulatory effect (by about 5 DPM/mg fb) but SH-extract showed an inhibitory effect of 15 DPM/mg fb on lipid synthesis from the fat body. Figure 6.2 shows the interaction of SH-extract and AKH with different stages of *S. mauritia* on lipid synthesis by the fat body. It becomes clear that both hormones react differently with different stages of development.

Fig. 6. 1. Effect of syn AKH and SH - extract on [1^{14} C] acetate incorporation into lipids by fat body from *S.mauritia*

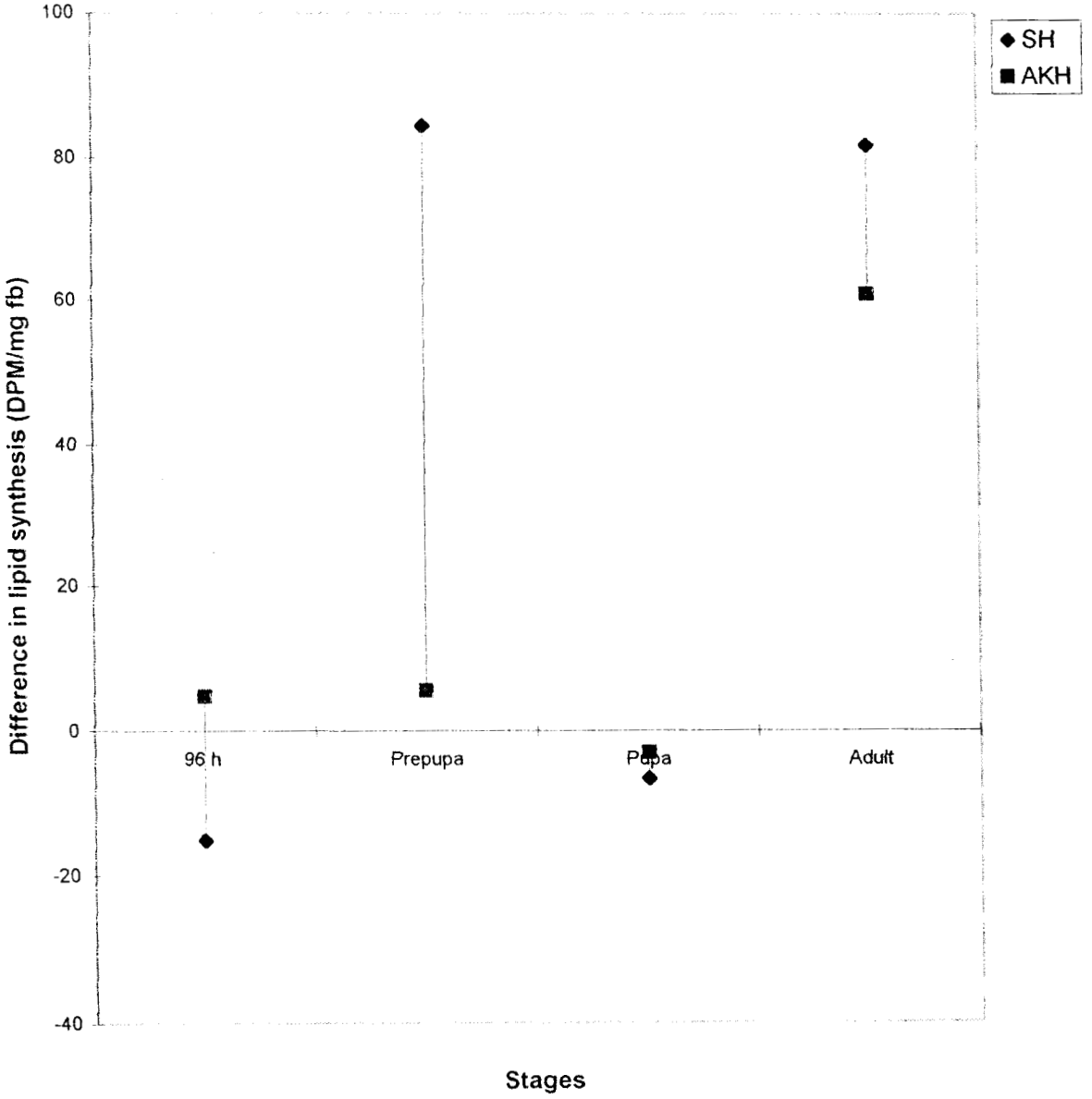


Fig. 6.2. Graph showing the interaction of syn AKH and SH- extract with different stages of *S.mauritia* in lipid synthesis

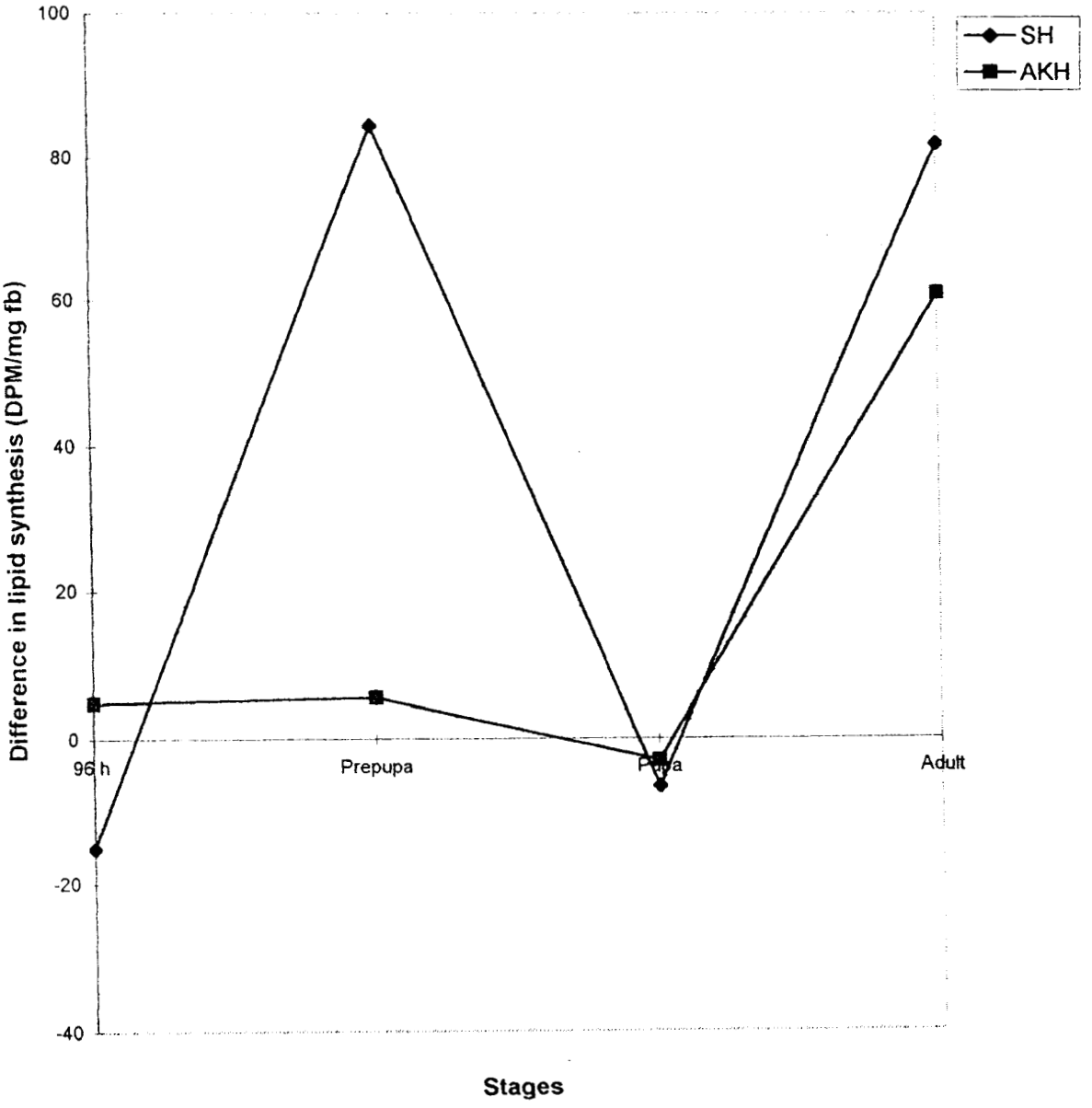


Figure 6.3 presents the amount of lipids synthesized (in terms of DPM/mg fb) in the experimental incubations in various stages of *S. mauritia*. Maximum incorporation of labelled acetate was found in the prepupal fat body followed by adult fat body both treated with AKH (175 and 161 DPM/mg fb respectively). A similar higher rate of incorporation were seen in the fat body from the same stages were seen in the SH-extract treated experiments (123 and 141 DPM/mg fb respectively). In both the cases, fat body from 96 h and pupa showed very poor rate of label incorporation. The effect is more evident in Fig. 6.4. From the observations it is clear that both hormones have similar effect on lipid synthesis in different stages of *S. mauritia*.

VI.3. Discussion

The synthetic activity of lipids appears to take an alternating fashion during development. It has been found from the quantitative measurement of lipids (Chap. VII) towards the end of day 4, the quantity of lipids in the larva declines which eventually reverses the condition and accumulates more lipids during prepupal stage (Fig. VII.1). The observation made regarding the effect of hormone on lipid synthesis also reveals that SH extract stimulates lipid synthesis in the prepupal stage considerably. This is suggestive of an increased rate of lipid synthesis during the prepupal stage. Here, AKH was not very effective. In pupa the rate of lipid synthesis and response to the hormone is

Fig. 6.3. Effect of syn AKH and SH- extract on lipid synthesis in the fat body from various stages of *S.mauritia* (n=6)

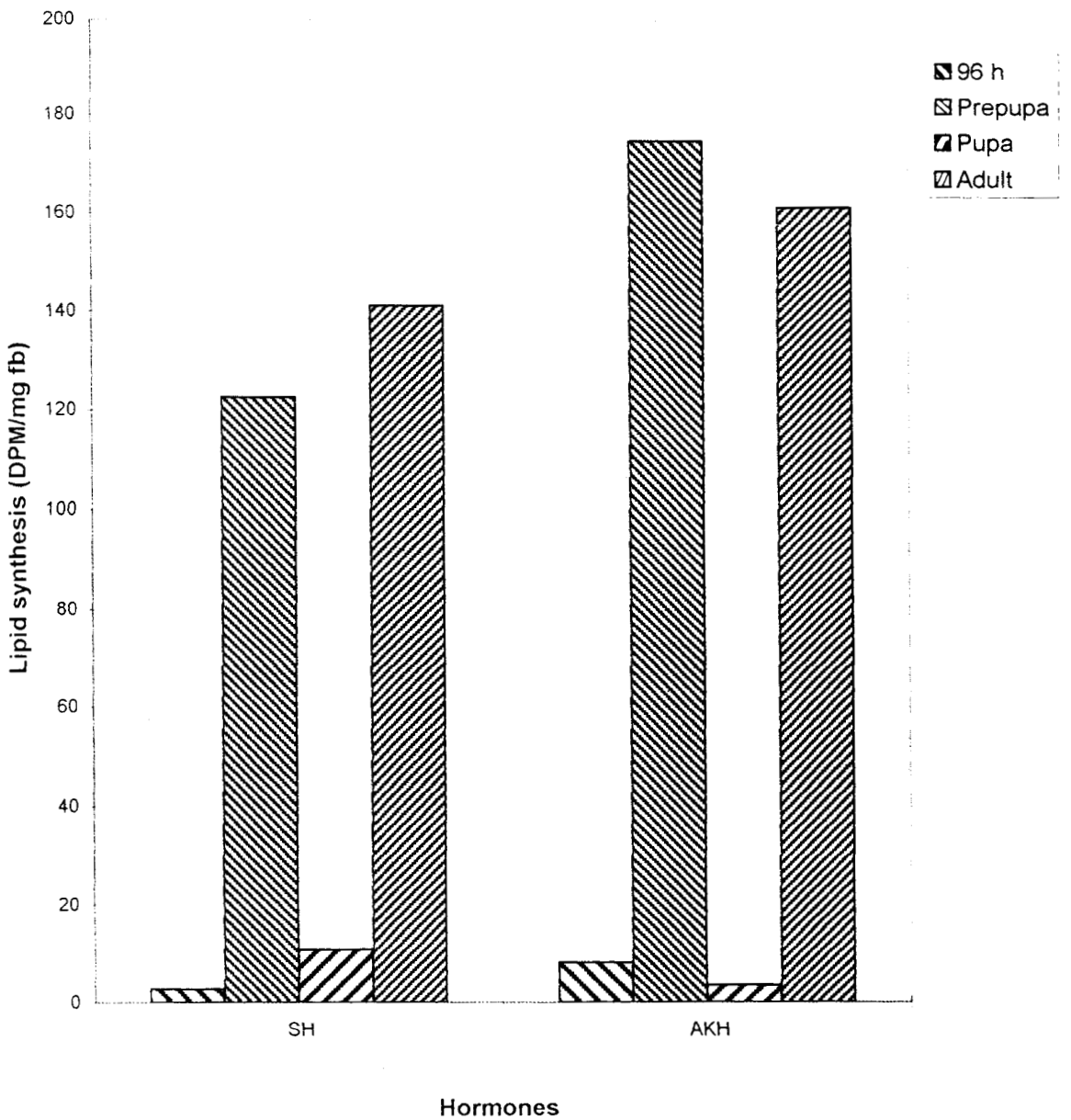
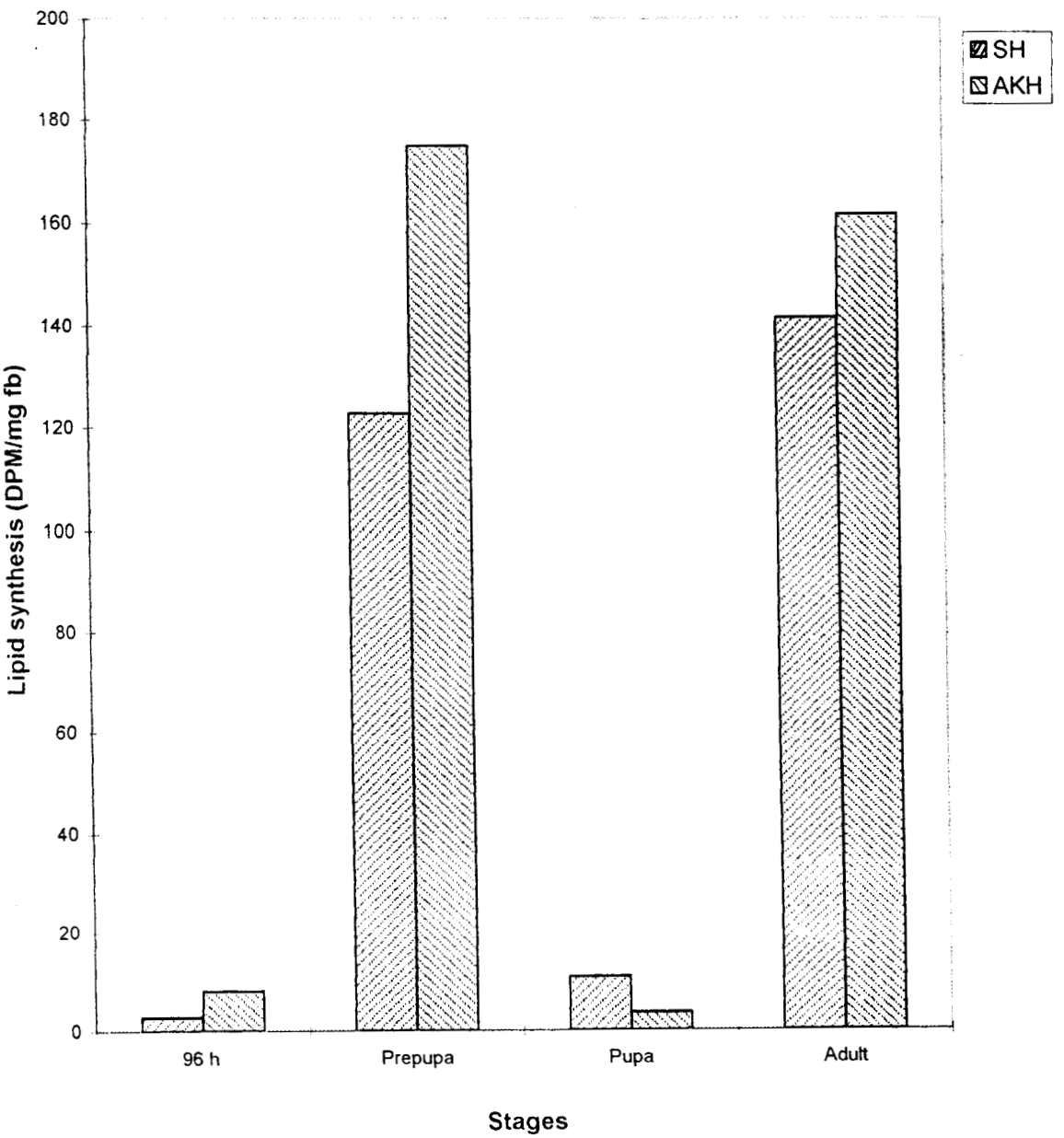


Fig. 6.4. Variation among different stages of *S.mauritia* in the effect of syn AKH and SH- extract on lipid synthesis (n=6)



very low. The synthetic activity gets to higher rates again in the adults. Unlike the prepupal fat body, adult fat body is sensitive to both SH-extract and AKH. Both the hormones stimulates lipid synthesis, here again SH-extract showing better stimulation. These results shows that those stages which are sensitive to the hormones also are the stages where maximum accumulation of lipids takes place (Chap. VII). This again points to possible involvement of the hormones in the SH-extract in the lipid synthetic activity of *S. mauritia.*

Various hormones in insects have been found to be involved in lipid synthesis and accumulation. For example, juvenile hormone secreted by CA (Pfeiffer, 1945; Hill, 1972) and peptides secreted by CC (Pimley and Langley, 1981; Gokuldas *et al.*, 1988). These hormones naturally come into action when they are needed for the physiological regulation. The physiological state of the insects changes during the growth and metamorphosis, especially in the case of holometabolous insects. Synthesis, storage and mobilization occur as and when required, sometimes the latter has to take place unexpectedly in situations like starvation. In order to meet such emergencies, insects should have the mechanism to switch over to the required pathway by means of a switch over in the production of the necessary hormone. In order to make this possible, the necessary hormone-mediated transducing elements should be present.

Experiments on lipid synthesis indicate that SH-extract and AKH are able to influence the synthetic pathway. It is possible that the hormones act on the acetyl-CoA synthase, acetyl-CoA carboxylase or fatty acid synthetase in the fat bodies of prepupae and adults to bring about timely regulation.

CHANGES IN TOTAL LIPID CONTENT DURING GROWTH AND DEVELOPMENT OF S. MAURITIA

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

Chapter VII

CHANGES IN TOTAL LIPID CONTENT DURING GROWTH AND DEVELOPMENT OF *S. MAURITIA*

VII.I. Introduction

Lipids constitute the most economical energy reserves in insects and form substrate for energy production required by all energy consuming processes such as growth, metamorphosis, oogenesis and embryogenesis etc. (Gilbert, 1967). Insects are particularly rich in lipids, most of which is stored in the fat body, an organ analogous to mammalian liver. The importance of lipids lies in the fact that, in terms of weight and storage space it is the most efficient form in which energy can be stored which is extremely important for migratory and diapausing insects. Storage of lipids during the larval development for subsequent utilization during metamorphosis of several insects was reported (Fast, 1970). Lipids play structural roles in cellular and intracellular membranes and appear to be metabolically active in most cellular activities such as energy production and storage, protein synthesis and cellular respiration.

Studies on the lipid composition of a number of insects have been carried out. Most of these studies have been confined to whole insects. Since fat body is the major site of synthesis and storage in insects, the major part of lipid is found in the fat body (Wlodawer and Baranska, 1965, Crone *et al.*, 1966; Thomas, 1974). Total lipid content of a large number of insects of various orders, at various stages of development have been described by Fast

(1964) and discussed by Gilbert (1967). Fawzi *et al.* (1961) for the first time prepared samples of lipid extracts from fat body of *S. gregaria*. Later such studies have been undertaken on fat body and haemolymph lipids of *H. cecropia* (Chino and Gilbert, 1965 a, b), *B. mori* (Sridhara and Bhat, 1965 b), *L. migratoria* (Tietz, 1967), *Pyrrhocoris apterus* (Martin, 1969), *G. morsitans* (D'costa and Rutesaria, 1973) and *Oncopeltus fasciatus* (Thomas, 1974).

The amount of lipids present in insects is often high, making up, for example, 25% of the wet weight of the cocoon of the silkmoth and 49-69% of the dry weight of male locusts (Fawzi *et al.*, 1961). The larvae of the beetle, *Pachymerus dactris* is reported to contain nearly 50% of lipids (Collin, 1933). Quantitative estimation of the fat body lipids in *P. regina* shows that 52-69% of the fat body dry weight is lipid (Orr, 1964). In *Oryctes* larvae, 56.6% of the fat body dry weight is constituted by lipids (Nair *et al.*, 1967).

The content of lipids in the fat body shows changes during the reproductive cycle of female insects. It varies from 52-59% of the dry weight of the fat body during the reproductive cycle in the female blowfly, *P. regina* (Orr, 1964), 29-46% during oogenesis in female *L. maderae* (Gilbert, 1967) and 56-71% in the first reproductive cycle of female *S. gregaria* (Hill *et al.*, 1968). In males also, the lipid content of the fat body may be high, for

instance its quantity ranges from 10-30% of the wet weight during the adult development in males of the desert locust, *S. gregaria* (Walker *et al.*, 1970). Several dimorphism of fat body lipids has been reported in several species (Nelson *et al.*, 1967; Bhakthan and Gilbert, 1972) and changes associated with diet, metamorphosis, aging and exercise also have been noted (Wigglesworth, 1942; Nelson, *et al.*, 1967; Martin, 1969; Dutkowski and Ziajka, 1972).

The experiments described in this chapter were intended to quantify the lipids in the whole body of various stages of *S. mauritia* and find out any correlation between the level of lipid content and the receptiveness of fat body cell membranes for hormone molecules if the hormone had any lipid mobilizing or synthesizing potential.

VII.2. Results

Pattern of lipids during development of *S. mauritia*

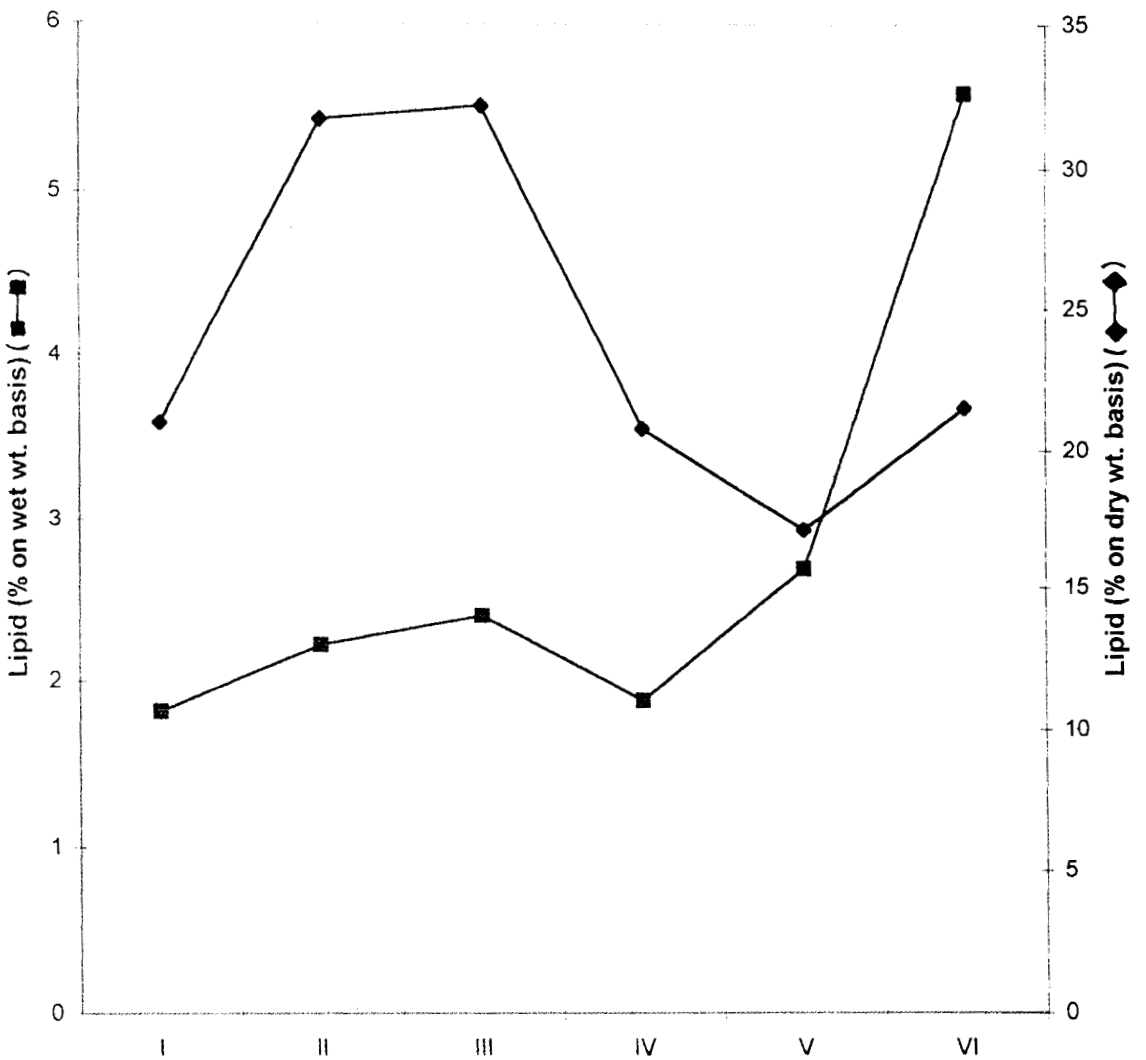
Changes in the patterns of total lipids during growth and development of sixth instar larva, pupa and adult of *S. mauritia* are presented in Tables VII 1-3. The total lipids present in whole insect were measured gravimetrically. In the sixth instar larvae, the percentage of total lipids calculated on wet weight basis showed a slight but gradual increase from 1.82% to 2.4% during day I to day 3 and declined afterwards (1.89%) until the 4th day. But later,

Table VII.1. Changes in total lipids during the growth of sixth instar larva of *S. mauritia.*

Age of larva (days)	Weight of fresh larva (mg)	Weight of dry larva (mg)	Weight of lipids (mg)	% of lipids (wet wt. basis)	% of lipids (dry wt. basis)
I	226.5 ± 7.2	19.9 ± 0.9	4.1 ± 0.2	1.82 ± 0.11	20.96 ± 1.26
II	398.0 ± 16.0	27.9 ± 1.3	8.8 ± 0.4	2.22 ± 0.11	31.73 ± 1.49
III	589.8 ± 13.2	44.5 ± 1.6	14.1 ± 1.0	2.4 ± 0.17	32.21 ± 2.80
IV	674.2 ± 19.0	60.6 ± 2.5	12.6 ± 1.1	1.89 ± 0.17	20.77 ± 1.44
V	295.6 ± 7.0	49.0 ± 1.7	8.2 ± 0.4	2.69 ± 0.09	17.13 ± 0.98
VI	209.1 ± 6.1	54.4 ± 2.0	11.8 ± 0.71	5.6 ± 0.27	21.52 ± 0.86

Values are expressed as means ± SEMs (n = 10).

Fig. 7.1. Changes in the quantity of total lipids during growth of the sixth instar larva of *S.mauritia*



Each point represents the mean of 10 determinations

the total lipids showed a sharp increase from day 4, until day 6 (about 5.6%, Fig.7.1). Change in total lipid calculated on a dry weight basis shows more or less same pattern. The lipid content increased from day 1 to day 3 (from 20.96% to 32.21%). The increase was sharp when compared to values obtained from a wet weight basis measurement. There is a decline afterwards until day 5 (17.13%). However, after day 5, there was another phase of increase in total lipids to about 21.5% (Fig.7.1).

Figure 7.2 represents changes in the quantity of total lipids during the non-feeding stage, the pupa. There was an initial decline in the amount of total lipids during first 2 days, from 7.57% to 6.14% on wet weight basis and 30.7% to 23.7% on dry weight basis until day 3, the amount of lipid remained more or less the same. But from 3rd day on there was a sharp increase in total lipids calculated on the basis of both wet weight and dry weight, and reached 8.76% and 34.5%, respectively. This plateau remained until the 5th day (8.9% and 38.4% respectively). Afterwards there was a sharp decline during the next day (6) about 3.6% in wet weight basis and 17.6% on dry weight basis. The lipid content further declined to very low amounts during 7th day, upto 2.6% on wet weight basis and 12.8% on dry weight basis (Fig.7.2).

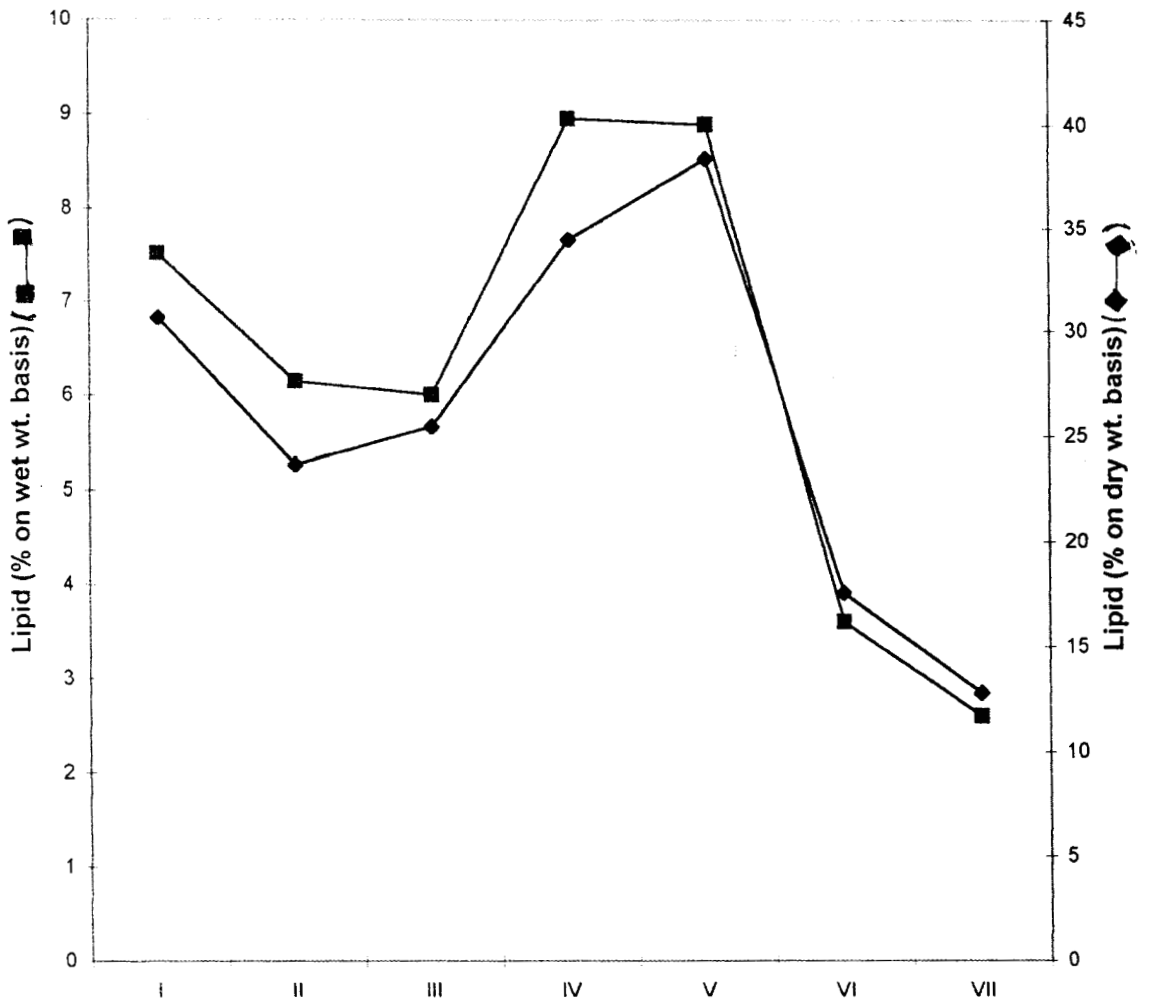
In the case of adult, the total lipids on wet weight basis showed a gradual increase from Ist day to 4th day (6.1% to 8.4%). But lipids calculated

Table VII.2 Changes in total lipids during pupal stage of *S. mauritia*

Age of pupa (days)	Weight of fresh pupa (mg)	Weight of dry pupa (mg)	Weight of lipids (mg)	% of lipids (wet wt. basis)	% of lipids (dry wt. basis)
I	285.2 ± 9.5	70.6 ± 3.5	21.5 ± 0.8	7.57 ± 0.28	30.7 ± 0.98
II	262.5 ± 5.9	67.5 ± 2.0	16.1 ± 1.0	6.14 ± 0.37	23.7 ± 1.14
III	251.5 ± 5.4	59.8 ± 1.7	15.0 ± 1.9	6.00 ± 0.74	25.5 ± 3.39
IV	241.7 ± 8.2	64.2 ± 2.4	21.6 ± 3.8	8.96 ± 1.54	34.5 ± 6.89
V	222.1 ± 9.3	52.5 ± 2.6	20.1 ± 3.6	8.90 ± 1.50	38.4 ± 6.72
VI	212.1 ± 6.4	43.5 ± 1.7	7.7 ± 0.7	3.60 ± 0.25	17.6 ± 1.07
VII	156.6 ± 7.6	31.1 ± 1.8	4.00 ± 0.5	2.60 ± 0.28	12.8 ± 1.39

Values are expressed as means ± SEMs (n = 10).

Fig. 7.2. Changes in the quantity of total lipids during pupal stage of *S.mauritia*



Agé of pupa (days)

Each point represents the mean of 10 determinations

on dry weight basis showed a slightly different pattern. There was a small initial decline in the amount of total lipids during the first 2 days (from 18.0% to 16.7%). From day 2 onwards, the amount of lipids increased and reached a high level during the 4th day of adult (20.9%). On the basis of dry weight and 8.4% on the basis of wet weight on the whole, the adult stage had low lipid content in comparison with larval and pupal stages (Fig. 7.3).

VII. 3. Discussion

The changes in total lipid content during growth and development of the various stages of *S. mauritia* show patterns that reflect the various life activities during these periods. The larval stage show a gradual accumulation of lipids in this insect. On the basis of both wet weight and dry weight of the larva it has been found that there was an increase in the total lipid content during the first 3 days (2.4% and 32.2% of the total body weight respectively). Similar results were obtained from the observations of Kapur *et al.* (1983). They found that in the larvae of *Chilomenes sexmaculata*, the total lipid and triglyceride content showed a high level in the last larval instar. The results indicate that as the larva grows old the rate of lipid synthesis also increases. This is in close agreement with the results obtained from *H. cecropia* (Gilbert and Schneiderman, 1961) and *P. regina* (Wimer and Lumb, 1967). During larval period, large amount of reserve materials are stored for metamorphosis

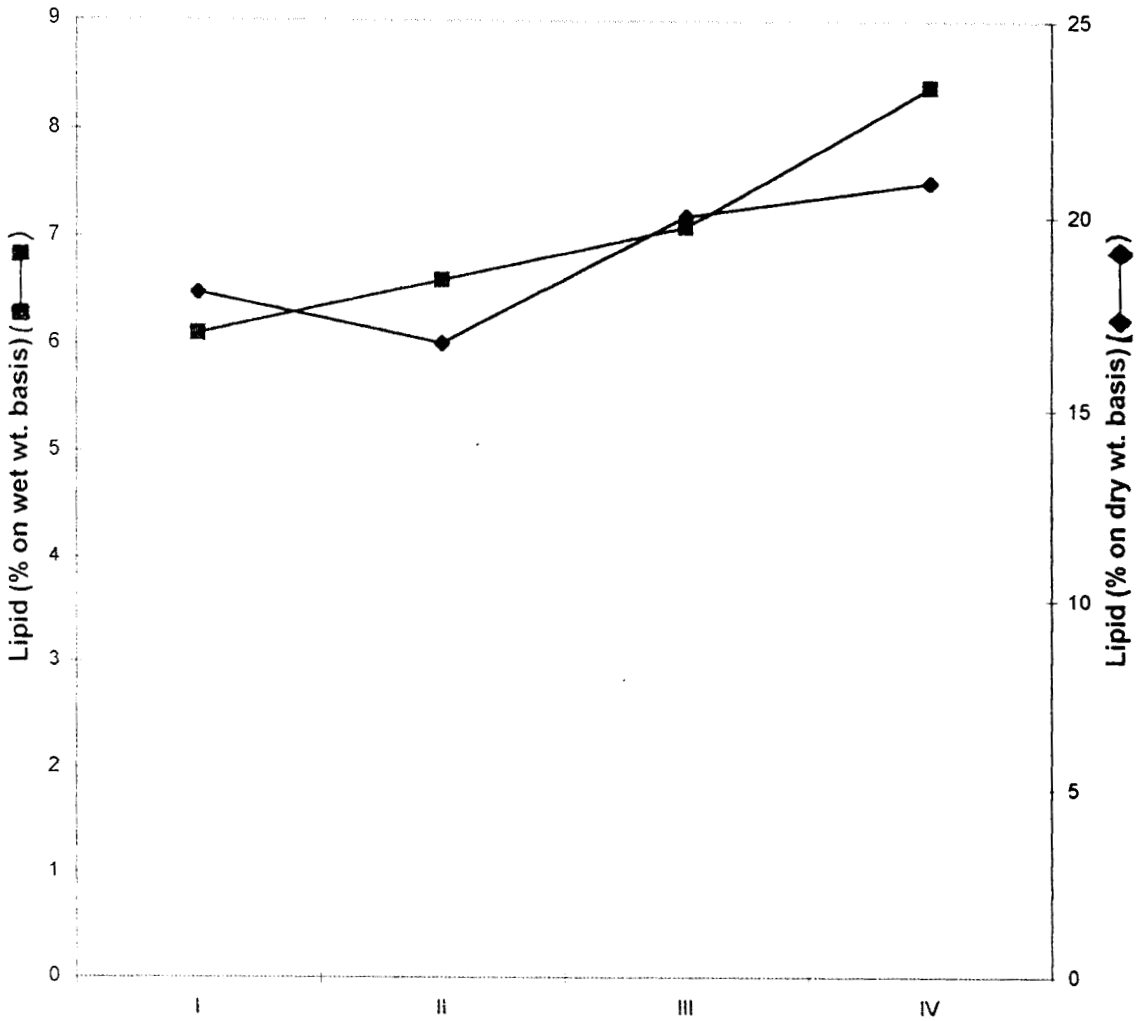
Table VII.3 Changes in total lipids during development of adult *S. mauritia*

Age of adult (days)	Weight of fresh adult (mg)	Weight of dry adult (mg)	Weight of lipids (mg)	% of lipids (wet wt. basis)	% of lipids (dry wt. basis)
I	69.0 ± 4.9	23.7 ± 2.0	4.2 ± 0.4	6.1 ± 0.421	18.0 ± 1.34
II	122.3 ± 10.4	50.0 ± 5.6	7.8 ± 0.7	6.6 ± 0.56	16.7 ± 1.46
III	125.2 ± 11.2	45.2 ± 3.5	8.8 ± 1.3	7.1 ± 0.89	20.0 ± 2.36
IV	61.1 ± 2.8	24.2 ± 1.2	5.1 ± 0.5	8.4 ± 0.75	20.9 ± 1.42

Values are expressed as means ± SEMs (n = 10).

125B

Fig. 7.3. Changes in the quantity of total lipids during development of adult *S.mauritia*



Age of adult (days)

Each point represents the mean of 10 determinations

H 9

(Wimer, 1962; Sridhara and Bhat, 1965 b; Wlodawer and Baranska, 1965). After 3rd day there was a sudden decline in total lipid content (to 1.89% on wet weight basis and 20.77% on dry weight basis). The reduction in lipid content after 3rd day is probably due to the low rate of lipid synthesis as is clear from Fig. 6.1. This is suggestive of a phase of reduced lipid synthesis during which the insect transforms gradually to the pupal stage during the next two days. The total lipid content calculated on wet weight basis attained the highest peak on the last larval day (5.6%). The lipid content calculated on dry weight basis (Fig.7.1) also showed a similar pattern of variation. But the per cent content was more than these amounts calculated on wet weight basis which was due to the higher content of water in the larval stage. After day 3, observed drop in the lipid content could be due to further reduction in water content in the whole body resulting from the non feeding towards the end of larval stage. On the day 5, the content of lipids again showed an increase (to about 21.52%) before pupation. The significant increase in lipid content during late larval period was probably the result of synthesis of lipids from carbohydrates. The above observation is in agreement with the findings of Mukherji and Guppy (1973). They found that in *P. unipuncta*, the lipid content decreased at the beginning of 4th instar, then increased and attained a high level until pupation. Niemierko et al. (1956) observed similar changes

in *B. mori*, Demainovsky and Zubova (1956) in *Antheraea pernyi*, Strogaya (1961) in *Pieris rapae* and *P. napae* and Gilbert and Sehneiderman (1961) in *H. cecropia*. Henson *et al.* (1972) have also found similar increase in lipid content until pupal stage during metamorphosis of the boll weevil, *Anthonomus grandis*. The drop during the 4th day (wet weight basis, 1.89%) and 5th day (dry weight basis, 17.13%) in the quantity of lipids might be due to a high rate of synthesis and accumulation of nonlipid compounds such as glycogen and protein towards the end of larval stage. Wimer and Lumb (1967) reported that in *P. regina* there was an increase in triglyceride content during development. The average lipid content calculated on dry weight basis 32.21% (Table VII.1) on 3 day old larva. According to Fast (1964) the average lipid content of larvae is about 30%. It is suggested that active feeding take place during the first 3 days, and feeding decreases afterwards and stops at 96 h larval stage. After 96 h, there is an increase in the amount of lipid content during prepupal stage. The results agreed with the results obtained by Kapur *et al.* (1983). The lipid accumulation in the late phase of larvae is because of the fact that the energy for the subsequent pupal stage need to be met from the savings of this stage. Most of the lipids synthesized during the larval period are stored in the fat body. Since the fat body is considered as the major site of lipid storage, it is expected that its lipid content

would be higher. Most of these stored lipids are utilized at the pupal stage for the formation of adult structures. Ingested nutrients like carbohydrates are utilized during the larval activity and they are also transformed to lipid for storage (Lohr and Gaede,1983). The increased level of total lipid in larval stages may act as a potential reserve sources for pupa–adult development (Pant and Kumar,1979). Mandal and Chaudhuri (1992) reported that in holometabolous insects, the high level of lipids could also be due to high synthetic rate.

There was a very high degree of fluctuation in the amount of total lipids during the pupal period. The initial higher values showed a downward trend during the 2nd and 3rd days and thereafter increased and reached a plateau during 4th and 5th days (Fig.7.2). This period having high lipid content probably represent the period of formation of more structural lipids from lipid and nonlipid precursors because it is towards the end of pupation that adult tissues are formed. Similar results have been reportred by Mukherji and Guppy (1973). Their studies indicate that the accumulation was probably the result of synthesis of lipids from carbohydrates (Walker and Bailey,1970 a, b; Storey and Bailey, 1978 a). According to Gilbert (1967) the increase of lipids during pupation may reflect a release of lipids firmly bound to the proteins used in construction of cocoons or may be due to enhanced

lipid synthesis. The sharp decline from 5th to 7th day of pupal stage both on wet weight (from 8.96% to 2.60%) and dry weight (from 38.4% to 12.8%) basis probably marks the depletion of more quantity of lipids for energy used for the formation of nonlipid components of newly formed tissue. The results agreed with the results obtained by Kapur *et al.* (1983) from *C. sexmaculata*, where the level of lipids decreased during pupation from prepupa to late pupa. Pupa is a closed non-feeding entity and hence utilises reserves stored during larval stage. Our results also showed similarity with the results obtained by D'costa and Birt (1966). They found that in *L. sericata* the neutral lipids decreased immediately after pupation (by 3 μmoles /insect) maintained for a period in mid pupal life, then declined rapidly (by 4 μmoles/insect) until adult emergence. In *Lucilia* the larval fat reserves are utilised for pupal and adult development. A similar decline was noticed in *Lucilia* by Evans (1932) and in the *Tribolium* by Villeneuve and Limonde(1963). Finch and Birt (1962) reported that the fatty acids provide energy for some of the metabolic events occurring during true pupal life, in which oxidative metabolism is possible. During pupal stage most of the lipids is utilised as energy fuel and for tissue transformation during histogenesis. The low profile in lipid content agrees with the results represented in Fig.6.1.

(the results represented in Fig.6.1. for the pupa were obtained from analysis of lipid synthesis in the fat body taken from 6-7 day old pupa).

During the adult period, the total lipid content calculated on a wet weight basis showed a gradual increase from day 1 to day 4 (Fig.7.3). But on dry weight basis the values declined from the first day, (to 16.7%), then showed an increase thereafter and reached a high value on 4th day (20.9%). The increase in total lipid content in adult may be due to the increase in the rate of lipid synthesis in the adult stage. In the adults, since the diet is mainly carbohydrate rich, relatively more lipids, required to meet the demand during flight and reproduction are to be synthesised. The results also agree with the lipid synthesis studies (Fig.6.1), where adult showed an increased rate of lipid synthesis. The synthesised lipids are meant for use for most of the physiological processes. The lipids are mobilised from the fat body and transported to flight muscles and developing oocytes (in females). Adult fat body synthesises lipids from carbohydrates and stores lipids as energy reserves. Similar results obtained from *L. migratoria* by Walker *et al.* (1970), suggest that the lipid reserves increases between 6 and 8 days after emergence. Walker and Bailey (1970 a, b) also reported that fat body synthesises lipid from carbohydrate during adult development. A similar pattern of lipid changes during development of the male desert locust has been reported by

Odhiambo (1966). The stored lipid is subsequently utilised during adult life together with the lipid and carbohydrate ingested from the diet for activities such as flight. The findings thus agree with the latest reports from Zera *et al.* (1994) that the lipid in *Gryllus firmus* increased during adult development.

The results obtained, thus suggests that the pattern of lipid accumulation in *S. mauritia* is similar to any other insect, i.e., lipid accumulates during intensive feeding stage and is utilised during phases of non-feeding and new tissue formation.

VIII.I. Introduction

The presence of hyperglycaemic hormones (HGHs) involved in the regulation of carbohydrate metabolism have been reported in many insects. The HGHs were first reported in the cockroach, *P. americana* (Steele, 1961). Subsequently many other insects were also found to possess these hormones (Ziegler, 1979; Witten *et al.*, 1984; Gaede and Rinehart, 1987 a; Gaede *et al.*, 1988). The HGHs are peptide hormones that activate glycogen phosphorylase in the animal's principal metabolic tissue to convert glycogen reserves into circulating carbohydrates (glucose in vertebrate liver; trehalose in insect fat body) as an energy source in peripheral tissues (Childress and Sacktor, 1970; Downer, 1979; Steele and Hall, 1985). Factors with hyperglycaemic activity have been demonstrated in both storage and glandular lobe regions of the locust CC (Mordue and Goldsworthy, 1969). Locusts utilize fat body glycogen as an important fuel for initial stages of flight (Van Marrewijk *et al.*, 1980; Beenakkers *et al.*, 1984). The mobilization of glycogen is dependent on glycogen phosphorylase, whose activity is a prerequisite for the synthesis of trehalose, the transport form of carbohydrate in insect haemolymph. Glycogen phosphorylase activity is controlled by AKH. During flight AKH is released from the CC into the haemolymph (Orchard and Lange, 1983 a; Goldsworthy and Wheeler, 1984) and induces the activation of phosphorylase in the fat

body (Gaede, 1981b; Van Marrewijk *et al.*, 1983, 1985, 1986). In *P. americana,* the injection of AKH induced the fat body to release trehalose (Siegert *et al.*, 1986).

Fat body glycogen phosphorylase in the tobacco hornworm, *M. sexta,* is activated by a peptide from the CC, the glycogen phosphorylase activating hormone (Ziegler, 1979; Siegert and Ziegler, 1983). Changes of haemolymph carbohydrate concentrations observed during the late larval development (Siegert, 1987 a) and starvation of 5th instar larva of *M. sexta* (Siegert, 1987b) are due to the regulation of glycogen phosphorylase activity and/or the release of GPAH from the CC. Feeding insects generally contain low percentages of active phosphorylase (Ziegler and Gaede, 1984; Siegert *et al.*, 1986); this level may be exceeded during metabolic stress, e.g., flight (Van Marrewijk *et al.*, 1980) or starvation (Siegert and Ziegler, 1983). Goldsworthy (1983) reported that locusts use besides lipids, carbohydrates at the beginning of flight and to a smaller degree during prolonged flight.

Although levels of haemolymph trehalose remained virtually constant during a 45 min flight regime of *C. erythrocephala,* cardiactectomized blowflies showed a steep depletion of blood trehalose concentration (Normann and Duve, 1969). Injections of CC-extracts failed to produce hypertrehalosaemia in *C. erythrocephala* (Normann and Duve, 1969) and *P.*

regina (Friedman, 1967). The CC-extracts of *P. regina* exhibited a small hypertrehalosaemic effect when injected into *P. americana* (Friedman, 1967).

In *P. americana* flight activity results in an increase of blood carbohydrates of 24 μg/μl (King *et al.*, 1986) and about the same increase is found when synthetic hypertrehalosaemic hormones of the cockroach are injected into *P. americana* (Gaede, 1986). Wilps and Gaede (1990) reported that in *P. terraenovae*, stress of 1 h of flight activity results in the release of the hypertrehalosaemic factor causing activation of phosphorylase, breakdown of glycogen, and an increase of blood carbohydrates. The HTHs of the cockroach, *P. americana* not only stimulate the release of trehalose from the fat body, but also possess cardioacceleratory and myoactive properties (Steele, 1961; Scarborough *et al.*, 1984; O'Sea *et al.*,1984). Synthetic HTH stimulate glycogen phosphorylase activity in *B. discoidalis* fat body, and glycogen reserves decline as haemolymph trehalose levels increase (Lee and Keeley , 1994). Administration of syn Bld-HTH to adult male *B. discoidalis* increases both haemolymph trehalose levels and fat body trehalose synthesis by 3 to 4 times their respective basal levels (Keeley *et al.*, 1991, 1995). Bld-HTH also increases fat body glycogen phosphorylase activity (Park and Keeley, 1995). The responses agree with similar increases in fat body glycogen phosphorylase activity by syn HTHs or AKHs in other insect species such as the American

cockroach, *P. americana* (Orr *et al.*, 1985), the locust, *L. migratoria* (Gaede, 1981 b; Van Marrewijk *et al.*, 1991), and the tobacco hormworm, *M. sexta* (Ziegler *et al.*, 1990). On *B. discoidalis*, the Bld-HrTH increases specific expression of the gene for cytochrome P450 and increases protein biosynthesis in the fat body (Keeley *et al.*, 1994).

Thus, it has now become evident that the hyperglycaemic factors is of wide occurrence among insects. The order Lepidoptera to which our present experimental insect *S. mauritia* belongs, also have been found to have hyperglycaemic factors. The experiments in this chapter have been assigned to find out whether the insect neuronal tissue contain any factors regulating sugar release into the haemolymph. All stages of this holometabolous insect have been tested viz ., the larval, prepupal, pupal and adult stages. Cross-reactivity studies for AKH (Locust) and *Spodoptera* hormones were also tested in the fat body of *P. americana.*

VIII.2. Results

VIII.2.1. Sugar release from the fat body of various stages of *S. mauritia* incubated with different concentrations of SH-extract *in vitro*

Fat body from various stages viz., 96 h larva, prepupa, pupa and adult of *S. mauritia* were incubated for 30 min with different concentrations of SH-

extract and quantitative changes in total sugar released over control were measured as described in Materials and Methods section. The results obtained were subjected to ANOVA as described and the data presented in Table VIII.1. Table VIII.1.1 analyses the data obtained for the experimentals where the incubation mixture contained different hormone concentrations. The first row of values is the significance of response for various stages of *S. mauritia*. The F-value obtained (83.11) when compared to the Table F-value (2.61) shows that there is a significant difference between the different stages with respect to the sugar release from the fat body. Likewise, the second row of values shows that the calculated F-value is higher (34.02) than the Table F-value (2.10). From this observations it is suggested that the different hormone concentrations have significantly different activities on fat body with respect to sugar release. Third row of values which represent the analysis of the combined effect of the stages and concentrations also shows high significance with respect to sugar release (F-value calculated 35.15 and tabulated 1.57).

Similarly, Table VIII.1.2 presents the analysis of data for fat body incubations without the hormone (controls). Here the release of sugar is dependent on stages because they are highly significantly different in various stages (F-value 45.64 compared to Table F-value of 2.61). Since the controls contained only distilled water in place of the hormones, the significant

136A

Table VIII.1. ANOVA of data for sugar release from the fat body of various stages of *S. mauritia* incubated with different concentrations of SH-extract *in vitro*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	284.9413	94.98043	83.11	2.61
Conc. (C)	3	116.6467	38.88224	34.02	2.10
S x C	9	361.5162	40.16847	35.15	1.57
Error	112	127.9995	1.14285		

2. Control

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	156.4808	52.16028	45.64	2.61
Fat body (Fb)	3	50.65466	16.88489	14.77	2.10
S x Fb	9	152.7828	16.97587	14.85	1.57
Error	112	127.9996	1.142854		

3. Difference

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	8.925314	2.975105	2.60	2.61
Conc. (C)	3	19.13079	6.376932	5.58	2.10
S x C	9	12.68929	1.409921	1.23	1.57
Error	112	127.9999	1.1429857		

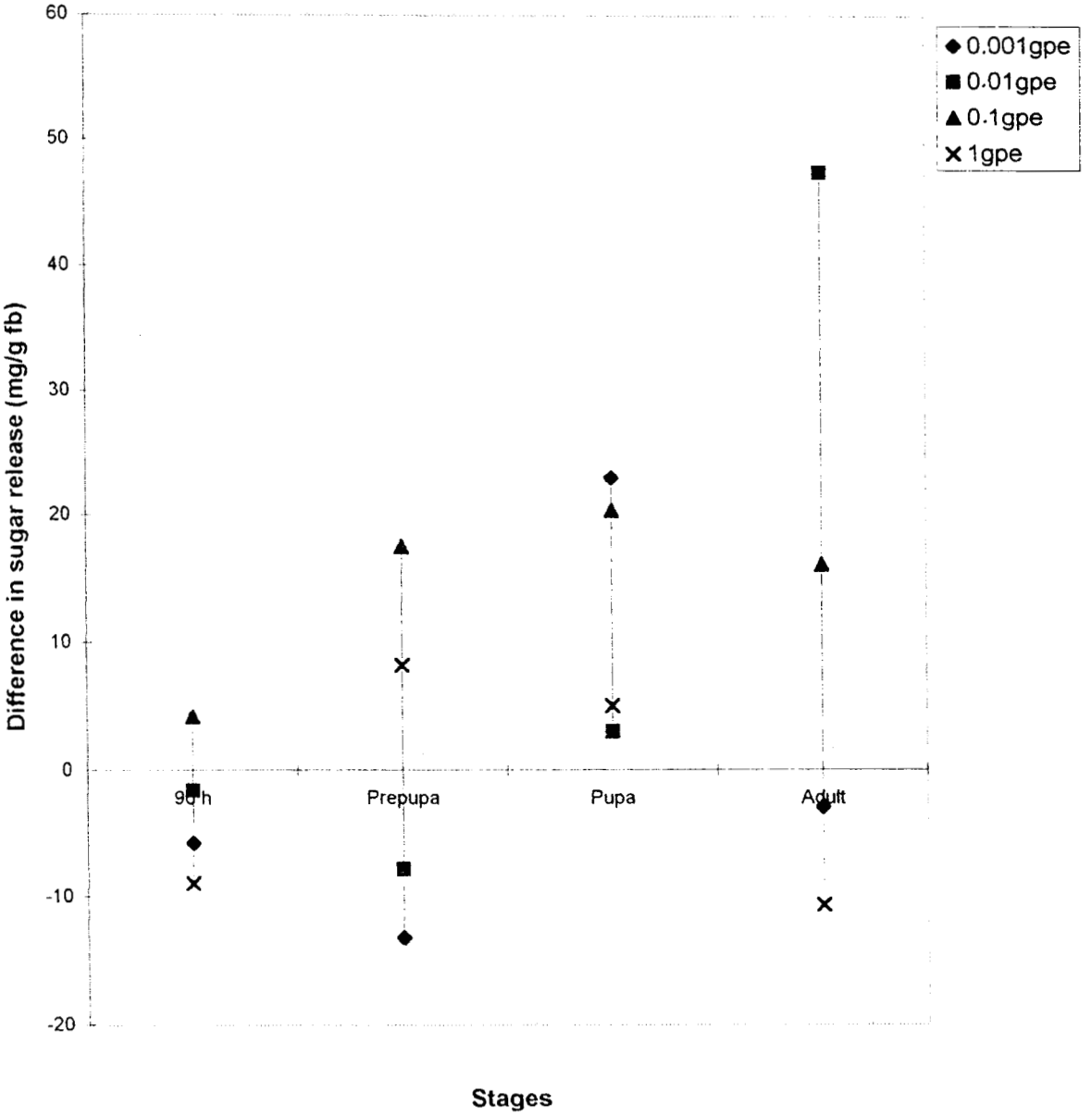
The data were obtained from analysis of sugar release using various stages of *S. mauritia* at different concentrations of SH-extract (Total cases considered are 128).

difference can only be due to the difference brought about by the different fat bodies, the inherent physiological difference between the fat body from different individuals (F-value 14.77 compared to Table F-value 2.10).

Table VIII.1.3. provides the analysis of data for the difference between experiments and controls. The first row of values shows the difference between different stages (F-value is 2.60 compared to the Table F-value 2.61). This indicates that sugar release in different stages are not significantly different. The second row of values shows that the different concentrations of the hormone have significantly different effect on sugar release (F-value calculated 5.58 and Table F-value 2.10). The third row of values represent the combined effect of stages and concentrations on the sugar release. The F-value is lower than the (1.23) tabulated F-value (1.57) showing that the effect is not significant.

Figure 8.1 presents the effect of SH-extract on sugar release from the fat body of different stages of *S. mauritia*. It appears that the SH-extract had a stimulatory effect on fat body sugar release. In the case of 96 h larva, stimulation was shown by a concentration of 0.1 μM by about 4 mg/g fb. All the other concentrations, 0.001, 0.01 and 1.0 gpe shows negative effect on sugar release (1-8 mg/g fb). In prepupal fat body both stimulatory as well as inhibitory effect on sugar release from the fat body were shown by the

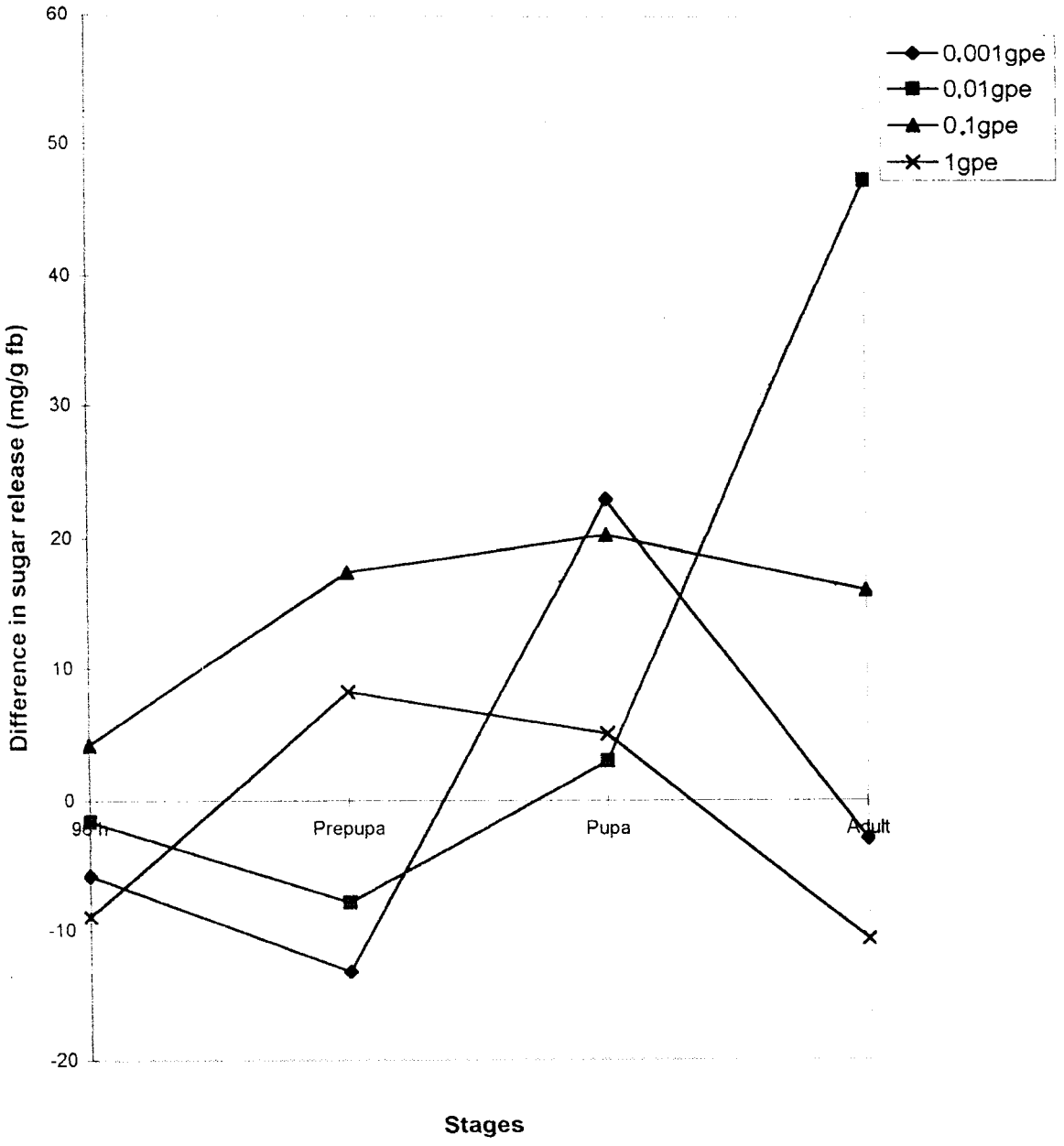
Fig. 8.1. Effect of different concentrations of SH- extract on sugar release *in vitro* from the fat body of various stages of *S. mauritia*



hormone. The maximum amount of sugar was released (17 mg/g fb) with a concentration of 0.1 gpe followed by a concentration of 1.0 gpe by about 8 mg/g fb. The other two concentrations (0.001 and 0.01 gpe) elicited negative response by the fat body. Sugar released was about 8-13 mg/g fb. In the pupal fat body all the hormone concentrations elicited good stimulatory response. Maximum release of sugar occurred with the lowest concentration 0.001 gpe (23 mg/g fb) followed by 0.1 gpe (20 mg/g fb). Concentrations of 1.0 and 0.01 gpe showed slight positive effect on fat body sugar release (about 5 mg/g fb and 3 mg/g fb respectively). In the case of adult, stimulation of sugar release was maximum with a hormone concentration of 0.01 gpe. There was an increase in sugar by about 47 mg/g fb. With 0.1 gpe of the hormone, the fat body sugar release was 16 mg/g fb. Concentrations of 0.001 and 1.0 gpe gave inhibitions between 3-11 mg/g fb respectively. Thus in brief, different hormone concentrations had different pattern of activity with respect to sugar release. The overall activity of the hormone in different concentrations seemed to be, stimulatory.

Figure 8.2 shows the interaction between different stages of *S. mauritia* with different concentrations of SH-extract. It indicates that most of the hormone solutions had significant stimulatory effect with respect to sugar

Fig. 8.2. Graph showing the interaction between various stages of *S.mauritia* and different concentrations of SH - extract



release. Different hormone concentrations had different pattern of activity in different stages of *S. mauritia* as seen in the Fig. 8.2.

Figure 8.3 provides the average sugar release from the fat body of *S. mauritia* using different hormone concentrations (experimentals). It appears that the maximum amount of sugar released in experimental observations was in the adult fat body and was about 132 mg/g fb with a hormone concentration of 1.0 gpe. Other concentrations of the hormone with similar high stimulatory effect were 0.1 and 0.01 gpe. The sugar released into the medium were 128 mg/g fb and 129 mg/g fb respectively. The hormone at 0.001 gpe concentration had a lesser effect (56 mg/g fb). In 96 h larval fat body, the maximum sugar release was measured at the concentrations of 0.01 gpe (56 mg/g fb) and 1.0 gpe (57 mg/g fb) followed by a concentration of 0.1 gpe (42 mg/g fb). In the case of prepupa the maximum amount of sugar was released at a concentration of 0.1 gpe (76 mg/g fb). Less pronounced effect on prepupal fat body was observed at a concentration of 0.01 gpe. Pupal fat body showed good response to 0.001 gpe concentration. The sugar released was 109 mg/g fb. Other concentrations showed only slight effect on sugar release from the fat body. Figure 8.4 provides the graph showing the amount of sugar released from various stages at the different hormone concentrations. From the bar diagram it is clearly seen that different concentrations of the

Fig. 8.3. Average sugar release from the fat body of *S.mauritia* incubated with different concentrations of SH- extract in various stages (n=8)

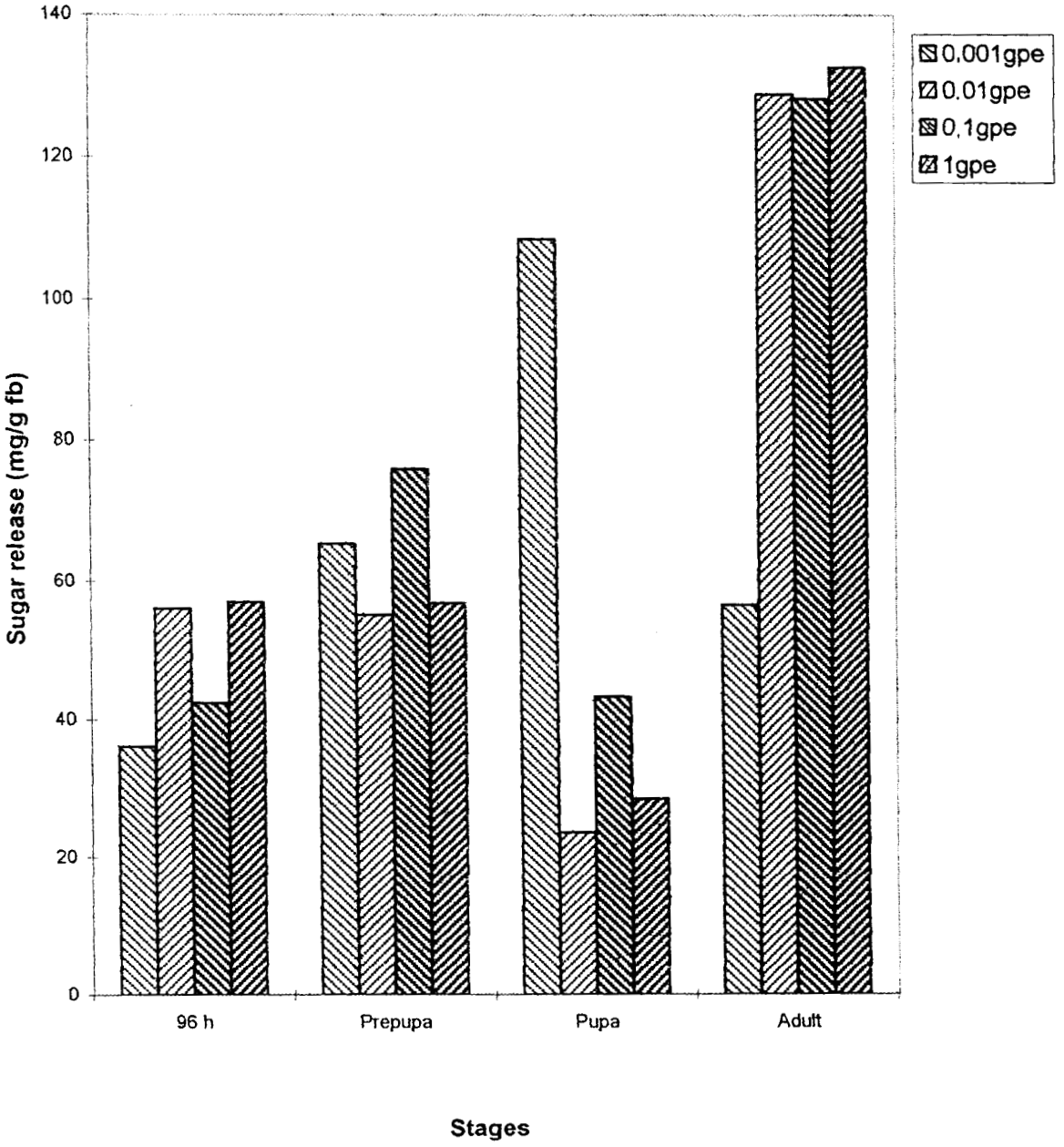
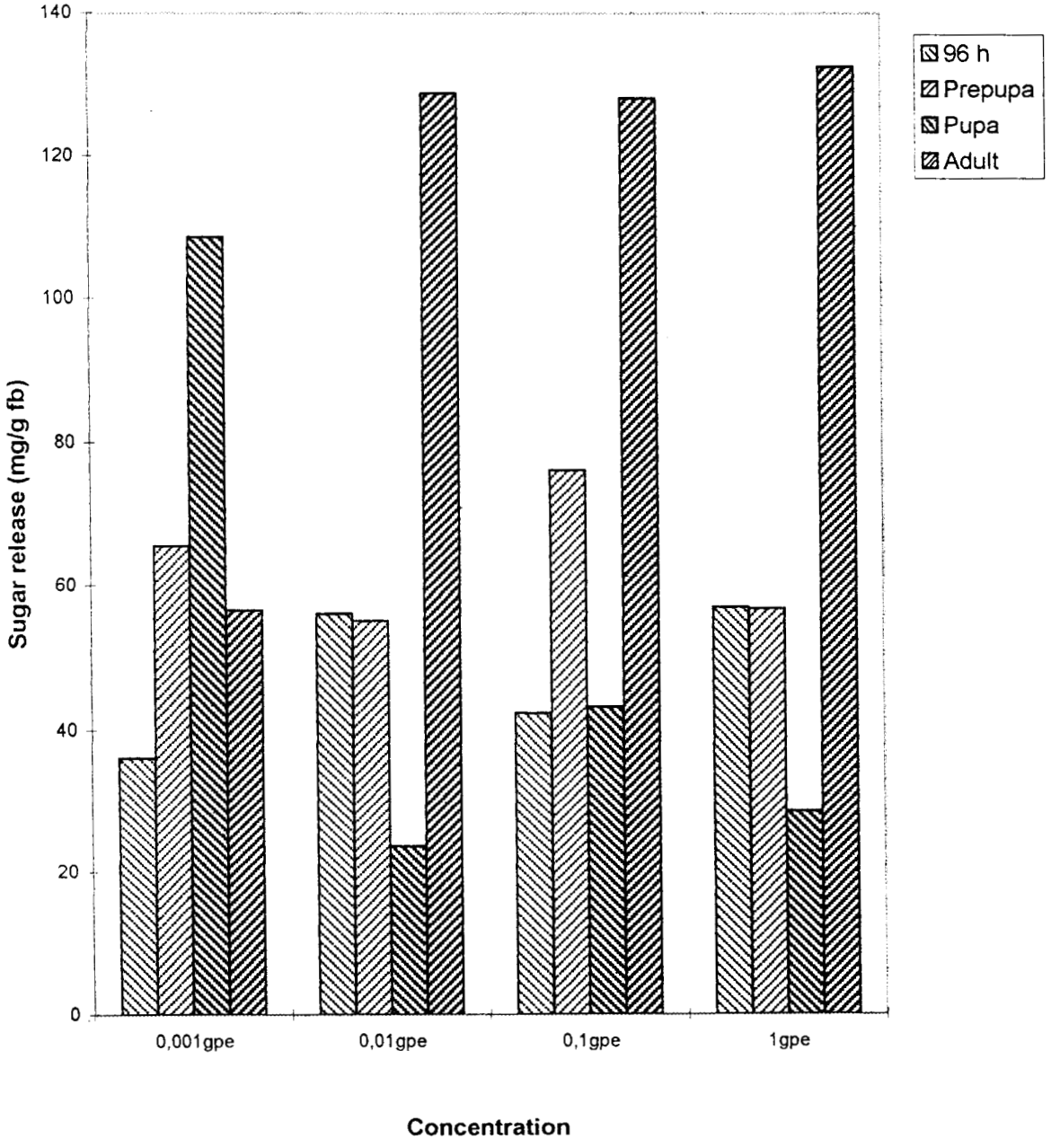


Fig. 8.4. Average sugar release from the fat body of various stages of *S.mauritia* at different concentrations of SH- extract (n=8)



hormone had significant and different effect on sugar release from *S. mauritia*. Adult fat body showed better release with all concentrations except the lowest concentration (0.001 gpe). This concentration was effective only on pupal fat body.

VIII.2.2. Sugar release from the fat body of various stages of *S. mauritia* incubated with different concentrations of syn AKH *in vitro*

Fat body from various stages of *S. mauritia* viz., 96 h larva, prepupa, pupa and adult were incubated for 30 min with different concentrations of syn AKH and quantitative changes in total sugar released over control were measured as described in materials and methods section. The results obtained were subjected to ANOVA as described and the data is presented in Table VIII.2. Table VIII.2.1 analyses the data obtained for the experimentals where the incubation mixture contained hormone at various concentrations. The first row of values represents data for various stages of *S. mauritia*. The F-value obtained (12.09) when compared to the Table F-value (2.61) shows that there is a significant difference between various stages with respect to the sugar release from the fat body. Similarly, the second row of values obtained with various concentrations, shows that the calculated F-value is higher (5.40) than the Table F-value (2.10) and therefore different hormone concentrations have

Table VIII.2. ANOVA of data for sugar release from the fat body of various stages of *S. mauritia* incubated with different concentrations of syn AKH *in vitro*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	41.44934	13.81645	12.09	2.61
Conc. (C)	5	30.87695	6.175391	5.40	2.10
S x C	15	74.24426	4.949617	4.33	1.57
Error	168	192.0001	1.142858		

2. Control

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	108.8635	36.28784	31.75	2.61
Fat body (Fb)	5	30.30823	6.061646	5.30	2.10
S x Fb	15	57.56116	3.83741	3.36	1.57
Error	168	191.9999	1.142856		

3. Difference

Source	df	SS	MSS	F	Tab. F
Stages (S)	3	9.785206	3.261735	2.85	2.61
Conc. (C)	5	46.80383	9.360765	8.19	2.10
S x C	15	48.25604	3.217069	2.81	1.57
Error	168	192	1.142857		

The data were obtained from analysis of sugar released from the fat body of various stages of *S. mauritia* at different concentrations of syn AKH (Total cases considered are 192).

significantly different response on fat body sugar release. Third row of values which represent the analysis of the combined effect of the stages and concentrations also shows that the difference is highly significant (F-value calculated 4.33 and tabulated 1.57).

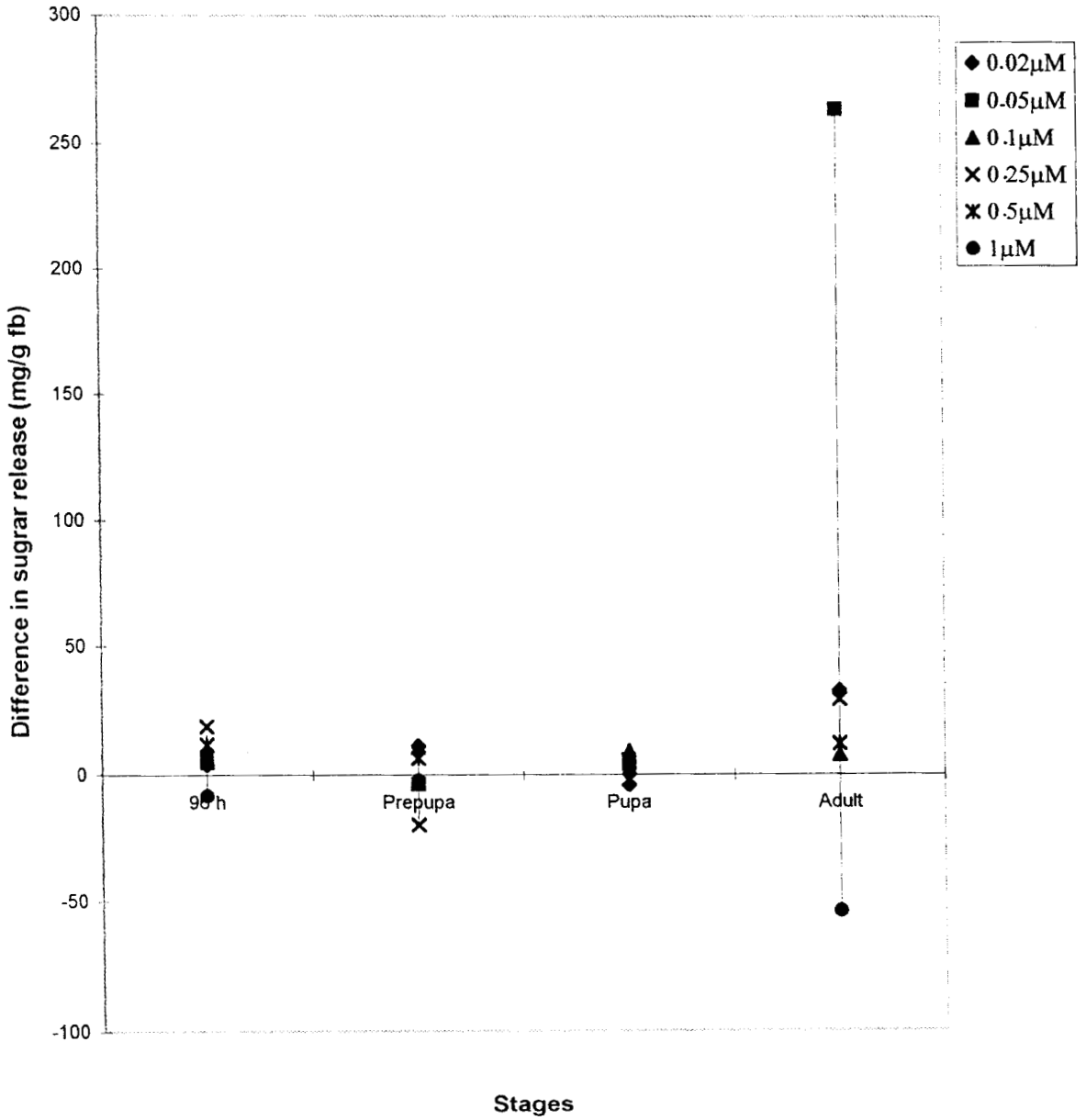
Table VIII.2.2 likewise, presents the analysis of data for fat body incubations without the hormone (controls). Here the release of sugar is shown to be dependent on the stage i.e., they are significantly different in different stages (F-value 31.75 compared to Table F-value of 2.61). Since the controls contained only distilled water in place of the hormones, the significant difference can only be due to the difference brought about by different fat bodies, the inherent physiological difference between the fat body from different individuals (F-value 5.30 compared to Table F-value 2.10).

Table VIII.2.3 presents the analysis of data for the difference between experiments and controls. The data shows that the difference between experimental and control observations is significant. The first row of values shows the significance of difference between different stages of the insect (F-value is 2.85 compared to the Table F-value 2.61). The second row of values shows the data for the difference between experiments and controls with different concentrations. (F-value calculated is 8.19 and Table F-value 2.10). The third row of values represent the difference of the values for the combined

effect of stages and concentrations. This value also shows significant difference (F-value 2.81 compared to Table F-value 1.57).

Figure 8.5 presents the effect of AKH on sugar release from the fat body of various stages of *S. mauritia*. It is seen that only the adult fat body responded to the synthetic hormone solutions. In 96 h larval fat body, the hormone at all the concentrations tested except 1.0 μM, showed significant hyperglycaemic effect though to a small extent. The maximum stimulatory effect on fat body sugar release in this case (by about 19 mg/g fb), was given by 0.25 μM. Next better concentrations are 0.5 and 0.05 μM (release of about 12 mg/g fb and 7 mg/g fb respectively). Other concentrations had very little or no effect. In the case of prepupa, the hormone had only very little effect on fat body sugar release. Different concentrations of the hormone used gave sugar release of about 7-12 mg/g fb. A concentration of 0.25 μM showed negative effect on sugar release (20 mg/g fb). Other concentrations which showed slight negative effect are 0.05, and 1.0 μM (4 mg/g fb and 2 mg/g fb respectively). In the pupal stage, the effect of hormone on sugar release is less prominent. Maximum stimulatory effect (by about 9 mg/g fb) was shown by a concentration of 0.1 μM followed by 0.05 μM (by about 6 mg/g fb). With lower concentration of the hormone (0.02 μM), there is an inhibition of sugar release (4 mg/g fb). Higher concentration (1.0 μM) also seemed to have no

Fig. 8.5. Effect of different concentrations of syn AKH on sugar release *in vitro* from the fat body of various stages of *S.mauritia*



effect on the stimulation of sugar release from the fat body cells. With adult fat body, a hormone concentration of 0.05 μM, showed very high stimulatory effect by about (264 mg/g fb). Other concentrations such as 0.02, 0.1, 0.25 and 0.5 μM also showed positive effect and sugar release ranged from 7 to 32 mg/g fb. The adult fat body on the other hand, also showed an inhibitory activity of about 54 mg/g fb with a hormone concentration of 1.0 μM. Thus it becomes clear that the effect of various concentrations of the hormone had varying activity on sugar release from the fat body of *S. mauritia*, although these effects were significant.

Figure 8.6 shows the interaction between various stages of *S. mauritia* and AKH on sugar release from the fat body. From the graph it is clear that the pattern of activity in various stages is more or less similar with respect to sugar release. No stages except adult showed any appreciable response to the hormone with respect to the release of sugar from the fat body. In adult only 0.05 μM elicited good response. There was a stimulation of sugar release by 264 mg/g fb with this concentration. The highest concentration of 1.0 μM showed inhibition.

Figure 8.7 provides the average release of sugar from the fat body of *S. mauritia* in the experimental observations. It appears that the adult fat body

Fig. 8.6. Graph showing the interaction between various stages of *S.mauritia* and different concentrations of syn AKH

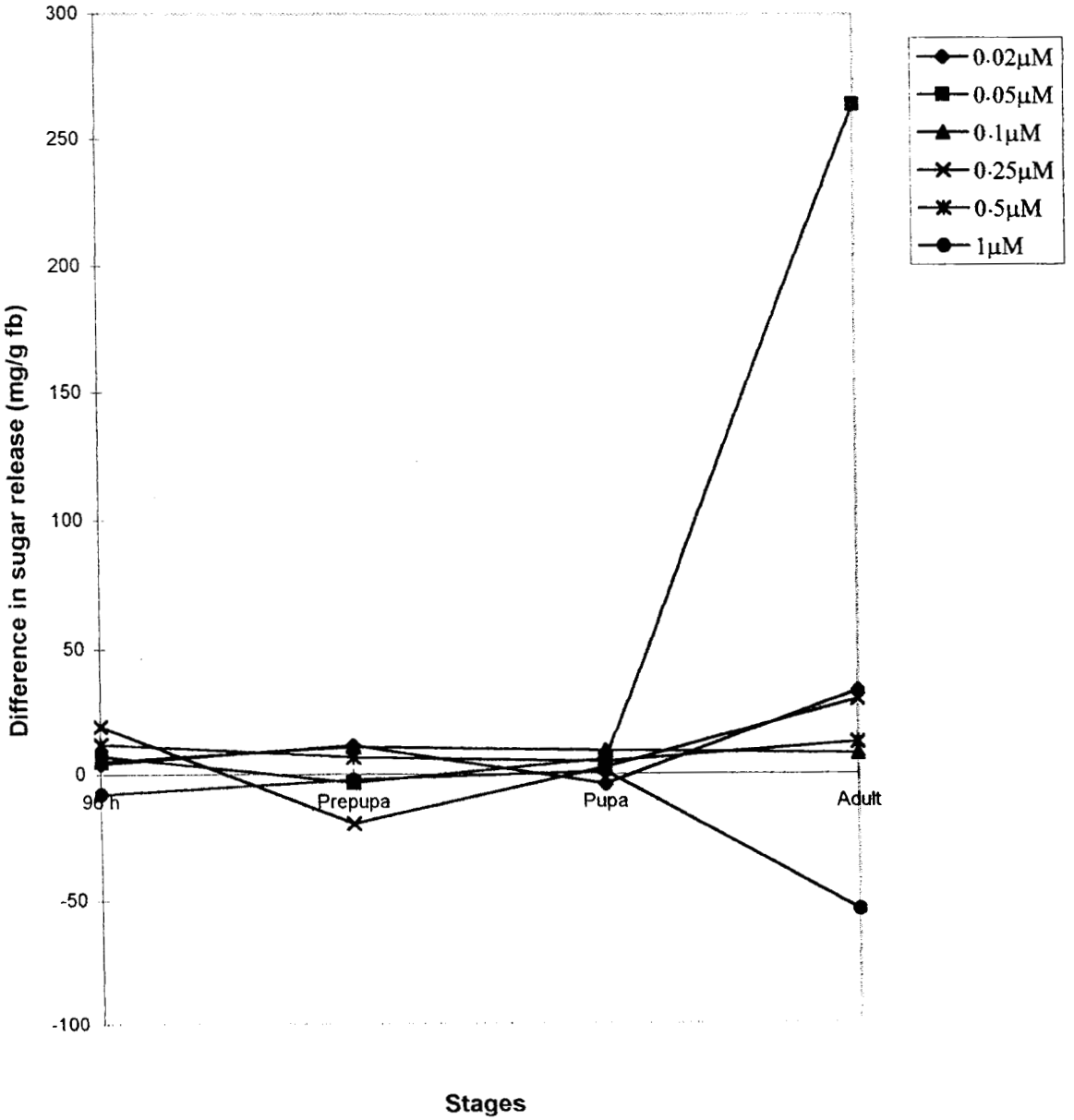
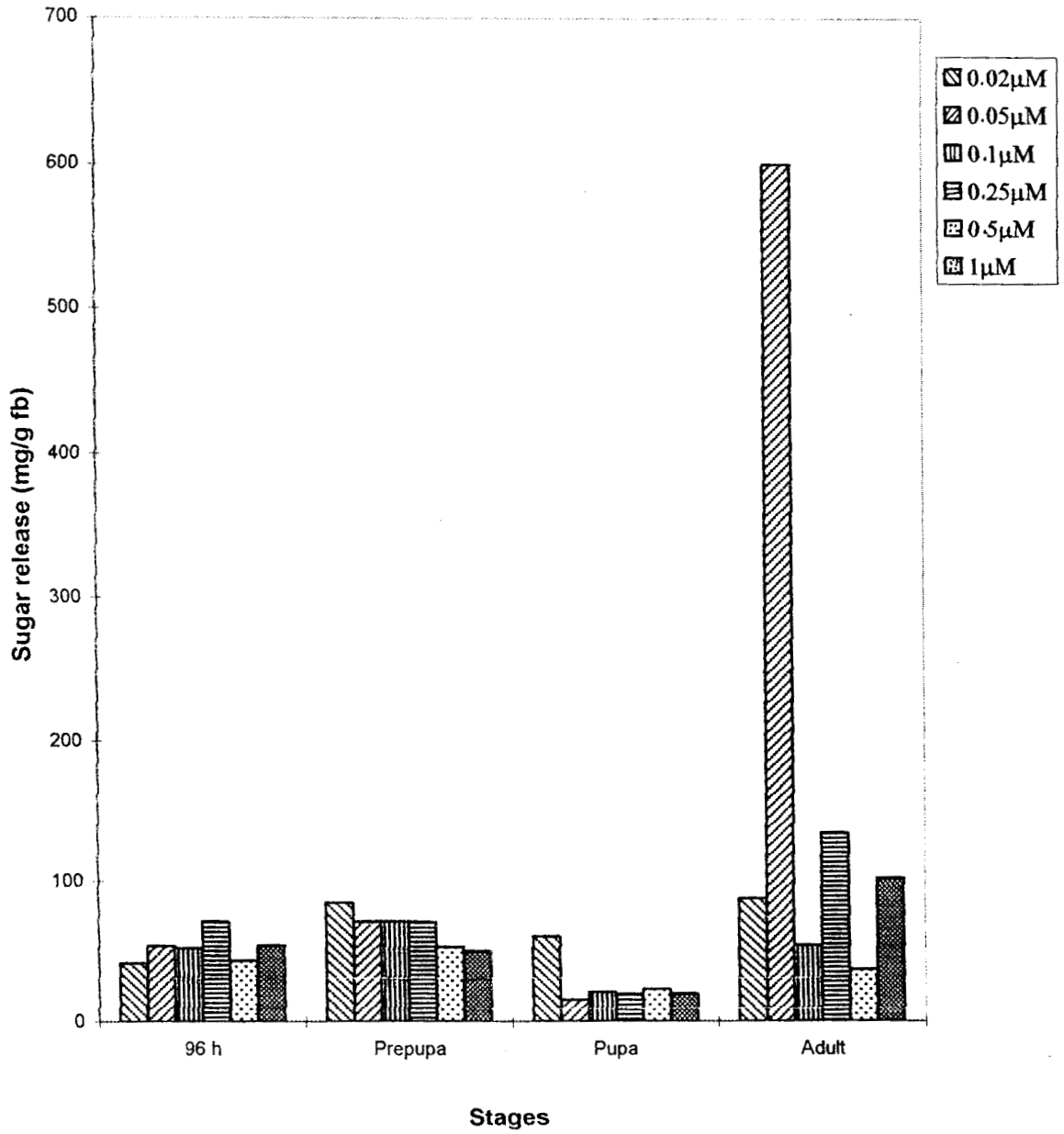


Fig. 8.7. Average sugar release from the fat body of *S.mauritia* incubated with different concentrations of syn AKH in various stages (n=8)

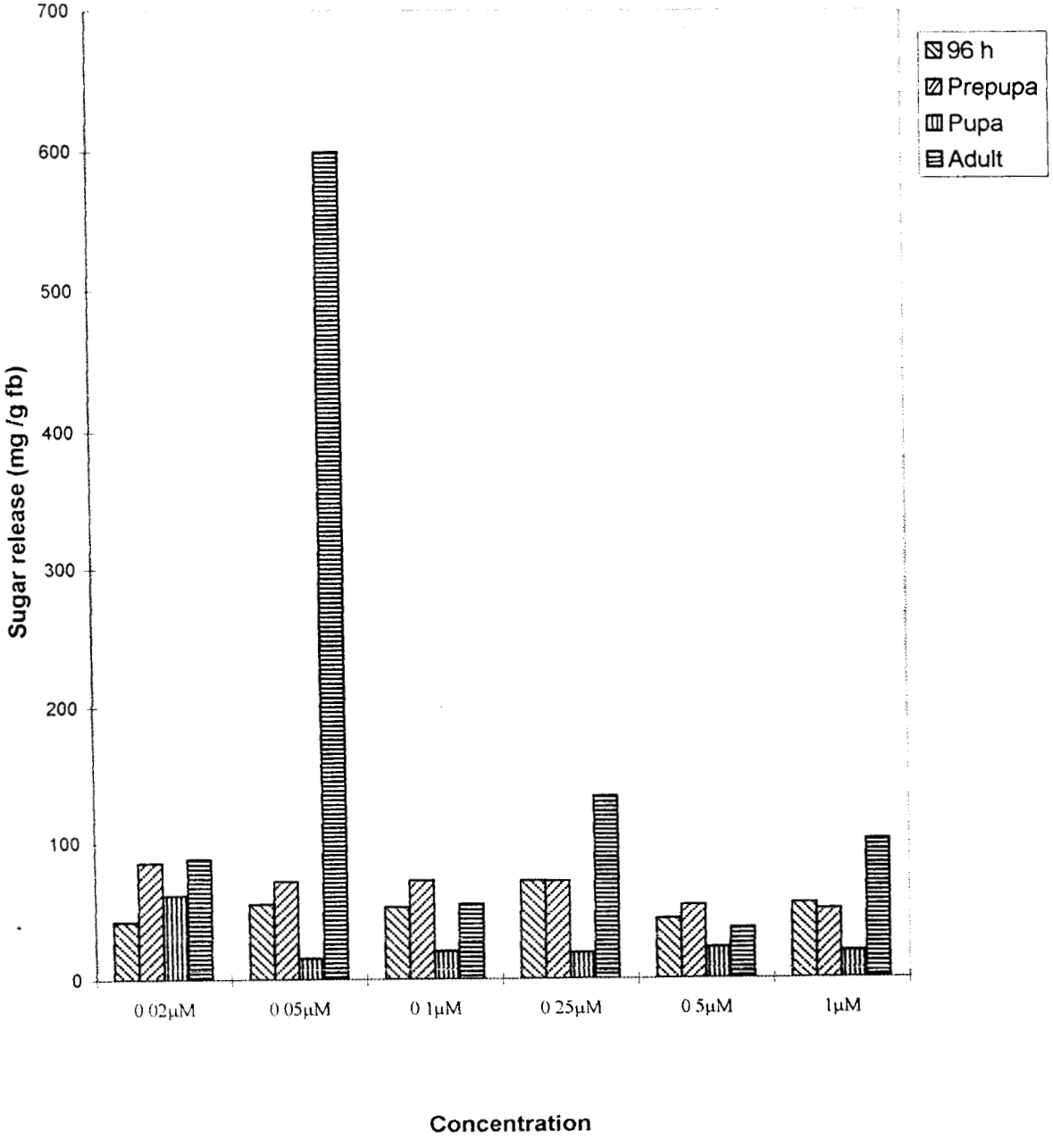


showed maximum release compared to all of the other stages where the sugar release was below and around 100 mg/g fb. Pupal stage showed the lowest amount of sugar release except the lowest concentration tested (see also Fig. 8.8).

VIII.2.3. Sugar release from the fat body of *P. americana* incubated with different concentrations of SH-extract *in vitro*

Different concentrations of SH-extract were tested for their effects on sugar release *in vitro* from the fat body of *P. americana*. The results subjected to ANOVA, are presented in Tables VIII.3. Table VIII.3.1 contains data obtained for the experimentals where the incubation mixture contained hormone at different concentrations. The F-value obtained (1.13) is smaller than the Table F-value (2.45) which shows that there is no significant difference in sugar release from the fat body with different concentration of the hormone. Table VIII.3.2 likewise presents the analysis of data for control experiments. The incubation mixture contained distilled water instead of hormone. The F-value obtained is 9.85 and the Table F-value is 2.45. The significant difference between the different incubations may be due to the difference between the fat bodies used, as they contained no hormones but only distilled water. Table VIII.3.3 presents the difference between experimental and control observations. The F-value is 2.93 compared to Table

Fig. 8.8. Average sugar release from the fat body of various stages of *S.mauritia* at different concentrations of syn AKH (n=8)



F-value of 2.45 and shows that the effect of different concentrations of SH-extract on sugar release from the fat body of *P. americana* is significant.

Figure 8.9 presents the actual direction of activity of SH-extract on sugar release from the fat body of *P. americana*. It appears that the hormone has stimulatory effects at 0.1, 1.0 and 2.0 gpe concentrations and inhibitory effects at 0.001 and 0.01 gpe concentrations. A concentration of 1.0 gpe gave maximum stimulation of sugar release (12 mg/g fb) followed by 2 gpe (6 mg/g fb). With a concentration of 0.1 gpe, the sugar release from the fat body was 7 mg/g fb. The extent of inhibition of sugar release was so high with a concentration of 0.01 gpe (43 mg/g fb). Still lower concentration of the hormone (0.001 gpe) showed lower inhibitory activity on sugar release from the fat body. Figure 8.10 presents a picture of average of total sugar released from the fat body in the experimental incubations. It appears that there is a gradual increase in sugar release with increasing hormone concentrations of 0.001, 0.01, 0.1 and 1 gpe (41, 59, 71 and 96 mg/g fb). In the incubation with the highest hormone concentration (2 gpe) there is a decreased sugar release from the fat body (28 mg/g fb).

Fig. 8.9 . Effect of different concentrations of SH - extract on sugar release *in vitro* from the fat body of *P.americana*

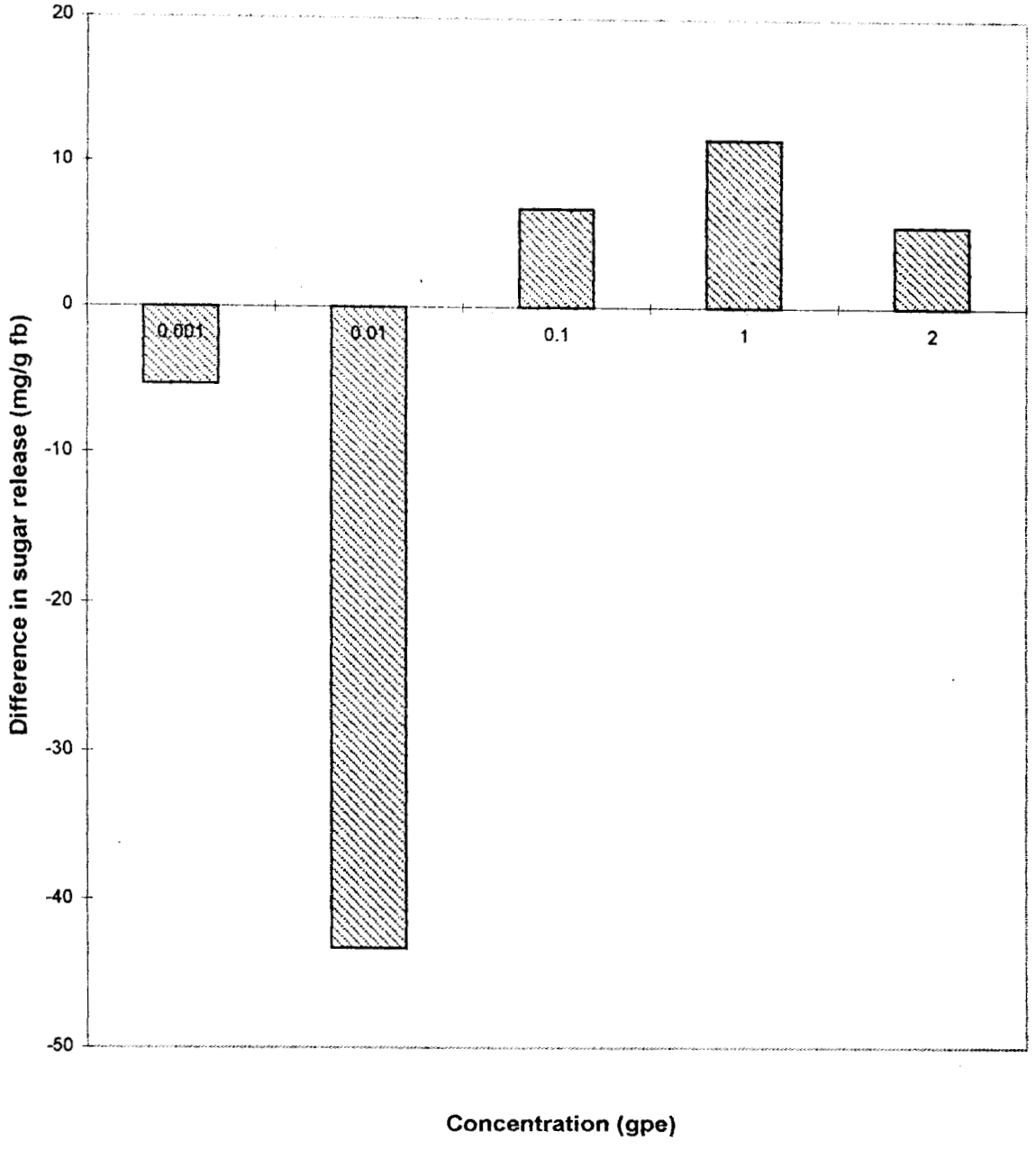
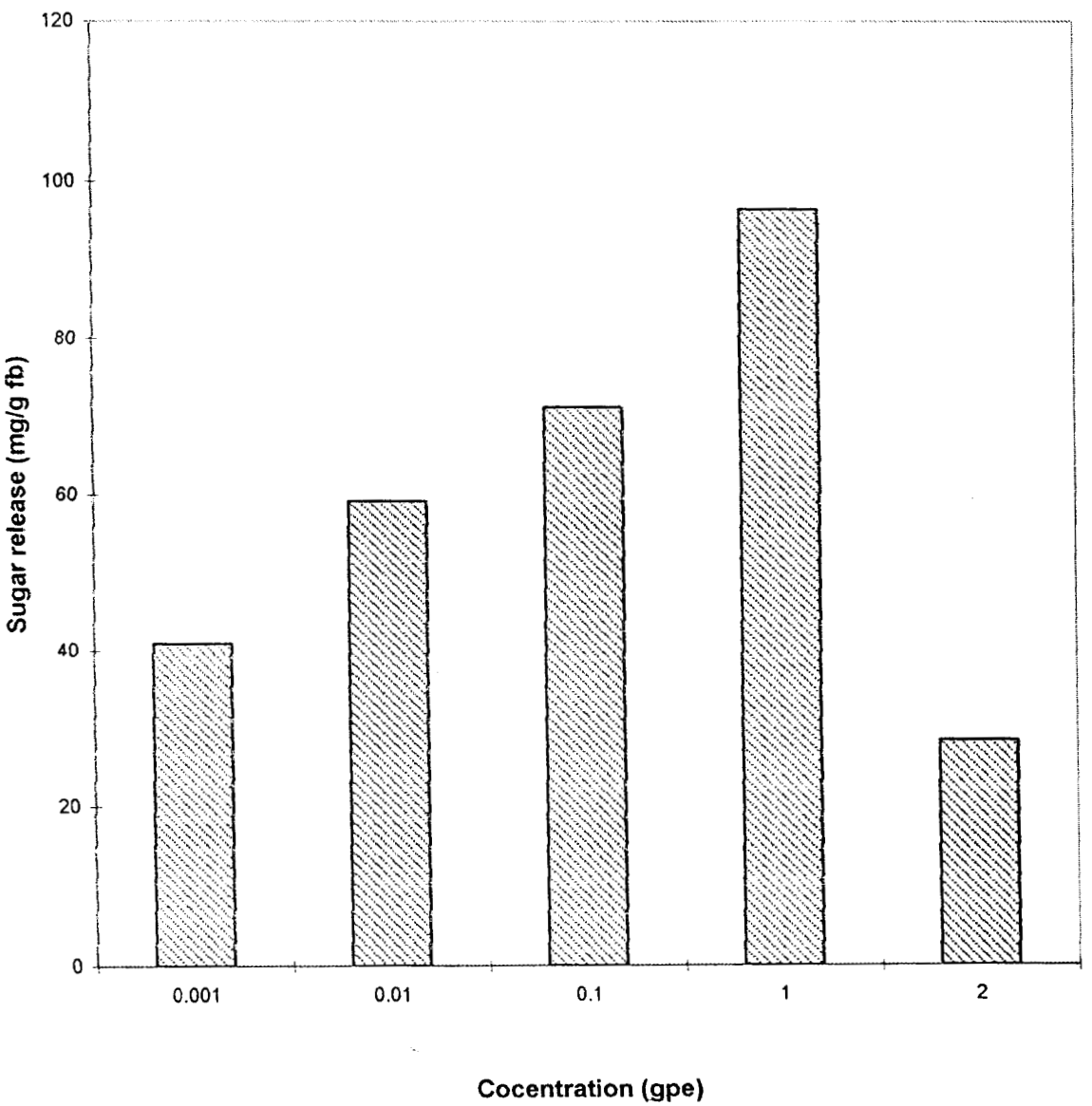


Fig. 8.1.0. Average sugar release from the fat body of *P.americana* incubated with different concentrations of SH- exctract *in vitro* (n=8)



VIII.2.4. Sugar release from the fat body of *P. americana* incubated with different concentrations of syn AKH *in vitro*

Different concentrations of syn AKH were tested for their effects on sugar release from the fat body of *P. americana*. The results were subjected to ANOVA and are presented in Table VIII.4. Table VIII. 4.1 analyses the data obtained for the experimentals where the incubation mixture contained hormone at various concentrations. The F-value obtained (5.38) when compared to the Table F- value (2.45) shows that there is a significant effect for the hormone on fat body sugar release. Table VIII.4.2 presents the analysis of data for fat body incubations without the hormone (controls). The difference (significant) between the different sets may be due to the difference in fat body cells taken for the experiment. The F-value is 3.74 which is greater than the Table F-value of 2.45. Table VIII. 4.3 presents the difference between experimental and control observations. The F-value obtained (2.44) when compared to the Table F-value (2.45) shows that, although sugar is released from the fat body, the experimentals are not significantly different from the controls.

Figure 8.11 presents the effect of AKH on sugar release from the fat body of *P. americana*. It appears that the synthetic hormone at lower concentration has inhibitory effect on sugar release. But this inhibitory effect

Table VIII.4. ANOVA of data for sugar release from the fat body of *P. americana* at different concentrations of syn AKH *in vitro*

1. Experiment

Source	df	SS	MSS	F	Tab. F
Conc. (C)	4	24.5729	6.143227	5.38	2.45
Error	35	39.99997	1.142856		

2. Control

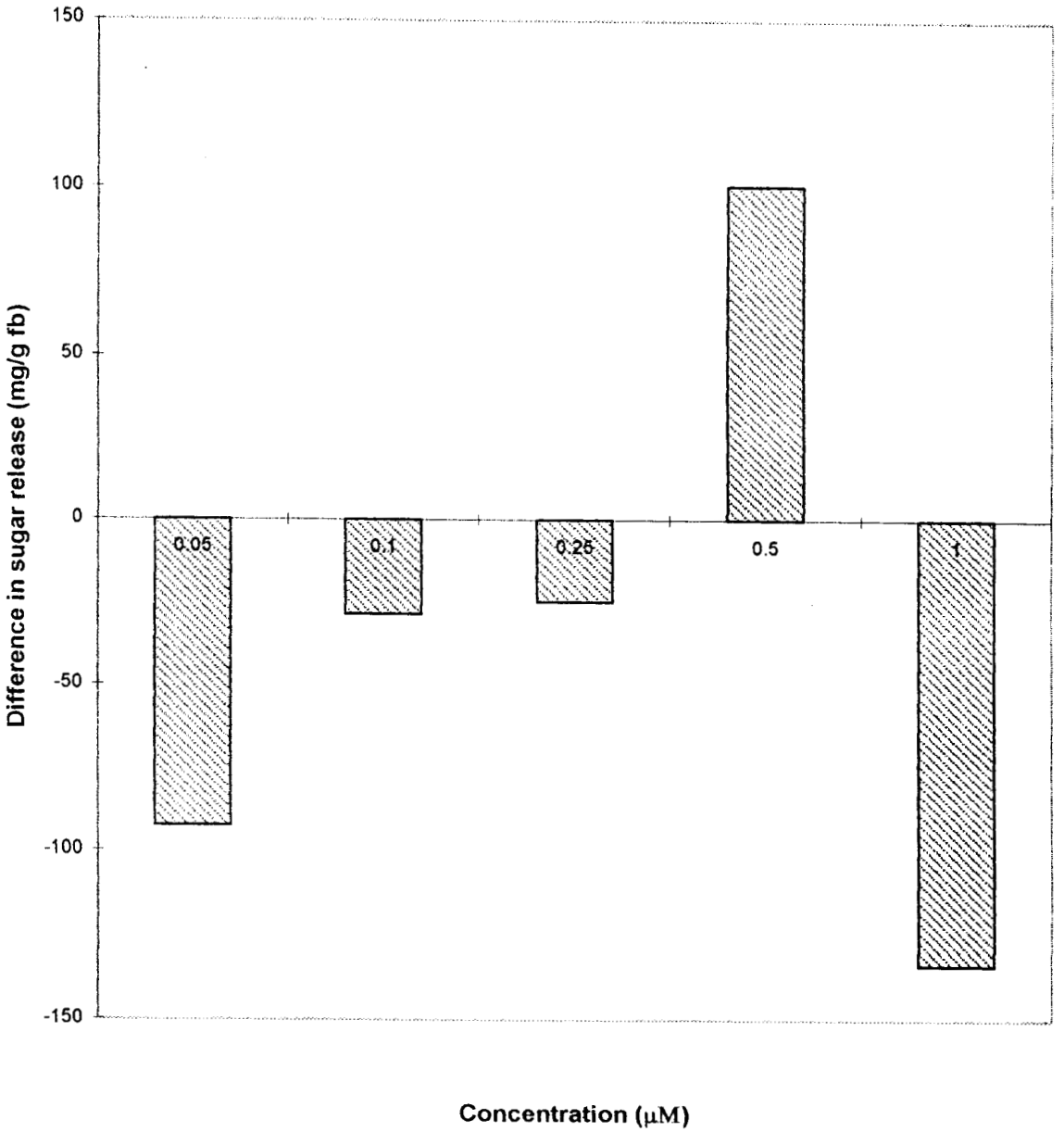
Source	df	SS	MSS	F	Tab. F
Fat body (Fb)	4	17.09251	4.273129	3.74	2.45
Error	35	40.00005	1.142859		

3. Difference

Source	df	SS	MSS	F	Tab. F
Conc. (C)	4	11.13977	2.784942	2.44	2.45
Error	35	40	1.142857		

The data used for ANOVA were obtained from analysis of sugar release from *P. americana* at different concentrations of syn AKH (Total cases considered are 40).

Fig. 8.11. Effect of different concentrations of syn AKH on sugar release *in vitro* from the fat body of *P. americana*



is gradually lost as the concentration is increased. At 0.5 μM concentration there is stimulation (101 mg/g fb). But still higher concentration (1.0 μM) again shows inhibition (133 mg/g fb) to a considerable extent. Concentrations of 0.1 and 0.25 μM showed inhibitions between 0.24 and 0.28 mg/g fb. Still lower concentration of the hormone (0.05 μM) showed high inhibitory activity (92 mg/g fb). Figure 8.12 shows the quantity of sugar released in the experimental samples with various hormone concentrations. Here the maximum amount of sugar released is about 291 mg/g fb at a concentration of 0.5 μM. Samples with lower concentrations of the hormone (0.05, 0.1 and 0.25 μM) were having better sugar release although controls had still higher values (143-211 mg/g fb). A concentration of 1.0 μM showed only a moderate release whereas the control had a far better response to the hormone, thus bringing about a negative net effect.

VIII.2.5. Sugar release from the fat body of *P. americana* injected with different concentrations of SH-extract *in vivo*

Different concentrations of SH-extract were tested *in vivo* for their effects on sugar release from the fat body of *P. americana* and the results are subjected to ANOVA and are presented in Table VIII.5. The results show that

Fig. 8.12. Average sugar release from the fat body of *P. americana* incubated with different concentrations of syn AKH *in vitro* (n=8)

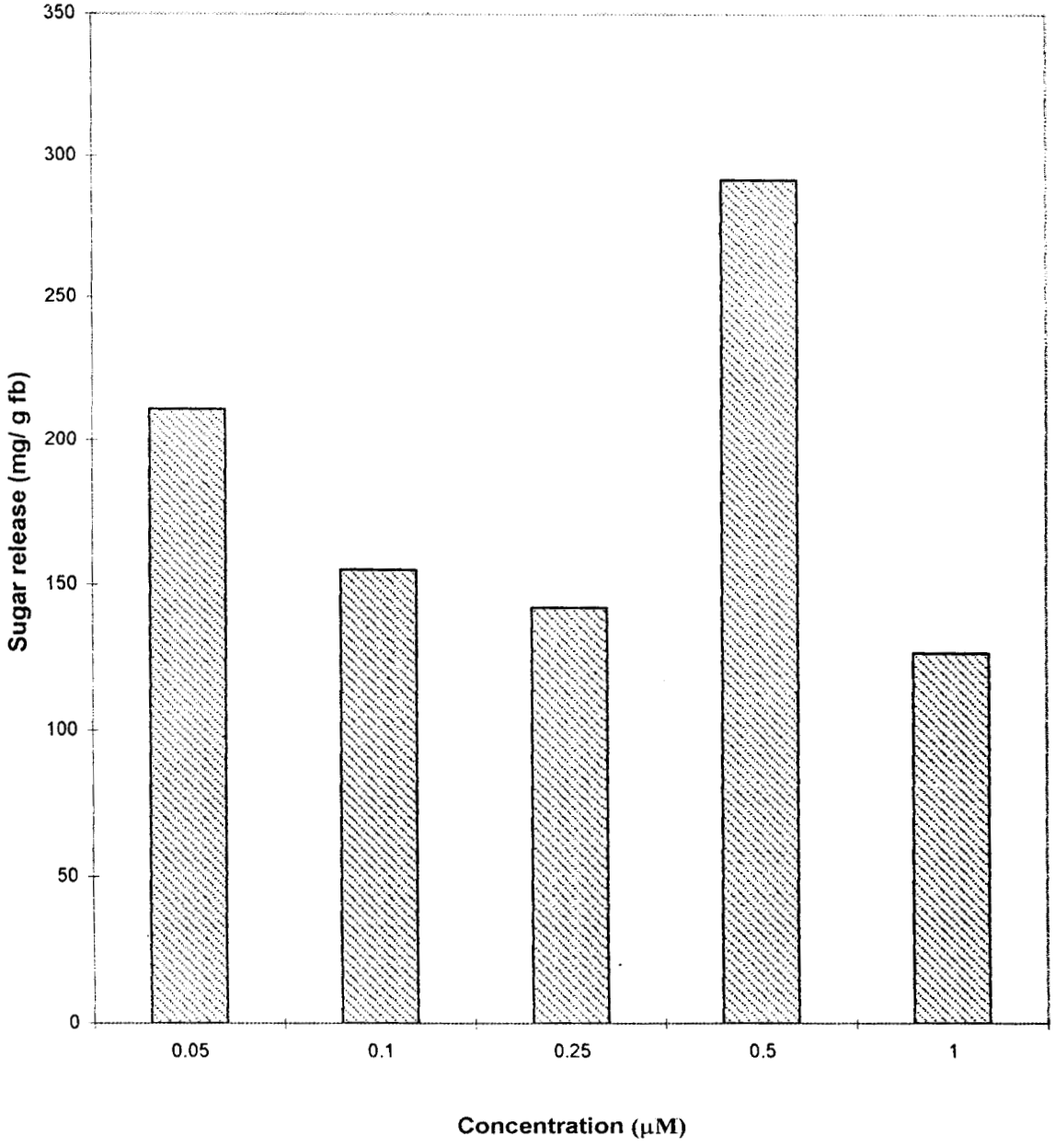


Table VIII.5. ANOVA of data for sugar release from the fat body of *P. americana* at different concentrations of SH-extract *in vivo*

Experiment

Source	df	SS	MSS	F	Tab. F
Conc. (C)	5	150662.8	30132.57	4.29	2.55
Error	30	210822.8	7027.427		

The data used for ANOVA were obtained from sugar release from *P. americana* at different concentrations of SH extract (Total cases considered are 30).

there is a significant effect of hormone on fat body sugar release. The F-value obtained (4.29) is greater than the Table F-value (2.45).

Figure 8.13 shows the effect of SH-extract *in vivo* on sugar release from the fat body of *P. americana*. There was an increasing effect with the increase in the hormone concentration. The maximum amount of sugar was released 15.9 mg/g fb at the maximum concentration of the extract tested (2 gpe). Other concentrations were not very good at sugar release stimulation when compared to the release brought about by distilled water injection. At a concentrations of 0.001 gpe the sugar release increased by about 6.1 mg/g fat body. The other hormone concentrations (0.01, 0.1 and 1 gpe) showed a gradual increase on sugar release (by about 1.8 mg/g fb, 2.1 mg/g fb and 4.2 mg/g fb) from the fat body, which were however, less than the increase effected by distilled water injection.

VIII.2.6. Sugar release from the fat body of *P. americana* injected with different concentrations of syn AKH *in vivo*

Different concentrations of syn AKH were tested *in vivo* for their effects on sugar release from the fat body of *P. americana* and the results obtained were subjected to ANOVA and are presented in Table VIII.6. The results shows that there is a significant effect for the hormone on the fat body sugar release

Fig. 8.13. Effect of different concentrations of SH- extract on sugar release *in vivo* from the fat body of *P.americana*

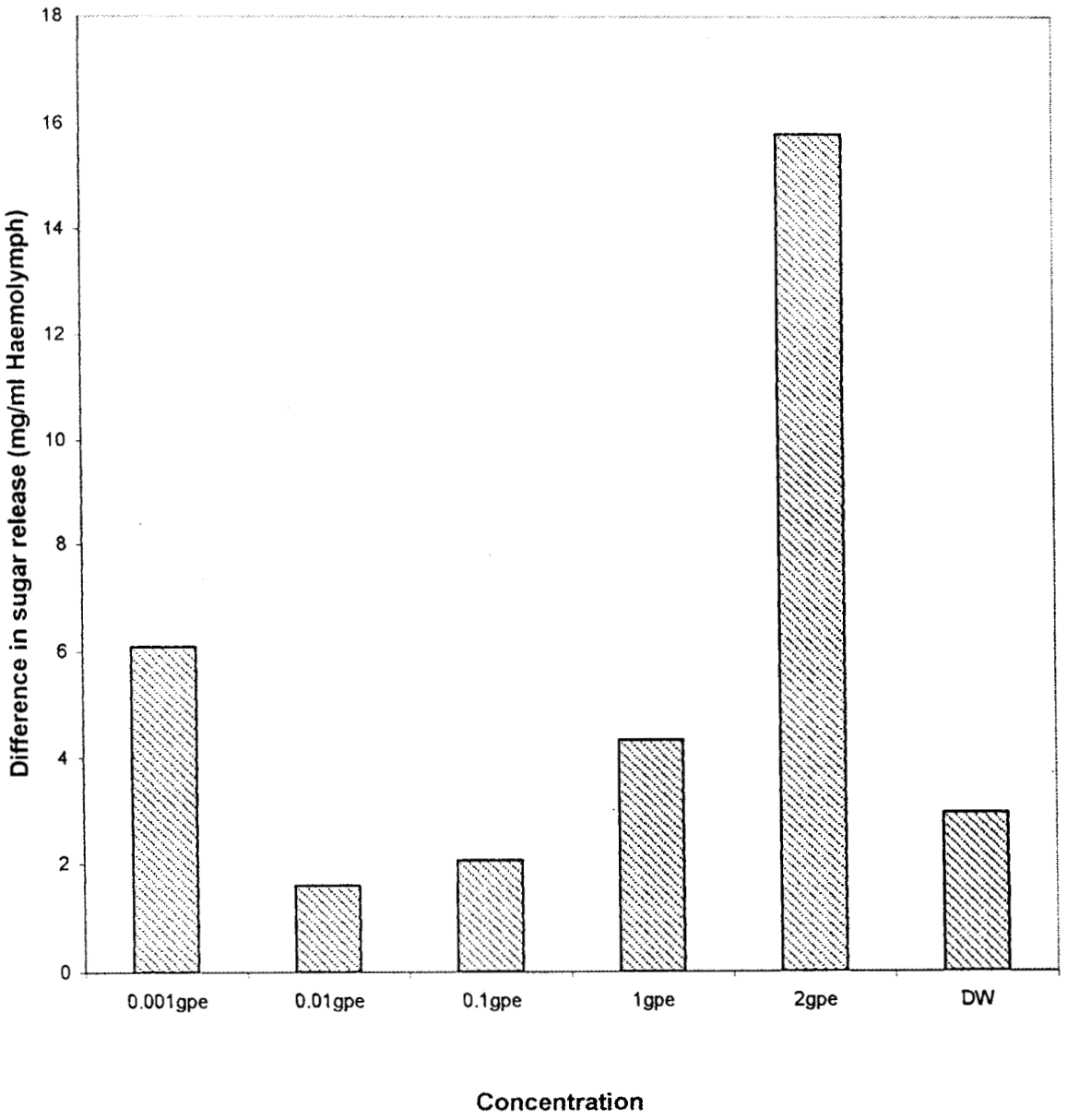


Table VIII.6 ANOVA of data for sugar release from the fat body of *P. americana* at different concentrations of syn AKH *in vivo*

Experiment

Source	df	SS	MSS	F	Tab. F
Conc. (C)	5	48028.67	9605.733	5.62	2.55
Error	30	51289.33	1709.644		

The data used for ANOVA were obtained from analysis of sugar release from *P. americana* at different concentrations of syn AKH (Total cases considered are 30).

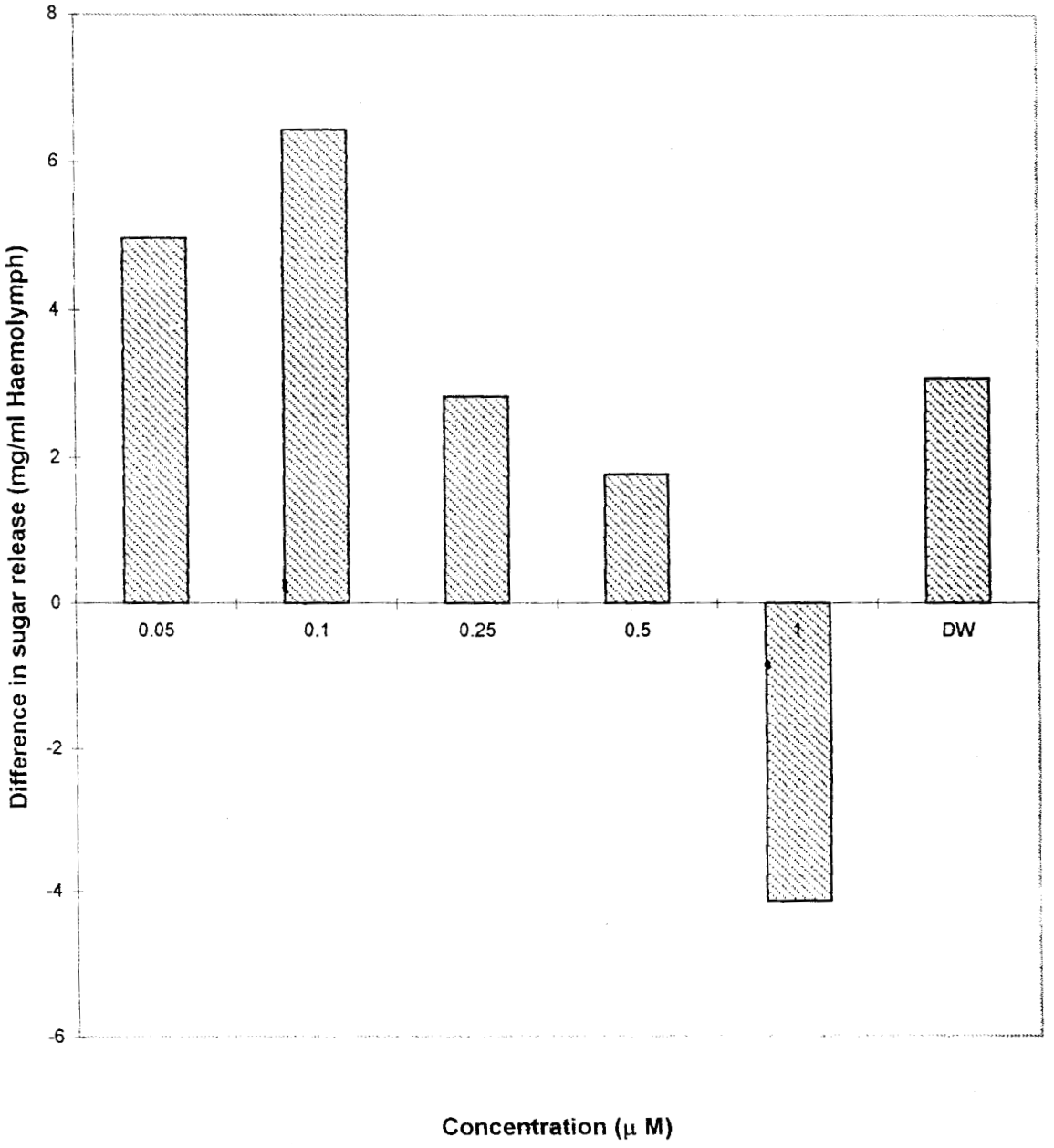
in *P. americana in vivo*. The F-value obtained is 5.62 and the Table F-value is 2.45.

Figure 8.14 shows the effect of AKH on sugar release from the fat body of *P. americana in vivo*. It appears that maximum stimulation is elicited by a concentration of 0.1 μM (by about 6.3 mg/g fb). Further increase in the concentration resulted in a gradual loss of stimulatory effect on sugar release from the fat body. At a concentration of 0.25 μM the release was 2.8 mg/g fb and at 0.5 μM, it was 1.7 mg/g fat body. At higher concentration (1.0 μM) instead of stimulation, there was an inhibition of sugar release (by about 4.1 mg/g fb). Lower concentration of the hormone (0.05 μM) showed a stimulation of about 5 mg/g fb sugar release from the fat body. When distilled water was injected instead of the hormone the sugar release was about 3.1 mg/g fb.

VIII.3. Discussion

Hyperglycaemic activity of SH-extract and syn AKH on the fat body of *S. mauritia* were found to be significant when tested *in vitro*. Activity was found to be prominent in the adult compared to other stages, although this was not true with all the tested concentrations. In the adult, AKH at the highest concentration (1.0 μM) showed inhibitory activity whereas all other

Fig. 8.14. Effect of different concentrations of syn AKH on sugar release *in vivo* from the fat body of *P.americana*



concentrations showed stimulation to various extents. SH-extract on the other hand, elicited hyperglycaemia to various extent with all the stages, but not with all the concentrations. Some concentrations showed inhibitory activities also. In the case of AKH, when compared to the maximum stimulation obtained (with a concentration of 0.05 μM) with the adult fat body (264 mg/g fb) the stimulation obtained in other stages are negligible, although these were all statistically significant. Maximum stimulation obtained with SH-extract on adult fat body (47 mg/g fb) was not much higher than the stimulation shown by other stages with other concentrations of the hormone whereas in the case of AKH, all stages except adult with all the concentrations the effect was similar, having only one tenth or one twentieth of the stimulation found in the adult fat body. In the case of SH-extract also the difference between minimum and maximum stimulation were similar. The difference in total amount of sugar released obtained between SH-extract and AKH only reflects the difference between the different batches of insect used. Neglecting this difference it appears that SH-extract contained more than one compound which is able to elicit hyperglycaemia in stages other than adults with different concentrations which may be perhaps due to the presence of more components than AKH-like compound. It is not sure whether the activity seen with SH-extract is elicited with the component having similar structure as

syn AKH or a different component. Because the crude extract of insect brain and associated structures have been found to contain large number of active components having similar chromatographic characteristics.

The fat body from *P. americana* showed stimulatory effect with both SH-extract and AKH but only with higher concentrations. SH-extract at a concentration of 1.0 gpe showed a stimulation of sugar release by about 12 mg/g fb. With AKH on the other hand, 0.5 μM elicited the maximum release of 101 mg/g fb and a concentration of 1.0 μM showed inhibition. The results obtained from *P. americana* is not fully agreeing with the earlier reports published on hyperglycaemia in the cockroach. Gaede (1989) demonstrated dose dependent hyperglycaemic activity with HGH-1and HGH-11. Our results showed positive response only to 0.5 μM AKH and 0.1, 1.0 and 2.0 gpe of SH-extract, however, exhibited a dose-response *in vitro*. Both SH-extract and AKH had different hyperglycaemic activity pattern *in vivo*. AKH showed better activity with all the concentrations except 1.0 μM with a maximum at 0.01 gpe. With SH-extract 2.0 gpe showed maximum hyperglycaemic activity with a minimum at 0.01 gpe. The better activity observed *in vivo* could be due to the presence of all the necessary complements required for the metabolic release of sugar into the haemolymph.

Investigations carried out on the hyperglycaemic activity of SH-extract thus revealed that *S. mauritia* has active hyperglycaemic factors in its neuronal tissues of the insect. These factors are active in *Spodoptera* as well as in *P. americana*. Though it was not able to demonstrate a smooth dose-response, the activities were found to be statistically significant when ANOVA was carried out.

GENERAL DISCUSSION

P. Shylaja Kumari “Neurohormonal regulation of metabolism in the paddy pest spodoptera mauritia boisd.(noctuidae : lepidoptera) ” Thesis. Department of Zoology , University of Calicut, 1999

GENERAL DISCUSSION

Experiments carried out to investigate the presence of active factors in the brain and associated glands of the Lepidopteran pest, *S. mauritia* indicated that the neuronal tissues contained some factors which had adipokinetic and hyperglycaemic activities. Adipokinetic hormones (synthetic) of locusts also showed similar activities. High performance liquid chromatographic separation showed that the extract contained compounds that had similar retention time as that of syn AKH-I, which gave support to the assumption that the active factors had similarity to AKH in their molecular characteristics too (Chapter IV). The extract was tested for its activity on lipid release from fat body of *S. mauritia* and *I. limbata*, on sugar release from the fat body of *S. mauritia* and *P. americana* and on lipid synthesis in the fat body of *S. mauritia*. The effect on metabolic activities in various developmental stages of *S. mauritia* were tried to correlate the activity with the quantitative changes taking place in the whole body of the insect.

It is becoming increasingly evident that insects belonging to various orders contain metabolically active peptides in their brain and associated structures. These peptides by virtue of their similarity in amino acid content and sequence, carry out similar functions. In many cases they also show cross-reactivity. Slight change in the amino acid sequence of the hormones

from different insects sometimes do not have much effect on their activity, and this is especially so if the changes in the amino acids are conservative.

The Noctuid moth, *S. mauritia* which we studied belonging to the order Lepidoptera, is found to contain some active neuronal factors which take part in the regulation of carbohydrate and lipid metabolism in some or other stages in its life cycle. Many other Lepidopteran species have been similarly found to possess peptides which are involved in metabolic regulations. For example, *M. sexta* (Ziegler and Schulz, 1986; Fox and Reynolds, 1990), *D. plexippus* (Dallmann and Herman, 1978) and *P. unipuncta* (Orchard *et al.*, 1991). However quantitative studies on these factors in Lepiodoptera have been done only in a few cases. Fox and Reynolds (1990) reported that the CC of adult *M. sexta* contained 33 pmole/CC and in larva about 3.5 pmole/CC. Orchard *et al.* (1991) quantitated adipokinetic peptide from the CC of *P. unipuncta* males and found that there was approximately 17.6 pmole of the peptide. These values are very much less when compared to the quantities measured earlier from some Orthopteran species such as *Schistocerca* (varies from 400-1000 pmoles between various stages and sexes) and *Locusta* (200-540 pmoles , Stone *et al.*, 1976; Orchard, 1987).

On the basis of the area of the peak obtained for SH-extract on an HPLC, the quantity of the component calculated to be present in a brain-CC-CA- complex worked out to be 7.35 nmoles which seems to be very high compared to the already reported values. This could be either due to the co-elution of other inactive components including some of the propeptides, along with the active peptides. It is well established that change in the amino acid sequence can bring about considerable reduction or loss of activity. This can also happens if the peak was given by many components having same polarity but having no or negligible activity. This lack of activity could be due to their difference in amino acid composition from those active components. Various hormones and their analogues have been tested for their activity to study the importance of some of the amino acid residues in variant and invariant positions for maximum activity (Stone *et al.*, 1978; Gaede, 1990 a). It is therefore not possible to say anything of sure regarding the actual quantity of the active component until the material is separated and purified for structural studies.

Variation in the hyperlipaemic activity of SH-extract on fat body of different stages reflects the physiological state of the insect during these stages. The appreciable response of the fat body of younger stages to lower concentrations suggests that the fat body in younger stages responds to the

hormone and they are sensitive to the available very low titre of hormones. It has been reported in many cases that the quantity of hormone produced and released is lower in the younger stages which increases during development to reach maximum in the adults (Siegert and Mordue, 1986). For example, in the case of the locust *S. gregaria* there was an increase of more than 6 fold in the quantity of AKH-I during development of the female 1 day 5th instar larva to the adult stage . Similarly in the case of *L. migratoria* there was nearly an 8 fold increase. Similar increase was also noted in the case of males during development. The quantity of AKH-II also showed a similar pattern (Siegert and Mordue,1986). The fact that the younger stages of various insects also contained hormones therefore suggests that they are involved in the regulation of metabolism (Goldsworthy *et al.*1973; Pannabecker *et al.*1987; Gokuldas *et al.*, 1988) and that they cannot be described as adult hormones as has been believed earlier. The locust adipokinetic hormone when described for the first time (Mayer and Candy, 1969) and found to be released during sustained flight was believed to be a hormone regulating flight metabolism. But the hormone was detected in young locusts and the hormone was also found to be involved in a variety of metabolic activities other than flight (Carlisle and Loughton, 1986; Gokuldas *et al.*, 1988; Kodrik and Goldsworthy, 1994). In *S. mauritia,* though the titre of the hormone was found to be very low in the

larvae, the fat body responded to the hormone added /given externally indicating that the fat body is all set to respond to the regulatory hormones which are to come into action in the later stages of the insect.

Quantitative analysis of lipid content in *S. mauritia* have shown that the growing larvae accumulates large amount of lipids during growth which get stored in the fat body. This lipids are used up during non feeding stages such as prepupae and pupae. Total lipids have been found to rise during prepupal stage (Fig.7.1). The fat body was responding reasonably well to adipokinetic factors (Figs.5.1 and 5.5). Similarly towards the 6th and 7th day of pupa, which were used for lipid release studies, there was very poor response to the hormones (Fig.5.1 and 5.5) which is explained by the very low lipid content at that stage (Fig.7.2). In adults on the other hand the steady but slow increase in lipids was associated with a balanced titre of hypo- and hyperlipaemic hormones ready to act in different situations that may arise in natural conditions.

The difference in the pattern of activity between SH-extract and syn AKH may be due to the presence of many other related compounds in the SH-extract. This is possible especially when the extract was prepared from the brain-CC-CA complex. The neurosecretory cells in the pars intercerebralis secretes and store their secretory materials in the CA which are known to

possess activities opposite to those secreted by the secretory cells in the CC. Orchard and Loughton (1980) obtained evidence for the presence of a hypolipaemic factor in the storage lobe of the CC which opposes the action of AKH in *L. migratoria.* Also the quantity of material required for eliciting response is different between the two sets of hormones. For the regulation of physiological activities, such counteracting hormones seems to be necessary as exemplified by the role of JH and ecdysone in moulting and metamorphosis. It is possible that, similarly CA contains some hypolipaemic factors whereas CC contain hyperlipaemic factors.

AKH showed hyperlipaemic activity only on prepupal and adult fat body whereas SH-extract showed activity in all the stages though none of these stages showed a dose-response. The presence of adipokinetic factors in the SH-extract is more evident when tested in *I. limbata* where a higher concentration, however, showed a negative response. The adipokinetic response by the fat body of *I. limbata* to SH-extract also give support to the cross-reactivity found among peptide hormones from different insect groups. It is possible that the active component in the SH-extract might be having structural similarity with similar factors present in the CC of *Iphita* and is also suggestive of the presence of such adipokinetic hormone responding system in *Iphita.* It is possible that *Iphita* depends mainly on lipids for its energy

requirements since it appears to possess a well developed AKH responding system. Some of our *in vivo* and *in vitro* experiments with *Iphita* have shown that fat body release lipids in a dose-dependent manner, when syn AKH-I was used (Vrinda, 1997, unpublished).

SH-extract also appeares to contain hyperglycaemic factor/s which had activity in all stages but to various extents. Adult fat body showed better response (with 0.01 gpe) than other stages. With AKH also adult fat body showed the maximum response. Here also lower concentration (0.05 μM) was showing better activity. It thus becomes apparent that the adult fat body responds to lower concentrations of AKH and hyperglycaemic factors present in the SH-extract. However, when tested in *P. americana* both SH-extract and AKH showed a different pattern of activity. Lower concentrations showed inhibitory effect on sugar release from the fat body. Maximum sugar release was effected with a reasonably higher concentration of the hormone. These results thus point to the fact that SH-extract contain many active component with activities similar to AKH and that the extract also contain components with opposite activities. Certain hormones are active at lower concentrations and without activity at higher concentrations. The same hormone exhibits different activities at different concentrations is exemplished by locust AKH. At lower concentrations, which is insufficiant to elicit adipokinetic effect it

inhibited protein synthesis (Carlisle and Loughton, 1986) whereas higher concentrations stimulated lipid release. It is also likely that the hyperglycaemic factors in the SH-extract and syn AKH are not as potent as the HGH of cockroach as the HGH in *P. americana* show a smooth dose-response (Gaede, 1990 b). Another interesting aspect which comes out in *S. mauritia* is that earlier stages in the life cycle seems to be less reactive to hyperglycaemic factors whereas better activity appears in pupal and adult fat body. The hyperglycaemic activity in pupal fat body could possibly be an indicator of the activity of the fat body of adults which were to emerge soon (within about 24 hrs) as the pupa selected for the study was mostly 6-days old which means the fat body almost have completed reorganisation into adult fat body. Adult stage seems to have a natural tendency to have more carbohydrates based metabolic activities as they feed on nectar or honey. Such a discriminative type of activity is found in other insects such as *M. sexta* where the fat body exhibit stage-specific sensitivity to AKH peptides. The peptide regulates mobilization of carbohydrates in larvae and lipids in adults (Ziegler *et al.*, 1984; Ziegler, 1990). In *M. sexta* there is also a discriminatory regulation of cytochrome synthesis involved in mitochondrial separation and trehalose synthesis. This may be achieved via fat body cell receptors which can produce different intracellular transducing mechanisms. For example, HTH in *P. americana*

mobilizes glycogen through a Ca^{2+} mediated mechanism and inhibiting heme synthesis through α-amanitin and gene involved processes (Keeley *et al.*, 1991).

It has been reported in *M. sexta* that the precentage of fat body glycogen phosphorylase which can be induced by native peptides changes considerably during development (Siegert and Mordue, 1994). In different stages, the maximum activation of glycogen phosphorylase induced varied (60-80% in 5th instar larvae and pharate pupae and 40% in prepupae, pupae and 1and 2 day old adults). The quantity of hormone required for the activation also varied between stages (2 pmoles for younger larvae and 20 pmoles for larger larvae) (Siegert and Mordue, 1994). This probably suggests the variation in the density of receptor protein on fat body cell membrane during different stages. A similar situation can be suggested for *S. mauritia.* A single hormone having different functions have been found bind to different receptor proteins and generate different second messengers to mediate their activities (Schramm and Selinger, 1975).

Since a similar difference in lipid release and sugar release activities for different concentrations of the hormone in different stages have been noticed, it is possible that in *Spodoptera* different peptide hormones present in the neuronal tissues are involved jointly in the regulation of carbohydrate and

lipid metabolism as per demand. In the feeding stages of the insect due to the ingestion of more carbohydrate rich food, hormones and enzymes involved in the conversion of simple carbohydrates into polysaccharides and lipids are likely to increase in titre and activity. Naturally a reduction in the hyperlipaemic activity and associated phenomena is what is expected.

Several distinct AKH-related peptides co-exist in the CC of insects with distinct physiological activities. For example, in locusts, AKH-II elevates more cAMP than AKH-I (Goldsworthy *et al.*, 1986) and AKH-II also influences hyperglycaemia while AKH-I effects lipid mobilization (Chino, 1985). In *P. attratus* one AKH-peptide is hyperglycaemic and other one is hypoglycaemic (Jaffe et al., 1989). This type of co-existing hormones help insects to keep up homostasis in the metabolic pool where changes in food, internal as well as external environment etc. change several times during development. This seems to be well- applicable in the case of *S. mauritia* also as the SH-extract (as well as AKH) show variation in their activities (both on lipid and carbohydrate metabolism) with different concentrations and at various developmental stages.

SUMMARY

The presence of components that can influence the metabolism of lipids and carbohydrates in the neuronal tissues of the noctuid moth, *Spodoptera mauritia*, the paddy armyworm moth, were tested. Extract prepared of the neuronal tissues - corpora cardiaca - corpora allata -brain complex - was run on an HPLC in an isocratic system and compared the peak obtained with a similarly obtained peak for synthetic Lom-AKH-1. It has been found that there was a peak shown at the elution time corresponding to syn AKH. This suggested the presence of a similar compound or a mixture of compounds in the extract. However, whole extract was used for further testing the biological activities of the extract. The extract was tested on lipid release from the fat body of *S. mauritia* itself (*in vitro*) and *Iphita limbata* (*in vivo* and *in vitro*). The result was compared with experiments carried out with syn AKH-I, *in vitro* in *S. mauritia*. Hyperglycaemic activity was tested in *S. mauritia* (*in vitro*) and *P. americana* (*in vivo* and *in vitro*). The above experiments were carried out with different concentrations of both the hormones. The effect of the hormones were also tested for their influence on lipid synthesis from labelled acetate. The response by the fat body to the hormones were tried to correlate with the quantitative changes in the lipids stored in the fat body.

Results obtained give strong indication that the neuronal tissues of *S. mauritia* contains (a) component/s that exhibit both hyperlipaemic and hyperglycaemic activity. It also appears that at certain stages, the hormones present in the extract act differently making the regulatory mechanisms possible. The hormones were also found to be involved in the regulation of lipid synthesis (from ^{14}C-acetate incorporation studies). However, from the experiment, it has been seen that different concentrations of the hormone including the syn AKH used for comparison, did not give a smooth dose-response curve, making it impossible to find out the optimum concentration required for optimal activity.

The chemical identity and structure are to be further studied on a gradient system of RP-HPLC, making it possible to fractionate all the components and to locate the different active fractions to be collected for further analysis and structural studies. The lack of an HPLC equipment at our reach and disposal made this impossible for the time being.

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