# BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AN ADIPOKINETIC NEUROPEPTIDE FROM THE MANGO LEAF WEBBER, *ORTHAGA EXVINACEA* HAMPSON (PYRALIDAE: LEPIDOPTERA)

Thesis
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**DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY**

*By*

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# DECLARATION

I, **Umadevi. D.,** do hereby declare that the thesis entitled "**Biochemical and Molecular Characterization of an Adipokinetic Neuropeptide from the Mango Leaf Webber, *Orthaga exvinacea* Hampson (Pyralidae: Lepidoptera)"** is an authentic record of the research work carried out by me in the Department of Zoology, University of Calicut, Under the guidance of **Dr. M. Gokuldas.** I further declare that no part of this thesis has been submitted previously for any other Degree.

Calicut University Campus, **UMADEVI, D.**
Date.

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**UMADEV**
**I. D**.

Dedicated to My Family

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AKH | Adipokinetic hormone |
| APRP | AKH-precursor-related peptide |
| Bommo-AKH | *Bombyx mori* AKH |
| BSA | Bovine serum albumin |
| CA | Corpora allata |
| cAMP | Cyclic AMP |
| CC | Corpora cardiaca |
| CCAP | Crustacean cardioactive peptide |
| DAG | Diacylglycerol |
| gDNA | Genomic DNA |
| GPCRs | G-protein coupled receptors |
| gpe | Gland pair equivalent |
| HrTH | Hypertrehalosaemic hormone |
| HoTH | Hypotrehalosaemic hormone |
| iP3 | Inositol 1,4,5-trisphosphate |
| JH | Juvenile hormone |
| Lom-AKH/Locmi-AKH | *Locusta migratoria* AKH |
| LTP | Lipid transfer particle |
| MALDI-TOF | Matrix assisted laser desorption ionization time of flight |
| MS | Mass spectrometry |
| Mas-AKH/Manse-AKH | *Manduca sexta* AKH |
| NCC | Nervous corporis cardiaci |
| PCR | Polymerase chain reaction |
| pGlu | Pyroglutamic acid |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| RPCH | Red-pigment concentrating hormone |
| RP-HPLC | Reverse phase-high pressure liquid chromatography |
| Spofr-AKH | *Spodoptera frugiperda* AKH |
| TAG | Triacylglycerol |
| TFA | Trifluoroacetic acid |
| Vanca-AKH | *Vanessa cardui* AKH |

# CHAPTER I
# INTRODUCTION

# INTRODUCTION

Neuropeptides make up the largest and the most diverse class of signaling molecules used in nervous system communication. Because these polypeptides play a key role in the regulation of nearly all physiological processes, it is of great interest to characterize this diverse assortment of molecules and to determine what effects they elicit on neural circuitry. Most neuropeptides are, in fact, hormones, which control a number of physiological processes. Hence, the neuroendocrine system represents a form of communication between cells, tissues and organs, other than the classical nervous and endocrine systems. Nervous control mechanisms act rapidly through synapses, releasing neurotransmitters into the synaptic cleft and generating action potentials of short duration. The classical endocrine control is slower acting but of longer duration since the hormones are released into circulation often a long distance away from the target organ and it takes some time before the hormones are degraded.

The importance of insects as biochemical models is supported by the fact that many discoveries in insects are applicable to vertebrate systems as well. The basic physiological processes of digestion, muscle contraction, and nervous transmission, as well as important metabolic and developmental pathways, are almost identical in insects and vertebrates (Law and Wells, 1989). Studies of a number of insect species have provided invaluable information for understanding the function and the evolution of neuropeptides. Due to the overwhelming complexity of mammalian nervous

systems, simpler model organisms are often used to study basic principles of neuronal function. Because arthropod nervous systems contain a manageable number of neurons, many of which exhibit consistent morphological and physiological properties between animals, these organisms provide excellent model systems for investigating neuromodulation in well-defined networks. Further more, these organisms contain a rich repertoire of neuropeptides, which are categorized into super families of structurally related isoforms. Some of these peptide families, such as RFamides, tachykinin-related peptides and kinins, are also present in mammals.

Earlier studies on insect neuropeptides have used large physiological model species (i.e., locust, cockroach and moth) and these have provided the groundwork for identifying the active signaling molecules. Further characterization of neuropeptides have been provided by recent genetic studies in *Drosophila melanogaster*, examining the genetic null mutants and cell ablations of specific peptidergic cells (McNabb *et al*., 1997; Park *et al*., 2002; Kim and Rulifson, 2004; Isabel *et al*., 2005; Kim *et al*., 2006). Thus, they are very useful models for understanding biological processes in general (Klowden, 2003). Research on insects has immensely contributed towards our understanding of neuroendocrinology. More studies on structure, metabolism, hydrolysis, release, receptor binding, mode of action and analogues of neuropeptides in insects would be useful in developing target specific and eco-friendly insecticides (Couillaud and Peypelut, 1995; Hoffmann *et al*., 2001; Gaede and Goldsworthy, 2003).

The intermediary metabolism in insects is regulated by small neuropeptides of the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) family (Gaede *et al.*, 1997). The structure of AKH was determined for the first time from the locusts, *Schistocerca gregaria* and *Locusta migratoria* (Stone *et al*., 1976). To date, the structures of around 40 different peptides of this family are known from insects belonging to most of the insect orders with seemingly overlapping functions (Gaede *et al.*, 1996). They are structurally very similar to the red pigment-concentrating hormone (RPCH) of crustaceans (Fernlund and Josefsson, 1972). Common characteristics of the known AKHs are that the peptides have a length of 8-10 amino acids, are amino terminally blocked by a pyroglutamate residue and end in a carboxy terminal amide. They contain at least two aromatic amino acids, tryptophan in position 4. These peptides are mainly uncharged, but some charged members were sequenced from certain dipteran and scarabaeoid beetles (Gaede *et al*., 1997). One of the two AKHs from the butterfly *Vanessa cardui* is unusual, having a length of 11 amino acids and a non-amidated COOH-terminus (Kollisch *et al*., 2000). Information on the physiological actions and roles that AKHs play, are available mainly for locusts, moths and beetles. Not much are known for peptides isolated from many other species. In lepidopterans, an AKH was first sequenced from the tobacco hornworm moth *Manduca sexta* (Ziegler *et al*., 1985) and this nonapeptide was code named Mas-AKH (pQLTFTSSWGamide). Mas-AKH was also found in the silkmoth, *Bombyx mori* (Ishibashi *et al*., 1992).

The AKHs exert a wide range of effects, many of which are analogous to the vertebrate glucagons (Goldsworthy *et al*., 1994). The major function of AKH is to mobilize stored products (fat, glycogen or proline) by the activation of phosphorylases or lipases. Besides the mobilization of energy reserves, these hormones are involved in additional physiological actions such as inhibition of synthesis of proteins (Carlisle and Loughton, 1979; Cusinato *et al*., 1991), lipids (Gokuldas *et al*., 1988; Ziegler, 1997; Lorenz, 2001) and mRNA (Kodrik and Goldsworthy, 1995). They also modulate the neuro or myoactivity (Scarborough *et al*., 1984; Milde *et al*., 1995; Socha *et al*., 1999) and inhibit glycolytic pathway (Becker and Wegener, 1998). Carlisle and Loughton (1986) suggested that AKH also inhibits protein synthesis in the aorta and the midgut, two tissues which are not involved in lipid mobilization, suggesting that receptors for AKHs are present in many tissues. An additional effect of AKH is in connection with immune response in locusts. Injections of laminarin (a major component of fungal cell walls) in to the haemolymph of the migratory locust activate the prophenoloxidase cascade, generating quinines that are toxic to microbes (Lavine and Strand, 2002). Activation of the enzyme cascade is prolonged by co-injection of Locmi-AKH-I together with laminarin (Goldsworthy *et al*., 2002, 2003 a). These actions can have significant impact on insects but their exact roles need to be determined.

Ortholog neuropeptide genes show a high degree of divergence in their overall amino acid sequences while only small portions of the genes have been highly conserved, namely, those regions coding for mature peptides or

even only the motif within the peptide sequence that is required for biological activity (Liu *et al*., 2006 a). Understanding of the evolution of the peptidergic signaling molecules has thus been hampered by the discontinuity of available data on evolutionarily related taxa, as well as by incomplete data within a particular species. Another powerful approach to gain insight in the evolution of the peptidergic signaling systems is based on the phylogeny of the cognate receptors for the neuropeptide ligands. G-protein coupled receptors are large transmembrane proteins that have been well conserved and that carry more informative sequences allowing the evolutionary analysis of the respective genes. This phylogenetic analysis, which of course assumes that ligands and cognate receptors co-evolve, allows evolutionary grouping of insect neuropeptides (Park *et al*., 2002; Hauser *et al*., 2006 a, b; 2007). New techniques have recently emerged to identify the genes encoding neuropeptides. These new approaches include bioinformatic tools to predict the genes from whole genome sequences and expressed sequence tag (EST) libraries, and direct detection of the processed mature peptides using mass spectrometry (MS), also known as peptidomics (Hewes and Taghert., 2001; Baggerman *et al*., 2002; Riehle *et al*., 2002; Predel *et al*., 2004; Hummon *et al*., 2006; Liu *et al*., 2006 b). Upon identification of putative peptide encoding genes from the genome sequence, the transcription of these predicted genes can be confirmed by reverse-transcription PCR (RT-PCR) and by EST data. The amino acid sequence of the mature peptides obtained by peptidomic approaches can be compared with the predicted sequences, thereby revealing the mature peptide after post translational processing of the prepropeptide. As

such, genomics, transcriptomics and peptidomics approaches mutually support and supplement each other and lay the foundation for further functional investigations of the neuropeptides.

**Relevance of the present study**

Recent development in genome projects of various insect species have allowed for the rapid discovery of novel genes including those coding for bioactive peptides. Genome sequences have been completed and are available for the fruit fly *Drosophila melanogaster* (Adams *et al*., 2000), two mosquitoes, *Anopheles gambiae* (Holt *et al*., 2002) and *Aedes aegypti* (Nene *et al*., 2007), the honeybee *Apis mellifera* (The Honeybee Genome Sequencing Consortium, 2006), and the flour beetle *Tribolium castaneum* (*Tribolium* Genome Sequencing Consortium, 2008). Genomic studies on neuropeptides and their receptors in these insects have been highly successful and have provided crucial information about their development, physiology, behavior and evolutionary relationships (Coates *et al*., 2000; Hewes and Taghert, 2001; Park *et al*., 2002; Riehle *et al*., 2002; Hummon *et al*., 2006; Zitnan *et al*., 2007; Hauser *et al*., 2008; Li *et al*., 2008).

Lepidoptera represents a diverse and important group of agricultural insect pests that cause widespread economic damage to food and fiber crop plants, fruit trees, forests and stored grains. The understanding of certain genes in Lepidoptera may enable the design of new, targeted pesticides. Several insect neuropeptides that have important roles in the regulation of endocrine mechanisms or behavior were first identified in *Bombyx* (Tanaka

and Kataoka, 2006). However, genomic studies on neuropeptides and their receptors have not yet been carried out, although a draft sequence for the genome of *Bombyx* has been reported (Mita *et al*., 2004; Xia *et al*.,2004). Mimetic analogues are valuable tools for the development of future pest management strategies. Chemical and conformational requirements for neuropeptide-receptor interactions represent a template from which agonist/antagonist peptide mimetics, with a potential to disrupt critical insect processes can be developed. The design of biologically active pseudopeptide analogues in which amino acids were replaced by non-peptide moieties represented a milestone in the development of non-peptide mimetic analogues (Nachman *et al*., 1995)

All known AKH precursors have similar general organization; a signal peptide followed by a single copy of AKH peptide, canonical amidation and dibasic cleavage signals and by an AKH-precursor-related peptide (APRP). The cDNAs encoding AKH precursors in Lepidoptera have been cloned from *Manduca* (Bradfield and Keeley, 1989) and from the fall armyworm *Spodoptera frugiperda* (Abdel-latief and Hoffmann, 2007). In *Bombyx*, a nonapeptide identical with *Manduca* AKH has been chemically identified (Ishibashi *et al*., 1992) and the orthologue of the *Drosophila* AKH receptor (AKHR) has been cloned (Staubli *et al*., 2002). The *Bombyx* genome contains a triplet of paralogous genes encoding adipokinetic hormones. *Bombyx* AKH1 is identical to nonapeptides found only in moths, while *Bombyx* AKH2 is closely related to many other AKH/HrTH decapeptides. *Bombyx* AKH3 has charged amino acids (Arg-Asp) at residues 6 and 7.

Sequence similarity among different genes from insects suggests that there are at least two distinct ancestral forms of AKH, and *Bombyx* has two distinct ancestral forms of AKH (Li *et al*., 2008). *Spodoptera* has four AKHs closely related to *Bombyx* AKH1 and AKH2, while their similarity to *Bombyx* AKH3 is less obvious. These suggest that the AKH gene family evolved differently in the two lepidopteran insects, *Spodoptera* and *Bombyx*. Within lepidopterans, the nonapeptide *Manduca sexta* AKH (Manse-AKH) represents a widely occurring AKH, whereas the decapeptide Helze-HrTH (at first isolated from *Helicoverpa zea*) seems to be restricted to moths. The determination of primary structure of adipokinetic neuropeptides of *Iphita limbata* and *Aularches miliaris* indicated the presence of more than one adipokinetic factor in them (Ajaykumar and Gokuldas, 2011 a, b).

The recently reported molecular biological techniques leading to the cloning of certain AKH receptors will surely allow the elucidation of more receptors in future. Especially for those species that produce three endogenous AKHs, it will be important to know whether each AKH has its own receptor. Cloned AKH gene sequences will facilitate studies on the synthesis of AKH peptides and provide insight into molecular evolution. With a cloned AKH structural gene sequence, we can begin to examine the cellular events leading from AKH transcript modulation to release of a biologically active insect neuropeptide. These studies will be of broad interest, because the physiologically important AKH family may be ubiquitous among insects and other arthropods. We decided to isolate AKH gene sequence from the mango leaf webber, *Orthaga exvinacea*, because this species is one of the important

models for insect physiology and biochemistry, and also it represents the Lepidoptera, a large and important insect pest group.

Insect hormones and interventions of insect endocrine processes have been propagated as possible pest management tools ever since investigations on insect neuroendocrinology have been initiated. Confronted with the problem of developing insect resistance to conventional pesticides, there is a critical need for developing new concepts and alternative approaches in controlling pest insects. The basic premise of this research points to that those neuropeptides that can serve as potent messengers in insects to regulate vital functions. New, selective control measures may be developed in designing metabolically stable mimics of those neuropeptides that actively inhibit or overstimulate the functions regulated by them, resulting in sustained disruption of the internal insect homeostatic environment. This appears to be one of the rare attempts of the molecular characterization of AKH neuropeptide from a lepidopteran insect. The fact that different isoforms of AKH are being used by both pest and beneficial insects to regulate critical life processes increases the likelihood that, control measures based on this neuropeptide can be developed that can be highly selective. The work is one step closer to (tries to) the development of practical neuropeptide-like substances that will be effective in controlling pest insects in an environmentally friendly fashion. Comparative analysis of the amino acid and nucleotide sequence shed light on their evolution.

**Objectives of the study**

- Detection of biological activity of neuropeptides in *Orthaga exvinacea* both *in vivo* and *in vitro* on,

a) Lipid metabolism

b) Carbohydrate metabolism and

c) Protein metabolism.

- Separation of peptides from the extracts of retrocerebral complexes of *O. exvinacea* by reverse phase high performance liquid chromatography (RP-HPLC).

- Testing the effects of different HPLC fractions on lipid, carbohydrate and protein release using bioassays.

- Identification and sequencing of adipokinetic neuropeptides from the extracts by MALDI-TOF-MS and MS/MS analysis.

- Molecular level analysis for the characterization of AKH gene by polymerized chain reaction (PCR), and gene sequencing.

- To study the evolutionary relationship of adipokinetic neuropeptide among Lepidoptera and among other insect groups.

- To study whether the insect AKH has any lipokinetic effects on vertebrate liver.

# CHAPTER II
# REVIEW OF LITERATURE

# REVIEW OF LITERATURE

Insecta, the hexapod class of arthropods, is the biggest group of animals among all other animals on earth. Among the several factors that constitute for their successful invasion of all possible habitats, their ability to manage fuel storage and utilization is one of the most important. They have developed special systems for efficient and fast use of energy stores in processes like reproduction, embryogenesis, metamorphosis and flight. The reproduction is usually sexual, although in many insect groups eggs sometimes develop without fertilization by sperm. Embryogenesis is a developmental process that usually begins once the egg has been fertilized. It involves multiplication of cells (by mitosis) and their subsequent growth, movement, and differentiation into all the tissues and organs of a living insect. All insect growth involves a metamorphosis, a transformation in form and in way of life, and has three/four stages: egg, larva, pupa (only n holometabolous group), and adult.

Flying ability is one of the primary reasons that insects have been successful in nature. Flight assists insects in escaping from danger, finding food, locating mates and dispersal. It involves the highest metabolic rate in nature, which increases 50 to 100-fold compared to that at rest (Beenakkers *et al*., 1984, 1985). The dramatic increase in the fuel demand is accomplished by fat body, which is the main storage organ of highly energy-rich molecules and is comparable to the adipose tissue and liver of vertebrates (Kilby, 1963; Prince, 1973; Wyatt and Pan, 1978; Wyatt, 1980). The insect fat body is the major

center for intermediary metabolism and is involved in the homeostatic maintenance of haemolymph proteins, lipids, and carbohydrates. Glycogen, proteins and lipids are the primary reserve food materials, of which lipids constitute a major portion. Triacylglycerol (TAG) forms the major storage lipid of insect fat body. During locomotion and other sustained activities, TAG reserves in the fat body are mobilized and released in to the haemolymph mainly as diacylglycerol (DAG) and are transported to the sites of utilization. The mobilization, release, transport and utilization of lipids are regulated primarily through the mediation of adipokinetic neuropeptide hormones.

Insects are unique among invertebrates in that they are the only invertebrates performing true flight. For this reason insect flight and flight metabolism has attracted much attention of insect physiologists and biochemists (Sacktor, 1975). Insect flight commands a very high rate of fuel utilization and is considered the most metabolically demanding form of animal kingdom. The higher energetic cost of flight is explained by the higher frequency of contraction in flight muscle than in non-flight muscle (Harrison and Roberts, 2000). Respiratory rates have been shown to increase as much as 50-100-fold in some insect species when going from rest to flight compared to a 14-fold increase in birds and a 5-fold increase in vertebrate skeletal muscles from rest to maximum work (Kammer and Heinrich, 1978; Beenakkers *et al*., 1985)

**Flight fuels**

On initiation of flight, the fat body releases carbohydrates, lipids, the amino acid proline, or mixtures thereof in to the haemolymph, which are oxidized by flight muscles to fuel flight (Gaede and Auerswald, 2003).

**Carbohydrates**

Carbohydrates are stored in the fat body mainly in the form of glycogen, which can be rapidly hydrolyzed to release trehalose into the haemolymph (Wyatt, 1967; Candy, 1985; Candy *et al*., 1997; Thompson, 2003). Trehalose is transformed into glucose and used by the flight muscles when energy is required. Trehalose can be synthesized in the fat body from monosaccharides of dietary origin or by gluconeogenesis from a variety of precursors. It is the major sugar in insect haemolymph and most important for flight. It is a non-reducing, less reactive sugar and therefore has an advantage over glucose and other reducing sugars. It occurs in the haemolymph in concentrations between 5 and 50 mM, which differ considerably between species. Regulation of trehalose synthesis occurs at two levels: hormonal regulation of glycogen conversion to trehalose and feedback inhibition by trehalose. Insects belonging to the orders Hymenoptera, Diptera and Blattodea are examples of insects using carbohydrates as fuels for flight.

**Lipids**

Lipids are highly reduced, and therefore hold more energy per gram than glycogen. In addition, lipids can be stored in compact form, as they have very little water associated to them compared to glycogen. Therefore,

migratory insects which fly for long distances generally use lipids to fuel flight (Cheeseman *et al*., 1976; Ziegler and Schulz, 1986). Haemolymph lipid content and composition vary with the physiological state of the animal. Diacylglycerol (DAG) is the major lipid component of haemolymph in most insect species (Beenakkers *et al*., 1985). Hormonal activation leads to the release of lipids from the fat body, which is oxidized by flight muscle cells (Candy *et al*., 1997). In locusts, the initial stage of a flight is mainly dependent on fuels stored in the muscle cell, phosphoarginine and muscle glycogen, which are rapidly supplemented by haemolymph trehalose (Wegener, 1996). During prolonged flight, fuel utilization is gradually shifted from carbohydrates to the oxidation of lipids that are mobilized from locust fat body. Storage of energy in the form of lipids is also advantageous for insects that do not feed as adults. In insects, the lipid content can vary from 1% to 50% of the wet weight, and is influenced by many factors including stage of development, nutritional state, sex, environmental temperature, diapauses and migratory flight. Gross fluctuations in lipid content have been noticed in different orders of insects (Fast, 1964, 1970; Gilbert, 1967; Bailey, 1975). In most insects studied, triacylglycerol (TAG) comprises more than 90% of total fat body lipid. Several studies have indicated that fatty acid (FA) profile of the fat body is influenced by the FA composition of the diet (Beenakkers *et al*., 1985).

**Ketone bodies**

The haemolymph of the locust, *Schistocerca gregaria*, has been shown to contain acetoacetate and smaller amounts of 3-hydroxybutyrate (Bailey *et al*., 1971). Ketone bodies appear to be good energy substrates for brain in insects as they are in mammals (Candy *et al*., 1997). Acetoacetate might be important during flight in locusts, as its concentration in the haemolymph increases during flight or upon injection with brain-corpora cardiaca (CC) extracts. However, not much is known about the use of ketone bodies in other species.

**Amino acids**

During insect flight a number of amino acids can be oxidized to provide energy; in a number of insects, proline is the most important. Proline oxidation by the tsetse fly, *Glossina morsitans* flight muscle was first discovered by Bursell (1963, 1981). Later, it was found that in many beetles proline exclusively or in combination with carbohydrates is utilized as a fuel for flight activity (Weeda *et al*., 1979; Gaede and Auerswald, 2002). Proline is partially oxidized in the muscle cells, and the alanine which is formed in the process is transported to the fat body where it is reconverted to proline. The two carbon atoms required for the reconversion are derived by β–oxidation of fatty acids to acetyl-CoA. Regulation of proline synthesis is under hormonal control (Candy *et al*., 1997). Use of proline as a flight fuel has many advantages. It can be stored at high concentrations in flight muscles and haemolymph. Conversion of proline into alanine and vice versa does not

release ammonia and no specific carrier molecules are necessary for their transport.

**Insect metabolic neuropeptides**

Functionally, insect hormones have been classified as metamorphic hormones and metabolic hormones. The former includes the hormones involved mainly in the metamorphosis such as ecdysone and prothoracic hormones secreted from the prothoracic glands and the corpora allata respectively. The variation in the titre of these hormones regulate moulting and growth. Metabolic hormones include mainly peptide hormones synthesized in the neurosecretory cells of the brain and corpora cardiaca and secreted from the corpora cardiaca and corpora allata. These hormones are involved in the regulation of various key metabolic pathways.

Neuropeptides and protein hormones are produced in endocrine cells or neurons as large precursors. These precursors (prepropeptides) are cleaved and further modified to yield mature peptides that are secreted in to the extracellular environment. Peptides exert their action by binding to membrane receptors, mostly being G-protein coupled receptors (GPCRs), although some of them are receptor tyrosine kinases. Neuropeptides represent the largest single class of regulatory compounds in invertebrates, as well as in vertebrates. In insects there are various neuropeptide families, the members of which mostly occur in multiple forms and display pleiotropic actions. They are either released from the nervous system in to the haemolymph as circulating neurohormones or more specifically released as neurotransmitters

at specific target sites. The existence of neuropeptides which regulate physiological, developmental and behavioral events in insects have been known for a long time. Proctolin, the first insect neuropeptide identified (Brown, 1967) and isolated from the proctodaeum of *P. americana* (Brown and Starratt, 1975), and widely distributed among insects, is a pentapeptide. Another group of neuropeptides, which regulate carbohydrate and lipid metabolism, have been identified from the brain-corpora cardiaca (CC) complexes of insects as well as from the neurosecretory X-organ sinus gland complex of the eyestalks of crustaceans. The first reported among these hormones was the hyperglycaemic factor (comparable to glucagons of vertebrates) from the CC of *P. americana* (Steele, 1961). In locusts, a hyperglycaemic response was demonstrated in male insects, six days after the final moult, but not in insects of other ages (Goldsworthy, 1969). There is also evidence for the presence of a hypoglycaemic factor released from the neurosecretory cells of the brain in honey bee (Dixit and Patel, 1964) and blowfly (Norman, 1975). Another peptide hormone identified is the adipokinetic hormone (AKH) concerned with mobilization of lipids from the fat body, its transport and oxidation in flight muscles. A factor with hypolipaemic activity has also been found to be released from the storage lobe of locust CC, when nervous corporis cardiaci II (NCC-II) was electrically stimulated (Orchard and Loughton, 1980). Holwerda *et al*. (1977) found that the CC extract from locusts exhibited both hypolipaemic and hyperlipaemic activities. Other neurohormones identified in insects include the cardioacceleratory peptides, myotropic peptides, gastrin-cholecystokinin like

peptides, FMRF-amide like peptides, neurophysin, somatostatin etc. It is only during the last two decades, that a great number of neuropeptides have been isolated, purified and their primary structures completely elucidated (Gaede, 1997 a; Gaede *et al*., 1997).

**Adpipokinetic peptides**

Adipokinetic hormones form a major group of neuropeptides, which regulates physiological homeostasis. The first members were discovered in 1960s in the American cockroach and in locusts, where they are involved in the control of carbohydrate and lipid breakdown, respectively. It is now known that these peptides are members of a large family of structurally related peptides which are found in crustaceans and insects (Gaede, 1996). Such peptides became known under the acronym AKH/RPCH family peptides on the basis of the first members of this family to be fully characterized, viz., an adipokinetic hormone from locusts (Stone *et al*., 1976), now called Locmi-AKH-I according to the nomenclature proposed by Raina and Gaede (1988), and a chromatotropic peptide from the prawn, *Pandalus borealis* (Fernlund and Josefsson, 1972), the red pigment concentrating hormone (code name: Panbo-RPCH). These peptides are present in the neurosecretory X-organ/sinus gland complex in the eyestalks of crustaceans and in the intrinsic neurosecretory cells of the corpora cardiaca of insects. Both of these structures are neurohaemal organs, analogous to the vertebrate hypothalamo/hypophyseal system and it can thus be inferred that the peptides can be released from the neurohaemal organs in to the circulation and thus,

act as true hormones. However, release has been demonstrated only in a few cases, for example, during flight in locusts and the moth, *Manduca sexta* (Gaede, 1992).

Adipokinetic hormones and related peptide hormones, known as hypertrehalosaemic hormones (HrTHs), hypotrehalosaemic (HoTHs), or hyperlipaemic peptides (AKHs)-herein generically referred to as AKHs, are included in one of the most extensively characterized peptide families in insects with almost 40 isoforms identified (Gaede, 1988 a; Gaede, 2004; Van der Horst, 2003; Gaede and Marco, 2011). These peptide hormones may even be part of a superfamily that includes the growth hormone-releasing factors (GRF) and glucagons in vertebrates (Clynen *et al*., 2004). All AKHs are hydrophobic compounds and are stable at high temperatures. They are detected and isolated from almost all insect orders and have great importance in recent years (Auerswald *et al*., 2005; Gaede *et al*., 2005, 2006, 2007 a, b). Most insects have only one AKH peptide, but some species have two, three or four isoforms (Stone *et al*., 1976; Siegert *et al*., 1985, 2000; Gaede, 1990 b, c; Oudejans *et al*., 1991; Gaede *et al*., 1994; Siegert, 1999). The AKH precursor begins with a signal peptide that is followed by a single AKH of 8-10 amino acids in length and then an AKH-precursor-related peptide (APRP). Prior to secretion, the AKH region is enzymatically cleaved from the ARPP and modified at the amino and carboxy termini to a pyroglutamic acid and amide, respectively (Gaede and Auerswald, 2003). The number of AKHs known to exist in a particular insect varies from one in *Drosophila melanogaste*r (Isabel

*et al*., 2005; Lee and Park, 2004; Schaffer *et al*., 1990) to four in *Locusta migratoria* (Siegert, 1999; Van der Horst, 2003). Generally, AKHs are present in all life stages, and they are secreted from a distinct region of the corpora cardiaca (CC), a neurohemal gland connected to the brain, that contains intrinsic neurosecretory cells (Diederen *et al*., 2002). Cells in the brain and other ganglia also secrete, such peptides, as shown by immunocytochemistry in the mosquito *Aedes aegypti* (Brown and Lea, 1988) and other insect species (Schooneveld *et al*., 1983, 1985). Mobilization of metabolites during energy expensive activities, like flight is regarded as the primary endocrine action of AKHs in insects. For example, injection of species-specific AKH increased the levels of circulating trehalose in the American cockroach, *Periplaneta americana* (Oguri and Steele, 2003), mobilization of lipids in the migratory locust, *L. migratoria* (Gaede and Auerswald, 2003; Van der Horst, 2003) and, similarly, proline in the fruit beetle, *Pachnoda sinuata* (Gaede and Auerswald, 2002). Other functions have also been ascribed to these peptides (Gaede, 2004; Gaede and Auerswald, 2003). Genetic manipulation of AKH gene expression altered not only circulating levels of trehalose and lipid in *D. melanogaster* larvae but also affected general locomotor activity and survival of adults during starvation (Isabel *et al*., 2005; Lee and Park, 2004). A possible immune function for AKH was described in locusts (Mullen and Goldsworthy, 2003; Mullen *et al*., 2004).

As for AKH signal transduction, the biochemical characterization of an AKH receptor was first reported for the tobacco hornmoth, *Manduca sexta*

(Ziegler *et al*., 1995) and later, AKH G-protein coupled receptors (GPCR) were cloned for *D. melanogaster* (Park *et al*., 2002; Staubli *et al*., 2002); the silkworm *Bombyx mori* (Staubli *et al*., 2002); and the cockroach, *P. americana* (Wicher *et al*., 2006), and expressed in cells for ligand binding studies. Other elements in the intracellular signaling pathway also are known for insects (Gaede, 2004). Studies on the flight performance and metabolism of the African malaria mosquito, *Anopheles gambiae*, revealed that both carbohydrates and lipids were used for flight (Kaufmann and Briegel, 2004). These results suggest that an AKH may play an important role in the mobilization of nutrients for flight by mosquitoes, as shown in other insects (Van der Horst, 2003). For other Diptera, AKHs and their bioactivity have been reported. For example, the horse fly, *Tabanus attratus* (Jaffe *et al*., 1989; Woodring and Leprince, 1992), blowfly *Phormia terraenovae* (Gaede *et al*., 1990), fleshfly, *Neobellieria bullata* (Verleyen *et al*., 2004) and *D. melanogaster* (Isabel *et al*., 2005; Lee and Park, 2004).

**Hormonal regulation of lipid metabolism**

The major class of storage lipids of insect fat body is TAG (Downer, 1985; Arrese and Wells, 1997; Canavoso *et al*., 1998). The lipid stored should be mobilized and released in to the haemolymph to be transported to the site of metabolism. Fat body lipolytic enzymes hydrolyse stored TAG into DAG and fatty acids. The release form of lipids has been difficult to generalize, because it has been found that in different insects, it is either TAG (Martin, 1969), DAG (Beenakkers and Gilbert, 1968; Downer and Steele, 1972;

Thomas, 1974) or free fatty acids (Wlodawer and Lagwinska, 1967). Under the influence of lipid mobilizing hormones, TAG lipase gets activated and hydrolyses TAG in to DAG, primarily *sn*-1, 2-DAG. After the formation of DAG, it is moved from the fat droplet of cytosol to the plasma membrane possibly by a cytosolic carrier protein that binds to DAG and also interacts with the membrane for delivering to the haemolymph (Arrese *et al*., 2001). The DAG is translocated in to the haemolymph and loaded in to pre-existing HDLP (Arrese *et al*., 2001). The transfer of DAG from to the HDLP is catalysed by a complex lipoprotein called lipid transfer particle (LTP) (Van Heusden and Law, 1989). This lipoprotein has been identified and purified from the haemolymph of several species including *L. migratoria* (Hirayama and Chino, 1990), *M. domestica* (Capurro and De Bianchi, 1990), *P. americana* (Takeuchi and Chino, 1993) and *B. mori* (Tsuchida *et al*., 1997). LTP has been found to be synthesized in the fat body and secreted in to the haemolymph in *M. sexta* (Van Heusden *et al*., 1996).

Studies have demonstrated the involvement of AKH in the control of lipid metabolism during flight, the hormone being released from CC within about 2 to 5 minutes of flight (Goldsworthy and Wheeler, 1989). It has been proposed that the initial stimulation for the release of AKH involves receptors associated with wing or air movement, tarsal contact or metabolic levels (Goldsworthy, 1983) and is controlled by secretomotor centers in the lateral areas of protocerebrum. In *L. migratoria*, Orchard and Loughton (1981) have shown that electrical stimulation of nervi corporis cardiaci II (NCC-II) results

in the release of lipid during the first 10 to 20 minutes of flight. Excitation caused by handling stress in *L. migratoria* (Orchard *et al*., 1981) and *Acheta domesticus* (Woodring *et al*., 1989) and heat and chemical stress in *S. gregaria* (Davenport and Evans, 1984) have been found to cause hyperlipaemia.

Starvation is another physiological variable influencing lipid metabolism in insects. An increase in ketone bodies, acetoacetate 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 hormonally induced (Jutsum *et al*., 1975; Mwangi and Goldsworthy, 1977; Cheeseman and Goldsworthy, 1979). In *Romalea microptera*, starvation blocks the RCC- stimulated hyperlipaemia (Spring and Gaede, 1987). Studies by Orchard *et al*. (1982) suggested that the hyperlipaemic condition of starved locusts is due to octopamine. Downer (1985) demonstrated sequencial mobilization of carbohydrates and lipids in starved cockroaches. Gokuldas (1989) has reported that in *S. gregaria*, starvation reduced the capacity of fat body to synthesise lipid from acetate and refeeding reversed the condition. It is suggested that the decrease in lipid synthesis during starvation could be due to elevated circulating AKHs.

**Source of AKH**

The AKH are synthesized in the intrinsic neurosecretory cells of corpora cardiaca (CC) of insects. The adipokinetic cells are neuron like

unipolar cells with short cell processes (Cassier and Fain-Maurel, 1970; Orchard and Shivers, 1986). The glandular region of CC contains approximately 6,000 to 10,000 adipokinetic cells (Hekimi and O'Shea, 1987; Hekimi *et al*., 1989; Schulz-Aellen *et al*., 1989). These cells contain large number of electron dense granules (Rademakers and Beenakkers, 1977; Krogh and Norman, 1977) and the AKH reside in these dense granules (Stone and Mordue, 1979). Neurosecretory cells in the brain and other ganglia also secrete adipokinetic peptides directly, as shown by immunocytochemistry in the mosquito *Aedes aegypti* (Brown and Lea, 1988) and other species (Schooneveld *et al*., 1985). The presence of AKH-I in the locust brain is indicated by Schooneveld *et al*. (1983) and has been substantiated by Moshitzky *et al*. (1987 a, b) and Bray *et al*. (1993). Similarly, using radioimmunoassay, *Manduca* brain was found to contain *Manduca*-AKH (Fox and Reynolds, 1990). AKH-like material is found in other insect neural tissues (Schooneveld *et al*., 1983; Ziegler *et al*., 1988), but only in small quantities. The fourth AKH identified from *L. migratoria* was found in the storage lobe of CC and hence is presumed that they are probably synthesized in the brain (Siegert, 1999).

**Synthesis and release**

Flight activity is the only natural stimulus for the release of AKH (Mayer and Candy, 1969; Cheeseman and Goldsworthy, 1979; Orchard and Lange, 1983 a, b; Diederen *et al*., 2002). AKH producing cells continuously synthesize AKH and the synthesis is not affected by its release during flight

(Harthoorn *et al*., 2001). Release of AKH upon flight was established by using biological assays (Cheeseman and Goldsworthy, 1979). Receptors associated with wing movements or wind receptors or change in metabolite levels could be involved, but none has been established (Goldsworthy, 1976, 1983). Both neuronal and hormonal factors are involved in the release of AKH. *Locusta* tachykinins and crustacean cardioactive peptide (CCAP) stimulate AKH release from CC *in vitro*. The most potent AKH in the brain extract of the desert locust, *S. gregaria* has been identified and sequenced as PFCNAFTGC-$NH_2$ (Veelaert *et al*., 1997). A detailed biosynthetic pathway of two adipokinetic hormones from *S. gregaria*, including the characterization of prohormone, has been elucidated by direct protein chemical methodologies, and by molecular cloning techniques (Gaede *et al*., 2006).

Octopamine is involved in AKH release as the neurotransmitter of the secretomotor neurons that make synaptic contact with the adipokinetic cells (Orchard *et al.,* 1993). Diederen *et al*. (2002) also suggested a humoral role for octopamine since the haemolymph titer of octopamine is known to increase three fold at the onset of flight (Goosey and Candy, 1980; Goldsworthy, 1983). Passier *et al*. (1995) identified that octopamine only potentiates the release-initiating factors that stimulates adipokinetic cells resulting in AKH release and that it cannot induce the AKH release on its own. Dopamine, serotonin and tyramine also potentiate the AKH release in a similar way. Secretion of AKH is believed to be controlled by conventional neurons from the brain, since severing the nervous connection between brain

and CC prevents the flight induced elevation of haemolymph lipid titers (Goldsworthy *et al*., 1972), while electrical stimulation of these nerves in isolated CC causes the release of AKH (Orchard and Loughton, 1981). AKHs are transported in the haemolymph without any carrier proteins (Oudejans *et al*., 1996). To terminate the signal, the peptides of AKH/HrTH family are cleaved by endopeptidases in to fragments, which are susceptible to further degradation by exopeptidases (Isaac, 1988; Ryane and O'Shea, 1992; Oudejans *et al*., 1996).

Adipokinetic hormone release can be artificially induced by certain insecticides (Samaranayaka, 1974; Singh and Orchard, 1982), anticholin-esterases (Samaranayaka, 1976) and by proctolin administration (Clark *et al*., 2006). Release of AKH *in vitro* has been found to get inhibited by several factors. For example, trehalose was shown to inhibit AKH release (Passier *et al*., 1997). The tetrapeptide FMRF-amide, decapeptide Schisto-FLRF amide (Vullings *et al*., 1998) and the locust myoinhibiting peptide, Lom-MIP (Harthoon *et al*., 2001) are also inhibitors of AKH release.

**Inactivation**

After releasing to the haemolymph, AKHs are attacked by peptidases or proteases (Issac *et al*., 1978). Half lives for various AKHs are determined with near physiological and sometimes totally non-physiological concentrations of peptides. In locusts, when tritiated AKHs with a high specific radioactivity are used, half lives for the three AKHs during resting conditions and after flight are short but different for each peptide and are differently affected by flight. Locmi-AKH-III possesses a short half life, and

breakdown of Locmi-AKH-II is apparently almost not affected by flight at all (Oudejans *et al*., 1996). One active peptidase having similar action and properties to mammalian endopeptidase has been reported, but it is enigmatic how one peptidase can cleave the three AKHs at different rates.

## Physiological Functions of AKH

### Hyperglycaemic activity

Hormonal control of carbohydrate metabolism has been studied in many insect species (Gaede, 1990 a; Gaede, 1991 a; Raina *et al*., 1995; Keeley *et al*., 1996; Becker and Wegener, 1998). Carbohydrate mobilization is an important function of AKH. Although lipids are the main fuels for long distance flight in insects, carbohydrates are used during the initial phase of flight and still contribute substantially during the later phase (Beenakkers *et al*., 1985). They are mobilized mainly from the glycogen reserves of fat body resulting in an increased level of soluble carbohydrates in the haemolymph. Haemolymph trehalose level is maintained by the activity of phosphorylase, whose activity is regulated by hormone-induced cascade of reactions (Gaede, 1988 b). The brain-CC extract when injected in to haemolymph, was found to elevate the level of glycogen phosphorylase (Steele, 1963) and haemolymph carbohydrates (Goldsworthy *et al*., 1972) in *P. americana*. Injection of locust retrocerebral extract has been shown to induce hyperglycaemia in cockroaches (Holwerda *et al*., 1977) and causes an elevation in haemolymph lipid in locusts (Goldsworthy *et al*., 1972). In *L. migratoria* each of the three AKHs (AKH-I, II, III) is capable of mobilizing both glycogen and TAG

(Oudejans *et al*., 1992). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in larvae and lipids in adults (Ziegler, 1984; Ziegler *et al*., 1990).

**Hyperlipaemic activity**

The target of the adipokinetic signal of AKH peptides during mobilization of triacylglycerols (Ogoyi *et al*., 1998) from the fat body of locusts and moths is the enzyme TAG lipase. TAG lipase catalyses the first step of TAG breakdown to diacylglycerols and free fatty acids. Similar reports have been corroborated for the moth, *M. sexta*, which uses lipids as the main fuel for flight muscle contraction (Ziegler, 1995). The peptides characterized from various locusts and from moths are very closely related.

After the establishment of initial phase by carbohydrate molecules, the flight is regulated by lipids (Beenakkers *et al*., 1985). This patterns for the use of fuels during flight have been found in the brown locust, *Locustana pardalina* (Gaede, 2008), and in the pyrgomorphid grasshopper, *Phymateus morbillosus* (Gaede *et al*., 1996). 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, and are carbohydrates (as in cockroaches, Steele, 1961) or lipids (as in locusts, Mayer and Candy, 1969). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in larvae and lipids in adults (Ziegler, 1984). When the brain-CC extract was injected in to locusts or in to

lepidopteran insects, it showed an adipokinetic effect (Goldsworthy, 1983; Beenakkers *et al*., 1985; Gaede, 1990 a, c; Cusinato *et al*., 1991; Kollisch *et al*., 2000). A significant hyperlipaemic response was shown by the fat body of *I. limbata* injected with CC extracts of *Spodoptera mauritia* (Kumari and Gokuldas, 2001) *I. limbata* (Rasheed and Gokuldas, 2002) and *O. nitidula* (Ajaykumar and Gokuldas, unpublished data). But there were no such effects in *Tenebrio molitor*, *P. americana*, *Carausius morosus* and *Gryllus bimaculatus*, after injection of the hormone extract from their retrocerebral complexes.

**Hyperprolinaemic activity.**

Mobilization of the third important fuel for flight in insects besides carbohydrates and lipids is the amino acid proline. The major difference between the adipokinetic and hyperprolinaemic modes of action is the use of different end products of TAG. In locusts and moths, 1,2-DAG is produced and released from the fat body in to the haemolymph (Arrese and Wells, 1997; Van der Horst *et al*., 1999) and in beetles, it is speculated that free fatty acids are produced by the action of TAG lipase and these undergo β-oxidation, the resulting acetyl CoA together with alanine, are used for re-synthesis of proline (Auerswald and Gaede, 1999). Proline synthesis was found to be stimulated *in vitro* by CC extracts from various insects as well as by synthetic Locmi-AKH-I (Weeda, 1981). It was shown that injection of crude CC extract decreased the haemolymph alanine concentration in the beetle *in vivo*. Conspecific injections of synthetic peptide in low

concentrations elevated the proline titer in the haemolymph (Gaede, 1997 b, c). Similar results have been obtained for the African fruit beetle, *P. sinuata*. Hyperprolinaemia associated with a decrease in alanine concentration in the haemolymph, after the injection of synthetic Melme-CC was studied by Auerswald (1997) and also demonstrated that this response is dose dependent.

The metabolic rate of proline during flight is different in different species. For example, only a little proline is metabolized during the onset of flight in the blowfly, *Phormia regina*, to provide tricarboxylic acid intermediates necessary for maximal oxidation of pyruvate (Sacktor and Childress, 1967). In the tsetse fly, *Glossina morsitans*, proline is present in strikingly high concentration in the flight muscles and is thought to be the exclusive fuel during flight (Bursell, 1981). Proline is only partially oxidized, and the alanine formed is transported to the fat body for re-synthesis of proline. Extracellular $Ca^{2+}$ plays an essential role in hyperprolinaemic signaling: endogenous AKH causes an immediate influx of extracellular $Ca^{2+}$ *in vitro* (Auerswald and Gaede, 2001 a). This is probably achieved by modulating $Ca^{2+}$ channels via a G-protein and results in a stimulation of proline synthesis. An increased proline production is measured when $Ca^{2+}$ influx was mimicked by using the ionophore A 23187 in a series of *in vivo* and *in vitro* experiments (Auerswald and Gaede, 2001 b). Furthermore, by incubating fat body pieces of *P. sinuata* in $Ca^{2+}$ free medium, cAMP elevation induced by the endogenous AKH is dramatically reduced, when compared

with $Ca^{2+}$ containing medium. Calcium from intracellular stores is also important in hyperprolinaemic signaling (Auerswald and Gaede, 2001 b).

**Other functions**

Like many other neuropeptides, AKHs are also multifunctional. Other known physiologiocal effects observed include cardioacceleration in cockroaches and migration of tegumentary and retinal distal pigments in crustaceans. In *D. melanogaster*, the conspecific AKH has a cardio-acceleratory effect in prepupae (Noyes *et al*., 1995), and two research groups found that AKH had influence on locomotory activity, trehalose titre in the haemolymph and length of the larvae, survival during a period of starvation by genetically manipulating the expression of the AKH gene in larval fat body (Isabel *et al*., 2005; Lee and Park, 2004). AKH also induces transcription of the cytochrome P450 gene in the fat body of cockroaches, and the expression of a gene encoding fatty acid binding protein in the flight muscle of locusts.

AKH plays a crucial role in connection with immune response in locusts. Injections of laminarin, a major component of fungal cell walls in to the haemolymph of the migratory locust activate the prophenol oxidase cascade, generating quinines that are toxic to microbes (Lavine and Strand, 2002). Activation of the enzyme cascade is prolonged by the co-injection of Locmi-AKH-I together with laminarin (Goldsworthy *et al*., 2003 a, b). Interestingly, lipopolysaccharides from gram-negative bacteria activate the prophenol oxidase system only when coinjected with Locmi-AKH. The effect of AKH on the locust immune system may be partly explained through its

effect on apolipophorin III, which is a component of the lipid mobilization system. Studies by Moshitzky and Applebaum (1990) suggested that in locusts AKH not only inhibits general protein synthesis but preferentially inhibits vitellogenin synthesis.

**AKH and reproduction**

The ligand of the gonadotropin-releasing hormone receptor (GnRHR) of *D. melanogaster* and *B. mori* has been identified as adipokinetic hormone (Staubli *et al*., 2002). It is reasonable to suggest that the hypothalamic-pituitary-gonadal axis has been conserved in insects and that the most ancient function of AKH might be related to reproduction (Lindemans *et al*., 2009). The AKH-GnRH signaling system probably arose very early in metazoan evolution and that its role in reproduction might have been developed before the divergence of protostomians and deuterostomians. Analogous to both insect AKH receptor and vertebrate GnRH receptor signaling, the GnRH receptor in *C. elegans* (Ce-AKH-GnRH) activated its receptor through a Gαq protein with calcium as second messenger. Gene silencing of Ce-GnRHR, Ce-AKH-GnRH, or both resulted in a delay in the egg-laying process, comparable to a delay in puberty in mammals lacking a normal dose of GnRH peptide or with a mutated GnRH precursor or receptor gene. It is interesting to note that in the fall armyworm *S. frugiperda*, AKH mRNAs occur in ovaries, midgut, fat body, accessory glands and muscle tissues, suggesting that AKH genes may play a role in the regulation of oocyte maturation (Abdel-latief and Hoffmann, 2007). Also in *Anopheles gambiae* females, AKH receptor

transcripts were abundant in the head, thorax, dorsal and ventral abdomen walls to which most of the fat body is attached and in ovaries (Kaufmann and Brown, 2006).

The vitellogenin synthesis in *L. migratoria* is repressed by Lom-AKH-I *in vitro* at the end of the vitellogenic cycle when the oocytes are fully mature (Moshitzky and Applebaum, 1990). In addition, synthesis of vitellogenin can be reactivated *in vitro* by washing pieces of fat body in the incubation medium. The reactivation of vitellogenin synthesis fails when Lom-AKH-I is added. It is believed that, the juvenile hormone III (JH-III) activates a transcription factor that subsequently participates in the regulation of JH-dependent genes, such as the vitellogenin gene. AKH may play a role in inactivating this transcription factor, thereby repressing the transcription of vitellogenin (Glinka and Wyatt, 1996). In the cockroach, *Blattella germanica*, transcription of the vitellogenin gene in fat bodies from cardioallatectomized females is activated by JH-III *in vitro*, and the addition of the endogenous AKH at $10^{-8}$M profoundly inhibits the response to JH (Comas *et al*., 2001).

**Structure of AKH**

The first physiological data indicating the presence of a lipid mobilizing hormone in the brain retrocerebral complex of *L. migratoria* and *S. gregaria* were obtained by Mayer and Candy (1969) and Beenakkers (1969). The primary structure of this AKH (AKH-I, a decapeptide) was elucidated by Stone *et al*. (1976) and appeared to be identical for both locust species. Carlsen *et al*. (1979) and Gaede *et al*. (1984) obtained indications for

the presence of another AKH in these locust species. This AKH-II is an octapeptide differing in one amino acid residue between *S. gregaria* and *L. migratoria* (Siegert *et al*., 1985). A third one from *L. migratoria* (AKH-III, an octapeptide) was isolated and sequenced by Oudejans *et al*. (1991). Siegert (1999) identified an AKH-type peptide from the storage part of the corpus cardiacum of *L. migratoria*, which has no biological activity usually associated with AKHs. Lom-AKH-I is the most potent for lipid mobilization. Lom AKH-II is more powerful for carbohydrate mobilization. AKH-III mobilizes both lipids and carbohydrates but is less active than Lom AKH-I and II. The primary structures of Locmi-AKH-I and crustacean peptide Panbo-RPCH are strikingly similar.

The common characteristics of these peptides include – a chain length of 8 to10 amino acids, N-terminally blocked by a pyroglutamic acid (pGlu) residue and C-terminally blocked by a carboxyamide, by the occurrence of aromatic amino acids at positions 4 (Phe or Tyr) and 8 (Trp), and by a glycine residue at position 9 (Gaede, 1996, 2004). Representatives of the AKH/RPCH family have been found in most orders (Table-II. 1).

In a number of taxa, gene duplication has taken place and two to three AKH peptides are found in one species. In contrast to crustaceans, insects show a high degree of variability in isoforms of AKH peptides. The majority of AKHs are not charged under physiological conditions, but some charged members were sequenced from certain Diptera and scarabaeoid beetles (Gaede *et al*., 1997). In lepidopterans, an AKH was first sequenced from the

tobacco hornworm moth *Manduca sexta* (Ziegler *et al*., 1985) and this nonapeptide was code-named Manse-AKH. Manse-AKH was also found in the silk moth, *B. mori* (Ishibashi *et al*., 1992), whereas the noctuid moth, *Heliothis zea* contains Manse-AKH and a decapeptide called Helze-HrTH (Jaffe *et al*., 1986, 1988). There is indirect evidence that various other moth species also contain Manse-AKH (*Pseudaletia unipuncta*; Orchard *et al*., 1991; *Hippoteoneson*; Liebrich and Gaede, 1995). In contrast to moths, the chemical identity of AKH peptides in butterflies is unclear. In the painted lady butterfly *Vanessa cardui*, the major AKH present is Manse-AKH (Kollisch *et al*., 1999). In addition to Manse-AKH, *V. cardui* contains the non-amidated undecapeptide Vanca-AKH (Kollisch *et al*., 2000). Vanca-AKH showed significant adipokinetic activity in adult specimens of *V. cardui*.

The AKH structure and peptide identities were deduced from comparative RP-HPLC analysis of the native peptides with synthetic peptide standards, as well as from the data obtained by MALDI-TOF mass spectrometry (MS) and nano-electrospray-quadrupole TOF tandem MS or by electron spin ionization spectrometry (ESI).

**Structure – activity relationships**

The structure of the peptides is remarkably stable with only exchanges by a few selective amino acids occurring. The reason for such stability probably lies in the interdependency of hormone and receptor. A change in the peptide molecule that will alter conformational properties will consequently result in no receptor and thus no action. But when the structure

of the peptides is rather similar and stable, different biological functions can be achieved by differentiation of the specific receptor or receptor binding sites during evolution. Attempts at indirect characterizations of the receptors for AKHs have made use of both the variety of naturally occurring analogues, which are known, and the chemical synthesis of analogues (Ford *et al*., 1988; Gaede, 1990 c; Hayes and Keeley, 1990; Ziegler *et al*., 1991; Lee and Goldsworthy, 1995 a; Caers *et al*., 2012). It is clear from such studies that the pGlu$^{1}$, Phe$^{4}$ and Trp$^{8}$ residues are very important for biological activity. For example, a study on *Blaberus* HrTH has shown that replacement of Phe$^{4}$ or Trp$^{8}$ either with alanine or D-amino acids result in biologically inactive analogues (Ford *et al*., 1988), although D-Phe$^{4}$ in HrTH is tolerated, but with lower potency (Hayes *et al*., 1994). Studies on the secondary structure of AKH showed the existence of a 2-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 (Figure II.1). Replacements of 5-10 residues resulted in reduced activity (Mordue and Morgan, 1985).

The deletion of pGlu$^{1}$ from AKH-I results in a peptide (des-pGlu-AKH-I) which is essentially inactive (Gaede, 1990 c), but acetylation of the N-terminal leucine restores some activity in the lipid mobilization and acetate uptake assays (Lee *et al*., 1997). In AKH-I, pGlu$^{1}$ can also be replaced by hydroxyphenyl propionate (HPP), with only a minor reduction in potency (Lee *et al*., 1997), but HPP-[Pro$^{1}$]-*Blaberus* HrTH is virtually inactive (Hayes *et al.*, 1994). Ziegler *et al*. (1991) found that [Gly$^{1}$] and [Tyr$^{1}$]-*Manduca*-

AKH were unable to activate glycogen phosphorylase in the fat body from *Manduca sexta* larvae, but as is the case with locust AKH-I, the acetylated peptides [AcGly$^1$] *Manduca*-AKH (Ziegler *et al*., 1991) and [AcAla$^1$]-*Blaberus*-HrTH (Ford *et al*., 1988) retained activity.

The importance of the C-terminal carboxyamide of AKH-I in influencing potency has been investigated by Lee *et al*. (1996). Replacement of this amide by free acid causes a severe loss in potency in both lipid mobilization and acetate uptake assays (100-fold *in vivo* and 230-fold *in vitro*), as noted for other AKHs (Ford *et al*., 1988; Gaede, 1990 c). Replacement of C-terminal carboxyamide with a methyl ester was tolerated best of all the carboxyamide modifications studied, with only a 12-fold loss of potency in the lipid mobilization assay, and only a five-fold loss in the acetate uptake assay. Replacement of one carboxyamide hydrogen by a methyl or a phenyl group, or both carboxyamide hydrogens by methyl groups results in very dramatic reductions of potency, although these modifications are better tolerated in the acetate uptake assay (60-160-fold reduction in potency) than the lipid mobilization assay (580-1050-fold reduction in potency). It appears that the C-terminal carboxyamide group of AKH-I is important for potency in both the lipid and acetate uptake assays.

**Evolutionary relationships**

Even though the peptide structures of AKH family have been characterized from representatives of many insect orders (Gaede *et al*., 1997; Gaede, 2009), little is known about the evolutionary connections of AKH

structures and functions within insect orders or even between insects and animals from other phylogenetic levels. The studies on the evolutionary relationships of AKH peptides indicate that there exists a family or order specificity. The first attempt to relate phylogenetic relationships to structural differences of AKHs was made for cockroaches (Gaede, 1989). Fourteen species of Blattodea have been investigated with respect to hypertrehalosaemia, retention time of peptides in RP-HPLC, amino acid composition of bioactive peak material and in the peptide sequence. The results suggest that the members of the family of Blattidae contain two hypertrehalosaemic octapeptides - Pea-CAH-I and II in their corpora cardiaca, whereas members of the families Blaberidae and Blattellidae contain the hypertrehalosaemic decapepide Bld-HrTH. Subsequently, more detailed phylogenetic analyses were done with some species belonging to the orders Odonata (Gaede and Marco, 2005) and the Orthoptera: Ensifera (Gaede *et al*., 2003) and Caelifera (Gaede and Marco, 2009). Studies on orthopteran insects revealed the presence of more than three AKH/RPCH peptides, suggesting that the genes coding for this AKHs are highly conserved during the course of evolution (Ajaykumar and Gokuldas, unpublished data). Recently, an extensive study dealing with the phylogenetic relationship of several neuropeptides including AKHs within Dictyoptera, Blattoptera became available (Roth *et al*., 2009). In addition, a few species from the order Heteroptera have been analyzed (Kodrik *et al*., 2010). An expanded heteropteran AKH database was compiled by incorporating sequences from various species as well as from previous studies and documented the

phylogeny of the molecular structure of AKHs. The distribution of Panbo-RPCH in Crustacea and some Heteroptera could be interpreted as direct symplesiomorphic retention of a crustacean peptide solely by a relatively advanced group of paraneopteran insects. It would be possibly due to the loss of numerous genes concerned in the other insect groups, convergent evolution (homoplasy, a potential synapomorphy of several pentatomoid groups) of the peptide in some Heteroptera, re-expression of silent or blocked genes, parallel evolution based on an unidentified 'underlying synapomorphy' or horizontal transfer of the genes concerned.

**Signal transduction of AKH**

The AKH receptors have been cloned from the fruitfly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Park *et al*., 2002; Staubli *et al*., 2002). The receptors are G protein coupled and are structurally and evolutionarily related to the gonadotropin releasing hormone receptors from vertebrates. More than one such AKH receptor types can be present in an insect (Staubli *et al*., 2002). AKH peptides are believed to act by binding to the receptor which changes conformation and interacts with a G-protein. This, subsequently, transduces the signal to an enzyme, which produces a second messenger in the cytoplasm. Signal transduction of AKH peptides has only been studied in a few insects and many details remain far from clear, nevertheless a general picture has been deduced (Van der Horst *et al*., 1999; Gaede and Auerswald, 2003). In general, in hypertrehalosemia, peptides bind to the G-protein-coupled-receptor and activate phospholipase C (PLC)

increasing inositol 1,4,5-trisphosphate (IP3) levels. This induces the release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores which leads to the initiation of the capacitative $Ca^{2+}$ entry into the cytosol. The increased $Ca^{2+}$ concentration results into phosphorylation and activation of glycogen phosphorylase by phosphorylase kinase. AKH further enhances the efflux of $Ca^{2+}$ from the cytosol to reach the normal basal level. In *P. americana*, production of DAG along with IP3 has been proposed. DAG in conjunction with $Ca^{2+}$ then activates protein kinase C (PKC), which, in turn, activates glycogen phosphorylase by phosphorylation (Sun and Steele, 2001, 2002). In general, in hyperlipemia, binding of AKH leads to a conformational change in a Gs protein which, in turn, activates an adenylate cyclase, resulting in an increase of intracellular cyclic AMP levels. Cyclic AMP stimulates lipase activity, most likely via activation of a protein kinase A (PKA). The influx of extracellular $Ca^{2+}$ is also essential for the adipokinetic effect. In moths, release of $Ca^{2+}$ from IP3-insensitive intracellular stores causes an increase in the hemolymph lipid titers (Arrese *et al*., 1999).

The mode of action of AKH during hyperprolinemia appears to be similar to that during hyperlipaemia. It seems that AKH binds to the receptor to cause a conformational change of a Gs-protein which, in turn, activates an adenylate cyclase. The increase in cAMP levels might activate triacylglycerol lipase (TGL) and consequently TAG breakdown to release FAs. AKH seems to activate $Ca^{2+}$ release from intracellular stores and also the capacitative $Ca^{2+}$ entry into the cytosol. Free FAs produced can undergo β-oxidation, the

resulting acetyl-CoA, together with alanine, are used for re-synthesis of proline.

**Quantitation of AKH**

Determination of the amounts of AKH in the brain-CC (or in the CNS) and also in the haemolymph are important for the interpretation of the results of experiments to understand the physiological role of AKH. On the basis of amino acid analysis, Stone *et al*. (1976) estimated the AKH content of CC to be between 400-700 pmol in *S. gregaria* and 200-500 pmol in *L. migratoria*. They also noticed that, in *S. gregaria* AKH-I increases from 190 pmol (early $5^{th}$ instar) to 920 pmol (in adult males, 6 weeks old) and to 1200 pmol (in adult females, 6 weeks old). Likewise, AKH-II increased from about 50 pmol to 180 pmol in males and 260 pmol in females. The maximum amount of AKH-I found in adult *L. migratoria* was about 550 pmol in males and about 750 pmol in females. AKH-II was around 100 pmol and 125 pmol in males and females respectively. At any 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. The AKH content in the brain-CC of *M. sexta* was estimated by radioimmunoassay to be 33 pmol per CC pair in adults and 35 pmol per CC pair in larvae (Fox and Reynolds, 1990). Ziegler (1984) suggested that the *Manduca* CC contained 10-20 times less adipokinetic peptide than that in the locust and it increase during adult life (Bray *et al*., 1993). The smaller quantity of hormone in adult *Manduca* compared with the quantity of AKH-I in the adult locust indicate the difference between the two species in the time

spent in sustained flight as well as the greater longevity of the adult locust. A similarity with the locusts is that a considerable increase in the peptide content of the CC occurs during metamorphosis. The CC of *Pseudaletia unipuncta* males contains about 17.6 pmol of *Manduca*-AKH equivalents (Orchard *et al*., 1991). This is almost similar to the amounts observed in other Lepidoptera (Ziegler *et al*., 1985, 1990) but considerably lower than the amount (500-1000 pmol) reported for the locusts (Orchard, 1987). Using RIA, Candy (2002) estimated the AKH titre of *S. gregaria* haemolymph. However, the highest reported content of AKH in the CC is found, paradoxically, in the flightless grasshopper *Romalea microptera*, in which about 2700 pmol of AKH are recorded in a single pair of CC from an adult male (Spring and Gaede, 1991).

There is a continuous increase in the amounts of this hormone with the age of females (Kodrik *et al.*, 2003). Studies on the total content of three different AKHs (Lom-AKH-I, II, III) in the CC of larvae and adults of *L. migratoria* also showed a continuous increase in the amounts of these hormones (Bray *et al*., 1993; Oudejans *et al*., 1993), which was reflected by an increase in the number of secretory granules, at least ageing in adults (Diederen *et al*., 1992). The amounts of AKH-I and AKH-II continuously also increased in the CC during the life cycle of *S. gregaria* (Hekimi *et al*., 1991). However, the AKH content in the CC of the house cricket *Acheta domesticus* (Grb-AKH) remained unchanged through the first 9 days following adult ecdysis (Woodring *et al.*, 1990).

## Identification of AKH

### Bioassay

Synthetic peptides were assayed for lipid and carbohydrate mobilizing activity in the homologous bioassay (Gaede, 1980). Bioassays generally measure the increase in the concentration of haemolymph diacylglycerols (Mayer and Candy, 1969), trehalose (Steele, 1961) and proline (Gaede and Auerswald, 2002). It is thus necessary to establish the distribution of hormonal activity throughout the nervous system and to ascertain whether these activities from different sources are linked to the same molecules.

### Extraction of AKH

For the isolation and extraction of peptides, the central nervous system consisting of brain with CC and CA attached were dissected. Homogenization of tissues can be performed with tissue grinders of the potter-Elvejhem type, glass homogenizer and with ultra sonicators to disrupt the tissues. Three to four sonications are sufficient to extract AKH-I and AKH-II from a single pair of locust neuronal tissues (brain-CC/CA complex) (Siegert and Mordue, 1986).

### Structural characterization

### HPLC analysis

HPLC instruments are now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds

that are present in any sample that can be dissolved in a liquid. Today, trace concentrations of compounds, as low as “parts per trillion” (ppt), are easily obtained. HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to provide a characteristic retention time for the analyte. With HPLC, a pump provides the higher pressure (up to 400 atmospheres) required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography. A typical column has an internal diameter of 4.6 mm and a length of 150 to 250 mm. There are two variants in use in HPLC depending on the relative polarity of the solvent and the stationary phase. In normal phase HPLC, the column is filled with tiny silica particles, and the solvent is non-polar like hexane. Polar compounds in the mixture being passed through the column will stick longer to the polar silica, than the non-polar compounds will. The non-polar ones will therefore pass more quickly through the column. In reversed phase HPLC (RP-HPLC), the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface (typically with either 8 or 18 carbon atoms in them). A polar solvent like a mixture of water and methanol is used. There will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column and therefore these polar molecules travel through the column more quickly.

Peptides are injected on to the column in a predominantly aqueous solution, and the hydrophobic parts of the molecule bind to the C-8 or to C-18 residue present in the column. A normal gradient for reverse phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes, depending on how hydrophobic the analyte is. The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. At a particular percentage of organic modifiers, the peptide detaches from the stationary phase, elutes from the column and is detected with a UV spectrophotometer at wavelengths around 210 nm (peptide bonds) and 280 nm (aromatic amino acids like phenyl alanine and tryptophan). Organic solvents frequently used for the isolation of insect peptides are acetonitrile and methanol, since it can be easily removed by gentle heating under reduced pressure. If a low pH is required. 0.1% trifluoroacetic acid (TFA) can be used. The output will be recorded as a series of peaks – each one representing a compound in the mixture passing through the detector and absorbing UV light.

**Analysis of primary structure**

After the identification and purification by HPLC, the primary structure can be elucidated, this includes the analysis of the amino acid sequence and whether the N or C-terminals are modified or blocked. From this, the purity of the preparation can be judged and the molecular weight of the peptide can be calculated. The composition data are very essential for the

interpretation of the results from the structure analysis. Prior to the identification and quantification of amino acids, the peptides must be hydrolyzed. This is important because, amino acids such as tryptophan or proline can be destroyed or derivatized when the peptide is hydrolyzed in 6 M HCl. Mild hydrolysis in 4 M methane-sulfonic acid mixture containing 0.2% tryptamine protects tryptophan and allows its determination (Ziegler *et al*., 1985). After hydrolysis, the amino acids are reacted with O-phthalaldehyde (OPA) (Turnell and Cooper, 1982) or phenylthiohydantoin (PTH). Both of them are equally sensitive, but PTH derivatives of amino acids are more stable. Amino acids in the hydrolysate are then identified and quantified using RP-HPLC and reference standards.

**Edman degradation**

This method is widely used to establish the amino acid sequence of peptides and proteins. It requires free N-terminus. This is coupled to the matrix of the sequencer using a special coupling agent (initial step of degradation). The conditions are then changed to cleave the first amino acid from the N-terminus of the peptide chain (second step), the released amino acid is converted to a PTH derivative, because these are very stable (final step), and the derivatives are subsequently determined using conventional amino acid analysis. The cleavage of amino acid is repeated and the sequence of the peptide is determined.

**Determination of N-terminus**

Edman degradation can not be performed on peptides containing a blocked N-terminus. In most of the insect neuropeptides like AKH, RPCH and HGH-I are N-terminally blocked. In such situations pyroglutamyl aminopeptidase enzymes are used to cleave N-terminal modification.

**Mass spectrometry techniques**

The elemental composition of the unknown adipokinetic peptide and its amino acid sequence was determined by a combination of various mass spectrometric techniques. Mass spectrometry is a powerful technique for identifying unknowns, studying molecular structure, and probing the fundamental principles of chemistry. The mass spectrometer as a whole can be separated into distinct sections that include the sample inlet, ion source, mass analyzer, and detector. A sample is introduced into the mass

spectrometer and is then ionized. The ion source produces ions either by electron ejection, electron capture, cationization, deprotonation or the transfer of a charged molecule from the condensed to the gas phase. MALDI and ESI have had a profound effect on mass spectrometry because they generate charged intact biomolecules into the gas phase. In comparison to other ionization sources such as APCI, EI, FAB, and CI, the techniques of MALDI and ESI have greatly extended the analysis capabilities of mass spectrometry to a wide range of compounds with detection capabilities ranging from the picomole to the zeptomole level.

**Fast atom bombardment mass spectrometry (FAB-MS)**

FAB-MS is a predominant technique for the elucidation of peptide containing 8-10 amino acids. The molecule is ionized and the ions subsequently separated and analyzed according to their mass charge ratio (m/z). The primary structures of many AKH/RPCH peptides are elucidated by this technique. FAB-MS elucidates the entire structure of a peptide including any modification to the N and C-terminals.

Previously, a combination of antibody-based techniques and Edman degradation was commonly used for the study of the peptides. But, antibodies are rarely specific to one neuropeptide and Edman degradation requires large amounts of purified sample. Because neuropeptides exist in complex biological matrices at low concentrations, more specific and sensitive mass spectrometry (MS) based strategies have become popular in peptidomic research. With the advent of electron spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI), it became possible to detect

biological entities with molecular specificity in very limited samples such as single organs or single cells (Predel *et al*., 2004; Redeker *et al*., 1998; Neupert and Predel, 2005).

**Sample preparation strategies**

The sample for mass spectral analysis can be prepared by homogenizing the tissue and extracting the analyte of interest, or it can be investigated directly by MALDI analysis of freshly dissected and mounted tissue samples with minimal preparation. An extraction based strategy results in neuropeptide rich samples via pooling of many organs or cells. The sample can then be enriched for a specific peptide family via immune-precipitation, desalted using $C_{18}$ columns, and fractionated to reduce sample complexity prior to ionization by ESI or MALDI. By reducing the sample complexity through fractionation, the ionization suppression decreases, thus enabling more comprehensive peptide profiles to be obtained, as evidenced by studies in honey bee (Audsley and Weaver, 2006), Crab (Fu *et al*., 2005 a, b) and fruit fly (Baggerman *et al*., 2005). Alternatively, placing the tissue on the MALDI plate, coating with matrix and irradiating the co-crystallized tissue to cause desorption or ionization of the analyte may enable direct tissue MALDI analysis. This approach requires less sample manipulation and also the spatial localization of neuropeptides in the tissue can be preserved. In order to reduce the impact of the high salt content associated with biological samples, tissues are commonly rinsed with water (Predel *et al*., 2001) or with matrix solution (Kutz and Schmidt, 2004; Li *et al*., 2003; Takeuchi *et al*., 2003). Additionally,

rinsing the tissue in acidified methanol prior to direct analysis may lead to more efficient peptide extraction and ionization processes (Kutz and Schmidt, 2004). It is possible to analyse a small piece of tissue rinsed in dilute 2, 5-dihydroxybenzoic acid matrix and acidified methanol by MALDI-Fourier transform mass spectrometry (FTMS). The high resolving power of FTMS is beneficial for distinguishing unique neuropeptides in complex nervous system tissue. While the dried droplet method of matrix application is sufficient for decapod crustacean neuron analysis, methods that allow application of nanoliters volumes of matrix are more effective at limiting peptide dilution in insect tissue (Predel *et al*., 2001; Balau *et al*., 2004) by limiting the applied matrix volume to just 20 nanoliters of solution.

**Tandem mass spectrometry:**

In insects like fruit fly, mosquito and honey bees the genome has been sequenced, an exact mass measurement may be employed in conjugation with database searching to identify the amino acid composition of a given neuropeptides (Audsley and Weaver, 2006). But the prediction of a final bioactive peptide structure can be challenging due to post translational modifications, tissue specific prohormone processing and unusual processing sites. Therefore, fragmentation techniques such as post source decay (PSD) and collissionally induced dissociation (CID) are often required to obtain peptide fingerprint or *de novo* sequence information. Accurate mass measurements and fragmentation information can be used in conjugation with web-based tools to facilitate the process of *de novo* sequencing (Eisenacher *et*

*al*., 2006) and aid in the discovery of novel neuropeptides. Much recent neuropeptide sequencing work has been performed on ESI-quadrupole time-of-flight (QTOF) mass spectrometers (Baggerman *et al*., 2005; Fu *et al*., 2005 a; Predel *et al*., 2005; Takeuchi *et al*., 2003). This instrumental configuration is especially effective for tandem MS because ESI commonly results in multiply charged peptides, which more readily produce detectable fragments than singly charged ions. In addition, CID coupled with TOF mass analysis encourages the production and detection of amino acid specific immonium ions, which are valuable for elucidating the peptide composition. As evidenced by the identification of almost 60 neuropeptides, 23 of which were *de novo* sequenced in a *Cancer productus* neuroendocrine organ extract (Fu *et al*., 2005 b), ESI-QTOF MS/MS can be used as a powerful tool for peptidomic characterization. For the analysis of small tissue samples, direct tandem MS can be performed with MALDI. For example, direct single organ neuropeptide fragmentation has been performed by MALDI-TOF-PSD on the cockroach and flesfly (Predel *et al*., 2005), MALDI-CID of neuropeptides directly from single organs has been performed on cockroach using Q-TOF and lobsters and crabs bt FTMS (Kutz and Schmidt, 2004). MALDI-TOF PSD of putative SIFamide peptide in a single *Drosophila melanogaster* neuron produced sufficient sequence information for this peak to be confidently assigned (Predel *et al*., 2004). A combination of PSD and CID fragmentation can also be used to *de novo* sequence, a novel insect periviscerokinin peptide with the sequence PALIPFPRV-$NH_2$ from direct analysis of a single cell (Neupert *et al*., 2005). The high energy CID uniquely

available for a MALDI-TOF/TOF instrument produced the side chain specific w-fragment ion necessary to distinguish between the isobaric amino acids Leu/Ile (Nachman *et al*., 2005) and this is the first reported demonstration of *de novo* sequencing of a peptide from a single-cell preparation of arthropod.

Konig *et al*. (2005) analysed several AKHs from different insect species and their particular ionization behavior with respect to their sequence and they observed following results. AKHs exhibit characteristic ion pairs, $(M+Na)^+/(M+H)^+$ in MALDI-MS and $(M+H+K)^{2+}$, $(M-17+H)^+$, $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$ in ESI-MS. Their affinity for Na+ and K+ alkali cation is observed after reversed phase purification. AKHs rarely form doubly charged ions with protons or sodium while the $(M+H+K)^{2+}$ ion is often abundant, suggesting a special conformation of larger metal ion complex possibly related to its size.

**Characterization of AKH gene**

The sequence changes in the genomes of organisms can be used for their molecular barcoding and unambiguous identification. DNA barcoding is usually used for identifying gene sequence and for species identification studies using a short standardized DNA. In this, a short genetic marker in an organisms DNA is used to identify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to be determining classification, but to identify an unknown sample in terms of a known classification. The use and relevance of barcodes for insect studies investigated the barcode sequence of *Diatraea saccharalis*. This sequence has

a high level of homology (99%) with the barcode sequence of the Crambidae (Lepidoptera). The sequence data can be used to construct relationships between species, allowing a multidisciplinary approach for taxonomy, which includes morphological, molecular and distribution data, all of which are essential for the understanding of biodiversity.

Recently, the genome of certain insect species has been elucidated, for example, *B. mori, A. aegypti and T. castaneum*. The search for structural homologs of AKHs in the databases of these species revealed the presence of such peptides. Such kinds of predicted peptides were later elucidated from the same insects (*A. aegypti* and *T. castaneum*) with MALDI-MS analysis (Gaede *et al*., 2008).

The biochemical characterization of AKH receptor was first reported for the tobacco hornworm, *M. sexta* (Ziegler *et al*., 1995), and later, AKH G-protein coupled receptors (GPCR) were cloned for *D. melanogaster* (Park *et al*., 2002; Staubli *et al*., 2002); the silkworm *B. mori* (Staubli *et al*., 2002); the cockroach *P. americana* (Wicher *et al*., 2006); and African malaria mosquito *A. gambiae* (Kaufmann and Brown, 2006) and expressed in cells for ligand binding studies. Molecular approaches have also resulted in the characterization of the structure of the AKH/RPCH precursors (a signal peptide, AKH/RPCH, a Gly-(Lys/Arg)-Arg sequence, and an AKH/RPCH-associated peptide (AAP/RAP) - in this order) of *S. gregaria*, *S. nitans*, *M. sexta*, *D. melanogaster* and *Carcinus maenas* (Noyes and Schaffer, 1990, 1993; Noyes *et al*., 1995; Bradfield and Keeley., 1989; Schulz-Allen *et al*.,

1989; Fischer *et al*., 1993; Linck *et al*., 1993). In insect species where two or more different peptides of AKH family are present (e.g: in *S. gregaria*, *S. nitens*, *L. migratoria* or *A. gambiae*), each of the respective prohormone precursors is represented by a distinct mRNA (cDNA). In *S. gregaria*, the immediate precursors are dimers (O'Shea and Rayne, 1992). After cleaving off the signal peptide, two independently translated monomers of the pro-Locmi (Schgr)-AKH-I (or two monomers of pro-AKH II or one of each) are oxidized to a precursor dimer. Thereafter, the precursor is processed to two monomeric peptides (for example, Locmi (Schgr)-AKH I) and one dimeric molecule of an AKH precursor related peptide.

In *Bombyx*, a nonapeptide AKH-I have been first identified (Ishibashi *et al*., 1992), and quite recently Roller *et al*. (2008) identified another two distinct cDNAs encoding the prepro-*Bombyx* AKH II, and III. *Bombyx* AKH-I is identical to nonapeptides found only in moths, while *Bombyx* AKH-II is closely related to many other AKH/HrTH decapeptides. The application of recombinant DNA techniques to the study of neuropeptide biosynthesis is essential because these techniques facilitate structural analyses and evolutionary studies, clarify biosynthetic pathways, provide the necessary background and probes for analysis of synthesis regulation at the levels of mRNA and gene transcription and assist in classical genetic experimentation.

# CHAPTER III

# MATERIALS AND METHODS

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## MATERIALS

### Experimental insects

*Orthaga exvinacea* Hampson (Pyralidae: Lepidoptera) (Plate I. a) is a serious caterpillar pest of mango trees infesting during the period from December to April of the year. Larvae were collected from their natural habitat, i.e., mango trees and were transferred to plastic basins kept in the insectary and reared by feeding mango leaves. The basins were covered with cotton clothes and kept in large insect cages with three sides covered with metal wire gauze and there was a glass door in the fourth side. The larvae of the same age group were kept in plastic jars and provided with mango leaves. They were protected from ants by water barriers. The plastic basins were cleaned in alternate days. The colony was maintained at 27± 2°C and 70%-80% RH. Adults were fed with diluted honey. Mating occurred at night and the females laid eggs in masses. Neonate larvae were fed on fresh mango leaves. Sixth instar larvae were separated from the colony and used for experiments.

Adults of the polyphagous bug, *Iphita limbata* Stål (Pyrrhocoridae: Heteroptera) (Plate I. b) were locally collected and maintained in cages in the insectary on a diet of germinating seeds of green gram (*Phaseolus radiatus*) and pieces of various tropical fruits. Mature adults were used for the experiments.

### Vertebrate liver tissue

Fresh chicken liver tissue was procured from a local poultry farm. They were packed in ice immediately and transported to the laboratory for use.

Rat liver was collected from already sacrificed animals in the University Animal House (maintained strictly as per CPCSEA regulations).

**Equipments**

| | | |
|---|---|---|
| 1. | Agarose gel electrophoresis unit | (Biorad, USA) |
| 2. | Binocular stereozoom microscope | (ZEISS, Germany) |
| 3. | Deep freezer | (Labline, India) |
| 4. | DNA sequencer | (Applied Biosystems,USA) |
| 5. | Filtration membrane (0.45 µm) | (Millipore, USA) |
| 6. | High speed refrigerated centrifuge | (Plastocraft, India) |
| 7. | HPLC | (Shimadzu, Japan) |
| 8. | HPLC fraction collector | (Biorad, USA) |
| 9. | HPLC solvent filtration unit | (Millipore, USA) |
| 10. | Magnetic stirrer | (REMI, India) |
| 11. | MALDI-TOF and MALDI-MS | (Bruker Daltonics, Germany) |
| 12. | Microliter syringe (10 µl and 20 µl) | (Hamilton, Switzerland) |
| 13. | Micropipettes | (Accupipet, Finnpipet) |
| 14. | PCR | (Eppendorf, Germany) |
| 15. | Refrigerator | (LG, India) |
| 16. | Sample filtration unit | (Millipore, USA) |
| 17. | Shaker water bath incubator | (Promega, India) |

| | | |
|---|---|---|
| 18. | Ultrasonicator | (Sonics & Materials, USA) |
| 19. | UV-vis spectrophotometer | (Shimadzu, Japan) |
| 20. | Vacuum concentrator | (Savant, USA) |
| 21. | Vacuum pump | (Rivotek, India) |

**Chemicals**

| | | |
|---|---|---|
| 1. | Agarose | (Merck) |
| 2. | Acetonitrile (HPLC grade) | (SRL and Merck) |
| 3. | Anthrone | (SRL) |
| 4. | Bovine serum albumin (BSA) | (Himedia) |
| 5. | Calcium chloride | (Merck) |
| 6. | Chloroform | (SRL) |
| 7. | Concentrated sulphuric acid | (Merck) |
| 8. | Disodium hydrogen phosphate | (Qualigens) |
| 9. | Folin-Ciocalteu phenol reagent | (SRL) |
| 10. | Glucose | (Merck) |
| 11. | Glycerol trioleate | (Merck) |
| 12. | HEPES buffer | (SRL) |
| 13. | HPLC water | (Merck) |
| 14. | Hydrochloric acid | (SRL) |
| 15. | Magnesium sulphate | (SRL) |
| 16. | Methanol (HPLC grade) | (SRL andMerck) |
| 17. | Nucleo Spin column DNA Gel Extraction kit | (Macherey-Nagel, Germany) |
| 18. | Orthophosphoric acid | (Merck) |

| | | |
|---|---|---|
| 19. | Phosphovanillin | (SRL) |
| 20. | Potassium chloride | (Merck) |
| 21. | Quick Extract DNA extraction solution | (Epicentre Biotechnologies, USA) |
| 22. | Sodium chloride | (Merck) |
| 23. | Sucrose | (Himedia) |
| 24. | Synthetic Locmi-AKH-I | (GenScript Corp., USA) |
| 25. | Trifluoroacetic acid | (SRL) |
| 26. | Vanillin | (SRL) |

**Reagents**

**1. Insect saline**

The saline solution used in controls in the *in vivo* and *in vitro* bioassays contained NaCl, 130 mM; KCl, 5 mM; $Na_2HPO_4$, 1.9 mM and $K_2HPO_4$, 1.7 mM, the pH was adjusted to 7.5.

**2. 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; $Na_2HPO_4$, 1 mM; $CaCl_2$, 1 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.

**3. Physiological saline**

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

**4. Phosphate buffered saline (PBS).**

Fat body incubations for lipid release from vertebrate liver were done in a buffer having the following composition: NaCl, 10 mM; KCl, 12 mM; $MgSO_4$, 2 mM; $Na_2HP0_4$, 1 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

**5. Phosphovanillin reagent**

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

**6. Anthrone reagent**

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

**7. Lowry reagents**

Reagent 1 (Alkaline copper reagent): Mixed one volume of reagent B (0.5% copper sulfate pentahydrate, 1% sodium potassium tartrate) with 50 volumes of reagent A (2% sodium carbonate, 0.4% sodium hydroxide)

Reagent 2 (Folin reagent): Diluted commercial Folin-Ciocalteu phenol reagent with an equal volume of water.

**8. Lipid standard solution**

The lipid standard solution was prepared by dissolving 500 mg of chromatographically pure glycerol trioleate in 50 ml of chloroform. A working standard solution was prepared by diluting 1.0 ml of stock solution to 10 ml with chloroform so as to get 1.0 µg lipid/1.0 µl. Appropriate volumes of this solution were taken for calibration and for quantification of lipid samples in various experiments.

**9. 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.

**10. BSA standard solution**

Stock solution of BSA was prepared by dissolving 3 mg of BSA in 12 ml of distilled water just before the experiment. From this, standard protein solution containing different quantities of BSA (10 µg to 150 µg) were taken in test tubes.

**11. Synthetic Lom-AKH-1 solution**

Synthetic Lom-AKH-1 was purchased from GenScript Corp., USA. A stock solution of 100 µmol of hormone was prepared dissolving 1.0 mg of hormone in 8.625 ml of 80% methanol (HPLC grade). Solutions of required concentrations of the hormone were prepared in 80% methanol for HPLC analysis and in 20% methanol for bioassay.

**METHODS**

**Preparation of brain-CC complex of the insect**

A crude extract of brain-CC complex of *O. exvinacea* was prepared by a modified method described by Gaede (1994). Sixth instar larvae of approximate 45 days old were used for collecting brain-CC complexes. A superficial incision was made on the dorsal surface of the head capsule, the incision was broadened and the brain-CC complex was exposed. The tissue complex was removed and immediately put in to ice cold 80% methanol

(HPLC grade) and stored at -4°C until extraction. Tissue complexes were sonicated for 1 min on ice with an ultrasonicator. The tissue homogenate was centrifuged at 4°C and 10,000 rpm for 10 min. After centrifugation, the supernatant was transferred to another eppendorf tube. The residue was re-extracted with 500 µl of methanol and again centrifuged. The supernatants were combined and vacuum dried. The dried supernatants were stored at 4°C until used for bioassay studies, HPLC separations and mass spectrometric analysis. Samples were redissolved in appropriate solvents. For HPLC, the required volumes were filtered using Millipore filter and filtration unit.

## *In vivo* bioassays

### Detection of biological activity of the brain-CC extract on lipid, carbohydrate and protein metabolism

The heterologous *in vivo* bioassay for hyperlipaemic activity was done in the plant bug, *Iphita limbata*, in which increase of vanillin-positive material (=total lipids) in the haemolymph, was measured. The dried methanolic brain-CC extract prepared as mentioned above was dissolved in insect saline to get a final concentration of one gland pair equivalent (gpe) per 5 µl. A sample of haemolymph (2 µl) was collected directly from the cut end of antenna in to a precalibrated capillary tube. The sample was transferred in to the bottom of a small test tube. An aliquot of the extract (5 µl) was then injected in to the acceptor plant bug, *Iphita limbata*, through the intersegmental membrane between thoracic and first abdominal segment. The needle was kept in position for a while to allow mixing of the material with haemolymph and to

avoid loss through oozing haemolymph droplet. After 60 min, another sample of haemolymph was collected (2 µl) directly from the cut end of the other antenna in to another capillary tube and was then transferred in to the bottom of another test tube. Haemolymph samples taken before injections were taken as controls and 60 min after injection as experimentals. Similar experiments were carried out using 5 µl of insect saline and synthetic AKH (equivalent to 2 nmoles) instead of extract. Haemolymph samples thus collected were used for the quantitation of lipids, carbohydrates and proteins.

**Quantitation of haemolymph lipids**

Total lipids in the haemolymph samples were determined using phosphovanillin reagent (Frings *et al*., 1972). To the haemolymph samples collected in various experiments and kept at the bottom of small test tubes, concentrated sulphuric acid (50 µl) were added, heated in a boiling water bath for 10 min, cooled to room temperature and 2 ml each of phosphovanillin reagent were added. The tubes were thoroughly shaken to mix the content. Optical densities of the pink complex formed were measured within 5 min using UV-vis spectrophotometer at 540 nm against a reagent blank. The tubes containing haemolymph samples collected from insects injected with insect saline instead of the hormone extract were processed similarly and the change in lipid level was measured which served as controls.

**Quantitation of haemolymph sugar**

The hyperglycaemic effects of the extracts were measured by analysing the difference between sugars in the experimental and control samples, colorimetrically using anthrone method (Mokrasch, 1954). Anthrone reagent

(3 ml) were added to the haemolymph samples collected as described above, mixed well and kept in a boiling water bath for 10 min. Samples were cooled and the absorbance were measured at 620 nm. Estimation of haemolymph sugar was also performed using insect saline instead of the extract to serve as controls.

**Quantitation of haemolymph protein**

The change in haemolymph protein concentration was studied before (control) and 60 min after injection (experiment) of the extract, as mentioned above. The amount of protein present in the samples were measured, using Lowry's method (1954). The haemolymph samples (2 µl) collected in the control and experimental sets were diluted to 0.5 ml using distilled water. To the samples thus prepared, 2.5 ml of alkaline copper solution was added and allowed to stand for 10 min. Added 0.25 ml of Folin's reagent rapidly with immediate mixing. After 30 min incubation, the optical densities were taken at 750 nm. Tubes containing insect saline instead of the haemolymph samples were processed in a similar manner and the haemolymph protein concentration was measured.

## *In vitro* bioassays

**Preparation of fat body for *in vitro* incubation**

Fat body from appropriate stage of *O. exvinacea* were removed and pooled. They were washed in insect saline and blotted on filter paper to remove excess adhering saline. The fat body was placed on a polyvinyl disc and chopped gently with a sharp razor blade. The chopped fat body were

mixed using stainless steel needle and divided approximately in to two equal halves. One half served as experimental and the other half as control. These halves were then put in to preweighed incubation vials containing fixed volumes (200 µl) of incubation mixture and fat body weight were determined. The whole procedure was completed as quickly as possible (within 5-6 min).

**Investigation on the role AKH in lipid metabolism (hyperlipaemic activity)**

***In vitro* incubation**

For *in vitro* incubation flat bottomed glass vials (5 ml capacity) with bakelite screw caps were used. The incubation mixture contained 200 µl of the HEPES buffer and 20 µl of either the hormone extract or synthetic AKH (equivalent to 2 nmoles) or insect saline (experimentals) or 20 µl distilled water (control). The incubations were carried out for 30 min in a shaker water bath preset at 37°C.

**Extraction of lipids**

After the *in vitro* incubation, lipids released in to the medium were extracted by modified Bligh and Dyer (1959) procedure. Using a micropipette, 150 µl of the medium was drawn out from the incubation vials, taking care not to draw any fat body pieces, and transferred in to another vial. 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 centrifuged for 3 min at 1000 rpm to separate the organic and aqueous phases. The lower chloroform layer contained the extracted lipids. Lipid samples (100 µl each) were drawn from the lower organic phase using micropipette (without trapping any droplets of the upper phase) and transferred to test tubes for quantitation of lipids.

**Measurement of lipid release**

From the lipid samples collected as described above, chloroform was evaporated off by keeping them at room temperature. The amount of lipids in the samples was measured by using phosphovanillin reagent (Frings *et al*., 1972) as described elsewhere. The optical densities were measured at 540 nm using UV-vis spectrophotometer against a reagent blank. From the values obtained, absorbance per mg of fat body tissue was calculated using the absorbance value of standard lipid solution. From the values thus obtained from controls and experimentals, change in lipid release due to the hormone action has been estimated.

**Construction of lipid standard calibration graph**

Glycerol trioleate was used for preparing calibration graph. Known quantities of glycerol trioleate were taken in the bottom of test tubes and chloroform content was evaporated off. Concentrated sulphuric acid (50 µl) were added to each tube and were mixed thoroughly. These mixtures were heated in a boiling water bath for 10 min, brought to room temperature and phosphovanillin reagent (2 ml) was added. The tubes were thoroughly shaken to mix the contents. The optical densities of the samples were measured as

described above. The values obtained for different concentrations were used for constructing a calibration graph.

**Investigation on the role of AKH in carbohydrate metabolism (hyperglycaemic activity)**

***In vitro* incubation**

The amount of sugar released from the fat body in the presence or absence of adipokinetic hormone extract were measured colorimetrically using anthrone reagent. The fat bodies for incubation were prepared as described earlier. The experimental tube contained 200 µl of physiological saline and 20 µl of either the hormone extract or synthetic AKH (equivalent to 2 nmoles) or insect saline. In control tubes, 20 µl of distilled water were added instead of the hormone solution. The incubations were carried out for 30 min in a shaking water bath at 37°C.

**Measurement of sugar release**

After *in vitro* incubation, the tubes were taken out and cooled to room temperature and 25 µl of the medium from the experimental as well as control were pipetted out into another set of tubes. To these tubes 3 ml of anthrone reagent were added. Mixed well and kept in a boiling water bath for 10 min. Samples were cooled and the absorbances were measured at 620 nm together with a similarly processed standard solution of appropriate concentration.

**Construction of trehalose standard calibration graph**

Known concentrations (10 µg to 200 µg) of trehalose standard were taken in the bottom of test tubes, 200 µl of the physiological saline were added and incubated at 37°C for 30 min. After incubation, 3 ml each of

anthrone reagent were added and kept in a boiling water bath for 10 min. Tubes were cooled and absorbance read at 620 nm. The absorbance values were used to construct a calibration curve.

**Investigation on AKH activity on protein metabolism**

***In vitro* incubation**

The preparation of fat bodies and the incubation were done as described elsewhere and the estimation of protein present in the samples, using Lowry's method. The incubation mixture contained 200 µl of physiological saline and 20 µl of either the brain-CC extract or synthetic AKH (equivalent to 2 nmoles) or insect saline (experimental) or 20 µl distilled water (control). The incubations were carried out for 30 min in a shaker water bath preset at 37°C.

**Measurement of protein release**

After *in vitro* incubation of the fat body, the tubes were cooled to room temperature and 0.2 ml of the medium was drawn out from the experimental and control tubes. To the tubes, 2 ml of alkaline copper reagent was added and incubated for 10 min at room temperature. After incubation, 0.2 ml of Folin's reagent was added, mixed rapidly and incubated again for 30 min. The absorbances were read at 750 nm.

**Construction of protein standard calibration graph**

Known quantities of protein standard (10 µg to 150 µg) were taken in test tubes tubes and added 200 µl of incubation buffer and incubated at 37°C for 30 min. After incubation, 2 ml of alkaline copper reagent was added and incubated for 10 min. To the tubes, 0.2 ml of Folin's reagent was added with

thorough mixing. After 30 min incubation at room temperature, the optical densities were measured at 750 nm.

**Calculations**

From the absorbance values obtained for various samples, concentrations of lipids, sugars and proteins were calculated by applying the formula,

$$\text{Concentration of sample} = \frac{\text{Concentration of standard x Absorbance of sample}}{\text{Absorbance of standard}}$$

From these values, the amounts of the lipid, sugar and protein released in to the fat body of insects in various experiments were determined.

**High Performance Liquid Chromatography (HPLC) of extract of brain-retrocerebral complexes of *O. exvinacea***

The dried extract made from the retrocerebral complexes from *O. exvinacea* was resuspended in 80% methanol (HPLC grade). The extract was filtered using a sample filtration unit (Millipore, USA) with a filter of pore size 0.45 µm. A sample of 20 µl (2 gpe/µl) of the filtered brain-retrocerebral extract was directly injected into the instrument using a Hamilton micro-syringe. HPLC separations were carried out using Shimadzu system with a reversed phase column ($C_{18}$) of 250 mm long and 4.6 mm i.d. The separation was done in a binary gradient from 43% to 53% solvent B in 20 min with a flow rate of 1 ml/min. Trifluoroacetic acid (TFA) 0.01% in water (HPLC grade) was used as solvent A, solvent B was 60% acetonitrile in solvent A. All the solvents were filtered through 0.45 µm pore size Millipore filter. The eluants were monitored at 210 nm using UV-VIS detector. One minute

fractions starting from 1 min up to 20 min were collected with a fraction collector for testing their biological activities. The HPLC profiles were exported into Microsoft Word file and were used for further analysis of the data.

The synthetic peptide (100 pmol) Lom-AKH-I was injected (20 µl) into the HPLC instrument maintained in the same set up as before for the retrocerebral extracts of *O. exvinacea.* Similarity of retention time of any materials (appearing as peaks) of the extract of *O. exvinacea* with that of the synthetic locust AKH was confirmed by overlaying this profile with that obtained for synthetic Lom- AKH.

**Bioassays for testing the effects of different HPLC fractions on lipid, carbohydrate and protein release.**

The extracts of retrocerebral complexes of *O. exvinacea* were fractionated on HPLC and the fractions were collected with a fraction collector in 20 separate eppendorf tubes. These one minute fractions of HPLC eluants were dried in a vacuum concentrator. The dried fractions were resuspended in 200 µl of double distilled water and stored until use. Samples of these fractions (2 gpe/20 µl) were tested separately for their effects on lipid, sugar and protein release from the fat body employing the methods described for crude extracts. The hyperlipaemic and hyperglycaemic effects of these fractions were determined by *in vitro* bioassays using phosphovanillin and anthrone reagents respectively and the effects on protein release were measured by Lowry's method. The experimental and control samples were analysed by spectrophotometric methods.

## Mass spectrometric analysis

### Matrix Assisted Laser Desorption Ionisation-Time Of Flight-Mass Spectrometry (MALDI-TOF-MS)

The dried extract of neurohaemal tissues of *O. exvinacea* purified on HPLC present in fraction 10 was collected and subjected to mass spectrometric analysis. Mass spectrometric analysis were performed on an Ultra Flex mass spectrometer in reflectron ion mode, using a 90 ns time delay and a 25 kV accelerating voltage monitored in $Na^+$ mode. The system utilized 50 Hz pulsed voltage laser, emitting at 337 nm. The ion source and the flight tube were kept at pressure of about $7x10^{-7}$ mbar by turbo molecular pump.

The samples were prepared by mixing equal volumes of peptide solution (fraction 10) (1 ml) and a saturated solution of the matrix, dihydroxybenzoic acid in 1:1 (v/v) acetonitrile: water mixture and applied on a multisample target. The samples were measured in the reflectron mode within the mass range m/z 850-1250 Da, and the laser energy was reduced until an optimal resolution was obtained. The results of 10-20 shots were averaged to obtain the final spectrum. Acquisition of a mass spectrum can be accomplished in less than one hour. A standard peptide mixture was used for external calibration.

### Tandem- MS/MS

The amino acid sequence information of the peptide was obtained by employing MALDI-MS/MS analysis. During MS/MS or tandem mass

spectrometry, fragment ions are generated from a selected precursor ion. The amino acid sequence is determined by calculating the m/z difference (which corresponds to the mass of an amino acid) between the adjacent y-ion peaks and/or b-ion peaks.

The tandem mass spectra were acquired by selecting the precursor mass 1030.471 with a 10 Da window and fragments were generated in Post Source Decay (PSD) mode. The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct [M+$Na^+$] of peptide with mass 1008.471. The measured monoisotopic masses [M+$Na^+$] of known or conceptually derived peptides were compared to the calculated masses. Mass spectra were analysed by using Flex-analysis software.

**Interpretation of MALDI-MS/MS data**

The MS/MS data were interpreted by using Peptide Fragmentation Ion Analyser-II (PFIA-II) software (**http://hodgkin.mbu.IISC.ernet.in/~pfia/PFIA-II.html**). PFIA-II is a web tool for evaluating possible sequence specific product ion types for *de novo* sequencing of novel peptides. It also provides the side chain d-, v- and w- ions for Leu/Ile arising from high energy Collision Induced Dissociation (CID). Acyclic and cyclic peptide sequences with a maximal length of 25 residues can be queried. The system provides fragmentation pattern and fragmentation ion diagrams and the list of all sequence specific product ions (a, b, c, x, y, z and immonium ions) for the protonated adducts of queried sequence.

In the present study, the precursor ion selected has molecular mass 1030.471 Da in M+$Na^+$ mode. The sequence of the peptide was entered in to

the PFIA-II web tool as queried sequence. The fragment ions a, b, y and immonium ions obtained were used for determining the structure of the precursor molecule.

## Characterization of AKH gene

### Preparation of genomic DNA

The intestinal tissues (50 mg) of *O. exvinacea* were weighed out and the DNA was extracted using Quick Extract DNA extraction solution of Epicentre Biotechnologies as per the manufacturer's instruction. The tissue was ground in a mortar and pestle with 0.5 ml of Quick Extract DNA extraction solution. The mixture was vortexed for 15 sec and transferred the tube to 65ºC and incubated for 6 min. After incubation, the mixture was vortexed again and mixed for 15 seconds. The tubes were incubated at 98ºC for 2 min. A sample of the extracted DNA (3 µl) was used for PCR amplification.

### Primer Designing

The available sequences for adipokinetic hormone from different species of Lepidoptera (*B. mori* AKH-I and II, *M. sexta* AKH and *S. frugiperda* AKH-I, II and III*)* were used for primer designing. The sequences were obtained from GenBank. From those sequences, two upstream and two downstream primers were designed for the amplification of the AKH gene from *O. exvinacea*. All the primers used in the study were synthesized by Integrated DNA Technologies, Inc.USA.

### Genomic DNA amplification

A PCR was performed in a total reaction mix of 50 µl of the isolated genomic DNA from *O. exvinacea* to amplify the AKH gene. The PCR was performed with the primer pairs AKHF1-AKHR1, AKHF1-AKHR2, AKHF2-AKHR1, AKHF2-AKHR2 combinations of the forward and reverse primers. The PCR mixture contained 3 µl of isolated genomic DNA (200 ng) from *O. exvinacea*, 1 µl of each primer (100 pmol/µl), 2 µl 10 mM deoxy-ribonucleoside triphosphate, 5 µl 10xPCR buffer containing $MgCl_2$, and 1µl of 5 U/µl Taq DNA polymerase. The PCR was conducted with the initial denaturation at 94°C for 2 min followed by denaturation at 94ºC for 45 sec, annealing at 60°C for 60 sec and elongation at 72ºC for 2 min. These cycles were then followed by 34 cycles of denaturation, annealing and elongation was followed by an extended final elongation step at 72ºC for 10 min.

**Agarose gel electrophoresis**

The PCR product was electrophoresed in a 1% (w/v) agarose gel, stained with ethidium bromide and observed on a UV transilluminator. The amplicon was excised from the gel and the DNA was eluted from the gel slice by using the Nucleo Spin column DNA Gel extraction kit obtained from Macherey and Nagel, Germany, according to the manufacturer's specifications. Amplification of genomic DNA with degenerate primers forward AKHF1 and reverse AKHR1 yielded an amplicon of approximately 350 bp at an annealing temperature of 60ºC.

**AKH Gene sequencing**

Sequencing was done using the big dye terminator kit (Applied Biosystems) in 3730XL DNA ANALYSER. Nucleotide sequences spanning the ORF were obtained and compiled for comparison to the predicted gene sequences. Translations of ORF sequences were used for a Pileup of related protein sequences. The nucleic acid and deduced protein sequences were analyzed. The sequenced PCR product was analyzed online using BLAST (http://www.ncbi.nlm.nih.gov/blast). Nucleic acid and protein sequences of other AKH genes were obtained from NCBI. Alignments of sequences were carried out using CLUSTALW and Dendrogram by http://align.genome.jp/. The electropherogram data containing fluorescent peak trace chromatograms are obtained using Sequence Scanner v1.0.

**Evolutionary studies**

Phylogenetic analysis of AKHs of Lepidoptera was conducted using MEGA4 software (Tamura *et al.*, 2007). The systematic position and relationships of lepidopteran species, based on AKH structures, were studied by constructing phylogenetic trees. To reveal the amino acid relationship of *O. exvinacea* adipokinetic peptide with other adipokinetic peptide sequences, 10 putative AKH sequences (*B. mori* AKH-I and II, *M. sexta*, *H. zea, M. cinxia, S. frugiperda* AKH-I, II, III and IV and *V. cardui*-AKH) were collected from previous works for multiple alignment by ClustalW and phylogenetic analysis. The adipokinetic peptide sequence of *O. exvinacea* along with 10 other AKH sequences available were aligned. The tree was generated by using the Neighbour-joining algorithm.

The evolutionary analysis of AKHs performed in the present study is not limited to representatives of Lepidoptera. An expanded AKH dataset was compiled by incorporating the AKH sequences of various insect orders from previous studies and their molecular evolution trends compared. To evaluate the evolutionary relationship of Lepidoptera and Orthoptera (*L. migratoria*), the AKH structures were compared by constructing phylogenetic tree. The molecular evolution of Lepidoptera with respect to other insect groups was also demonstrated. AKH sequences of 51 members (about ten orders analysed) were assembled and the phylogenetic tree constructed. Even though the phylogenetic relationship analysed for some of the insects based on the primary structures of AKH peptides present in that group, this is the first attempt that the evolutionary aspects of various insect orders has been discussed.

**Effect of insect adipokinetic peptides on vertebrate liver**

The effect of insect adipokinetic hormone on vertebrate liver was studied using fresh liver tissue from chicken and rat. Samples of fresh chicken liver (10 mg) were weighed out, dipped in 0.9% saline and blotted dry on a filter paper. The tissue was chopped well on a polyvinyl disc with a sharp razor blade. The chopped tissue samples were put in to the bottom of incubation vials (5 ml capacity) with bakelite screw caps containing 200 μl of phosphate buffered saline (PBS) and considered as experimental. Similarly, another set of tubes were processed in the same manner and taken as control. To the medium in the experimental tube, 20 μl of the hormone extract was

added whereas the control tube contained 20 µl of distilled water instead of the extract. The whole process was completed as quickly as possible (within 5-6 min). The incubations were carried out for 30 min in a shaker water bath preset at 37°C. After incubation, the samples were brought to room temperature and lipids released in to the medium were extracted by modified Bligh and Dyer (1959) procedure. Using a micropipette, 150 µl of the incubated medium was drawn out from the incubation vials. 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 centrifuged for 3 min at 1000 rpm to separate the organic and aqueous phases. The lower chloroform layer contained the extracted lipids. Lipid samples (100 µl each) were drawn from the lower organic phase using micropipette and transferred to test tubes for quantitation of lipids.

The effect of brain-retrocerebral complex extract in lipid mobilization was also studied using mammalian (rat) liver. A fresh sample of rat liver tissue was weighed out and the lipid release is measured as mentioned above. From the values thus obtained from controls and experimentals, change in the lipid release due to the hormone action was estimated.

**Quantitation of lipid release from the liver tissue.**

From the lipid samples thus obtained from chicken and rat liver tissues, chloroform was evaporated off by keeping them at room temperature. The

amount of lipids in the samples was measured colorimetrically using phosphovanillin reagent as described elsewhere. The optical densities were measured using UV-vis spectrophotometer at 540 nm against a reagent blank. From the values obtained, absorbance per mg of liver tissue was calculated using the absorbance value of standard lipid solution.

**Statistical analysis and data presentation**

Values obtained from various bioassay experiments were subjected to statistical analysis for significance and the values are expressed as mean $\pm$ standard deviation as well as percentage difference of the experimental over controls (E/C%). The analyses were performed using SPSS Software (version 10). The graphical representation of change in lipid mobilization was plotted using Origin software and Microsoft Excel programme.

# CHAPTER IV
# RESULTS

# RESULTS

## 1. Detection of biological activity of hormones *in vivo*

### a) Lipid release from the fat body of *I. limbata* in response to crude brain-retrocerebral complex extract and synthetic Lom AKH-I by heterologous *in vivo* bioassay.

The hyperlipaemic effects of the extracts were measured by estimating change in the lipid content of the haemolymph after the injection of brain-retrocerebral complex extract. Lipids in the experimental and control samples were estimated by spectrophotometric methods. The results obtained is summarized in Table IV. 1. It was found that the hormone extract induced significant hyperlipaemia. There was an increase in the lipid content by 15% ($115.41 \pm 0.11$, $P < 0.001$).

The change in lipid concentration when injected with the synthetic peptide was estimated in the same way. It was observed that the synthetic peptide also produced significant ($P < 0.05$) hyperlipaemic effects. The lipid levels increased to $116.11 \pm 0.18$ per cent of controls. In the control, the injection of 5 µl of insect saline did not evoke any significant hyperlipaemic activity. On the other hand, there was a small hypolipaemic response (-8%).

Values obtained from the bioassay experiments were subjected to statistical analysis and expressed as mean ± standard deviation. The values are also expressed as percentage difference of the experimental over controls (E/ C %). The analysis shows that the brain-retrocerebral complex extract of *O.*

*exvinacea* as well as the synthetic hormone have significant hyperlipaemic effects on *I. limbata* fat body to release lipids in to the haemolymph.

**b) Sugar release from the fat body of *I. limbata* in response to brain-retrocerebral complex extract and synthetic Lom AKH-I by heterologous *in vivo* bioassay.**

The haemolymph sugar concentration was measured before (control) and 60 min after injection (experiment) of either the extract of appropriate concentration or synthetic AKH, as mentioned in the section on materials and methods. Table IV. 2 presents the result of hyperglycaemic bioassay. The data obtained were subjected to 't' test and was found that the difference between the amount of sugar released in the control and the experiment (i.e., before and after injection) was significant. The data showed that both the crude extract and synthetic peptide induced similar hyperglycaemic effects. Increases to the extent of 118.15 $\pm$ 0.18 per cent ($P < 0.05$) and 118.74 $\pm$ 0.17 per cent ($P < 0.05$) over the controls respectively were obtained, whereas in the saline injected controls, no significant effects were observed.

**c) Protein release from the fat body of *I. limbata* in response to brain-retrocerebral complex extract and synthetic Lom AKH-I by heterologous *in vivo* bioassay.**

Injection of brain-retrocerebral complex extract and synthetic AKH caused reduction in the amount of haemolymph proteins in the plant bug, *I. limbata*. The injection of crude extract resulted in a significant reduction of proteins to 53.31$\pm$2.92 per cent ($P<0.05$) over the controls. Synthetic AKH also produced a similar significant reduction in protein synthesis 49.12 $\pm$ 3.61

per cent (P<0.05). The control insects injected with insect saline instead of hormone extract showed a similar reduction. Here the inhibition rate was to 58.26 ± 2.11 per cent of controls. The results are provided in Table IV. 3. From the experiments it becomes evident that the synthesis of haemolymph proteins in the insect fat body is inhibited by the injection of the hormone extract as well as the saline in to the insect. However, the hormone extract seemed to be exerting a better inhibitory effect than the saline injection.

**2. Detection of biological activity of hormones *in vitro* on the fat body.**

**a) Effects of brain-retrocerebral complex extract and synthetic Lom AKH-I on lipid metabolism (hyperlipaemic activity)**

Effect of brain-CC extract on lipid metabolism was studied in terms of release of lipids from fat body *in vitro*. The extract showed significant effect on fat body lipid mobilization, an increase of about 18 per cent (117.63 ± 0.35) which was statistically significant (P <0.05) when compared with the values obtained using insect saline instead of the extract. The synthetic AKH also produced a similar effect on lipid mobilization from the fat body. Here the lipid release was stimulated to 119.45 ± 7.92 per cent of the controls which was found to be statistically significant (P <0.05). The results are presented in Table IV. 4.

**b) Effects of brain-retrocerebral complex extract and synthetic Lom AKH-I on carbohydrate metabolism (hyperglycaemic activity)**

The role of AKH in sugar release from the fat body was studied *in vitro*. The result indicated that the crude hormone extract produced 18 per cent increase (117.58 ± 1.49) in carbohydrate release in to the medium. The synthetic peptide also elicited sugar release to the same extent as that of crude extract (118.44 ± 1.08, $P < 0.05$). The result shows that there is an increase in the haemolymph sugars when compared to that observed in incubations with insect saline on fat body sugar mobilization. The results are presented in Table IV. 5.

**c) Effects of brain-CC extract and synthetic Lom AKH-I on protein metabolism**

Both the native hormone extract and synthetic peptide showed inhibitory effects (more than 50 per cent inhibition) on proteins released in to the incubation medium. The brain-retrocerebral complex extract produced an inhibition of about 56 per cent over the controls. The quantity of proteins was reduced to 43.36 ± 6.12 per cent of controls whereas the synthetic hormone produced an inhibition of nearly 57%. i.e., to 43.44 ± 5.58 per cent of controls. Both the inhibitions are highly significant ($P<0.001$). The results obtained is summarized in Table IV. 6.

Thus it appears that the synthetic as well as the native adipokinetic peptide (extracted from the brain-retrocerebral complex of *O. exvinacea*) have similar effects on the metabolism of lipids, carbohydrates and proteins. The hormones stimulate the release of lipids and carbohydrates in to the haemolymph (*in vivo*) or the incubation medium (*in vitro*). The hormone,

however, inhibits the synthesis and/or release of proteins in to the haemolymph or the incubation medium.

### 3. Characterisation of the adipokinetic peptide by HPLC

The dried methanolic extract of brain-retrocerebral complex of *O. exvinacea* prepared as described in materials and methods section and filtered through 0.45 µm pore size Millipore filter was subjected to RP-HPLC analysis. The elution profile of the chromatography was recorded. The elution profile clearly showed a cluster of a number of UV absorbing peaks in the initial stages of the run. An isolated large peak was seen eluted at 10 min of the run, and there were no major peaks observed afterwards except for a few minor peaks at 13, 15 and 18 minutes of the run.

The similarity of retention time of any materials of the extract of *O. exvinacea* with that of an already reported peptide, the synthetic locust AKH was tested by overlaying this profile with that obtained for synthetic AKH in a similar HPLC run. As seen in the chromatogram (Figure IV. 1), the large peak that had the retention time of 9.7 min was similar to that of the synthetic Lom-AKH. The peaks obtained at 10 min with the crude brain-retrocerebral complex extract and synthetic peptide had almost identical size.

### 4. Activity of HPLC fractions on lipid, carbohydrate and protein metabolism *in vitro*

One minute fractions from HPLC were collected using a fraction collector and were individually tested for their effects on lipid, sugar and protein metabolism. The samples were prepared by reconstituting each of the

freeze dried HPLC fractions of brain-CC extract in 200 µl of distilled water. Results showed that fractions 8, 10, 13, 17 and 19 have significant hyperlipaemic activity. Highest hyperlipaemic activity was shown by fraction 10, which induced lipid release by 35% ($P<0.001$) above the controls. The materials in the fractions 8, 13, 17 and 19 min showed significant adipokinetic activities with increase of lipids by 28% ($P<0.001$), 20% ($P<0.05$), 16% ($P<0.05$) and 20% ($P<0.001$) over the controls respectively. Though the fractions 7 and 16 also induced lipid release by 22%, and 29%, the results were not found to be statistically significant. Fractions 2 and 18 were found to be slightly hypolipaemic, but the effects were not significant. None of the other fractions showed any significant adipokinetic activity. The results are summarized in Table IV. 7 and Figure IV. 2.

The HPLC fractions when tested for their hyperglycaemic activity on fat body revealed that the fractions 6, 10, 12 and 18 induced hyperglycaemia. There were 30%, 38%, 18% and 14% increase in sugar release respectively, all of which were statistically significant. The hyperglycaemia induced by materials in other fractions was 23%, (7 min), 21% (9 min) and 25% (19 min), and none of these effects were statistically significant. Table IV. 8 and Figure IV. 3 shows the hyperglycaemic effects of the materials in different fractions separated on HPLC.

When tested for their effects on the protein turn over in the incubation medium, it was found that the protein levels were very much affected by almost all fractions compared to the control. A significant reduction in the amount of proteins was observed. Highest inhibition was shown by fraction

10 (up to 41%, $P<0.05$), which also caused the maximum lipid and sugar mobilization *in vitro*. Results are provided in Table IV. 9 and Figure IV. 4. From the data presented, it can be concluded that the materials in fraction 10 showed significant adipokinetic and hyperglycaemic activities with increase of lipid and sugar, whereas suppression in the amount of protein synthesized or released was also observed, over controls.

**5. Characterisation of the adipokinetic peptide by MALDI-TOF-MS**

The molecular masses of peptides belonging to AKH/RPCH family present in the retrocerebral complex extract prepared from *O. exvinacea* were determined by MALDI-TOF-MS analysis (Figure IV. 5). It was carried out in a reflector positive ($Na^+$) mode with an acceleration voltage of 25 kV and 50 Hz pulsed $N_2$ laser, emitting at 337 nm. The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct [$M+Na^+$] of peptide with mass 1008.471. This molecular ion peak has similar mass as that of already known AKH/RPCH peptide, *Manduca* AKH. The amino acid sequence information of the peptide was obtained by employing MALDI-MS/MS analysis (Figure IV. 6). The tandem mass spectra (MS/MS) was acquired by selecting the precursor mass (sodium adduct) 1030.471 Da with a 10 Da window and fragments were generated in post source decay (PSD) mode. The MALDI-TOF-MS/MS spectrum of the precursor ion [$M+Na^+$] =1030.471 Da is shown in the figure IV. 7. This data was compared with the observed MS/MS fragment ions. The theoretical fragment ions (a, b, y, x, y, z and immonium ions) are shown in the figure. The fragment ions a, b and y types were identified from the MALDI-PSD data. The identified N- terminal ‘a’ type

fragment ions are $a_1$ (m/z 84.04), $a_2$ (m/z 197.12), $a_4$ (m/z 445.24), $a_5$ (m/z 546.29), $a_6$ (m/z 633.32), $a_7$ (m/z 720.35) and $a_8$ (m/z 906.43); and the 'b' type fragment ions are $b_7$ (m/z 748.35), $b_8$ (m/z 934.43) and $b_9$ (m/z 991.45) respectively. The fragment ions $y_2$ (m/z 261.13), $y_3$ (m/z 348.16), $y_4$ (m/z 435.19), $y_5$ (m/z 536.24), $y_6$ (m/z 683.31) and $y_7$ (m/z 784.36) represent the identified C- terminal 'y' type ions. All these evidences obtained from bioassay, HPLC and mass spectral analysis clearly indicate that the precursor peptide is biologically active with a primary structure pE-L-T-F-T-S-S-W-G-$NH_2$.

The identity of the pE form was fully confirmed by MS/MS analysis. Fragmentation of the precursor ion produced an almost full series C-terminal y-ions ($y_2$, $y_3$, $y_4$, $y_5$, $y_6$, $y_7$) and prominent b-ions corresponding to $b_7$, $b_8$ and $b_9$ and a series of a-ions ($a_1$, $a_2$, $a_4$, $a_5$, $a_6$, $a_7$, $a_8$) and numerous other masses in confirmation. The characteristic y- and b-type product ions, in conjugation with diagnostic y-$NH_3$ and b-$H_2O$ ions characterized these peptides unequivocally as members of the AKH family. The presence of amino acids phenyl alanine at position 4, Leu at position 2, tryptophan and glycine at positions 8 and 9 unambiguously shows that the derived peptide belongs to the AKH family.

## 6. Characterization of AKH gene

### Amplification and sequencing

The AKH gene was amplified by polymerase chain reaction, performed on the genomic DNA extracted from the intestinal tissues of

*Orthaga exvinacea*. The PCR primers used were- forward primer (AKHF) 5'-AACAACAGCAGAGTTCGCGG-3' and the reverse primer (AKHR) 5'-ATGCGCTTCGACTCTGCGCT-3'. The resulting PCR products were separated in 1% agarose gel. The amplification of genomic DNA yielded in an amplicon of approximately 400 bp (Figure IV. 8). The electropherogram data containing fluorescent peak trace chromatograms are obtained using Sequence Scanner v1.0 (Figures IV.9 and IV.10).

The PCR product was sequenced and the sequences were analyzed online using BLAST (http://www.ncbi.nlm.nih.gov/blast) as shown in Figure IV.11. The AKH gene sequences used for BLAST analysis include *B. mori* AKH-II mRNA, *B. mori* neuropeptide receptor-A26 (NGR-A26) mRNA and *B. mori* neuropeptide receptor-A21 (NGR-A21) mRNA. Nucleotide sequences spanning the ORF were obtained and compiled for comparison to the predicted gene sequences. Translations of ORF sequences were used for a Pileup of related protein sequences. The nucleic acid and deduced protein sequences were analyzed. Nucleic acid and protein sequences of other AKH genes were obtained from NCBI. Alignments of sequences were carried out using CLUSTALW (Figure IV. 12) and Dendrogram by http://align. genome.jp/ (Figure IV. 13) showing the phylogenetic relationship of *O. exvinacea* AKH gene with *B. mori* AKH-II gene and with other two neuropeptide hormone receptor (AKHR) gene. From the results it is clear that *O. exvinacea* AKH gene identified is a novel one and it had 100% similarity with *B. mori* AKH-II mRNA and *B. mori* neuropeptide receptor-A26 (NGR-

A26) mRNA whereas 92% similarity obtained with *B. mori* neuropeptide receptor-A21 (NGR-A21) mRNA, indicating that they belongs to the same cluster.

The comparison of nucleotide sequence information with the known gene sequence from Genbank indicated that this gene could encode AKH. Gene sequence containing 184 bp was obtained from the genomic DNA and sequence analysis revealed partial homology to adipokinetic hormone gene in *Bombyx mori*. This result confirms the unique nature of this AKH gene as it shows variability among other AKH genes. The sequence provided is a part of an exon, and the translation given is of frame+3. The amino acid sequences 1 to 14 (PVFKGNLLVAESQR) showed high homology to that of AKH2 of the silkworm *Bombyx mori*.

The AKH gene sequence was submitted to NCBI GenBank (Figure IV. 14) and the sequence was accepted and put in the public database under the accession number: **HQ269419**

## 7. Evolutionary tree construction using peptide sequence

Phylogenetic analysis of AKHs of Lepidoptera was conducted using MEGA4 software (Tamura *et al*., 2007). To reveal the amino acid relationship of *O. exvinacea* adipokinetic peptide with other adipokinetic peptide sequences, 10 putative AKH sequences (*B. mori* AKH-I and II, *M. sexta*, *H. zea*, *M. cinxia*, *S. frugiperda* AKH-I, II, III and IV and *V. cardui*) were collected from previous works for multiple alignment using CLUSTALW algorithm as shown in Figure IV. 15 and for phylogenetic analysis. The

adipokinetic peptide sequence of *O. exvinacea* along with 10 other AKH sequences available was aligned. The conserved sites were highlighted (Figure IV. 16). The amino acids present in positions 1, 3, 4 and 8 are strictly conserved in all peptide sequences studied. The AKH sequence of *O. exvinacea* is identical with that of already known AKH peptides, *M. sexta* and AKH-I of both *B. mori* and *S. frugiperda*. Each of them contained the same nonapeptide, Q-L-T-F-T-S-S-W-G-$NH_2$.

Figure IV. 17 shows the details of the AKH peptide sequences in the MEGA format used to export into the sequence data explorer. The systematic position and relationships of lepidopteran species, based on AKH structures, are demonstrated in the cladogram of AKHs identified from the studied species of Lepidoptera. The phylogenetic tree was plotted using neighbor joining method. Figure IV. 18 provide the topology of the evolutionary relationship of AKH sequences of Lepidoptera. The phylogenetic tree is constructed in two formats *viz*. rectangular (Figure IV. 19 a) and circular formats (Figure IV. 19 b).

To evaluate the evolutionary relationship of Lepidoptera and Orthoptera (*L. migratoria*), the AKH structures were compared by constructing phylogenetic tree. The alignment of AKH peptides and the positions for conserved residues were presented in Figures IV. 20 and 21 respectively. Details of the AKH sequences in the MEGA format for the construction of the tree is given in Figure IV. 22. Topology of the evolutionary relationship of AKH sequences of Lepidoptera and *Locusta* is provided in Figure IV. 23. The lepodopteran database suggests that particular

AKH variants are family-specific when compared to orthopteran studied. The phylogenetic analysis of Lepidoptera and *Locusta* is provided in rectangular (Figure IV. 24 a) and circular formats (Figure IV. 24 b).

To study the molecular evolution of Lepidoptera with respect to other insect groups, the AKH sequences of various groups from previous works were assembled and the phylogenetic tree constructed. The AKH peptides of 51 members were aligned by multiple sequence alignment and analysed their constant sites as shown in Figures IV. 25 and 26. Sequences were converted to MEGA format (Figure IV. 27) to process in the sequence data explorer. Topology of the molecular evolution of Lepidoptera and other available insect groups were presented in Figure IV. 28. The expanded AKH dataset is given in rectangle shaped tree and circular shaped tree in Figures IV. 29 a and 29 b. The cladogram revealed their evolutionary relationships. The amino acids present in positions 1 and 8 are constant in all cases reflecting their structure-activity relationships.

## 8. Effect of insect AKH on vertebrate liver

The effect of insect adipokinetic hormone on lipid mobilization and release from the liver of two vertebrates, chicken and rat were studied by conducting *in vitro* experiments. Fresh chicken liver samples were treated with the hormone extract as described in materials and methods section. In the experimental tubes, the tissue samples were incubated with PBS buffer containing either insect saline or brain-CC extract, where as the controls contain equal amount of distilled water instead of these solutions.

The extract produced significant effects in lipid mobilization from chicken liver [141.46±9.60 ($P<0.05$)]. But no such effects were observed in similar experiments conducted using insect saline (-5%). The results are presented in Table. IV. 10.

The effect of brain-retrocerebral complex extract in lipid mobilization was also similar on rat liver. In this case, the hormone extract produced significant effects in lipid mobilization 107.78±1.86 ($P<0.05$), even though the effect was relatively very small. The results are summarized in Table IV. 11. The experiments using insect saline instead of hormone extract did not produce any hyperlipaemic effects (-4%).

# CHAPTER V
# DISCUSSION

# DISCUSSION

Attempts were made to establish that the brain-retrocerebral complex of the mango leaf-webber, *Orthaga exvinacea* belonging to the family Pyralidae of the insect order Lepidoptera, contains bioactive peptides that are involved in the regulation of metabolism of lipids, carbohydrates and proteins. Structural characterizations were also carried out to confirm whether these peptide hormones have structural similarity to several other such hormones reported from many insects. It has been found that hormones of neural origin in insects play important roles in regulating key metabolic pathways and are commonly present in insects of various orders. From bioassays conducted both *in vivo* and *in vitro*, and from structural studies, it was found that the hormones extracted from *Orthaga* regulates key metabolic pathways and that they belong to the AKH/RPCH family of peptides characterized from many insects from different groups.

Tested *in vivo* in another insect (*Iphita limbata*), the hormone extract showed good hyperlipaemic and hyperglycaemic activities. It also showed inhibitory effects on the amount of protein released into the haemolymph. Heterologous incubation was opted for the reason that lepidopteran larval stages are not suitable for injection of materials as they collapse and die of bleeding which is unable to be stopped with minimum tackling. Adult moths also could not be used because of the very low volume of extractable haemolymph and also the hairy and scaly clothing over the entire body. At the same time, *Iphita* (a polyphagous plant bug) has been found to be a good test

insect which has already been established to show good hyperlipaemic response to hormones extracted from other insects such as the paddy armyworm, *Spodoptera mauritia* (Kumari and Gokuldas, 2001) and the paddy grasshopper, *Oxya nitidula* (Ajaykumar and Gokuldas, unpublished observation). Other *in vivo* incubations for hyperglycaemic as well as protein inhibitory activities also were done with *Iphita.*

Cross reactivity of neuropeptide hormones has been reported early by several workers (Holwerda *et al*., 1977; Liebrich and Gaede, 1995). Moreover, it has been found that locust CC extracts caused hyperglycaemia in 6-day-old, adult male *L. migratoria* at the expense of fat body glycogen (Goldsworthy, 1969) whereas it only released lipids in the mature adults. Injection of locust retrocerebral extract has been shown to induce elevation of haemolymph sugar (hypertrehalosaemic effect) in cockroaches (Holwerda *et al*., 1977) and to cause elevation in haemolymph lipid (adipokinetic effect) in locusts (Goldsworthy *et al*., 1972). *Heliothis zea* hypertrehalosaemic hormone (Helze-HrTH) was shown to possess adipokinetic and hypertrehalosaemic activity in the lepidopteran insect, *H. zea* (Jaffe *et al*., 1988) and exhibited adipokinetic activity in at least three other moth species of the families Sphingidae, Saturniidae and Bombycidae (Liebrich and Gaede, 1995). In *M. sexta*, a single AKH regulates the mobilization of carbohydrates in the larvae and lipids in the adults (Ziegler, 1984; Ziegler *et al*., 1990). Manse-AKH is widely distributed in lepidopteran insects and many of them possess either adipokinetic or hyperglycaemic or both the effects. Manse-

AKH mobilizes lipids in the silkmoth, *B. mori* (Ishibashi *et al*., 1992) and the butterfly, *Vanessa cardui* (Kollisch *et al*., 2000), carbohydrate in the noctuid moth, *Heliothis zea* (Jaffe *et al*., 1988), and both lipids and carbohydrates in the tobacco hornworm moth, *Manduca sexta* (Ziegler *et al*., 1985). These facts suggests that AKHs trigger the particular mobilization pathways leading to the release stored energy.

In our study it was observed that, injection of the peptide hormone extracted from *O. exvinacea* in to the plant bug, *I. limbata* elicits both carbohydrate and lipid mobilization from the fat body in to the haemolymph. The hormone also reduces the quantity of proteins in the haemolymph (*in vivo*) or the incubation medium (*in vitro*). These suggest that in any given species, AKHs trigger the particular mobilization pathways leading to activation of energy reserves characteristic of that species. Thus it becomes apparent that the nature of response is a function of the target tissue and the developmental stage of the insect and not that of the hormone alone.

This phenomena, in fact, reflect the occurrence of the receptors concerned and the presence of related or similar peptides in those insects. Most of these peptides have similar structure, which in turn reveals the possibility that these hormones evolved from some common ancestral molecule, and that they can attach to a limited number of receptor molecules. It has also been shown that the signal molecules thus attached to the receptors need not necessarily elicit the same internal cascade as mentioned above. Cross reactivity of peptide hormones have even been shown to exist between

insects and crustaceans (Fernlund and Josefsson, 1972). However, it is to be noted that the efficiency or extent of activity in these cross reactions are not similar in the different insects tested. For example, the Lom AKH-1 elicits hyperlipaemic activity in very low concentrations (at pmole levels) in the locusts themselves, whereas they are active only at a few fold higher concentrations in insects such as *Iphita*. Quantitative estimation of the peptides present in the brain and associated glands have not been done in too many insects.

In our eperiments, the extract was prepared from brain-retrocerebral complex dissected out from the last instar larvae of *O. exvinacea* just before pupation. This stage had the maximum size for its brain and associated glands. The extract when used at a concentration of one gland pair equivalent (gpe) per injection or 4 gpe per *in vitro* incubation, gave responses which are comparable to the activity elicited by 2 nmole synthetic Lom AKH-I.

The *in vivo* and *in vitro* studies to investigate the effect of peptide hormones present in the brain-retrocerebral complex extract, in carbohydrate, lipid and protein metabolism revealed that this peptide hormone play a key role in stimulating carbohydrate and lipid mobilization, and at the same time, they act as potent inhibitors of protein synthesis or release. The question whether the mobilization of different energy substrates in different insect species by peptides of the similar structures is coupled with the presence of different species-specific types of AKH receptors responsible for various signal transduction events remains to be answered. It is noteworthy to

mention that the use of carbohydrates and proline occurs commonly in some beetles and flies, and that dual usage of carbohydrate and proline metabolism might be mediated by different receptors, or by receptor subtypes (Auerswald and Gaede, 1999). Dual use of carbohydrates and lipids seems to be more conserved because both locust (Beenakkers *et al*., 1981) and moths (Ziegler and Schulz, 1986) use carbohydrates and lipids to power flight activity.

Although many studies have revealed the hyperlipaemic and hyperglycaemic effects of peptides of AKH family, very little is known about their role in protein metabolism. Lom-AKH-I suppresses fat body protein synthesis in *Locusta*. Administration of CC extracts or synthetic Lom-AKH-I inhibits protein synthesis more than 60% in immature and adult locusts (Carlisle and Loughton, 1979). The inhibitory effect of Lom-AKH-I on protein synthesis was observed for adult locusts *in vivo* at doses that are lower than those needed for lipid mobilization (Carlisle and Loughton, 1986), and both arylphorin and vitellogenin synthesis were suppressed. The inhibitory action of Lom-AKH-I is also confirmed for *in vitro* protein synthesis by the muscle and gut, as well as the fat body. In preparations of dispersed fat body cells, synthetic Lom-AKH-I stimulated cAMP synthesis and lipid release but inhibited protein synthesis (Asher *et al*., 1984). The present investigation was an attempt to demonstrate the inhibitory activity of peptide hormones on protein metabolism in the lepidopteran insect both *in vivo* and *in vitro*. A significant inhibition (more than 50%) was observed in both the cases.

The AKHs of insects generally induce mobilization of flight substrates such as lipids (in grasshoppers, locusts, crickets, and butterflies), sugars (in cockroaches, flies and bees), proline (in tsetseflies and some coleopterans) or sometimes mixtures thereof from fat body stores and therefore play a crucial role in flight metabolism (Wheeler, 1989; Candy *et al*., 1997). AKHs mobilize diglycerides from the fat body to provide energy for sustained flight. In addition, it modifies the metabolic activity of the flight muscles so as to oxidise fatty acids preferentially. Adipokinetic peptides are not involved in the stimulation of protein synthesis and/or release. This neurohormone mostly suppresses the synthesis and/or release of protein metabolites. The protein inhibiting cascade of AKHs has been shown to be manifested at the mRNA level (Kodrik and Goldsworthy, 1995). If mRNA synthesis is blocked, protein synthesis also get suppressed. It is possible that this mechanism may help the insect to conserve all available energy substrates for the generation of energy for flight and other activities instead of getting them diverted to protein synthesis and storage. However, the mechanism appears to be complicated by the fact that the injection of saline in the controls also showed a good rate of inhibition of protein synthesis/inhibition although the inhibition obtained with the hormone was better than the controls. Further studies are necessary to establish the real mechanism of action of the peptides in inhibiting protein synthesis and/or release by these hormones.

The RP-HPLC separation of the neuronal tissue extract of *O. exvinacea* revealed that the hormone extract contained compounds that elutes

on an octadecyl silane column using acetonitrile as the solvent. It was found that the most active fraction, eluted at 9.7 min was having a close retention time as that of the synthetic Lom AKH-1, i.e., 10 min. This result provided additional evidence to the assumption that the biologically active component of the crude extract had similarity to the synthetic AKH in their chemical structures also. This may be due to the fact that the AKH peptide sequence of insects belonging to various orders are very similar in their amino acid content as well as in their biological functions. Moreover, slight changes in the amino acid sequence of AKH among different insects do not show much variation in their activity. But the amino acids present in the constant sites are conserved in most of the cases (pGlu[1], Phe [4] and Trp[8]).

The data obtained from our bioassay studies with the HPLC fractions of the hormone extract of *O. exvinacea* revealed that several fractions had hyperlipaemic activity. At least five fractions have significant activity among which the highest activity was shown by fraction 10. With regard to the hyperglycaemic activity also fraction 10 elicited maximum activity and four other fractions showed significant activity. Fractions tested for their effect on protein metabolism revealed significant inhibition by fraction 10 (up to 41%), which also showed significant lipid and sugar mobilization. Thus from the present investigation, it is evident that the materials in the fraction 10 showed significant adipokinetic and hyperglycaemic activities with increase of lipid and sugar and a significant suppression of protein synthesis or release.

Our investigation also included experiments to elucidate the primary structure of the adipokinetic peptide from the brain-retrocerebral complex extract of the mango leaf webber *O. exvinacea* using MALDI-TOF/MS/MS. The molecular ion peak m/z value 1030.471 Da is indicative of sodiated adduct [M+$Na^+$] of peptide with mass 1008.471. This result is in agreement with the studies conducted by Ziegler *et al*. (1985). The molecular ion peak obtained has similar mass as that of already known AKH/RPCH peptide, *Manduca* AKH. The identity of the pE form was fully confirmed by MS/MS analysis. Fragmentation of the precursor ion produced an almost full series C-terminal y- ions ($y_2$, $y_3$, $y_4$, $y_5$, $y_6$, $y_7$) and prominent b- ions corresponding to $b_7$, $b_8$ and $b_9$ and a series of a- ions ($a_1$, $a_2$, $a_4$, $a_5$, $a_6$, $a_7$, $a_8$) and numerous other masses in confirmation. The characteristic y- and b- type product ions, in conjugation with diagnostic y-$NH_3$ and b-$H_2O$ ions characterized these peptides as members of the AKH family. Mass spectrometric analysis of the biologically active fraction revealed the chemical composition of the compound is pE-L-T-F-T-S-S-W-G-$NH_2$. The presence of amino acids phenyl alanine at position 4, Leu at position 2, tryptophan and glycine at positions 8 and 9 unambiguously shows that the derived peptide belongs to the AKH family. Thus our results from bioassays using crude extract and HPLC fractions and mass spectrometry analysis show that the biologically active material contains a nonapeptide.

The determination of primary structure of adipokinetic neuropeptides of other insect species like *I. limbata* and *A. miliaris* in our laboratory

indicated the presence of more than one adipokinetic factor in them (Ajaykumar and Gokuldas, 2011 a, b). Gaede and Marco (2009) identified that the insects *L. migratoria*, *P. morbillosus*, *P. leprosus*, *D. spumens*, *Lamarkiana sparrmani*, *Zonocerus elegans* and *Bullacris discolor* contain more than three AKH peptides. Siegert (1999) demonstrated the presence of a fourth inactive AKH peptide in *L. migratoria*, which is active in the American cockroach, *P. americana*. Insects possessing more than one adipokinetic hormone in their CC may have physiological or functional significances. The potencies of AKHs tested vary within and between different assays. Lom-AKH-I is the most potent in the lipid mobilization assay (Lee and Goldsworthy, 1995 b), while Lom-AKH-III is the most active in RNA inhibiting bioassay (Kodrik and Goldsworthy, 1995) and in the acetate uptake test (Lee and Goldsworthy, 1995 a). On the other hand, release of carbohydrates from the locust fat body is affected by Lom-AKH-II and not by Lom-AKH-I (Loughton and Orchard, 1981).

Recent development in genome sequencing of various insect species have allowed for the rapid discovery of novel genes including those coding for bioactive peptides. Sequencing for many insects are in progress and complete sequences are now available for the fruit fly, *Drosophila melanogaster* (Adams *et al*., 2000), two mosquitoes, *Anopheles gambiae* (Holt *et al*., 2002) and *Aedes aegypti* (Nene *et al*., 2007), the honeybee *Apis mellifera* (The Honeybee Genome Sequencing Consortium, 2006), and the flour beetle *Tribolium castaneum* (*Tribolium* Genome Sequencing

Consortium, 2008). Genomic studies on neuropeptides and their receptors in these insects have been highly successful and have provided crucial information about their development, physiology, behavior and evolutionary relationships (Coates *et al*., 2000; Hewes and Taghert, 2001; Park *et al*., 2002; Riehle *et al*., 2002; Hummon *et al*., 2006; Zitnan *et al*., 2007; Li *et al*., 2008; Hauser *et al*., 2008). Cloned AKH gene sequences will facilitate studies on the synthesis of AKH peptides and provide insight into molecular evolution. With a cloned AKH structural gene sequence, we can examine the cellular events leading from AKH transcript modulation to the release of a biologically active insect neuropeptide. However, many more questions remain unanswered in insect studies that may be addressed by additional investigations at different biochemical, physiological and molecular levels. Sequence similarity among different genes from insects suggests that there exist distinct ancestral forms of AKH. *Bombyx* has two separate ancestral forms of AKH (Li *et al*., 2008) and *Spodoptera* has four AKHs closely related to *Bombyx* AKH-I and AKH-II (Abdel-latief and Hoffmann, 2007). These suggest that the AKH gene family might have evolved differently in the two lepidopteran insects, *Spodoptera* and *Bombyx*. The cDNAs encoding AKH precursors in Lepidoptera have been cloned from *Manduca* (Bradfield and Keeley, 1989) and from the fall armyworm *S. frugiperda* (Abdel-latief and Hoffmann, 2007).

The data obtained from our gene sequencing studies revealed that the sequence has partial homology with AKH gene of *Bombyx mori*. The

sequence obtained is a part of an exon and the translation given is of frame+3. The amino acid sequences 1 to 14 ("PVFKGNLLVAESQR") showed high homology to that of AKH2 of the silkworm *B. mori*. The peptide BLAST of the conceptual peptide of the nucleotide sequence of AKH gene of *O. exvinacea* showed 100% similarity (max ident) with *B. mori* AKH2 mRNA and *B. mori* neuropeptide receptor-A26 (NGR-A26) mRNA whereas 92% similarity obtained with *B. mori* neuropeptide receptor-A21 (NGR-A21) mRNA. The result confirms that there exist different ancestral forms of AKH and suggests that the genes coding for this AKHs are conserved during the course of evolution. The result obtained by PCR and gene sequencing produced AKH prepeptide sequences, while mass spectrometry produces only mature AKH peptide. This could be the reason for the difference in the peptide structure obtained by gene sequencing. The BLASTp analysis indicates that the AKH gene identified is a novel one. The adipokinetic hormone gene sequence of *O. exvinacea* obtained can be used as a molecular barcode of the species. The dendrogram reveals the phylogenetic relationship of *O. exvinacea* AKH gene with *B. mori* AKH2 gene and with other two neuropeptide hormone receptor (AKHR) genes, indicating that they belong to the same cluster.

The AKH sequence of *M. sexta* (Bradfield and Keeley, 1989), *B. mori* AKH-II (Roller *et al*., 2008) and *S. frugiperda* AKH-I (Abdel-latief and Hoffmann, 2007) were found to be identical. Our present study elucidated the primary structure of *O. exvinacea* peptide and found that the sequence was

identical with that present in the above species. The primary structure of *H. zea* (Jaffe *et al*., 1988) and *S. frugiperda* AKH-II (Abdel-latief and Hoffmann, 2007) are reported to be similar, indicating their phylogenetic relationship. Even though the peptide structures of AKH family have been characterized from representatives of many insect orders (Gaede *et al*., 1997; Gaede, 2009), little is known about the evolutionary connections of AKH structures and functions within insect orders or even between insects and animals from other phylogenetic levels. The studies on the evolutionary relationships of AKH peptides from insects indicate that there exists a family or order specificity. The first attempt to relate phylogenetic relationships to structural differences of AKHs was made for cockroaches (Gaede, 1989). Fourteen species of Blattodea have been investigated with respect to hypertrehalosaemia, retention time of peptides in RP-HPLC, amino acid composition of bioactive peak material and the peptide sequence. The results suggest that the members of the family of Blattidae contain two hypertrehalosaemic octapeptides - Pea-CAH-I and II in their corpora cardiaca, whereas members of the families Blaberidae and Blattellidae contain the hypertrehalosaemic decapepide Bld-HrTH. Phylogenetic analyses were also conducted with some species belonging to the orders Odonata (Gaede and Marco, 2005) and the Orthoptera: Ensifera (Gaede *et al*., 2003) and Caelifera (Gaede and Marco, 2009). Gaede and Heather (2005) analysed the phylogeny of the insect order Odonata, based on the primary structures of AKH peptides present in two species of *Phymateus*, *P. morbilosus* and *P. leprosus* and showed the presence of two different decapeptides (Phymo-AKH-I and Phyle-CC) in their corpora

cardiaca. Studies on Orthopteran insects revealed the presence of more than three AKH/RPCH peptides, suggesting that the genes coding for this AKHs are highly conserved during the course of evolution (Ajaykumar and Gokuldas, unpublished data).

The primary structures of peptides from the AKH/RPCH family have been used as additional data to aid in the construction of phylogeny in insect orders. Recently, an extensive study dealing with the phylogenetic relationship of several neuropeptides including AKHs within Dictyoptera, Blattoptera became available (Roth *et al*., 2009). In addition, a few species from the order Heteroptera have been analyzed (Kodrik *et al*., 2010). An expanded heteropteran AKH database was compiled by incorporating sequences from various species as well as from previous studies and documented the phylogeny of the molecular structure of AKHs. The distribution of Panbo-RPCH in Crustacea and some Heteroptera could be interpreted as direct symplesiomorphic retention of a crustacean peptide solely by a relatively advanced group of paraneopteran insects. It would be possibly due to the loss of numerous genes concerned in the other insect groups, convergent evolution (homoplasy, a potential synapomorphy of several pentatomoid groups) of the peptide in some Heteroptera, re-expression of silent or blocked genes, parallel evolution based on an unidentified ‘underlying synapomorphy’ or horizontal transfer of the genes concerned. But this is the first time that the evolutionary analysis has been analysed for lepidopteran species, 11 species analysed. The lepidopteran database

encompasses their phylogenetic relationship even though the biochemical, structural and functional studies conducted indicate the similarities in their function and peptide sequence. Among the insects studied, *S. frugiperda* AKH-IV (Abdel-latief and Hoffmann, 2007) and *Vanessa cardui* AKH (Kollisch *et al*., 2000) are unusual. The evolutionary similarities between Lepidoptera and *Locusta* also have been studied. From the phylogenetic tree constructed, it is obvious that AKH sequence of Lepidoptera had a common structural and functional pattern (and origin) which is different from *Locusta*. An expanded AKH dataset was compiled by incorporating sequences from previous studies and from the present study. The cladogram constructed for 51 insects (belonging to ten orders) revealed their molecular evolution. The amino acids present in positions 1 and 8 are same.

Energy homeostasis, a fundamental property of all organisms, depends on the ability to control the storage and mobilization of fat, mainly triacylglycerols (TAG), in special organs such as mammalian adipose tissue or the fat body of insects. Malregulation of energy homeostasis underlies the pathogenesis of obesity in mammals including human. Packaging of storage fat in intracellular lipid droplets, and the various molecules and mechanisms guiding storage-fat mobilization, are conserved between mammals and insects (Sebastian *et al*., 2007, Marlene *et al*., 2008). In insects, as we discussed, storage fat lipolysis is stimulated by adipokinetic hormone in various species suggesting a general role in insect energy balance control. Like in mammalian TAG mobilization, AKH-stimulated lipolysis in the insect fat body relies on

signaling via a G protein-coupled receptor (Staubli *et al*., 2002), increase in intracellular cAMP, and activation of protein kinase A (PKA) (Van der Horst *et al*., 2001; Gaede and Auerswald, 2003). Sebastian *et al*. (2007) generated a *Drosophila* mutant lacking the receptor of adipokinetic hormone (AKHR) signaling pathway, an insect lipolytic pathway related to â-adrenergic signaling in mammals, and provided *in vivo* evidence that AKHR is important for chronic accumulation and acute mobilization of fat as is the Brummer lipase, the homolog of mammalian adipose triglyceride lipase (ATGL). Simultaneous loss of AKHR causes extreme obesity and blocks acute storage-fat mobilization in insects. They demonstrated that the key components and regulatory mechanisms of lipolysis are evolutionary conserved between insects and mammals.

Our observations on the adipokinetic effect of the hormone extract on the lipid release from vertebrate liver suggests the possibilities of using this hormone effectively for therapeutic purposes for controlling obesity (Umadevi *et al*., 2012). The fat mobilizing effect of AKH was studied in vertebrate (chicken) liver as there has been a similarity of this hormone with vertebrate hormone at functional level (Goldsworthy *et al*., 1994). AKHs resemble glucagon (Alquicer *et al*., 2009), a peptide hormone from the α-pancreatic islets cells in vertebrates and the vertebrate catecholamine adrenalin (Gaede, 2004). Another vertebrate candidate whose function can be compared with AKHs is vertebrate adiponectin, a hormone discovered recently from vertebrate adipose tissue (Tsao *et al*., 2002) which increases the

oxidation of fat and thereby reducing the intracellular triglyceride content of the liver and muscle and an increase of the cellular sensitivity to insulin (Diez and Iglesias, 2003).

From our present investigation there has been conclusive evidence that AKH regulates intermediary metabolism in the fat body, resulting in hyperglycaemia and hyperlipaemia and acts as a potent inhibitor of protein synthesis and/or release. The *in vitro* experiments to study the fat mobilizing effect of AKH on chicken liver gave significant results. It is possible that the activity observed with the crude extract is elicited with the component having similar structure as that of leptin, a vertebrate protein with fat depleting effect. The brain-retrocerebral complex extract have been shown to contain more than one AKH-like compound that are able to elicit hyperlipaemia. The effect can also be unspecific, because of a different receptor. However, there are patents regarding the use of polypeptide compounds based on the structures of insect peptides of the AKH family to mobilize lipids in humans. Schacter and Schacter (US Patent. 6852693, 2005) invented methods of using polypeptide compounds based on the structures of insect peptides of the adipokinetic hormone family to mobilize lipids in humans. The compositions and methods described in the claim are useful for modulating human body weight, such as inducing weight loss. The patent also includes screening methods for identifying other compounds effective for modulating lipid mobilization in humans.

A single protein having different functions have been found to bind to different receptor proteins and generate different second messengers to mediate their activities (Schramn and Selinger, 1975). The fat depleting effect of insect AKH can be correlated with AKH receptor belongs to the same protein family as the gonadotropin releasing hormone receptor family (Lindemans *et al*., 2009). Staubli *et al*. (2002) pointed out that the AKH receptor is related to the human gonadotropin releasing hormone receptor. Gonadotropin releasing hormone and AKH receptors as well as the corazonin and AKH/corazonin-related peptide (ACP) receptors all belong to a very large receptor family. They are found in vertebrates (reviewed by Tello and Sherwood, 2009), insects, nematodes, crustaceans, mollusks, Echinodermata, primitive chordates and possibly even the placozoan *T. adherens* (up to 23% identity with some insect AKH receptors). The result can be confirmed only after *in vivo* experiments. Ziegler *et al*. (2011) compared the *M. sexta* AKH receptor with the known AKH receptors of other insects and with gonadotropin releasing hormone-like receptors of invertebrates.

Studies on vertebrate and mammalian systems showed that, this hormone can mobilize fat *in vitro.* By using the information available on these lines, in future, we can target this hormone effectively for therapeutic purposes for controlling obesity. In addition, this work provides a foundation for understanding how the adipokinetic hormone can impact normal lipid metabolism and provides the possibility of designing agonists, antagonists and even mimics of neuropeptides, leading to the development of neuroendocrine

based biopesticides. However, much more studies is still needed on the physiological role of AKHs during flight and rest, their signal transduction, the dynamics of release of the hormones from the CC into the haemolymph, or the receptor binding features, possibilities of targeting the fat depleting effect (using animal models) and new molecular strategies for pest management of this nona peptide identified from the lepidopteran insect, *O. exvinacea*.

Confronted with the problem of developing resistance to conventional pesticides by insects, there is a critical need for developing new concepts and alternative approaches in controlling pest insects. Insect hormones and interrupting the insect endocrine processes have been propagated as possible pest management tools ever since investigations on insect neuroendocrinology have been initiated. New, selective control measures may be developed in designing metabolically stable mimics of these neuropeptides that actively inhibit or over-stimulate the functions regulated by them, resulting in sustained disruption of the insect internal homeostasis.

**Suggestions for further research**

Based on the findings of the study, we suggest the following areas in which future researches can be carried out.

1) Fat mobilizing effect of this insect hormone may be tested *in vivo* in vertebrate animal models.

2) To identify AKH receptor (AKHR) mediated signaling pathways in order to obtain a better understanding of the role of AKH/AKHR in the regulation of the molecular events responsible for energy homeostasis and carbohydrate and lipid mobilization.

3) Cloning of AKH structural gene sequence for examining the cellular events leading from AKH transcript modulation to release of a biologically active insect neuropeptide. Colning based approach may also reveal the various functions of these molecules during development.

4) The mechanism of action of peptide hormones in the regulation of protein metabolism has to be further investigated.

5) Quantitation of adipokinetic hormone in the neurohaemal tissues by ELISA.

# CHAPTER VI
# SUMMARY

# SUMMARY

Investigations to detect the presence of biologically active compounds in the neuronal tissues of the mango leaf webber, *O. exvinacea*, that can influence the metabolism of lipids, carbohydrates and proteins were carried out. The activity of the brain-retrocerebral complex extract was tested *in vivo* in the plant bug, *I. limbata* (heterologous bioassay) which revealed that the extract and also the synthetic peptide, Lom-AKH-I can increase both lipid (adipokinetic) and carbohydrate (hyperglycaemic) in the haemolymph whereas they also showed slight inhibitory effects on haemolymph protein content. *In vitro* studies using fat body taken from *O. exvinacea* showed similar response of the fat body to the crude extract and the synthetic peptide. The RP-HPLC separation of the hormone extract showed the apperance of a cluster of large peaks in the first few minutes and an isolated single large peak eluted at about 10 min. However no activity were shown by fractions appeared in the initial stages. Hypolipaemic, hyperglycaemic and protein inhibitory activities were restricted to fractions collected from 6 min onwards. A comparison of HPLC profiles of the synthetic Lom-AKH1 with that of the retrocerebral complex extract *O. exvinacea* showed that the hormone extract contained materials having similar retention times as that of synthetic AKH. The *in vitro* bioassays using purified fractions for lipid and carbohydrate mobilization showed a prominent hyperlipaemic and hyperglycaemic effects. The effects on protein metabolism caused by both the extract and the

synthetic AKH were significant but inconclusive because of the similar effects shown by the solvent controls.

The primary structure of the bioactive peptides in *O. exvinacea* was elucidated by MALDI-MS/MS in PSD mode. The study revealed that the chemical composition of the peptide is pE-L-T-F-T-S-S-W-G-$NH_2$. The presence of amino acids phenyl alanine at position 4, leucine at position 2, tryptophan and glycine at positions 8 and 9 shows that the derived peptide belongs to the AKH family. Our studies also indicated that *O. exvinacea* contained the same adipokinetic hormone as found in several moth species, namely Manse-AKH. Manse-AKH is widely distributed in lepidopteran insects and many of them possess either adipokinetic or hyperglycaemic or both the effects. Although fragmentation by mass spectrometry did not produce all C-terminal and N-terminal ions of the peptides, bioassays of crude extracts of neurohaemal tissues conducted both *in vivo* and *in vitro*, RP-HPLC fractionation and subsequent bioassays of the separated fractions and comparison of HPLC profiles of synthetic AKH and crude extract provides substantial evidences for peptide confirmation. The peptide BLAST of the conceptual peptide of the nucleotide sequence of AKH gene of *O. exvinacea* showed 100% similarity with *B. mori* AKH-II and 92% similarity with other two neuropeptide hormone receptor (AKHR) genes in *B. mori*. The results indicate that the AKH gene identified is a novel one. The amplified fragment may contain the AKH prepeptide coding sequence, while mass spectrometry

produces only mature peptide. This could be the reason for the difference in the peptide structure obtained by gene sequencing.

The present investigation also provides a foundation for studying the possibilities of developing neuropeptide-like substances that will be effective in controlling pest insects in an environmentally friendly fashion. Knowledge of the gene sequence as well as the primary structure of neuropeptides in insect pests is very essential to design biotechnology based insect pest management strategies. The current work also tries to study the phylogenetic relationship among lepidopteran insects and also between Lepidoptera and *Orthoptera*. The molecular evolution of Lepidoptera with respect to various other insect groups were also studied. Comparative analysis of the amino acid and nucleotide sequence sheds light on their evolution.

Results of our studies on the effect of adipokinetic factors on the lipid mobilization from invertebrate liver tissues suggest the possibility of using insect peptides or their analogues in therapeutic purposes in higher vertebrates (especially human beings) to control obesity. This result thus lend support to the already reported such effects.

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# REFERENCES

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**Table IV. 1.**
**Lipid released from the fat body of *I. limbata* in response to crude brain-CC extract of *O. exvinacea* and synthetic AKH *in vivo***

| No. | Source | Lipid release (µg/µl) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment(E) | | |
| 1 | Insect saline | 3.53 | 3.27 | 92.63±2.77 | N.S |
| 2 | Brain- CC extract | 2.53 | 2.92 | 115.41±0.11 | <0.001 |
| 3 | Synthetic AKH | 2.53 | 2.93 | 116.11±0.18 | <0.05 |

Values are expressed as means ± standard deviation (n=6). * Indicates the significance of difference between the controls and experimentals. The haemolymph samples were collected for lipid estimation before (control) and 60 min after (experiment) the injection of either insect saline, brain-CC extract (1 gpe) or synthetic AKH (500 pmole).

**Table IV. 2.**
**Sugar released from the fat body of *I. limbata* in response to crude brain-CC extract of *O. exvinacea* and synthetic AKH *in vivo***

| No. | Source | Sugar release (µg/µl) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment(E) | | |
| 1 | Insect saline | 2.95 | 2.78 | 94.27±2.7 | N.S |
| 2 | Brain- CC extract | 2.72 | 3.21 | 118.15±0.18 | <0.05 |
| 3 | Synthetic AKH | 2.39 | 2.84 | 118.74±0.17 | <0.05 |

Values are expressed as means ± standard deviation (n=6). * Indicates the significance of difference between the controls and experimentals. The haemolymph samples were collected for carbohydrate estimation before (control) and 60 min after (experiment) the injection of either insect saline, brain-CC extract (1 gpe) or synthetic AKH (500 pmol).

**Table IV. 3.**
**Protein released from the fat body of *I. limbata* in response to crude brain-CC extract of *O. exvinacea* and synthetic extract *in vivo***

| No. | Source | Protein release (µg/µl) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment(E) | | |
| 1 | Insect saline | 13.01 | 7.58 | 58.26±2.11 | N.S |
| 2 | Brain- CC extract | 11.48 | 6.12 | 53.31±2.92 | <0.05 |
| 3 | Synthetic AKH | 10.40 | 5.15 | 49.12±3.61 | <0.05 |

Values are expressed as means ± standard deviation (n=6). * Indicates the significance of difference between the controls and experimentals. The haemolymph samples were collected for protein estimation before (control) and 60 min after (experiment) the injection of either insect saline, brain-CC extract (1 gpe) or synthetic AKH (500 pmole).

**Table IV. 4.**

**Effects of brain-CC extract and synthetic peptide on lipid release *in vitro* from the fat body of *O. exvinacea***

| No. | Source | Lipid release (µg/mg) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment (E) | | |
| 1 | Insect saline | 63.35 | 60.95 | 96.21±6.03 | N.S |
| 2 | Brain-CC extract | 83.22 | 97.90 | 117.63±0.35 | <0.05 |
| 3 | Synthetic AKH | 75.11 | 89.72 | 119.45±7.92 | <0.05 |

Values are expressed as means ± standard deviation (n=10). * Indicates the significance of difference between the controls and experimentals. For experimental, the fat body was incubated in HEPES buffer containing either insect saline, brain-CC extract (4 gpe) or synthetic AKH (2000 pmole), controls contained buffer plus equal amount of distilled water instead of other solutions.

**Table IV. 5.**
**Effects of brain-CC extract and synthetic peptide on sugar release *in vitro* from the fat body of *O. exvinacea***

| No. | Source | Sugar release (µg/mg) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment (E) | | |
| 1 | Insect saline | 27.99 | 25.79 | 92.12±3.74 | N.S |
| 2 | Brain-CC extract | 18.01 | 21.17 | 117.58±1.49 | <0.05 |
| 3 | Synthetic AKH | 18.77 | 22.24 | 118.44±1.08 | <0.05 |

Values are expressed as means ± standard deviation (n=10). * Indicates the significance of difference between the controls and experimentals. For experimental, the fat body was incubated in physiological saline containing either insect saline, brain-CC extract (4 gpe) or synthetic AKH (2000 pmole), controls contained buffer plus equal amount of distilled water instead of other solutions.

**Table IV. 6.**
**Effects of brain-CC extract and synthetic peptide on protein release *in vitro* from the fat body of *O. exvinacea***

| No. | Source | Protein release (µg/mg) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment (E) | | |
| 1 | Insect saline | 38.96 | 19.57 | 50.24±11.15 | N.S |
| 2 | Brain-CC extract | 36.00 | 15.97 | 44.36±6.12 | <0.001 |
| 3 | Synthetic AKH | 34.63 | 15.04 | 43.44±5.58 | <0.001 |

Values are expressed as means ± standard deviation (n=10). * Indicates the significance of difference between the controls and experimentals. For experimental, the fat body was incubated in physiological saline containing either insect saline, brain-CC extract (4 gpe) or synthetic AKH (2000 pmole), controls contained buffer plus equal amount of distilled water instead of other solutions.

**Table IV. 7.**
**Hyperlipaemic activity of HPLC fractions tested *in vitro* on fat body of *O. exvinacea***

| HPLC Fractions | Lipid release (µg/mg) | | E/C (%) | Significance* (P) |
|---|---|---|---|---|
| | Control (C) | Experiment (E) | | |
| 1 | 11.03 | 11.86 | 107.52±2.01 | N.S |
| 2 | 10.4 | 10.01 | 96.25±3.37 | N.S |
| 3 | 11.73 | 13.38 | 114.06±3.07 | N.S |
| 4 | 10.64 | 12.56 | 118.05±3.05 | N.S |
| 5 | 13.06 | 13.86 | 106.12±2.95 | N.S |
| 6 | 11.72 | 13.21 | 112.71±3.16 | N.S |
| 7 | 10.94 | 13.36 | 122.12±2.63 | N.S |
| 8 | 11.66 | 14.93 | 128.04±0.89 | <0.001 |
| 9 | 10.97 | 12.84 | 117.05±3.08 | N.S |
| 10 | 10.83 | 14.6 | 134.81±0.58 | <0.001 |
| 11 | 11.4 | 12.58 | 110.35±4.01 | N.S |
| 12 | 11.23 | 12.17 | 108.37±3.01 | N.S |
| 13 | 10.95 | 13.19 | 120.45±2.06 | <0.05 |
| 14 | 10.51 | 11.59 | 110.28±2.13 | N.S |
| 15 | 10.73 | 12.41 | 115.66±2.17 | N.S |
| 16 | 10.88 | 14.04 | 129.04±3.12 | N.S |
| 17 | 11.07 | 12.86 | 116.17±1.43 | <0.05 |
| 18 | 10.94 | 10.31 | 94.24±2.6 | N.S |
| 19 | 11.81 | 14.19 | 120.15±0.65 | <0.001 |
| 20 | 11.84 | 13.39 | 113.09±3.14 | N.S |

HPLC fractions were collected as described in the materials and methods section. E/C % values are expressed as means ± standard deviation (n=6).
* Indicates the significance of difference between the controls and experimentals.

**Table IV. 8.**
**Hyperglycaemic activity of HPLC fractions tested *in vitro* on fat body of *O. exvinacea***

| HPLC Fractions | Sugar release (µg/mg) | | E/C (%) | Significance* (P) |
|---|---|---|---|---|
| | Control (C) | Experiment (E) | | |
| 1 | 23.25 | 26.211 | 112.74±1.09 | N.S |
| 2 | 25.887 | 27.94 | 107.93±2.01 | N.S |
| 3 | 23.69 | 26.14 | 110.34±2.63 | N.S |
| 4 | 21.55 | 19.41 | 90.07±8.77 | N.S |
| 5 | 20.84 | 20.74 | 99.52±4.08 | N.S |
| 6 | 18.94 | 24.63 | 130.04±1.09 | <0.05 |
| 7 | 20.61 | 25.37 | 123.10±6.97 | N.S |
| 8 | 22.8 | 23.82 | 104.47±7.75 | N.S |
| 9 | 20.42 | 24.81 | 121.50±6.92 | N.S |
| 10 | 17.61 | 24.31 | 138.05±1.14 | <0.001 |
| 11 | 25.55 | 27.35 | 107.05±2.16 | N.S |
| 12 | 17.85 | 21.08 | 118.10±0.77 | <0.001 |
| 13 | 18.42 | 20.64 | 112.05±2.87 | N.S |
| 14 | 16.55 | 17.49 | 105.68±1.69 | N.S |
| 15 | 18.13 | 20.66 | 113.95±2.54 | N.S |
| 16 | 19.04 | 21.53 | 113.08±2.07 | N.S |
| 17 | 22.71 | 26.16 | 115.19±4.09 | N.S |
| 18 | 19.11 | 21.89 | 114.55±2.14 | <0.05 |
| 19 | 20.52 | 25.74 | 125.44±6.08 | N.S |
| 20 | 24.02 | 26.53 | 110.45±2.66 | N.S |

HPLC fractions were collected as described in the materials and methods section. E/C % values are expressed as means ± standard deviation (n=6).
* Indicates the significance of difference between the controls and experimentals.

**Table IV. 9.**
**Activity of HPLC fractions in protein metabolism on fat body of *O. exvinacea* tested *in vitro***

| HPLC Fractions | Protein release (µg/mg) | | E/C (%) | Significance* (P) |
|---|---|---|---|---|
| | Control (C) | Experiment (E) | | |
| 1 | 39.08 | 24.45 | 62.56±8.83 | <0.05 |
| 2 | 31.59 | 21.09 | 66.76±11.89 | N.S |
| 3 | 30.48 | 14.88 | 48.82±0.34 | <0.001 |
| 4 | 30.07 | 21.22 | 70.57±12.54 | N.S |
| 5 | 38.22 | 19.65 | 51.41±18.15 | N.S |
| 6 | 30.08 | 14.76 | 49.07±0.41 | <0.05 |
| 7 | 30.59 | 12.59 | 41.16±5.68 | N.S |
| 8 | 38.77 | 37.36 | 96.36±6.41 | N.S |
| 9 | 30.17 | 14.53 | 48.16±0.62 | N.S |
| 10 | 37.29 | 15.31 | 41.06±10.75 | <0.05 |
| 11 | 13.62 | 10.33 | 75.84±6.09 | N.S |
| 12 | 30.57 | 14.43 | 47.20±0.45 | <0.001 |
| 13 | 31.09 | 28.15 | 90.54±14.98 | N.S |
| 14 | 36.22 | 19.22 | 53.06±10.56 | <0.05 |
| 15 | 30.29 | 14.91 | 49.22±0.58 | <0.001 |
| 16 | 24.31 | 15.67 | 64.46±15.1 | N.S |
| 17 | 30.19 | 14.84 | 49.16±0.31 | <0.001 |
| 18 | 25.56 | 15.48 | 60.56±12.23 | N.S |
| 19 | 30.43 | 14.57 | 47.88±0.53 | <0.001 |
| 20 | 32.43 | 30.55 | 94.20±9.63 | N.S |

HPLC fractions were collected as described in the materials and methods section. E/C % values are expressed as means ± standard deviation (n=6).
* Indicates the significance of difference between the controls and experimental.

**Table IV. 11.**

**Effect of insect AKH on lipid release from rat liver *in vitro***

| No. | Source | Lipid release (µg/mg) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment (E) | | |
| 1 | Insect saline | 94.54 | 90.94 | 95.76±1.18 | N.S |
| 2 | Brain-CC extract | 94.54 | 101.90 | 107.78±1.86 | <0.05 |

Values are expressed as means ± standard deviation (n=6). * Indicates the significance of difference between the controls and experimentals. For experimental, the fat body was incubated in PBS buffer containing either insect saline or brain-CC extract, controls contain buffer plus distilled water instead of the solutions.

**Table IV. 10.**

**Effect of insect AKH on lipid release from chicken liver *in vitro***

| No. | Source | Lipid release (µg/mg) | | E/C (%) | Significance * (P) |
|---|---|---|---|---|---|
| | | Control (C) | Experiment (E) | | |
| 1 | Insect saline | 102.08 | 97.08 | 95.10±3.12 | N.S |
| 2 | Brain-CC extract | 99.19 | 140.32 | 141.46±9.60 | <0.05 |

Values are expressed as means ± standard deviation (n=6). * Indicates the significance of difference between the controls and experimentals. For experimental, the fat body was incubated in PBS buffer containing either insect saline or brain-CC extract, controls contain buffer plus distilled water instead of the solutions.

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

| SI. No. | Code Name | Species | Amino Acid sequences | | | | | | | | | | | Molecular weight (Da) | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | | |
| 1 | Pambo-RPCH | *Pandalus borealis* | pE | L | N | F | S | P | G | W | $NH_2$ | | | 929.43 | Fernlund and josefsson. (1972) |
| 2 | Locmi-AKH-I | *Locusta migratoria* | pE | L | N | F | T | P | N | W | G | T | $NH_2$ | 1158.54 | Stone *et al*. (1976) |
| 3 | Peram-CAH-I | *Periplaneta americana* | pE | V | N | F | S | P | N | W | $NH_2$ | | | 972.44 | Witen *et al*. (1984) |
| 4 | Peram-CAH-II | *Periplaneta americana* | pE | L | T | F | T | P | N | W | $NH_2$ | | | 987.48 | Witen *et al*. (1984) |
| 5 | Manse-AKH | *Melittea cinxia* | pE | L | T | F | T | S | S | W | G | $NH_2$ | | 1007.47 | Zigler *et al*. (1985) |
| 6 | Locmi-AKH-II | *Locusta migratoria* | pE | L | N | F | S | A | G | W | $NH_2$ | | | 903.42 | Siegert *et al*. (1985) |
| 7 | Schgr-AKH-II | *Schistocerca gregaria* | pE | L | N | F | S | T | G | W | $NH_2$ | | | 933.43 | Siegert *et al*. (1985) |
| 8 | Bladi-HrTH | *Blaberus discoidalis* | pE | V | N | F | S | P | G | W | G | T | $NH_2$ | 1073.49 | Hayes *et al*. (1986) |
| 9 | Grybi-AKH | *Gryllus bimaculatus* | pE | V | N | F | S | T | G | W | $NH_2$ | | | 919.41 | Gaede and Rinehart. (1987 a) |
| 10 | Carmo-HrTH-I I | *Carausius morosus* | pE | L | T | F | T | P | N | W | G | T | $NH_2$ | 1146.53 | Gaede and Rinehart. (1987 b) |
| 11 | Helize-HrTH | *Heliothis zea* | pE | L | T | F | S | S | G | W | G | N | $NH_2$ | 1077.48 | Jaffe *et al*.(1988) |
| 12 | Rommi-CC | *Romalea microptera* | pE | V | N | F | S | P | N | W | G | T | $NH_2$ | 1144.5 | Gaede *et al*.(1988) |
| 13 | Tabat-HOTH | *Tabanus attratus* | pE | L | T | F | T | P | G | W | G | Y | $NH_2$ | 1150.54 | Jaffe *et al*.(1989) |
| 14 | Tabat-AKH | *Tabanus attratus* | pE | L | T | F | T | P | G | W | $NH_2$ | | | 930.45 | Jaffe *et al*.(1989) |
| 15 | Libau-AKH | *Libellula auripenis* | pE | V | N | F | T | P | S | W | $NH_2$ | | | 959.45 | Gaede (1990) |
| 16 | Tenmo-AKH | *Tenebrio molitor* | pE | L | N | F | S | P | N | W | $NH_2$ | | | 986.46 | Gaede and Rosinsky. (1990) |
| 17 | Phote-HrTH | *Phormia terraenovae* | pE | L | T | F | S | P | N | W | $NH_2$ | | | 973.46 | Gaede *et al*.(1990) |

Contd...

| SI. No. | Code Name | Species | Amino Acid sequences | | | | | | | | | | | Molecular weight (Da) | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | | |
| 18 | Melme-CC | *Melolontha melolontha* | pE | L | N | F | S | P | N | W | $NH_2$ | | | 1003.45 | Gaede (1991b) |
| 19 | Emppe-AKH | *Empusa pennata* | pE | V | N | F | T | P | N | W | $NH_2$ | | | 986.46 | Gaede (1991 b) |
| 20 | Locme-AKH-III | *Locusta migratoria* | pE | L | N | F | T | P | W | W | $NH_2$ | | | 1072.5 | Oudejans *et al*. (1991) |
| 21 | Carmo-HrTH-I | *Carausius morosus* | pE | L | T | F | T | P | N | W | G | T | $NH_2$ | 1146.53 | Gaede *et al*. (1992) |
| 22 | Polae-HrTH | *Polyphaga aegyptica* | pE | I | T | F | T | P | N | W | $NH_2$ | | | 987.48 | Gaede and Kelner (1992) |
| 23 | placa-HrTH-I&II | *Platipura capensis* | pE | V | N | F | S | P | S | W | G | N | $NH_2$ | 1116.49 | Gaede and Janssens (1994) |
| 24 | Psein-AKH | *Pseudogrion inconspicuum* | pE | V | N | F | T | P | G | W | $NH_2$ | | | 929.43 | Janssens *et al*. (1994) |
| 25 | Anaim-AKH | *Anax imperator* | pE | V | N | F | S | P | S | W | $NH_2$ | | | 945.43 | Gaede *et al*.(1994) |
| 26 | Declu-CC | *Decaptoma lunata* | pE | L | N | F | S | P | N | W | G | N | $NH_2$ | 1157.52 | Gaede (1995) |
| 27 | Phyle-CC | *Phymateus leprosis* | pE | L | T | F | T | P | N | W | G | S | $NH_2$ | 1137.53 | Gaede and Kelner (1995) |
| 28 | Micvi-CC | *Microhodotermis viator* | pE | I | N | F | T | P | N | W | $NH_2$ | | | 1000.4 | Liebrich *et al*. (1995) |
| 29 | Phymo-AKH-I | *Phymateus morbilosus* | pE | L | N | F | T | P | N | W | G | S | $NH_2$ | 1144.53 | Gaede *et al*.(1996) |
| 30 | Oniay-CC-I | *Onitis aygulus* | pE | L | N | F | S | T | G | W | $NH_2$ | | | 983.41 | Gaede (1997a) |
| 31 | Scade-II | *Scarabaeus deludens* | pE | F | N | F | S | P | V | W | $NH_2$ | | | 1021.46 | Gaede (1997a) |
| 32 | Scade-I | *Scarabaeus deludens* | pE | F | N | F | S | P | N | W | $NH_2$ | | | 1036.44 | Gaede (1997a) |
| 33 | Locmi-HrTH | *Locusta migratoria* | pE | V | T | F | S | R | N | W | S | P | $NH_2$ | 1202.58 | Siegert (1999) |
| 34 | Erysi-AKH | *Erythmis simplicolis* | pE | K | N | F | T | P | S | W | $NH_2$ | | | 988.47 | Gaede and Kelner (1999) |

Contd...

| SI. No. | Code Name | Species | Amino Acid sequences | | | | | | | | | | | Molecular weight (Da) | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | | |
| 35 | Venca-AKH | *Vanessa cardui* | pE | K | T | F | T | S | S | W | G | G | K | 1208.5 | Kollisch *et al*. (2000) |
| 36 | Pyrap-AKH | *Pyrrhocoris apterus* | pE | L | N | F | T | P | N | W | $NH_2$ | | | 1000.4 | Kodrik *et al*. (2000) |
| 37 | Phymo-AKH-III | *Phymateus morbilosus* | pE | I | N | F | T | P | W | W | $NH_2$ | | | 1072.5 | Siegert *et al*. (2000) |
| 38 | Manto-AKH | *Mantophasmatodea(Order)* | pE | V | N | F | S | P | G | W | $NH_2$ | | | 915.42 | Gaede *et al*.(2005) |
| 39 | Anoga-AKH-II | *Anopheles gambiae* | pE | V | T | F | S | R | D | W | N | A | $NH_2$ | 1204.56 | Kauffman and Brown (2006) |
| 40 | Anoga-HrTH | *Anopheles gambiae* | pE | L | T | F | T | P | A | W | $NH_2$ | | | 944.47 | Kauffman and Brown (2006) |
| 41 | Corpu-AKH | *Corixa punctuata* | pE | L | N | F | S | P | S | W | $NH_2$ | | | 959.45 | Gaede *et al*.(2007a) |
| 42 | Letin-AKH | *Lethocerus indicus* | pE | V | N | F | S | P | Y | W | $NH_2$ | | | 1021.46 | Gaede *et al*.(2007b) |
| 43 | Nepce-AKH | *Nepa cenerea* | pE | L | N | F | S | S | G | W | $NH_2$ | | | 919.41 | Gaede *et al*.(2007b) |
| 44 | Trica-AKH | *Tribolium castaneum* | pE | L | N | F | S | T | D | W | $NH_2$ | | | 991.43 | Gaede *et al*.(2008) |
| 45 | Melcin-AKH | *Melittea cinxia* | pE | L | T | F | S | S | G | W | G | $NH_2$ | | 906.42 | Gaede *et al*., unpublished data |
| 46 | Bommo-AKH | *Bombyx mori* | pE | L | T | F | S | R | D | W | S | G | $NH_2$ | 1177.55 | Gaede *et al*.(2008) |
| 47 | Trica-AKH-II | *Tribolium castaneum* | pE | V | T | F | S | R | D | W | N | P | $NH_2$ | 1230.57 | Gaede *et al*. (2008) |
| 48 | Aedae-AKH-I | *Aedes aegypti* | pE | L | T | F | T | P | S | W | $NH_2$ | | | 960.47 | Kauffman and Brown (2008) |