

**IDENTIFICATION, CHARACTERISATION, AND FUNCTION OF
ADIPOKINETIC HORMONES AND RECEPTOR IN THE
AFRICAN MALARIA MOSQUITO, *ANOPHELES GAMBIAE*
(DIPTERA)**



Thèse

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Identification, characterisation and function of
adipokinetic hormones and receptor in the
African malaria mosquito, *Anopheles gambiae*
(Diptera)

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RESUME:

En utilisant la bioinformatique et la biologie moléculaire, nous avons pu identifier chez le principal vecteur africain de la malaria, le moustique, *Anopheles gambiae* deux hormones adipokinétiques (AKHs): l'octapeptide, Anoga-AKH-I (pQLTFTPAWa) et le décapeptide, Anoga-AKH-II, (pQVTFSRDWNAa). La fonction principale des AKHs est d'induire une hyperlipémie (effet d'adipokinétique), ainsi qu'une hypertrehalosémie et une hyperprolinémie. En tant que membres de la famille des AKH, les deux neuropeptides montrent des acides aminés aromatiques conservés en positions quatre et huit. Dans ce travail, nous avons caractérisé chez *A. gambiae* les deux neuropeptides AKH et nous avons également étudié leur fonction métabolique. Comme tous les neuropeptides, leur cadre de lecture ouvert est caractérisé par la présence d'un peptide signal, de l'hormone (AKH), et du peptide précurseur d'hormone. La différence observée entre les deux Anoga-AKHs indique une paralogie provenant d'une duplication à partir d'un gène ancestral. La comparaison avec d'autres AKHs connues montre que Anoga-AKH-I semble être un orthologue proche de l'AKH de *Drosophila melanogaster* (Drome-AKH). Quant à la séquence du neuropeptide Anoga-AKH-II, elle partage 80% d'homologie avec la quatrième AKH décrite chez *Locusta migratoria* (Locmi-HrTH). Ceci laisse penser que ces 2 AKHs sont probablement orthologues d'une AKH ancestrale.

Nous avons pu constater que la transcription de *Anoga-AKH-I* et *-II* était constante dans les œufs, les larves, les pupes, et les adultes d'*A. gambiae*. L'expression de ces gènes n'a été retrouvée que dans la tête et le thorax de l'insecte et jamais dans son abdomen. Des analyses plus poussées chez des femelles nourries avec du sucre ou du sang ont montré une transcription similaire et constante. De même, aucun changement n'a été détecté pendant le cycle gonotrophique des femelles en fonction du régime alimentaire. D'autre part, en utilisant l'immunocytochimie et la technique du «radioimmunoassay» (RIA), nous avons pu confirmer l'expression de *Anoga-AKH-I* et *-II* dans la tête et le thorax de l'insecte, et l'absence d'expression dans l'abdomen. La source principale d'Anoga-AKH-I est probablement localisée dans les cellules X du corps cardiaque et dans celles des ganglions thoraciques alors que la source d'Anoga-AKH-II se situerait dans les cellules neuro-sécrétrices latérales du protocerebrum. Les comparaisons avec les sources cellulaires de l'octapeptide orthologue d'Anoga-AKH-I chez *D. melanogaster* ont démontré que, dans la drosophile, la présence d'AKH n'est détectée qu'au niveau du corps cardiaque, tandis que chez le moustique, elle n'est pas limitée à cet organe neurohaemal, mais qu'elle se retrouve également dans les ganglions thoraciques. En ce qui concerne Anoga-AKH-II, la coloration des cellules neuro-sécrétrices latérales du protocerebrum observée en immunohistochimie n'est pas étonnante. En effet, comme ces cellules représentent probablement la source du décapeptide du moustique, ceci renforce l'hypothèse que l'AKH, Locmi-HrTH, décrite chez la locuste et Anoga-AKH-II sont orthologues; les deux hormones semblent être synthétisées dans le cerveau, et non dans le corps cardiaque.

Quelques récepteurs d'hormones adipokinétiques (AKHR) ont déjà été caractérisés, nous avons donc effectué des blasts parmi les séquences d'AKHR connues, ce qui nous a permis de

prédire un récepteur présumé pour les Anoga-AKHs. Ce récepteur, comme les autres AKHRs, appartient aux récepteurs couplés aux protéines G (GPCR). L'expression du gène de ce récepteur a été mise en évidence durant tout le cycle de vie d'*A. gambiae*. L'expression n'a pas changé au cours des tests expérimentaux que nous avons effectués avec des mâles et des femelles soumis à un régime alimentaire à base de sucre, ni pendant le cycle gonotrophique des femelles. La fonction principale des AKHs étant de mobiliser des éléments nutritifs comme les lipides, les hydrates de carbone et les acides aminés, nous avons émis l'hypothèse que les AKHRs étaient localisés dans le corps gras, tissus connu pour emmagasiner les principaux métabolites chez les insectes. Effectivement, l'expression des AKHRs n'a été décelée que dans les parois abdominales de l'insecte, là où le corps gras est présent. Une faible expression a cependant été également détectée dans les ovaires, dans lesquels, étonnamment, de l'ARN brut de *Anoga-AKH-I* a aussi été décelé. La question se pose alors de savoir si *Anoga-AKH-I* et son récepteur sont impliqués dans l'embryogenèse après la ponte?

Anoga-AKH-I provoque la mobilisation des sucres de l'hémolymph chez les femelles d'*A. gambiae* qu'elles soient nourries de sucre ou de sang, sans qu'aucun effet ne soit observé sur les lipides. Le trehalose étant le principal sucre de l'hémolymph utilisé par les moustiques, cette neurohormone, *Anoga-AKH-I*, devrait être renommée hormone hypertrehalosémique, *Anoga-HrTH*. D'autre part, comme cela a déjà été décrit pour la quatrième AKH de *L. migratoria*, aucune mobilisation spécifique de lipides ou d'hydrates de carbone n'a été détectée pour *Anoga-AKH-II*, le décapeptide d'*A. gambiae*. Cependant, la Locmi-HrTH a élevé le niveau d'hydrate de carbone chez la blatte américaine, *Periplaneta americana*, alors qu'aucun effet n'a été observé avec *Anoga-AKH-II*. Des tests similaires que nous avons effectués chez *L. migratoria* avec *Anoga-AKH-I* et -II ont révélé une hyperlipémie avec l'octapeptide du moustique, mais pas avec le décapeptide. De plus, en utilisant la technique d'interférence de l'ARN (RNAi) pour bloquer l'expression du gène du récepteur d'*Anoga-AKH-I* dans le corps gras, aucune hypertrehalosémie n'a été induite par *Anoga-AKH-I*. Nous avons ainsi montré, indirectement, qu'*Anoga-AKH-I* agit à travers ce récepteur GPCR.

L'ensemble de ce projet était basé sur le fait que *A. gambiae* utilise des lipides et des hydrates de carbone lors de ses vols. Les AKHs mobilisant ces métabolites de vol, comme cela a été montré chez d'autres insectes, nous avons étudié l'influence de ce type d'hormone sur le vol. Nous avons ainsi pu montrer, en utilisant un carrousel de vol, que seule *Anoga-AKH-I* induisait des vols plus performants pendant les premières heures. Après injection de l'*Anoga-AKH-I* à des femelles, les femelles décapitées ont montré une distance de vol statistiquement supérieure par rapport aux femelles contrôles, alors que les femelles intactes n'ont montré qu'une tendance vers des vols plus performants, sans que cela ne soit statistiquement significatif. En revanche, l'injection d'*Anoga-II* n'a entraîné aucune différence entre femelles traitées et contrôles. Ceci pourrait indiquer que *Anoga-AKH-II* ne bloquerait pas *Anoga-AKHR* et fonctionnerait probablement à travers un autre récepteur.

SUMMARY:

The main function of adipokinetic hormones (AKHs) is to induce hyperlipaemia and an adipokinetic effect, but also hypertrehalosaemia and hyperprolinaemia. This project consists of the identification and characterization, as well as the study of the central metabolic function of AKHs in the main African malaria vector, *Anopheles gambiae*. By using a combination of bioinformatics and common molecular biological tools, it was possible to characterize two AKH neuropeptides: i.e. the octapeptide, Anoga-AKH-I (pQLTFTPAWa) and the decapeptide, Anoga-AKH-II, (pQVTFSRDWNAa). As members of the AKH-family, both neuropeptides demonstrated two conserved aromatic amino acids at position four and eight. The peptides were blocked at the N- and C-termini by a pyroglutamate (pQ) and amide (a), respectively. Like all neuropeptides, their open reading frame was characterized by the presence of a signal peptide, the hormone (AKH), and the hormone precursor related peptide (APRP). Dissimilarity of both Anoga-AKHs indicates a paralogy from an ancient gene duplication. In comparison to known AKHs, Anoga-AKH-I seems to be closely orthologous to the *Drosophila melanogaster* AKH (Drome-AKH). Since the fourth AKH in *Locusta migratoria* (Locmi-HrTH) shares 80% homology with the neuropeptide sequence of Anoga-AKH-II, they probably are orthologs from an ancient ancestor AKH.

The gene expression profile of *Anoga-AKH-I* and *-II* was constant in eggs, larvae, pupae, and imago; they were only found in the head and thorax and not in the abdomen. Further analysis of sugar- and blood-fed females presented a similar and constant expression pattern; no changes were detected during the gonotrophic cycle compared to water- and sugar-fed females. In addition, using immunocytochemistry and radioimmunoassays (RIA), the expression was confirmed in the head and thorax only. The main source of Anoga-AKH-I is probably located in the X-cells and thoracic ganglia cells and Anoga-AKH-II in the lateral neurosecretory cells of the protocerebrum. Comparisons between the cellular sources of the octapeptide in *D. melanogaster* and *A. gambiae*, demonstrated that in the fruit fly, AKH is only found in the corpora cardiaca, whereas in the mosquito, it is not only in this neurohaemal organ, but is also found in the thoracic ganglia. The staining of the lateral neurosecretory cells was not surprising. Since it is probably the source of the mosquito decapeptide, it underlines the hypothesis that Locmi-HrTH and Anoga-AKH-II are orthologs; both seem to be synthesized in the brain, and not in the corpora cardiaca.

Since a few adipokinetic hormone receptors (AKHR) had already been characterized, it was possible to mine for the Anoga-AKHR, by blasting the known AKHR sequences, which resulted in the prediction of a putative Anoga-AKHR that, like all the other AKHRs, belongs to the G-protein coupled receptors (GPCR). Its expression was found in all life stages. The receptor expression pattern did not change during the experimental tests for sugar-fed males and females and during the gonotrophic cycle in females. Since the central function

of AKHs is to mobilize nutrients like lipid, carbohydrate, and amino acids, it is predicted that the AKHRs are located in the fat body, which is the main metabolite storage tissue in insects. After splitting the abdomen into ventral and dorsal body walls, digestive tracts, ovaries, and spermatheca, expression was only found in the body parts to which the most fat body were attached. Weak expression was detected in the ovaries, in which surprisingly the unprocessed RNA of Anoga-AKH-I was also found – a possible indication of the involvement of Anoga-AKH-I and Anoga-AKHR during the embryogenesis after oviposition?

Anoga-AKH-I induced the mobilization of haemolymph sugar in sugar- and blood-fed females of *A. gambiae*, and since trehalose is the main haemolymph sugar used in mosquitoes and while no effect on lipid was detected, this neurohormone should be renamed to hypertrehalosaemic hormone, Anoga-HrTH. On the other hand, just like for the fourth locust AKH, no species-specific mobilization of lipid or carbohydrate was detected for mosquito decapeptide. However, Locmi-HrTH elevated the carbohydrate level in the American cockroach, *Periplaneta americana*, whereas again, no effect was found for Anoga-AKH-II. Similar tests on the lipid level with Anoga-AKH-I and -II in the migratory locust revealed only hyperlipaemia for the mosquito octapeptide. In addition using RNA interference to silence the Anoga-AKHR gene, no hypertrehalosaemia was induced by Anoga-AKH-I treatment after knocking down of the receptor gene expression: an indirect prove that Anoga-AKH-I acts through this GPCR.

The whole project was based on the findings that *A. gambiae* use lipid and carbohydrate during flight. Since it was known that AKHs can mobilize those flight metabolites, as shown in other insects, its influence on flight in *A. gambiae* was studied. Flight experiments with a flight mill system using intact and decapitated females showed that only Anoga-AKH-I induced a stronger flight performance during the first few hours. Decapitated females revealed a statistically significant boost in distance flown after Anoga-AKH-I injection, whereas in intact females only a trend of stronger flights compared to the control females could be detected. The flight tests with Anoga-AKH-II did not indicate differences from the saline treated females, which could be an indication that Anoga-AKH-II is not blocking the Anoga-AKHR and probably functions through another receptor.

KEY WORDS - MOTS CLÉS:

Adipokinetic, hypertrehalosaemic, hypotrehalosaemic, neurohormone, metabolism, flight, immunocytochemistry, radioimmunoassay, irradiation, mosquito.

Adipokinétique, hypertrehalosémie, hyperprolinémie, neurohormone, métabolisme, vol, immunocytochimie, radioimmunoassay, irradiation, moustique.

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1. INTRODUCTION

More than a century has passed since the discovery of the first hormones (Oliver and Schafer, 1895; Bayliss and Starling, 1902). Today we know that hormones are involved in the regulation of metabolism, water balance, reproduction, molting, metamorphosis, diapause, cast determination, and even puberty and aging. Almost every aspect of life in vertebrates and invertebrates is under endocrine control. One of the first identified hormone was adrenaline, a catecholamine, which is commonly known as the stress hormone (Oliver and Schafer, 1895; Takamine, 1901a, b). This hormone is secreted from the adrenal medulla in response to fear, anger, or stress, and is responsible for increasing the heart rate, blood pressure, cardiac output, and carbohydrate metabolism. At around the same time, Bayliss and Starling (1902) described the vertebrate polypeptide hormone secretin. This hormone is synthesized and released in the small intestine and activates the secretion of pancreatic juice. They showed for the first time that a ‘chemical messenger’ controlled a physiological function. These ‘messengers’ have been named hormones, a word derived from the Greek verb ‘ὀρμαίνω’ (ormao) meaning “to arouse to activity”. Generally, hormones are synthesized in glands, released into the body fluid and transported to the receptive target organ or tissue, and finally result in activation or inhibition of a further cascade (Starling, 1905a, b, c, d). What started out small with the study of just a handful of hormones is today a large branch of science known as endocrinology. The progressive understanding of the endocrine system in animals and humans has increased our knowledge of the physiological processes in health and disease. One well-known example is the synthetic manufacture of the vertebrate hormone insulin (reviewed by Dominguez and Licata, 2001), which allows people diagnosed with diabetes type I, a disorder of carbohydrate metabolism, to live a normal life. This genetic disfunction causes a significant reduction in insulin production, resulting in an excessive amount of glucose in the blood and urine. Only a strict low sugar diet or injection of synthetic insulin prevents excessive thirst, weight loss, and supplementary destruction of small blood vessels leading to such complications as infections and gangrene of the limbs, or blindness. Insulin was isolated in 1921 by two Canadian physicians Banting and Best (1990). Soon after, various types of injectable insulin were accessible to treat diabetes. Today hormones are used to treat many diseases and hormonal ‘tragedies or malfunctions’. For example, they can help to treat human infertility. Contrarily, one of the most used hormone treatments in the world is the birth control pill, a hormone cocktail of oestrogen and progesterone, which prevents ovulation (Pincus, 1958; Pincus et al., 1958).

1.1 INSECT ENDOCRINOLOGY

The successful endocrinological work on vertebrates dominated the work on invertebrates, which were long believed to lack a hormone-like system. It took nearly two decades, after Starling’s discovery for Kopeć (1917, 1922) to propose that a secreted factor from the insect brain was responsible for metamorphosis. The idea of an endocrine function in

neuronal cells was new and it took almost an additional two decades until Wigglesworth (1934, 1939) demonstrated that neuronal cells are able to synthesize hormones. On the assumption that a brain hormone (neurohormone) activates the release of a molting hormone, which is involved in the control of the metamorphosis, neuroendocrinology was born. Nevertheless, it took nearly another two decades until the first neurohormone, the brain hormone of the silkworm *Bombyx mori*, was crystallized (Kobayashi and Kirimura, 1958; Kobayashi et al., 1962). It then became much more apparent that almost every aspect of life in invertebrates (and vertebrates) is under hormonal control.

Endocrine work on insects allowed the use of various experimental methods that were not ethically feasible to practice on vertebrates. Insects and other invertebrates became the perfect organisms to study neuroendocrinology. The fact that insects can survive several brain operations and manipulations in the ventral nerve cord, and still stay alive for days after decapitation provided the possibility of several basic endocrine experimentations. Parabiosis, a common used method, where two or more individuals share the same “blood” circulation, established the evidence of the brain hormone in experiments with assassin bugs and giant silkworms (Wigglesworth, 1934; Williams, 1946, 1952; Figure 1.1). An additional fundamental experimental method, which was only possible to be performed in insects, was the ectomy of an endocrine gland or neurosecretory cell cluster to switch off the hormonal related effect. For the ‘revitalization’ of the endocrine switch, the reimplantation of the ectomized organ or tissue established the function. Ectomy and reimplantation were used to find the source of the brain hormone and the molting hormone of *Rhodnius* spp. and other insects (Wigglesworth, 1934, 1939).

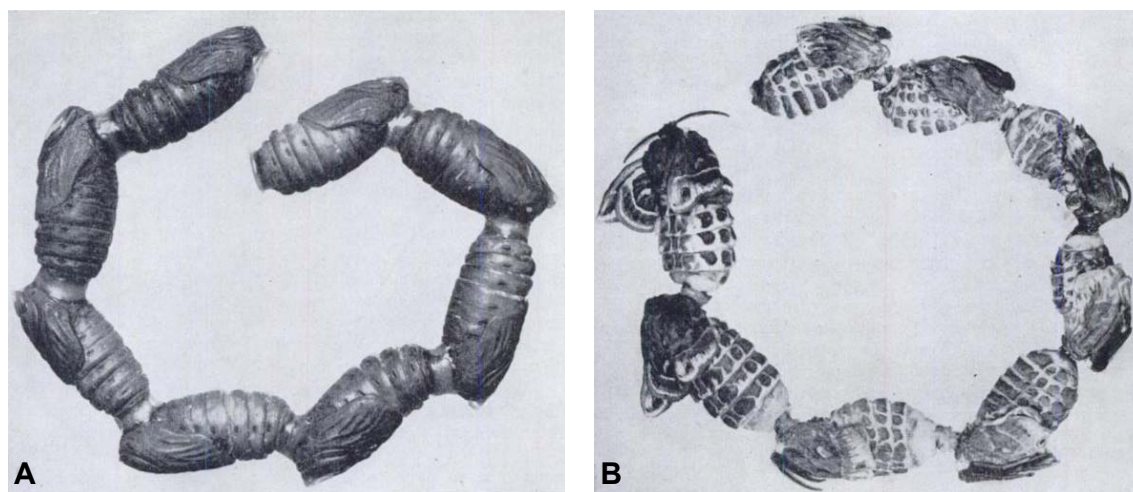


Figure 1.1: Parabiosis experiment of Williams (1952) with cecropia silkworms (*Platysamia cecropia*). The picture on the left (A) demonstrates a chain of debrained parabiosed pupae and on the right (B) the final metamorphosis after implantation of a single brain into the first pupa.

During the 1960s – 1980s, several neurohormones and hormones were discovered in insects. The basic endocrine regulation of metamorphosis in the tobacco hornworm, *Manduca sexta* and the giant silkworm, *Bombyx mori* was generally understood (Figure 1.2).

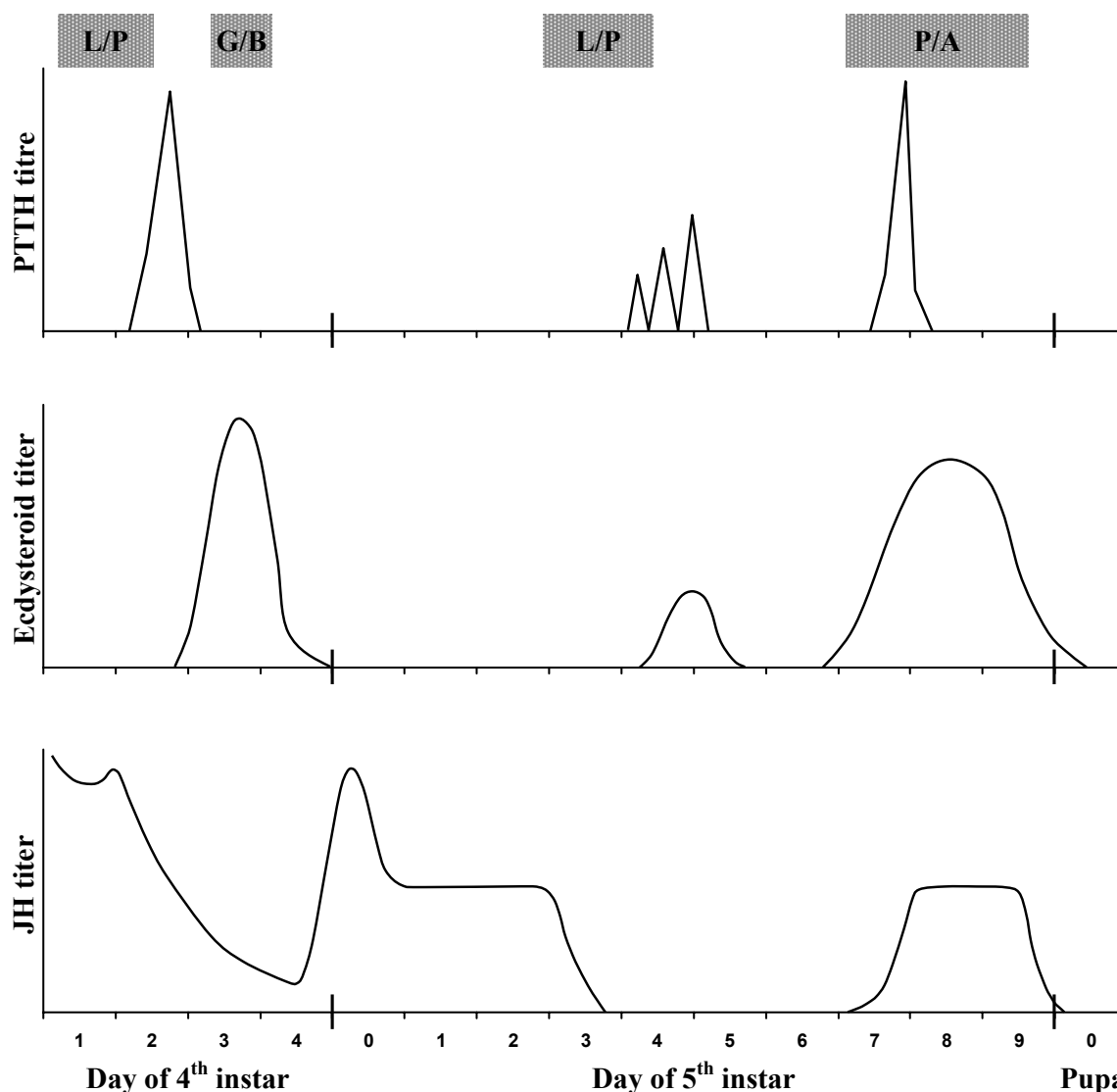


Figure 1.2: The juvenile hormone sensitive periods of the tobacco hornworm, *Manduca sexta* (last two larval instars before pupation). Relative hormone titres of PTTH (top), ecdysteroid (middle), and juvenile hormone titre (JH, bottom) are illustrated on a common daily time scale. The grey bars show the critical period for a different developmental stages: L/P, larval versus pupal determination; G/P, green versus black larval pigmentation; and P/A, pupal versus adult determination. If juvenile hormone is available, a PTTH peak is induced, which is responsible for the ecdysteroid peak. A larval molt follows. At the 3rd day in the fifth larval instar the juvenile hormone titre falls to zero, at this sensitive period the state of epidermis ‘status quo’ fails and finally the pupal and adult development starts, induced by the two ecdysteroid peaks (Based on Nijhout, 1994).

After the 1980s, many new techniques in chemistry, immunology, and molecular biology opened new doors in identifying and characterizing the function of hormones in several different species. In addition, the worldwide connection through the internet made it possible to use the online available genome databases of several organisms. Science experienced an unprecedented global connection. Soon after the completion of the human genome, the fruit fly, *Drosophila melanogaster* and the African malaria mosquito, *Anopheles gambiae* followed. Today several genomes of ‘medically important’ vertebrates and invertebrates are completed or are in progress, even our always-loyal friend the domestic dog, *Canis familiaris* is completed and available online (<http://www.ncbi.nlm.nih.gov>). More importantly, the basic local alignment search tool (blast, <http://www.ncbi.nlm.nih.gov/blast>) makes it possible to compare a known nucleotide or peptide sequence within all genome databases and can result in the discovery of similar sequences for the same or different species. The relationships between close hormone sequences help us to understand the evolutionary process in the different hormone families. Also, comprehension of the endocrine sources are crucial for the understanding the function and interaction of the endocrine system itself, which makes it absolutely necessary to know the anatomy of the animal being investigated.

1.2 INSECT HORMONE SYSTEMS

In insects, two different endocrine systems can be distinguished: the glandular and the neurosecretory system. In the glandular system, there are just two true endocrine glands known: the prothoracic glands and the corpora allata. Both glands are specialized for the synthesis and secretion of hormones, i.e. ecdysteroids in the prothoracic glands and juvenile hormones in the corpora allata. In the adult insect, ovaries and testes can also function as an endocrine gland. The other endocrine machinery is the neurosecretory system, characterized by a group of specialized neurons, the neurosecretory cells. Typically found in the central nervous system (brain and ventral nervous system), they produce neurohormones (small polypeptides) and neurotransmitters. The neuropeptides are released at the end site of the axon, called neurohaemal organ. Neurohormones often control the release of the hormones and vice versa. However, the nervous system also has a regulatory function in the endocrine system. The nervous and endocrine systems are closely linked and are strongly dependent on the developmental and physiological processes of the insect life (Figure 1.3).

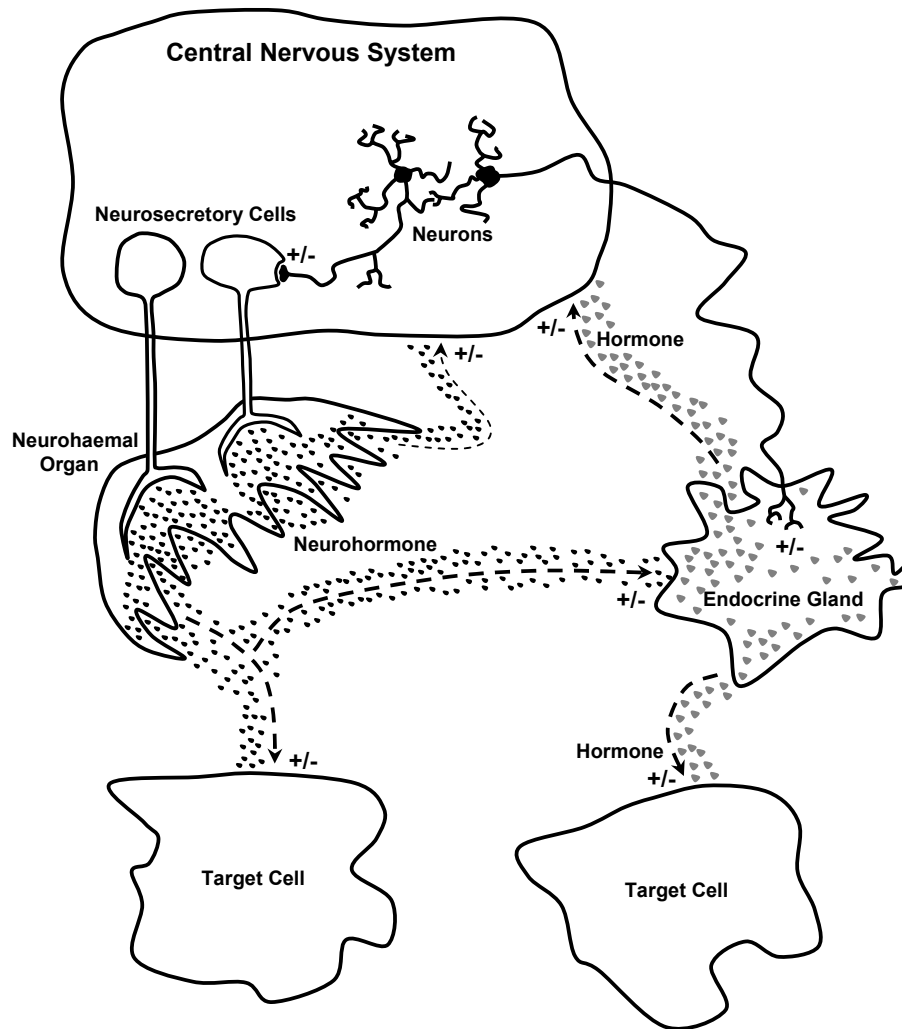


Figure 1.3: General scheme of the endocrine and nervous system in insects. Neurohormones, hormones, or neurotransmitters can react in a stimulation (+) or inhibition (-) of the endocrine gland or the target cell, but they can also be self-regulating (Based on Nijhout, 1994).

1.21 THE PROTHORACIC GLANDS

These endocrine glands, also called the head gland, molting gland, thoracic gland, or ventral gland, probably developed from a maxillary gland, an exocrine gland that evolved into an endocrine gland. In a few insects, part of the ‘head’ gland is still found in the head, but as its main name indicates, these glands are mainly situated in the pro-, but sometimes also in the mesothorax (Figure 1.4 A to D). In higher Diptera, the prothoracic gland fuses with the corpora cardiaca and corpora allata and form together the Weissmann’s ring (later known as the ring gland, Figure 1.4 E).

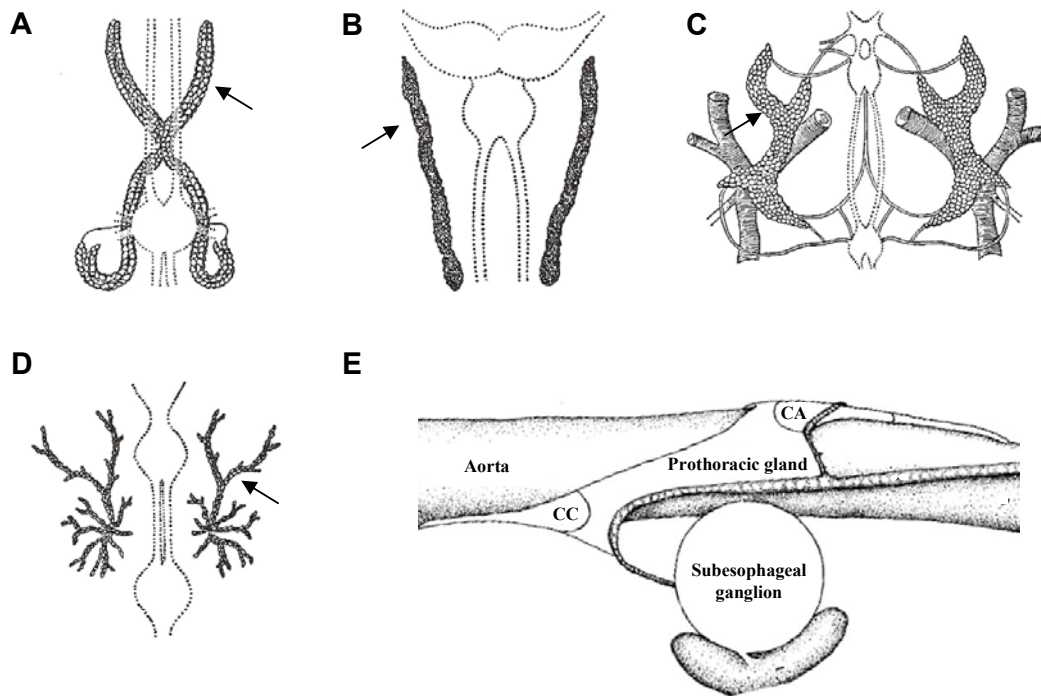


Figure 1.4: Various types of prothoracic glands (arrows): Blattodea (A), Hemiptera (B), Lepidoptera (C), Hymenoptera (D), and Diptera (E, prothoracic gland is enclosed in the Weissmann's ring gland together with the corpora cardiaca (CC) and corpora allata (CA) around the aorta); illustrations A-D provided by Novak (1975), and E by Meurant and Sernia (1993).

The morphology of the cells in the prothoracic gland is uniform throughout the insect orders (Sedlak, 1985; Nijhout, 1994). Generally the prothoracic gland is not present in the adult insect, except in insects that do not go through metamorphosis and instead still molt as an adult, e.g. the firebrat, *Thermobia domestica*, member of the wingless subclass Apterygota. Almost all other insects start with the apoptosis of these glands during or after the incomplete or complete metamorphosis. The prothoracic glands synthesize the ecdysteroids, which are not stored, but immediately secreted in the inactive α -form (α -ecdysone or ecdysterone). The hydroxylation to the active β -form (β -ecdysone or 20 hydroxy-(OH)-ecdysterone) takes place in the fat bodies. In the imagos of Pterygota, where the prothoracic glands are missing, the ovaries or testes are the sources of the ecdysteroids.

1.22 THE CORPORA ALLATA

The second true endocrine gland pair, the corpora allata, located in the neck region of insects, synthesizes and releases the juvenile hormones. Normally it is a pair of small glands (Figure 1.5 A to D, note, the CA is drawn out of proportion), but as mentioned above in larvae of higher Diptera the corpora allata is incorporated into the ring gland (Figure 1.4 E and 1.5 E). Serially arranged nerves connect the corpora allata with the brain and the corpora cardiaca

in between. Like the ecdysteroids in the prothoracic glands, the juvenile hormones are not stored in the corpora allata. Rich in mitochondria and endoplasmatic reticulum, the cells of the corpora allata are heavily interdigitated and form a compact organ which is surrounded by a membranous coating (Sedlak, 1985; Nijhout, 1994). The corpora allata also includes compounds of neurosecretory cells, but only their releasing sites. These neurohaemal organs in the corpora allata originate from the brain or the corpora cardiaca and regulate the juvenile hormone secretion activity. In a few species of Lepidoptera, the corpora allata also can act as a neurohaemal organ for neuropeptides coming from the brain.

1.23 THE NEUROSECRETORY SYSTEM

The neurosecretory system is located in the brain and ventral nervous system. Neurosecretory cells communicate with organs, different glands and the insect's periphery, or in some cases, they are self-regulating. Neurosecretory cells are different from neurons, because they do not release neurotransmitters from synaptic ends, but neurohormones from the neurohaemal organ. The general organization of a neurosecretory cell is illustrated in Figure 1.6. The synthesis of the neurohormones takes place in the perikaryon, the main cell body of the neurosecretory cell that contains the nucleus and organelles. The dendrites and collaterals are like those in the insect neuron, thus the neuropil, the grey matter of the brain, contains many axo-dendritic and axo-axonal contacts. The often very long axons branch in a pre-terminal bulge at their distal ending, the neurohaemal organ. The glial sheath around the neurosecretory cell and its axon supports their structure and provides insulation. There is less glial sheath found at the terminal end of the axon, where the neurohormones are released from the neurohaemal organ.

The peptide hormones are synthesized in the rough endoplasmatic reticulum of the perikaryon, and are later further processed in the Golgi bodies. Granules (carrier proteins) transport the active neuropeptides along the axon to its release site. In case of neurohormone overproduction, lysosomes reabsorb the abundance. The neurohormones are released by exocytosis into the haemocoel. The vesicles burst and release the neurohormones, the perikaryon then reabsorbs and reutilizes the used empty vesicles (Raabe, 1982; Nijhout, 1994).

The neurohaemal organ can also include hormone producing cells or glandular neurosecretory cells, like the adipokinetic hormone producing cells in the corpora cardiaca. Some insects even have the corpora cardiaca separated in a glandular and a storage part, i.e. in locusts (Figure 1.5 D), or the X-cells in mosquitoes (Kaufmann and Brown, 2006).

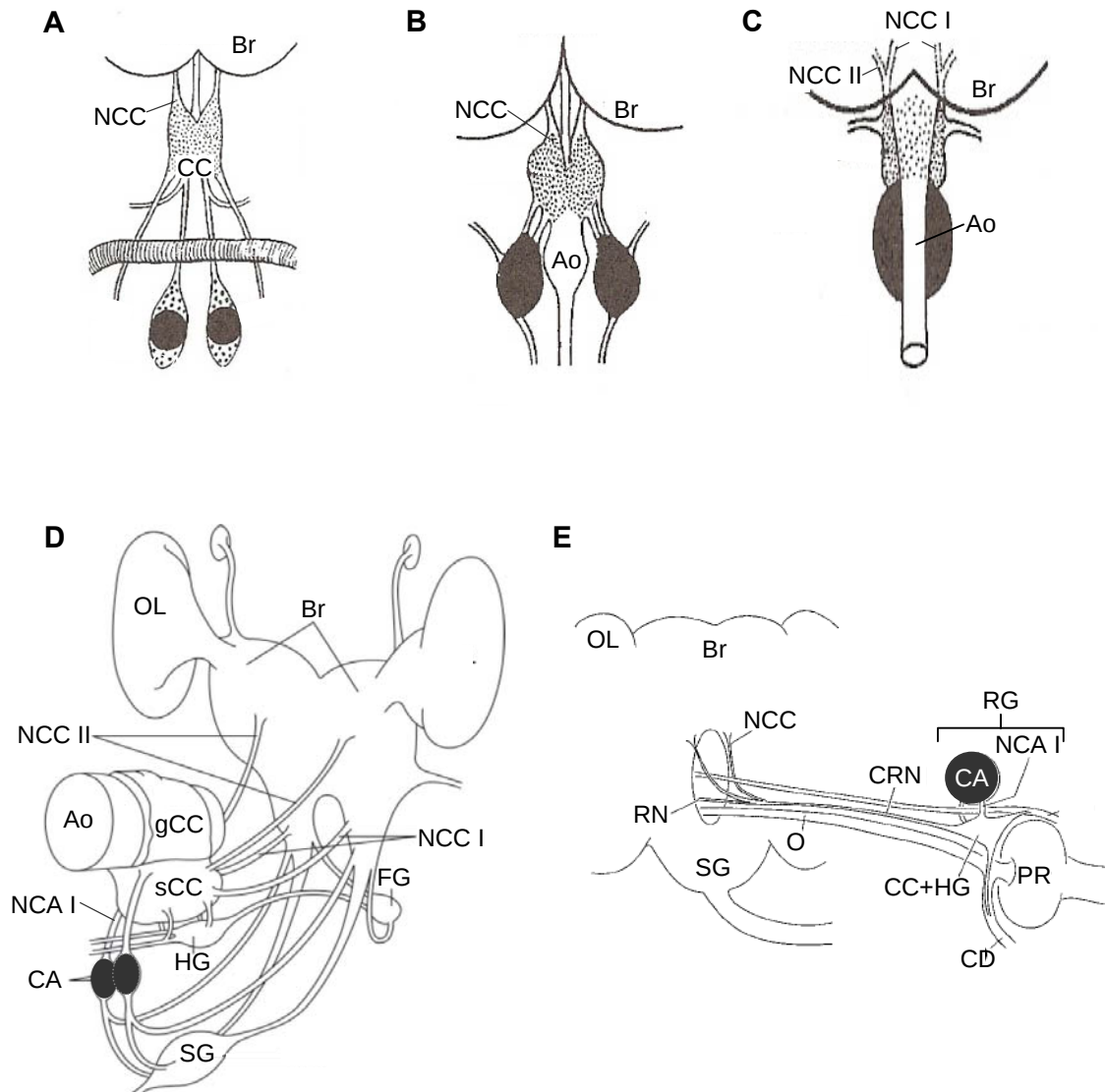


Figure 1.5: Location of the corpora allata (CA), and the corpora cardiaca (CC) in adult Diptera (A, mosquito), Blattodea (B, cockroach), and a hemipteran member (C). Below a more detailed adult brain anatomy of the desert locust, *Locusta migratoria* (D) and the blow fly, *Phormia terraenovae* (E). Abbreviations: aorta (Ao), brain (Br) corpora allata (CA), glandular (gCC) and storage (sCC) part of corpora cardiaca (CC), crop duct (CD), cardiac recurrent nerve CRN, frontal ganglion (FG), hypocerebral ganglion (HG), nervi corporis allati (NCA-I), nervi corporis cardiaci (NCC, NCC I, or -II), oesophagus (O), optical lobe (OL), proventriculus (PR), ring gland (RG), cardiac recurrent nerve (RN), and suboesophageal ganglion (SG). (Illustrations from Cymborowski (1992), A-C; Diederer et al. (2002), D; and Shiga (2003), E).

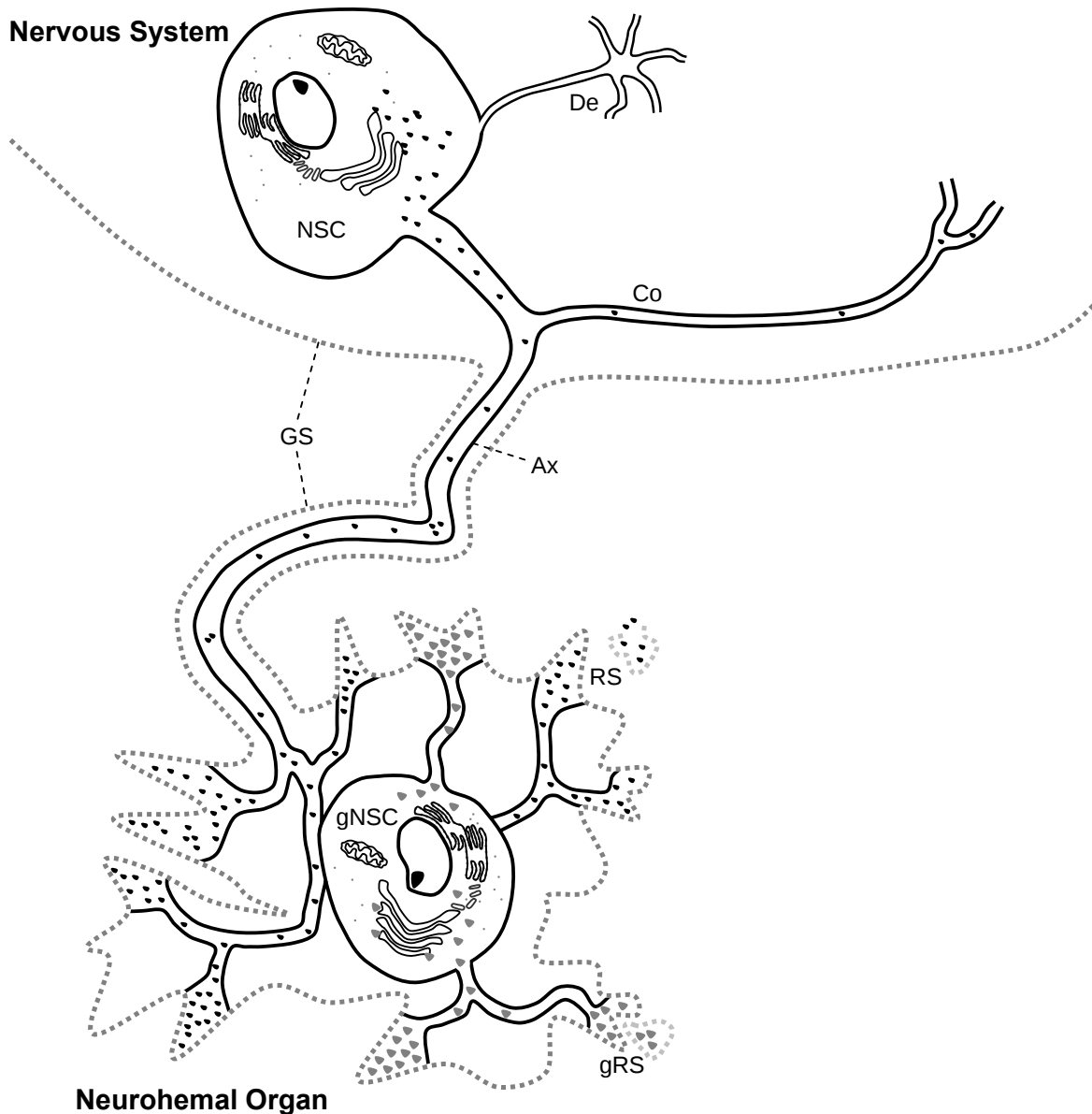


Figure 1.6: General structure of a neurosecretory cell (NSC) of the brain or ventral nervous system and the neurohaemal organ with a glandular neurosecretory cell (gNSC). In the perikaryon of both, the NSC and gNSC nucleus, rough endoplasmatic reticulum, Golgi apparatus, mitochondrion and other organelles are found. The neurohormones are illustrated in black and 'glandular'-hormones grey. The glial sheath (GS) insulates the nervous system (neuropil) and protects the axon (Ax), which connects the NCS with the neurohaemal organ. The glial sheath is reduced at the release site of the neurohormone, or hormone respectively (RS/gRS). NSC have like other neurons collaterals (Co) and dendrites (De). Illustration is based out of Raabe (1982).

1.231 DISTRIBUTION OF NEUROSECRETORY CELLS IN THE BRAIN

The amphophilic nature of neurosecretory cells, and the use of basic and acidic histochemical staining methods, makes it possible to stain the neurosecretory cells and axons, revealing their distribution in the brain and ventral nerve cord. Dyes like chrome-haematoxylin-phloxine, paraldehyde-fuchsin, or victoria blue (and many more) are used to identify neurosecretory cells by staining the neuropeptides, the granulas, tissues, nuclei and organelles. These staining methods involve a basic dye (e.g. chrome-haematoxylin), which fixes on the acidic structures, like nuclei, ribosomes and negative charged proteins, and an additional acidic dye (e.g. phloxine), which fixes on the basic structures, like mitochondria, collagen and positive charged proteins (Raabe, 1982). Neurosecretory products are generally basic and therefore stained by acidic dyes. Although basic and acidic dyes separate neurosecretory cells in to three to five different groups, these staining methods cannot be used to tell what kind of neurohormone is secreted. Immunocytochemistry can help to find the distribution and the cell source of a known neurohormone, but only if a specific antiserum for the peptide hormone exists.

The highest number of neurosecretory cells occurs in the brain and thus the important release sites are connected to and controlled by the brain. This complex, also known as the 'brain-retrocerebral neuroendocrine complex', demonstrates a close interaction between neurosecretory cells, neurohaemal organs, the endocrine glands, and the central nervous system (Figure 1.3). Together they manage the regulation of the synthesis, release, and inhibition of the hormones.

Generally, the insect brain is divided into three regions called the protocerebrum, the deutocerebrum, and the tritocerebrum (Figure 1.7). Collaterals, and possible synaptoid contacts, maintain coordination between different neurosecretory centres in the brain. The protocerebrum has two lobes and contains the pars intercerebralis in the middle. Each lobe is divided into the main and lateral protocerebrum. The vision (optic lobes and ocelli, if available) is also associated with the protocerebrum. Most neurosecretory cells are found in this part of the brain. The deutocerebrum contains the antennal lobes and processes the sensory information collected by the antennae. The tritocerebrum, together with the stomodaeal nervous system, regulates the function of the mouthparts, and links the brain with the rest of the ventral nerve cord. The stomodaeal nervous system, which is also referred to as the stomatogastric nervous system, contains the frontal, hypocerebral and the suboesophageal ganglion, and is linked to the tritocerebrum by the frontal commissure, the recurrent nerve, and the circumoesophageal connectives.

Some of the neurosecretory cell clusters in the brain (illustrated in Figure 1.7) are named due to their location. The median neurosecretory cells are found in the protocerebrum and normally have one or two paired clusters of cells on each side of the brain lobes. Their axons cross over to the other brain lobe through the deutocerebrum and the tritocerebrum, into the collateral located internal nervus corporis cardiaci or NCC-I, which runs to the corpora cardiaca and the thoracic endocrine organs. The anterior neurosecretory cells found in the pars

intercerebralis also contain two clusters of cells. Their axons do not cross over, but connect ipsilateral into the NCC-I. The other axonal bifurcation runs into the circumoesophageal connective and into the ventral nerve cord. One cluster of the intermediate neurosecretory cells is found between the median neurosecretory cells. Their axons run exclusively contralateral in the circumoesophageal connective posterior to the ventral nerve cord. The lateral neurosecretory cells show one or two clusters of cells and are situated dorsally and laterally of the protocerebrum in each brain lobe. Their axons do not cross over to the other lobe, but run together into the ipsilateral located external nervus corporis cardiaci or NCC-II, which runs towards the corpora cardiaca, like NCC-I. A third nervus corporis cardiaci, the NCC-III connects to neurosecretory cells found in the tritocerebrum. All three NCCs are bundled into the nervus corporis cardiaci (NCC), which runs in the direction of the corpora cardiaca. The corpora cardiaca act as an enormous neurohaemal organ, but not exclusively, it also contains neurosecretory cells and neurons. A last mentioned group of neurosecretory cells are the ventral neurosecretory cells found in the ventral and dorsal part posterior of the tritocerebrum. These neurosecretory cells are not found in all species, but in larvae of *M. sexta*, their axons run to the ventral nerve cord and have their release site at the terminal ganglion.

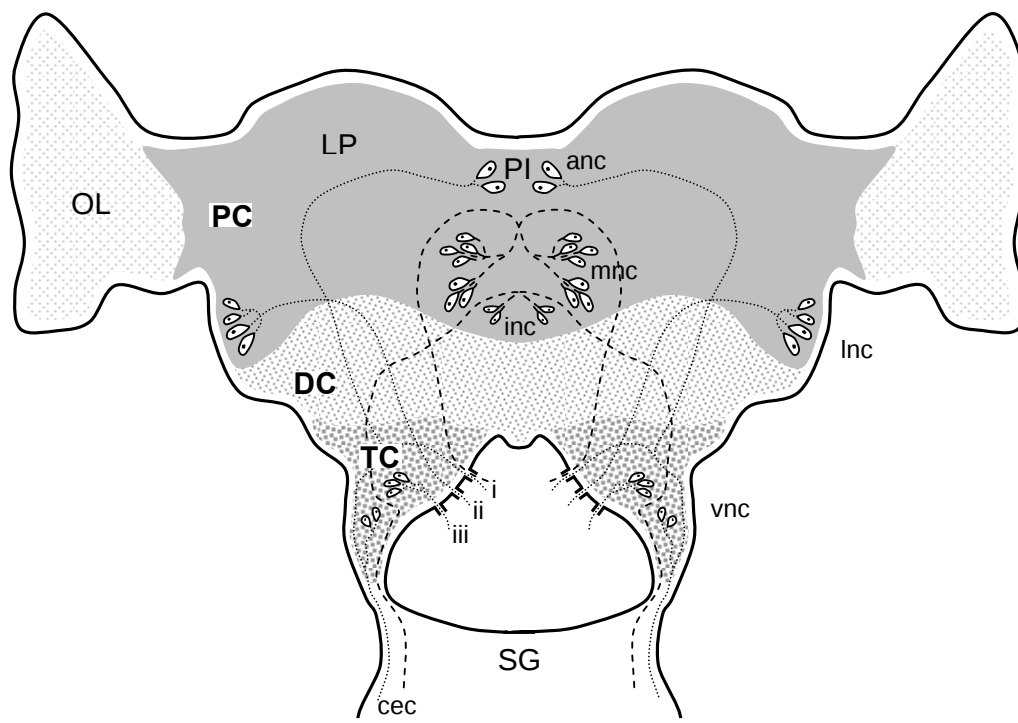


Figure 1.7: Distribution of the anterior (anc), median (mnc), intermediate (inc), lateral (lnc), and ventral (vnc) neurosecretory cells in an insect brain. The brain is divided into proto- (PC), deuto- (DC), and tritocerebrum (TC). Abbreviations: Cec, circumoesophageal connective; i, ii, and iii, nervous corpori cardiaci I, II, and III; OL, optical lobe; LP, lateral protocerebrum; PI, pars intercerebralis; and SG, suboesophageal ganglion. Ipsilateral axons are illustrated as dotted lines and contralateral as dashed lines (After Raabe, 1982; Clements, 1992; Nijhout, 1994).

1.232 CORPORA CARDIACA

The corpora cardiaca is named after the location of its releasing site, which is associated with the insect hearts. The corpora cardiaca is a conglomeration of several neurohaemal organs coming mainly from the brain, but also from the stomodaeal nervous system. The nervous corpori cardiaci (bundle of NCC-I, -II, and -III) runs from the brain to the corpora cardiaca. This large neurosecretory 'cable' ends in the form of the main insect neurohaemal organ, the corpus cardiacum (Figure 1.5 and 1.7). The release of the neurohormones from the protocerebrum (anterior, median, and lateral neurosecretory cells) and the tritocerebrum takes place mainly from this organ. The neurotransmitters released from synaptic connections, and the neurohormones released from intrinsic neurosecretory cells of the corpora cardiaca itself, can both have regulatory function on the corpora cardiaca. The hypocerebral ganglion or the recurrent nerve (depends on species) connects the stomodaeal nervous system and corpora cardiaca, and in some cases the hypocerebral ganglion and the corpora cardiaca are fused together. As mentioned above, the corpora cardiaca also is the source of intrinsic neurosecretory cells, which synthesize and release hormones locally. Some members of Orthoptera (and some Diptera) have the corpora cardiaca split into two different parts, a glandular and a storage lobe (gCC and sCC in Figure 1.5 D). The storage part contains two compartments, a neuronal one located to the haemocoel site and a neurohaemal site connected to the aorta (Vullings et al., 1995). The glandular lobe has neurohaemal organs, but also intrinsic neurosecretory cells, in which the main source of adipokinetic hormones synthesis is found (Diederer et al., 2002). These and other hormones are released from the corpora cardiaca into the aorta directly in the haemolymph. Other intrinsic neurosecretory cells of the corpora cardiaca have axons running towards the corpora allata. The connection between these two organs is called the nervi corporis allati I (NCA-I, Figure 1.5 D and E). The secretions from the corpora cardiaca may have regulatory function, but transection of the nervi corporis allati I did not affect the function of the corpora allata. Some members of Lepidoptera and Orthoptera contain a second nervi corporis allati, the NCA-II, which does not connect to the corpora cardiaca, but to the suboesophageal ganglion.

So far, there has been no evidence found to show that there are any neurohaemal organs coming from the ventral nervous system, connecting to, and releasing from the corpora cardiaca. However, in the ventral nerve cord, most neurosecretory cells either have a neurohaemal organ locally located around the ganglia, or extend their axonal connection that ends into a perisymphatic organ at the periphery.

1.24 NEUROENDOCRINE SYSTEM IN THE VENTRAL NERVOUS SYSTEM

The anatomy of the rope ladder like ventral nerve cord starts with the suboesophageal ganglion, then the thoracic ganglia, followed by the abdominal ganglia, and at the end the terminal abdominal ganglion (Figure 1.8 A). The connection between the brain and the ventral nerve cord is through the suboesophageal ganglion, which is often closely combined with the brain. In the thorax, there are three ganglia, one for each pair of legs. Noticeably, they are not

only responsible for a functional leg apparatus, but they also regulate the flight muscles and like all ganglia, they contain neurosecretory cells. Higher developed species show a fusion of the thoracic and/or abdominal ganglia. For example in most Culicidae, the thoracic ganglia are fused together, but each abdominal segment still contains one abdominal ganglion, except in the terminal ganglia where fusion is also found (Figure 1.8 B). Contrarily, in the housefly, *Musca domestica* all thoracic and abdominal ganglia are fused together (Figure 1.8 C). As mentioned above, all ganglia are sources of several various neurosecretory cells types. Most neurosecretory cells end in the segmental analogues of the corpora cardiaca, the perivisceral organs, also called the perisymphathetic organs, but some act locally on neighbouring cells or are of self-regulating nature. The axons of the perisymphathetic organs normally start on the posterior or anterior site of the ganglion and run out to the lateral periphery (Figure 1.8).

1.25 THE NERVOUS AND ENDOCRINE SYSTEM

The endocrine and nervous systems interact closely to regulate many animal life processes. These two systems have a regulatory nature and function with a messenger system that interacts with a specific target location. The nervous system is mainly responsible for the short-term regulation and action, whereas the endocrine system functions as a long-term regulator. Influences from the outside (i.e. temperature, humidity, water, characters of colours and surfaces, food sources, or light and darkness) generally activate the insects short-term system first. Insects have to act quickly, if a predator is approaching, or if the wind is changing during flight. Several neurons link receptors on the outside of the insect to the inside; e.g. mechanoreceptors detect wind changes by contact receptors, and insects are able to smell with olfactory chemoreceptors, located in the antennae and palpi. Thermo-, and hygroreceptors, and naturally the complete visual apparatus, with the compound eyes and the ocelli, recognize other kinds of messages from the outside. However, often after a short-term reaction, the endocrine response follows, showing that the nervous and endocrine system are dependent on each other. During and after an escape of a predator, hormones regulate the metabolism, e.g. by mobilizing metabolites for the leg or flight muscles and they are also responsible for resorting the storage nutrients after the succeeded escape, or initiate wound healing after a fight. Hormones can also cause a hunger response due to the lack of reserves. There are many behaviours that are under hormonal control, without mentioning the endocrine interactions that take place after mating, feeding, or during the gonotrophic cycle in haematophagous insects like mosquitoes.

1.26 THE MIDGUT ENDOCRINE SYSTEM

Often forgotten, the digestive tract in invertebrates and vertebrates is one of the largest endocrine sources. However, there has not been many reports published for invertebrates. In insects, the distribution of these endocrine cells was demonstrated in larvae, pupae, and imago of a diverse number of insects (Brown and Lea, 1988; Brown et al., 1999; Stanek et al., 2002; Garczynski et al., 2005; Garczynski et al., 2006). Antibodies, which were used in vertebrates

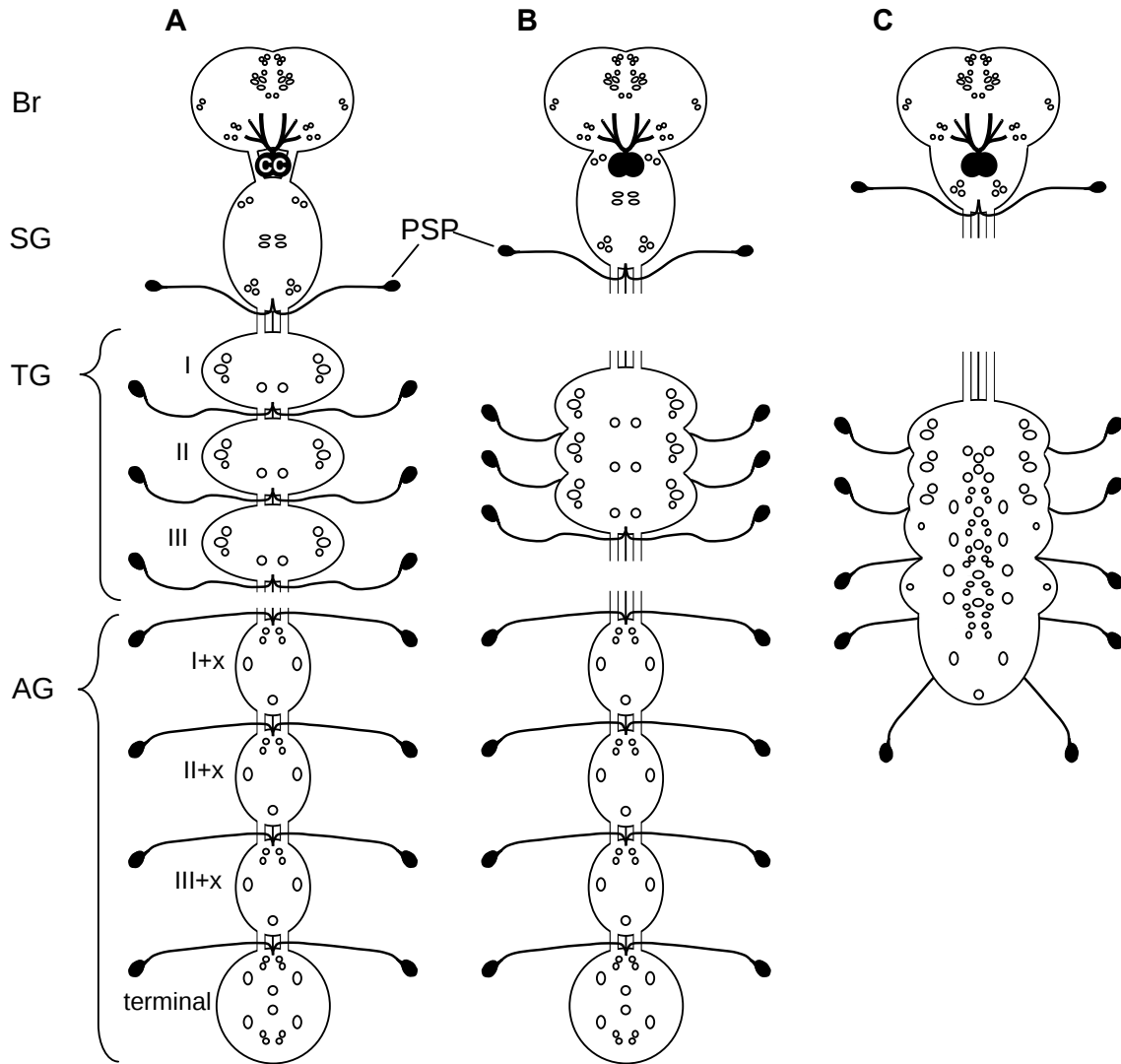


Figure 1.8: General arrangement of the neurohaemal organ system (black swellings). The corpora cardiaca (CC) are connected to the brain (Br) by the nervus corporis cardiaci. Neurosecretory cells from the ganglia run into axons that end in the perisymphathetic neurohaemal organ (PSP). The first illustration (A) shows the general neurohaemal-secretory system. The subesophageal ganglion (SG) fuses with the brain in more evolved insects, and the thoracic ganglia (TG, I, II, III) tend to fuse together (B, e.g. mosquitoes). Often the terminal abdominal ganglion is a fusion of two or three abdominal ganglia. In higher Diptera, the thoracic and abdominal ganglia (AG, varies in numbers) are fused together (C, e.g. house flies). Figure illustrated after Raabe (1982) and Nijhout (1994).

showed staining for several mammalian-like hormones, as shown for insulin, glucagons, somatostatin, β -endorphins and more. Recently it was demonstrated that neuropeptide F is found to be expressed in the brain and midgut (Brown et al., 1999; Stanek et al., 2002;

Garczynski et al., 2005). Neuropeptide F is involved in food intake and digestion. Another study showed that cardio accelerating hormone (CCAP) is secreted from midgut cells and is most probably involved in the release of α -amylase a digestive enzyme (Sakai et al., 2006). It is well reported that tachykinin, AKHs and other neuropeptides can induce midgut muscle contraction (Klowden, 2002), but no evidence has yet been found as to whether these neurohormones also activate the release of hormones that originate from the digestive tract.

1.3 INSECT HORMONES

Identical hormones can demonstrate different effects in the same organism, organ, or tissue. The determining factor is not only the messenger, but also the receptor, the genetics, and the biochemical machinery of the target cell. The pre-programmed target cell gives a specific and stereotypic response when the messenger (ligand) connects to the specific receptor. The same hormone can have an activating or inhibiting nature in the same tissue at different times or activates different enzymes, depending on the developmental stage. Such a typical example is the juvenile hormone, which maintains the status quo (existing condition) of the larval epidermis, but is also an important player in the vitellogenesis (egg protein production and uptake), and several more functions (some mentioned later). Often, the availability of specific receptors is the key for the stereotypic response.

Two different types of hormones can be distinguished, the non-polar, lipid hormones and the polar proteinaceous, peptide hormones. They have different surface and solubility conditions, and can connect at different places to the target receptor. Lipid hormones penetrate through cell membranes and act with receptors in the nucleus at the genome, whereas the peptide hormones cannot penetrate membranes and only interact with receptors on the cell membrane (Figure 1.9).

1.31 LIPID HORMONES

The non-polar and lipophilic nature of the lipid hormones makes it possible for them to pass through the cell membranes into the nucleus, where they often act directly at the chromosomal level. When bound to a nuclear receptor, lipid hormones activate or inhibit the expression of a specific gene. The regulation on the transcriptional level results in a physiological feedback with the consequences of a *de novo* synthesis of regulatory proteins, enzymes, or the disappearance of previously active proteins (Figure 1.9 A). The two main lipid hormones in insects are the ecdysteroids and the juvenile hormones.

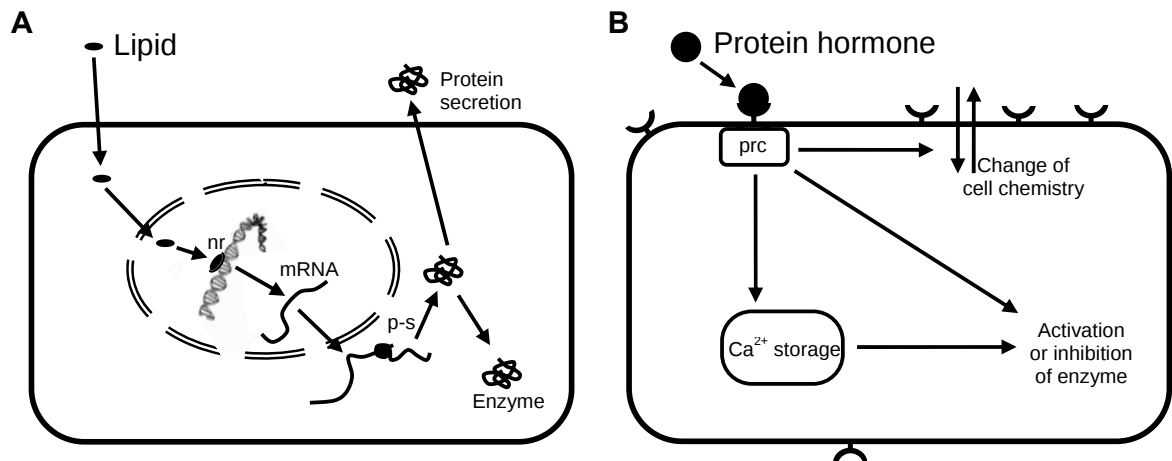


Figure 1.9: General mechanism of the action of a lipid (A), or protein (B) neurohormone. The lipid hormone penetrates through the cell membrane and nucleus membrane. It then binds to a nuclear receptor (nr) and activates or inhibits the transcription of a protein. In case of protein/enzyme synthesis (p-s) the protein can act in the cell itself or, by secretion, take action elsewhere in the organism. The protein hormone binds only to the cell membrane receptor (prc, protein hormone receptor complex), which activates or inhibits the already existing enzymes, or modifies the cell chemistry by ionic or osmotic changes (Nijhout, 1994).

1.311 ECDYSTEROID

Based on the prediction that a neurohormone controls the molting process (Kopeć, 1917, 1922), Hachlow (1931) demonstrated that the brain hormone was not the only factor responsible in the molting process. In experiments with pupae of the order Lepidoptera, it was realised that the thorax contains a gland that is essential for molting and metamorphosis, since only the pupae with the thorax developed into an adult. Later experiments demonstrated that the prothoracic gland was the endocrine source of the molting hormone (Wigglesworth, 1934, 1939; Fukuda, 1940). The molting hormone, today known as ecdysone, was the first structurally identified insect hormone (Butenandt and Karlson, 1954). Out of around 500 kg of *Bombyx mori* pupae, 25 mg ecdysone was purified. α -ecdysone and β -ecdysone were isolated, of which the latter represents the active form. Today over 60 different ecdysteroid analogues are characterized in insects. The synthesis of the inactive α -ecdysone takes place in the prothoracic gland. The precursor is a sterol, like cholesterol. Surprisingly, unlike most animals, insects cannot synthesize cholesterol from acetate precursors and have to get the cholesterol they require from their diet. Cholesterol is easily accessible for zoophagous insects, and especially for those of a haematophagous nature, whereas phytophagous insects have to convert campesterol, β -sitosterol, stigmasterol, or ergosterol into cholesterol. Phytophagous insects are also confronted with phytoecdysteroids (more than 100 known), which probably work as a natural insect repellent for the plants. The inactive α -ecdysone

penetrates through the cell membrane into the haemocoel. In the haemolymph, α -ecdysone binds rapidly to the ecdysone carrier protein that transports it to the fat body. The hydroxylation of α -ecdysone in the fat bodies results in the active β -ecdysone (Figure 1.10).

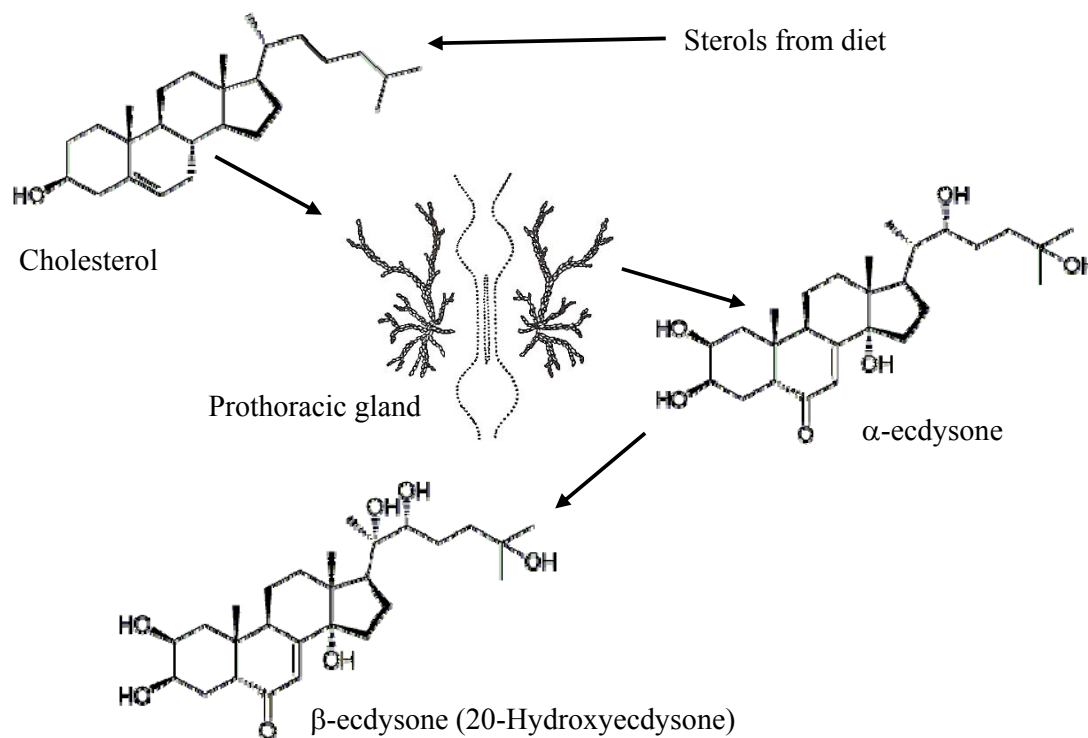


Figure 1.10: Structures of cholesterol, α , and β -ecdysone. Biosynthesis of ecdysteroids requires uptake of sterol within the diet. The main source are phytosterols (campesterol, β -sitosterol, stigmasterol, and ergosterol), or zoosterols (mainly directly cholesterol the precursor of ecdysteroids, but also coprostanol).

Experiments with *D. melanogaster* showed that ecdysteroids act directly on the transcriptional level. They cause two sequences of specific puffs in the giant polytene chromosomes (Figure 1.11). Puffs manifest the diffuse uncoiled regions of the polytene chromosome that are the sites of the RNA transcription. The first specific puffs induced by the ecdysteroids are responsible for the translation of the proteins that cause the second sequence of puffs. The translational products of the second puffs shut down the larval activities and make the insect ready for the pupal instar and the metamorphosis (Ashburner et al., 1974; Baehrecke, 1996).

In most Pterygota, the prothoracic gland degrades after adult life begins, and ecdysteroid production shifts into the ovaries or testes, but other sources also are described, like for example oenocytes. In the ovaries, the ecdysteroids are synthesized in the follicle cells and are incorporated into the eggs for the use during embryogenesis, or act on the fat body to activate the synthesis of yolk protein. In males, the steroid hormone is involved in

spermatogenesis, and acts like a sex hormone, as in mammals. However, the overall discussion on whether insects do have sex hormones, or not, is still a area of great controversy (de Loof and Huybrechts, 1998).

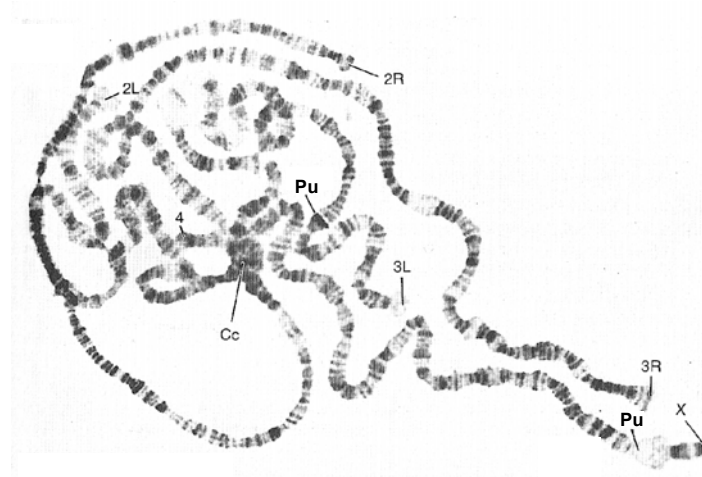


Figure 1.11: Polytene chromosome of *D. melanogaster* X, X-chromosome; Pu, Puff; Cc, centre of chromosome, 1-4 L/R, left or right arm of chromosome number 1-4. (light microscopic picture of Kloter and Gehring out of Wehner et al., 1995).

1.312 JUVENILE HORMONES

Wigglesworth (1934, 1939) first described the juvenile hormone's effect of inducing a status quo in the larval epidermis. However, juvenile hormones cause the incessant expression of larval characteristics and not the firstly presumed inhibition of metamorphosis. Conversely, the name 'juvenile hormone' certainly proved to be a misnomer after it was found that it has multiple effects and a wide variety of functions in an insect's life. The JHs (abbreviation found in the literature) influence development, reproduction, diapause, polyphenism, and behaviour. Juvenile hormones are probably the most adaptable hormones in insects. The only source of this lipid hormone is in the corpora allata. As a non-polar hormone, it can diffuse through cell membranes and acts directly at the transcriptional level like ecdysterone, but juvenile hormones can as well act through receptors on the outer cell membrane, like a peptide hormone (Figure 1.9). Juvenile hormones are methyl esters with a terminal or subterminal epoxide group. Röller et al. (1967) determined its sesquiterpenoid structure and today five structurally related forms are known in insects (Figure 1.12). The released juvenile hormone binds immediately with the juvenile hormone binding protein, which is responsible for the transport, but also protects the hormone from degradation by esterases in the haemolymph.

The nervous system controls the synthesis of juvenile hormones, but the secretion into the haemolymph is controlled by neuropeptides. Allatotropins are responsible for the release of the juvenile hormone, and like the nervous system, they also have a positive effect on its synthesis. Allatostatins, the antagonist of the allatotropins are of an inhibiting nature and stop

the juvenile hormone release from the corpora allata. Another polypeptide, known as allatoinhibin, inhibits the juvenile hormone release in *Manduca* spp. nonreversibly (Bhaskaran et al., 1990).

The switchover to the pupal commitment is one of the major roles of the juvenile hormone in holometabolous insects. The required ecdysteroid peak only occurs in the absence of juvenile hormone (Figure 1.2) and the reprogramming from larval to pupal molt and the complete metamorphosis is induced. In hemimetabolous insects, this mechanism is also described.

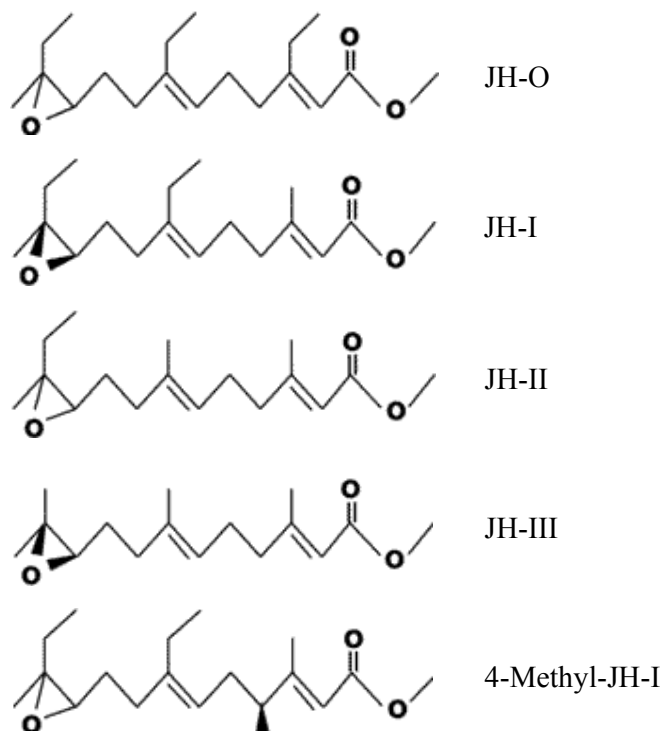


Figure 1.12: Structure of the five common juvenile hormones. JH-O $\equiv$ C-19 JH (contains 19 carbons in the structure); JH-I $\equiv$ C-18 JH; JH-II $\equiv$ C-17 JH; JH-III $\equiv$ C-16 JH; and 4-Methyl-JH-I $\equiv$ C-19 JH (Röller et al., 1967; Nijhout, 1994).

1.32 PEPTIDE HORMONES

Peptide hormones are small polypeptides and they cannot pass through cell membranes. Normally they act as a ligand on membrane receptors, which send the endocrine message further into the interior of a cell. The action of binding to a receptor instigates a conformational change that initiates a cascade, which ends in activation or inhibition of an enzyme, or in the change of the ionic level in the cytoplasm (Figure 1.9 B). Peptide hormones often bind to the G-protein coupled receptors (GPCR), which are widely dispersed in the animal kingdom. These GPCR's will send a signal transducer (second messenger) into the cell's interior, which activates pre-existing enzymes and proteins. Neuropeptides activate enzymes or proteins, which normally are already stored, whereas the lipid hormones on the

contrary are responsible for the new production of activators or inhibitors. Most known hormones in insects are peptide hormones, the following examples describe a list of important peptide hormones.

1.321 PROTHORACICOTROPIC HORMONE

This peptide hormone was the first described hormone in insects (Kopeć, 1917, 1922) and was named the brain hormone, but today more than one hormone source is known in the brain and it was renamed prothoracicotrophic hormone (PTTH). This polypeptide hormone interacts with the prothoracic glands and regulates the synthesis of ecdysteroids (Figure 1.13).

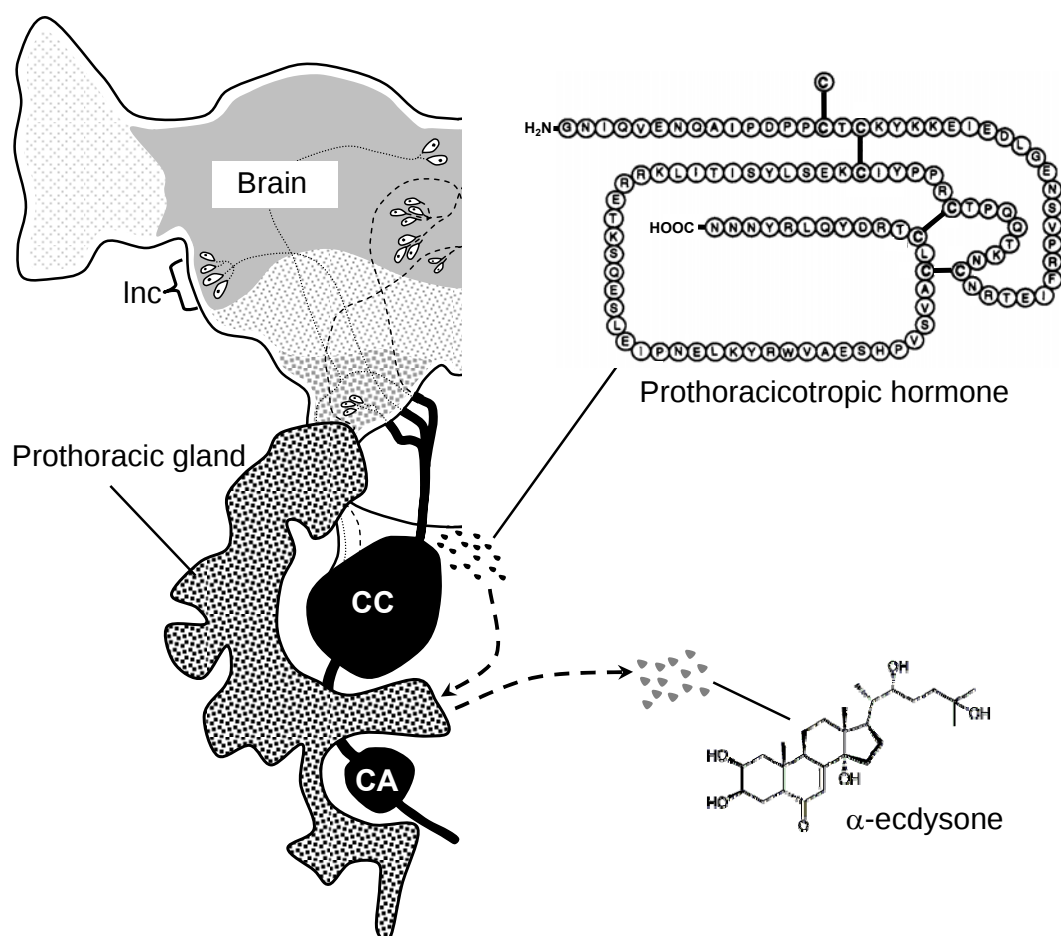


Figure 1.13: The prothoracicotrophic hormone (black dots) is synthesized in the lateral neurosecretory cells (Inc) and secreted from the corpora cardiaca (CC). Prothoracicotrophic hormone triggers the release of ecdysteroids (grey dots) of the prothoracic gland. Amino acid structure of *Bombyx mori* PTTH is given on the upper right (Ishibashi et al., 1994).

PTTH is synthesized in the lateral neurosecretory cells of the brain and is released from the corpora cardiaca, or in some species from the corpora allata into the aorta. In brain ectomized pupae, also referred to as “dauer” (German: long time) pupae, the lack of PTTH

keeps them in the pupal diapause. Such pupae can survive for up to 3 years until all nutrients are depleted. Reactivation of the prothoracic gland is only possible by injecting active PTTH or by connecting the debrained and intact pupa (parabiosis experiments of Williams, 1952), Figure 1.1). After stimulation of the prothoracic gland, ecdysteroid synthesis starts again, the pupal diapause ends, and the pupa undergoes the ‘normal’ adult development. *In vitro* experiments showed that PTTH is directly responsible for the ecdysteroid synthesis in the prothoracic gland (Bollenbacher et al., 1979).

Not many PTTHs were identified and characterized. In *B. mori* a small and big form was isolated. The small form showed a predicted effect in species like *Rh. prolixus*, but not in *B. mori*. Later it was renamed to bombyxin, which showed homology with the vertebrate insulin, but its effect in *B. mori* is not hypo-, but hypertrehalosaemic (Satake et al., 1997). Today bombyxin is a member of the insulin-like peptides, which are characterized in species of Orthoptera, Lepidoptera, and Diptera. The insulin-like peptides can affect developmental regulation, metabolism, longevity, and female reproduction (Wu and Brown, 2006).

1.322 DIURETIC AND ANTI-DIURETIC HORMONES

Most insects are terrestrial animals, which make them particularly susceptible to dehydration; therefore, they are more interested in maintaining water rather than expelling it. On the contrary, water living, herbivorous, and haematophagous insects are adapted to a water rich environment and are concerned with efficient water elimination. In general, diuretic hormones are responsible for the increased water loss, due to the enhanced secretion through the Malpighian tubes, and by inhibition of the reabsorption of water (Figure 1.14 A).

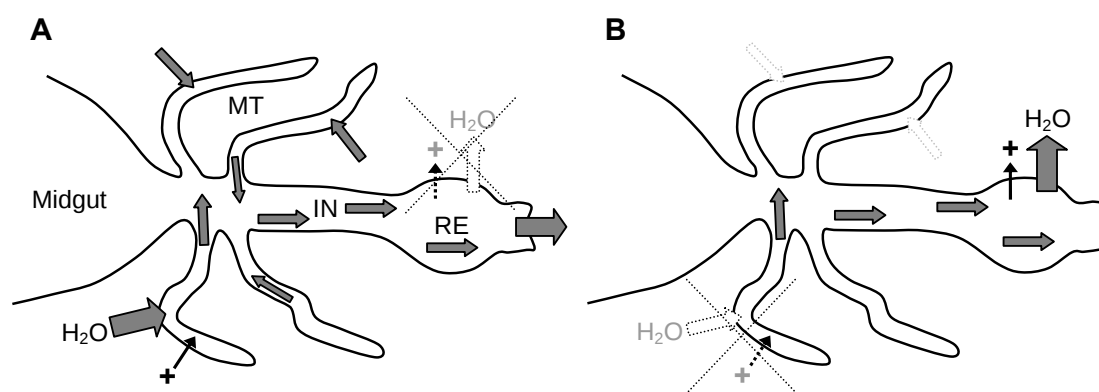


Figure 1.14: General illustration of the water balance in insects. (A) Induced by diuretic hormones and ionic pumps (+ ≡ cation pump, mostly a K^+/Cl^- pump) water is absorbed by the Malpighian tubes (MT), transported through the intestines (IN) and rectum (RE), furthermore excreted. (B) The reabsorption takes place in the rectum, enhanced by the anti-diuretic hormone (After Gäde et al., 1997).

The antagonists of the diuretic hormones, the anti-diuretic hormones inhibit the water excretion through the Malpighian tubes, and increase the reabsorption of water in the hindgut, especially in the rectum (Figure 1.14 B).

This water balance is closely linked with the insect's ion balance. Ion receptors in the Malpighian tubes pump ions from the haemolymph into their lumen and water is absorbed for excretion. Once the water is in the Malpighian tubes, it passes through the hindgut and the rectum, where it is finally excreted. To regain the water, insects reabsorb the water at the hindgut and rectum (Gäde, 2004; Schooley et al., 2005).

1.323 MUSCLE STIMULATING NEUROPEPTIDES

Proctolin, a pentapeptide, which functions as a neuromodulator and neurohormone, is involved in the contraction of the longitudinal muscles in the hindgut of the American cockroach, *Periplaneta americana* and also affects the visceral and skeletal muscle in a range of insect species (Isaac et al., 2004). This neuromodulator is found in the central and ventral nervous system. Another well-studied muscle stimulating neuropeptide group is the cardio-accelerating peptides. The CAPs (cardioactive peptides) are found in the abdominal ganglia and are released at the perisymphatic organs. These peptides not only accelerate heart muscle rate, but also activate the contraction of the hindgut (Tublitz et al., 1991) and play a crucial part in the ecdysis (see below and Figure 1.15). In addition, a cardioaccelerating effect is reported for members of the AKH-family (Orchard, 1987). It was also found that other neuropeptides, neuromodulators, or neurotransmitters have a cardioaccelerating effect, i.e. acetylcholine, 5-hydroxytryptamine, octopamine, neurohormone D*, periplanetins*, corazonin, and members of the leukosulfakinin family, as well as the above mentioned proctolin. However, this effect could occur as a secondary result, perhaps to enhance the transport in the haemocoel (Mordue and Morgan, 1985).

1.323 NEUROHORMONES ACTIVATE ECDYSIS AND SCLEROTIZATION

Ecdysis is a periodic shedding of the exoskeleton by arthropods, which allows growth. Juvenile hormone, prothoracicotropic hormone, and ecdysteroids are the regulators for keeping the larval status quo or initiating metamorphosis (Figure 1.2). In respect to maintaining the larval condition, ecdysis is the last crucial part for the next larval instar and is controlled by the eclosion hormone, the ecdysis-triggering hormone, and the crustacean cardioactive peptide. The eclosion hormone is synthesized in the ventral median neurosecretory cells (VM cells) of the protocerebrum and is released from the corpora cardiaca. The ecdysis-triggering hormone is synthesised and released from the endocrine cells located in the epitracheal glands, the inka cells. Both peptide hormones activate the release of each other. Before the actual ecdysis occurs, the release of ecdysis triggering hormone starts the pre-ecdysis, induced by ecdysteroid and other cuticular related cues (Figure 1.15).

*) Neurohormone D and periplanetins are found to be similar to the AKHs found in *Periplaneta americana*

Once the ecdysis-triggering hormone is released, a hormonal positive feedback mechanism occurs until the right proportion of ecdysis triggering hormone and eclosion hormone is reached, then the crustacean cardioactive peptide is secreted, thus activating the ecdysis program. After the successful ecdysis, the hardening of the cuticle follows. This tanning processes, called sclerotization, is activated by bursicon. This neuropeptide is synthesized in the same neurosecretory cells (located in the ganglia) as the crustacean cardioactive peptide, and is secreted into the haemocoel from the perisymphathetic organs. It is not yet known what activates bursicon and how it is activated, but it is vital that the tanning takes place after the old cuticle is shed (Mesce and Fahrbach, 2002).

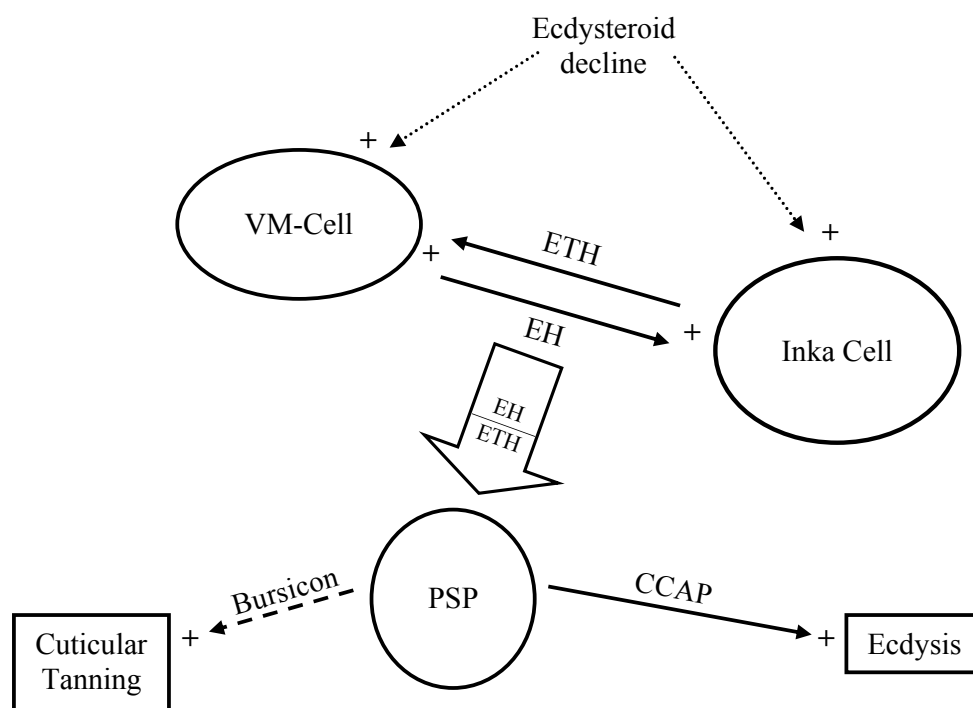


Figure 1.15: Graphic illustration of the hormonal positive feedback mechanism of the eclosion hormone (EH) and the ecdysis-triggering hormone (ETH). The decreased ecdysterone titre stimulates the VM-cells (release site at *corpora cardiaca*) and the inka cells to release EH and ETH respective. This release induces the additional secretion of EH and ETH (positive feedback) and at the right EH/ETH proportion the crustacean cardioactive peptide (CCAP) is released from the perisymphathetic organ (PSP). The crustacean cardioactive peptide activates the ecdysis motor program. Bursicon is synthesized in the same cell as CCAP and is also released from the perisymphathetic organ (Illustration according to Mesce and Fahrbach, 2002).

1.324 NEUROHORMONES ACTING AS NEUROTRANSMITTERS

The neurogenic amines are synthesised in neurosecretory cells and released at the neurohaemal organ and they can act as a neurohormone, -transmitter, or -modulator. Two well-identified neurogenic amines are octopamine and tyramine (Figure 1.16). Both are involved in metabolic and behavioural functions and are the only known non-peptide transmitter/hormone in invertebrates (Roeder, 2005). Octopamine, which may be analogue to the vertebrate hormone adrenalin, has been proposed to be an modulator in the releasing cascade of the neuropeptides of the AKH-family (Orchard and Loughton, 1981a; Passier et al., 1995).

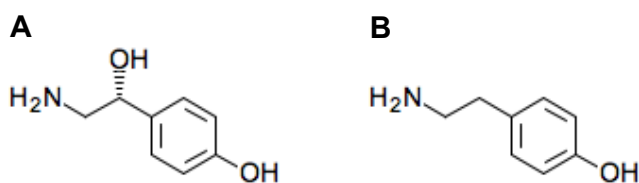


Figure 1.16: Structure of octopamine (A) and tyramine (B).

1.4 ADIPOKINETIC HORMONES

Adipokinetic hormones (AKHs) also known as hyperprolinaemic, or hypertrehalosaemic peptides – herein generically referred to as AKHs – are one of the most extensively characterized peptide hormone families in insects with more than 40 isoforms identified representing almost all insect orders (see reviews: Gäde et al., 1997; Gäde, 2004; Schooley et al., 2005). Mayer and Candy (1969) reported a hyperlipaemic effect by injection of corpora cardiaca extract into locust. In hyperlipaemia, the lipid content in the body fluid is elevated, also referred as an adipokinetic effect. Soon after, the purification and characterization of the first AKH in the migratory locust, *L. migratoria* was reported (Locmi-AKH-I; Stone et al., 1976a; b; Table 1.1). The classical AKHs are generally synthesized and secreted from a distinct region of the corpora cardiaca, found in all life stages. Prior to its secretion, they are modified at the amino and the carboxy termini to a pyroglutamic acid and amide, respectively; the two aromatic amino acid residues at position four (Phe/Try) and eight (Trp) typically are conserved (Table 1.1). Cells in the brain and ganglia may also synthesise and secrete such peptides; as shown in the mosquitoes *Aedes aegypti* (Brown and Lea, 1988), or *A. gambiae* (Kaufmann and Brown, 2006), and in other insect species (Schooneveld et al., 1983; 1985; Schooneveld and Veenstra, 1985), but none of those AKHs were proven to have any metabolic function and since they are also not synthesized in the corpora cardiaca, they are also referred to as non-classical AKHs (Siegert, 1999).

The evidence of multiple AKHs in the same species was first reported by Carlsen et al. (1979). Today it is known that each AKH has its own gene (Bogerd et al., 1995) and the number of AKHs in a particular insect varies from one, in phylogenetically lower insect orders such as Odonata, and up to four in orders like Blattodea and Orthoptera, whereas two or often only one in higher insect orders like Lepidoptera and Diptera. Why some insects evolved more than one AKH, is still a matter of speculation. In locusts they are probably crucial for the fine-tuning of the energy metabolism during migration (Vroemen et al., 1998); whereas in insects with no, or low migrating behaviour, AKHs might still play an important role, but may be underrepresented in their numbers. In Table 1.1, most of today's known AKHs are shown.

Table 1.1: List of known AKHs

Name:	Sequence:	Source	Order	Species	Reference:
Locmi-AKH-I	-pQLNFTPNGWGa--	CC	Orthoptera	<i>Locusta migratoria</i>	(Stone et al., 1976a; Bogerd et al., 1995)
	-pQLNFTPNGWGa--	CC	Orthoptera	<i>Schistocerca gregaria</i>	(Hekimi et al., 1989; Schulz-Aellen et al., 1989)
	-pQLNFTPNGWGa--	CC	Orthoptera	<i>Schistocerca nitens</i>	(Noyes and Schaffer, 1990)
	-pQLNFTPNGWGa--	CC	Orthoptera	<i>Melanoplus sanguinipes</i>	(Taub-Montemayor et al., 1997)
Locmi-AKH-II	-pQLNFSAGWa----	CC	Orthoptera	<i>Locusta migratoria</i>	(Siegert et al., 1985; Bogerd et al., 1995)
	-pQLNFSAGWa----	CC	Orthoptera	<i>Phymateus leprosus</i>	(Gäde and Kellner, 1995)
Locmi-AKH-III	-pQLNFTPWWa----	CC	Orthoptera	<i>Locusta migratoria</i>	(Oudejans et al., 1991; Bogerd et al., 1995)
Rommi-HrTH⁴⁾	-pQVNFTPNGWGa--	CC	Orthoptera	<i>Romalea microptera</i>	(Gäde et al., 1988)
Grybi-AKH	-pQVNFSTGWa----	CC	Orthoptera	<i>Gryllus bimaculatus</i>	(Gäde and Rinehart, 1987a)
	-pQVNFSTGWa----	CC	Orthoptera	<i>Acheta domesticus</i>	(Woodring et al., 1990; Cusinato et al., 1991)
	-pQVNFSTGWa----	CC	Orthoptera	<i>Gryllus sigillatus</i>	(Gäde, 1992a)
	-pQVNFSTGWa----	CC	Orthoptera	<i>Romalea microptera</i>	(Gäde et al., 1988)
Schgr-AKH-II	-pQLNFSTGWa----	CC	Orthoptera	<i>Schistocerca gregaria</i>	(Siegert et al., 1985; Gäde et al., 1986)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Schistocerca nitens</i>	(Gäde et al., 1986; Noyes and Schaffer, 1990)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Phymateus leprosus</i>	(Gäde and Kellner, 1995)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Phymateus morbillosus</i>	(Gäde et al., 1996)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Heterodes namaqua</i>	(Gäde, 1992a)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Acanthoproctus cervinus</i>	(Gäde, 1992a)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Libanasidus vittatus</i>	(Gäde, 1992a)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Anabrus simplex</i>	(Gäde et al., 1997)
	-pQLNFSTGWa----	CC	Orthoptera	<i>Dictyophorus spumans</i>	(Siegert et al., 2000)
Holal-AKH-II¹⁾	-pQLNFSTGWa----	CC	Hemiptera	<i>Holopterna allata</i>	(Gäde et al., 2006a)
Phyle-CC	-pQLTFTPNGWSa--	CC	Orthoptera	<i>Phymateus leprosus</i>	(Gäde and Kellner, 1995)
Phymo-AKH-I	-pQLNFTPNGWSa--	CC	Orthoptera	<i>Phymateus morbillosus</i>	(Gäde et al., 1996)
	-pQLNFTPNGWSa--	CC	Orthoptera	<i>Dictyophorus spumans</i>	(Siegert et al., 2000)

Phymo-AKH-III	-pQINFTPWWa----	CC	Orthoptera	<i>Phymateus morbillosus</i>	(Siegert et al., 2000)
	-pQINFTPWWa----	CC	Orthoptera	<i>Dictyophorus spumans</i>	(Siegert et al., 2000)
Polae-HrTH-I¹⁾	-pQLNFSPNwa----	CC	Blattaria	<i>Polyphaga aegyptiaca</i>	(Gäde and Kellner, 1989)
Bladi-HrTH	-pQVNFPSPGWGTa--	CC	Blattaria	<i>Blaberus discoidalis</i>	(Hayes et al., 1986; Lewis et al., 1997)
	-pQVNFPSPGWGTa--	CC	Blattaria	<i>Nauphoeta cinerea</i>	(Gäde and Rinehart, 1986)
	-pQVNFPSPGWGTa--	CC	Blattaria	<i>Leucophaea maderae</i>	(Gäde and Rinehart, 1990)
	-pQVNFPSPGWGTa--	CC	Blattaria	<i>Gromphadorina portentosa</i>	(Gäde and Rinehart, 1990)
	-pQVNFPSPGWGTa--	CC	Blattaria	<i>Blattella germanica</i>	(Gäde and Rinehart, 1990; Veenstra and Camps, 1990)
Peram-CAH-I	-pQVNFPSPNwa----	CC	Blattaria	<i>Periplaneta americana</i>	(Scarborough et al., 1984; Witten et al., 1984)
	-pQVNFPSPNwa----	CC	Blattaria	<i>Blatta orientalis</i>	(Gäde and Rinehart, 1990)
Lepde-HrTH-I ¹⁾	-pQVNFPSPNwa----	CC	Coleoptera	<i>Leptinotarsa decemlineata</i>	(Gäde and Kellner, 1989)
Tritr-HrTH-I ¹⁾	-pQVNFPSPNwa----	CC	Isoptera	<i>Trinervitermes trinervoides</i>	(Liebrich et al., 1995)
	-pQVNFPSPNwa----	CC	Isoptera	<i>Mastotermes darwiniensis</i>	(Liebrich et al., 1995)
Peram-CAH-II	-pQLTFTPnwa----	CC	Blattaria	<i>Periplaneta americana</i>	(Scarborough et al., 1984; Witten et al., 1984)
	-pQLTFTPnwa----	CC	Blattaria	<i>Blatta orientalis</i>	(Gäde and Rinehart, 1990)
Lepde-HrTH-II ¹⁾	-pQLTFTPnwa----	CC	Coleoptera	<i>Leptinotarsa decemlineata</i>	(Gäde and Kellner, 1989)
Pyrp-APH-II ¹⁾	-pQLTFTPnwa----	CC	Hemiptera	<i>Pyrrhocoris apterus</i>	(Kodrik et al., 2002)
Polae-HrTH-II	-pQITFTPnwa----	CC	Blattaria	<i>Polyphaga aegyptiaca</i>	(Gäde and Kellner, 1995)
Tenar-HrTH	-pQLNFSTGWGa--	CC	Hymenoptera	<i>Tenthredo arcuata</i>	(Lorenz et al., 2001)
Trica-AKH-I	-pQLNFSTDwa----	genome	Coleoptera	<i>Tribolium castaneum</i>	(Amare and Sweedler, 2007)
Libau-AKH	-pQVNFTPSwa----	CC	Odonata	<i>Libellula auripennis</i>	(Gäde, 1990)
	-pQVNFTPSwa----	CC	Odonata	<i>Ceratogomphus pictus</i>	(Gäde et al., 1997; Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Pantala flavescens</i>	(Gäde et al., 1997; Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Ictinogomphus ferox</i>	(Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Aphylla angustifolia</i>	(Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Somatochlora metallica</i>	(Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Brachythemis leucosticta</i>	(Gäde and Marco, 2005)
	-pQVNFTPSwa----	CC	Odonata	<i>Crocothemis erythraea</i>	(Gäde and Marco, 2005)

Libau-AKH (cont.)	-pQVNFTPSWa----	CC	Odonata	<i>Crocothemis sanguinolenta</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Orthetrum julia</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Orthetrum cancellatum</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Pachydiplax longipennis</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Sympetrum infuscatum</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Sympetrum vicinum</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Trithemis arteriosa</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Trithemis dorsalis</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Trithemis stictica</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Trithemis annulata</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Tramea basilaris</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Libellula auripennis</i>	(Gäde and Marco, 2005)
	-pQVNFTPSWa----	CC	Odonata	<i>Libellula vibrans</i>	(Gäde and Marco, 2005)
Anaim-AKH	-pQVNFSPSWa----	CC	Odonata	<i>Anax imperator</i>	(Gäde et al., 1994)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna subpupillata</i>	(Gäde et al., 1997; Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Anotogaster sieboldii</i>	(Gäde et al., 1997; Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Epiophlebia superstes</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna cyaneac</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna grandis</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna juncea</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna mixta</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Aeshna minuscule</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Nasiaeshna pentacantha</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Anax speratus</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Anax junius</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Phyllomacromia bifasciata</i>	(Gäde and Marco, 2005)
	-pQVNFSPSWa----	CC	Odonata	<i>Syncordulia gracilis</i>	(Gäde and Marco, 2005)
Ilyci-AKH ¹⁾	-pQVNFSPSWa----	CC	Hemiptera	<i>Ilyocoris cimicoides</i>	(Gäde et al., 2007)
Letin-AKH	-pQVNFSPYWa----	CC	Hemiptera	<i>Lethocerus indicus</i>	(Gäde et al., in press)

Psein-AKH	-pQVNFTPGWa----	CC	Odonata	<i>Pseudagrion inconspicuum</i>	(Janssens et al., 1994)
	-pQVNFTPGWa---	CC	Odonata	<i>Ischnura senegalensis</i>	(Janssens et al., 1994)
	-pQVNFTPGWa----	CC	Odonata	<i>Allocnemis leucosticta</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Ellatoneura frenulata</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Lestes tridens</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Lestes plagiatus</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Chlorolestes fasciatus</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Chlorolestes tessellatus</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Chlorolestes umbratus</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Platycypha caligata</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Platycypha fitzsimonsi</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Calopteryx splendens</i>	(Gäde and Marco, 2005)
	-pQVNFTPGWa----	CC	Odonata	<i>Phaon iridipennis</i>	(Gäde and Marco, 2005)
Erysi-AKH	-pQLNFTPSWa----	CC	Odonata	<i>Erythemis simplicicollis</i>	(Gäde and Kellner, 1999)
Carmo-HrTH-I²/II	-pQLTFTPNWGta--	CC	Phasmatodea	<i>Carausius morosus</i>	(Gäde and Rinehart, 1987b; Gäde et al., 1992a)
	-pQLTFTPNWGta--	CC	Phasmatodea	<i>Sipyloidea sipylos</i>	(Gäde, 1989)
	-pQLTFTPNWGta--	CC	Phasmatodea	<i>Extatosoma tiaratum</i>	(Gäde and Rinehart, 1990)
Manto-CC	-pQVNFTSGWa----	CC	Mantophasmatodea (unidentified species)		(Gäde et al., 2005)
Emppe-AKH	-pQVNFTPNWa----	CC	Mantodea	<i>Empusa pennata</i>	(Gäde, 1991a)
	-pQVNFTPNWa----	CC	Mantodea	<i>Sphodromantis spp.</i>	(Gäde, 1991a)
	-pQVNFTPNWa----	genome	<i>Phthiraptera</i>	<i>Pediculus humanus</i>	(personal blast)
Placa-HrTH-I²/II	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Platypleura capensis</i>	(Gäde and Janssens, 1994)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Munza trimeni</i>	(Gäde and Janssens, 1994)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Cacama valavata</i>	(Veenstra and Hagedorn, 1995)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Diceroprocta semicincta</i>	(Veenstra and Hagedorn, 1995)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Magicicada septendecim</i>	(Raina et al., 1995)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Magicicada septendecula</i>	(Raina et al., 1995)
	-pQVNFSPSWGNa--	CC	Hemiptera	<i>Magicicada cassini</i>	(Raina et al., 1995)
Corpu-AKH	-pQLNFSPSWa----	CC	Hemiptera	<i>Corixa punctata</i>	(Gäde et al., 2007)

Pyrap-AKH-I	-pQLNFTPNa----	CC	Hemiptera	<i>Pyrrhocoris apterus</i>	(Kodrik et al., 2000)
	-pQLNFTPNa----	genome	Coleoptera	<i>Tribolium castaneum</i>	(Abdel-latif and Hoffmann, in press)
Melme-AKH	-pQLNYSPDWa----	CC	Coleoptera	<i>Melolontha melolontha</i>	(Gäde, 1991c)
	-pQLNYSPDWa----	CC	Coleoptera	<i>Geotrupes stercorosus</i>	(Gäde, 1991c)
	-pQLNYSPDWa----	CC	Coleoptera	<i>Pachnoda marginata</i>	(Gäde et al., 1992b)
	-pQLNYSPDWa----	CC	Coleoptera	<i>Pachnoda sinuata</i>	(Gäde et al., 1992b)
Tenmo-HrTH	-pQLNFSPNa----	CC	Coleoptera	<i>Tenebrio molitor</i>	(Gäde and Rosinski, 1990)
	-pQLNFSPNa----	CC	Coleoptera	<i>Zophobas rugipes</i>	(Gäde and Rosinski, 1990)
	-pQLNFSPNa----	CC	Coleoptera	<i>Onymacris plana</i>	(Gäde, 1994)
	-pQLNFSPNa----	CC	Coleoptera	<i>Onymacris rugatipennis</i>	(Gäde, 1994)
	-pQLNFSPNa----	CC	Coleoptera	<i>Physadesmia globosa</i>	(Gäde, 1994)
	-pQLNFSPNa----	CC	Coleoptera	<i>Decapotoma lunata</i>	(Gäde, 1995)
Scade-CC-I	-pQFNYSPPWa----	CC	Coleoptera	<i>Scarabaeus deludens</i>	(Gäde, 1997)
	-pQFNYSPPWa----	CC	Coleoptera	<i>Gareta nitens</i>	(Gäde, 1997)
	-pQFNYSPPWa----	CC	Coleoptera	<i>Onitis aygulus</i>	(Gäde et al., 1984)
	-pQFNYSPPWa----	CC	Coleoptera	<i>Onitis pecuarius</i>	(Gäde et al., 1984)
Scade-CC-II	-pQFNZSPVWa----	CC	Coleoptera	<i>Scarabaeus deludens</i>	(Gäde, 1997)
	-pQFNZSPVWa----	CC	Coleoptera	<i>Gareta nitens</i>	(Gäde, 1997)
Trifa-CC³⁾	-pQINMTTGNa----	CC	Coleoptera	<i>Trichostetha fascicularis</i>	(Gäde et al., 2006b)
Declu-CC	-pQLNFSPNWGa--	CC	Coleoptera	<i>Decapotoma lunata</i>	(Gäde, 1995)
Oniay-CC	-pQYNFSTGNa----	CC	Coleoptera	<i>Onitis aygulus</i>	(Gäde et al., 1984)
	-pQYNFSTGNa----	CC	Coleoptera	<i>Onitis pecuarius</i>	(Gäde et al., 1984)
Micvi-CC	-pQINFTPNa----	CC	Isoptera	<i>Microhodotermes viator</i>	(Liebrich et al., 1995)
Manse-AKH	-pQLTFTSSWGa---	CC	Lepidoptera	<i>Manduca sexta</i>	(Ziegler et al., 1985; Bradfield and Keeley, 1989)
	-pQLTFTSSWGa---	CC	Lepidoptera	<i>Heliothis zea</i>	(Jaffe et al., 1986a; Jaffe et al., 1986b)
	-pQLTFTSSWGa---	CC	Lepidoptera	<i>Bombyx mori</i>	(Ishibashi et al., 1992)
	-pQLTFTSSWGa---	Br/CC/O	Lepidoptera	<i>Spodoptera frugiperda</i>	(Abdel-latif and Hoffmann, in press)
Apime-AKH¹⁾	-pQLTFTSSWGa---	CC	Hymenoptera	<i>Apis mellifera</i>	(Lorenz et al., 1999)

Helze-HrTH	-pQLTFSSGWNaa--	CC	Lepidoptera	<i>Heliothis zea</i>	(Jaffe et al., 1988b)
	-pQLTFTSSWGNaaa--	CC	Lepidoptera	<i>Spodoptera frugiperda</i>	(Abdel-latif and Hoffmann, in press)
SpoFr-AKH-I	-pQLTFSSGWaaaa--	Br/CC/O	Lepidoptera	<i>Spodoptera frugiperda</i>	(Abdel-latif and Hoffmann, in press)
Vanca-AKH	-pQLTFTSSWGGK--	CC	Lepidoptera	<i>Vanessa cardui</i>	(Köllisch et al., 2000)
	-pQLTFTSSWGGK--	CC	Lepidoptera	<i>Danaus plexippus</i>	(Köllisch et al., 2003)
	-pQLTFTSSWGGK--	CC	Lepidoptera	<i>Precis coenia</i>	(Köllisch et al., 2003)
	-pQLTFTSSWGGK--	CC	Lepidoptera	<i>Aglaia urticae</i>	(Köllisch et al., 2003)
	-pQLTFTSSWGGK--	CC	Lepidoptera	<i>Spodoptera frugiperda</i>	(Köllisch et al., 2003)
SpoFr-AKH-II	-pQLTFSSGWNCTs	Br/CC/O	Lepidoptera	<i>Spodoptera frugiperda</i>	(Abdel-latif and Hoffmann, in press)
Tabat-HoTH	-pQLTFTPGWGYaa--	CC	Diptera	<i>Tabanus atratus</i>	(Jaffe et al., 1988a)
	-pQLTFTPGWGYaa--	CC	Diptera	<i>Tabanus calens</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWGYaa--	CC	Diptera	<i>Tabanus lineola</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWGYaa--	CC	Diptera	<i>Tabanus proximus</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWGYaa--	CC	Diptera	<i>Tabanus sulcifrons</i>	(Woodring and Leprince, 1992)
Tabat-AKH	-pQLTFTPGWaaaa--	CC	Diptera	<i>Tabanus atratus</i>	(Jaffe et al., 1988a)
	-pQLTFTPGWaaaa--	CC	Diptera	<i>Tabanus calens</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWaaaa--	CC	Diptera	<i>Tabanus lineola</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWaaaa--	CC	Diptera	<i>Tabanus proximus</i>	(Woodring and Leprince, 1992)
	-pQLTFTPGWaaaa--	CC	Diptera	<i>Tabanus sulcifrons</i>	(Woodring and Leprince, 1992)
Anoga-HrTH	-pQLTFTPAWaaaa--	Br/CC/O	Diptera	<i>Anopheles gambiae</i>	(Kaufmann and Brown, 2006)
Aedae-HrTH	-pQLTFTPSWaaaa--	genome	Diptera	<i>Aedes aegypti</i>	(personal blast)
	-pQLTFTPSWaaaa--	genome	Diptera	<i>Culex pipiens</i>	(personal blast)
Anoga-AKH	-pQVTFSRDWNAAa--	Br/CC	Diptera	<i>Anopheles gambiae</i>	(Kaufmann and Brown, 2006)
	-pQVTFSRDWNAAa--	genome	Diptera	<i>Aedes aegypti</i>	(personal blast)
	-pQVTFSRDWNAAaaa--	genome	Diptera	<i>Culex pipiens</i>	(personal blast)
Trica-AKH-II	-pQVTFSRDWNPa--	genome	Coleoptera	<i>Tribolium castaneum</i>	(Amare and Sweedler, 2007)
Locmi-HrTH	-pQVTFSRDWSPa--	Br/CC	Orthoptera	<i>Locusta migratoria</i>	(Siegert, 1999)

Phote-HrTH	-pQLTFSPDWa----	CC	Diptera	<i>Phormia terraenovae</i>	(Gäde et al., 1990)
	-pQLTFSPDWa----	CC	Diptera	<i>Drosophila melanogaster</i>	(Schaffer et al., 1990; Noyes et al., 1995)
	-pQLTFSPDWa----	CC	Diptera	<i>Neobellieria bullata</i>	(Verleyen et al., 2004)
Panbo-RPCH	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Pandalus borealis</i>	(Fernlund, 1974)
	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Cancer magister</i>	(Gaus et al., 1990)
	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Carcinus maenas</i>	(Gaus et al., 1990; Klein et al., 1995)
	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Orconectes limosus</i>	(Gaus et al., 1990)
	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Callinectes sapidus</i>	(Klein et al., 1995)
	-pQLNFSPGWa----	Eyestalk	Decapoda	<i>Cherax quadricarinatus</i>	(Martinez-Perez et al., 2005)
Nezvi-AKH¹⁾	-pQLNFSPGWa----	CC	Hemiptera	<i>Nezara viridula</i>	(Gäde et al., 2003)
Nepci-AKH	-pQLNFSSGWa----	CC	Hemiptera	<i>Nepa cinerea</i>	(Gäde et al., in press)
Homsa-GLC	HSQGTFTSDYSKYLD	pr/gut	Primata	<i>Homo sapiens</i>	(Thomsen et al., 1972; White and Saunders, 1986)

AKH, adipokinetic hormone; Br, Brain; CAH, cardio acceleratory hormone; CC, corpora cardiaca; GLC, glucagon; HoTH, Hypotrehalosaemic hormone; HrPH, hypertrehalosaemic hormone; HrTH, hypertrehalosaemic hormone; O, ovaries; RPCH, red pigment concentrating hormone; pr, pancreas

¹⁾Codenames are not found in the literature, but it should indicate that identical peptide was found in different order – this untrue codenames are also found in the phylogenetic tree of Figure 5.1.

²⁾Residue 8 (Try) contains a hexose modification

³⁾Residue 7 (Thr) identified as phosphothreonin

⁴⁾exact modification of HrTH-I not known

1.41 SYNTHESIS, STORAGE AND RELEASE OF AKHS

Most insects synthesize AKHs in the corpora cardiaca, but it is also reported in the brain and the ventral nerve system. AKH synthesis is a continuous process and is independent of life stages (Diederer et al., 1992; Diederer et al., 2002; van der Horst, 2003). It has been shown that newly synthesized AKH is preferably secreted into the haemolymph (Diederer et al., 1992; Sharp-Baker et al., 1995), and as a result only a small amount of the stored AKHs will ever be used; consequently, the older an insect, the more stored AKH (Sharp-Baker et al., 1996; Harthoorn et al., 2002).

The biosynthesis of AKHs is conserved in all insects and well reported in the desert locust, *Schistocerca gregaria* (O'Shea and Rayne, 1992). The AKH precursor (pre-pro-hormone) begins with a signal peptide, followed by a single AKH of typically eight to ten amino acids in length and then the AKH-precursor-related-peptide (APRP, Figure 1.17).



Figure 1.17: Scheme of the peptide pre-pro-hormone structure: Signal peptide, hormone (AKH), followed by the hormone precursor related peptide (APRP, AKH-precursor related peptide).

After translation in the rough endoplasmic reticulum (r-ER), the signal peptide directs the pre-pro-hormone into the lumen of the endoplasmic reticulum. In the lumen, subsequent to the cleavage of the signal peptide, disulfide bridges create dimers of the AKH pro-hormones (Figure 1.18; Sossin et al., 1989; O'Shea and Rayne, 1992). The appropriate cysteines for the disulfide bridges were found in the APRP (O'Shea and Rayne, 1992; Huybrechts et al., 2002). In *L. migratoria*, which synthesizes three different AKHs in the same cells of the glandular part of the corpora cardiaca (Diederer et al., 1987; Hekimi et al., 1991; Bogerd et al., 1995; Diederer et al., 2002), not only homo-, but also heterodimers of pro-Locmi-AKH-I and -II were described (O'Shea and Rayne, 1992; Baggerman et al., 2002). For the third locust AKH (Locmi-AKH-III), parallel and anti-parallel homo-dimers were found, bound by two disulfide bridges (Huybrechts et al., 2002). After the dimerization, the dimers are packed in vesicles and transported to *cis* part of the Golgi apparatus. The modifications to the bioactive AKHs are considered to occur in the post-*trans* Golgi compartment (Figure 1.18; O'Shea and Rayne, 1992; Rayne and O'Shea, 1994). Then, the bioactive AKHs are packed in vesicles and stored, or prepared for the release by exocytosis (Jutsum and Goldsworthy, 1977; Rademakers, 1977a; Diederer et al., 2002). Prior to secretion, the N- and C-termini of the AKH are modified to a pyroglutamate and amide, respectively (Figure 1.18; Martinez-Perez et al., 2002; Gäde and Auerswald, 2003; Table 1.1; only Vanca-AKH is non-amidated, reported by Köllisch et al., 2000). This blockage gives stability to the active form and prevents the neurohormone from being attacked by exopeptidases in the haemolymph (Lee et al., 1997).

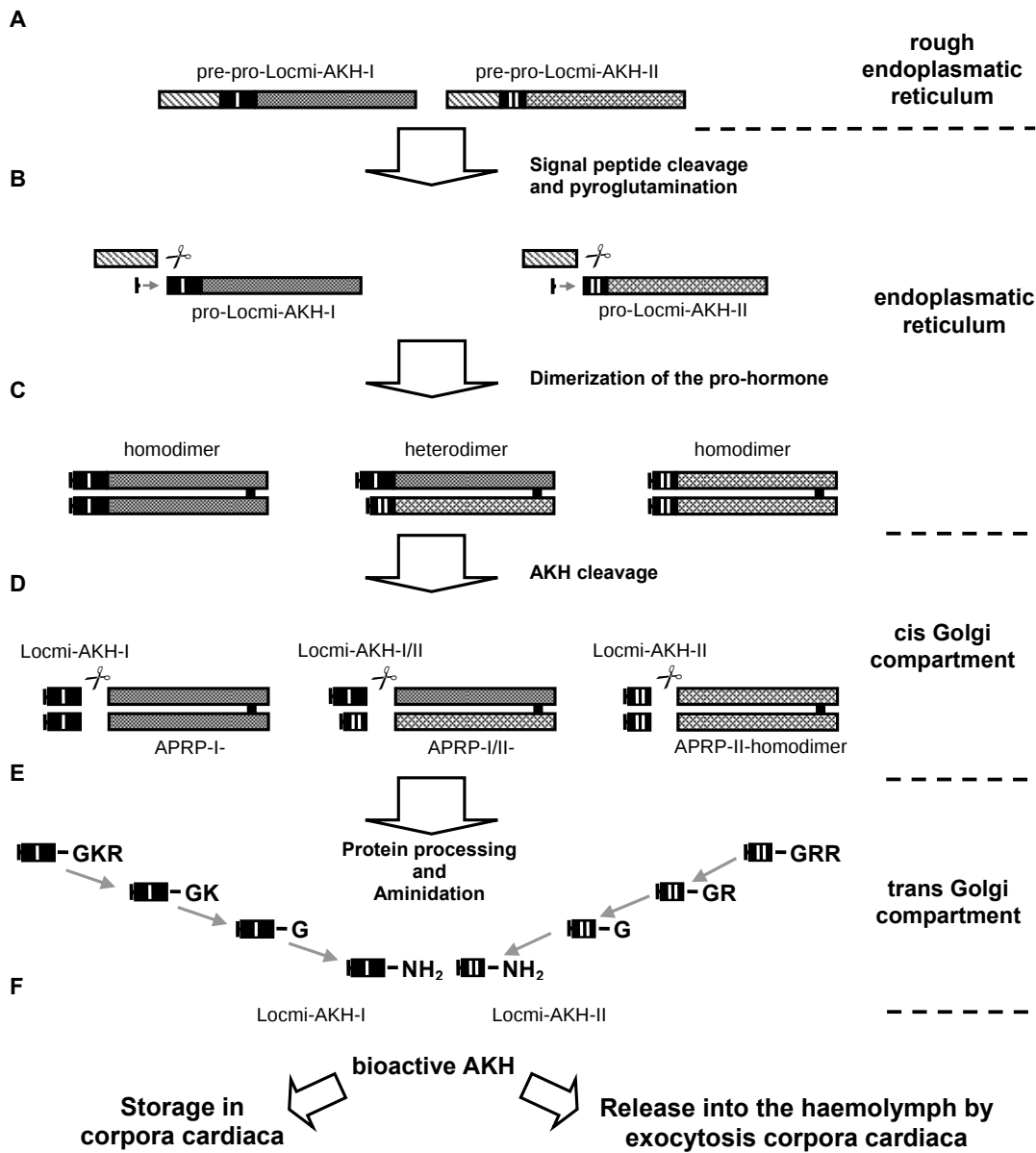


Figure 1.18: A model of locust AKH (Locmi-AKH-I and -II) biosynthesis:

- Pre-pro-hormone (signal peptide, AKH, and APRP) translation and transport in the rough endoplasmatic reticulum
- Cleavage of signal peptide and pyroglutamination of the N-terminal glutamine residue of the AKH peptide in the endoplasmatic reticulum: Pre-pro-hormone cleaved to pro-hormone (AKH and AKH-precursor related peptide, APRP)
- Dimerization of pro-hormone, formation of homo- (pro-Locmi-AKH-I or -II) and heterodimers (pro-Locmi-AKH-I and -II)
- Cleavage of the hormone in the cis-Golgi compartment
- Protein processing and aminidation of the AKH's C-terminus
- Storage or release of the bioactive AKH

For neurohormones, it is crucial to resist the degradation as long as possible. The half-life for AKH is dependent on the stability against the endopeptidases. Only a few studies report about the AKH half-life that varies from a few minutes to one hour (Oudejans et al., 1996), but in some cases the metabolic effect at rest can last for up to around 8 hours (Gäde, 1992b, 1993). During flight, degradation takes place quicker, an indication of the presence of more than one protease during extended actions (Rayne and O'Shea, 1992; Oudejans et al., 1996; Woodring et al., 2002).

The identity of the releasing factor for AKH is still a matter of discussion, but it is assumed to be of a neuronal and hormonal nature. Implantation of the corpora cardiaca in locusts does not result in any increase in AKH release during flight, which implies a neuronal releasing factor (Rademakers, 1977a). Further histological studies supported the neuronal activation. Neurons from the lateral part of the protocerebrum transport messengers through the NCC-II to a synaptic ending, which is connected to the glandular part of the corpora cardiaca (Rademakers, 1977b) and electrical stimulation of the NCC-II resulted in AKH release (Orchard and Loughton, 1981b; Orchard and Lange, 1983b). In studies with *L. migratoria* the neurohormones tachykinin-I and -II (Locmi-TK-I, and -II) induced AKH release (Nassel et al., 1995). With a tachykinin antiserum, synaptoid connections at the glandular corpora cardiaca were stained – an indication that Locmi-TK-I, and -II probably function more as a neurotransmitter than a neurohormone (Schoofs et al., 1993; Nassel et al., 1995; Nassel, 1999). Other immunocytological studies with an antiserum for FMRFamide-related peptides (FaRPs) indicated staining for the same cells and synapses as reported for the Locmi-TKs. FaRPs did not induce a release of AKH, but may act as a possible neuromodulator for the fine-tuning of the AKH release and act more as a inhibitor (Vullings et al., 1998; Vullings et al., 1999). *In vitro* studies with crustacean cardioactive peptides (CCAP), high potassium concentration, or the 3-isobutyl-1-methylxanthine (IBMX) also activated the release of AKHs, which supports a hormonal release of AKHs (Passier et al., 1997; Veelaert et al., 1997). The neuromodulator octopamine potentiates the effect of IBMX, but no AKH increase is detected on its own (Passier et al., 1995; Veelaert et al., 1997). On the other hand, it was found that high trehalose levels in the haemolymph inhibits AKH release (Cheeseman et al., 1976; Passier et al., 1997). Since AKHs act as multifunctional hormones, their release may be controlled differently depending on its need; especially for AKH cells that originate in the brain or ventral nerve cord.

1.42 WHY MORE THAN ONE AKH IN THE SAME INSECT?

As mentioned above, for several insects, more than one AKH member is identified (Table 1.1). In *L. migratoria* four AKHs were reported, of which three are known to have a metabolic function, especially during flight. The identical exon structure of the *Locmi-AKH-I* and *-II* suggests that they evolved through gene duplication of a common ancestral gene (Noyes and Schaffer, 1993). The expression for the *Locmi-AKHs* differ; it has been shown that the amount of *Locmi-AKH-I* gene is expressed nearly twice the amount of *Locmi-AKH-II*

(Fischer-Lougheed et al., 1993). The presence of the Locmi-AKH-I peptide is around 4.5 times greater than the Locmi-AKH-II (Hekimi et al., 1991), but all these ratios vary depending on instar. However, it is clear that Locmi-AKH-I and -II, differ in their potency: Locmi-AKH-I is more involved in lipid metabolism and Locmi-AKH-II in carbohydrate metabolism (Orchard and Lange, 1983a; Goldsworthy et al., 1986). Hence, it is reported that Locmi-AKH-II is responsible for the mobilization of the primary flight metabolites, the carbohydrates and Locmi-AKH-I activates the mobilization of the secondary flight metabolites, the lipids (Vroemen et al., 1997).

The different metabolic needs during the mandatory flight of the migratory phase acquired a hormonal interaction and the need for more than one AKH evolved. Whereas in other insects with no energy expensive actions like migration, swimming, and ball rolling, AKHs may just play a 'secondary' role, as reported for Locmi-AKH-III, which is speculated to be more active during rest providing the locust with energy (Vroemen et al., 1998).

The presence of a fourth non-classical AKH is reported in *L. migratoria* (Siegert, 1999). The classical locust AKHs (Locmi-AKH-I, -II, and -III) are synthesized and stored in the glandular part of the corpora cardiaca and all show hyperlipaemia and -trehalosaemia in locust, whereas the fourth locust AKH is found in the storage part of the corpora cardiaca, which indicates of an 'external-corpora-cardiaca' synthesis site, probably in the brain. In addition, no metabolic function was detected for this non-classical AKH in locust. Tests in the *P. americana* demonstrated a weak hypertrehalosaemia, which is responsible for its nomenclature: Locmi-HrTH (Siegert, 1999).

1.43 FUNCTION OF AKHS

The members of the AKH family are typical examples of multifunctional neurohormones. The main function of the AKHs is linked to the insect's nutrient metabolism (Figure 1.19), but not all members of the AKH-family are involved in hyperlipaemia, some induce hypertrehalosaemia (hypertrehalosaemic hormone, HrTH) or hyperprolinaemia (hyperprolinaemic hormone, HrPH). Since vertebrates lack the proline oxidation, just the hyperlipaemic and hypertrehalosaemic hormone are quoted to have analogue effects like the vertebrate glucagon.

On the contrary, it is also demonstrated that AKH members can inhibit lipid (Gokuldas et al., 1988), protein (Carlisle and Loughton, 1979; Cusinato et al., 1991), and RNA synthesis (Kodrik and Goldsworthy, 1995). Other functions induced by AKHs are reported, in *M. sexta*, where similar to octopamine, species-specific AKH injection increased motor activity of the mesothoracic muscle (Milde and Wallstein, 1994; Milde et al., 1995), whereas in different cockroaches, acceleration of the heart rate is reported (Scarborough et al., 1984; Baumann et al., 1990; Keeley et al., 1991). In *P. americana* AKH-family members induce an intracellular Ca^{2+} -influx and consequently amplify the spontaneous spike frequency in the dorsal unpaired median (DUM) neurons of the terminal ganglion (Wicher and Reuter, 1993; Wicher et al., 1994). An increase of the cytochrome P450 (P4504C1) was reported for the discoid

cockroach, *Blaberus discoidalis* after HrTH injection (Bradfield et al., 1991). Cytochrome P450 was also enhanced in starved animals, which is probably derived from an up-regulation of AKHs during starvation (Bradfield et al., 1991; Lu et al., 1995, 1996; Sowa et al., 1996). In locusts, there is even a possible immune function attributed to AKH enhancing indirectly the phenoloxidase and the nodule formation (Mullen and Goldsworthy, 2003, 2006 Mullen et al., 2004).

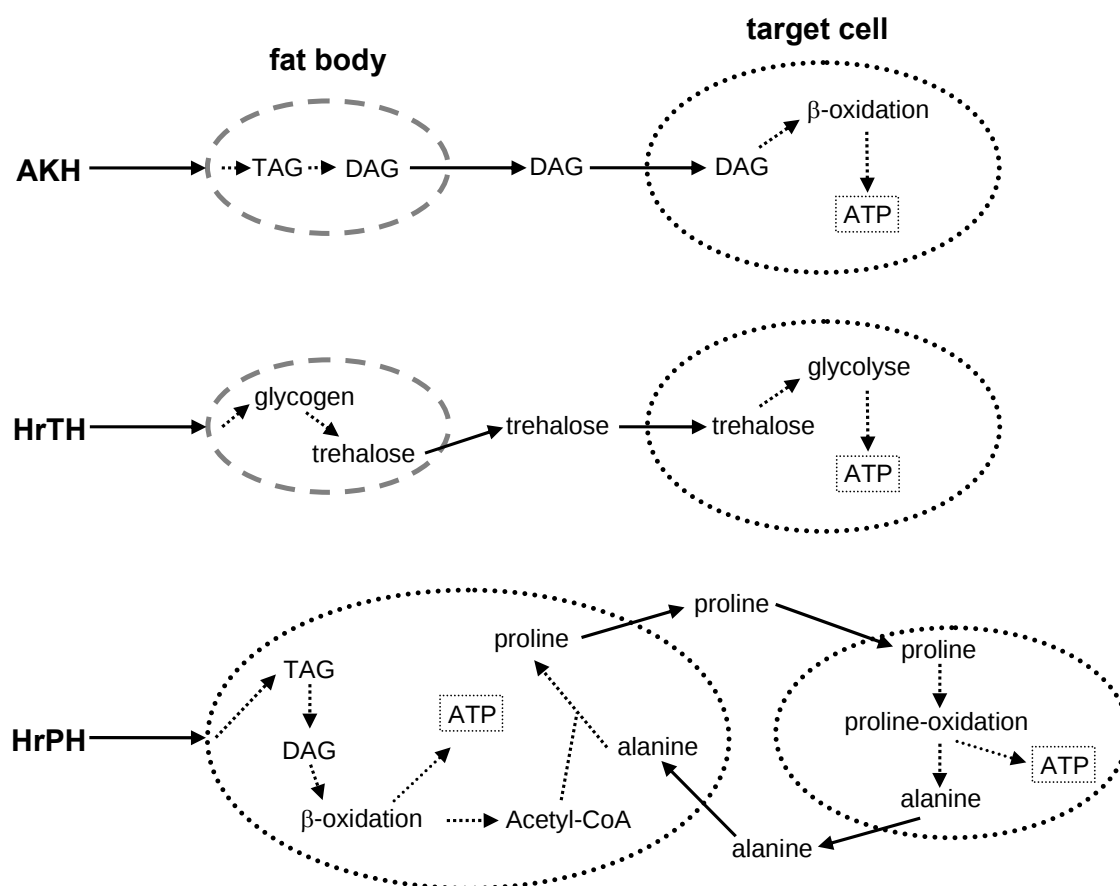


Figure 1.19: Summarized metabolic pathways of the adipokinetic or hyperlipaemic hormone (AKH), hypertrehalosaemic hormone (HrTH), and hyperprolinaemic hormone (HrPH, involved in hyperlipaemia and hyperprolinaemia). Abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; ATP, adenosine triphosphate; CoA, co-enzyme A.

1.44 AKH AND ITS RELATED PEPTIDES

The AKH-family is often reported as the AKH/RPCH-family, but today most authors separate them into two families, ‘insect’ adipokinetic hormones and the ‘crustacean’ red pigments concentrating hormones (RPCH) family. However, similar amino acid components and the hyperlipaemic or hyperglycaemic effect of RPCH in insects or the pigment aggregation by AKH treatment in crustacean clearly supports their evolutionary relationship

(Herman et al., 1977; Mordue and Stone, 1977; Josefsson, 1983; Gäde, 1986; Gaus et al., 1990). In addition, an immunocytological study with a locust AKH-antisera demonstrated staining in RPCH cells of different crustaceans (Schooneveld et al., 1987b). And recently, a report identified an AKH in the stinkbug, *Nezara viridula* (Gäde et al., 2003), which has already been characterized in the crustacean, *Pandalus borealis* (Panbo-RPCH, Table 1.1; Fernlund, 1974). The function of Panbo-RPCH differs in the shrimp (aggregation of the pigment granules in the chromatophores) and in the stinkbug (hyperlipaemic effect). However, speculation as to whether this hormone reveals a neuromodulatory function in both representatives could provide evidence to the close evolutionary link between Insecta and Crustacea (Giribet et al., 2001; Gäde et al., 2003).

After the first genes of AKHs and RPCHs were described, not only the similarity of the peptides, but also conservation of amino acids were demonstrated in the signal peptide and especially in the precursor sequences (Linck et al., 1993; Bogerd et al., 1995; de Kleijn and van Herp, 1995; Klein et al., 1995). RPCHs are known as conserved octapeptides (Gaus et al., 1990), whereas the insect AKHs vary from octa- to decapeptide (Gäde et al., 1997). A similar structure in the signal peptide was illustrated for AKH, RPCH and APGWamide-like genes, the latter are involved in the reproductive behaviour of molluscs (Martinez-Perez et al., 2002). Another theory suggests an evolutionary relationship between LWamide (regulates metamorphosis in Hydrazoa; Gajewski et al., 1996) and RFamide (regulates feeding behaviour in Insecta; Dockray, 2004), and the AKH-, RPCH-, and APGWamide-like-family by means of probable DNA loss (Figure 1.20; Martinez-Perez et al., 2007).

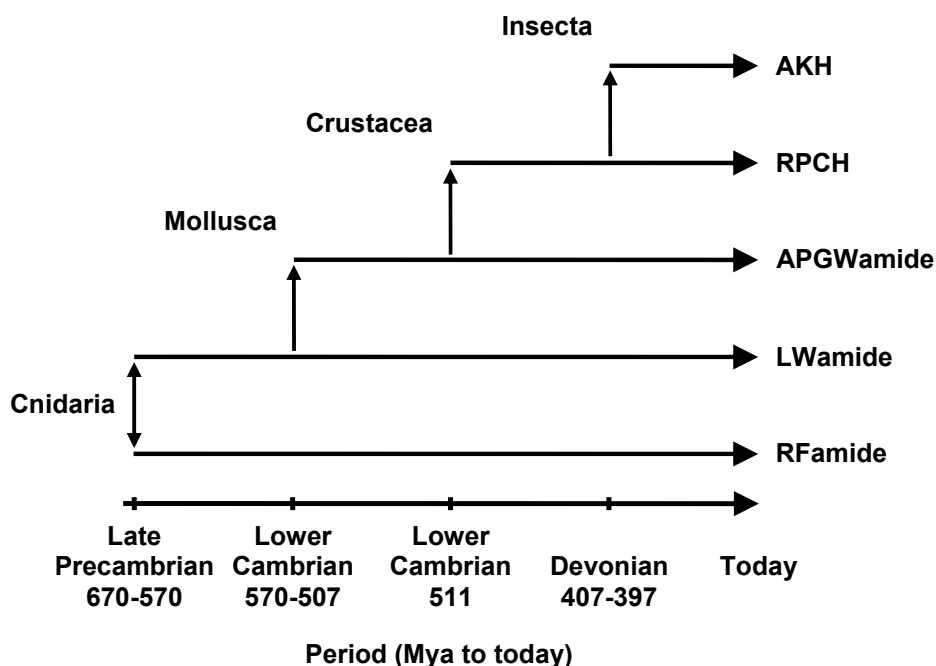


Figure 1.20: The appearance of neuropeptides in different Phyla through evolution provided evidence of the DNA loss theory (Martinez-Perez et al., 2007).

Corazonin, a conserved neuropeptide found in insects, is synthesised and released from the corpora cardiaca (Veenstra, 1989). In *Drosophila* it was found that corazonin was released from the same cells as the Drome-AKH, but synthesized in median neurosecretory cells in the protocerebrum (Choi et al., 2005). Its structure shows similarities to the members of the AKH-family, which could have also evolved from a common ancestral gene by gene duplication (Veenstra, 1994). Functions are described for *L. migratoria* and *B. mori*, where corazonin is involved in the colour polymorphisms (Tanaka et al., 2003) or in *P. americana* it described to work as a muscle-acceleratory peptide (O'Shea et al., 1984). The same study demonstrated that the bioactivity of the hormone is dependent on the last few amino acids, which are similar to some AKHs. In *M. sexta*, corazonin also is referred to trigger pre-ecdysis (Kim et al., 2004).

AKHs are often compared with the vertebrate hormone controlling glycogen and blood sugar homeostasis (Table 1.1; Orchard, 1987; Kim and Rulifson, 2004). In vertebrates, insulin and glucagon regulate the homeostasis of the major nutrients in the blood. Insulin is generally responsible for storing carbohydrates, but also fat and protein, and its antagonist glucagon, mobilizes them. In insects, such a conserved system like in vertebrates is not yet entirely described. The adipokinetic and hypertrehalosaemic hormones action may be compared to glucagon, whereas a hypotrehalosaemic hormone like insulin is not described yet in the AKH family. Some evidence of such a homeostatic regulator is described for insulin-like peptides. Four of eight residues are identical between some AKHs and glucagon, and at least two are conserved (Table 1.1; Scarborough et al., 1984; Orchard, 1987; de Loof and Schoofs, 1990; Clynen et al., 2004; Kim and Rulifson, 2004). Structurally, AKHs may even be part of the superfamily of the growth hormone-releasing factors that include also glucagon (Scarborough et al., 1984; Clynen et al., 2004).

1.45 AKH-RECEPTORS (G-PROTEIN COUPLED RECEPTORS)

As mentioned above, the AKH-receptor (AKHR) is a G-protein coupled receptor (GPCR). This chapter will give a general view of GPCRs, but only the common pathways are illustrated (Figure 1.21). GPCRs are the largest protein family known (Park and Adams, 2005). These receptors can sense photons (e.g. rhodopsin), small molecules (e.g. histamine), or small and large proteins (e.g. AKHs and interleukins). These integral membrane receptors are characteristic for their seven transmembrane helices, an extracellular N-terminus and intracellular C-terminus. In Figure 1.21, a generalized model of a GPCR is illustrated. GPCRs transduce extracellular signals into intracellular responses. The extracellular ligand binding sites can vary, generally small ligands appear to bind at the pockets formed by the extracellular transmembrane helices (Vaidehi et al., 2002), whereas larger ligands normally bind to the extracellular N-terminus and the transmembrane loops (Bockaert and Pin, 1999). The binding of the ligand activates the heterotrimeric G proteins ($\alpha\beta\gamma$ -subunits), especially the α -subunit. The active state of the α -subunit binds to GTP (guanosine triphosphate), which refers to the name 'G protein' (GTP-binding protein). After the α -subunit is activated, it

moves away from the heterotrimeric protein complex. The transduction from the external signal activates the intracellular second messenger, which can vary depending on the receptors nature.

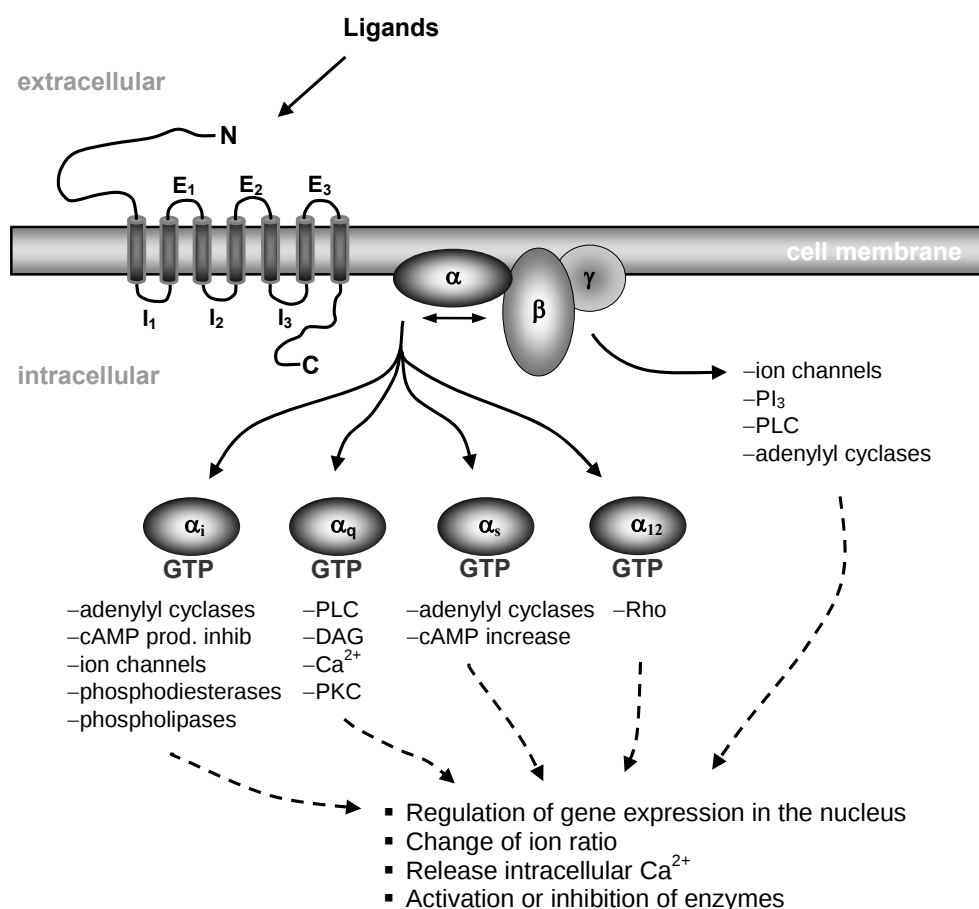


Figure 1.21: Generalized model of a G-protein coupled receptor. Characteristic are the seven transmembrane helices, the extracellular located N-terminus and intracellular C-terminus. The transmembranes are connected by three internal (I₁ to I₃) and three external (E₁ to E₃) loops. After the binding of the ligand, binding of GTP (guanosine triphosphate) to the α-subunit activates the heterotrimeric G-protein, which then disconnects from the β- and γ-subunit and activates a cascade or a 2nd messenger. Depending on receptor and target cell, a specific pathway starts that either regulates gene expression, activates/inhibits enzymes, or closes/opens ion channels. The presented activated α-subunits in the model (α_i, α_q, α_s, and α₁₂) illustrate the different classes of effector-targets (listed below the subunit).

Abbreviations: DAG, diacylglycerol; cAMP, cyclic adenosine monophosphate; IP₃, inositol 1,4,5-triphosphate; PKC, protein kinase C; PLC, phospholipase C; and Rho, member of the Ras-superfamily, which acts as a molecular switch for a variety of cellular signalling (Park et al., 2005).

Common second messengers are cyclic adenosine monophosphate (cAMP), inositol 1,4,5-triphosphate (IP₃), 1,2-diacylglycerol (1,2-DAG), and Ca²⁺ (Figure 1.21). For example, IP₃ is activated when the G protein unit links with the phospholipase C (PLC); this second messenger (IP₃) mobilizes intracellular Ca²⁺ stored in the endoplasmic reticulum. The Ca²⁺ increase can activate or inactivate protein synthesis. In the case of the cAMP as a second messenger, the α-subunit links with the adenylate cyclase, and like Ca²⁺, cAMP can activate or inactivate protein synthesis. The α-subunit can also be responsible for opening or closing ion channels, which affects the intracellular ion composition.

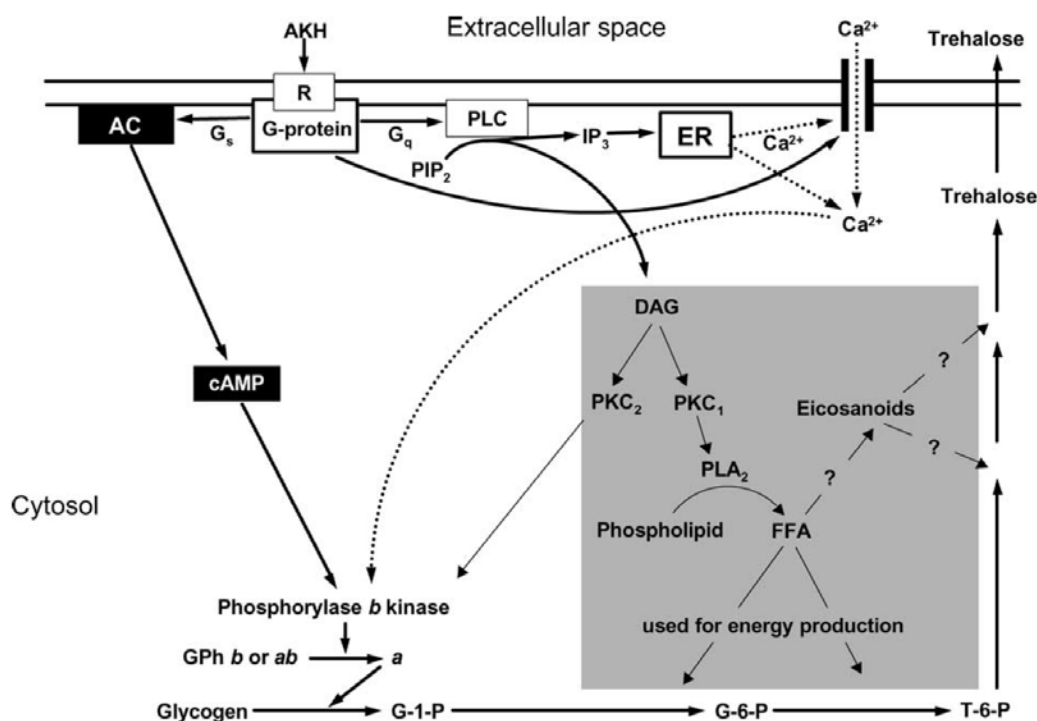


Figure 1.22: Proposed scheme of the transduction of the hypertrehalosaemic signal of AKH peptides in the fat body of locusts, cockroaches, and beetles (Figure taken from Gäde and Auerswald, 2003).

Abbreviations: AC, adenylyl cyclase; cAMP, cyclic AMP; DAG, diacylglycerol; ER, endoplasmic reticulum; FFA, free fatty acids; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; GPh, glycogen phosphorylase; IP₃, inositol 1,4,5-triphosphate; PIP₂, phosphatidylinositol-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; R, receptor; and T-6-P, trehalose-6-phosphate. White letters on black background indicate pathway in locusts only. Events in grey area are only reported for *P. americana*.

Biochemical characterization of an AKHR was first reported for *M. sexta* (Ziegler et al., 1995). Later ‘AKH-GPCRs’ were cloned and expressed in cells for ligand binding studies for *D. melanogaster* (Park et al., 2002; Staubli et al., 2002), *B. mori* (Staubli et al., 2002), *P.*

americana (Hansen et al., 2006; Wicher et al., 2006), and *A. gambiae* (Belmont et al., 2006). The identification of AKHR helped to test the bioactivity of the AKHs; prior studies already showed that the conserved amino acids at position 1, 4, and 8 (Table 1.1) are important for the potency and activity of the neurohormone (Hayes and Keeley, 1990; Gäde, 1992b). The protein structure and conformation is essential for the optimal binding to the receptor (Hayes and Keeley, 1990; Fox and Reynolds, 1991). For some AKHs, a β -turn conformation has been associated with more potency for the hyperlipaemic response (Cusinato et al., 1998), whereas AKHs that lack this conformation are more potent as a hypertrehalosaemic hormone, but do not exclude hyperlipaemia as shown for Locmi-AKH-II and Manse-AKH (Ziegler, 1990; Cusinato et al., 1998; Vroemen et al., 1998).

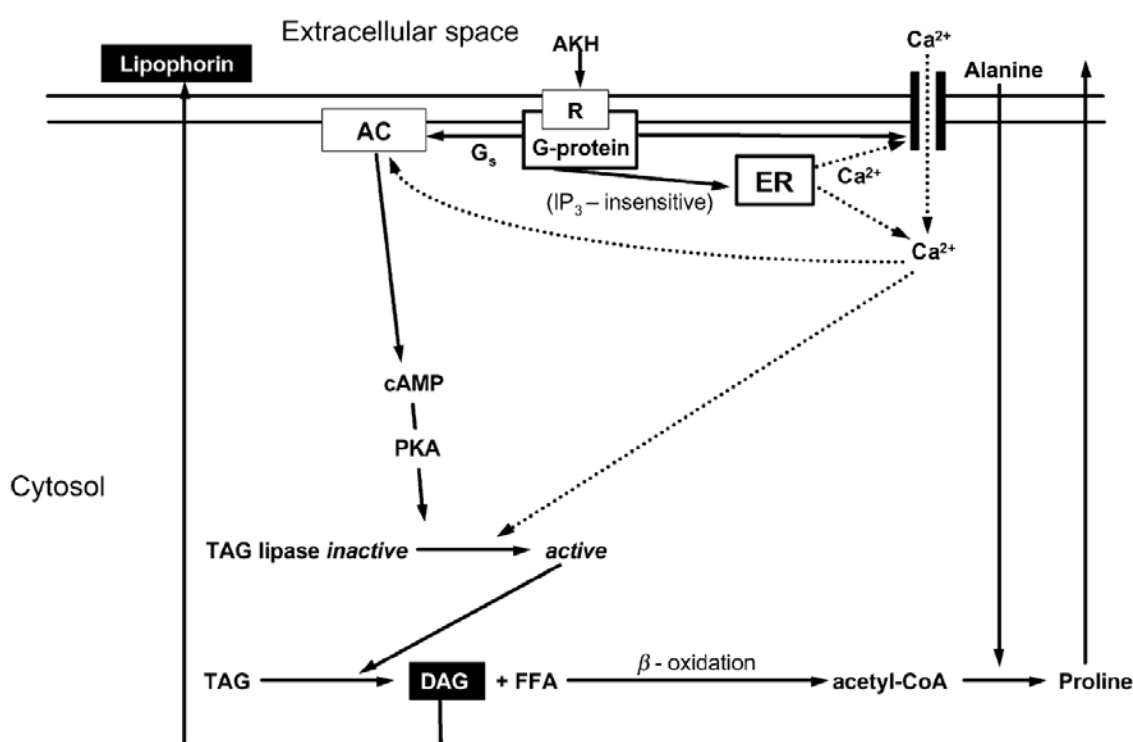


Figure 1.23: Proposed scheme of the transduction of the hyperlipaemic/hyperprolinaemic signal of AKH peptides in the fat body of locusts, moths, and beetles (Figure taken from Gäde and Auerswald, 2003).

Abbreviations: AC, adenylyl cyclase; cAMP, cyclic AMP; CoA, co-enzyme A; DAG, diacylglycerol; ER, endoplasmic reticulum; FFA, free fatty acids; IP3, inositol 1,4,5-triphosphate; PKA, protein kinase A; R, receptor; and TAG, triacylglycerol. White letters on black background indicate pathway in locusts only.

The signal transduction cascade is observed only for a few insects and in some only parts of it (Schooley et al., 2005), but apart from the above mentioned conformation-theory,

the signal transduction of the adipokinetic, hypertrehalosaemic, and hyperprolinaemic effect seemed to be more conserved and consistent. Generally, the adipokinetic response acts through the GPCR's second messengers IP₃, or cAMP, and Ca²⁺. In most cases, the hypertrehalosaemic hormone activates the phospholipase C (PLC), IP₃, and Ca²⁺, whereas the hyperprolinaemic hormone uses cAMP as a second messenger (Gäde and Auerswald, 2003; Schooley et al., 2005). The AKH induced cascade differs depending on species and receptor, which makes it practically impossible to predict the metabolic function without a bioassay. In Figure 1.22 and 1.23, a summary of today's known AKH signal transduction pathways in insects are illustrated (Gäde and Auerswald, 2003).

1.46 AKHS IN DIPTERA

All identified AKHs in Diptera induce hypertrehalosaemia, which is probably related to the main flight metabolite carbohydrate (Sacktor, 1976). The presence of such a hyperglycaemic factor in insects was first described by Steele (1961). He reported a hyperglycaemic effect in cockroaches after injection of species-specific corpora cardiaca extract. Steele was inspired by the various demonstrated metabolic effects produced by sinus gland extract in crustaceans, and its similarity with the corpora cardiaca in insects. Ironically, there is a crustacean hyperglycaemic factor present in the sinus gland of crustaceans (Fanjul-Moles, 2006), but RCPH (AKH analogue), which is not active on the metabolic level in crustaceans, is also synthesized in the sinus gland of the eyestalk. However, further experiments by Steele (1963) demonstrated that the insect hyperglycaemic hormone activates fat body glycogen phosphorylase, which catalyzes the mobilization of glucose from glycogen (Figure 1.22). He also showed that only the fat bodies mobilize glycogen, whereas muscle tissue was not affected by the hormone extract treatment. Muscle tissue even showed a trend of storing the mobilized carbohydrates from the fat bodies – an initial indication of moving carbohydrates from the fat bodies in the form of trehalose (a hypertrehalosaemic effect) to the muscle tissues in the thorax (Steele, 1963). This bioassay was used in various species of different orders and demonstrated the metabolic effect of a hormone stored and released from the corpora cardiaca, although these effects are not clearly associated with members of the AKH family, because the corpora cardiaca is the releasing site of many different neurohormones. However, a summary of such experiments for Diptera is shown in Table 1.2. Most studies showed a 'hypertrehalosaemic' effect, and in a few cases, hyperlipaemia was detected. Hypoglycaemia was observed in the blow fly, *Calliphora erythrocephala*, and the mosquito *Aedes taeniorhynchus*, but in these two dipterans, the median neurosecretory cells were ectomized, rather than undergoing the previously mentioned bioassay. Today it is assumed that insulin-like peptides are synthesized in the median neurosecretory cells and secreted from the corpora cardiaca, whereas the AKHs are mainly synthesized in and released from the glandular part of the corpora cardiaca. Interestingly, in the tsetse fly, *Glossina morsitans* studies with fat bodies *in vitro* in a medium, showed hyperlipaemic effect and inhibition of lipid synthesis when treated with species specific corpora cardiaca extract

(Pimley, 1984), whereas in *in vivo* studies only hypertrehalosaemia was reported and no effect on the lipid level was found (Mwangi and Awiti, 1989).

Table 1.2: Metabolic effects of corpora cardiaca extract in Diptera.

Species	Effect	Reference
<i>Phormia regina</i>	hypertrehalosaemic	(Friedman, 1967)
<i>Calliphora erythrocephala</i>	hypertrehalosaemic ¹	(Normann and Duve, 1969)
<i>Musca domestica</i>	hypertrehalosaemic	(Liu, 1974)
<i>Tabanus spp.</i> ²	hyperlipaemic and -trehalosaemic ²	(Woodring and Leprince, 1992)
<i>Glossina morsitans</i>	hyperlipaemic (<i>in vitro</i>)	(Pimley, 1984)
<i>G. morsitans</i>	hypertrehalosaemic (<i>in vivo</i>)	(Mwangi and Awiti, 1989)
<i>Calliphora erythrocephala</i>	hyperglycaemic and -terhalosaemia ³	(Duve, 1978)
<i>Aedes taeniorhynchus</i>	glycogenic and lipolytic ⁴	(Van Handel and Lea, 1970)

¹electrostimulation of the CC

²*T. calens*, *T. lineola*, *T. proximus*, and *T. sulcifrons*, effects shown with corresponding AKHs HPLC fraction

³removal of median neurosecretory cells in the protocerebrum (connected with the corpora cardiaca)

⁴removal of median neurosecretory cells demonstrated an increase in glycogen reserves and a decrease in lipid reserves; no haemolymph nutrient titres were analyzed

As mentioned above the first characterized AKH was reported in the desert locust, *L. migratoria* (Stone et al., 1976a; b); the first dipteran representative was extracted and identified in *T. atratus* (Jaffe et al., 1988a), followed by the blow fly, *Phormia terraenovae* (Gäde et al., 1990), the fruit fly, *D. melanogaster* (Schaffer et al., 1990) and recently in the flesh fly, *Neobellieria bullata* (Verleyen et al., 2004). Table 1.3 presents the identified dipteran AKHs and their metabolic function. The identification of the amino acid structure of AKHs made it possible to produce synthetic AKHs and test AKH specifically instead of the whole corpora cardiaca extract. With the knowledge of the known hormone sequence, antisera against AKHs can now be produced and used to make maps of the hormonal distribution in insects using immunocytological assays. Furthermore, by comparing the different sequences, more can be found about the inter- and intraspecific evolutionary relationships.

AKH gene sequences were identified in some species, but at present in Diptera, only one AKH gene is fully characterized: i.e. the *D. melanogaster* AKH, *Drome-AKH* (Noyes et al., 1995). The corresponding receptor in the fruit fly was characterized seven years later (Park et al., 2002; Staubli et al., 2002), and it represented the only identified AKHR in Diptera at the beginning of this project.

Table 1.3: Members of the AKH family known for Diptera (Bold letters illustrate conserved amino acids).

Name	Sequence	Species	Source	Effect
Tabat -AKH	pQLTFTPGWa	<i>Tabanus spp.</i> ¹⁺²	CC	A/Hr
-HoTH	pQLTFTPGWGYa		CC	A/Hr [*]
Phote-HrTH	pQLTFSPDWa	<i>Ph. terraenovae</i> ³	CC	Hr
Drome-AKH	pQLTFSPDWa	<i>D. melanogaster</i> ⁴	CC	A/Hr ⁺
	pQLTFSPDWa	<i>N. bullata</i> ⁵	CC [#]	-

¹ *T. atratus* (Jaffé et al., 1988a); ² *T. calens*, *T. lineola*, *T. proximus*, and *T. sulcifrons* (Woodring and Leprince, 1992); ³ (Gäde et al., 1990); ⁴ (Schaffer et al., 1990); ⁵ (Verleyen et al., 2004)

Abbreviations: A, adipokinetic or hyperlipaemic effect; CC, corpora cardiaca; CNS, central nervous system; Ho, hypotrehalosaemic effect; Hr, hypertrehalosaemic effect

^{*}effects only shown in *T. calens*, *T. lineola*, *T. proximus*, and *T. sulcifrons* (see also Table 1.2)

[#]HPLC extraction of larval central nervous system (immunological studies were done by Clottens et al. (1989), who found staining in the brain and CC only)

⁺test done in larvae only

1.461 EVIDENCE OF AKHS IN MOSQUITOES

Unfortunately, there are only reports for mosquitoes showing the effects of ectomization of the median neurosecretory cells or allatectomy (removal of corpora allata – no metabolic effect found; Van Handel and Lea, 1970); no tests were published where the corpora cardiaca was removed or species-specific corpora cardiaca extract was injected (Table 1.2). It was found that mosquitoes use carbohydrates for flight (Rowley, 1970; Nayar and Sauerman, 1971, 1973; Nayar and Van Handel, 1971; Briegel et al., 2001a; b; Kaufmann and Briegel, 2004), however, lipid (Briegel et al., 2001a; Kaufmann and Briegel, 2004) and proline (Scaraffia and Wells, 2003) can also be utilized, depending on the observed species. These results indicate that an AKH or AKHs might be involved in flight metabolism. It had already been shown for a couple of insects that flight is controlled by members of the AKH-family (Köllisch et al., 2001; van der Horst, 2003; Gäde et al., 2004; Gäde et al., 2006a) and for Diptera it is shown in the blow fly, *Ph. terraenovae* (Wilps and Gäde, 1990). In addition, after the release of the genome of the African malaria vector, *Anopheles gambiae*, a putative mosquito AKH was reported (Riehle et al., 2002), which provided the initial preliminary information for the project.

1.5 HYPOTHESIS AND OBJECTIVES :

The main objective of the project was to characterize on the molecular and physiological level the adipokinetic hormone(s) in the mosquito *A. gambiae*:

- Many insects have more than one AKH (Gäde et al., 1997) and members of the order Diptera were reported with more than one AKH (Woodring and Leprince, 1992). It is therefore suggested that there are at least two AKH genes present also in *A. gambiae*.

Therefore the identification of AKHs in this mosquito will be carried out by using available molecular tools like ‘blasting’ the genomic database with already identified AKH sequences. Identified genes will be characterized by cloning and sequencing.

- Changes of gene expression in different body parts of mosquitoes are expected, especially during the life cycle and depending of the different feeding condition.

The expression levels in larvae, pupae, and imagos of sugar- and blood-fed females will be studied by using reversed transcriptase PCR.

- The synthesis and storage of AKH is generally expected to be in the corpora cardiaca. However, since Brown and Lea (1988) showed staining in the head, thorax, and abdomen of *A. aegypti*, it is suggested that this might also be the case in *A. gambiae*.

Using immunocytochemistry with different AKH-antisera and using radioimmunoassay with radiolabeled synthetic AKHs their localisation in neurosecretory cells will be verified and by using PCR the localisation of AKH in different body parts will be confirmed.

- Since AKH is involved in the mobilisation of different metabolites, changes of the AKH-titre in the haemolymph under varying feeding conditions have to be assumed.

Using a radioimmunoassay, the AKH haemolymph titres will be determined under varying feeding conditions,

- Since AKH interacts with an AKH-receptor (AKHR), such a receptor has also to be present in *A. gambiae*.

Using blasts with already known AKHR sequences a putative AKHR for *A. gambiae* will be identified and its expression in larvae, pupae, and imagos of sugar- and blood-fed females will be characterized. The expression of the putative AKHR shall be attempted in a mammalian cell system.

- Since during flight *A. gambiae* is using carbohydrate and lipid (Kaufmann and Briegel, 2004), it is believed that AKH will induce hypertrehalosaemia and hyperlipaemia and will also affect the flight performance.

Treatments of sugar- and blood-fed females with synthetic AKHs will be done *in vivo* and *in vitro* in order to test carbohydrate and lipid mobilisation. *In vivo* flight experiments using flight mills combined with AKH treatment will demonstrate the possible effect on flight performance of female *A. gambiae*.



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Adipokinetic hormones in the African malaria mosquito, *Anopheles gambiae*: Identification and expression of genes for two peptides and a putative receptor

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Abstract

Adipokinetic hormones (AKHs) are neuropeptides that mobilize stored fuels for flight in insects, and thus, may regulate flight by mosquitoes that transmit pathogens of human diseases. Our study of AKHs in the African malaria mosquito, *Anopheles gambiae*, identified and characterized the expression of genes encoding two AKHs, Anoga-AKH-I (pQLFTPAWa) and Anoga-AKH-II (pQVTFSRDWNaa), and a putative homolog for an AKH G-protein coupled receptor. Gene transcripts for both Anoga-AKHs and the AKH receptor were present in eggs, larvae, pupae, and adults of *An. gambiae*. In females, these transcripts were apparent in heads and thoraces for up to 72 h after blood or sugar feeding, as revealed by RT-PCR. With immunocytochemistry, a cluster of neurosecretory cells posterior to the corpus cardiacum and specific cells in the brain and thoracic ganglia of females were immunostained with an AKH antibody, which recognizes both Anoga-AKHs. Accordingly, Anoga-AKH-I was detected in extracts of female heads and thoraces by HPLC and an AKH radioimmunoassay, whereas Anoga-AKH-II was detected only in heads.

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Keywords: Insect; Neuropeptide; Immunocytochemistry; Radioimmunoassay; Metabolism

1. Introduction

Adipokinetic hormones and related peptide hormones, known as hypertrehalosaemic hormones (HrTHs), hypotrehalosaemic (HoTHs), or hyperlipaemic peptides (AKHs)—herein generically referred to as AKHs, are one of the most extensively characterized peptide families in insects with almost 40 isoforms identified (Gäde, 2004; Van der Horst, 2003). These peptide hormones may even be part of a superfamily that includes the growth hormone-releasing factors (GRF) and glucagon in vertebrates (Clynen et al., 2004). 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 APRP and modified at the

amino and carboxy termini to a pyroglutamic acid and amide, respectively (Gäde and Auerswald, 2003). The number of AKHs known to exist in a particular insect varies from one in *Drosophila melanogaster* (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 (Diederer et al., 2002). Cells in the brain and other ganglia also may secrete such peptides, as shown by immunocytochemistry in the mosquito *Aedes aegypti* (Brown and Lea, 1988) and other insect species (Schooneveld et al., 1985, 1983).

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 levels of circulating trehalose (a hypertrehalosaemic effect) in the American

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cockroach, *Periplaneta americana* (Oguri and Steele, 2003), mobilization of lipids in the migratory locust, *L. migratoria* (Gäde and Auerswald, 2003; Van der Horst, 2003) and, similarly, proline in the fruit beetle, *Pachnoda sinuata* (Gäde and Auerswald, 2002). As well, other functions have been ascribed to these peptides (Gäde, 2004; Gäde 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 by 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 hawkmoth, *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, *Pe. americana* (Wicher et al., 2005), and expressed in cells for ligand binding studies. Other elements in the intracellular signaling pathway also are known for insects (Gäde, 2004).

Recent research on the flight performance and metabolism of the African malaria mosquito, *Anopheles gambiae*, found 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 the horse fly, *Tabanus atratus* (Jaffe et al., 1989; Woodring and LePrince, 1992), blow fly, *Phormia terraenovae* (Gäde et al., 1990), flesh fly, *Neobellieria bullata* (Verleyen et al., 2004), and *D. melanogaster* (Isabel et al., 2005; Lee and Park, 2004). Previously, a putative AKH gene had been identified in the genome of *An. gambiae* (Riehle et al., 2002). In the present study, the expression of this and another AKH gene and a putative AKHR are characterized in the life stages and in females in response to sugar and blood meals, because they are the sources of nutrients used for reproduction and flight (Fernandes and Briegel, 2005; Kaufmann and Briegel, 2004). Furthermore, the existence of both AKH peptides was confirmed in body extracts, and their cellular sources identified by immunocytochemistry. These results provide a foundation for future examination of the flight dynamics and dissemination of malaria pathogens by this mosquito.

2. Materials and methods

2.1. Mosquitoes

The colony of *Anopheles* (*Cellia*) *gambiae* s.s. (Giles, strain 16c55 Lagos, Nigeria) was maintained at $26 \pm 1^\circ\text{C}$ under long-day conditions (16 h light, 8 h dark). Larvae were raised in trays ($24 \times 16 \times 6$ cm) with 350 ml distilled

water and fed pulverized Tetramin® daily (Timmermann and Briegel, 1993). Adults ($200\text{--}300/24 \times 19 \times 18.5$ cm cage) had access to 10% fructose solution or distilled water, depending on experimental conditions. For experiments and egg production, females were given blood meals from a human arm.

2.2. Cloning and sequencing of peptide and receptor cDNAs

The genes for *Anoga-AKH-I* (Riehle et al., 2002) and *Anoga-AKH-II* were identified in the ENSEMBL EST database (http://www.ensembl.org/Anopheles_gambiae/), based on BLAST searches with other known AKH peptide sequences. The putative gene for an AKH receptor, *Anoga-AKHR*, was identified similarly, based on BLAST searches with the protein sequence of the predicted, extracellular region of the N-terminus from the AKHR identified in *D. melanogaster* (Drome-AKHR, Staubli et al., 2002; Park et al. 2002).

To obtain cDNA for template in PCR, heads, thoraces, and abdomina were dissected from female *An. gambiae* (1 day old) in saline solution (128 mM NaCl, 4.7 mM KCl, and 1.9 mM CaCl_2), immediately transferred into 100 μl TRIzol® (Invitrogen) on ice, and processed quickly as 10 body parts/tube. Total RNA was extracted with the TRIzol® Reagent kit (Invitrogen) and treated with DNase (Ambion). cDNA was synthesized from body part RNA using either the BioRad iScript™ cDNA Synthesis Kit with a oligo (dT) and random hexamer mix or the Clontech cDNA kit with the oligo-dT₁₈-Not I (0.2 μM) reverse primer. Products covering the open reading frame (ORF) of *Anoga-AKH-I*, *Anoga-AKH-II*, and *Anoga-AKHR* were amplified by PCR from body part cDNA with the following forward (fwd) and reverse (rev) primer pairs (0.2 μM): *Anoga-AKH-I* fwd 5'-tct aat taa tct acc gcc gag atc ttt-3' and rev 5'-ATG CTT TGC GCC AGC ACT CCA TA-3'; *Anoga-AKH-II* fwd 5'-GGC AGC TAG TGT GAT CGG TGT TAT-3' and rev 5'-CGG TCA GCG TAC TAT ATG TCC CAT-3'; and *Anoga-AKHR* fwd 5'-CAG CCA GCC AGC CAG AAC-3' and rev 5'-GGA GCG TTA GTA ACA TGG AAT GAA GTG-3'. Conditions were set according to Eppendorf® MasterMix (2.5x) PCR kit: initial denaturation, 2 min at 94°C ; amplification for 20 s at 94°C ; 20 s at 60°C for *Anoga-AKH-I* and *Anoga-AKHR* or 55°C for *Anoga-AKH-II* for annealing; and 1 min for *Anoga-AKH-I* and *Anoga-AKH-II* or 1 min 30 s for *Anoga-AKHR* at 65°C for 32 cycles, followed by a 5 min 65°C incubation. Products from PCR were separated in 1.5% agarose gels, and bands of the expected size were cut out and treated on GenElute™ Minus EtBr Spin Columns (Sigma). These products were cloned (TOPO TA Cloning Kit, Invitrogen), and plasmids isolated (QIAquick PCR Purification Kit) for sequencing at the Integrated Biotechnology Laboratories (IBL, University of Georgia, Athens, GA). Nucleotide sequences spanning the ORF were obtained and compiled for comparison to the predicted gene sequences. For confirmation of intron sequences in

the *Anoga-AKH-I* gene, E.N.Z.A.® Mollusc DNA kit was used for the isolation of genomic DNA, which was used as template for PCR, as described above.

The 3'-untranslated regions (UTRs) of *Anoga-AKH-I*, *Anoga-AKH-II*, and *Anoga-AKHR* mRNA were obtained from body part cDNA, after reverse transcription of total RNA with the primer, oligo-dT₁₈-Not I. The respective forward primers and the reverse primer Not I (5'-AAC TGG AAG AAT TCG CGG CCG CAG GAA-3', $T_a = 63^\circ\text{C}$) were used for PCR as above, and aliquots of the PCR mixtures (1 μl PCR mix/1000 μl nano pure water) were used to amplify "nested" products by PCR, as above with an annealing temperature of 55°C , a reverse primer (5'-GGG GAG GCT CGA GT₁₇-), and the forward primers: *Anoga-AKH-I* nes 5'-ATG TTG ATC ACC GAA GCT CTG ACC-3', *Anoga-AKH-II* nes 5'-TAT GAA TTC AAT CAG CTC GTC CCG-3', and *Anoga-AKHR* nes 5'-CTT TCT GAT GAT GCC ACT GGA GA-3'. PCR products of expected sizes were cloned and sequenced as above, and the nucleotide sequences were compiled with ORF sequences. Translations of ORF sequences were used for a Pileup of related protein sequences (HostExplorer for Win32, Version: 8.0.0.0 at the UGA Research Computing Resource).

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

To examine *Anoga-AKH-I*, *Anoga-AKH-II*, and *Anoga-AKHR* expression in different life stages and in females after sugar and blood meals, cDNA was prepared as above (BioRad iScript™ cDNA Synthesis Kit) from body regions or tissues. Specific products were amplified by PCR with the primers given above that span the ORF and conditions from the following sets of templates.

Set 1 for the different life stages: fresh eggs, hatched first instar larvae, fourth instar larvae, pupae and adults.

Set 2 for time points after females (5 days old with access to 10% fructose solution) had ingested a blood meal: 2, 6, 12, 24, 36, 48 and 60–72 h (oviposition occurred between 60 and 72 h post blood meal).

Set 3 for time points after females (starved for 12 h after eclosion) had access to 10% fructose for 2 h: 2, 6, 12, 24, 36, 48 and 72 h.

For each experimental set, three different cohorts of mosquitoes were used for the analysis of results. For each cohort used in the above sets, cDNAs (1.0 μl) for all body regions of the stages or time points were all prepared together with single primer and "no template" controls and subjected to PCR at the same time. Integrity of the body region cDNA was checked by amplification of an *actin* gene (GeneID: 1275175, Locus tag: ENSANGG00000012550) product by PCR with specific primers (for 5'-CCC GCT GAA CCC GAA GGC TAA CC-3' and rev 5'-GTA CCA CCG GAC AGG ACA G-3' ($T_a = 60^\circ\text{C}$) and all samples in a cohort. To verify absence of genomic DNA contamination, PCR with *Anoga-AKH-I*

primers and total RNA as template was performed for the cohorts. Pictures of gels with PCR products were taken with GeneSnap 6.03 (SynGene) and processed with Photoshop 7.0 (Adobe).

2.4. Radioimmunoassay (RIA)

For antigen, synthetic *D. melanogaster* AKH (pQLTFSPDWa; Drome-AKH) was conjugated to porcine thyroglobulin by glutaraldehyde and used to immunize rabbits, following a standard procedure (Cao and Brown, 2001). Synthetic Tyr (Y) analogs of *Anoga-AKH-I* (pQYTFTPAWa) and *Anoga-AKH-II* (pQYTFSRDWNAA) were radiolabeled with ¹²⁵I and purified by HPLC (Crim et al., 2002) for use in antiserum binding assays. It was determined radiolabeled Y-*Anoga-AKH-II* and an antiserum from one rabbit (30C, third bleed) were best for the RIA of both *Anoga-AKHs*. When used at a final dilution of 1:3000–6000, the Drome-AKH antiserum gave a bound/free ratio of ca. 1 of radiolabeled Y-*Anoga-AKH-II* (~10,000 cpm/tube) in the absence of unlabeled peptide. This same antiserum was used for immunocytochemistry with *An. gambiae* tissues. The *Anoga-AKH* analogs were synthesized on an Applied Biosystems 433 synthesizer (Clark et al., 2005), and purified by HPLC (Jupiter C18, 10 mm particle size, 21.2 mm $\times$ 25 cm, Phenomenex Inc., Torrance, CA). Peptide structures were confirmed by MALDI-TOF mass spectrometry (Clark et al., 2005).

To compare the binding of *Anoga-AKHs* and other AKHs in the RIA, standard amounts (per 100 μl) of synthetic *Anoga-AKH-I*, *Anoga-AKH-II*, unlabeled Y-*Anoga-AKH-II*, Drome-AKH, *T. atratus* hypotrehalosae-mic hormone (Tabat-HoTH), *Heliothis zea* AKH (Helze-AKH), *L. migratoria* AKH-I (Locmi-AKH-I) and Locmi-AKH-II (Table 2) were set up in triplicate tubes with radiolabeled Y-*Anoga-AKH-II* (~10,000 cpm/100 μl), and Drome-AKH antiserum (1:1000–2000 dilution/100 μl ; total 300 μl /tube; RIA buffer: 0.05 M Tris-HCl, pH 7.2; 0.1% bovine serum albumin; and 0.02% sodium azide). After overnight incubation at 4°C , bound and free radiolabeled AKH were separated by centrifugation after the addition of a mixture of activated charcoal dextran goat serum to each tube, and the pellets counted in a Packard Cobra II gamma counter. For each AKH peptide, a curve was plotted from the log values of the standard amounts and their averaged bound/free ratios.

2.5. Extraction and HPLC of AKH from female body parts

Heads, thoraces, and abdomina of 200 females were collected separately and homogenized in 4 ml of an ice cold solution of methanol/water/acetic acid (90:9:1 v/v/v). The solution was briefly sonicated and centrifuged at 15,000g for 15 min at 4°C . Methanol was removed from the supernatant solutions by vacuum centrifugation, and the remaining solutions were stored at -80°C . These solutions were thawed, centrifuged as above, and passed through C₁₈

cartridges (3 g sorbent mass, Mega Bond Elut, Varian, Harbor City, CA), preconditioned with acetonitrile (CH₃CN) followed by 0.1% trifluoroacetic acid (TFA) in water (2 ml each). Afterwards, the cartridges were washed with TFA solution (2 ml), and absorbed material was step eluted with 10% and 80% CH₃CN in TFA solution (2 ml each). The same cartridge was washed with CH₃CN, and the procedure repeated. As determined by the RIA of aliquot dilutions, only the 80% CH₃CN eluates contained AKH-immunoreactive material.

After reducing the volume by vacuum centrifugation, the 80% CH₃CN eluates of body parts were subjected to analytical HPLC (Beckman 126/166 system). Each eluate was mixed with an equal volume of the initial mobile phase, injected onto a C₈ column (Alltech Macrosphere 300, 7 µm 300 Å matrix, 250 mm × 4.6 mm), and eluted with a CH₃CN gradient (mobile phase A, 0.1% TFA in water, and mobile phase B, same with 80% CH₃CN; gradient of 20 100% B over 45 min; flow rate of 1 ml/min; and monitored at 280 nm). To test the column for contaminating AKH-like material, a blank injection was made before the first body part eluate, and the fractions (1 ml) were collected for RIA. In addition, a gradient (same mobile

phases as above; 20 100% B, 15 min; 100 20% B, 15 min) was run between the body part injections. Lastly, synthetic Anoga-AKH-I and AKH-II (2 µg each) were injected together and eluted as above (monitored at 206 nm); the elution time for each peptide had been determined previously by HPLC. Fractions (1 ml) were collected in polypropylene tubes separately for each body part eluate and the synthetic peptides, and aliquots for RIA were taken before freezing and lyophilized.

For RIA, aliquots of the HPLC fractions were rehydrated with RIA buffer (225 µl) and briefly sonicated to solubilize the material. Duplicate tubes (100 µl of solubilized sample/tube) were prepared for each fraction, and radiolabeled Y-Anoga-AKH-II and diluted Drome-AKH antiserum were added. Standard amounts of Anoga-AKH-II were prepared in triplicate tubes as above for the RIA. After separation of bound and free radiolabeled AKH, as above, and counting of the pellets, a standard curve was plotted for Anoga-AKH-II, and a regression line was calculated for its linear range (250–10,000 fmol) and used to determine relative AKH amounts from the bound/free ratios obtained for the fraction samples.

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1  TCTAATTAATCTACCGCGAGATCTTTGCCGGTGCCGCGCATATGGATACCGTGAAGC
1                                     M D T V K
61  TGTTCACCGTGCTGCTGATTGTGCCTCTTTGATGTTGATCACCGAAGCTCAGcTGACCT
6  L F T V L L I C A S L M L I T E A Q L T
121 TCACACCGGCTGGGGCAAGCGGTGCGAAGTGCGATGGGAATTAATCCGCTCGGCAGTA
26  F T P A W G K R S Q G A M G I N P L G S
181 CCTTCGGGCAGGACGCGTGCAAAACACCGGTTGATTGCTGCTCGTCATCTACCGCATGA
46  T F G Q D A C K T P V D S L L V I Y R M
241 TTCAggCCGAAGCGCAGAAGATTGTTGATTGCGAGCAGAAGTAGACACCAACGCCACCA
66  I Q A E A Q K I V D C S Q K *

301 GCCTATGGAGTGCTGGCGCAAAGCATCGCGCCCGTGGCCACCGTTTGCCCGCGCTTTCT
361 TGCCCGATGCGCGCTCCACGCTCTTCATACAGTCGGTTGCTCGATTATCCCGCAGGCAG
421 GATGTACACAGATTTTCTCACCGTTTGTTCGTTTCGTTTCGGCTCACTGGGCAACAAAGA
481 TAGCTCTTGTTCGAGGACCACTTCACCCCATCATGTTTCGGCCTTGTTCGGGGTTG
541 CGCTGTATTGTAATAACTCGTGCTGCTCTATCATCGACGGAAGGATTCAATGTGACAAA
601 GTCAAAACAAACAAATCAGAAAAAATAAACAAATGGCATAAGTTAAAAAATAAAAAA
608 AAAAAAA

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Intron 1 (99 bp)

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1  GTAAGCGCAGATGGATGGATGATGTTTACCGCGGCGCGGCGAGTTTCGCGGGCCCTAGCA
61  CCACCCCGCAGCAGGTACTAATAGAAATGCCGTTTCAG

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Intron 2 (116 bp)

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1  GTAGGGTTGGGGAATGGGTGGCGAAACCGGTGAGCGAAGCGAAGCGAATGTCATGG
61  TACAAACCGGTGACAAATGCATATAACTTTCTCTCTCCCTATTGGGAATGCACAG

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Fig. 1. Nucleotide sequence of gDNA and body part cDNA encoding open-reading frame for *An. gambiae* AKH-I (*Anoga-AKH-I*) with flanking sequences (3' polyadenylation signal underlined). Intron sequences (positions indicated between small underlined letters in the cDNA sequence) are below the diagram of the *Anoga-AKH-I* gene structure. In the prepropeptide sequence, signal peptide is in italics, and Anoga-AKH-I is in the black box with the processing site in the gray box, followed by the AKH-precursor-related-peptide (APRP).

2.6. Immunocytochemistry

Tissues from females (10–15 days old) were dissected in 4% paraformaldehyde in phosphate buffered saline (PBS) and incubated for 1.5 h at room temperature. Tissues were permeabilized for 30 min in 0.1% Triton X-100 in PBS at room temperature and washed with PBS 2 × 10 min at room temperature. For blocking, 5% goat serum and 0.1% Tween-20 in PBS (PBS-GS-T) was added to the tissues and incubated for 2 h at 4 °C. Antiserum to Drome-AKH (30C; 1/500 1/2,000) was added directly to the blocking solution for an overnight incubation at 4 °C. Tissues were washed 3 × 30 min with PBS-GS-T at 4 °C, and then secondary antibody (1/2000; Molecular Probes; Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG, 2 mg/ml) was added for an overnight incubation at 4 °C. Tissues were washed 3 × 30 min with 0.1% Tween-20 in PBS at 4 °C and mounted on slides in a 1:1 solution of PBS:glycerol. As negative control, diluted antiserum was incubated with 20 µg of synthetic Anoga-AKH-I overnight, prior to immunocytochemistry. Processed tissues from more than three females were observed for immunostaining. Images were acquired with Auto Montage software (5.01; Syncroscopy) through a digital camera (JVC KY-F70B) attached to an Olympus BX60 microscope equipped with an epifluorescent light source.

3. Results

3.1. *Anoga-AKH* and *Anoga-AKHR* cDNAs

The predicted genes, *Anoga-AKH-I* (GeneID: 88595937, GenBank: DQ388479) and *Anoga-AKH-II* (GeneID: 3290616, Locus tag: ENSANGG00000021979, GenBank: DQ396550), are located on chromosome 3R and 2R, respectively. After cloning specific PCR products obtained from body cDNAs, the product sequences were compiled for *Anoga-AKH-I* and *Anoga-AKH-II* (Figs. 1 and 2). The ORF of *Anoga-AKH-I* is encoded by 240 bp, and the gene contains two introns of 99 and 116 bp (confirmed by sequencing PCR products from gDNA, Fig. 1). The ORF of *Anoga-AKH-II* is encoded by 342 bp, and the gene contains two introns of 92 and 794 bp (not confirmed). Predicted amino acid sequences for the AKH ORFs were identical to that of the genes or ESTs in the *An. gambiae* ENSEMBL database, and they are compared to sequences of AKH prepropeptides from other insects in Table 1.

A putative *Anoga-AKHR* (GeneID: 1269400, Locus tag: ENSANGG00000016873, GenBank: DQ396551) with high sequence similarity to *Drome-AKHR* is located on chromosome 2R and is predicted to have six introns of 81, 87, 73, 80, 72 and 76 bp (not confirmed). After cloning specific products obtained from body cDNA and compiling

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1  GGCAGCTAGTGTGATCGGTGTTATCGAATCGTGTATCCTTCCGGAACGTGTGTGATGATTT
61  CCGAACTATTTCAACAATCATTATGAATTCATCACTCGTCCGTCATCTTGCGGGCTA
1  M N S I S S S R H L A A
121 AGCTCTTCTGCTGGTCCGCTGTGTCCGTGCTGCTTCCGGTCCGAGCGCTGGACAGG
14  K L F L L V A L C A V L L P V P S A G Q
181 TAACGTTTCCGCGATTGGAATGCGGCAAGCGGCGATGCCGACTCGCCGGTCAGCG
34  V T F S R D W N A G K R A M P D S P V S
241 GGTGGCCGAATGCTCTGCCATCTGGCGTCCGGTGAACCACTGTGCGCGGAGTAACga
54  G V A E C S A I W R P V N N L C A A V T
301 AAAATATTCAACACCTAACGCTGTGCGAAACACGCTCCCTGCTTAAATCACTGCAACCGG
74  K N I Q H L T L C E T R S L L K S L Q T
361 ATGAGGCATCGATGAAAGCAACAGCGGGAACCACTTGCCAATGTTTCCAAACAACATA
94  D E A S M E S N S G N N L P M P S N N H
421 TCTAACCTGCATCTAACTGTTTGTATGGAAGAAGAAAAACAGCACTGTAGACTTAAGCA
104 I *

481 GTGCTGGTAGTATCTGAGACCGTTTGCAATGGGACATACGCTGACCGAATTCGCTTCAAT
541 TACAACTAGAGAGAGAAAAATCCTTCTATTAAATCCCAATTGAATAATGTTCCATTT
601 CCTTAATACTTCTGTAACCTGCCTTTACATTGAAATCCACACAAACACACATTTGTTTT
661 AATGTATCCAGTAATGATTGCATCGTACACCTTCTAAACAAATGAGCATGTCCCAATTGC
721 GCATAGTGTAGCATAACATATTGCATCGCGCAAGCAAGAAAAACATACAAAAA
781 AAATAATGACAAAAAATAAAGTAAAAATACAGTCATAATAATAAAAAAAAAAAAAAA
841 AA

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Fig. 2. Nucleotide sequence of body part cDNA encoding open-reading frame for *An. gambiae AKH II* (*Anoga-AKH-II*) with flanking sequences (3' polyadenylation signal underlined). Predicted positions of the first intron (92 bp) and the second one (794 bp) indicated between small underlined letters in the cDNA sequence and in the diagram of the *Anoga-AKH-II* gene structure. In the prepropeptide sequence, signal peptide is in italics, and *Anoga-AKH-II* is in the black box with the processing site in the gray box, followed by the AKH-precursor-related-peptide (APRP).

Table 1
Comparison of AKH prepropeptide sequences (Pile Up program) known for different insects.

Name	Sequence
Dros-AKH	-----NPKSEVLIAAFLFLLACVQCQTESEDTAKRSVGGRGPGTFPEF-QQCKTSNEMLELFFP-VQSQDGLFLCKHRE-----
Anoga-AKH-I	-----NTVKLFTEICASMLITEQDFTFAKRSQGMGINPLGSGDACKTPVDSLVIVFM-FOAERQIVICQK-----
Locmi-AKH-II	-----TQSCTEFVAVVAVLALATQMSAGD-SEFYD-----P-----NDDPAPLAYEL-FOIARILAGD-----
Locmi-AKH-I	-----MYQCAEVLELVAVAAALCSQAMFTNTMTSGKDA-----D-----FADPYSTFAYEL-FOAERRVSCGN-----
Bladi-HrTH	-----MHLVAVLVVAVLAVLCEQVMSFGCTGKASV-----QDSPCKGSEBSMYTKKI-VQNEKQILCEKFPSSN-----
Locmi-AKH-III	-----QVRAVEVLAVLAVATSRQDNGFTWQK-EALGA-----PAAGDCVQSPQALGILNAAQAVVQILDCGRFTSEANS-----
Manse-AKH	-----MYKTEFEMFIAVLAEQVETSSG-SES-AM-----TNSISCRNDEALAAEK-AQNEERFINGEN-----
Anoga-AKH-II	-----MNSISSEHLEAKLFELVACAVLPPPSAGQVTSRDVWAGK-AMPDSPVSGVAECSAIWRPVNVC-----AAVTKKIQHTTCTETRLLESLOTDEASNESNGNLPMFNNHI-----
con	m k lvvllvvavllal aQlnPsp Wg gtr am ad l iyrl iq eaqlidcs

Conserved residues are illustrated with black, similar with dark grey, and identical with light grey background by a threshold of 0.50. The consensus row (con) shows the conserved amino acids in capital letters and identical in small letters. Anoga, *Anopheles gambiae*; Bladi, *Blattella discoidalis*; Drome, *Drosophila melanogaster*; Locmi, *Locusta migratoria*; Manse, *Manduca sexta*.

sequences for the *Anoga-AKHR*, the predicted amino acid sequence for the ORF (1065 bp; Fig. 3) differed by only one residue in the first intracellular loop at position 74 (Arg instead of Gln) from that of the gene in the *An. gambiae* database. The putative *Anoga-AKHR* is predicted to have seven transmembrane regions, an amino (N)-terminus of 50 residues, and a carboxy (C)-terminus of 27 residues (Fig. 3). This protein sequence is compared to that of other insect AKH receptors in Table 3.

3.2. *Anoga-AKH* and *Anoga-AKHR* transcript expression profiles

As the first step to evaluate changes in transcript abundance during important physiological processes in females, *Anoga-AKH-I*, *Anoga-AKH-II*, *AKHR*, and *actin* products were amplified by PCR from female head cDNA for 22, 32, 42 cycles (Fig. 4A). The density of the PCR band corresponding to each gene of interest was intermediate for 32 cycles, thus this number of cycles was used for RT-PCR to determine whether there were observable differences in band intensity in different body regions of females during the course of sugar and blood digestion. Although only representative gels are shown in Fig. 4A E, the control PCR reactions with the *Anoga-AKH-I*, *Anoga-AKH-II*, and *AKHR* primers alone and with no-template or total RNA were consistently negative, and those with the *actin* primers, positive, across all samples in the cohorts used in the experimental sets described below.

Set 1 for different life stages (Fig. 4B): *Anoga-AKH-I* and *Anoga-AKH-II* transcripts of the expected processed size (326 and 529 bp, respectively) were present in freshly hatched larvae and heads and thoraces of fourth instar larvae, pupae, and adults of both sexes. An additional band of 541 bp for *Anoga-AKH-I* was amplified in eggs and only in abdomens of the other life stages. Presumably, this product was amplified from cDNA of unspliced *Anoga-AKH-I* mRNA, because the total RNA for all body parts was treated with DNase, and there is no evidence of gDNA after PCR with *Anoga-AKH-I* primers and total RNA as template (Fig. 4B, gel on the bottom). A subsequent study determined the tissue source of such transcripts in female abdomens (see below, Fig. 4E).

Anoga-AKHR transcripts of the expected size (1110 bp) were present in the three body regions of all life stages (Fig. 4B), but heads appeared to have lower transcript abundance in comparison to other body regions. This distribution of *Anoga-AKHR* transcripts likely reflects that of fat body, which is present throughout the mosquito body but predominantly in the thorax and abdomen (see below).

Set 2 for females post-blood meal (Fig. 4C): The distribution of *Anoga-AKH-I*, *Anoga-AKH-II*, and *AKHR* transcripts described above for non-blood-fed females also was evident in the body regions of females taken at different times after a blood meal and oviposition, the span of a gonotrophic cycle. Again, the abundance of *AKHR*

N terminus

1 CAGCCAGCCAGCCAGAAACAATGCCCAACACAATGGCCGCCACATCAACCAGCGCATCGA
1 M P N T M A A H I N Q R I E

61 GGACCATCGGAATTGGCCGACTGGTCTGACTATGCGAACGAAACGGCCGGCGAGGAGTA
15 D H R N L A D W S Y Y A N E T A G E E Y

121 CTACGAGATGCCGATCGATATGCGCTTCAACTCCGGCCACATCCTCTCGATCATGGTGTA
35 Y E M P I D M R F N S G H I L S I M V Y

TM 1

181 CA~~ct~~LACTGATGGTGTTCGGCCACGGGCAACCTTACGGTGCTCAGCATACTGGCCCA
55 T T L M V F S A T G N L T V L S I L A R

IC 1

241 GCGGAAGGTGCGCGCTCGTCGGGATCAATATCATGTTGGCGCATCTAGCGATTGCAGA
75 R K V R A S S R I N I M L A H L A I A D

TM 2

301 TCTGCTggTCACCTTTCTGATGATGCCACTGGAGATTGGATGGGCGTACACGGTTAGATG
95 L L V T F L M M P L E I G W A Y T V R W

EC 1

361 GACGGCCGGTGACTTGATGTGTCGCGTGATGGCGTTCCTCCGACCTTCGGGCTGTATCT
115 T A G D L M C R V M A F F R T F G L Y L

TM 3

IC 2

421 GTCCAGCTTCATTCTGATCTGCATATCGGTGGACagaTACTTTGCTGTGTGAAACCGCT
135 S S F I L I C I S V D R Y F A V L K P L

TM 4

481 CAAGGTGCACGAACATCGGGCGTGCTGATGATAGCAGCGGCTGGATAATGTCGGACT
155 K V H E H R A V L M I A A A W I M S G L

541 GTGCAGTTTACCTCaggCGTTTATCTTTACCTGGAAGGACATCCAAACATTACGGGATA
175 C S L P Q A F I F H L E G H P N I T G Y

EC 2

601 CCAGCAGTGCGTAACGTACCACTACTTTGAGGAGGAGATCTACCAGATCATCTACAACGT
195 Q Q C V T Y H Y F E E E I Y Q I I Y D V

TM 5

661 GCTGGTAATGTGCTGATGTACACCTTCCCGCTGATGTGATCCTCTACTGCTACGGGTC
215 L V M C L M Y T F P L I V I L Y C Y G S

IC 3

721 TATCTACTACGAAATATTTAGTCGTACCAATCCACGCAATTTAgAGAGCTTTCGCCGCTC
235 I Y Y E I F S R T N P R N L E S F R R S

781 GTCGATCGATGTGCTTGGCGAGCGAAGCGAAAGACGCTACGGATGACCATAATGATCGT
255 S I D V L G R A K R K T L R M T I M I V

TM 6

841 GATCGTGTTCGTCTGCTGGACGCCCTACTACGTATGTCACCTATggTACTGGCTCGA
275 I V F V V C W T P Y Y V M S L W Y W L D

EC 3

901 CAAGGAGTCGACCAAAAATGTGGACCAGCGAATACAGAAGGGACTGTTTCTGTTCCGCCAG
295 K E S T K N V D Q R I Q K G L F L F A S

TM 7

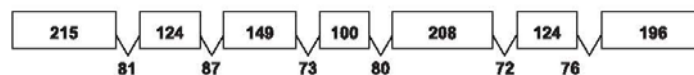
961 CACGAACAGCTGCATGAACCCGGTGGTGATCGGTGTCTTTAACGTGCGCAAGAAACACAC
315 T N S C M N P V V Y G V F N V R K K E T

1021 TAAAAAATGCTAAAGACGACACACGAGAAAAGCTGCGGCAGTCATCTGACGATGAGGGC
335 K K L L K T T H E K S C G S H L T M R A

C terminus

1081 G~~TA~~AGAGCGTTAGTAACATGGAGTGAAGTGTGTGTTGGGGAGGGAGAGATTTTAAGATG
355 *

1141 TTTGCCCCATTTTATAGCATAGGATAGCGTTACAACCTTACAATTACACTACTCACGGTCT
1201 GAAATACTCTGTATGTAGCGGCTATGTACGCAATAAAACCCACGATTCCGTTGGACGTATCC
1261 ATGAAATGGAAGGATCATAAAAA



transcripts appeared to be lower in heads than in the other body regions, and the 541 bp band for *Anoga-AKH-I* was present only in abdomens of all sampled females. As surveyed for three cohorts of blood-fed females, there were no observable differences in *Anoga-AKH-I*, *Anoga-AKH-II*, and *AKHR* transcript abundance/body region between any of the sampled times during a gonotrophic cycle.

Set 3 for females post-sugar meal (Fig. 4D): The distribution of *Anoga-AKH-I*, *Anoga-AKH-II*, and *AKHR* transcripts in body regions of females did not differ from that described above, and there were no evident differences in transcript abundance for up to 72 h after a sugar meal.

An additional RT-PCR study determined the tissue sources in female abdomens of transcripts for *Anoga-AKH-I* and *AKHR* (Fig. 4E). Different parts or tissues in abdomens were processed for cDNA, which was used for PCR as above, and the larger *Anoga-AKH-I* product was amplified only from ovary cDNA. To confirm the identity of this larger PCR product, another product with the 3' untranslated region was amplified from abdominal cDNA with the *Anoga-AKH-I* forward primer and a oligo-dT₁₈-Not I reverse primer, followed by *Anoga-AKH-I* "nested" forward primer and Not I reverse primer, cloned, sequenced, and found to contain the two introns – another indication that this product originates from stable, unspliced *Anoga-AKH-I* mRNA. In addition, the *Anoga-AKHR* product was amplified in abundance from dorsal and ventral abdominal wall cDNA, but less so from ovary cDNA (Fig. 4E), and not from other tissues.

3.3. Radioimmunoassay (RIA)

In a test study, an antiserum made to Drome-AKH RIA bound radiolabeled Y-Anoga-AKH-II, thus a RIA was developed for the Anoga-AKHs. The specificity of the RIA was determined by competitive displacement of the radiolabeled Y-Anoga-AKH-II by different amounts of Anoga-AKHs and related insect peptides (Fig. 5): unlabeled Y-Anoga-AKH-II > Drome-AKH > Anoga-AKH-I = Helze-AKH = Tabat-HoTH > Anoga-AKH-II. Locmi-AKH-I and Locmi-AKH-II showed no binding.

3.4. Extraction and HPLC of AKH from female body parts

Female head, thorax, and abdomen extracts were subjected separately to reversed-phase HPLC to separate the Anoga-AKHs, so that their presence in the different body regions could be determined with the AKH RIA.

Wash gradients were performed between each HPLC of the body region extracts and the final HPLC of synthetic Anoga-AKHs to prevent carryover of AKHs. Based on the elution and detection of the synthetic Anoga-AKHs (Fig. 6), both Anoga-AKHs were present in the head extract, but only Anoga-AKH-I was in the thorax extract. As calculated from the RIA results and the relevant dilutions, the amount of Anoga-AKH/female body part was estimated: Anoga-AKH-I, 32 fmol/head and 115 fmol/thorax, and Anoga-AKH-II, 25 fmol/head. Neither Anoga-AKH was detected in the abdomen extracts after HPLC (results not shown).

3.5. Immunocytochemistry

Since the Drome-AKH antiserum recognizes both Anoga-AKHs, it was used for immunocytochemistry to identify cell sources (Fig. 7). Preabsorption of the antiserum with Anoga-AKH-I blocked immunostaining (Fig. 7A), thus demonstrating its relative specificity in this method. In female brains, only pairs of lateral neurosecretory cells (Fig. 7B and C) and their axons in the protocerebrum were immunostained with the antiserum. Immunostaining was evident in the neurohemal region of the CC (Fig. 7B) and in the separate "intrinsic CC cells" or "X cells" (Clements et al., 1985; Meola and Lea, 1972) and their axons extending anteriorly to the CC (Fig. 7C and E). In addition, immunostained axons extended posteriorly from the CC along the tubular anterior midgut and onto the posterior midgut (Fig. 7F). No immunostaining was observed in the abdominal ganglia or in midgut endocrine cells.

4. Discussion

Many AKHs are known for a great variety of insects, and now the characterization of genes encoding two different AKHs and their expression is reported for the first mosquito, *An. gambiae*. A putative AKH GPCR also was characterized, and its ubiquitous gene expression in all stages and body regions suggests that it has a key role in AKH signaling. Our results provide a basis for comparing the AKH signaling system in a mosquito to that in other Diptera and insects in general.

4.1. Structural comparisons to other insect AKHs

The identification of *Anoga-AKH-I* and *Anoga-AKH-II* brings the number of AKH genes known for Diptera to

Fig. 3. Nucleotide sequence of the body part cDNA from *An. gambiae* encoding a putative G-protein-coupled receptor for AKH (*Anoga-AKHR*) with flanking sequences (3' polyadenylation signal underlined). Positions for the predicted six introns indicated between small underlined letters in the cDNA sequence and in the gene structure diagram (exons boxed and linked introns with base pair numbers). Amino acid sequence of the open-reading frame starts with the extracellular amino terminus (N-terminus) and ends with the intracellular carboxy terminus (C-terminus) and between these regions, the predicted seven transmembrane helices (TM 1–7) are boxed in gray. ▼ indicates putative glycosylation site, and ● indicates phosphorylation sites. Boxed residues predicted for receptor transduction and G-protein coupling. IC, intracellular loop, EC, extra cellular loop. Above structural predictions were made with the bioinformatics tools on the CBS server (<http://www.cbs.dtu.dk/biotools/>).

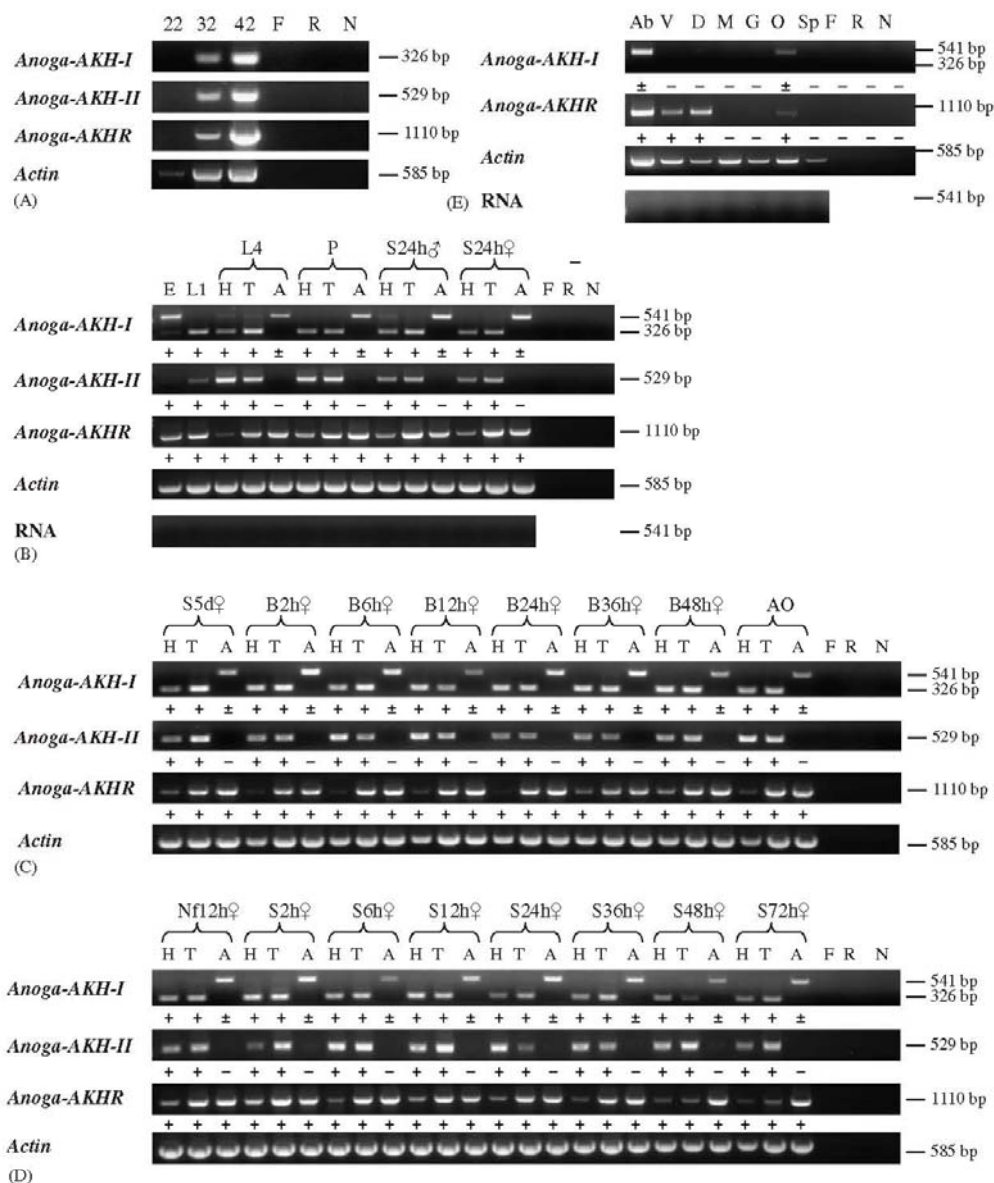


Fig. 4. Survey of transcript expression for *Anoga-AKH-I*, *Anoga-AKH-II*, and *Anoga-AKHR* in *An. gambiae* by reverse-transcriptase PCR shown in representative gels with specific PCR products (size to the right). Primers, template, and conditions (32 cycles for PCR amplification, except for A) as given in Section 2. A representative gel showing *actin* products is included with each set to demonstrate cDNA integrity of the samples, and a representative gel is included in B and E showing absence of gDNA contamination after *Anoga-AKH-I* PCR with total RNA used to make cDNA, as template. Top of gels: H, head; T, thorax; A, abdomen; S, sugar-fed; Nf, non-fed; B, blood-fed; AO, after oviposition; E, eggs; L, larvae; ♀, female; ♂, male; Ab, abdomen; V, ventral abdominal body wall; D, dorsal abdominal body wall; M, midgut; G, hindgut; O, ovaries; Sp, spermatheca; F, forward primer control; R, reverse primer control; N, no template control. Below each gel in B-E is a summary of product presence for three cohorts: +, present in majority, and -, absent in majority; ±, only upper band for *Anoga-AKH-I*. (A) Increased PCR product density after amplification for 22, 32, and 42 cycles (numbers on top) with cDNA template from heads of 1 day old sugar fed females. (B) Life stages and body regions. (C) Time course for females before and after blood feeding and oviposition (60–72h post-blood meal). (D) Time course for females before and after sugar feeding. (E) Transcript expression for *Anoga-AKH-I* and *Anoga-AKHR* in abdomen tissues of female *An. gambiae* (10 day old sugar-fed), and total RNA⁺ as template to demonstrate absence of gDNA contamination.

three. *Anoga-AKH-I* and *Drome-AKH* appear to be orthologs, because they encode related octapeptide AKHs and have an intron between residues 1-Gln and 2-Leu of the AKHs (Fig. 1). *Anoga-AKH-I*, however, has another

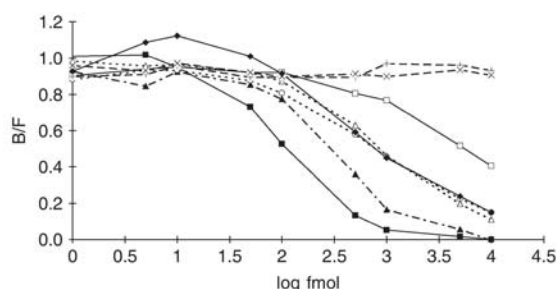


Fig. 5. Radioimmunoassay demonstrating Drome-AKH antiserum binding to related synthetic AKHs: Tyr-Anoga-AKH-II (■), Anoga-AKH-I (◆), Anoga-AKH-II (□), Drome-AKH (▲), Tabat-HoTH (△), Helze-AKH (○), Locmi-AKH-I (+) and Locmi-AKH-II (×). Curves show displacement of ^{125}I -labeled Tyr-Anoga-AKH-II from Drome-AKH antiserum by increased amounts of synthetic peptides (log values, X-axis) plotted as a bound/free ratio (Y-axis) calculated from two radioimmunoassays.

intron that is not present in *Drome-AKH* (Noyes et al., 1995). *Anoga-AKH-II* has two introns in different positions than the two in *Anoga-AKH-I*. Other AKHs have been extracted and biochemically characterized for dipteran species (Table 2). Interestingly, both octapeptide and decapeptide AKHs have been identified in species from the lower two Suborders Nematocera (*An. gambiae*) and Brachycera (*T. atratus*) of Diptera, but only octapeptide AKHs are reported for species (*D. melanogaster*, *Ph. terraenovae*, and *N. bullata*) from the higher Suborder Cyclorrhapha. The questions, as to whether these higher flies have lost the decapeptide AKH gene, or it has yet to be identified, need to be examined.

The dipteran octapeptide and decapeptide AKHs have the same amino acids at residues 1, 3, 4, and 8 and conserved substitutions at residue 2 and 5 (Table 2). More notable are the different amino acids found at residues 6 and 7, specifically the charged ones (Arg-Asp) in *Anoga-AKH-II*, and at residue 6 (Asp) in the “Fly AKHs”. Only a few AKHs from other insects are known to have charged amino acids in these positions, e.g. Locmi-AKH-IV (Table 2; Gäde et al., 1990; Schaffer et al., 1990; Verleyen et al., 2004). This locust AKH and

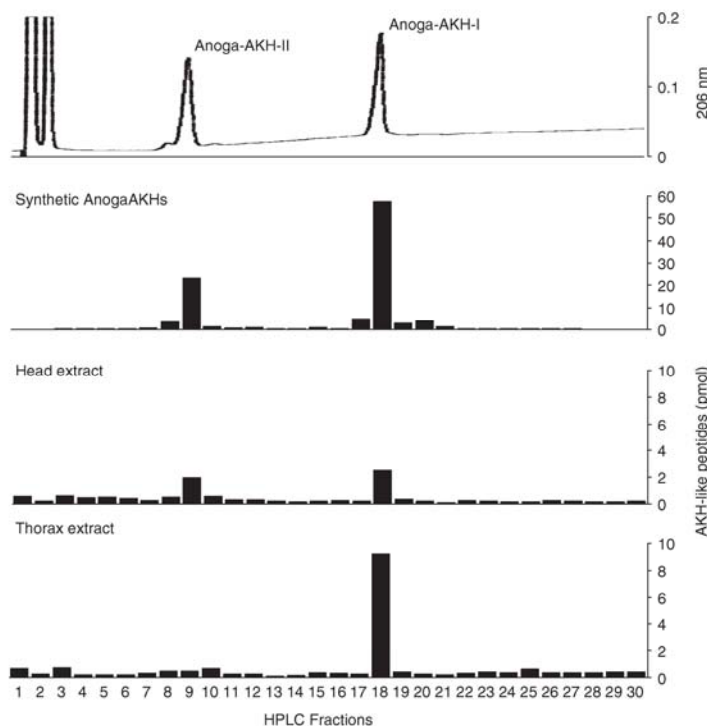


Fig. 6. Separation of Anoga-AKH-I and Anoga-AKH-II-like peptides in extracts of heads and thoraces from female *An. gambiae* (5 days old, sugar-fed) subjected to reversed-phase HPLC and Drome-AKH radioimmunoassay. Top panel: absorbance profile for separation of synthetic Anoga-AKH-I and Anoga-AKH-II (each 2 μg) by the same HPLC column and conditions (see Section 2) used for body part extract solutions. Second panel: quantities of AKH immunoreactive material detected by radioimmunoassay of fractions from HPLC separation of synthetic Anoga-AKH-I and Anoga-AKH-II. Third panel: as above, HPLC separation of head extract. Fourth panel: as above, HPLC separation of thorax extract. In the last two panels, immunoreactive material/fraction = approximately 80 body parts.

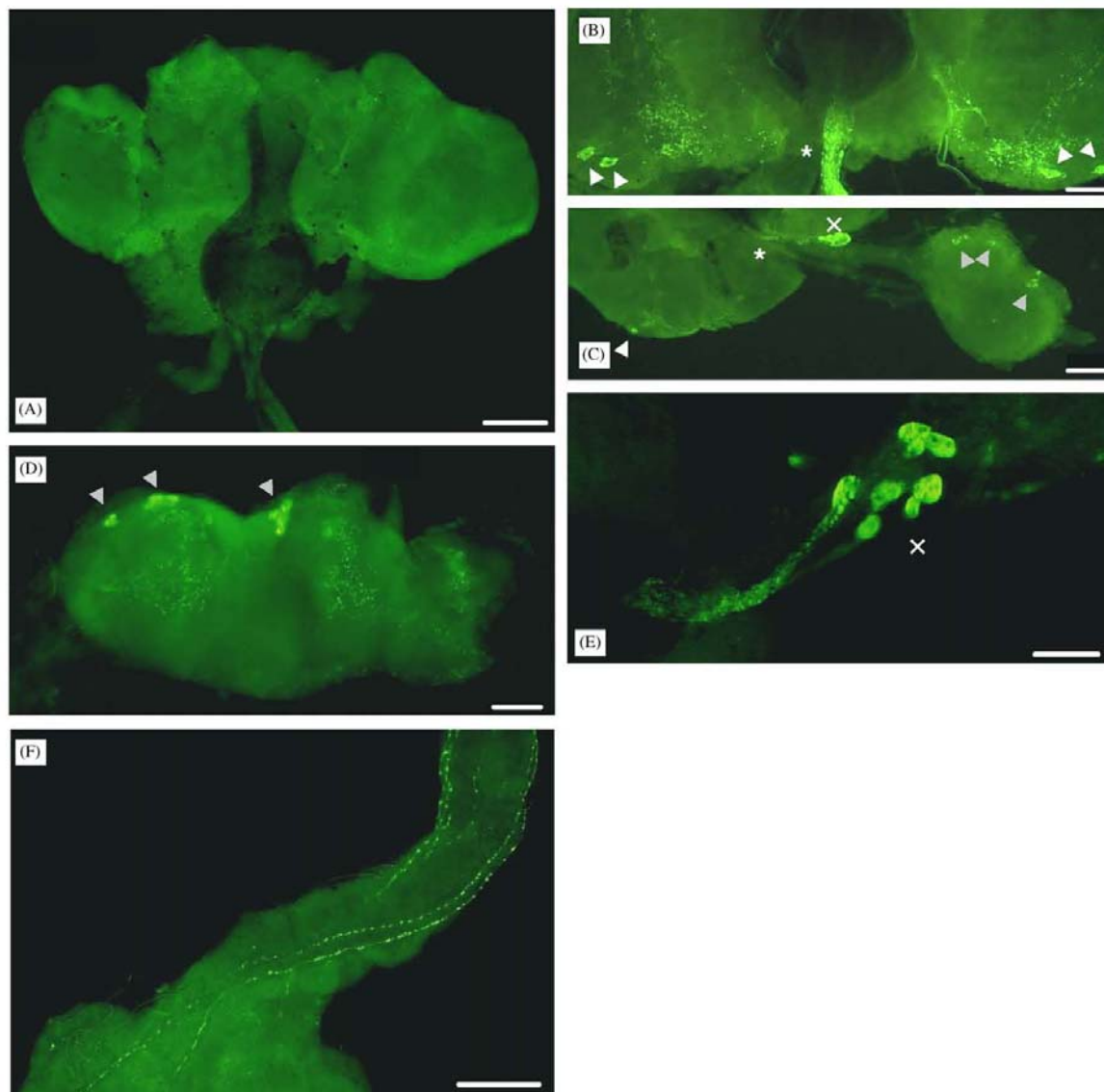


Fig. 7. Localization of AKH-like peptides in whole tissues of female *An. gambiae* (10–15 days old, sugar and blood-fed) by immunocytochemistry with the Drome-AKH antiserum. (A) Antiserum preabsorbed with Anoga-AKH-I showed no staining of brain (dorsal, top; bar = 100 µm). (B) Immunostaining of two pairs of lateral neurosecretory cells (▷) and their axons in brain and of the nervi corporis cardiaci (*) exiting the brain (dorsal, top; bar = 50 µm). (C) Immunostaining in the X-cells (×) and their axons, cells in the thoracic ganglia (►), and brain lateral neurosecretory cells (anterior, right; bar = 50 µm). (D) Immunostaining of cell clusters in the ventral region of the pro- and mesothoracic ganglia (anterior, right; bar = 50 µm). (E) Detail of immunostaining in the X cells (×) and their axons (anterior, right; bar = 50 µm). (F) Immunostaining in bilateral axons along the anterior and posterior midgut (anterior, top; bar = 100 µm).

Anoga-AKH-II have the same eight amino acid sequence at the amino terminus, and to date, there is no reported function for Locmi-AKH-IV. In addition, nonapeptide AKHs have been identified in two lepidoteran species (Jaffe et al., 1986; Ziegler et al., 1985), and recent studies of

Odonata species found only octapeptide AKHs (Gäde and Marco, 2005).

By comparing the insect AKH prepropeptides, it was found that Anoga-AKH-I (Table 1) shares the highest percentage (47.5%) of identical residues with that of

Drome-AKH and next (31.6%) with that of Locmi-AKH-II, but only 13.4% with the Anoga-AKH-II prepropeptide. The APRP region of these peptides contains conserved Cys residues that form inter-molecular disulfide bonds for homo- or heterodimerization between these peptides during storage in cells (O'Shea and Rayne, 1992). With the exception of the APRPs for Locmi-AKH-I and AKH-II, which contain only one Cys, all others have at least two Cys, thus indicating that dimerization may occur through two disulfide bonds, as known for Locmi-AKH-III (Huybrechts et al., 2002). In a further analysis of the APRP region alone, an identity/similarity matrix of insect APRPs was constructed, and the APRP of Anoga-AKH-I was more related to that of the AKH from the death-head cockroach, *Blaberus discoidalis* (42.9%) than to the Drome-AKH APRP (27.7%), and again least related to the Anoga-AKH-II APRP (3.0%). The prepropeptide of Anoga-AKH-II has the highest residue identity (21.8%) with those of Locmi-AKH-III and Anoga-AKH-I and next (18.5%) with that of Drome-AKH. The APRP of Anoga-AKH-II is the longest of the ones characterized to date (Table 1), and shares low identity with the other APRPs (6.6–11.1%). These structural comparisons suggest that the *Anoga-AKH* genes likely did not arise from a recent duplication in the *An. gambiae* genome, because of differences in intron placement and the APRP sequences, and as more insect AKH genes are characterized, their evolutionary relationships can be discerned.

4.2. *Anoga-AKH* transcript expression

After the genes for *Anoga-AKH-I* and *AKH-II* were identified, their expression in *An. gambiae* was examined by RT-PCR in different life stages and in females in response to blood and sugar meals (Fig. 4). Based on the amplification of specific products, *Anoga-AKH-I* and *AKH-II* expression was evident in all life stages and both sexes, but limited to the head and thorax regions. For comparison, a recent microarray analysis of gene expression profiles in *An. gambiae* showed that *Anoga-AKH-II* has its highest expression in males in comparison to last instar larvae and females, where expression was more variable in non-blood-fed ones and low and stable through 96 h post-blood-feeding (<http://www.angagepuci.bio.uci.edu>; Marinotti et al., 2005); no information was given for *Anoga-AKH-I*. In our study, this same stable pattern of *Anoga-AKH-II* and *Anoga-AKH-I* expression was apparent in heads and thoraces of females for up to 48 h after the ingestion of blood meal, which triggers egg development. The same results were observed for the expression of both genes in females for up to 72 h after a sugar meal.

The lack of any observable and consistent differences in the expression of *Anoga-AKH-II* and *Anoga-AKH-I* in females before and after ingestion of these meals was surprising. It was expected that low PCR product densities would be evident in females before the meals and at some time later, and this would reflect the storage of AKHs in

cells and a low level of gene expression. Conversely, higher PCR product densities would be evident when the AKHs were released from cells in response to the ingestion of the meals, because gene expression would be increased to secrete more of the AKHs or to replenish the peptide stores. This last expectation was based on the presumption that at least one of the *Anoga-AKHs* regulated one or more aspects of nutrient metabolism. In general for insects, AKH gene transcription occurs in relatively few cells associated with the nervous system, and such cells have the capacity to store AKHs for relatively long periods of time or to secrete them continuously at low levels or in pulses of higher levels in response to specific cues (Van der Horst, 2003). Future studies of *Anoga-AKH* transcription may best be revealed with quantitative PCR methods, once the release cues and functions of the peptides are known.

The discovery of a large PCR product for unprocessed *Anoga-AKH-I* transcripts was unexpected, especially since the large PCR product was predominant only in early eggs and abdomens of fourth instar larvae, pupae, and both adult sexes. A subsequent RT-PCR test of different tissues in female abdomens showed that the large PCR product was amplified only from ovary cDNA, thus suggesting that at some time during egg maturation or embryogenesis, these transcripts may be further processed and translated into a peptide product with a role. It should be noted that no AKH-like immunoreactivity was detected or resolved by HPLC in abdomen extracts from sugar-fed females. The more puzzling phenomenon, however, is the presence of these transcripts in the abdomens of earlier stages.

4.3. Cellular sources of AKHs

For insects, the intrinsic cells of the CC are regarded to be both the site of synthesis and release for AKHs, in contrast to many peptide hormones, which are synthesized in more distant brain neurosecretory cells and released from axons terminating in the CC. Northern blotting was used to show that the CC contained *HrTH* transcripts in the cockroach, *B. discoidalis* (Lewis et al., 1997), but not brain, ventral nerve cord, or fat body. Recent studies of *D. melanogaster* used molecular techniques to confirm that the CC is the sole source of Drome-AKH (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004). As well, immunocytochemical studies with Locmi-AKH-I antisera show that the CC generally are immunostained in a variety of insects, but positive cells also have been observed in the brain and ventral nerve cord of *L. migratoria*, *Ae. aegypti*, *Pe. americana* and other insects (Brown and Lea, 1988; Schooneveld et al., 1985, 1983).

Immunoassays with an antiserum to *D. melanogaster* AKH were used to stain cells containing AKH-like peptides and to quantify and identify the two *Anoga-AKHs* in heads and thoraces of female *An. gambiae*, thus confirming the expression of both *Anoga-AKH* genes. AKH-immunostained cells were observed in the brain and

Table 2

Comparison of the sequences for the processed/bioactive forms of the hypertrehalosaemic (HrTH), hypotrehalosaemic (HoTH), cardioactive (CAH), and adipokinetic hormones (AKH) from selected insect species

Name	Sequence	Species
Cockroach HrTH	p QVNFSPGWG Ta	<i>B. discoidalis</i> ^a , <i>Bl. germanica</i> ^a and <i>N. cinerea</i> ^a
Peram-CAHI/Blaor-HrTH-I	p QVNFSPNa	<i>Pe. americana</i> ^a and <i>Bl. orientalis</i> ^a
Peram-CAHII/Blaor-HrTH-II	p QLTFTPNWa	<i>Pe. americana</i> ^a and <i>Bl. orientalis</i> ^a
Locmi-AKH-I	p QLNFTPNWG Ta	<i>L. migratoria</i> ^a
AKH-II	p QLNF S AGWa	<i>L. migratoria</i> ^a
AKH-III	p QLNFT P WWa	<i>L. migratoria</i> ^a
AKH-IV	p QVTF S RDW S Pa	<i>L. migratoria</i> ^b
Helze-HrTH	p QLTFSSGWGNa	<i>H. zea</i> ^a
Moth AKH	p QLTFTSSWG a	<i>M. sexta</i> ^a and <i>H. zea</i> ^a
Tabat-HoTH	p QLTFTPGW G Ya	<i>T. atratus</i> ^a
AKH	p QLTFTPGWa	<i>T. atratus</i> ^a
Fly AKH	p QLTFSPDWa	<i>D. melanogaster</i> ^a , <i>Ph. terraenovae</i> ^a and <i>N. bullata</i> ^c
Anoga-AKH-I	p QLTFTPAWa	<i>An. gambiae</i>
AKH-II	p QVTF S RDWNa a	<i>An. gambiae</i>

Bold letters illustrate conserved amino acids.

^aGäde et al. (1997).

^bSiegert (1999).

^cVerleyen et al. (2004).

thoracic ganglia of females (Fig. 7), and axons from the brain cells were observed in the CC and along the anterior and posterior midgut of females. Presumably, Anoga-AKHs are released from these axonal sites that delineate part of the stomatogastric nervous system in adult mosquitoes (also described as the cerebral and cardiacal neurosecretory systems, Clements, 1992). Most notably, AKH-immunostaining was localized in a cluster of neurosecretory cells, just posterior to the CC that have axons extending to the CC. These cells are known as “X-cells” and were hypothesized to be homologous with the intrinsic cells of the CC in other insects (Clements, 1963; Meola and Lea, 1972). They are located more in the thorax than in the heads of females, thus the higher amount of Anoga-AKH-I in the thorax extract (Fig. 6) suggests that these cells are the primary source of this peptide. Anoga-AKH-I in the head extract may be due to its transport to the CC, which is positioned more in the head than in the thorax. Anoga-AKH-II was detected only in the head extract, thus indicating its synthesis in the AKH-stained lateral neurosecretory cells in the brain. The Locmi-AKH-IV with high sequence similarity to Anoga-AKH-II also was extracted from the brain and not the CC (Siegert, 1999). In situ hybridization with specific probes for *Anoga-AKH-I* and *AKH-II* mRNA would provide a definitive answer as to which of the AKH immunostained cells are the source of a particular AKH in this mosquito.

4.4. Identification of a putative AKH receptor

Recent efforts to pair known peptide ligands with peptide GPCRs annotated for *D. melanogaster* revealed an AKHR (Staubli et al., 2002, GenBank: AF077299; Park et al., 2002, GenBank: AAN10047), and it is structurally

related to the class of gonadotropin-releasing-hormone receptors (GnRH) in mammals, birds, amphibians, and molluscs (Rodet et al., 2005). Related AKHRs (Table 3) were identified in *B. mori* (Staubli et al., 2002, GenBank: AAL95712) and *Pe. americana* (Wicher et al., 2005; GenBank: AAQ17230) and predicted for the honeybee, *Apis mellifera* (GeneID: 551388). Following these molecular leads, a related GPCR was identified in the genome database of *An. gambiae* (Table 3). The extracellular N-terminus and loops, along with the seven transmembrane regions, of the Anoga-AKHR and the other AKHRs have high sequence similarity, but the intracellular carboxy terminus of Anoga-AKHR is similar to that of the honeybee and much shorter than the others.

As determined by RT-PCR, transcripts for the *Anoga-AKHR* gene were present in the head, thorax, and abdomen of all mosquito life stages (Fig. 4), presumably reflecting the distribution of the fat body, which is the primary tissue involved in nutrient storage and metabolism. In females, *Anoga-AKHR* transcripts were abundant in the dorsal and ventral abdomen walls to which most of the fat body is attached, and less abundant in ovaries (Fig. 4E). In addition, its transcript expression appeared constant in all body region of females for up to 72 h after sugar or blood ingestion (Fig. 4C and D). For comparison, the microarray analysis of gene expression in *An. gambiae* showed that *Anoga-AKHR* expression was comparably high in last instar larvae, males, and non blood-fed females, and lowest in females at 3 and 24 h post blood meal but increased thereafter (<http://www.angagepuci.bio.uci.edu>, Marinotti et al., 2005).

The fat body is known to be the primary tissue site for AKH action, but only two other studies have examined the expression of the AKHR in insect tissues. With radio-

Table 3
Comparison of amino acid sequences (Pile Up program) for known and putative AKH receptors (AKHR)

Name	Sequence
Anoga-AKHR	MPNTMAAHINORIEDHRNLA--DWSYYANETAGEEYEMFIDMRFN SCHILS MVVYITLM
Apime-AKHR	----MESSI--KIITTTGLE--NWR--VNNSNYTEL--LPIDMRFN ECHIVSIVFYSVLM
Drome-AKHR	-----MAKVABE-NDHRDLS--NWS-NVNDTNGT--IHLTKMVFNDCHRLSITVYSILF
Bommo-AKHR	-----MDIDKVS GPGGASQKNWSHLHVN--TVDELPLEMRFNYSHMVSM TVYSVLM
Peram-AKHR	--MALSSCSDQTLNCTAVITMATPTPSPTVLM EY--MTDDMKFNDCHRMSIITYSILM
	1.....10.....20.....30.....40.....50.....
Anoga-AKHR	VFSATGNLTVLS-ITARRKVRASSRINIMLAHLA ADLVITFLMMPLEIGWAYTVRWTAG
Apime-AKHR	IISAI GNTTVLI-LITCRKRVSKRIHIMLMHLA ADLVITFLMMPLEIGWAI TVSWKAG
Drome-AKHR	VISTIGNSTVLY-LITKRRRLRGPLRIDIMLMHLA ADLMVITLLMPMEI VAWTVQWLST
Bommo-AKHR	VISATGNLTVLYQLVRRRAKRASRLDILLMHLA VADLMVITFLMMPLEIAWACTVQWFAG
Peram-AKHR	VVSAGNSTVLITILKRGRTLR YGN-NYMFMLA ADLVITFLMMPLEIAWNITVSWKGC
	61.....70.....80.....90.....100.....110.....
Anoga-AKHR	DIMCRVMAFERIFGLYLSSFILICTSVDRYFAVLKPL-KV-H--EHRVLMIAAAWIMSG
Apime-AKHR	DVMCRIMAFERMFGLYLSSFVLVCI SMDRYAVIKPL-QL-WDVKRKGKIMLSFAWIGSV
Drome-AKHR	DIMCRIMSFERVFGLYLSSYVMVICISLD RYFAVLKPL-K---RSYNGRIMLACAWLGSV
Bommo-AKHR	DIMCRVMMFERIFGLYLSSFVLICTIAVD RYVAILKPL-NVTWEATVERAIIIV--AWVCAG
Peram-AKHR	DIMCRIMLFERIFGLELSSFVIVICISLDRCVAILRPM SKLLNVARRGKLMITVAVILAT
	121.....130.....140.....150.....160.....170.....
Anoga-AKHR	LCSLPQAFIFHLEGHPNITGYQOCV TYHYEEIEYQIIYNVLVMCLMYTEPLIVILCYG
Apime-AKHR	VCSLPQTIVFHEHPNVTWYSQCVTFNAFPTIYTHEITYSLFGMIMMYWPLVVIITVT
Drome-AKHR	VCSLPQAFIFHLEHPAVTGYFQCVIFNSERSD PDEKLYQAASMCSMYAFPLIMFIYCYG
Bommo-AKHR	LASLPQSFIHVEEHPVKGYNQCVSYGSLPTEKHEFAFLVNMILMYVIPLEVSTLYCSC
Peram-AKHR	LCSLPQAVIFHEPHPNVTWYEQCVSENFESTKMEFTYRVLCMVMMYGLPLVIVISLA
	181.....190.....200.....210.....220.....230.....
Anoga-AKHR	SIYYEIFSRT---NPRNLESRRSSIDVLGRARKRTL RMTIMIVIVFVVCWIPYYVMSL
Apime-AKHR	SILLEIRRRS---KKS EDDKIRRSSIGFLTRAKIRTLKMTVIIIAVFFICWIPYYVMSL
Drome-AKHR	AIYYEIIYRKSRVLKD VIAERERRSNDDVLSRAKKRTLKMTITIVTFIICWIPYYTISM
Bommo-AKHR	AALFEIIRANTAN----DKMRRSGIGLLGRARATLKMTVTIIVLVFFTCWSPYYCYCT
Peram-AKHR	CITLGEIIRRYQL---SPDSSRRSSLVFLNRARNRTLKMAIIIFVFFICWIPYYVMCL
	241.....250.....260.....270.....280.....290.....
Anoga-AKHR	WYWDKESAKNVDQRIQKGLFLFAS TNSCMNPVYGVFNVRKKHTKKLLKT THE-KSCGS
Apime-AKHR	WYWDNRNSAYKIDQRIQKGLFLFAC TNSCMNPVYGA FNIRDRNKTSARPITTIETRVTP
Drome-AKHR	WYWDKHSAGKINPLLRKALFIFAS TNSCMNPVYGLYNI RGRMNN--NPSVNNRHTSL
Bommo-AKHR	WYWDKESIKNLDPALCKAMWTFSC TNSCANP VYGVFN---RNRWNWRAGKFQNGRCRS
Peram-AKHR	WYWDERSAETVDHREVKALFLFAS TNSCMNPVYGYNF LGRGSGYGAT---GGRVGQ
	301.....310.....320.....330.....340.....350.....
Anoga-AKHR	HTIMRA-----
Apime-AKHR	SLSLKLLD-----
Drome-AKHR	SNRLDSSNQLMQKQLTNNSL LNGRGQVMAAAVSATTKLANVVS LKTANGNCSAAAAGTV
Bommo-AKHR	GSGRKGSRLPHGEST EISAATLSRARHSNGSDHNGRRDSSYANQNGPKHWN TINNNHVT
Peram-AKHR	QHHQNVVALSGNSTGLNSRRGSNSSIYRNNSNQSMSWKNSLRSGRRNSRETEHLHPLP
	361.....370.....380.....390.....400.....410.....
Anoga-AKHR	-----
Apime-AKHR	-----
Drome-AKHR	PITPPLTVTIAPLATDDEANDDSCLSAVTIRCQDQSPIRQK-----
Bommo-AKHR	NGMV-----
Peram-AKHR	HRNSAQAITNVNGRDDQHQLHTNSNKT PNVLEDASNSNSKNALTSVICR
	421.....430.....440.....450.....460.....

Identical amino acid residues are highlighted in black, partly identical in grey, and light grey indicates residues with one missense mutation only. End of the predicted extracellular amino terminus, ▼, and the beginning of the predicted intracellular carboxy terminus, ▲. Drome, *Drosophila melanogaster*; Anoga, *Anopheles gambiae*; Apime, *Apis mellifera*; Bommo, *Bombyx mori*; Peram, *Periplaneta americana*.

receptor assays, AKH binding was localized to membranes of the pterothoracic ganglion and fat body from last instar larvae in *M. sexta*, whereas the brain was devoid of such binding (Ziegler et al., 1995). Later, the GPCR for AKH was identified first in *D. melanogaster* (Staubli et al., 2002), but its tissue expression has not been reported, although functions for AKH have been suggested based on the genetic ablation of AKH-secreting cells and misexpression of the AKH gene (Isabel et al., 2005; Lee and Park, 2004). More recently, AKHR transcripts were detected by RT-PCR in the brain and ventral nerve cord, digestive tract, ovaries, and trachea of the cockroach, *Pe. americana*, and the AKHR was localized in specific neurons and fat body with immunoassays using AKHR antisera (Wicher et al., 2005).

4.5. Function of AKHs in mosquitoes

The identification and cloning of genes for *Anoga-AKH-I*, *Anoga-AKH-II*, and the putative *Anoga-AKHR* provides the key elements needed to investigate the functions of these neuropeptides in *An. gambiae*. *Anoga-AKHs* likely play an important role in regulating nutrient mobilization during flight, as in other insects (Gäde and Auerswald, 2003; Van der Horst, 2003). The precedent of a hypertrihaloemic (or hyperglycaemic) effect by AKHs is known for a few dipteran species (Gäde et al., 1990; Isabel et al., 2005; Lee and Park, 2004), as is that of a hyperlipaemic effect (Isabel et al., 2005; Jaffe et al., 1989; Lee and Park, 2004; Woodring and Leprince, 1992). Further studies of the *Anoga-AKHR* are required to determine whether it binds both *Anoga-AKHs* and to define a signaling pathway. More importantly, insight into how nutrient metabolism is regulated in female *An. gambiae* to meet the demands of flight and reproduction may lead to novel strategies that interfere with the dissemination of malarial pathogens that continue to devastate human populations worldwide.

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3. REGULATION OF CARBOHYDRATE METABOLISM AND FLIGHT PERFORMANCE BY A HYPERTREHALOSAEMIC HORMONE IN THE MOSQUITO *ANOPHELES GAMBIAE*

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3.1 ABSTRACT

The role of adipokinetic hormones (AKHs) in the regulation of carbohydrate and lipid metabolism and flight performance was evaluated for females of the African malaria mosquito, *Anopheles gambiae*. Injection of various dosages of synthetic Anoga-AKH-I increased carbohydrate levels in the haemolymph and reduced glycogen reserves in sugar-fed females but did not affect lipid levels. Anoga-AKH-I enhanced the flight performance of both intact and decapitated sugar-fed females, during a 4 hour flight period. Anoga-AKH-II had no effect on carbohydrate or lipid levels or flight performance, thus its function remains unknown. Targeted RNA-interference lowered Anoga-AKH receptor expression in sugar-fed females, consequently injections of Anoga-AKH-I failed to mobilize glycogen reserves. Taken together, these results show that a primary role for the neurohormone, Anoga-AKH-I, is to elevate trehalose levels in the haemolymph of female mosquitoes.

3.2 INTRODUCTION

Energy expenditure while flying, running, and swimming requires the mobilization of metabolites in insects, and the storage and release of carbohydrates, lipids, proteins, and amino acids are strongly under endocrine control (Gäde, 2004). Neuropeptides in the adipokinetic hormone family are important regulators of energy metabolism, and more than 40 peptide isoforms in this family – herein called AKHs – have been extensively characterized across the insect orders (Gäde et al., 1997; van der Horst, 2003; Gäde, 2004). The number of AKHs known to exist in a particular insect species varies from one in the lower orders, such as Odonata, to as many as four in Blattodea and Orthoptera, and in the higher order of Diptera, only one or two AKHs are known. AKHs occur as octa-, nona-, or decapeptides and are characterised by a pyroglutamate at the amino-terminus, two conserved aromatic amino acid residues at position 4 (in most cases a phenylalanine) and a tryptophan at position 8, and an amidated carboxy-terminus. The blocked termini make it only accessible to endopeptidases. Present in all life stages, these peptides are synthesised and secreted from a distinct region of the corpora cardiaca (CC), a neurohaemal gland connected to the brain that also contains intrinsic neurosecretory cells. Other cell types in the brain and ganglia may also synthesize such peptides, as shown by immunocytochemistry in females of the yellow fever mosquitoes *Aedes aegypti* (Brown and Lea, 1988), and of the African malaria mosquito *Anopheles gambiae* (Kaufmann and Brown, 2006), as well as in other insect species (Schooneveld et al., 1985; Kodrik et al., 2003).

The primary endocrine function of AKH is to mobilize metabolites from storage to the haemolymph, and this has been demonstrated in several different insects. In the migratory locust, *Locusta migratoria*, injection of species-specific AKH mobilized lipids, an adipokinetic or hyperlipaemic effect (van der Horst, 2003). In the American cockroach, *Periplaneta americana*, it increased the trehalose in the haemolymph, a hypertrehalosaemic effect (Scarborough et al., 1984). Similarly, it mobilized proline in dung beetles, *Scarabaeus* spp. and the fruit beetle, *Pachnoda sinuata*, revealing a hyperprolinaemic effect (Gäde and Auerswald, 2002). In larvae of *D. melanogaster*, manipulation of AKH gene expression altered not only circulating levels of trehalose and lipid, but also affected general locomotor activity and survival of imagos during starvation (Lee and Park, 2004).

The expression of two AKH genes (Anoga-AKHs) and a putative G protein-coupled receptor for AKH (Anoga-AKHR) in female *A. gambiae* (Kaufmann and Brown, 2006) suggest that at least one of the AKHs may be involved in the mobilization of metabolites for flight, as known for other insects (Gäde and Auerswald, 2002; van der Horst, 2003). Both carbohydrates and lipids are used as flight substrates in females of this species (Kaufmann and Briegel, 2004). Other dipteran AKHs have been identified (Table 3.1), and their bioactivity reported for the blow fly, *Phormia terraenovae* (Gäde et al., 1990), the horse fly (*Tabanus* spp., Woodring and Leprince, 1992), the tsetse fly, *Glossina morsitans* (Pimley, 1984; Mwangi and Awiti, 1989), and the fruit fly, *D. melanogaster* (Lee and Park, 2004). In

this study, we first tested the synthetic Anoga-AKHs for effects on metabolite mobilization in female *An. gambiae*, and then examined whether these peptides also affected female flight performance. In addition, this *in vivo* approach was combined with targeted RNA interference of Anoga-AKHR gene expression to link the endocrine effect of an Anoga-AKH to the putative AKHR.

Table 3.1: AKH family members known for Diptera

Name	Sequence	Species
Tabat -AKH	pQLTFTPGWa	<i>T. atratus</i> ¹ and other <i>Tabanus spp.</i> ²
-HoTH	pQLTFTPGWGYa	<i>T. atratus</i> ¹ and other <i>Tabanus spp.</i> ²
Phote-HrTH	pQLTFSPDWa	<i>Ph. terraenovae</i> ³ , <i>D. melanogaster</i> ⁴ , and <i>N. bullata</i> ⁵
Anoga-AKH-I (Anoga-HrTH)	pQLTFTPAWa	<i>A. gambiae</i> ⁶
-AKH-II	pQVTFSRDWNa	<i>A. gambiae</i> ⁶ , <i>A. aegypti</i> ⁷ , and <i>C. pipiens</i> ⁸
Aedae-HrTH	pQLTFTPSWa	<i>A. aegypti</i> ⁷ and <i>C. pipiens</i> ⁸

Bold letters illustrate conserved amino acids.

¹(Jaffe et al., 1988a); ²AKHs in *T. calens* *T. lineola* *T. proximus* *T. sulcifrons* same as in *T. atratus* (Woodring and Leprince, 1992); ³(Gäde et al., 1990); ⁴(Schaffer et al., 1990); ⁵(Verleyen et al., 2004); ⁶(Kaufmann and Brown, 2006); ⁷Predicted in the *A. aegypti* genome data base, Aedae-HrTH: GeneID, [AAEL011996](#); and AKH-II same in *A. gambiae* and *A. aegypti*: GeneID, [AAEL010950](#); ⁸Predicted in the *C. pipiens* genome data base, Aedae-HrTH or Culpi-HrTH: GeneID, [145648990](#); and AKH-II same in *A. gambiae*, *A. aegypti*, and *C. pipiens*: GeneID, [145648990](#).

3.3 MATERIAL AND METHODS

3.31 MOSQUITOES

The colony of *Anopheles (Cellia) gambiae* s.s. (Giles), strain 16CSS from Lagos, Nigeria, was maintained at 26 ± 1°C under long-day conditions (16 h light, 8 h dark). Larvae were raised in trays (24 x 16 x 6 cm) with 350 ml distilled water and fed pulverized Tetramin[®] daily (Timmermann and Briegel, 1993). Imagos (200 – 300 in cages of 24 x 19 x 18.5 cm) had access to 10% fructose solution or distilled water, depending on experimental conditions. For experiments, large females with wing lengths between 3.2 to 3.5 mm (measured from the alula to the tip, including the fringes) were used. For egg production and experiments, females were given blood meals from a human arm.

3.32 INJECTION OF ANOGA-AKHs

Synthetic Anoga-AKH-I and -II (Table 1) were dissolved first in dimethyl sulfoxide (DMSO) and then diluted to 2.5% DMSO in *Aedes* saline (7.5 g NaCl, 0.35 g KCl, and 0.21 g CaCl₂ in 1 l nanopure H₂O; adjusted to pH 6.5). For control injections, 2.5% DMSO in *Aedes* saline alone was used. Known amounts of peptide in 0.25 µl of DMSO/saline were injected with fine glass needles into the first abdominal segment of females to ensure the least damage.

Since in blood-fed females the haemolymph content increases to around twice the amount in the first few hours (2.4 μ l in *Aedes aegypti*) and protein and lipid transport takes place first in the gonotrophic cycle (Clements, 1992), an injection of 250 nl of saline should not disturb the lipid transport system. The needles were made from borosilicate glass capillaries (TW100-6, World Precision Instruments) on a Flaming/Brown Micropipette puller (Model P-97, Sutter Instruments Co.). After injection, females were caged, held at 27°C in a humidified chamber, and not given access to fructose solution or water.

3.33 BIOCHEMICAL ANALYSES

To quantify nutrient reserves, two abdomens from experimental sugar-fed females were pooled, and for experimental blood-fed females, a whole individual or haemolymph from two females was used. Haemolymph was collected by incubating two individuals together in 100 μ l *Aedes* saline on ice; each abdomen was carefully 'opened' at the segmental line between the last two abdominal segments, this open gap was exposed to the saline, allowing the haemolymph to bleed into the saline. After 10 minutes bleeding, 90 μ l was used for the assay. Sugar, glycogen and total lipids were measured in the samples using a modified version of Van Handel and Day (1988): For the separation of sugar and lipids 1.6 ml CHCl_3 -MeOH (v:v, 1:1) and 0.6 ml of distilled H_2O was used. Glycogen in the precipitate and sugar in the aqueous fraction were measured with the hot anthrone reaction (2 ml anthrone/tube), with glucose standards (Merck 8337) 0.1% in EtOH (25%). Lipid was quantified by a vanillin-phosphoric acid reaction (1 ml vanillin (Merck 818718)/tube) with 0.1% soybean oil (Sigma S-7381) in chloroform as a standard. Absorbance values for 100 μ l/well from processed experimental females and standard samples were measured in 96 well plates by a microplate reader (SpectraMax, at $\lambda = 630/525$ nm for carbohydrate/lipid), and converted to μ g per female, based on a formula calculated from a regression line derived from the standard sample values (Note, flight metabolites in Figure 3.5 were further converted to Joules). Bioassays were replicated for two or three cohorts (N = 8 to 10 individuals per cohort). Statistical analyses were performed with ANOVA (JMP Version 5.1.2).

3.34 FLIGHT-MILL SYSTEM

To determine whether Anoga-AKH affects female flight performance, a modified flight-mill system, originally described by Rowley et al. (1968), was used to measure the flight distance over a certain time period (Kaufmann and Briegel, 2004). A female mosquito was mounted on one arm of the flight-mill, and a female unable to fly (wings glued together) acted as a counterbalance on the other arm. The number of revolutions (32.7 cm circumference) powered by the female was registered by a computer at 30 s intervals to give total flight distances over a known time period and simultaneously the temporal pattern of flight activities. Resting periods were also recorded; for additional details, see Briegel et al. (2001a). Flight-trials were set up with 5 to 8 cohorts of up to 10, 4 day old females, given continuous access to a 10% fructose solution after eclosion. It was found that decapitated females flew as

well as intact ones; and since after decapitation hormonal sources released from the brain and CC were eliminated, decapitated females were also the subjects of the flight experiments. After decapitation, females were held for 1 h to allow the wound to close, and then half of these or the intact females were injected with saline and the others with 50 pmol of the Anoga-AKHs. Flights started at around 3:00 pm and lasted for 20 h at room temperature ($24 \pm 2^\circ\text{C}$) in a natural light regimen. Only flights of more than 500 m per 20 h trial were included in the data set that was analyzed for just the first 4 h of flight. Statistical analysis for all time points (1, 1.5, 2, 4 h) were performed with 2-way ANOVA ($N = 10-15$, JMP Version 5.1.2). In addition, flight metabolites in females given 10% fructose for up to 5 days after eclosion were quantified after 4 hours on the flight mill, as described in Kaufmann and Briegel (2004).

3.35 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION AND RNA INTERFERENCE

To obtain a cDNA template for the reverse transcriptase polymerase chain reaction (RT-PCR), abdominal body walls were dissected in *Aedes* saline, immediately transferred into 50 μl RNeasyTM (Sigma) for storage at 4°C overnight and then at -80°C until processed. Total RNA was extracted with the RNeasy[®] Mini kit, treated with DNase digestion (Qiagen), and used to make cDNA with the Clontech AdvantageTM RT-for-PCR Kit and the oligo (dT)₁₈ reverse primer.

For the RNA interference (RNAi) experiment, cDNA fragments (~200 bp) of the amino (N)- and carboxy (C)-termini of the *Anoga-AKHR* (GeneID: 1269400, Locus tag: ENSANGG00000016873, GenBank: [DQ396551](#)) were amplified by RT-PCR from the abdomen cDNA and specific primer pairs tailed by the T7-promotor sequence (Table 3.2).

Table 3.2: Primers (0.2 μM) used for RT-PCR

Primer name:	Sequence 5'-3'	ORF (bp)
AKHR-for	CAG CCA GCC AGC CAG AAC	1110
AKHR-rev	GGA GCG TTA GTA ACA TGG AAT GAA GTG	
Actin-for	CCC GCT GAA CCC GAA GGC TAA CC	585
Actin-rev	GTA CCA CCG GAC AGG ACA G	
N-term-T7-for	T7- CAG CCA GAA CAA TGC CCA ACA CAA	267
N-term-T7-rev	T7- GCC AGT ATG CTG AGC ACC GTA A	
C-term-T7-for	T7- TAT GGT ACT GGC TCG ACA AGG AGT	253
C-term-T7-rev	T7- ATG TTA CTA ACG CTC CTA CGC CCT	
T7-sequence	TAA TAC GAC TCA CTA TAG G	-

ORF: open reading frame spanning of the forward (for) and reverse (rev) primer pair; AKHR: adipokinetic hormone of *Anopheles gambiae* (Kaufmann and Brown, 2006); N(C)-term-T7 primer set represent the forward and reverse primer set of the N- and C-terminus of the AKHR, T7 sequence was added for the further dsRNA synthesis.

Conditions were set according to Titanium Tag PCR kit: initial denaturation, 4 min at 95°C ; denaturation for 20 s at 95°C ; 20 s at 60°C for annealing, and 45 s extension for the

amplification at 72°C for 32 cycles, followed by a 5 min 72°C incubation. dsRNA was generated from the *Anoga-AKHR* cDNA fragments with the MEGAscript® RNAi Kit (Ambion). Concentrations of dsRNA were estimated after running a 1.5% agarose gel with a low mass leader as marker (Invitrogen, Cat. No. 10068-013). Approximately 200 ng *Anoga-AKHR* dsRNA/0.25 µl were injected into freshly eclosed females from three different cohorts (N = 2 to 5 individuals/cohort), and the same amount of enhanced green fluorescent protein (EGFP) dsRNA was injected into females, as a control because this gene does not exist in the mosquito genome. The females had access to 10% fructose, and two days after dsRNA injection, *Anoga-AKHR* expression in females was visualized by RT-PCR of the ORF regions of the *Anoga-AKHR* gene. PCR conditions were the same as above, except an amplification time of 1 min and 30 s was used, and set up included lanes for single primer and “no template” PCR controls, as well as amplification of an *Anoga-actin* gene (GeneID: [1275175](#), Locus tag: ENSANGG00000012550, primers in Table 3.2) to check cDNA integrity. PCR products were separated in 1.5% agarose gels, and pictures of the gels were taken with GeneSnap 6.03 (SynGene) and transferred to Photoshop 7.0 (Adobe).

3.4 RESULTS

3.41 METABOLIC EFFECTS OF ANOGA-AKHs

Synthetic Anoga-AKH-I and -II were injected over a dose range of 1 to 100 pmol separately into groups of four day old *A. gambiae* females given access to 10% fructose solution for the first three days. After 1.5 h, total glycogen and lipid content of abdomens and sugar content in haemolymph were assayed. Anoga-AKH-I doses of 5 pmol and higher significantly reduced glycogen levels but not lipid levels, relative to that of control females (Fig. 3.1 A). As well, haemolymph trehalose content was significantly increased in females given 1 pmol and higher doses of Anoga-AKH-I, whereas no effect on the lipid level was detected (Fig. 3.1 B). Anoga-AKH-II had no effect on glycogen, lipid, or trehalose levels in females over the dose range shown in Figures 3.1 A and B (data not shown).

To determine the temporal efficacy of Anoga-AKH-I treatment, 4 day old females, previously given access to the fructose solution, were injected with 50 pmol and assayed at different times thereafter. This high dose was administered with the hope that its action would persist for a few hours, because the half-life of AKHs is limited to tens of minutes by the action of endopeptidases in locusts (Oudejans et al., 1996). By 1 h post injection, glycogen levels had significantly decreased relative to that of control females. This effect persisted up to the 4 h post injection, whereas with the 6 h post injection there was no difference in the glycogen levels of saline and peptide-treated females (Fig. 3.2 A). We speculate that the half-life of Anoga-AKH-I is short, since its effect was undetectable after 6 hours incubation time and the mobilized carbohydrates were reabsorbed into the fat body or burned in the basal metabolism. Again, this peptide had no effect on lipid levels over the 6 h period and since there was plenty of carbohydrates stores available, lipid was not used for the basal

metabolism (Fig. 3.2 B). Anoga-AKH-II at this same dose had no effect on carbohydrate or lipid levels for up to 6 h post injection (data not shown).

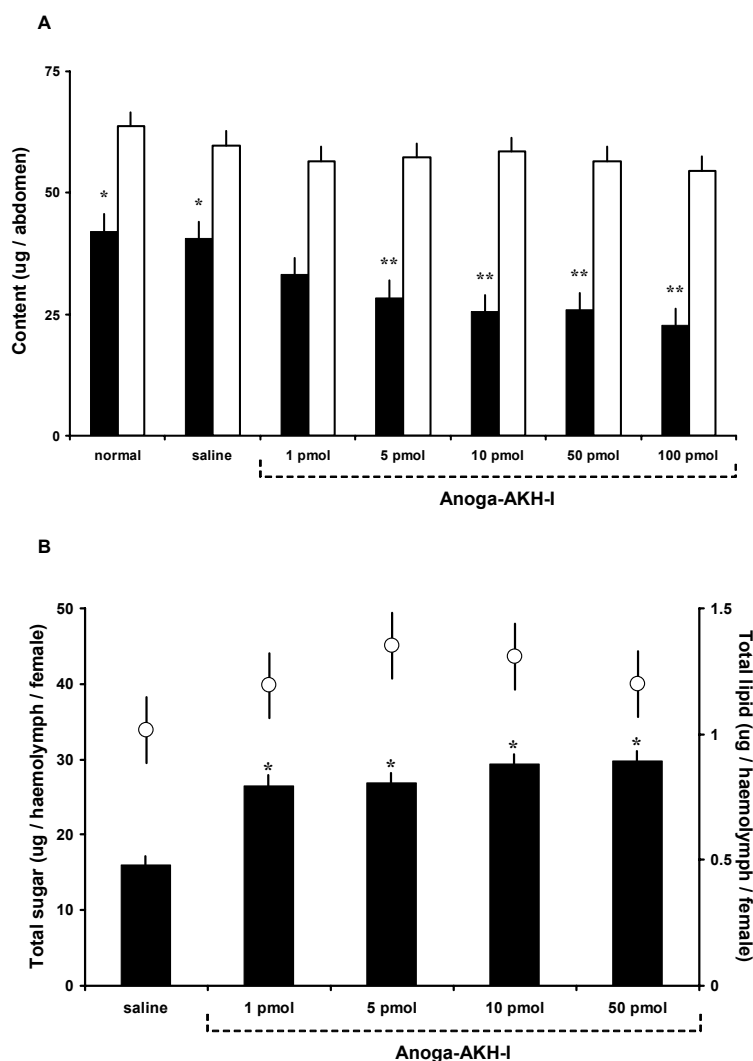


Figure 3.1: Profiles of maximal responses of female *A. gambiae* treated with Anoga-AKH-I.

- (A) Black bars represent the glycogen levels and white bars the lipid levels of the abdomen after 1.5 h incubation. Columns with one asterisk differ significantly from columns with two asterisks (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 3 cohorts, $n = 10/\text{cohort}$).
- (B) Black bars represent the haemolymph sugar levels and white circles the lipid levels of haemolymph after 1.5 h incubation. The sugar increased significantly with all Anoga-AKH-I injections 1.5 h post injection. Lipid levels, with an average of 1.1 ± 0.2 ug / female, showed no statistical difference. Asterisks indicate significant differences to saline controls (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 3 cohorts, $n = 8-10/\text{cohort}$).

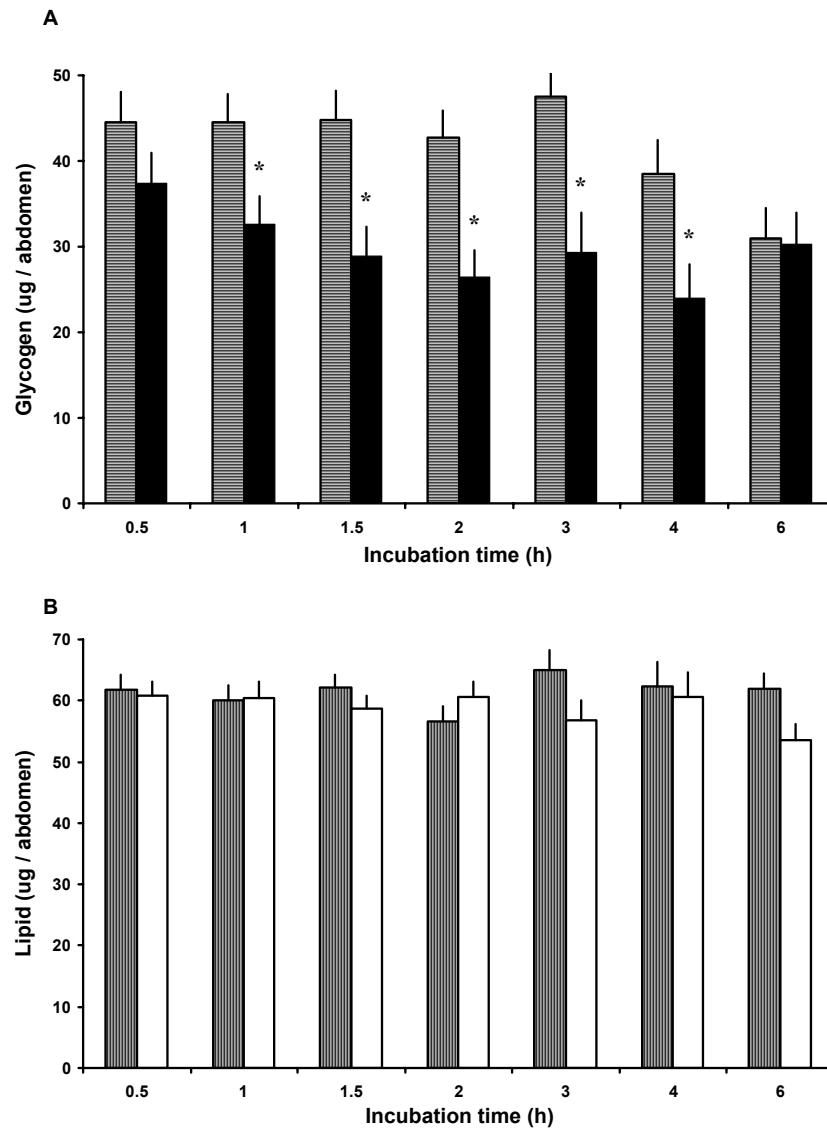


Figure 3.2: Profiles of responses to 50 pmol Anoga-AKH-I injection in female *A. gambiae*, 4 day-old with continuous access to 10% fructose: **A)** Horizontal shaddings present glycogen values with saline injection, in comparison to females with Anoga-AKH-I injection (black bars). The total glycogen content significantly decreased between 1 to 4 hours post injection. Asterisks indicate significant differences to the saline control (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 3 cohorts, $n = 8/\text{cohort}$). **B)** Vertical shadings present lipid values with saline injection, in comparison to females with Anoga-AKH-I injection (white bars).

The bioactivity of the two peptides also was tested in females given only water after blood-feeding and oviposition, so that the nutrient reserves were obtained from the blood meal and not a sugar meal (Briegel, 1990). As before, a 50 pmol dose of Anoga-AKH-I significantly decreased glycogen levels and increased haemolymph carbohydrate levels after 1.5 h incubation (Fig. 3.3), but had no effect on lipid levels in haemolymph.

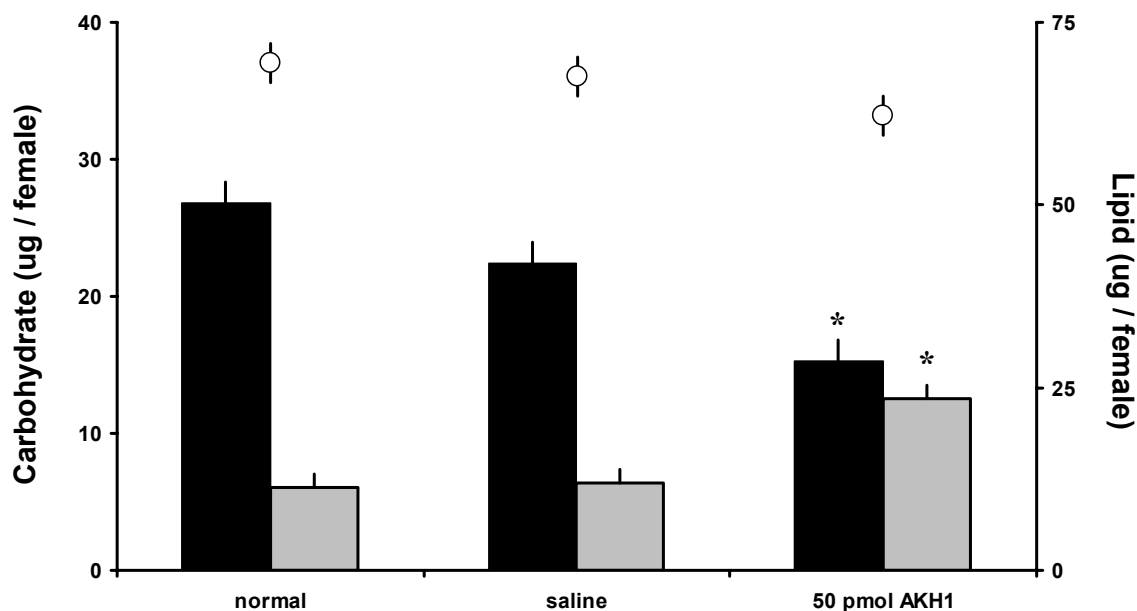


Figure 3.3: Carbohydrates and lipid levels in 5 day-old blood-fed *A. gambiae* after oviposition. Injections of saline or Anoga-AKH-I are compared to untreated females, all after 1.5 h incubation periods. Black bars for glycogen, grey bars for sugar; lipid levels are shown in white circles. All three components measured in individual females. Anoga-AKH-I increased carbohydrate levels in the haemolymph and reduced glycogen reserves. Asterisks indicate significant differences to the other treatments (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 3 cohorts, $n = 10/\text{cohort}$).

The mosquito AKHs were assayed for bioactivity in *L. migratoria* and *P. americana* to examine their structure-activity relationships for mobilizing metabolites (Gäde, 1992b, 1993). These tests were performed in laboratory of Prof. G. Gäde following the procedure of Gäde (1980). Anoga-AKH-I stimulated a hyperlipaemic response in *Locusta* (Table 3.3). Anoga-AKH-II was tested because it shares 80% similarity with the fourth *Locusta* AKH (Locmi-HrTH), which showed hypertrehalosaemic activity in *P. americana* (Siegert, 1999), and in the assay with this species, the mosquito AKH decapeptide was inactive (Table 3.4).

3.42 AKHS AND FLIGHT PERFORMANCE

Given that Anoga-AKH-I mobilized glycogen stores in relatively immobile females, the next step was to determine whether this peptide would enhance female flight performance. As shown above (Fig. 3.2 A), the effect of a 50 pmol dose of Anoga-AKH-I persisted for at least 4 h in females, so both intact and decapitated females were injected with the AKHs separately at this dose, prior to being mounted on the flight mills. Another reason for using this higher dose was that the half-life of AKHs in flying locusts is much shorter than in resting ones (Oudejans et al., 1996). To optimize the effect of injected peptides, females were decapitated to remove the CC, the putative source of Anoga-AKH-I, and the brain, the source

of Anoga-AKH II (Kaufmann and Brown, 2006) and other hormonal sources. By pulling the head back and stretching the neck, the CC is pulled away from the thorax, so that when the head is cut off, both AKH sources are removed, along with other neurohaemal factors that may be released in response to flight.

The total distance flown after 4 h was approximately the same for the peptide- and saline-injected intact and decapitated females (Fig. 3.4). The mean distance flown was 1.12 ± 0.20 km and 1.50 ± 0.21 km for intact and decapitated females respectively, thus showing that the flight capacity of decapitated females was not diminished by head removal. The mean difference in distance flown over the time points 1, 1.5, 2, and 4 h was for saline vs. Anoga-AKH-I injected intact or decapitated females 0.35 ± 0.12 km and 0.44 ± 0.10 km, respectively.

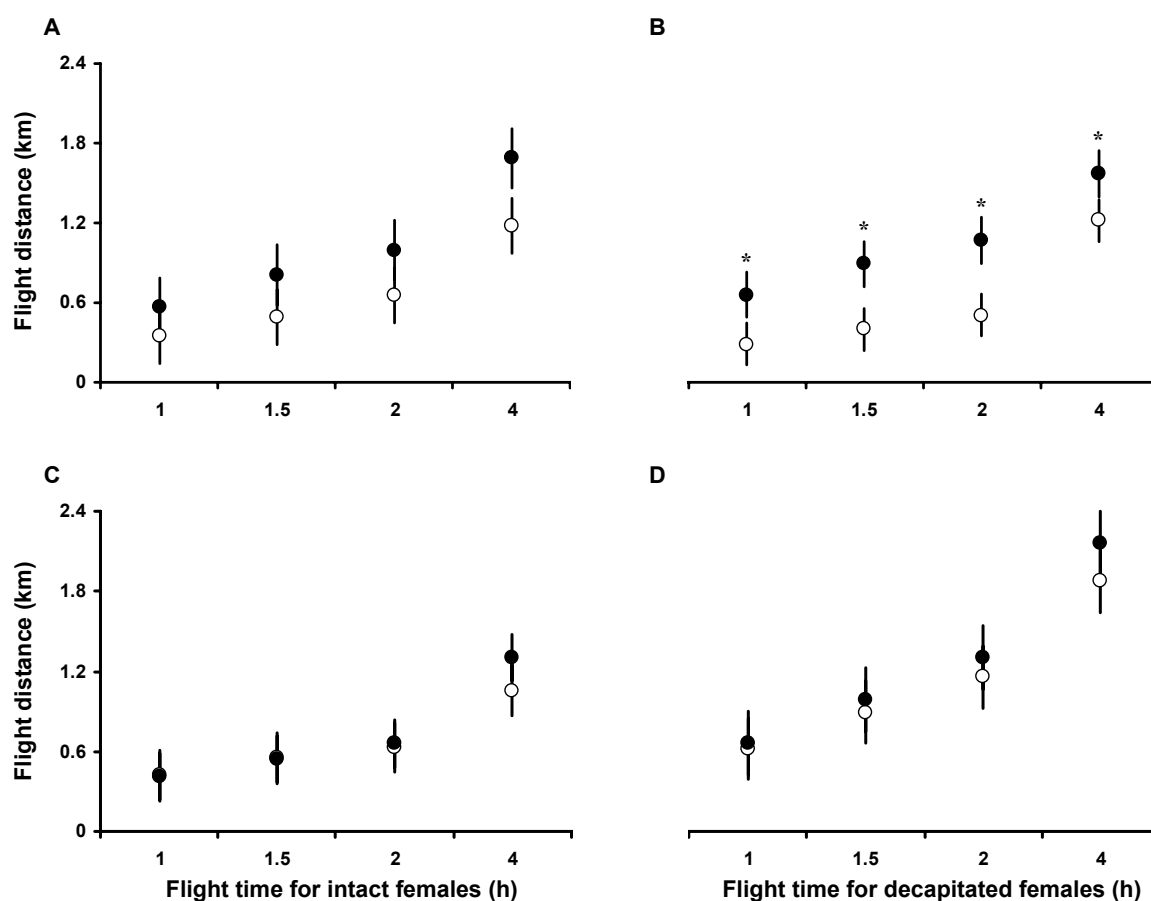


Figure 3.4: Total flight distances at different time points for 4 day-old intact and decapitated female *A. gambiae* with access to 10% fructose. Empty circles for saline treated females, full circles for siblings after Anoga-AKH-I treatments (**A**, intact; **B**, decapitated) or Anoga-AKH-II treatments (**C**, intact; **D**, decapitated). A significant difference was only detected for Anoga-AKH-I treated females: i.e. for intact (**A**) $p \leq 0.03$, as well as for decapitated ones (**B**) $p \leq 0.0002$ (2-way ANOVA; $M \pm S.E.$; $n = 9-14$).

The distance flown by decapitated females was significantly increased by the injection of Anoga-AKH-I, relative that of control females, over the 4 h period (Fig. 3.4 B; $p \leq 0.0002$, 2-way ANOVA). This effect was less pronounced in intact females injected with this peptide (Fig. 3.4 A, $p \leq 0.03$, 2-way ANOVA). The flight performance of intact and decapitated females was not significantly affected by the injection of Anoga-AKH-II (Figure 3.4 C and D). For saline vs. Anoga-AKH-II treated intact or decapitated females the mean difference in distance flown was 0.06 ± 0.12 km and 0.14 ± 0.11 km, respectively.

An additional experiment showed that carbohydrates were the main flight substrate used during a 4 hour flight by intact females up to 5 days old (Fig. 3.5). Total energy expenditure (Joules used per km flown) increased as females aged from day 1 to day 5, as did the use of lipids. When averaged for the three age groups of females, 74% of the energy used for flight per km was carbohydrate (glucose and glycogen combined) and 26% lipid. This result suggests that the enhanced flight performance of decapitated females injected with Anoga-AKH-I is due to the hypertrehalosaemic effect of this peptide.

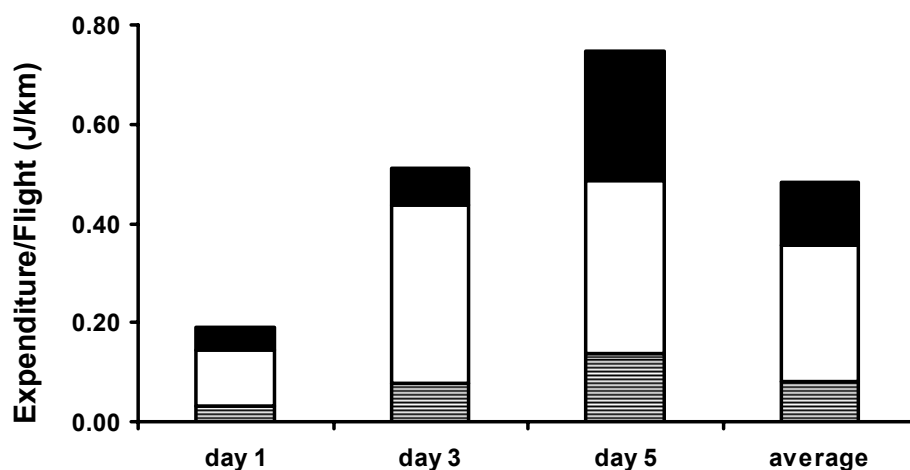


Figure 3.5: Energy expenditure by sugar-fed female *A. gambiae* at day 1, 3, and 5 after eclosion (N = 11-13 per day) for 4 hours of flight. Total energy expenditure during flight (J/km): glucose (hatched), glycogen (white), and lipid (black).

3.43 RNA-INTERFERENCE

To establish the functional pairing of Anoga-AKHR and Anoga-AKH-I, we used RNA interference to silence the *Anoga-AKHR* gene to test whether the hypertrehalosaemic effect of injected peptide was diminished. Double-stranded RNA for the N- and C-termini of the Anoga-AKHR was injected into newly emerged *A. gambiae* females, and 48 h later, transcription of the gene in abdomens was greatly reduced, as judged by the faint *Anoga-AKHR* PCR products (Fig. 3.6 A). In comparison, the gene products were clearly evident, when amplified from the cDNA of females injected with the control EGFP dsRNA (EGFP; Fig. 3.6 A), as was the expression of an *actin* gene in females injected with the receptor dsRNA (Fig. 3.6 A). When Anoga-AKH-I (50 pmol/female) was injected into dsRNA-treated

females from the same cohort as those with lowered *Anoga-AKHR* expression, it failed to lower glycogen levels in females receiving the receptor dsRNA (N-, and C-terminus; Fig. 3.6 B). In those females treated with the EGFP dsRNA, it significantly lowered glycogen levels in comparison to the levels in females injected with saline after the earlier treatment with EGFP and *Anoga-AKHR* dsRNA (Fig. 3.6 B). These results show that the action of Anoga-AKH-I requires a high expression level of AKHR in female abdomens, and as such indicates that this peptide is a likely ligand for the receptor *in vivo*.

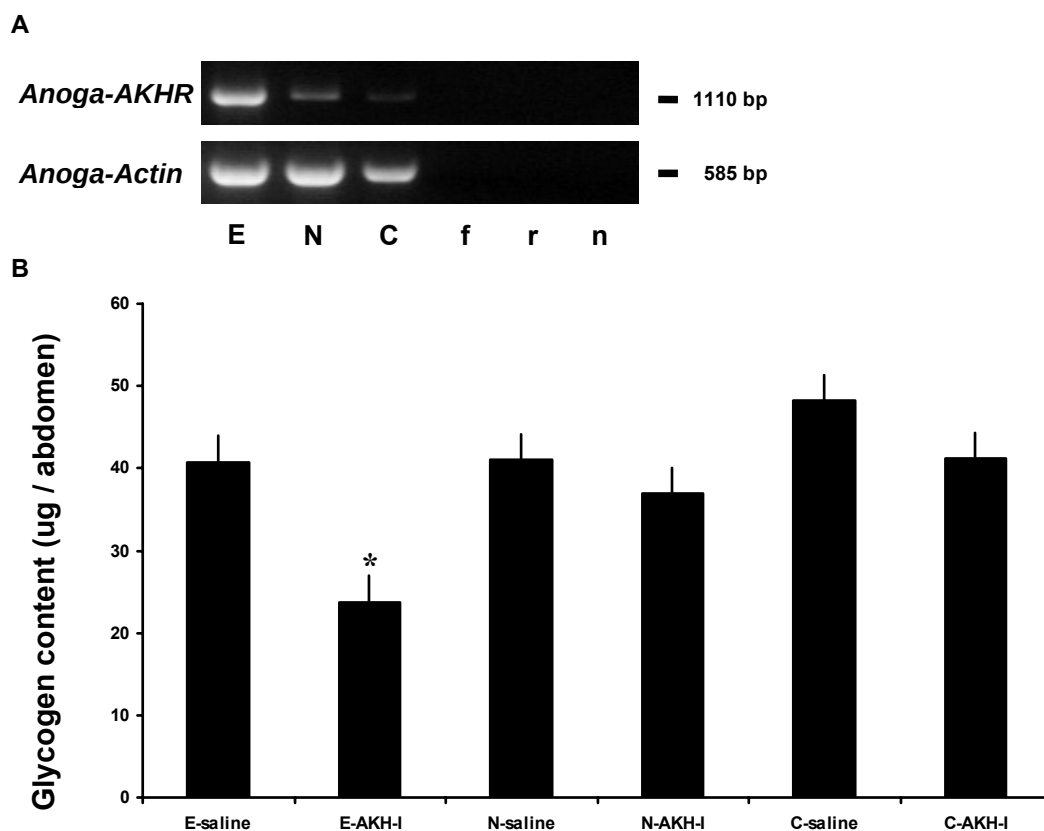


Figure 3.6: Representative RT-PCR gels (A) and metabolic glycogen analysis after Anoga-AKH-I treatment (B) of sugar-fed *A. gambiae* two days after silencing the Anoga-AKHR using RNAi:

- (A) Representative gels of *AKHR* and *actin* PCR products for abdomens of two day-old females treated with dsRNA-fragments: E, EGFP control; N, dsRNA of AKHR N-terminus; C, dsRNA of AKHR C-terminus; f, forward primer (AKHR-for); r, reverse primer AKHR-rev) and n, no template.
- (B) Glycogen content of female *A. gambiae* injected with 50 pmol AKH-I or saline, 2 day post dsRNA treatment: Fragments of dsRNA for EGFP (E), or N-terminus (N), and C-terminus (C) for Anoga-AKHR. Only EGFP-dsRNA treated *A. gambiae* females show significant decrease in glycogen after 1.5 h by AKH-I injection. Asterisk indicates a significant difference to saline controls (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 2 cohorts, $n = 8-10/\text{cohort}$).

3.5 DISCUSSION

The primary endocrine function of AKH-related peptides across the great diversity of insects is to mobilize metabolites stored in tissues for energy-requiring activities (Gäde et al., 1997). This includes hyperglycaemia and hyperlipaemia that resulted from the injection of a species-specific CC extract, first reported for the cockroach (Steele, 1961) and locust (Mayer and Candy, 1969) respectively, and later from a synthetic AKH, after its isolation and characterization from *Locusta* (Stone et al., 1976b). Carbohydrates are mobilized mainly from glycogen reserves in the fat body, resulting in an increased level of soluble carbohydrates in haemolymph. If an AKH induces this mobilization, it is defined as a hypertrehalosaemic hormone (Gäde, 1991b), because the main 'blood-sugar' in insects is trehalose and not glucose (Wyatt, 1967). As reported herein, Anoga-AKH-I induced this effect in female *A. gambiae*. A single injection of 1 pmol resulted in a significant increase in soluble carbohydrates in haemolymph, but to achieve an equivalent decrease in glycogen, 5 pmol was required (Fig. 3.1). A 1 pmol dose is approximately equivalent to the Anoga-AKH content of 4 to 6 female thoraces containing the CC, as determined with a radioimmunoassay based on an antiserum to the *D. melanogaster* AKH (Kaufmann and Brown, 2006). Anoga-AKH-I had no effect on lipid levels stored in the body or circulating in the haemolymph of the experimental females (Fig. 3.1). As in other insects, trehalose is the main carbohydrate in the haemolymph of adult mosquitoes (Van Handel, 1969), and injection of up to 50 pmol of Anoga-AKH-I increased haemolymph carbohydrates to as high as 30 µg/female (Fig. 3.1B). These results lead us to designate Anoga-AKH-I as a hypertrehalosaemic hormone in female *A. gambiae* (abbreviated as Anoga-HrTH). This hormone-induced mobilization of the carbohydrates is similar to the action of glucagon in mammals, and both the pro-peptide and processed forms of insect AKHs share sequence homology with the glucagon peptide family of vertebrates (Clynen et al., 2004).

3.51 DIPTERAN AKHS AND FUNCTION

3.511 MOBILIZATION OF CARBOHYDRATE AND LIPID STORES

Relatively few AKHs are known for Diptera, including two now identified in the genome database of another mosquito, *A. aegypti* and *C. pipiens* (Table 3.1). In mosquitoes (Suborder Nematocera) and horse flies, *Tabanus spp.* (Suborder Brachycera), there are two processed forms of AKH, an octapeptide and a decapeptide (Jaffe et al., 1988a; Kaufmann and Brown, 2006, Table 3.1). In tests with an intact adult tabanid species (*T. lineola*), *T. atratus* AKH decapeptide at 100 pmol/fly induced a significant hyperlipaemic and hyperglycaemic response; and the *T. atratus* AKH octapeptide (same dose), only a weak hyperglycaemic response (Woodring and Leprince, 1992). To date, only a processed AKH octapeptide with the same sequence has been identified in three higher dipteran species (Suborder Brachycera): blow fly, *Ph. terraenovae* (Gäde et al., 1990), fruit fly, *D. melanogaster* (Schaffer et al., 1990), and flesh fly, *Neobellieria bullata* (Verleyen et al.,

2004) (Table 3.1). This AKH octapeptide caused a hypertrehalosaemic effect in male blow flies (0.75 to 3 pmol/fly) and did not affect lipid levels (Gäde et al., 1990). Only for *D. melanogaster* larvae are both a hypertrehalosaemic and a hyperlipaemic effect ascribed to this AKH (Lee and Park, 2004). These effects were not achieved by AKH injection, but indirectly by gene ablation of AKH producing cells in the CC and by over expression of the AKH gene in the fat bodies. In the tsetse fly, *G. morsitans*, only a hypertrehalosaemic response was obtained with species-specific CC extract injected into females (Mwangi and Awiti, 1989), but *in vitro* studies with female fat bodies and crude CC extracts produced a hyperlipaemic effect (Pimley, 1984). The processed structure of AKH octapeptides is highly conserved across the insects, so much so that Anoga-HrTH produced a significant hyperlipaemic response in *Locusta* (Table 3.3), as does another dipteran AKH octapeptide (Gäde, 1993).

Table 3.3: Lipid levels in the haemolymph of males of *Locusta migratoria* (14 to 20 days old). Lipid levels (mg/ml haemolymph) were measured before, and 1.5 h after treatment of the same individuals. (M $\pm$ S.E., n = 8, paired t-test; bioassay described in Gäde, 1980).

Treatment	Incubation time		difference	paired t-test
	0 h	1.5 h		
Control	16.3 $\pm$ 4.4	15.1 $\pm$ 4.1	-1.2 $\pm$ 3.8	p $\leq$ 0.55
0.1 pCC Locmi	14.4 $\pm$ 2.6	57.4 $\pm$ 13.5	43.0 $\pm$ 11.5	p $\leq$ 0.000015
35 pmol Anoga-HrTH	17.7 $\pm$ 7.0	26.4 $\pm$ 5.2	8.7 $\pm$ 4.4	p $\leq$ 0.00080
70 pmol Anoga-HrTHI	17.6 $\pm$ 6.4	41.8 $\pm$ 7.2	24.2 $\pm$ 11.7	p $\leq$ 0.00065
control	11.5 $\pm$ 2.6	14.9 $\pm$ 5.1	3.4 $\pm$ 3.7	n.s.
0.1 pCC Locmi	11.5 $\pm$ 2.2	41.2 $\pm$ 10.7	29.7 $\pm$ 10.3	p $\leq$ 0.00008
52 pmol Anoga-AKH-II	11.1 $\pm$ 4.1	13.8 $\pm$ 5.7	2.7 $\pm$ 2.1	n.s.

The mosquito AKH decapeptide had no activity in our bioassays. This lack of bioactivity is surprising given the reports that the only other AKH decapeptide known for Diptera does affect lipid and carbohydrate levels in adult flies, as described above, and even in the cricket, *Acheta domesticus* (Woodring and Leprince, 1992). Although its processed sequence is dissimilar to that of Anoga-HrTH and other dipteran AKH octapeptides (Table 3.1), the *Anoga-AKH-II* gene structure clearly shows it to be a member of the AKH family (Kaufmann and Brown, 2006). Interestingly, the Anoga-AKH-II sequence is highly similar to that of *Locusta* AKH IV (pQVTFSRDWSPa), which has no known function in *Locusta*. Both decapeptides have no Gly at position 9 that is typical for larger AKHs (Gäde et al., 1997), which might be the reason why no detectable specific metabolic effect was seen. The locust peptide induced a weak hypertrehalosaemic effect in the cockroach and was named Locmi-HrTH (*P. americana*; Siegert, 1999), but Anoga-AKH-II had no such activity in the same cockroach (Table 3.4). Since it has been speculated that both of these decapeptides are synthesised in the brain and not the corpora cardiaca, they are also referred to as non-classical

AKHs (Siegert, 1999; Kaufmann and Brown, 2006), but might have another non-metabolic function. Future studies of mosquitoes and other insects are likely to aid the discovery of a function for AKHs or to de-orphanize a G-protein coupled receptor for this peptide through binding assays, which in turn could lead to a function for this mysterious non-classical AKH.

Table 3.4: Carbohydrate levels in the haemolymph of males of *Periplaneta americana* (unspecified age). Carbohydrate levels (mg/ml haemolymph) were measured before, and 2 h after treatment of the same individuals. ($M \pm S.E.$, $n = 5$, paired t-test; bioassay described in Gäde, 1980).

Treatment	Incubation time		difference	paired t-test
	0 h	2 h		
control	10.7 ± 2.9	11.7 ± 4.3	1.0 ± 2.0	n.s.
0.1 pCC Peram	13.7 ± 2.8	28.0 ± 9.1	14.3 ± 8.5	$p \leq 0.04$
52 pmol Anoga-AKH-II	14.9 ± 2.7	16.0 ± 3.5	1.1 ± 2.0	n.s.

3.512 FLIGHT METABOLISM

For several insect species, AKH octapeptides play an important role during flight (Gäde and Auerswald, 2002; van der Horst, 2003) and other energy consuming activities, such as walking, ball rolling, or swimming (Gäde and Auerswald, 2002; Gäde et al., 2007). Locusts have two phases of flight metabolism (van der Horst, 2003). For flights of a short duration, they mainly depend on carbohydrates, and then for a longer flight, there is a transition to mobilization of lipids for energy, which is regulated by these AKHs.

Typically, adult dipterans do not engage in long flights, but even for short flights, different metabolites provide energy. For the tsetse fly, the main flight substrate is proline (Bursell et al., 1974). Mosquitoes normally utilize carbohydrates during flight (Briegel et al., 2001a), but when attached to a flight mill, *A. gambiae* females can fly up to 22 h and use both lipids and carbohydrates over this period (0.38 and 0.29 J/km respectively; (Kaufmann and Briegel, 2004). In our experiments, females of this species used only a third of the lipid (0.13 J/km, Fig. 3.5) and an equal amount of carbohydrate (0.33 J/km; Fig. 3.5) for short flights (4 h) compared to longer flight. These results suggest that when *A. gambiae* females are forced to fly on a mill, carbohydrates are primarily used during the first few hours, and when these metabolites are exhausted, lipids are mobilized and used. The mechanism for mobilizing lipids is not known at this time, and it is unlikely that Anoga-HrTH plays a role, because it clearly had no hyperlipaemic effect even in carbohydrate starved, blood-fed, females, which have high lipid and low carbohydrate reserves in comparison to sugar-fed females (Kaufmann and Briegel, 2004). It is also possible that *A. gambiae* females may use proline as a flight metabolite, as reported for *A. aegypti* (Scaraffia and Wells, 2003). If the used lipid during flight was involved in proline oxidation, then this may indicate that the Anoga-HrTH induces

hyperprolinaemia and not hyperlipaemia. During hyperprolinaemia the mobilized lipid is oxidised or stays in the fat body, and since in our experiment the females were resting all the time (Fig. 3.1-3), there might be no use to synthesise proline and the hyperprolinaemic effect was silent, since the amount of proline in the haemolymph could be saturated.

Since the half-life of AKHs has been reported to vary from a few minutes to one hour and during flight (Oudejans et al., 1996) and our observation of a hypertrehalosaemic effect over 4 hours only (Fig. 3.2 A), a pharmacological dose of Anoga-HrTH (50 pmol/female $\leq$ 50 μ M in a female containing approximately 1.0 μ l of haemolymph) was used to achieve a long-lasting hypertrehalosaemic response in the flight experiments (Fig. 3.2 and 3.4). Decapitated females injected with this dose of Anoga-HrTH had a significantly stronger flight performance and in total flew an average of 0.4 ± 0.1 km more than the control females, and than the control females. This enhanced performance is due to the hypertrehalosaemic effect of the Anoga-HrTH. Interestingly, flight performance was enhanced but not statistically significant in intact females injected with Anoga-HrTH, relative to controls, which suggests that other factors released from the brain or increased protease action may counter the effect of the injected peptide.

3.52 RNA INTERFERENCE

RNA interference (RNAi) is a highly favoured method for determining the function of gene products in mosquitoes (Brown and Catteruccia, 2006), because injection of gene-specific dsRNA reduces expression of the gene and creates a 'temporary null mutation'. This approach was used to silence expression of a putative *A. gambiae* AKHR, previously identified as a homolog of the *D. melanogaster* AKHR that is generously expressed throughout the body of all *A. gambiae* life stages (Kaufmann and Brown, 2006). Expression of the *Anoga-AKHR* likely is fat body-specific in females, and genes in this tissue are amenable to RNAi. Heterologous expression of this receptor did verify that Anoga-HrTH is a ligand (Belmont et al., 2006), but its *in vivo* action was not reported. As demonstrated in this study, *Anoga-AKHR* expression was reduced in females by dsRNA injection (Fig. 3.6A), and a subsequent injection of Anoga-HrTH had no significant effect on their carbohydrate metabolism. Together these results indicate that high expression of the identified AKHR is necessary for Anoga-HrTH to activate a signalling pathway that mobilizes carbohydrates. To our knowledge, this is first time that this approach has been used to investigate peptide hormone/receptor signalling in insects.

This study establishes a foundation for future studies of AKH signalling and function in *A. gambiae* and other mosquito species. The fitness of female mosquitoes depends on their ability to fly, so that they can find mates, nectar or blood sources, and sites for eggs. Flights to accomplish these activities may be short or long in duration, and as demonstrated herein, the AKH octapeptide mobilizes carbohydrates most likely for short duration flights. Longer flights require lipids as previously demonstrated for *A. gambiae* females (Kaufmann and Briegel, 2004), and proline may be used, as reported for *A. aegypti* (Scaraffia and Wells,

2003). These metabolites may be readily used by females in the field where nutrients are more limited. Investigation of proline and lipid metabolism during long flights or in wild females may reveal that AKH signalling is also involved, as reported in the fruit beetle, *P. sinuata* (Gäde and Auerswald, 2002). Defining the AKH signalling pathway and where it intercedes to mobilize carbohydrates in mosquitoes will allow us to understand the degree to which these mechanisms are conserved across insects.

ACKNOWLEDGEMENTS:

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4. FLIGHT PERFORMANCE OF FERTILE AND STERILE MALE *ANOPHELES GAMBIAE*

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4.1 INTRODUCTION:

Several techniques are used to fight malaria. Integrated control, which involves all available control methods that are dependent on the field situation, is likely to be the key function in 'rolling-back' malaria (Utzinger et al., 2002). One course of action is to negatively affect the vector population itself. A controversial and widely discussed method is the release of genetically modified (GM) mosquitoes, which can have different effects on the population. Another, more environmentally friendly control method is the sterile insect technique (SIT), in which the release of irradiated, sterile male mosquitoes reduces the mosquito population (Esteva and Mo Yang, 2005). Recent reports suggest that a combination of both methods might be the safest method i.e. the release of sterile (irradiated) GM male mosquitoes (Catteruccia et al., 2005; Williams, 2005; Knols et al., 2006). Before following one of these courses of action and rearing masses of male mosquitoes, several questions have to be answered. At present, there are several studies in progress to figure out which rearing method is most appropriate, and what kind of food enhances the survival of the larvae and results in competitive adults. Yet only a few answers are known. The dispersal of sterile males is of great importance to the success of the SIT based control method. A recent report provides answers about the minimal irradiation dose for the sterilization of *Anopheles arabiensis* male mosquitoes (Helinski et al., 2006), and we are interested if this irradiation affects the flight performance. The aim of the study is to compare the flight performance of sterile males (gamma-irradiated) with their fertile siblings using flight mills. The effect of irradiation on the male nutrient levels is also analyzed, and is shown to be the key argument for the potency to compete with the wild male anopheline.

4.2 MATERIAL AND METHODS

4.21 MOSQUITO COLONIES

Mosquitoes were provided by the Malaria Research and Reference Reagent Resource Center (MR4) collection in Atlanta, GA USA. Experiments were done with males of two strains of the malaria mosquito *A. arabiensis* (DONGOLA and KGB) and one strain of *A. gambiae* (G3). Larvae were reared at $27^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $70 \pm 10\%$ relative humidity using the feeding schedule according to Benedict (1997). For the experiments, the mosquitoes were held in small cups (5 cm x 10 cm; diameter and high) with access to 10% sucrose solution. The different characteristics of the genital lobe, which is less distinct and smaller in females, was used to separate male from female pupae.

4.22 IRRADIATION

Assisted by Dr. Mark Benedict and the CDC Radiation Safety Officer, Dr. Paul Simpson, males of late pupae and one day-old adults were irradiated using a Gamma-Cell irradiator, located at the Centers for Disease Control and Prevention in Atlanta, GA, USA. For all irradiations, 80 Gy was used, which is an appropriate dose for sterilization of both pupae and adults (Helinski et al., 2006; data out of the International Atomic Energy Agency (IAEA) Mosquito SIT program for *A. arabiensis*).

4.23 THE FLIGHT-MILL SYSTEM

Our flight-mills were built according to Rowley et al. (1968), with a flight path circumference of 32.7 cm; the number of revolutions was registered by a computer at 30 s intervals. Further details are described by (Briegel et al., 2001a; b). Flight-experiments lasted for 16 h and usually started between 6.00 and 7.00 pm at $27 \pm 2^{\circ}\text{C}$ and $70 \pm 10\%$ relative humidity under long-day conditions (12 h light, 12 h dark). Dusk and dawn were stimulated by dimming light. Each flight trial provided information on the total flight distance covered during this period, the temporal pattern of flight activities, i.e. bursts of continuous flights, or erratic flight pulses, and pauses for each male tested.

We tested one and five day-old males. Each flight cohort included ten males, i.e. five sterile and five fertile males. All experiments were repeated for several cohorts. Statistical analyses were performed with 'All Pairs Tukey-Kramer' ($p \leq 0.05$) and the 'Students t-Test' (JMP Version 5.1.2).

4.24 BIOCHEMICAL ANALYSES

Sugar, glycogen and total lipids were measured in the same individual males by a modified version of Van Handel and Day (1988); i.e. we used 1.6 ml CHCl_3 -MeOH (v : v; 1 : 1) and 0.6 ml of distilled H_2O . Glycogen in the precipitate, and sugar in the aqueous fraction, were both measured with the hot anthrone reaction (2 ml anthrone/tube), with glucose (Merck 8337) 0.1% in EtOH (25%) as standard. The lipid content was quantified by a vanillin-

phosphoric acid reaction (1 ml vanillin/tube). The vanillin-reagent (Merck 818718) was used to initiate the colour reaction. Soybean oil (Sigma S-7381) 0.1% in chloroform was used as a standard. The photometric readings were measured in a 96 wells plate and finally converted to μg per male.

For biochemical analyses, males were fixed after termination of the flight trials and only males with flights of more than 300 m per 16 h trial were included in the results. Two different controls were used. Siblings of same body sizes were fixed as pre-flight controls at the time when the flights started, and as a second control, siblings were kept individually in test tubes, restrained by cotton plugs to very narrow spaces to prevent flight movements (un-flown controls). After the flight trials ended, all males were fixed for analysis in test tubes (10x75 mm) by heating in 100 μl EtOH for 10 min at 90°C. Utilization of a given substrate for flight was obtained by subtraction of post-flight caloric values from pre-flight values. Alternatively, the rate of substrate utilization was calculated by expressing these absolute differences per hour of actual flight activities. The same was achieved for survival data (basal metabolism) when pre-flight values were compared to the values of un-flown, resting controls.

4.3 RESULTS:

4.31 FLIGHT PRE-TESTS:

For the KGB strain of *A. arabiensis*, 5 cohorts were tested. The total average distance flown for KGB males was 243 ± 348 m, independent of age and sterilization. In Table 4.1, the different cohorts are illustrated in more detail. The other *A. arabiensis* strain (DONGOLA) showed an equally weak flight performance and a lower average total distance flown at 151 ± 232 m. (see also Table 4.2). Neither strain showed any strong flight performances. Tests with females of the DONGOLA strain showed an average of 192 ± 283 m total flight distance, and only 25% of the tested females ($n = 8$) flew more than 300 m per the 16 h trial. Based on these poor results, we wanted assurance that the flight-mills were working properly at the CDC location so we tested the flight of a large North American anopheline, *An. freeborni* provided by the MR4 facility (Table 4.3) The high rate of strong flight of this species reasonably confirmed that the mills were operating properly. Therefore, we suspected the low flights we had observed might be a specific characteristic of these strains of *An. arabiensis*.

Because we had relevant flight experience with *An. gambiae* females elsewhere (Kaufmann and Briegel, 2004), we tested strain G3 of this species. We expected that any radiation effects might vary in magnitude, but not quality, in this closely related species. Note that *An. gambiae* was treated by a different larval diet (brewer's yeast for the first 3-4 days followed by 1:1:1 brewer's yeast, lactalbumin, and ground new world monkey chow). The tests with the G3 strain of *A. gambiae* (Table 4.4) showed better flight performances than *A. arabiensis*. These circumstances, our experience with female *A. gambiae* flight (Kaufmann

and Briegel, 2004) and more importantly the availability of energy reserve data from *A. gambiae* males (Fernandes, 2003), let us to decide, in consultation with Bart Knols, to continue with *A. gambiae* instead of *A. arabiensis*.

Table 4.1: Distance flown by fertile and sterile male *A. arabiensis* (strain: KGB), irradiated as (A) pupae and (B) adults. Strong flights indicate the percentage of flights that reached more than 300 m total distance in 16 h flight time (n.a., no data taken).

	Day 1		Day 2		Day 5	
A	Distance (km)	Strong flights	Distance (km)	Strong flights	Distance (km)	Strong flights
fertile	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
sterile	0.215 ± 0.376	20% (n = 10)	n.a.	n.a.	n.a.	n.a.
B	Distance (km)	Strong flights	Distance (km)	Strong flights	Distance (km)	Strong flights
fertile	0.037 ± 0.018	0% (n = 5)	0.041 ± 0.020	0% (n = 5)	0.322 ± 0.475	30% (n = 10)
sterile	0.093 ± 0.185	20% (n = 5)	0.473 ± 0.195	80% (n = 5)	0.357 ± 0.386	40% (n = 10)

Table 4.2: Distance flown by fertile and sterile male *A. arabiensis* (strain: DONGOLA), pupal irradiation only. Strong flights indicate the percentage of flights that reached more than 300 m total distance in 16 h flight time.

	Day 1		Day 5	
	Distance (km)	Strong flights	Distance (km)	Strong flights
fertile	0.139 ± 0.100	0% (n=5)	0.028 ± 0.013	0% (n=5)
sterile	0.101 ± 0.143	20% (n=5)	0.081 ± 0.083	0% (n=5)

Table 4.3: Distance flown by fertile and sterile male *A. freeborni*, adult irradiation only. Strong flights indicate the percentage of flights that reached more than 300 m total distance in 16 h flight time.

	Day 7	
	Distance (km)	Strong flights
fertile	1.301 ± 0.522	100% (n= 5)
sterile	0.695 ± 0.432	80% (n= 5)

Table 4.4: Distance flown by fertile female and male *A. gambiae* (strain: G3) no irradiation. Strong flights indicate the percentage of flights that reached more than 300 m total distance in 16 h flight time.

	Day 1 to 3	
	Distance (km)	Strong flights
female	0.780 ± 0.337	100% (n= 3)
male	0.452 ± 0.394	67% (n= 12)

4.32 FLIGHT PERFORMANCE OF FERTILE AND STERILE MALE *A. GAMBIAE* (G3)

A summary of the flight experiments is illustrated in Figure 4.1 and Table 4.5. Each graph shows the different experimental conditions. We divided the flights into five categories to describe the distribution of the flight distances and to remove the weak flyers from this analysis. As illustrated, the first category includes all flights measured; the next one includes all flights that reached more than 100 m per 16 h flight trial, followed by > 200, 250 and 300 m per trial.

With one exception, no statistical difference in total distance flown between the fertile and sterile males of *A. gambiae* was found. Adult irradiated males, at one day post eclosion, show a statistically significant decrease in flight performance compared to the fertile males, but only for flights higher than 250 m. Overall, the fertile males showed trend of a better flight performance at the day after eclosion, but at 5 days post eclosion no such trend was detected. Interestingly 5 day-old males (fertile and sterile) illustrated a weaker flight performance. To make sure the number of strong and weak flyers were evenly spread for the different categories the amount of tested flyers for each category is shown in Table 4.5.

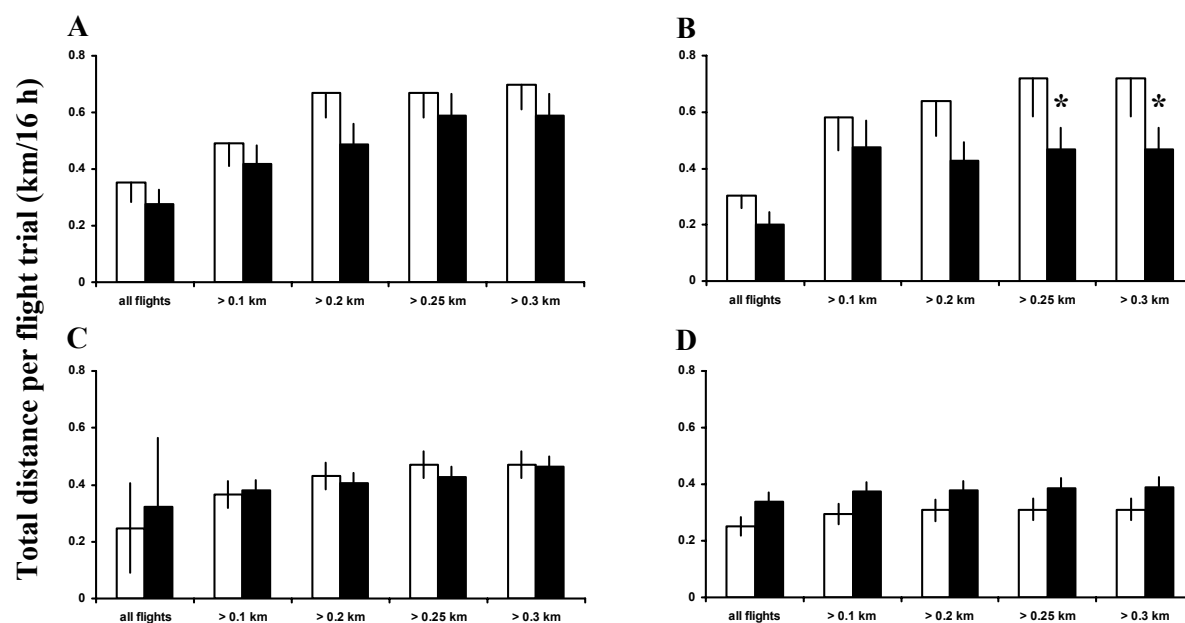


Figure 4.1: Total distance flown by fertile (white bars) and sterile (black bars) of 1 and 5 day-old male *A. gambiae* (G3) with access to 10% sucrose. One day old males irradiated as (A) pupae or (B) adults. Five-day old males irradiated as (C) pupae or (D) males. Asterisk indicates a significant difference of total distance flown between fertile and sterile males (All pair Tukey-Kramer, $p < 0.05$; $M \pm S.E.$; $n = 8-30$).

Table 4.5: Percentage of male *A. gambiae* that fulfil the terms for each different category.

The last row illustrates the maximal total distance flown for each condition.

Pupal-irradiation (day 1)	all flights	> 0.1 km	> 0.2 km	> 0.25	> 0.3 km	max-flights
n = 30 (fertile) %	100	70	47	47	40	0.676 km
n = 30 (sterile) %	100	63	50	37	37	0.921 km
Adult-irradiation (day 1)						
n = 30 (fertile) %	100	50	43	37	37	1.592 km
n = 30 (sterile) %	100	50	40	33	33	1.055 km
Pupal-irradiation (day 5)						
n = 25 (fertile) %	100	64	48	40	40	1.407 km
n = 25 (sterile) %	100	84	76	68	56	1.107 km
Adult-irradiation (day 5)						
n = 20 (fertile) %	100	60	45	40	40	0.771 km
n = 20 (sterile) %	100	75	70	60	55	0.766 km

4.33 STORAGE METABOLISM

As shown in Figure 4.2, there is no indication that irradiation affects the level of stored energy reserves. During the first 4 days no differences in storage were detected under any condition. This is consistent with the observation that there was no increase in total distance flown between 1 and 5 day-old males.

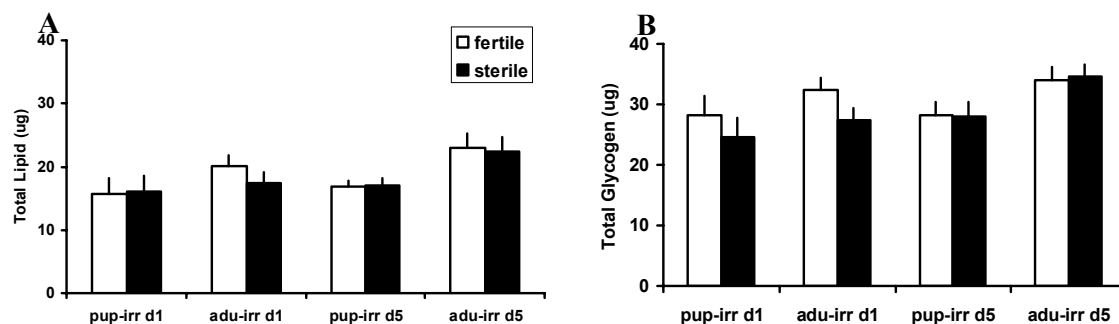


Figure 4.2: Lipid (A) and glycogen (B) content of 1 (d1) and 5 day-old (d5) male *An. gambiae*. Fertile males are illustrated by white and sterile males by black bars. Pupal irradiation (pup-irr) and adult irradiation (adu-irr) show no significant difference for lipid and glycogen storage (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; $n = 11-20$).

4.34 HAEMOLYMPH AND CROP CARBOHYDRATES

The soluble carbohydrate measurements include the haemolymph trehalose and the stored sugar (sucrose) of the crop. In contrast to stored reserves above, there was a significant difference detected between irradiated and non-irradiated adults (Figure 4.3). A higher amount of sugar was registered for the sterile males, which probably reflected sugar storage in the crop. It seems that irradiated adults have the need to consume liquid after irradiation (this

effect was found only for adult irradiated males, Figure 4.3). Note, the fertile control males were transported and treated in exactly the same way as their irradiated siblings, the only difference was the irradiation process.

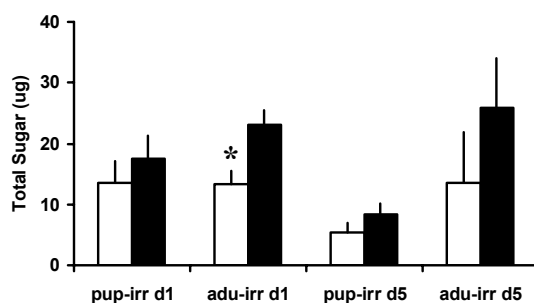


Figure 4.3: Sugar content of 1 (d1) and 5-day old (d5) male *A. gambiae*. Fertile males are illustrated by white and sterile males by black bars. Asterisk indicates a significant difference between pupal irradiation (pup-irr) and adult irradiation (adu-irr) (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; $n = 11-20$).

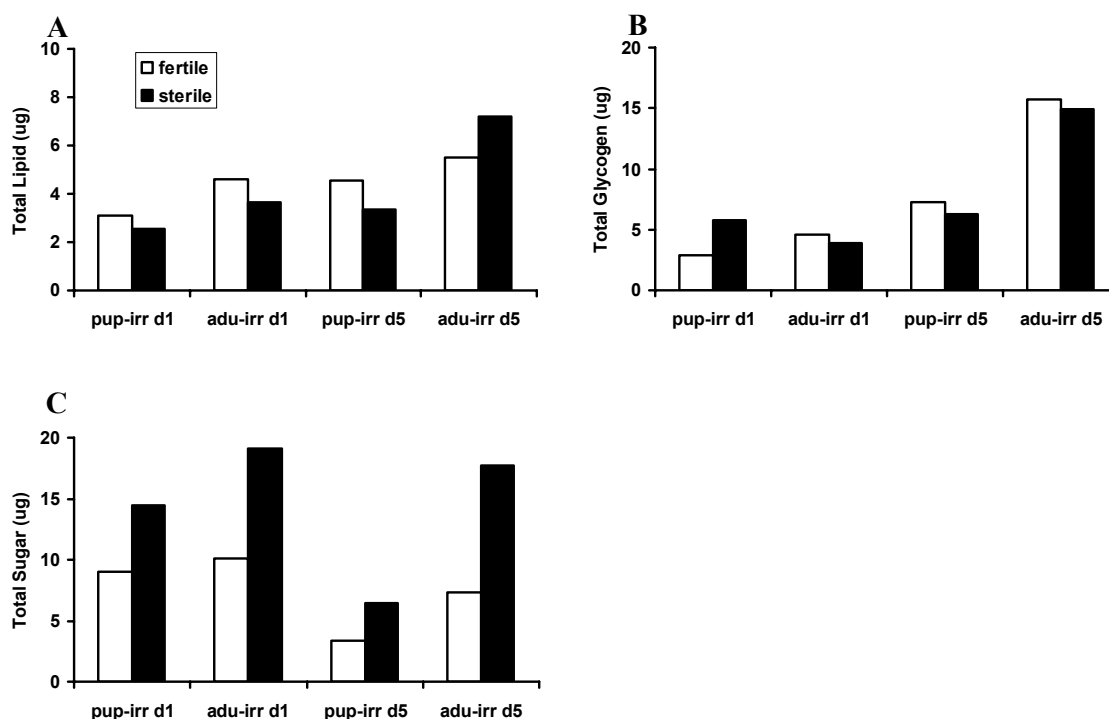


Figure 4.4: Basal metabolism as indicated by content of lipid (A), glycogen (B) and sugar (C) of male 1 (1d) and 5 day (5d) old *An. gambiae* during the 16 h rest. Fertile mosquitoes are indicated by white bars and sterile ones by black bars. Rates were calculated by subtracting the means from the pre-flight and un-flown controls (pup-irr, pupal irradiation; adu-irr, adult irradiation). Asterisk indicates a significant difference of total distance flown between fertile and sterile males (All pair Tukey-Kramer, $p < 0.05$; $M \pm S.E.$; $n = 8-30$).

4.35 BASAL METABOLISM

The net amount of energy a male used during the 16 hours of rest can be stated as the basal metabolism and was calculated by the subtraction of the un-flown from the pre-flight control (Figure 4.4). With 3-5 μg per male, the lipid contents seem to be constant, except that 5 day-old adult irradiated males used a higher amount of lipid (7.2 μg per male). Glycogen was used equally by the fertile and sterile males; note that the 5 day-old adult irradiated males showed again a higher amount of glycogen used, but the fertile controls also used a similar amount. It seems that the irradiated males used more sugar than their fertile siblings, but this did not reflect in increased glycogen storage nor in a stronger flight performance.

4.36 FLIGHT ENERGY

To measure the flight energy, we compared the total nutrient content between the flown and un-flown controls. Sufficient data remained after excluding the data of males that flew less than 300 m (Figure 4.5 and Table 4.5). It shows that the main energy used was carbohydrate, but also lipids. Surprisingly was the data for the 5 day-old adult irradiated flyers, it demonstrated that the un-flown control used more lipid than the flown siblings. We conclude that there was a problem with the gained energy reserves, as mentioned above – this may result from the lack of increase in lipid or carbohydrate storage (see discussion later).

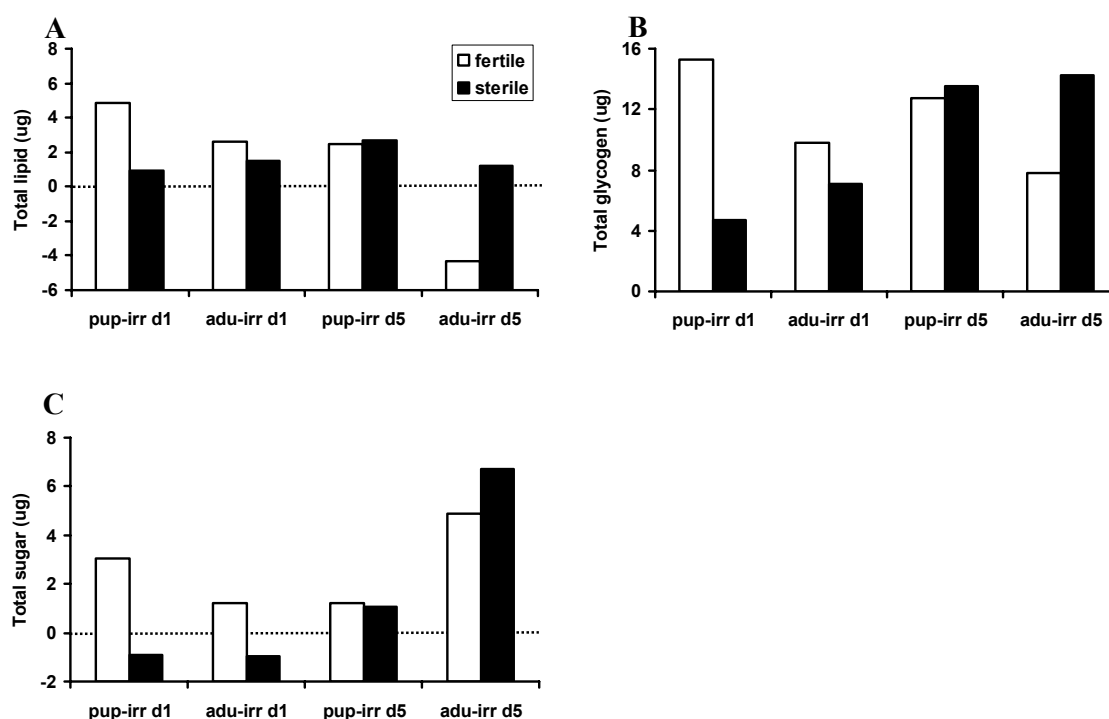


Figure 4.5: Content of lipid (A), glycogen (B) and sugar (C) of male *A. gambiae* used for the flight metabolism during the 16 h trial. The data was calculated by the subtraction of the means from the un-flown controls and flown males (pup-irr, pupal irradiation; adu-irr, adult irradiation, dashed line in graph A and C illustrate the zero line; n = 8 -12).

4.37 METABOLISM PER HOUR

The overall comparison between basal and flight metabolism is deceiving because by looking at Figure 4.4 and 4.5, one could think more energy was used for basal metabolism, whereas not much energy was left for flight. It is important to illustrate the amount of energy used per hour rest/flight. Figure 4.6 clearly demonstrates that more energy was used for flying than resting (Note: y-axis values are different for each graph). Both, lipids and carbohydrates were used for flight. With this result we can conclude that the study was a success, and with additional fine tuning (especially the larval diet and caging), we should be able to demonstrate whether irradiation has an influence on flight performance/activity for older males.

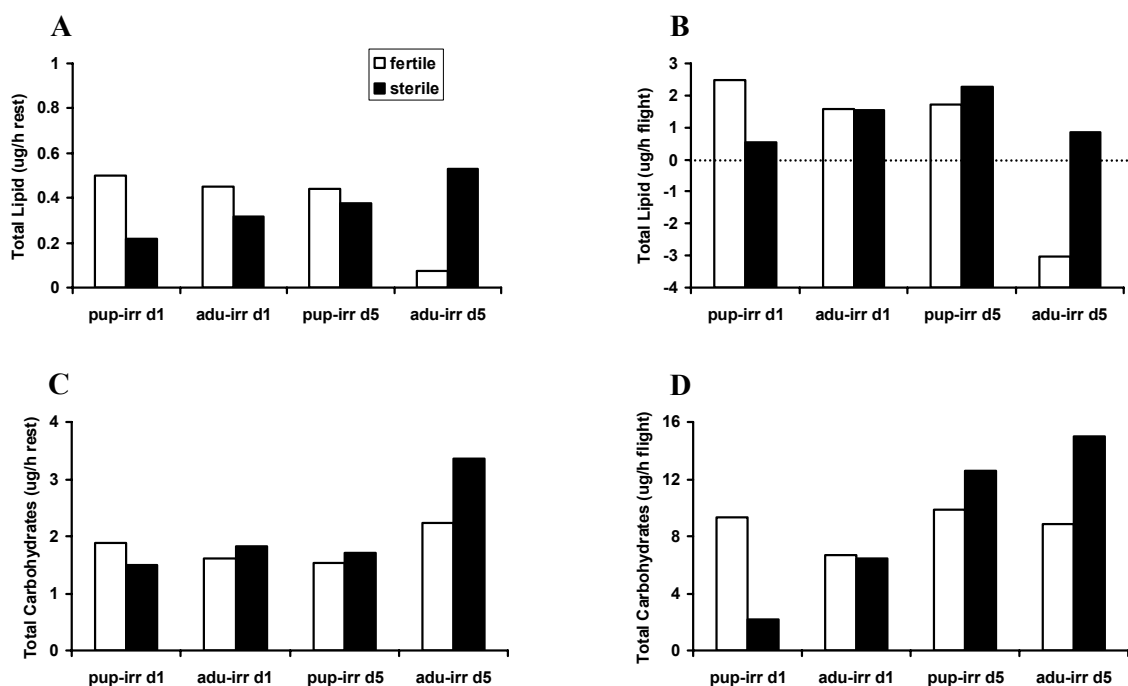


Figure 4.6: Basal and flight metabolism per hour of fertile (white bars) and sterile (black bars) male *A. gambiae*: Top row shows content of lipid used per hour rest (A) and used per hour flight (B); bottom row shows content of carbohydrates used per hour rest (C) and used per hour flight (D) (pup-irr, pupal irradiation; adu-irr, adult irradiation, dashed line in graph B illustrates the zero line; n = 8 -12).

4.4 DISCUSSION

When using the sterile insect technology (SIT) to fight against a disease like malaria, it is of great importance to know as much as possible about the vector of interest; in our case the main African malaria mosquitoes of the genus *Anopheles*. According to the recent study about optimal life stage for sterilization of male mosquitoes, we know that 80 Gy seems to be the optimal (minimal) dose for pupal and adult irradiation (Helinski et al., 2006). In this study we show, that male irradiation can affect flight performance, nutrients, and flight metabolism.

4.41 METABOLISM

The biochemical analysis reveals the probable reason as to why this flight study was not a total success. Our males did not reach the same teneral (freshly eclosed) amount of lipid reserves shown in other studies. Fernandes (2003) reported about twice the amount of teneral lipid reserves in male *A. gambiae* (approx. 44 µg/teneral male at 2.9 mm wing length) than in our 1 day-old males (between 15 – 20 µg/1 day-old male, wing length 3.0 mm). The glycogen amounts were approximately equal compared to Fernandes results. However, we would expect an increase in nutritional levels for our males, because they had one day access to 10% fructose, whereas the total lipid usually does not increase during the first 24-48 hours. The low amount of reserves, which probably caused the weak flights, could be the result of undernourished larvae. The other finding that 5 day-old males still have the same amount of nutritional storage levels could be an indication that the cages were too small, which may have stressed the male mosquitoes? This might be the reason why 5 day-old adult irradiated males demonstrated a negative amount in lipid consumption during flight. The negative flight energy values for the sugar were dependent on a full or empty crop. All negative values were nearly zero, indicating that rested and flown mosquitoes used up all their soluble sugar reserves. Helinski et al. (2006) found that, the median survival time for irradiated *A. arabiensis* given a dose of 80 Gy was normally 10 days or higher. Even if we did not work with the same species, *A. gambiae* is more radiation-resistant (Andreasen and Curtis, 2005) than *A. arabiensis* and we could expect a higher survival rate, which makes us certain that after 5 days post eclosion irradiated *A. gambiae* males were not over-aged.

Adult irradiated males showed an increase of liquid (in our case 10% fructose solution) uptake. As mentioned above, we added to this experiment an additional cohort of 20 fertile and 20 sterile males, to confirm this increase. The additional data verifies this increase of sugar uptake. Considering also the higher sugar usage during rest (Figure 4.4 and 4.6) of irradiated males, it was surprising that this does not reflect in an increase of stored glycogen or lipid. Adult irradiation definitely affects liquid uptake, but this, one should think, cannot affect the fitness negatively.

Compared to the energy used for survival, threefold the amount of lipids and eightfold carbohydrate were used for flight, which ratio is roughly equal to that of females (Kaufmann and Briegel, 2004). Males use lipid and carbohydrates for flight, as already known for female *A. gambiae* (Kaufmann and Briegel, 2004). Overall, the flight energy is covered by approximately one third of lipids (≈ 0.015 cal/h) and the rest (≈ 0.03 cal/h) by carbohydrates (proteins not included). In female *A. gambiae* this ratio is different, they use approximately the same amount of lipid and carbohydrates for flight (0.07 – 0.09 cal; Kaufmann and Briegel, 2004, which is nearly three- and fivefold the amount males use, respectively).

4.42 FLIGHT PERFORMANCE

The flight performances of male *A. gambiae* did not show any strong flight behaviour, but better performances than the two *A. arabiensis* strains. Considering the significant

difference in total flight distance for the adult irradiated one day-old males, we have to note that one flyer under this condition showed an 'excellent' flight performance with nearly 1.6 km. The statistic difference calculated is only due to this single male; therefore, with more data the difference would disappear or result in a trend only. However, one day-old pupal irradiated males also demonstrated a 'loss in fitness' trend. This study definitely illustrates a lack in strength of one day-old irradiated males, independent of irradiation stage. As a matter of fact this trend disappears for the 5 day-old males. Considering the discussed metabolic data above, we assume that the 5 day-old males suffered under-nourishment because no increase in storage metabolites was detected. To manage this starvation issue, the experimental mosquitoes could be kept in larger cages and the larval diet should be adjusted to achieve optimal conditions for the newly eclosed imago as already shown for anophelinae and culicinae (Timmermann and Briegel, 1993). After establishment of optimal teneral conditions, flight studies will provide a good tool to demonstrate if irradiation causes a loss of fitness.

In a female mosquito, generally three typical flight behaviours/strategies are known: i.e. find host (and/or food), find place for (resting and) laying eggs, and find a mating partner (the order is situation dependent). Males, on the other hand, probably have only two main flight behaviours, which are finding food and mating. Females can show strong flight performances, especially during host seeking behaviours, but males normally stay at the breeding site, where they seek mating partners, which could be a freshly eclosed female or a female that returns for laying its eggs (Klowden, 1999; Pates and Curtis, 2005; Yuval, 2006). These different male flight behaviours suggest that other methods than the flight mill, which records the absolute total flight distance (under lab condition), could be used. A method that measures the flight activity of a single and/or crowded male/s may be a better tool for measuring the difference between fertile and sterile males. Such a technique was used for measuring the flight activity of different mosquito species whereby flight activity was measured by recording the sound of the wing beats (Jones et al., 1967; Braack et al., 1994).

4.5 CONCLUSIONS AND RECOMMENDATIONS

1. The teneral reserves of our males demonstrated a much lower lipid level than that observed by Fernandes (2003). This could be due to different larval densities or diet amount or quality. Measurement of teneral lipid alone as an outcome variable to determine the quality of test diets should be considered.
2. The lack of increase in stored energy (lipid and glycogen) indicates stress or an inefficient feeding tool. Changes in caging and trials with different sugar concentrations could help (Briegel, 2003).
3. Adult irradiated males show an increase in uptake of more liquid than the non-irradiated control males. This is indicated by higher amount of sugar measured with

the hot anthrone reaction, a direct determination of drinking activity as a function of irradiation should be performed.

4. Irradiation diminishes flight performance on the first day after eclosion when adults are irradiated (Figure 1). Pupal irradiation shows a similar but non-significant trend.
5. Independent of the irradiation stage, the 5 days old males should not be included in the study because they probably were undernourished (Fenandes, 2003), biochemical analysis optimal treatment during larval and adult stages of males during the first few days should be considered.

5. GENERAL DISCUSSION

Adipokinetic hormones have been identified in a wide diversity of insects, they are most probably found in all insect orders. In Diptera, a few AKHs have been described (Table 1.3 and 3.2), and with the characterization of two neuropeptides in the main African malaria vector, *A. gambiae*, two new dipteran AKHs were identified. These two AKHs were the first reported for the phylogenetically lower dipteran suborder Nematocera. One is a classical octapeptide and the other a non-classical decapeptide member of the AKH-family. In addition, a putative AKHR (adipokinetic hormone receptor) was characterized as a G-protein coupled receptor (GPCR). The gene expression of the neurohormones and its receptor were omnipresent during all different experimental conditions, which indicates a continuity of AKH signalling during all life stages, as described for the homeostatic vertebrate hormone glucagon or insulin.

5.1 AKHS GENE AND STRUCTURE IN *A. GAMBIAE*

The availability of the *A. gambiae* genome and the methods in bio-informatics, like 'basic local alignment search tool' (blast), made it possible to blast known AKH sequences through the database to receive sequence predictions, which may originate from an orthologous gene. The advantage of using bioinformatics, instead of the Edman degradation of corpora cardiaca HPLC fractions, is the identification of the complete open reading frame of the gene. By characterizing the transcript levels in different tissues, at altered conditions and life stages, the constancy and origin of synthesis can be ascertained. However, analysis of the HPLC fractions of the corpora cardiaca provides native AKH(s), which can be used for injection assays, but the following Edman degradation provides only the amino acid sequence information of the neuropeptide. In addition, HPLC extractions are easy to process from large insects like locusts, which, compared to small insects like mosquitoes, have large and easily accessible corpora cardiaca. This possibility of mining (blasting) putative peptides or transcripts makes it possible to bypass the biochemical analysis. The gene expression can be documented via RT-PCR or microarray assays.

Blasting parts of the pre-pro-peptide sequences of Drome-AKH (Noyes et al., 1995) and the Locmi-AKH-I, -II, and -III (Bogerd et al., 1995) and Locmi-HrTH (neuropeptide sequence only, Siegert, 1999), two AKHs were identified in *A. gambiae* with blast: i.e. the classical octapeptide, Anoga-HrTH; and non-classical decapeptide, Anoga-AKH. Both *Anoga-AKH* genes have two introns, but since their positions are dissimilar and their AKH sequences have the typical dipteran amino acid residues in common, they probably not evolved from a recent gene duplication. Comparison between the pre-pro-neurohormone, which consists of a signal peptide, the AKH, and the AKH-precursor related peptide (APRP), showed that both mosquito AKHs share only 18.4% amino acid residues. The pro-neurohormone, which was cleaved from the signal peptide showed 15.9% similar amino acid

residues, and the APRPs just shared 8.7% identity. However, both have a blocked N-terminus (pyroglutamate) and C-terminus (amide), position four and eight consist of a phenylalanine and tryptophan respectively, therefore these two peptides can clearly be identified as members of the AKH family.

In Diptera only one other AKH gene has been fully characterized: i.e. the *D. melanogaster* AKH (*Drome-AKH*; Noyes et al., 1995). Comparison of the fruit fly AKH gene and the two malaria vector AKH genes showed that *Anoga-HrTH* and *Drome-AKH* appear to be orthologs. Both encode an AKH-octapeptide and share an intron between residues 1 - Gln and 2 - Leu of the AKHs (Noyes et al., 1995; Kaufmann and Brown, 2006), their transcript of the pre-pro-hormone shares 47.5% identity, however *Drome-AKH* lacks a second intron in the APRP. Interestingly, *Anoga-HrTHI* showed more similarity to the *Locmi-AKH-II* (31.6%) than to *Anoga-AKH*. The open reading frame of *Anoga-AKH* has only 16.8% similarity with *Drome-AKH* and no second AKH has been identified in *D. melanogaster*. It seems at present that Diptera lack an ortholog for *Anoga-AKH*, but predictions made from the *A. aegypti* genome implies that this decapeptide may be conserved in mosquitoes; surprisingly it does share the first eight amino acid residues with the fourth AKH member of the migratory locust, *Locmi-HrTH* (Siegert, 1999).

Most of the seven identified and two predicted dipteran AKH members (Table 3.2) were extracted and biochemically characterized. Only the mosquito AKHs were predicted by blasting the genomic database. Octa- and decapeptide AKH members have been identified or predicted in species of the suborders Nematocera (*A. gambiae*, *A. aegypti*, and *C. pipiens*) and Brachycera (*Tabanus* spp.), whereas only one AKH in the form of an octapeptide has been reported in the higher flies (Cyclorrhapha: *Ph. terraenovae*, *D. melanogaster*, and *N. bullata*). Blasts in the genome of *D. melanogaster* did not reveal second AKH (personal blasts), nor did HPLC fractions of the thorax, which encompasses the corpora cardiaca, indicate any presence of a decapeptide AKH (Schaffer et al., 1990). In *Ph. terraenovae* and *N. bullata* HPLC fractions of the corpora cardiaca or the complete ring gland also revealed only the above mentioned AKH member (Gäde et al., 1990; Verleyen et al., 2004). It is speculation as to whether these higher flies have lost the gene for the decapeptide AKH, or whether it has yet to be identified.

Identical amino acid residues of the characterized and predicted dipteran AKHs (Table 3.2) were found at four positions (1, 3, 4, and 8), and two are conserved substitutions (2 and 5). Compared to the AKHs of most other insect orders only three conserved amino acids were found at position 1, 4, and 8 (Table 1.1). In general AKHs are represented either in the form of an octa-, or decapeptide and are blocked at the N- and C-terminus by a pyroglutamate and an aminidation, respectively. Exceptions are found in the order Lepidoptera, where a conserved nanopeptide was characterized in the moths, *M. sexta*, *H. zea*, and *B. mori* (Ziegler et al., 1985; Jaffe et al., 1986a; Jaffe et al., 1986b; Ishibashi et al., 1992). Recent reports described a conserved bioactive hendecapeptide (eleven amino acid peptide, C-terminus not aminidated) in *Vanessa cardui* and other lepidopterans, as an AKH member (Vanca-AKH,

Table 1.1; Köllisch et al., 2000; Köllisch et al., 2003). In Diptera no nano- or hendecapeptide has yet been reported.

5.2 GENE EXPRESSION OF *ANOGA-HrTH* AND *ANOGA-AKH*

In *A. gambiae*, the expression of *Anoga-HrTH* and *-AKH* was assayed using the reverse transcriptase polymerase chain reaction (RT-PCR) in different life stages and in females fed on sugar- or blood-meals (Kaufmann and Brown, 2006). The expression for both specific amplification products was limited to the head and thorax only. Surprisingly, unprocessed *Anoga-HrTH* was amplified in the abdomen, whereas in the head and thorax no such PCR products were detected. These unprocessed *Anoga-HrTH* were predominantly found in early eggs and abdomens of fourth instar larvae, pupae, and in both adult sexes. RT-PCR of different tissues in female abdomens showed that the large PCR product was amplified only from ovary cDNA. It is still matter of speculation as to whether this unprocessed mRNA will be further processed into an active hormone during egg maturation or embryogenesis.

In general, the expression of processed *Anoga-HrTH* and *-AKH* was more or less stable and constant, which was surprising because a difference before and after a sugar meal was expected. This observation, however, corresponds with the general continuous synthesis of AKHs in insects that likely does not respond to nutrient metabolism (Diederer et al., 2002; van der Horst, 2003). Continuous synthesis results in the constant storing of AKH(s) in the glandular part of the corpora cardiaca, of which preferably newly synthesised AKHs are released (Diederer et al., 2002). A recent microarray analysis, which represented a genome-wide analysis of gene expression in larvae, male and female (sugar-fed and during gonotrophic cycle) *A. gambiae* (<http://www.angagepuci.bio.uci.edu>, Marinotti et al., 2005), showed the highest expression of *Anoga-AKH* in males in comparison to larvae and females. It was also reported that blood-fed females had less variability in their expression than sugar-fed siblings; no information was given for *Anoga-HrTH*. This difference in variability stands in contrast with Kaufmann and Brown (2006), but could be explained by the experimental feeding conditions of the sugar-fed males and females, which consisted of raisins and water *ad libitum* resulting in variable sugar concentrations (Marinotti et al., 2005). Since the *Anoga-AKH* is characterized as a non-classical member of the AKH-family and most probably synthesized in the lateral neurosecretory cells and released from the corpora cardiaca (Kaufmann and Brown, 2006), its expression profile might be not constant. However, in the study of Kaufmann and Brown (2006), all individuals were fed a consistent sugar concentration and no such trend was observed from the RT-PCR results. The expression was stable for *Anoga-HrTH* and *-AKH* for all time points examined after the sugar- or blood-meal in females and for the experimental tested sugar fed males. In addition, the expression for the actin gene was also stable, which indicates a similar amount of extracted total RNA for each sample of all three cohorts tested. Since AKH expression generally appears to be constant, the

expression of potential AKH releasing factors is probably influenced more by the different feeding conditions and nutrient metabolism. When the expressions of the predicted tachykinin neurohormone and tachykinin receptor were compared, using the above mentioned database microarray study, an increase of products was detected from sugar-fed individuals only, whereas blood fed females only demonstrated a weak increase in the 96 hour observation period of the gonotrophic cycle. Other potential releasing factors, like FMRFamides and CCAP were not as much elevated as tachykinin. Again, this microarray data would have allowed better predictions if the experimental individuals would have been held under constant feeding conditions. However, the elevation of tachykinin could be another indication of the involvement of the mosquito AKHs in the sugar metabolism, whereas during the gonotrophic cycle it was silenced. On the other hand, in *A. gambiae* an elevation of AKH-like peptides during the first 6 hours after blood meal was demonstrated (Annexe, Figure A-IX), which is in contrast to the above mentioned expression profile of the predicted releasing factors.

5.3 CELLULAR SOURCES OF AKH PEPTIDES

In general, AKH producing cells are neurosecretory cells found in the corpora cardiaca and, in contrast to many peptide hormones, they are synthesized and released at the same place. In some insects like the locust, the corpora cardiaca is divided into a glandular and a storage part. The classical locust AKHs are only found in the glandular part, which surrounds the aorta, the release site of the neurohormones. To detect the source of AKH producing cells HPLC studies of these neurohaemal glands were performed. However, AKH positive HPLC assays may not provide information about the origin of synthesis of the peptide, since the neurohormones of the corpora cardiaca or perisymphathetic organs may be supplied by axons of neurosecretory cells originating in the brain or the ventral nerve cord. In the cockroach, *B. discoidalis*, northern blot studies showed that *Bladi-HrTH* transcripts were also found only in the corpora cardiaca, and not in the brain, ventral nerve cord or fat bodies (Lewis et al., 1997). In *D. melanogaster*, molecular and immunological techniques confirmed the corpora cardiaca as the sole source of AKH (Kim and Rulifson, 2004; Lee and Park, 2004; Isabel et al., 2005), as in *B. discoidalis*. Immunocytochemical studies with different Locmi-AKH antisera established the theory of general AKH synthesis in the corpora cardiaca in a variety of insects, but positive cells have also been observed in the brain and ventral nerve cord of *L. migratoria*, *A. aegypti*, *P. americana* and other insects (Schooneveld et al., 1983; 1985; Schooneveld and Veenstra, 1985; Brown and Lea, 1988). However, staining with AKH antisera may stain peptides similar to the native AKH(s), as shown by the immunocytochemistry assays with the locust AKH antisera 311 C and E in *A. gambiae* (Annexe, Figure A-I), which showed staining of several cells in the brain and ganglia, even the perisymphathetic neurohaemal organs were stained, but there was no clear evidence of staining of the corpora cardiaca. Since no specific binding to Anoga-HrTH or -AKH could be proved, the stainings in Figure A-I could show

peptides that are not AKHs; thus demonstrating the importance of establishing specific binding of the antisera to the native neuropeptide before assaying.

With a *D. melanogaster* AKH-antisera, which was proven to recognize both mosquito AKHs, the stained cells allowed quantification and identification of the two Anoga-AKHs in the heads and thoraces of female *A. gambiae*, hence confirming the expression of both *Anoga-AKHs*. Stained cells were observed in the brain and thoracic ganglia of females; stained axons ran from the brain cells into the corpora cardiaca and along the anterior and posterior midgut of females. Presumably, Anoga-AKHs are released from these axonal sites that delineate part of the stomatogastric nervous system in adult mosquitoes (also described as the cerebral and cardiacal neurosecretory systems, Clements, 1992). In mosquitoes (shown for *Aedes* spp.) the main NCC divides into dorsal and ventral branches, Meola and Lea (1972) reported the releasing site of peptides prior to this branching. The dorsal branch probably runs into the X-cells, which were hypothesized to be homologous with the intrinsic cells of the corpora cardiaca in other insects (Clements, 1963; Meola and Lea, 1972). These X-cells were clearly immunostained and since their location is more often in the thorax than in the head, the higher amount of Anoga-HrTH in the HPLC thorax fractions (Figure 6, Kaufmann and Brown, 2006) indicates the primary source of the octapeptide. Thus the assumption can be made that the source of Anoga-HrTH is found mainly in the X-cells and the thoracic ganglia with the releasing site along the bundle of the three nervi corpori cardiaci, which is also part of the mosquito corpora cardiaca along the aorta (Meola and Lea, 1972). Anoga-HrTH in the head extract may be due to its transport to the corpora cardiaca releasing site, which is positioned more in the head than in the thorax. Anoga-AKH, however, probably originates from the lateral neurosecretory cells in the brain only and the weak Anoga-AKH immunopositive results in the thorax HPLC fractions may reveal the axons of the midgut only. The fourth locust AKH with high sequence similarity to Anoga-AKH was detected in the storage lobe of the corpora cardiaca in *Locusta* (Locmi-AKH-I, -II, and -III all are found in the glandular part only), which proves its synthesis origin in the brain and not the neurosecretory cells of the corpora cardiaca (Siegert, 1999). The RIA results for Anoga-AKH stand in contrast to the RT-PCR experiments, where expression was found in the head and thorax, whereas the HPLC fractions of the thorax only show weak or no evidence for the presence of the decapeptide. Definitive answers as to which of the AKH immunostained cells are the sources of a particular AKH in this mosquito could be provided in further experiments using *in situ* hybridization specific for *Anoga-HrTH* and *-AKH* mRNA.

Furthermore, no AKH-like immunoreactivity was detected or resolved by HPLC in extracts of abdomens from sugar-fed females; HPLC experiments with late ovaries may provide answers as to whether AKHs are involved in embryogenesis. However, it still does not explain the presence of these transcripts in the abdomens of the larvae, pupae and male mosquitoes. Unfortunately, no closer observations in males were made; could this unprocessed form be expressed in the male genital organs, and even be involved in the glycolytic pathway of sperm movement?

5.4 IDENTIFICATION AND FUNCTION OF AKH-RECEPTOR IN *A. GAMBIAE*

Adipokinetic hormone receptors (AKHRs) are identified for only a few species, like *D. melanogaster* (Staubli et al., 2002), *B. mori* (Staubli et al., 2002), and *P. americana* (Wicher et al., 2006). All described AKHRs are known to be G-protein coupled receptors (GPCRs) and they are probably structurally related to the gonadotropin-releasing-hormone receptors (GnRH) of mammals, birds, amphibians, and molluscs (Rodet et al., 2005). Predictions of AKHR sequence were also achieved by blasting genomic databases, using the sequences of the extracellular N-terminus and the extracellular loops, the potential AKH binding sites. Using this method, predictions of AKHRs were made for the red flour beetle, *Tribolium castaneum* (Hansen et al., 2006), the honeybee, *Apis mellifera* (GeneID: [551388](#)), the yellow fever mosquito, *A. aegypti* (GenBank: [EAT36594](#)), and in this study for *A. gambiae* (Kaufmann and Brown, 2006). The potential binding sites of the Anoga-AKHR, along with the seven transmembrane regions, demonstrated high sequence similarity with the *D. melanogaster* AKHR, but the intracellular C-terminus, which further transduces the hormonal message, was more similar to that of the honeybee and much shorter than the others, as also predicted for the AKHR in *A. aegypti*.

As showed by RT-PCR, the *Anoga-AKHR* expression was present in the head, thorax, and, unlike the hormonal expression, in the abdomen of all mosquito life stages (Kaufmann and Brown, 2006). The appearance of abdominal *Anoga-AKHR* reflects the distribution of the fat body, the primary tissue involved in nutrient storage and metabolism. Most presumably, Anoga-HrTH mobilizes glycogen from the fat body via Anoga-AKHR, which is transported in the form of trehalose to the flight muscles (see later). In the split female abdomen *Anoga-AKHR* was mainly expressed in the dorsal and ventral abdominal walls to which the fat body is attached, less abundant but still present in ovaries and otherwise absent in the digestive tracts and spermatheca. Throughout all different experimental conditions, it seemed that the receptor expression appeared constant. The microarray analysis of gene expression in *A. gambiae* indicated a high expression in the last larval instar, 'raisins-water' fed males and females, a decline of 50% of the expression followed immediately after the blood-meal, which started to increase 24 h post blood feeding and after the gonotrophic cycle the same level as demonstrated for sugar-fed females was reached again (<http://www.angagepuci.bio.uci.edu>, Marinotti et al., 2005). Interestingly, the RT-PCR results of the thoracic tissues in our study showed similar expression levels to that in the abdomen (Figure 4; Kaufmann and Brown, 2006). It is still a matter of speculation as to whether the *Anoga-AKHR* expression in eggs reflects receptors on the outside of the ovaries or inside the eggs. After ovipositing the eggs, its expression is certainly not on the outside of the hardened chorion, which could explain the necessity of the unprocessed Anoga-HrTH, but no immunostaining or evidence of these neuropeptides were found in the abdominal HPLC fractions. In addition, we demonstrated that Anoga-HrTH treatment does not influence yolk deposition (Annexe, Table A-II), which is another indication that the *Anoga-AKHR* is probably situated inside the

ovaries only, where it may have an unknown role for the unprocessed Anoga-HrTH I during the gonotrophic cycle or most probably during embryogenesis after oviposition.

Most of the fat bodies are situated in the abdomen, but as mentioned above, an equal expression was detected in the thoraces that represent the flight muscle tissues; surprisingly there was no hypertrehalosaemia reported in the thorax. However, no report verifies AKHR expression in muscle tissues. Two studies have examined the expression of the AKHRs in the fat bodies (Ziegler et al., 1995; Wicher et al., 2006). With radio-receptor assays, the binding of AKH was localized to membranes of the pterothoracic ganglion and fat body from last instar larvae in *M. sexta*, whereas the brain was devoid of such binding (Ziegler et al., 1995). In *P. americana*, the AKHR transcripts, detected by RT-PCR or immunoassays, showed a similar picture as for *Anoga-AKHR* in *A. gambiae*. The expression of the *Peram-AKHR* gene was shown in the fat bodies, the brain and ventral nerve cord, the ovaries, and trachea of the cockroach. It also showed expression in the digestive tract, which differs to the *Anoga-AKHR* gene expression (Wicher et al., 2006). For *D. melanogaster* an AKHR is also identified as a GPCR (Staubli et al., 2002), but its tissue expression has not yet been reported.

5.5 AKHS AND ITS INDUCED METABOLISM IN DIPTERA

In the order Diptera, all known reports of AKH related bioactivity showed mobilisation of carbohydrates and since in most insects the circulating sugar in the haemolymph is mainly trehalose and not glucose (Wyatt, 1967), we conclude that the dipteran AKHs generally induce hypertrehalosaemia. An AKH-octapeptide was identified in the blow fly, *Ph. terraenovae* and its effects on metabolism and flight performance were analysed and the results further support the presence of a hypertrehalosaemic effect and its involvement in flight (Gäde et al., 1990; Wilps and Gäde, 1990). This corresponds to the classical hypothesis that Diptera are exclusively carbohydrate users, especially during flight (Sacktor, 1976). In the tabanids, however, AKH related hyperlipaemia was reported (Woodring and Leprince, 1992) and the exclusive use of proline for flight in the tsetse fly (Bursell et al., 1974) does not support the theory that dipteran AKHs are exclusively involved in the carbohydrate metabolism. The tsetse fly is not the only exception in Diptera, the yellow fever mosquito *A. aegypti* has utilized sugar (Briegel et al., 2001a), as well as proline during flight (Scaraffia and Wells, 2003). In addition, the recent flight study of an African (*A. gambiae*) and potential European (*A. atroparvus*) malaria vector demonstrated that *A. atroparvus* dominantly uses carbohydrate for flight and that *A. gambiae* utilizes both, lipids and sugar, which is untypical for the suborder Nematocera (Kaufmann and Briegel, 2004), but was also reported for *A. vexans* (Briegel et al., 2001b).

The AKHs likely play an important role in regulating the carbohydrate and lipid basal and flight mobilization in *A. gambiae*. With the identification of the *Anoga-HrTH* and *-AKH* genes, we were able to synthesize the two peptides and investigate the functions of these neurohormones in *A. gambiae* on the metabolic level, as already shown in a variety of insects

(Gäde and Auerswald, 2003; van der Horst, 2003). All metabolic measurements in this study represented total amount analysis of lipids, glycogen, sugar, or fructose. With the simplicity of these methods, it was possible to show the direct metabolic effect induced only by Anoga-HrTH after its injection under all tested conditions. However, in other studies the metabolic effects were analysed by measuring the involvement of the different enzymes like glycogen phosphorylase for glycogen mobilization or triacylglycerol lipases for lipid mobilization (Gäde and Auerswald, 2003). The demonstrated increase for a particular enzyme indirectly proved glycogen or lipid mobilization, respectively. The enzymatic methods have the advantage of showing an effect shortly after AKH treatment, whereas with the direct measuring method a longer incubation time has to occur. In addition, enzymatic methods can be more sensitive and the variation of the direct method, which compares metabolic levels of AKH and saline treated cohorts, appears to be relatively high. A combination of the direct and indirect method would probably demonstrate optimal results.

However, the experiments clearly showed that only the octapeptide (Anoga-HrTH) is involved in metabolism and, as demonstrated for *Ph. terraenovae* (Gäde et al., 1990), it mobilizes only carbohydrates, inducing a hypertrehalosaemic effect after treatment. The maximal increase of haemolymph sugar was saturated by injections of 1 pmol Anoga-HrTH, which corresponds approximately to 4-6 corpora cardiaca extracts (according to Kaufmann and Brown, 2006). The consequent maximal decrease in glycogen was achieved by 5-10 pmol injection of Anoga-HrTH. The decapeptide, Anoga-AKH did not cause any metabolic changes. We conclude that both, Anoga-HrTH and -AKH have no species-specific hyperlipaemic activity and that only Anoga-HrTH causes a metabolic effect in *A. gambiae* females. Since this effect results in hypertrehalosaemia only, the adipokinetic hormone was renamed from its original nomenclature Anoga-AKH-I (Kaufmann and Brown, 2006) to Anoga-HrTH (Kaufmann and Brown, submitted to Journal of Insect Physiology).

The lack of lipid mobilization was surprising, given its involvement in flight metabolism (Kaufmann and Briegel, 2004) and the evidence that some Diptera mobilize lipid after AKH treatment, as shown in the horse flies, *Tabanus* spp. (Woodring and Leprince, 1992). Even the hypothesis that Anoga-HrTH could induce hyperlipaemia after depletion of the carbohydrate reserves was not confirmed. In blood-fed, but otherwise starved females, which have low carbohydrate reserves stored (Kaufmann and Briegel, 2004), only hypertrehalosaemia was induced by Anoga-HrTH, but no hyperlipaemia, supporting that Anoga-HrTH is only involved in carbohydrate metabolism. However, tests with Anoga-HrTH in *Locusta* showed hyperlipaemia with 35 or 70 pmol injection of the mosquito octapeptide. Similar tests with the other known dipteran AKHs showed comparable results. The ‘higher fly’ AKH (Phote-HrTH, sometimes also called Drome-AKH) and Anoga-HrTH both reached the 50% potency (ED_{50}) of the species-specific adipokinetic response in *Locusta* only by high dose injections. Maximal response for the ‘higher fly’ AKH was between 400 and 600 pmol treatment (Gäde, 1993); for Anoga-HrTH no such test was performed. According to Gäde’s studies, the two tabanid AKHs (Tabat-AKH/HrTH) demonstrated a lower ED_{50} than Phote-

HrTH; especially Tabat-AKH, which at 8.4 pmol was around three times more potent than Tabat-HrTH (24.8 pmol), and five times more than Phote-HrTH (39.5 pmol), but surprisingly both tabanid AKHs did not reach maximal response, only around 70% of the species-specific Locmi-AKH-I treatment was achieved (Gäde, 1993). All known dipteran classical AKH members have a proline at position 6, which could indicate a β -turn in their secondary protein structure, which may be crucial for binding (Cusinato et al., 1998). Interestingly, the negative amino acid residue in the ‘higher fly’ AKH did not interfere with binding and the response in the locust.

Similar tests with Anoga-AKH, like the fourth locust AKH, resulted in no adipokinetic response (Table 3.3; Siegert, 1999). Both of these non-classical AKHs probably do not originate from the corpora cardiaca. The non-classical mosquito AKH is almost certainly synthesized in the lateral neurosecretory cells of the protocerebrum in the mosquito (Kaufmann and Brown, 2006) and the discovery of the non-classical locust AKH in the storage part of the corpora cardiaca, leads to its origin in the brain (Siegert, 1999). The immunocytochemical evidence of ‘external-corpora-cardiacal’ AKH sources in *Locusta migratoria* (Schooneveld, 1984) confirms this. The fourth locust AKH demonstrated weak hypertrehalosaemia in the American cockroach, which was responsible for the non-species-specific nomenclature Locmi-HrTH (Siegert, 1999). Apart from this misnomer, the 80% similarity between Locmi-HrTH and Anoga-AKH clarifies that the last two amino acid residues are not responsible for binding or for a hypertrehalosaemic effect of Anoga-AKH in the same tests (Table 3.4).

For a few characterizations of the AKHRs it was proved that the species-specific AKH(s) bind to their AKHR. In *Drosophila*, only one AKHR is reported, but the metabolic effect induced by indirect experiments with the ablation/mutation of the AKH-cells demonstrated hypertrehalosaemia and hyperlipaemia (Lee and Park, 2004; Isabel et al., 2005). Could it be possible that only one receptor is responsible for both metabolic mobilizations? Drome-AKH was not directly injected into the fruit fly, which probably resulted in only hypertrehalosaemia as seen with the same AKH (Phote-HrTH) in the blow fly (Gäde et al., 1990). In *P. americana* only one AKHR is reported and both AKHs (Peram-CAH-I and -II; CAH, cardio acceleratory hormone) induce hypertrehalosaemia only (Scarborough et al., 1984; Barrett and Loughton, 1987). In contrast, in *L. migratoria*, three of the four Locmi-AKHs, (Locmi-AKH-I, -II, and -III) are bioactive. Locmi-AKH-I and -II differ in their potency. It has been demonstrated that Locmi-AKH-I is more involved in lipid metabolism of the long-term flight and Locmi-AKH-II in carbohydrate utilization during the primary flight phase (Orchard and Lange, 1983a; Goldsworthy et al., 1986). Locmi-AKH-III, is speculated to be more active during rest providing the locust with energy (Vroemen et al., 1998). These different metabolic needs during the gregarious (migratory) phase are, among others, regulated by these three locust AKHs. The question as to whether one or more AKHRs are involved in this regulation has not yet been answered, but evidence for multiple GPCRs was provided by experiments with double injections (Gäde, 1993). If Anoga-AKH binds to a

GPCR it is most certainly not the Anoga-AKHR, because in the metabolic assay and in flight studies the injection of the high dose Anoga-AKH (50 pmol) would have surely blocked the Anoga-AKHR. The hypertrehalosaemic effect would have been inhibited, which should have resulted in hypotrehalosaemia – an increase in glycogen or decrease in haemolymph sugar level. This was not reported and furthermore would also negatively affect the flight activity, since the main flight metabolite mobilization of the mosquito HrTH is carbohydrate.

Recently, a bioluminescence receptor binding assay with Anoga-AKHR demonstrated that Anoga-AKH binds (Belmont et al., 2006), but its function was not reported. When *Anoga-AKHR* silenced females, via RNA interference, were treated with Anoga-HrTH there was no change in carbohydrate metabolism. For the first time, it was shown that the physiological response of an AKHR was reduced by RNAi, and that the Anoga-AKHR and the Anoga-HrTH, as its ligand, transduce hypertrehalosaemia in this mosquito. One of the dsRNA fragments (C-terminal) silenced the *Anoga-AKHR* transcript expression stronger than the other (N-terminus), which is not only visible in the weaker band, but also in the increase of the total glycogen content (chapter 3). A small trend towards hypotrehalosaemia was detected, which could be due to the lack of Anoga-AKHRs. Again, this effect could indicate that Anoga-HrTH is not only involved in the hypertrehalosaemic effect, which boosts flight activity, but also in regulating the carbohydrate mobilization during rest as described for the vertebrate hormone glucagon. In the future, studies with *Anoga-AKHR* gene silenced females on the flight mill could provide more information as to what other metabolites could be utilized for flight.

5.6 AKH, FLIGHT, AND FLIGHT-METABOLISM IN DIPTERA

Prior to the flight studies, the time response experiment showed a significant decrease in glycogen at 1 h post injection of 50 pmol Anoga-HrTH, while after 6 h post injection the differences disappeared, indicating a 3-4 h period of hypertrehalosaemic activity. Considering the flight experiments, which showed that Anoga-HrTH treatment (50 pmol) resulted in an enhanced flight activity during the first 4 hours, the bioactivity at this dose was confirmed. With this unusually high dose of Anoga-HrTH, (50 pmol corresponds to approx 200-300 CC extracts) we achieved this long-lasting maximal hypertrehalosaemic response during.

By comparison of this study and the report of *Ph. terraenovae* (Wilps and Gäde, 1990), it seems that both of these dipteran utilize carbohydrates for flight (Sacktor, 1976). In *Ph. terraenovae*, the hypertrehalosaemic factor, Phote-HrTH induced a decrease in glycogen and an increase in haemolymph sugar during stress (cage shaking for 20 min; Wilps and Gäde, 1990) and during one hour flights on flight mills. In *A. gambiae*, we showed the utilization of carbohydrates during 4 and 22 hour flights and a boost of flight by Anoga-HrTH treatment in the first 4 hours (chapter 3 and Kaufmann and Briegel, 2004). For *A. gambiae*, however, lipid consumption was also detected during flight. Comparison of the short- (4 h) and long-term (22 h) flight study showed that the total distance flown was not significantly

different. It can be concluded that *A. gambiae* forced to fly on a flight mill probably already reached their mean maximum after around 4 hours. Under both conditions the same amount of carbohydrate was utilized, whereas in the long-term study more lipid was used. Considering that the same total flight distance was achieved under both conditions, the additional use of lipid in the long-term study was most likely not utilized during flight, but may have been due to the stressed situation when resting on the flight mill. However, we still cannot explain the involvement of the 26% lipid used during the short-term flight. As shown above, Anoga-HrTH did not induce any hyperlipaemic response in sugar- and blood-fed females, but theoretically hypertrehalosaemia or Anoga-HrTH may be involved in proline oxidation. Firstly, after mobilization of trehalose and its movement to the thoracic flight muscles, trehalose is either stored in the form of glycogen. In the case of its use it will provide energy by the glycolysis and the citric acid cycle in the target cell. Acetyl-CoA, the intermediate between glycolysis and the citric acid cycle could also be used in proline oxidation (Bursell et al., 1974). If, *A. gambiae* utilizes proline during flight, as reported in *A. aegypti* (Scaraffia and Wells, 2003), additional acetyl-CoA may be provided by the β -oxidation, which could explain the involvement of lipid during flight of *A. gambiae*. Secondly, Anoga-HrTH may induce a hyperprolinaemic effect, which would provide additional evidence regarding the above-mentioned possibilities of the various involved metabolic pathways and the utilization of lipid, but hyperlipaemic effect might just be achievable during or after extensive flight.

5.7 PHYLOGENETICAL COMPARISONS OF AKHS IN DIPTERA AND OTHER INSECTS

Considering that AKH studies are only available in a few dipteran representatives, it is rather difficult to make any phylogenetical assumptions. As mentioned above, some dipterans only have one AKH, especially the genera in the ‘suborder’ Cyclorrhapha, where only evidence of the shorter AKH type has been found. On the other hand for *Glossina*, which is also a member of Cyclorrhapha, two AKHs have been predicted (Pimley, 1984). Even the use of a different classification-key, which only divides Diptera into the two suborders Brachycera and Nematocera, shows that suborder Brachycera is divided into superfamilies Asilomorpha, Muscomorpha, and Tabanomorpha, and the suborder Nematocera consists mainly of the superfamily Culicimorpha. There have been no AKHs yet identified in Asilomorpha. In Tabanomorpha, two AKHs (tabanid AKHs) have been reported, whereas in Muscomorpha, which contains the superfamilies Oestroidea (*Phormia* and *Neobellieria*) and Ephydroidea (*Drosophila*), only one AKH member has been identified. In the superfamily Hippoboscoidea (*Glossina*) two AKHs have been predicted. In the suborder Nematocera (mosquitoes) two AKHs for *A. gambiae* have been identified, but in *A. aegypti* two ortholog AKH genes have been predicted (www.ensembl.org; Aedae-HrTH (octapeptide), Vectorbase gene ID: [AAEL011996](http://www.ensembl.org); Aedae-AKH (decapeptide, similar to Anoga-AKH), Vectorbase gene ID: [AAEL010950](http://www.ensembl.org)), and personal blasts in the www.flybase.org confirmed that *C. pipiens* has the same octa- and decapeptide as predicted in *A. aegypti* (Table 1.1 and Fig. 5.1).

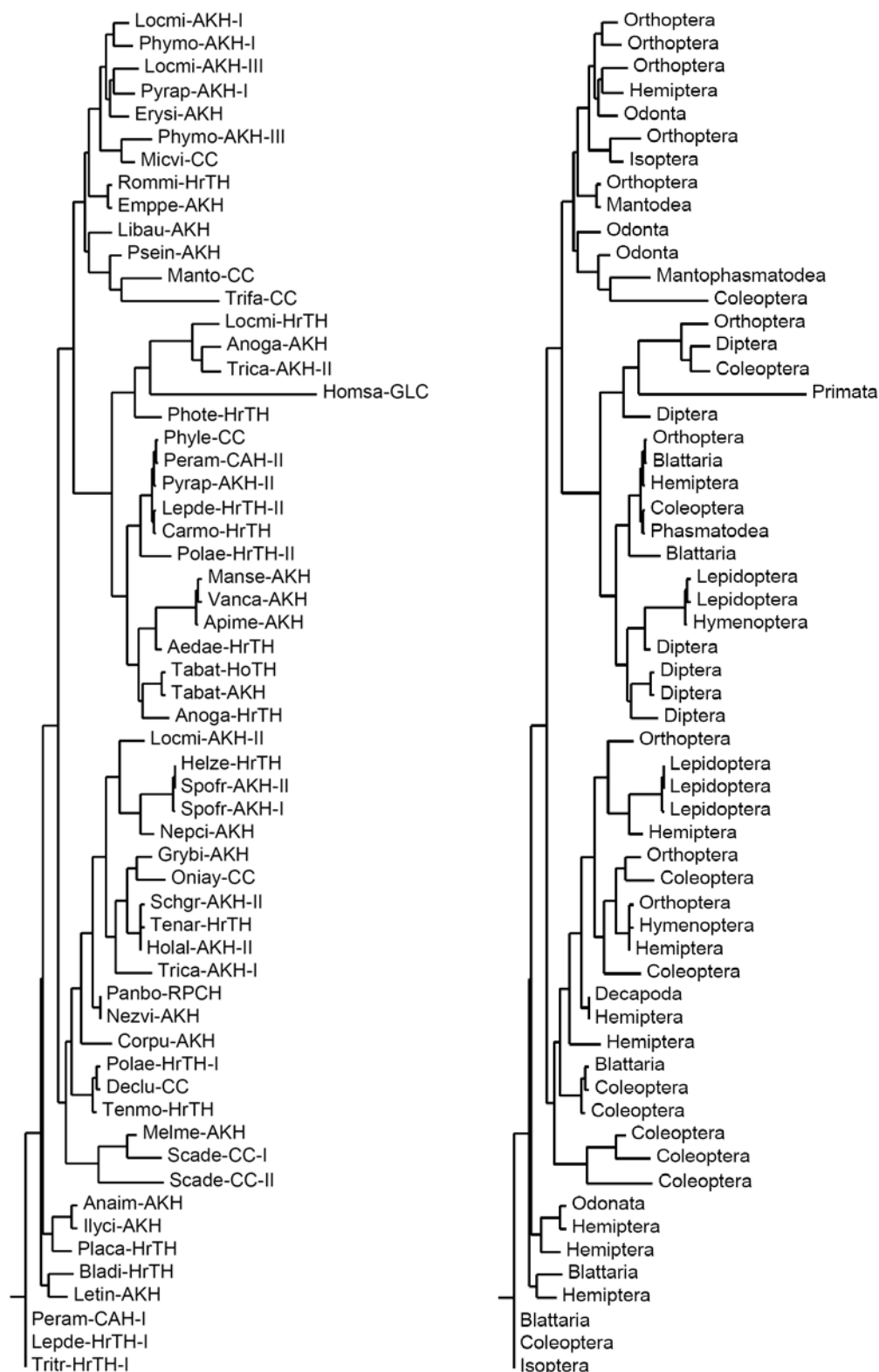


Figure 5.1: Phylogenetic tree from listed AKHs in Table 1.1. Left side illustrates the AKH-codenenames and on the right, the corresponding order is shown. (Phylogenetic tree provided by <http://iubio.bio.indiana.edu/treecapp>; Phylo dendron by D.G. Gilbert version 0.8d,)

Interestingly, it seems that the tabanid AKHs probably developed from a recent gene duplication, whereas the two mosquito AKHs appear not to originate from a recent duplication, which reveals different evolutionary paths. In addition, in both mosquitoes, the blow, fruit, and flesh fly no evidence was found for a gene duplication of their octapeptide. The AKH gene in *Ph. terraenovae*, *D. melanogaster* and *N. bullata* are most certainly orthologs to Anoga-HrTH (and Aedae-HrTH), and since Anoga-AKH is probably ortholog to Locmi-HrTH, we assume that in higher Diptera this decapeptide is lost. One of the recently identified AKHs in the coleopteran *Tribolium castaneum* (red flour beetle, Trica-AKH-II, Amare and Sweedler, 2007) is probably ortholog to Locmi-HrTH and Anoga-AKH. Trica-AKH-II shares 80% similarity to Locmi-HrTH and 90% to Anoga-AKH (Table 1.1 and Fig. 5.1). We speculate that those three non-classical AKHs originate all from the brain and have the corpora cardiaca as releasing site, but their function is probably not of metabolic nature.

The future characterization of the two *Glossina* AKH members could help reveal two AKH paralogs from a recent gene duplication as found in the tabanids or they may contain a classical and non-classical AKH, like in the mosquitoes *A. gambiae*, *A. aegypti*, and *C. pipiens*. Since bioactivity is reported for both *Glossina* AKH-extracts (Pimley, 1984), they probably resulted from a recent gene duplication. However, only a few species have been analyzed in this insect order, which makes it difficult to make any prediction, but in the future, when more dipteran AKHs are identified, combined with their involvement in their species-specific metabolism, it may be possible to prove the mentioned hypotheses above.

To illustrate the close relationship between the different dipteran AKHs, a phylogenetic tree was plotted in Figure 5.1 with all known AKH-members of Table 1. Before making any interpretation of this phylogenetic tree, it has to be stated that the length of the peptides compared is between 8 and 13 amino acid residues only, which is relatively short; a comparison of the complete pre-pro-peptide surely would represent a better platform. However, to make the distribution of the different orders clearer the same phylogenetic tree is illustrated again, but instead of the AKH codenames, their corresponding taxa were plotted. It shows that all the dipteran AKHs are found on the same branch on the tree. The non-classical AKHs (Locmi-HrTH, Anoga-AKH, and Trica-AKH-II) were, because of their similarity, found close together. Not surprisingly, some of the AKHs characterized in Lepidoptera are found close with Diptera, which probably makes sense, since both orders are members of the same infraclass, the Endopterygota. Coleoptera and Hymenoptera, also members of Endopterygota, were more dispersed in the phylogenetic tree, but these two orders, especially Coleoptera, are known as being older than Diptera and Lepidoptera, which are both found in the ‘younger’ superorder Mecopteroidea. Overall the infraclass Orthopteroidea dominates in most branches, which probably is due because most AKHs are characterized in this infraclass. Again, these conclusions are only a matter of speculation, more AKHs have to be identified and characterized on their genomic level, to make more defined phylogenetic assumptions

5.8 CONCLUSION

This project was the first attempt of AKH-research in mosquitoes. It proved the existence of two AKHs and one AKHR in *A. gambiae*. Expression for the peptides was present in the head and thorax, but not in the abdomen, which was confirmed by immunocytochemistry and radioimmunoassay experiments, whereas the mosquito AKHR was found in all body parts. Only one AKH demonstrated a metabolic response; this was the shorter octapeptide, whose treatment resulted in hypertrehalosaemia in sugar- and blood-fed females, and increased flight activity of decapitated females.

Additional experiments, which aided the success of this project, are included in chapter 6. The molecular and physiological characterisation of AKHs in *A. gambiae* surely supplements the understanding of metabolic functions in this mosquito. Similar AKHs were predicted for the Yellow fever mosquito, *A. aegypti*. This work, however, will not directly contribute to vector control and fight against its transmitted diseases, but it will certainly provide additional clues as to the basic understanding of *A. gambiae*. On the other hand, the flight study of irradiated and non-irradiated male *A. gambiae*, provides some elementary information, which may aid in the development of sterile male mosquitoes in the fight against malaria and other vector born diseases. This flight study clearly showed that this kind of vector control is dependent on the understanding of the insect's basic physiology; if the irradiated males cannot compete with the wild male population, then even the most advanced molecular or genetic tools are useless.

6. ANNEXE

6.1 ADDITIONAL EXPERIMENTAL DATA

6.11 AKH-ANTIBODY SENSITIVITY AND PROOF OF SPECIFIC BINDING

Subsequent to the positive immunocytochemistry results, we were interested to find out if the used antibodies also bound specifically. The following four procedures were used to illustrate specific binding of AKH-antibodies to the synthetic Anoga-AKHs, i.e. Dot-Blot, ELISA, RIA and pre-absorption in immunocytochemistry. The latter method was used as a control in each immunocytochemistry experiment (1° antibody was pre-treated with antigen, in our case Anoga-AKHs, further described Kaufmann and Brown, 2006).

6.111 DOT-BLOT

This technique is used to detect biomolecules, like small peptides. It can replace a Southern-, Northern-, or Western-blot. However, it does not provide us with the product size, but only confirmation of its presence. Of interest to us was evidence that the AKH-antibody bound to the Anoga-AKHs.

Our goal was to prove that the antibodies (Locmi-Anti-AKH-311C and E, Sasek et al., 1985; Brown and Lea, 1988); Drome-Anti-AKH-29C, and -30C, Kaufmann and Brown, 2006) show specific binding. Unfortunately, with this method, no specific binding for Anoga-AKH-I, or -II was demonstrated.

Dot-Blot protocol

- Peptides were dissolved in 10% acetonitrile [CH_3CN , J.T.Barker, Lot: V18474] and diluted from a stock solution [$100 \mu\text{g}/\mu\text{l}$] in steps of ten down to a concentration of $1 \text{ fg}/\mu\text{l}$.
- $1 \mu\text{l}$ of each of the peptide dilutions were applied to a nitrocellulose membrane (Protran)
- Covered with tinfoil, the membrane was baked at $80 - 120^\circ \text{C}$ for 30 min
- The membrane was bathed in tris saline buffer + 2% GS (goat serum) in 1% BSA (bovine serum albumin) for 30 min
- Overnight incubation with the 1° antibody (in tris saline buffer + 2% GS in 1% BSA)
- 3 x washed for 5 - 10 min in tris saline buffer + 2% GS in 1% BSA
- 2 - 3 h incubation of 2° antibody (1/1000) in tris saline buffer + 2% GS 1% BSA
- 3 x washed 5 - 10 min in tris saline buffer
- 3 x washed 5 min in tris buffer
- Add staining solution (SIGMA FAST™; DAB with metal enhancer tablet; D0426)
- Staining solution removed and washed with distilled water
- The membrane was dried

In the first Dot-Blot, the 1° antibody Locmi-Anti-AKH-311E (dilution factor: 1/50'000) was tested on eight different AKHs, i.e. Tabat-AKH, Tabat-HoTH, Bladi-HrTH, Manse-AKH, Helze-HrTH, Locmi-AKH-I, Drome-AKH, and Anoga-AKH-I (Abbreviations for species verification see Table 1.1). Tabat-AKH, Tabat-HoTH, Bladi-HrTH, Manse-AKH, Helze-HrTH gave positive results, whereas no staining was observed for Locmi-AKH-I, Drome-AKH, and Anoga-AKH-I. Surprisingly no antibody binding was detected for the locust AKH, the specific antigen (data not shown).

In the second Dot-Blot, a different locust AKH antiserum (Locmi-Anti-AKH-311C) and two *Drosophila* anti-AKHs (Drome-Anti-AKH-29C and -30C) were used. With a dilution factor of 1/1000, these 1° antibodies were tested on four different peptides (Locmi-AKH-III, Drome-AKH, Anoga-AKH-I, and -II). Again, the locust antibody did not show any staining, whereas the two *Drosophila* anti-AKHs only reacted with the Drome-AKH (data not shown).

Unfortunately, the two Dot-Blot tests did not provide any evidence that the antibody used for the immunocytochemistry (Drome-Anti-AKH-30C) bound to the Anoga-AKHs.

6.112 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

The enzyme linked immunological technique is unlike the radioimmunoassay using an enzyme-catalyzed colour reaction instead of radiolabelling. The ELISA, however, is similar to the other technique in that it also uses two antibodies, i.e. a specific 1° antibody, which binds to the detectable probe (antigen) and a 2° antibody, also known as enzyme-linked antibody, which binds to 1° antibody and reacts in a colour change after initiation of the enzyme-catalyzed colour reaction.

ELISA protocol

- Pipette 50 µl of the different peptide dilutions into the plate and let it dry out (overnight)
- Add 50 µl coating solution overnight
- Add 100 µl block solution (phosphate buffered saline Tween 20 (PBS-T) in 1% BSA) for 2 h.
- Wash 3 times with pBSA
- Add the 1° antibody (diluted in PBS-T 1% BSA) at 4° C for overnight
- Wash 3 times with pBSA
- Add the 2° AB (Anti-Rabbit IgG, Sigma, Lot: 012K91691; 1/5000 in PBS-T-1% BSA) at room temperature for 2 h
- Wash 4 times with pBSA
- Add the Enzyme (p-Nitrophenyl Phosphate Tablets, Sigma, Lot: 014K8205: one Tablet in 5 ml 10% Diethanolamine)
- Yellowish coloration appears, measure the OD ($\lambda = 405\text{nm}$) at different time intervals

For the quantitative ELISA, the optical density of the colour reaction was interpolated into a standard curve, which is typically a serial dilution of the target and can be used as a tool for proving antibody binding. As in the Dot-Blot above, the tested peptides were dissolved in 10% acetonitrile. However, they were not fixed on a nitrocellulose membrane, but ‘coated’ in a 96-well microtiter plate.

In Table A-I, a summary of the different ELISAs are illustrated. We tested the same 1° antibodies as in the second Dot-Blot (311C, 29C and 30C) on Anoga-AKH-I and -II, but also on Drome-AKH, Locmi-AKH-I and -III. The results demonstrated that both *Drosophila* antibodies (29C and 30C) bound with the Anoga-AKHs; the affinity to the Anoga-AKH-I was higher, which is probably because Drome-AKH and Anoga-AKH-I are both octapeptides and differ only by two amino acids (Table 1.3).

Table A-I: Summary of the results for the antibody-binding test using ELISA.

Antibody [dil. fac.]	Antigen	Sensitivity [ng/μl]	Incubation (min)
311C [1/5 K]	Locmi-AKH-I	0.002 - 1	0 - 240 (45)*
311C [1/5 K]	Anoga-AKH-I	no binding	0 - 240
311C [1/1 K]	Anoga-AKH-I	no binding	0 - 240
311C [1/1 K]	Anoga-AKH-II	no binding	0 - 240
29C [1/1 K]	Locmi-AKH-III	no binding	0 - 240
29C [1/0.5, 1, 2 K]	Drome-AKH	0.01 - 1	0 - 240 (120)*
29C [1/0.5, 1, 2 K]	Anoga-AKH-I	0.01 - 1	0 - 240 (25)*
29C [1/0.5, 1, 2 K]	Anoga-AKH-II	0.01 - 0.2	0 - 240 (90)*
29C [1/5, 10 K]	Anoga-AKH-I	weak/no binding	0 - 240
29C [1/5, 10 K]	Anoga-AKH-II	weak/no binding	0 - 240
30C [1/0.5, 1, 2 K]	Drome-AKH	0.01 - 1	0 - 240 (120)*
30C [1/0.5, 1, 2 K]	Anoga-AKH-I	0.01 - 1	0 - 240 (25)*
30C [1/0.5, 1, 2 K]	Anoga-AKH-II	0.01 - 0.3	0 - 240 (90)*
30C [1/5, 10 K]	Anoga-AKH-I	weak/no binding	0 - 240
30C [1/5, 10 K]	Anoga-AKH-II	weak/no binding	0 - 240

*Incubation time in brackets demonstrates the start of significant colour changes

6.113 RADIOIMMUNOASSAY (RIA)

With this method (more details in Kaufmann and Brown, 2006) it was clearly demonstrated that both Anoga-AKHs bound with the *Drosophila* antibody 30C, which we used for the immunocytochemistry. Several different peptides were tested for bindings, i.e. Anoga-AKH-I, and -II, Y²-Anoga-AKH-II, Tabat-HrTH, Helze-HrTH, Locmi-AKH-I, and -II. All except the locust AKHs showed binding (Figure 4 in Kaufmann and Brown, 2006).

ELISA, RIA and the pre-absorption in immunocytochemistry all illustrated that the *Drosophila* AKH antibody 30C bound to both of our synthetic manufactured *A. gambiae* AKHs. This, and the pre-absorption of the immunocytochemistry, proved that Drome-Anti-AKH-30C was sensitive for Anoga-AKHs in the immunocytochemistry assays and the RIA of the HPLC extractions.

6.12 IMMUNOCYTOCHEMISTRY

As mentioned above several AKH-antibodies were available. The Locmi-Anti-AKH-311 series (kindly provided by Professor Dr. R.P. Edele, University of Minnesota, Minneapolis MN and mentioned in Sasek et al. (1985) was used first. With the antiserum 311E, very nice stainings were achieved, but only a small amount of this antiserum was left. Unfortunately, the E-serum was not available anymore, but additional new antiserum 314 and 315 was provided kindly by the Edele laboratory. Regrettably, no staining was achieved using the 314 and 315 antisera. Staining was successful by using antiserum 311C, but, as shown above, no specific antibody-antigen binding was observed. Antibodies provided kindly by Professor H. Schooneveld (alpha-Anti-AKH-R351A and B, and others), and by Professor D. van der Horst and Professor K.W. Rodenburg (Drome-Anti-AKH, Anti-AKH (pool), AKH 3 ascites, Locust Anti-AKH 1, Locust AAP1-3 – the latter were described in Harthoorn et al. (1999) specific for the AKH-precursor-related-peptide) did not show any binding in the mosquito immunocytochemistry. The protocol for this method is described in Kaufmann and Brown (2006). However, Figure A-I shows the stainings for the 311 antisera C and E. The antiserum 311E demonstrated excellent staining in the abdominal ganglia; in each ganglion, three neurosecretory cells were stained along with the axons to the neurohaemal organ, the perisymphatic neurohaemal organ. This is probably the first picture taken for a perisymphatic neurohaemal organ in mosquitoes. Even with no other evidence, this staining probably shows AKH-like peptide(s), and therefore further studies will be required to find out what kind of AKH-like peptides are synthesized in the abdominal ganglia and released from the peripheral located sympathetic neurohaemal organs!

Using the same antisera (311 E), staining was also found in the brain, interestingly only weak or no staining was found for the corpora cardiaca region, whereas several cells in the optical lobes, frontal region of the pars intercerebralis, and medina, ventral and lateral neurosecretory cells were stained. Males and females seem to have similar cells stained in the brain (Figure A-I). Similar staining in several insect orders were described in the reports of the Schooneveld groups (Schooneveld et al., 1983; 1984; 1985; 1986, 1987a; Schooneveld, 1984; Schooneveld and Veenstra, 1985). The antiserum 311 C showed similar staining in the optical lobes, but in the brain and corpora cardiaca, almost no staining was demonstrated. Interestingly staining was achieved in the junction between the fore- and midgut (Figure A-I, F), which was also indicated in the study with *A. aegypti*, where also a locust AKH-antibody was used (Brown and Lea, 1988). Both antisera did not show any staining in the ovaries (data for 311 C is only shown). In picture I of the same figure, it is nicely demonstrated that the terminal ganglion is a fusion of two or three ganglia, since it is bigger and more than three cells were stained.

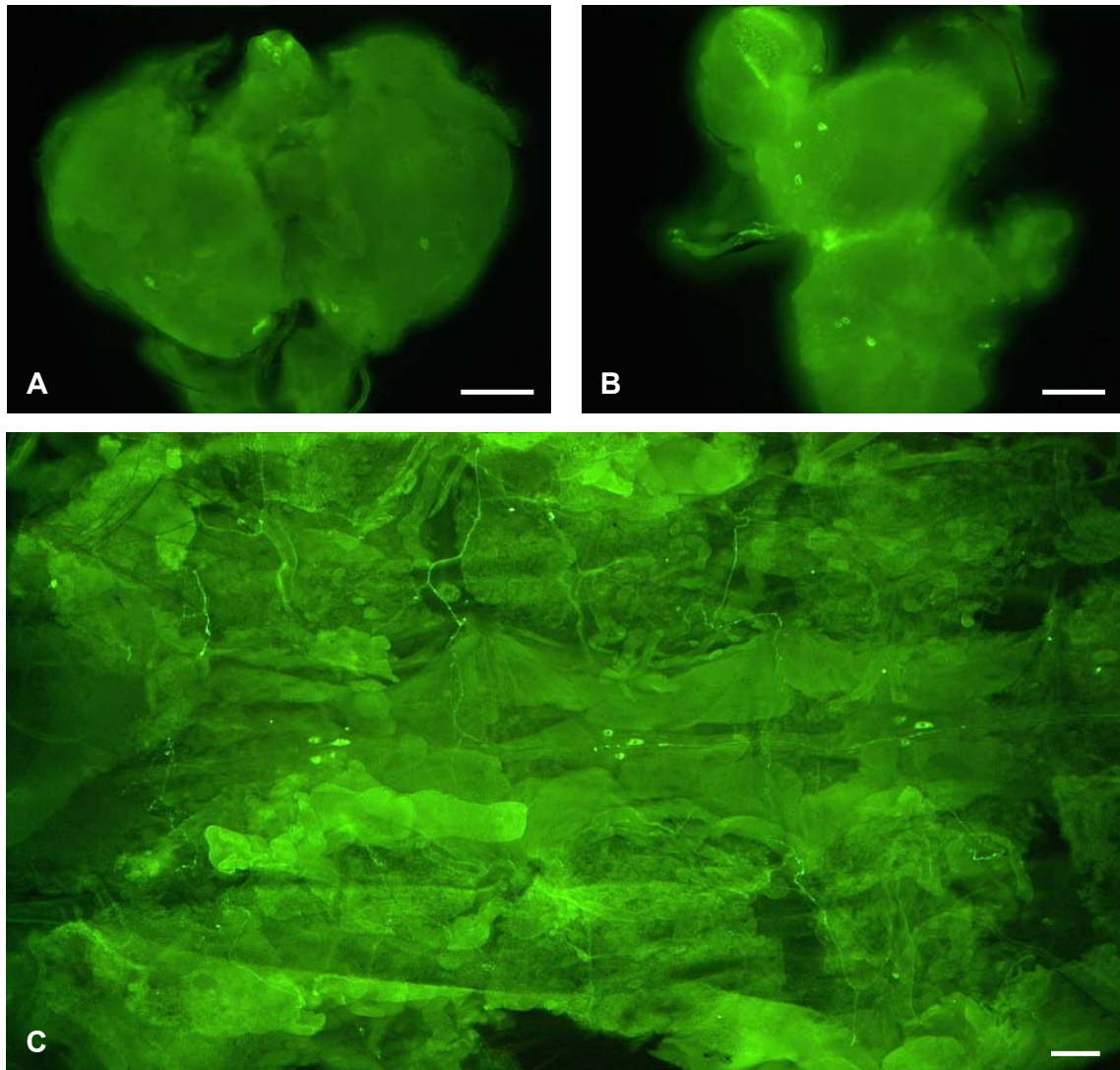
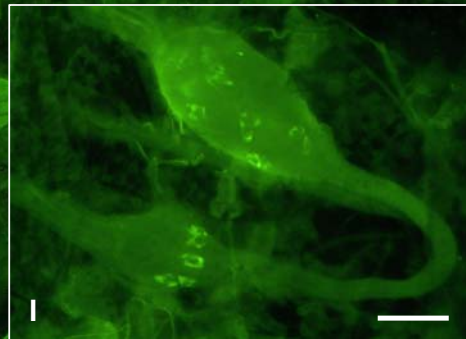
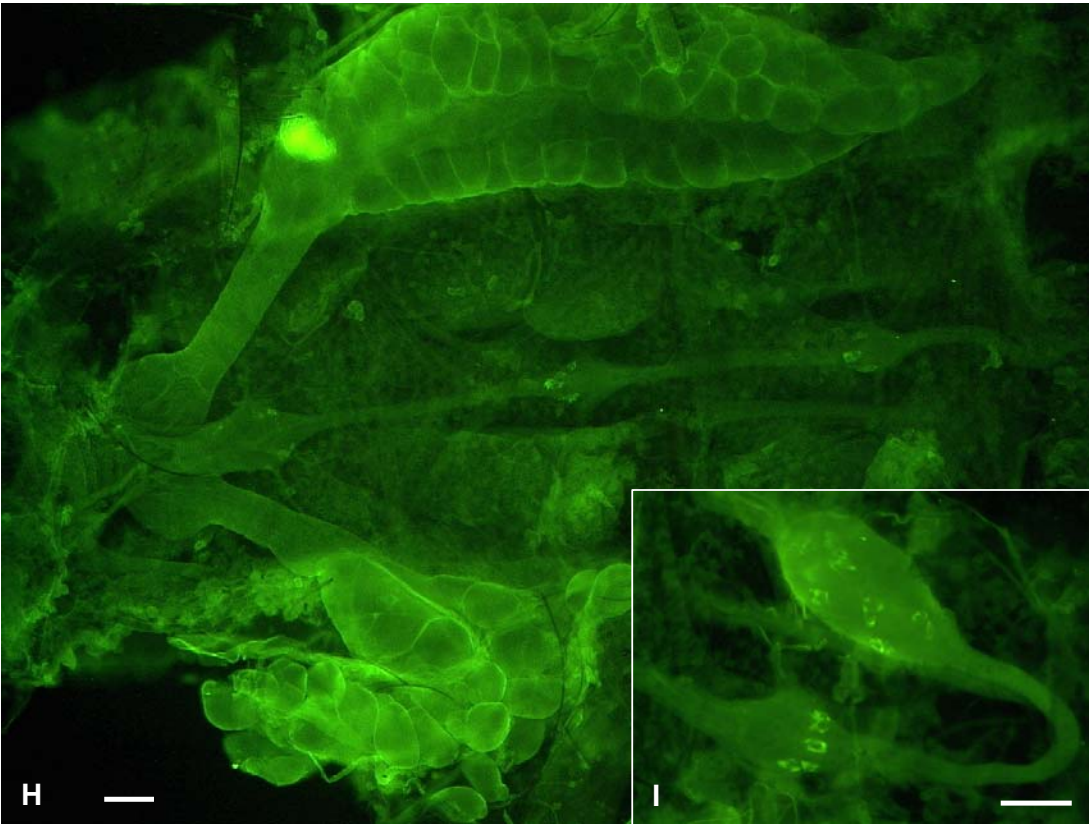
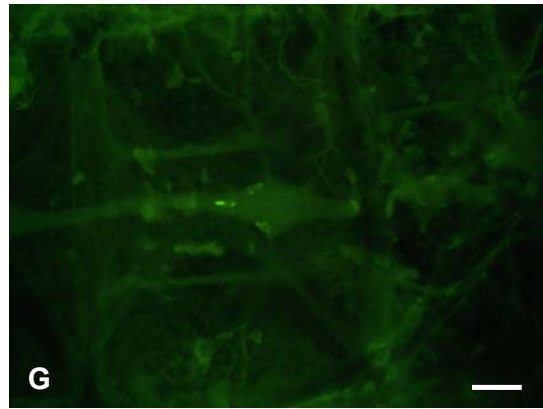
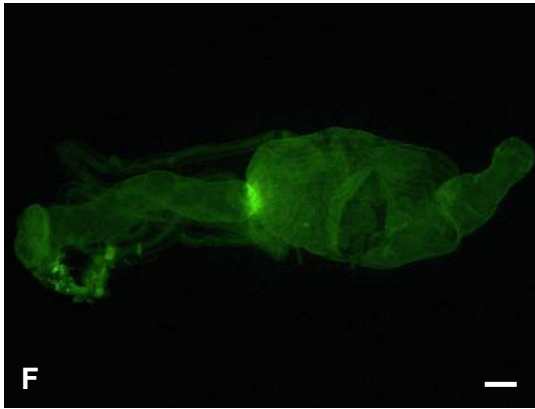
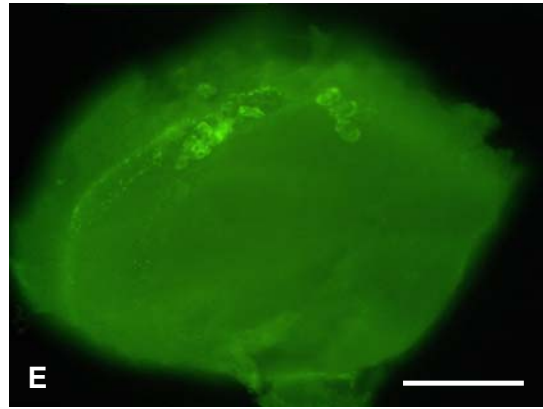
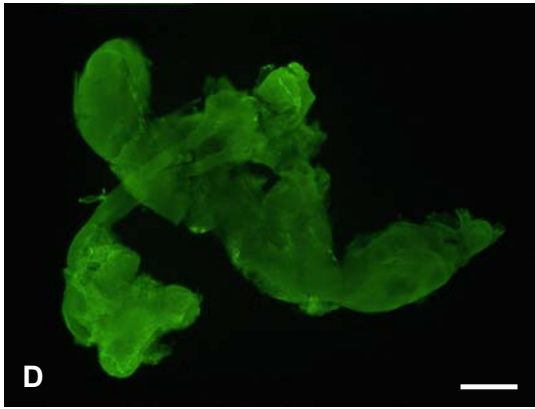


Figure A-I: Localization of AKH-like peptides in whole tissues of female *A. gambiae* (10–15 days old, sugar and blood-fed) by immunocytochemistry with two locust AKH antisera, Locmi-anti-AKH-311E and C (white bar indicates always 100 μ m). Above (antiserum 311E): (A) brain, (B) brain (male), (C) abdominal ganglia Below: (antiserum 311C): (D) brain, (E) optical lobe, (F) fore- and mid-gut, (G) abdominal ganglion, (H) abdominal ganglia, (I) terminal abdominal ganglia.



6.13 AKH-OVIPOSITION EXPERIMENT

A recent report showed that species specific AKH injections result in a change in the ratio of nutritional storage metabolites in eggs of the two-spotted cricket, *Gryllus bimaculatus* (Lorenz, 2003, 2004). In our study, we were interested in whether Anoga-AKH-I or -II have any influence on egg growth. To do this the yolk deposition of females *An. gambiae* was determined by measuring the yolk length, but no significant difference was found.

Oviposition protocol

- *A. gambiae* female, 4 to 7 days post eclosion, access to 10% fructose solution
- Injection of *Aedes*-Saline (AS), Anoga-AKH-I, or -II (different concentrations tested) were given before or/and after the blood meal
- All beheaded females were decapitated immediately after the blood meal
- Yolk deposition (yolk length) was measured 24 h post blood meal

In the pre-experiments, several injections of saline and AKH were given, as described in Lorenz et al. (2003). However, more than one injection of 0.25 μ l, (method described in chapter 3) during a time interval of 6 hours (approx. half-life period of Anoga-AKH-I at 50 pmol at rest) and also 12 hours, was lethal for the mosquitoes. The cricket, *G. bimaculatus* is approximately 10 to 20 times the size of a female mosquito and more robust for undergoing such procedures. We decided to continue using only one injection, but we tried to interfere at different time points during the first 24 hours of the gonotrophic cycle.

Unfortunately, we could not demonstrate a difference in yolk deposition (Table A-II), and after several cohorts, we stopped the experiment. However, further tests could be undertaken to investigate the influence of AKHs on the egg-laying rate, or the storage metabolite ratio in eggs, as Lorenz et al. (2003) demonstrated in crickets. For these kinds of experiments, it is of great importance that the reading conditions are constant at times; and the experimental mosquito females need to be the same size.

Table A-II: Measurement of yolk length (μ m) of *A. gambiae* after injection of *Aedes* saline (AS), or Anoga-AKH-I, or -II (p-BM, post blood meal; b-BM, before blood meal) at different incubation periods. Student's t-tests (Microsoft Office, Excel 2003) were performed for each experiment (Ex-1 to Ex-16) separately, always in correspondence to the AS-injected control sisters.

Injection	Follicle length	Age (d)	N females	Measure time (h); Notes
AS (16 h p-BM)	360 $\pm$ 108	5	5 intact	41, control, Ex-1
AKH-I (100 pmol, 16 h p-BM)	300 $\pm$ 187	5	5 intact	41, ns, Ex-1
AKH-II (100 pmol, 16 h p-BM)	24 $\pm$ 54	5	5 intact	41, females nearly dead, Ex-1
AS (12 h p-BM)	126 $\pm$ 49	6	5 intact	27, control, Ex-2
AKH-I (100 pmol, 12 h p-BM)	86 $\pm$ 22	6	5 intact	27, ns, Ex-2
AKH-II (100 pmol, 12 h p-BM)	126 $\pm$ 71	6	5 intact	27, ns, Ex-2

AS (16 h p-BM)	80 ± 45	5	5 decapitated	41, control, Ex-3
AKH-I (100 pmol, 16 h p-BM)	48 ± 44	5	5 decapitated	41, ns, Ex-3
AKH-II (16 h p-BM)	0 ± 0	5	5 decapitated	41, females nearly dead, Ex-3
AS (12 h p-BM)	68 ± 16	6	5 decapitated	27, control, Ex-4
AKH-I (100 pmol, 12 h p-BM)	50 ± 33	6	5 decapitated	27, ns, Ex-4
AKH-II (100 pmol, 12 h p-BM)	18 ± 25	6	5 decapitated	27, weak nearly dead, Ex-4
AS (12 h p-BM)	147 ± 89	6	10 intact	27, control, Ex-5
AKH-I (100 pmol, 12 h p-BM)	90 ± 71	6	10 intact	27, ns, Ex-5
AS (½ h p-BM)	131 ± 50	6	10 intact	26, control, Ex-6
AKH-I (50 pmol, ½ h b-BM)	63 ± 32	6	3 intact	26, some died, ns, Ex-6
AKH-I (100 pmol, ½ h b-BM)	37 ± 19	6	7 intact	26, p < 0.0001, Ex-6
AS (½ h p-BM)	99 ± 29	7	8 intact	24, control, Ex-7
AKH-I (1 pmol, ½ h b-BM)	40 ± 49	7	7 intact	24, p < 0.03, Ex-7
AKH-I (10 pmol, ½ h b-BM)	42 ± 40	7	5 intact	24, p < 0.03, Ex-7
AKH-I (100 pmol, ½ h b-BM)	25 ± 22	7	6 intact	24, p < 0.0002, Ex-7
AS (½ h p-BM)	70 ± 55	7	6 intact	24, control, Ex-8
AKH-I (0.01 pmol, ½ h b-BM)	98 ± 53	7	6 intact	24, ns, Ex-8
AKH-I (0.1 pmol, ½ h b-BM)	63 ± 39	7	6 intact	24, ns, Ex-8
AKH-I (1 pmol, ½ h b-BM)	62 ± 38	7	9 intact	24, ns, Ex-8
AS (½ h p-BM)	60 ± 59	6	8 intact	24, control, Ex-9
AKH-I (0.05 pmol, ½ h b-BM)	43 ± 34	6	6 intact	24, ns, Ex-9
AKH-I (0.5 pmol, ½ h b-BM)	27 ± 46	6	5 intact	24, ns, Ex-9
AKH-I (5 pmol, ½ h b-BM)	24 ± 33	6	6 intact	24, ns, Ex-9
AS (½ h p-BM)	146 ± 50	1	5 intact	24, control, Ex-10
AKH-I (0.05 pmol, ½ h b-BM)	75 ± 57	1	4 intact	24, ns, Ex-10
AKH-I (0.5 pmol, ½ h b-BM)	30 ± 38	1	4 intact	24, p < 0.005, Ex-10
AKH-I (5 pmol, ½ h b-BM)	133 ± 84	1	4 intact	24, ns, Ex-10
AS (½ h p-BM)	152 ± 23	4	5 intact	24, control, Ex-11
AKH-I (0.01 pmol, ½ h b-BM)	113 ± 70	4	6 intact	24, ns, Ex-11
AKH-I (0.1 pmol, ½ h b-BM)	146 ± 39	4	9 intact	24, ns, Ex-11
AKH-I (1 pmol, ½ h b-BM)	122 ± 64	4	10 intact	24, ns, Ex-11
AKH-I (10 pmol, ½ h b-BM)	80 ± 59	4	10 intact	24, p < 0.005, Ex-11
AKH-I (50 pmol, ½ h b-BM)	96 ± 64	4	8 intact	24, p < 0.05, Ex-11
AKH-I (100 pmol, ½ h b-BM)	80 ± 55	4	7 intact	24, p < 0.01, Ex-11
No injection	94 ± 30	4	10 intact	24, p < 0.001*, Ex-11
AS (½ h p-BM)	140 ± 71	4	7 intact	24, control, Ex-12
AKH-I (0.01 pmol, ½ h b-BM)	173 ± 39	4	7 intact	24, ns, Ex-12
AKH-I (0.1 pmol, ½ h b-BM)	148 ± 51	4	8 intact	24, ns, Ex-12
AKH-I (1 pmol, ½ h b-BM)	135 ± 46	4	8 intact	24, ns, Ex-12
AKH-I (10 pmol, ½ h b-BM)	114 ± 19	4	7 intact	24, ns, Ex-12
AKH-I (100 pmol, ½ h b-BM)	127 ± 41	4	7 intact	24, ns, Ex-12
AS (½ h p-BM)	128 ± 48	4	9 intact	24, control, Ex-13
AKH-I (0.5 pmol, ½ h b-BM)	105 ± 51	4	12 intact	24, ns, Ex-13
AKH-I (5 pmol, ½ h b-BM)	107 ± 49	4	11 intact	24, ns, Ex-13
AKH-I (50 pmol, ½ h b-BM)	94 ± 45	4	10 intact	24, ns, Ex-13

AS (½ h p-BM)	148 ± 59	4	9 intact	24, control, Ex-14
AKH-I (0.5 pmol, ½ h b-BM)	99 ± 58	4	7 intact	24, ns, Ex-14
AS (½ h p-BM)	88 ± 28	6	9 intact	24, control, Ex-15
AKH-I (50 pmol, ½ h b-BM)	26 ± 34	6	9 intact	24, p < 0.0007, Ex-15
AS (½ h p-BM)	142 ± 42	3	6 intact	24, control, Ex-16
AKH-I (0.01 pmol, ½ h b-BM)	127 ± 64	3	12 intact	24, ns, Ex-16
AKH-I (0.1 pmol, ½ h b-BM)	125 ± 63	3	10 intact	24, ns, Ex-16
AKH-I (1 pmol, ½ h b-BM)	112 ± 75	3	10 intact	24, ns, Ex-16
AKH-I (10 pmol, ½ h b-BM)	146 ± 46	3	10 intact	24, ns, Ex-16
AKH-I (100 pmol, ½ h b-BM)	74 ± 49	3	8 intact	24, p < 0.02, Ex-16

*Note: Females with no injections did show significant differences with the AS-injected females

6.14 ADDITIONAL METABOLIC TESTS

6.141 MODIFICATION OF THE NUTRIENT SEPARATION

- (1) Homogenize individual mosquitoes in 100 µl of 4% NA₂SO₄-solution (12 x 75 mm glass tube, Tube A)
- (2) Add 1.6 ml CHCl₃-MeOH and vortex
- (3) Centrifuge at max speed for 10 min
- (4) Transfer supernatant into Tube B and keep the pellet (Glycogen and soluble Protein in Tube A)
- (5) Add 0.6 ml H₂O into Tube B (12 x 75 mm glass tube), vortex and centrifuge for 10 min at max speed
- (6) Pipette upper phase (take just 0.5 ml aliquots) into 1.5 ml plastic Tube C (soluble Sugars) and keep the organic phase in Tube B (Lipid)
 - Tube A: Glycogen (and soluble Protein) – Hot anthrone test
 - Tube B: Lipid – Vanillin test
 - Tube C: soluble Sugars – Cold or hot anthrone test

To prove the metabolic test worked, known quantities mix of lipid, glycogen and glucose were tested and compared with the standard (Figure A-II, -III, IV).

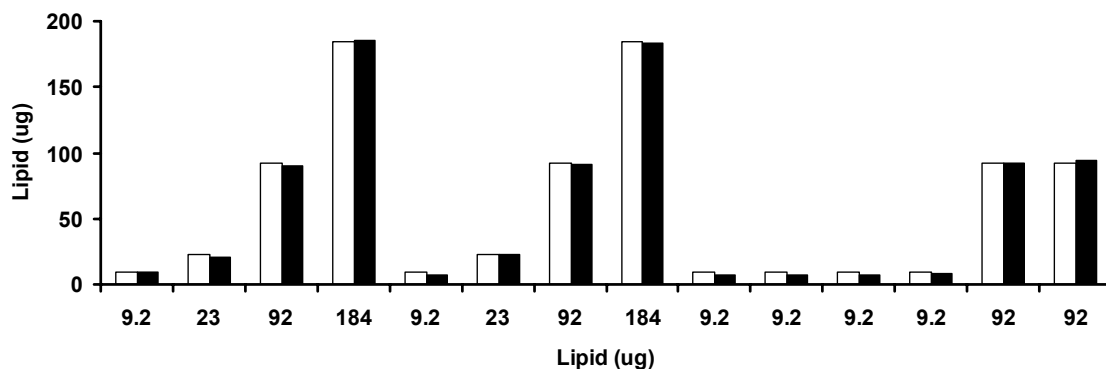


Figure A-II: Vanillin tests with known lipid amounts. The x-axis demonstrates the initial added amount and the y-axis the measured results (black bars). The standard amounts are demonstrated by the white bars.

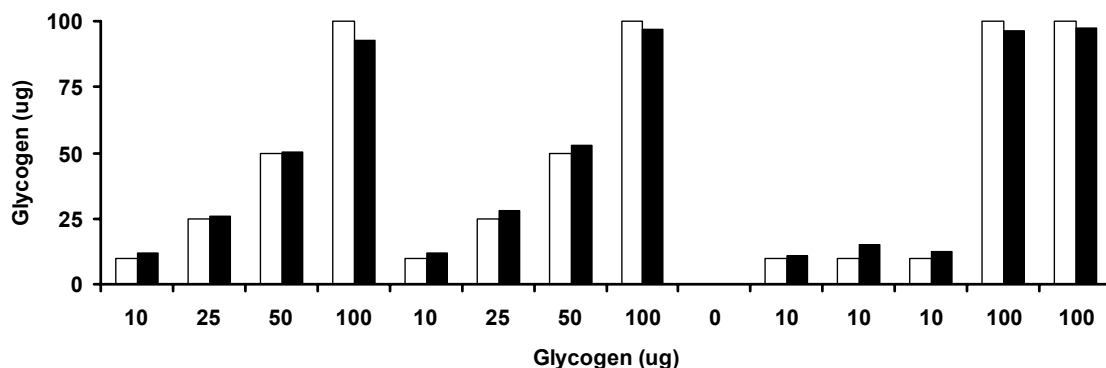


Figure A-III: Hot anthrone tests with known glycogen amounts. The x-axis demonstrates the initial added amount and the y-axis the measured results (black bars). The standard amounts are demonstrated by the white bars.

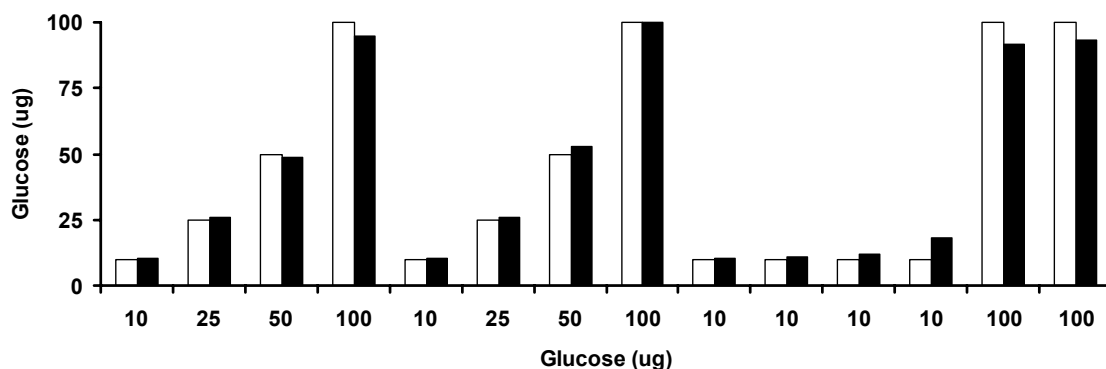


Figure A-IV: Hot anthrone tests with known glucose amounts. The x-axis demonstrates the initial added amount and the y-axis the measured results (black bars). The standard amounts are demonstrated by the white bars.

6.142 AKH INJECTION OF DECAPITATED MOSQUITOES

Female *A. gambiae* reached maximal levels for lipid and carbohydrate reserves after 3 to 4 days continuous access to 10% sugar solution, kept at 27° C and 60 – 85% relative humidity (Kaufmann and Briegel, 2004; Fernandes and Briegel, 2005). Females of this age were used for the first pre-tests with the modified biochemical analysis of Van Handel and Day (1988). Initially, we worked on achieving optimal conditions for the peptide injections. The first findings showed that only injection of Anoga-AKH-I produced a metabolic effect, later called a hypertrehalosaemic effect (chapter 3).

Considering the source of AKHs was either in the brain or the corpora cardiaca (see immunocytochemistry, chapter 2), we tested the effect of AKH injections on decapitated females. We were hoping to achieve a more intense hypertrehalosaemic effect by injecting Anoga-AKH-I, but to our surprise Anoga-AKH-I, nor -II demonstrated any significant changes on glycogen or total lipid levels (Figure A-V).

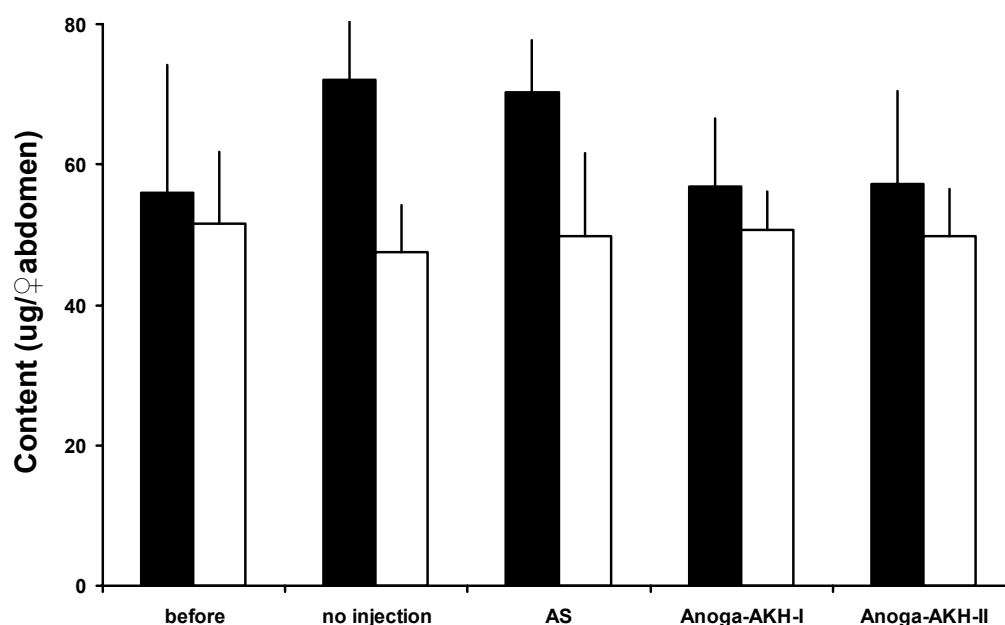


Figure A-V: Glycogen (black bars) and total lipid (white bars) levels in abdomen of 4 day-old female *A. gambiae* (10% fructose-fed) after decapitation. Injections of saline or Anoga-AKHs were done after one hour healing. Nutrient levels of females before decapitation (before) and 2.5 h after (no injection) are shown; in addition saline (AS), Anoga-AKH-I, and -II (50 pmol) treated females are compared (1.5 h incubation time). No significant differences was found (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 2 cohorts, $n = 8/\text{cohort}$, two abdomens were pooled together for analysis).

Van Handel and Lea (1970) showed, in female mosquitoes, an increase of glycogen and a decrease of lipid levels after removing the median neurosecretory cells or decapitation.

The Anoga-AKH-I injection was probably not strong enough to compete with this hormonal related change after decapitation; nevertheless, injections of higher concentrations also did not alter the result (data not shown).

In an additional series of experiments, we demonstrated the predicted increase for glycogen after decapitation of female *A. gambiae* (Figure A-VI).

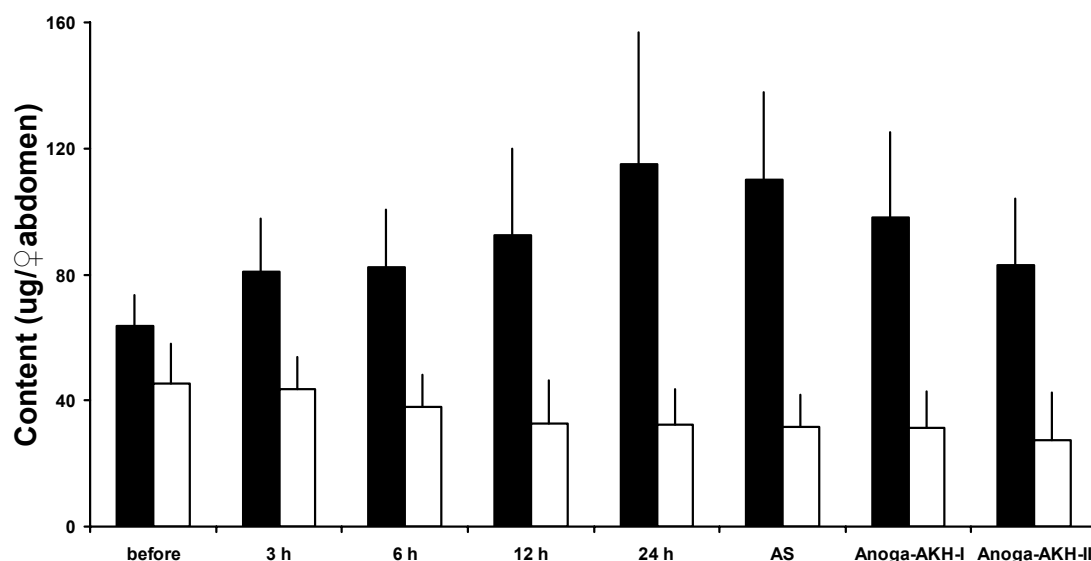


Figure A-VI: Profile of glycogen and total lipid levels in abdomina of 4 day-old female *A. gambiae* (10% fructose-fed) for the first 24 hours after decapitation. Injections of saline or Anoga-AKHs were done 24 after decapitation. Nutrient levels (glycogen in black and lipid levels in white bars) of females before decapitation (before) and at different time points are illustrated (3, 6, 12, and 24 h after decapitation). In addition saline (AS), Anoga-AKH-I, and -II (50 pmol) treated females are compared (1.5 h incubation time). No significant differences were found, but a clear trend of the increasing glycogen levels was observed (Tukey-Kramer HSD test, $p \leq 0.05$; $M \pm S.E.$; 1 cohort, $n = 8$).

The small decrease in the total lipid levels is probably due to the nutritional use of the basal metabolism (Note that in decapitated mosquitoes there is no external nutritional source). Injection of Anoga-AKH-I and -II 24 hours after decapitation did not affect glycogen or lipid levels.

Experiments with different concentrations of both AKHs were also unsuccessful, even decapitated females with a blood meal prior to decapitation did not show any effect from the species specific AKHs. In summary, we decided that decapitation was not the tool to maximize the conditions for an optimal response after AKH injection.

6.143 ANALYSIS OF THE HYPERTREHALOSAEMIC EFFECT

Only treatment with Anoga-AKH-I produced a metabolic effect, and as mentioned above, a decrease of glycogen was observed. To prove the existence of a hypertrehalosaemic effect, an increase in the level of haemolymph-sugar is required. The first experiments showed a large variation in the amount of sugar found in the females. It appeared that females had different amounts of sugar in their crop. To overcome this problem we starved (access to water only) the females for 24 hours until the crop was empty. Two pre-tests were done to prove that the crop was empty after 24 hours. In the first experiment, 2 day-old females with access to water only, were fed with 10% fructose for 2 h. Only the females with a full crop were tested in the experiment, in which we analysed the fructose in the crop, the sugar in the haemolymph, the glycogen, and the total lipid. The methods for sugar, glycogen and total lipids were described above, for analysing the amount of fructose, the cold anthrone technique was used (Van Handel, 1967). The fructose and total sugar were measured from the same sample. At first, the cold anthrone was used for measuring the amount of fructose, and after that, the hot anthrone was used with the same sample. Using this method we obtained the amount of fructose and total sugar. Standards were done for both. The subtraction of the fructose (sugar in crop) from the total sugar resulted in the amount of haemolymph sugar. After 12 hours all of the fructose appeared to have been digested (Figure A-VI). For the second experiment, 4 day-old females, that were kept for 3 days on 10% fructose and 1 day with access to water only, were tested, and after 24 hours the fructose level was negligible at $1.05 \pm 0.25 \mu\text{g}$.

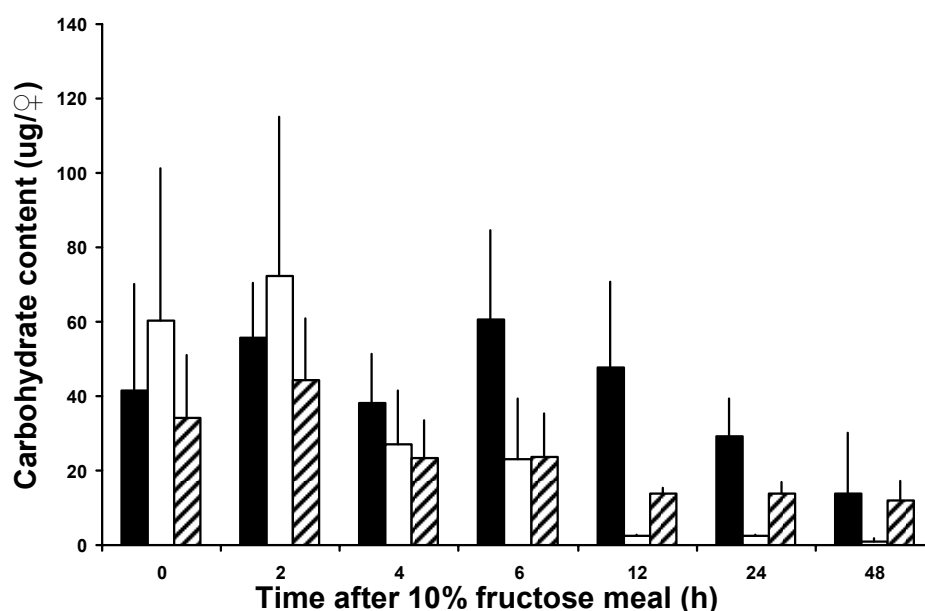


Figure A-VII: Content of glycogen (black bars), crop sugar (fructose, white bars), and haemolymph sugar (bars with diagonal lines) levels of 4 day-old female *A. gambiae* (3 d fructose-fed, 1 d with access to water). After 12 hours the crop of most females was empty ($M \pm S.E.$; 1 cohort, $n = 5$).

After these series of experiments, we tried to test the hypertrehalosaemic effect on 4 day-old females, sugar fed for 3 days and the fourth day with access to water only. Unfortunately, most females were positive for fructose after 24 hours access to water only. We decided to change our strategy.

Another technique we used to measure the haemolymph sugar was to let the mosquitoes bleed into saline (method described in chapter 3). After the right adjustments, this *in vitro* experiment was successful. At the beginning, we incubated three to five abdomens and thoraces in saline and in saline treated with Anoga-AKH-I and -II for up to 4 hours. We demonstrated a hypertrehalosaemic effect, but after one hour incubation the glycogen levels increased as well, probably due to the ongoing damage of the incubated cells (Table A-III). We adjusted the experiment and simply let the females bleed for 5 - 10 min on ice by a small opening at the abdomen 1.5 h post injection. Using this strategy, we could demonstrate the hypertrehalosaemic effect of Anoga-AKH-I.

At the same time, we also tried to find another way to bypass the fructose contamination. The key was to use blood-fed females, which only had access to water. In chapter 3, the decrease in glycogen and increase in sugar can be seen.

Table A-III: Content of glycogen at different time points after *in vitro* incubation. Three *A. gambiae* abdomens were incubated in *Aedes* saline, with the opening on the surface.

Time point	30 min	60 min	120 min	180 min
Glycogen content (μg)	1.15	5.23	2.97	5.84

6.15 AKH TITRE IN HAEMOLYMPH

After the experiments where we extracted and detected Anoga-AKH-I and -II in the head and thorax, but not in the abdomen (Kaufmann and Brown, 2006), we were interested in isolating the peptides from the haemolymph to prove their presence in the body fluid, and therefore their function as a (neuro-) hormone.

This following procedure explains how the AKH titre of un-flown and flown female *A. gambiae* was obtained. The principle of the test was simple: haemolymph was taken from resting and flying females. The resting females were held in a cage under a black towel, whereas the flying sisters were in a cage on a shaker. To acquire the haemolymph the last two segments of the abdomen were ripped off and up to six females bled into *Aedes* saline on ice (5 - 10 min, the bleeding technique is described in more detail in chapter 3). The saline was treated with a protease inhibitor (0.02 M acetic acid with protease inhibitor, Roche mini tablets) and always kept on ice. After collection of the haemolymph, the ‘rested’ and ‘flown’ samples were freeze dried and stored at -20° C. Each sample was mixed with an equal volume (800 μl) of the initial mobile phase B, injected onto a C8 column (Alltech Macrosphere 300, 7 mm 300 Å matrix, 250mm x 4.6 mm) and eluted with a CH₃CN gradient (mobile phase A: 0.1% TFA in water; mobile phase B: same with 80% CH₃CN; gradient of 15-40% B over 40

min, and 45-100% B over 5 min; flow rate of 1 ml/min; and monitored at $\lambda = 210$ nm). Fifty fractions were collected, of which the first 30 were centrifuged in a speed-vacuum centrifuge and freeze-dried for the AKH radioimmunoassay (material and method for HPLC and RIA, see chapter 2). Prior to the haemolymph samples, a background run was performed and lastly synthetic Anoga-AKH-I and -II (each 3 μ l of a 1 μ g/ μ l stock peptide) were injected together.

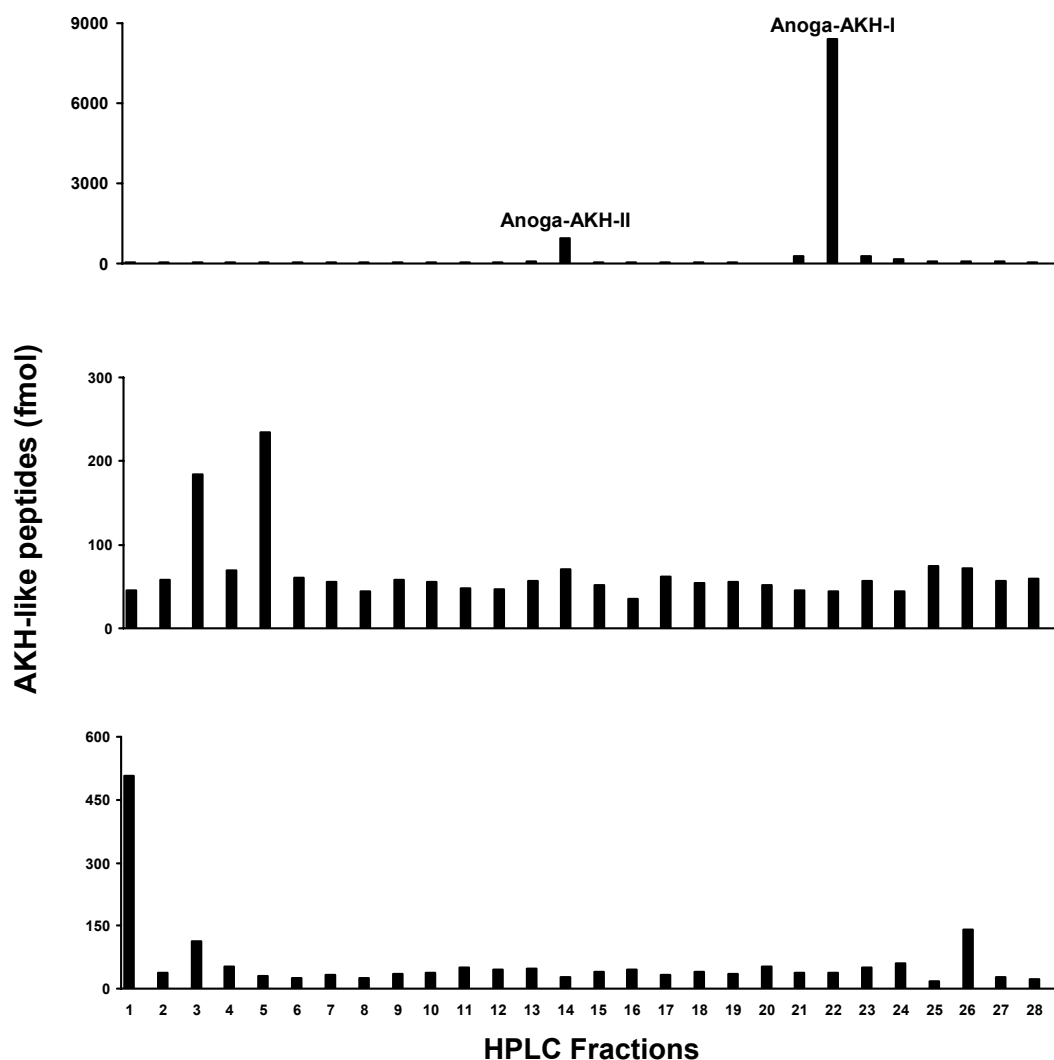


Figure A-VIII: Separation of Anoga-AKH-I and -II-like peptides in haemolymph extracts of rested and flown female *A. gambiae* (2 days old, sugar-fed) subjected to reversed-phase HPLC and RIA. Top panel illustrates quantities of AKH immunoreactive material detected by radioimmunoassay of fractions from HPLC separation of synthetic Anoga-AKH-I and Anoga-AKH-II. In the second and third panel the same as illustrated above, HPLC separation of rested and flown samples respectively. In the last two panels, immunoreactive material per fraction represents the haemolymph of approximately 45 females.

In the first experiment haemolymph of 90 females, and in the second, 180 females for each sample were tested. The run for the synthetic Anoga-AKHs peaked at fraction 14 (Anoga-AKH-II) and fraction 22 (Anoga-AKH-I), AKH presence was proved by RIA. The corresponding fraction in the rested and flown samples did not show any elevation in bound over free, in the RIA (Figure A-VIII). However, binding was detected in fraction 1, 3, and 5, which could be the pro-hormone form of the AKH, but as far as it is known, only the active AKH should be found in the haemolymph.

The experiment with the rested and flown females did not work out as predicted. The problem was that there was no AKH detected at all. Next, a trial with blood-fed females was tested. Before collecting haemolymph for the HPLC separation, RIA was done directly on the haemolymph at different stages during the gonotrophic cycle. Haemolymph was collected from 1, 2, and 3 day-old sugar-fed females (20 individuals each). After three days access to sugar, a blood meal was given and haemolymph was taken immediately 6, 12, 24, and 48 h post blood meal, and after oviposition. In Figure A-IX an increase of AKH during the first few days and after the blood meal is demonstrated. This increase decreases between 6 and 12 hours after the blood meal to the same amount that is measured after 2 days of sugar. After successful repetition of this experiment, we decided to investigate females between 3 and 6 hours after the blood meal.

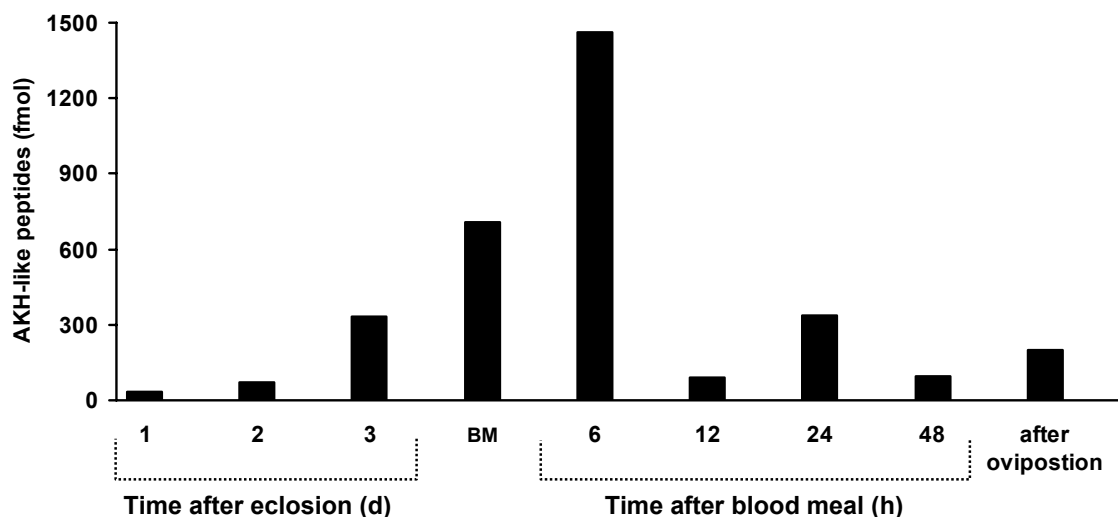


Figure A-IX: Anoga-AKH-like peptides in haemolymph extracts of female *A. gambiae* (sugar- and blood-fed). Black bars illustrate quantities of AKH immunoreactive material detected by radioimmunoassay of the haemolymph of 20 females. Blood meal (BM) was given 3 days post eclosion.

For the HPLC separation, haemolymph of 300 females were collected. The procedure was the same as described above. Alas, nothing was detected for the representative fractions where the Anoga-AKHs were expected (Figure A-X), but again, just like the rested and flown

haemolymph fractions, immunoradioactivity was found in fraction 5. After the failure of these experiments, we stopped trying to acquire the AKH-titre in the haemolymph.

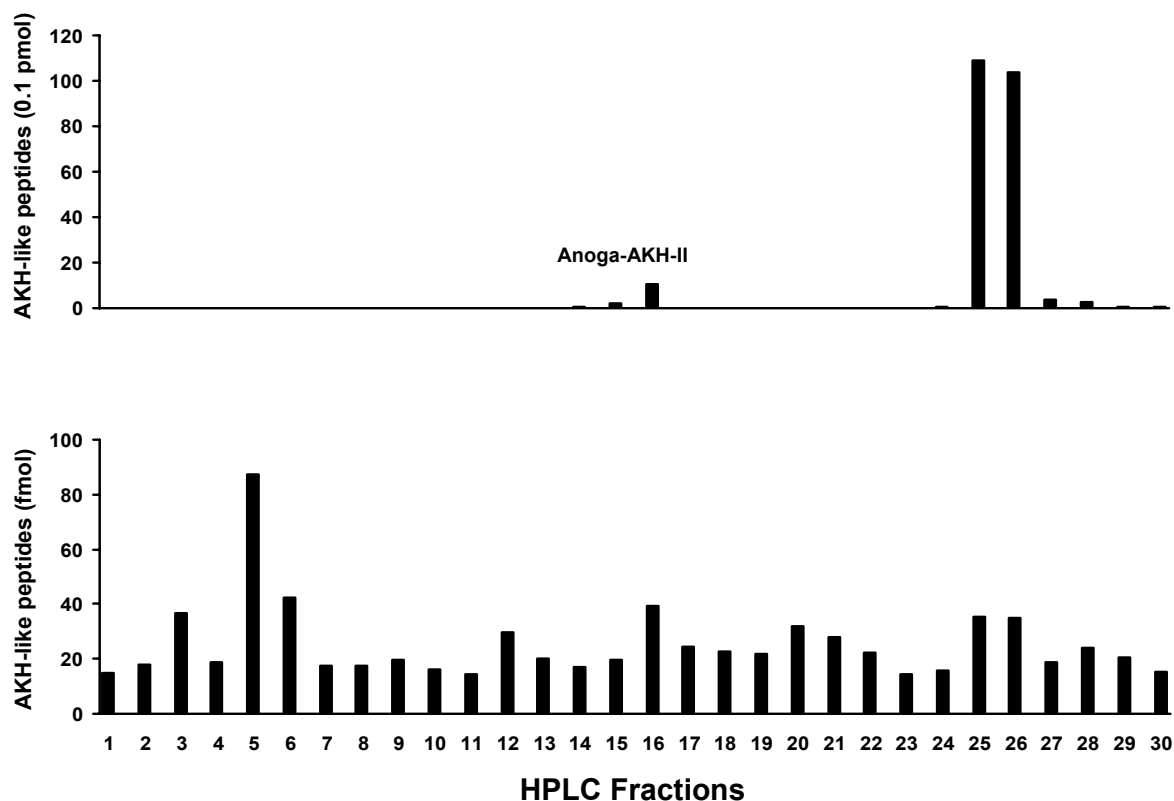


Figure A-X: Separation of Anoga-AKH-I and Anoga-AKH-II-like peptides in haemolymph extracts of blood-fed female *A. gambiae* (3 days old, sugar-fed and 3 to 6 h after the blood meal given) subjected to reversed-phase HPLC and RIA. Top panel illustrates quantities of AKH immunoreactive material detected by radioimmunoassay of fractions from HPLC separation of synthetic Anoga-AKH-I and Anoga-AKH-II. In the second panel the same as illustrated above, HPLC separation of the haemolymph sample from the blood-fed females. Immunoreactive material per fraction represents the haemolymph of approximately 150 females.

6.16 RECEPTOR BINDINGS

Kaufmann and Brown (2006) predicted a G-protein coupled AKH receptor for *A. gambiae* (Anoga-AKHR). As for the adipokinetic peptides, a prediction was made possible using BLAST as a search tool. AKHRs are known for *M. sexta* (Ziegler et al., 1995), *B. mori* (Staubli et al., 2002), *D. melanogaster* (Park et al., 2002; Staubli et al., 2002), and *P. americana* (Hansen et al., 2006; Wicher et al., 2006). Predictions were done for the honey bee, *Apis mellifera* (Consortium, 2006), the red flour beetle, *Tribolium castaneum* (Hansen et al., 2006) and *A. aegypti* (GenBank: [EAT36594](#)), for more details see Discussion and Kaufmann and Brown (2006).

To prove that the predicted Anoga-AKHR really is the receptor for the AKH, ligand binding to the receptor must be demonstrated. For this procedure, the genomic message had to be transferred from the mosquito genome into the genome of a mammalian cell line (in our case, Chinese hamster ovary (CHO) cells), which were then used for the binding studies. At first, total RNA was extracted from abdomina of 5 day-old sugar-fed female *A. gambiae*, copied into cDNA and cloned into a vector with the desired restriction sites. This procedure is described in Kaufmann and Brown (2006), the only divergence is the use of a different vector (pCR[®]2.1-TOPO II; Invitrogen). Ten positive *Escherichia coli* colonies were picked and tested for the insertion and its direction. For this, a reverse-transcriptase polymerase chain reaction (RT-PCR) with M13 reverse primer (3'-CAG GAA ACA GCT ATG AC-5') at the 3'-end as forward and Anoga-AKH rev (5'-GGA GCG TTA GTA ACA TGG AAT GAA GTG-3') at the 5'-end as reverse primer ($T_a = 55^\circ \text{C}$, program and method described in chapter 3.1; Kaufmann and Brown, 2006). PCR products were separated on 1.5% agarose gels (Bio Rad). After the proof that the receptor insert was in the right direction in the plasmids, the AKHR needed to be excised from the pCR[®]2.1-TOPO II vector and transferred into a vector that could transfect mammalian cells. Using the two restriction endonucleases BamHI and XbaI, the AKHR insert was cut out and ligated into the mammalian expression vector pcDNA3.1+ (Invitrogen). The restriction enzymes were from Promega and the suitable buffer can be found at http://www.promega.com/guides/re_guide under 'compatible buffers'. For the digestion, 20 μl plasmid, 20 μl nanopure water, 5 μl of 10x buffer E, and 2.5 μl of each restriction enzyme was used. This total 50 μl reaction was held for 3 h at 37°C and the product was separated on 1.5% agarose gels (Figure A-XI).

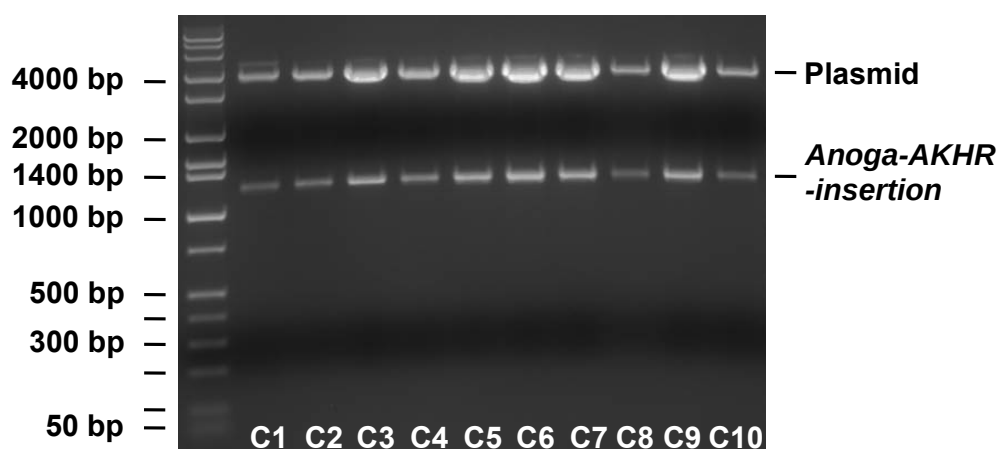


Figure A-XI: Profile of plasmid (pCR[®]2.1-TOPO II) and insert after digestion by two restriction enzymes (Bam-HI and Xba-I). Ten plasmids were cloned (P1-P10). The band on top shows the plasmid (3837 bp) without the Anoga-AKHR insertion (1204 bp), which is found in the lower band. The whole gel is presented to illustrate the clear digestion.

The bands with the AKHR insert were cut out of the agarose gel and cleaned with Sigma Gen elute columns. 10 µl of each product was sent for sequencing at Integrated Biotechnology Laboratories (IBL) at the University of Georgia, Athens GA, USA and the rest was used for the ligation into the mammalian expression vector. We used 1 µl of vector, 5 µl of AKHR insert, 2 µl of nanopure water, 1 µl of 10x ligation buffer, and 1 µl of ligase. The ligations were performed overnight at 4° C. This cDNA construct was used for transfecting CHO-K1 cells (American Type Culture Collection, CCL-61) according to the protocol for six well tissue culture plates using LipofectAMINE reagent (Life Technologies Inc.). Briefly, CHO-K1 cells were grown in RPMI-1640 media (Fisher) containing 1mM pyruvate (Fisher) and 10% fetal bovine serum (FBS; Fisher Scientific) at 37° C with 6% CO₂ in six well tissue culture plates to a density of approximately 75% confluency. DNA complexes were formed by adding LipofectAMINE reagent to the cDNA constructs (1 µg) diluted in RPMI-1640 media. The DNA-LipofectAMINE reagent complexes were added to the CHO-K1 cells containing RPMI-1640 media and incubated for 3 h at 37° C with 6% CO₂. After 3 h, RPMI-1640 media containing 1mM pyruvate and 10% FBS was added, and the cells were incubated for 48 h at 37° C with 6% CO₂. For stable expression of Anoga-AKHR, the transfected CHO-K1 cells were selected for 3 weeks, with 800 µg/ml G418 (Fisher) added to the tissue culture media. Expression of Anoga-AKHR cDNA in CHO-K1 cells was determined by RT-PCR as described above.

Analysis of AKH Binding to Anoga-AKHR

With a radio-receptor approach, we tried to determine if the Anoga-AKHR is a functional receptor for the Anoga-AKHs. The membranes from the Anoga-AKHR transfected CHO-K1 cells should express the potential *A. gambiae* AKH-receptor cDNA. By adding radiolabeled ¹²⁵I-Anoga-AKH (the same as used in Kaufmann and Brown, 2006) and unlabelled Anoga-AKH at different concentrations, the binding of the labelled AKH should be displaced. The procedure works with the same principle as is described for the RIA above. Unfortunately, no binding was achieved by the iodide-labelled AKH: the reason for the lack of binding was probably due to the iodide being responsible for a conformational change and the labelled AKHs were not able to bind to the AKHR.

Another group identified this receptor as a real Anoga-AKHR during the same work period (Belmont et al., 2006), which led us to the decision to stop this experiment. They used a different technique, where the binding of native AKH on the AKHR (also in mammalian cell line) induces a bioluminescent reaction. However, additional experiments with the Anoga-AKHR demonstrated that this receptor is responsible for the hypertrehalosaemic effect in *A. gambiae* and showed indirectly that this receptor must be involved with the Anoga-AKH-I.

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