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Five functional adipokinetic peptides expressed in the corpus cardiacum of the moth genus *Hippotion* (Lepidoptera, Sphingidae)

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ABSTRACT

This is the first study that finds five adipokinetic hormones (AKHs) in the corpus cardiacum of an insect. From two species of the sphingid moth genus *Hippotion*, *eson* and *celerio*, three novel and two known AKHs were isolated and sequenced by deduction from multiple MS^n electrospray mass data: two octapeptides are pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp amide (denoted Hipes-AKH-I) and its Thr^7 analogue (Hipes-AKH-II); two nonapeptides are pGlu-Leu-Thr-Phe-Thr-Ser-Ser-Trp-Gly amide (Manse-AKH) and its Thr^7 analogue (Hipes-AKH-III), as well as a decapeptide pGlu-Leu-Thr-Phe-Ser-Ser-Gly-Trp-Gly-Gln amide (Manse-AKH-II). All sequences were confirmed by identical behaviour of natural and synthetic peptides in reversed-phase HPLC and liquid chromatography coupled to electrospray mass spectrometry, resulting in identical retention times and tandem mass spectral data. High resolution mass spectrometry and retention time data also confirmed that the amino acid at position 10 in Manse-AKH-II is Gln and not the isobaric Lys. Conspecific injections of all five peptides in synthetic form and low doses caused hyperlipaemia in *H. eson*. Our results and pertaining literature suggest that five genes code for the mature peptides, which are very likely released during flight to provide energy for long distance migration in this genus via lipid oxidation; as all five peptides are active at low doses in a conspecific bioassay, it may be speculated, but not proven, that there is only one AKH receptor present in *Hippotion* that can bind all five peptides with high affinity.

1. Introduction

Insects can show impressive flight performances: either as short, intermittent powerful flights lasting up to a few minutes, or as long-distance migratory flights of several hours. A number of moths of the family Sphingidae are a prime example of such migratory flights. Their bodies have a streamlined form, they have powerful, narrow and pointed forewings contracted by a strong and large thoracic muscle mass. Their flight is fast and straight, but most are also able to hover and take in nectar via their long proboscis [1]. In Europe, for example, at least 9 species of Sphingidae are surmised to be migratory, viz. death's head hawk moth, *Acherontia atropos*, convolvulus hawk moth, *Herse convolvuli*, oleander hawk moth, *Daphnis nerii*, striped hawk moth, *Hyles livornica*, hummingbird hawk moth, *Macroglossum stellatarum*, spurge hawk moth, *Hyles euphorbiae*, bedstraw hawk moth, *Hyles galii*, pine hawk moth, *Hyloicus pinastri*, and the silver-striped hawk moth, *Hippotion celerio* [2,3]. In South Africa two species of the genus *Hippotion* occur: the common striped hawk moth, *H. eson*, and *H. celerio*. The common striped hawk moth has been described as a good hovering flyer [4]. In the laboratory, tethered hovering flights of 15 min duration can easily be induced [5]. During such a flight episode the lipid concentration in the haemolymph was significantly diminished from a high resting value of about 37 mg ml^{-1} to about 21 mg ml^{-1}; during a subsequent rest period of 15 and 45 min respectively, lipid levels reached the pre-flight niveau or were even 20% higher respectively [5]. In contrast, although the concentration of carbohydrates in the haemolymph decreased from about 19 mg ml^{-1} to about 11 mg ml^{-1} in 15 min, no significant restoration of the pre-flight levels of carbohydrates occurred during 45 min of recovery after flight [5]. In conjunction with the known haemolymph volume of the striped hawk moth, it was calculated that the contribution of carbohydrates is negligible to energy provision during flight, and lipids are the main fuel component for flight [5]. The whole scenario is reminiscent of another sphingid, the model lepidopteran species, i.e. the tobacco hornworm moth, *Manduca sexta* [6,7].

Since insects store only small amounts of energy in their haemolymph or flight muscles which are depleted after a few minutes of intense flight, substrates for oxidation by the contracting flight muscles in the thorax must be replenished by mobilisation of stored fuel components in the fat body. Small peptide hormones of the so-called adipokinetic hormone (AKH) family, which are the most abundant peptides in the corpus cardiacum (CC) neurohaemal organ in insects, control these mobilisation

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processes (reviewed by [8]). This peptide family of about 60 known members is structurally recognised by mostly being non-charged peptides of a chain length of 8 to 10 amino acids with characteristically post-translationally modified termini (pGlu at the N-terminus; carboxyamide at the C-terminus), aromatic amino acids at position 4 (Phe or Tyr) and 8 (Trp), and a Gly residue at position 9 in the longer peptides (reviewed by [9]).

For *M. sexta* and other sphingid moths, such as the large elephant hawk moth, *Deilephia elpenor*, poplar hawk moth, *Laothoe populi*, eyed hawk moth, *Smerinthus ocellata*, and *A. atropos* it was recently shown by mass spectrometric analyses that these sphingids all contain not only the nonapeptide Manse-AKH (pELTFTSSWG amide) in their CC but also a second peptide which was identified as a decapeptide denoted Manse-AKH-II (pELTFSSGWGQ amide) [10]. For *H. eson*, we have only preliminary results on its endogenous AKHs from a previous study [5]: data on bioactivity plus the comparison of retention times of active fractions (after chromatography) with those of then-known AKH family members, suggested that the common striped hawk moth contains Manse-AKH and a second AKH which is possibly identical to Helze-HrTH (pELTFSSGWGN amide), i.e. a peptide isolated and previously sequenced from the CC of the noctuid corn earworm moth, *Heliothis zea* [11].

The current investigation unravels the amazing complement of five AKHs in the CC of both *Hippotion* species: one decapeptide, two nonapeptides and two octpeptides, of which three are novel members of the AKH family. In addition, it is established that all five variants are biologically active and have a hyperlipaemic function. From these facts a number of interesting questions follow about number of genes and receptors.

2. Material and methods

2.1. Insects

Different larval stages (mostly 2nd to 4th) of the common striped hawk moth, *H. eson*, were caught on the property of the University of Cape Town (Rondebosch, Cape Town, Western Cape Province of South Africa) and in private gardens in the suburb of Mowbray, and in Lotus River on the Cape Flats of Cape Town during the austral winter months May to July when feeding on the leaves of arum lilies (*Zantedeschia aethiopica*). All stages were kept in insect cages in a constant temperature room at the University of Cape Town's Biological Sciences Department at 27 °C, 62% relative humidity and under a 14 h light:10 h dark photocycle regime; larvae were fed daily with fresh leaves of arum lilies. Pupae were kept under the same conditions until adult emergence, which took place about 17 days after pupation. After emergence, it became clear that occasionally adults of the sister species *H. celerio* had emerged; these adults were kept separately from *H. eson* and their corpora cardiaca (CC) were separately dissected and analysed by mass spectrometry (see Section 2.4 and Results) since it is known from other genera that species-specific AKH forms occur, such as in the genus *Phymateus* [12,13] and *Tribolium* [14]. Adult moths of both sexes were used during the first two days after eclosion (90% actually after the first day) for either dissection of CC (see Section 2.2) or biological assays (see Section 2.3).

Adult male locusts, *Locusta migratoria*, were purchased from a commercial dealer and maintained as previously outlined [15].

2.2. Tissue preparation and peptide isolation

The CC of *H. eson* were dissected with the aid of a stereomicroscope and iridectomy scissors and freed from all contaminating muscle and fat tissue. The dissected CC material was placed into 80% methanol, and crude extracts were prepared by sonication and then evaporated to dryness as described previously [16]. Aliquots of such a dried extract, equivalent to 8 pairs of CC, were sent for mass spectrometric measurement (see Section 2.4). This was also done for extracts prepared from one pair of CC that had been dissected from either *H. eson* or *H. celerio* individual moths. Other aliquots of the dried CC extracts were used for the purification of the active peptides on reversed-phase high performance liquid chromatography (RP-HPLC) as outlined previously [17], for comparing retention times on RP-HPLC of natural and synthetic peptide material (see Section 2.4), and for heterospecific bioassays to monitor the function of the peptides (see Section 2.3).

2.3. Bioassay

Bioassays in locusts were performed as described previously [15]. Fractions after RP-HPLC (see Section 2.4) were collected, dried in a vacuum centrifuge and subsequently reconstituted in 100 µl of distilled water. An aliquot (10 µl, which represented the amount of maximally 2 gland equivalents) was injected into 6 to 8 locusts (see also Fig. 1).

Adult *H. eson* specimens of both sexes that had emerged during the night were used for biological assays on the following day; only occasionally (about 10% of the cases) were adults used on the 2nd day post-emergence. Individual moths were kept under a funnel at 19 to 21 °C for at least one hour prior to experimentation to ensure resting conditions. The scales on the mid-line of the abdomen were gently removed with tissue paper to reveal the abdominal dorsal vessel, which is clearly visible as a green line. Haemolymph (0.5 µl) was sampled with a glass microcapillary from this blood vessel and the content of the capillary was blown into a test tube containing 100 µl of concentrated sulphuric acid. The moth was then injected ventro-laterally into the abdomen with 3 µl of either a control water solution, or

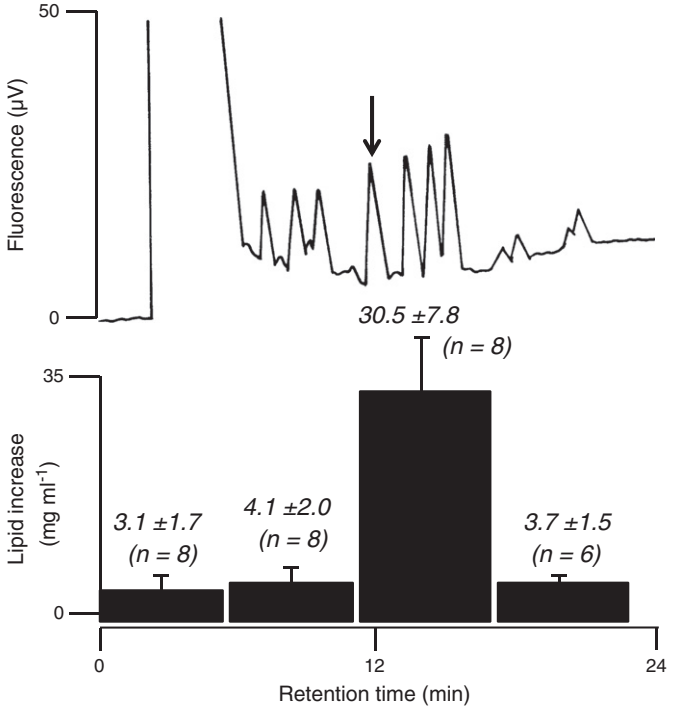

Fig. 1. RP-HPLC separation of a crude extract of hawk moth CC extract and biological activity of collected fractions. Reversed-phase high performance liquid chromatographic separation of material in a methanolic crude extract of 20 *Hippotion eson* corpora cardiaca applied to a Nucleosil 100 C-18 column, monitored by fluorescence (excitation at 276 nm and emission at 350 nm). The column was developed with a linear gradient of 0.01% trifluoracetic acid (TFA) in water (solvent A) and 0.1% TFA in 60% acetonitrile (solvent B) from 43% to 53% B in 20 min at a flow rate of 1 ml min^{-1}. The arrow represents the retention time of synthetic Manse-AKH on this chromatographic system. Fractions were collected during the separation process, dried by evaporation and then reconstituted in water for injection into resting *Locusta migratoria* at a dose of 2 gland equivalents. The difference in lipid concentration in the haemolymph (mg ml^{-1}) was measured before, and 90 min after injection of the fractions and are shown in the bar graph.