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The first decapeptide adipokinetic hormone (AKH) in Heteroptera: A novel AKH from a South African saucer bug, *Laccocoris spurcus* (Naucoridae, Laccocorinae)

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ABSTRACT

A novel peptide of the adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) family has been elucidated by mass spectrometry from the corpora cardiaca of an African saucer bug species, *Laccocoris spurcus*. It is the first decapeptide member found in the species-rich taxon Heteroptera, has the primary sequence pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-Gly amide and is denoted as Lacsp-AKH. The first eight amino acids are identical to the octapeptide Anaim-AKH of the European saucer bug, *Ilyocoris cimicoides*. The synthetic peptide Lacsp-AKH elevates lipids upon injection into the hemolymph of *L. spurcus* at a low dose of 3 pmol. Swimming activity in this saucer bug also causes a significant increase in the lipid concentration in the hemolymph. Thus, both results point to an apparent function of the endogenous new decapeptide Lacsp-AKH in *L. spurcus*, namely, to regulate lipid mobilization. Isolation of an AKH peptide from the corpora cardiaca of the water bug *Aphelocheirus aestivalis* (Aphelocheiridae) resulted in the assignment of the octapeptide Anaim-AKH, supporting current phylogenies on the infraorder Nepomorpha.

1. Introduction

Adipokinetic hormones are among the most abundant neuropeptides in most insect orders (see reviews [4,6]). These octapeptides to decapeptides are synthesized in intrinsic neurosecretory cells of the retrocerebral corpora cardiaca (CC) glands. Structurally they are quite diverse but all share an aromatic amino acid at position four from the N-terminus (Phe or Tyr) and at position eight (Trp) and have the post-translational modifications at the N-terminus, a pyroGlu residue, and a carboxyamide at the C-terminus (see [6]). Further unique modifications are a C-mannosylation at the Trp residue [10,19] and a phosphorylation at the Thr residue at position 6 [12] in some members of the large AKH family. Functionally, these peptides are mostly known for their action to regulate the mobilization of stored energy substrates such as triacylglycerols, glycogen and proline during strenuous physical exercise (flight, swimming) when the demand for energy is too high to be met by readily available energy-rich phosphates. It becomes more clear, however, that AKHs can be regarded as general regulators of homeostasis of energy metabolism, for example, during egg production, larval growth and molting (reviewed by [18]).

Heteroptera is the largest suborder of the most species-rich exopterygote order Hemiptera. Although we have studied the structure of AKHs from quite a number of "water bugs" (infraorder: Nepomorpha) such as the backswimmer *Notonecta glauca* [7], the water scorpions *Nepa cinerea* and *Ranatra linearis* [13], the giant water bug *Lethocerus indicus* [13], the water boatman *Corixa punctata* [14] and the saucer bug *Ilyocoris cimicoides* [14], these have been restricted to European species. In the current investigation we studied structure and function of an AKH from an African saucer bug, *Laccocoris spurcus* (family: Naucoridae). This species is quite abundant in fast-flowing streams that originate in mountainous areas; characteristically, *L. spurcus* lives in the run of the stream where a hard bottom made up of large pebbles occurs. Although we have not found any records of dispersal flight for this particular species in the literature, the adults are winged and it is anticipated that flight episodes take place; the hind legs are modified for swimming, and personal observations confirm that specimens are good swimmers. For comparative purposes and to find out the relatedness of AKHs from the infraorder Nepomorpha, we also analysed the AKH from *Aphelocheirus aestivalis*, a representative of the family Aphelocheiridae. *A. aestivalis* is a peculiar water bug that lives preferentially in fast flowing streams with a gravel bed, in the zone with little organic substrate and vegetation. Thanks to a dense plastron system, the bug extracts oxygen from the surrounding water and never surfaces for respiration; locomotion is mainly by walk-

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ing along the riverbed, as the metathoracal legs are only marginally developed for swimming; in most individuals wings are micropterous and, thus, dispersal flight (or flight in general) cannot occur [16]. Males have continual spermatogenesis, can always copulate and these features may be the reason that *A. aestivalis* is a semivoltine species in central Europe, and even during winter all developmental stages (eggs, nymphs of all instars, adults) can be found in the natural habitat [20].

2. Materials and methods

2.1. Insects

Adult specimens of both sexes and unspecified age of the saucer bug or creeping water bug, *L. spurcus* (family: Naucoridae; subfamily: Laccocorinae), were collected from a number of small fast flowing streams originating in mountainous regions of the Western Cape Province in South Africa during February to April of 2008, 2009 and 2010. Saucer bugs were collected by kick sampling, and individuals were transported in cold stream water (8–12 °C) to the laboratory. Conspecific bioassays (see Section 2.3) and swimming experiments were performed latest 48 h after arrival in the laboratory and acclimation to 20 °C. Adult specimens of undetermined age and gender of *A. aestivalis* were collected from the middle of a medium-fast flowing, shallow stream, the "Wallensteingraben" in Germany, in July 2009; water temperature was 18–20 °C.

For heterologous bioassays (see Section 2.3), adult male American cockroaches, *Periplaneta americana*, of unspecified age, were used. Rearing and maintenance of these insects are outlined elsewhere [3].

2.2. Tissue preparation and peptide isolation

Corpora cardiaca (CC) of both species under investigation were dissected with the help of a stereomicroscope and iridectomy scissors. The CCs of *L. spurcus* are located in the proximal part of the prothorax, just behind the brain. The paired CCs are tiny, tear-shaped structures lying well-separated from each other and clearly visible by the Tyndall blue effect as spongy entities next to the white cerebral lobes. In *A. aestivalis* the relatively small CCs are dorsally obscured by blue, translucent sacs that, presumably, contain digestive solutions (enzymes or toxins). The glandular (CC) material was placed into 80% methanol, and crude extracts were prepared as outlined previously [8]. An aliquot of such a dried extract (equivalent to 12 pairs of CC in the case of *L. spurcus* and 25 pairs of CC for *A. aestivalis*) was sent for mass spectrometric analyses (see Section 2.4). An aliquot of the *L. spurcus* extract was used for conspecific and heterologous bioassays (see Section 2.3) and used for the purification of the active peptide on reversed-phase high-performance liquid chromatography (RP-HPLC) as outlined previously [2], and for comparison of retention times on RP-HPLC with synthetic peptide.

2.3. Bioassays

The heterologous bioassays in cockroaches were performed as described previously [1]. For conspecific bioassays, *L. spurcus* individuals had a body mass of 71.35 ± 12.72 mg (mean ± S.D., $n = 14$). To keep bugs under resting conditions and prevent major activity including swimming, they were removed from the water and individually wrapped in wet tissue paper 2 h prior to experimentation at about 20 ± 1 °C (see also [7]). Immediately prior to taking a 0.5 μl hemolymph sample, the bug was unwrapped and the body area for sampling was dried using tissue paper. The hemolymph was sampled with a glass microcapillary after penetrating the membrane at the base of a leg with a needle under a stereomicroscope (Nikon, 1.1-fold magnification, 10× lens magnification); the 0.5 μl hemolymph sample was collected into 100 μl of concentrated sulphuric acid for determination of resting levels of metabolites. Injection of 3 μl of either distilled water (as injection control) or a specific dose of the synthetic *L. spurcus*-specific AKH (Lacsp-AKH) into the abdomen was done with a 10 μl Hamilton syringe, the insect was then re-wrapped in wet tissue paper and, 90 min after injection, a second sample of hemolymph (0.5 μl) was taken. The hemolymph was mixed thoroughly with the sulphuric acid; the total anthrone-positive material (=carbohydrates), or vanillin-positive material (=lipids) was measured in the mixture as previously described [1].

For activity experiments, 12 specimens of *L. spurcus* were put into a small beaker with stream water (50 ml), this was then placed on a shaker for continuous motion for 1 h. Insects were observed over the entire period, and those that tried to cling to each other or seemed to stop active swimming (i.e. those who were only floating), were prodded with a stick to continue swimming. A 0.5 μl hemolymph sample was collected from each bug after the full swimming period and analysed for lipid concentration as described earlier. As a control group, a second group of bugs was kept inactive, wrapped in wet tissue paper for 1 h and a hemolymph sample was collected for analysis at the end of the 1 h.

Statistical analyses of the data were performed as stated in the text and in footnotes of the tables and figure.

2.4. Liquid chromatography and mass spectrometry

A dried CC extract of *L. spurcus* or *A. aestivalis* was taken up in 50 μl of aqueous 0.05% formic acid; a 5 μl aliquot containing about 1.2–2.5 gland equivalents was injected into a 150 mm × 1 mm i.d. RP Jupiter Proteo HPLC column (Phenomenex Inc., Torrance, USA) coupled directly to a linear quadrupole ion trap mass spectrometer (LTQ XL: Thermo Fischer, San Jose, USA) equipped with an electrospray ion source operated at 4 kV, a capillary temperature at 300 °C and positive ion detection. A gradient elution from 15% to 60% B within 12 min was used at a flow rate of 50 μl min^{-1} and a column temperature of 35 °C; solvent A was 0.1% aqueous formic acid and solvent B was 0.05% formic acid in acetonitrile. Full mass range scanning of m/z 850–1650 was used for peptide detection, combined with data dependent collision-induced dissociation (CID) MS^2 scan of the detected $[M+H]^+$ ions. For further detailed parameters see [17].

2.5. Synthetic peptides

The novel peptide discovered in *L. spurcus* (code-name Lacsp-AKH) was custom-synthesized by GenScript Corporation (Piscataway, USA). The synthetic cardioacceleratory hormone I (also known as hypertrehalosemic hormone I) of the American cockroach and code-named Peram-CAH-I was purchased previously from Peninsula Laboratories (Belmont, USA). Synthetic adipokinetic hormone of the dragonfly *Anax imperator*, Anaim-AKH, was custom-synthesized previously by Dr R. Kellner (Merck AG, Darmstadt, Germany).

3. Results

3.1.1. Presence of adipokinetic/hypertrehalosemic neuropeptide material

First we established that a crude methanolic extract of the CC from the saucer bug had hypertrehalosemic activity, when injected at a dose of 1 pair equivalents of CC into cockroaches. This heterologous assay revealed that the CC extract of *L. spurcus* was