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Predicted versus expressed adipokinetic hormones, and other small peptides from the corpus cardiacum–corpus allatum: A case study with beetles and moths

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ABSTRACT

This mass spectrometric study confines itself to peptide masses in the range of 500–1500 Da. Adipokinetic hormones (AKHs) that are predicted from the genome of the red flour beetle, *Tribolium castaneum*, and the silk moth, *Bombyx mori*, are shown to exist as expressed peptides in the corpora cardiaca (CC) of the respective species as evidenced by various mass spectrometric methods. Additionally, some related species were included in this study, such as the tenebrionid beetles *Tribolium brevicornis* and *Tenebrio molitor*, as well as the moths *Spodoptera frugiperda*, *Spodoptera littoralis*, *Mamestra brassicae* and *Lacanobia oleracea*, to investigate whether AKH peptides are structurally conserved in the same genus or family. Interestingly, the AKH peptide of *T. brevicornis* is identical to that of *T. molitor* but not to the ones of its close relative *T. castaneum*. Moreover, other peptides in *T. brevicornis*, such as various FXPRL amides (=pyrokinins), also match the complement in *T. molitor* but differ from those in *T. castaneum*. All the CC of beetles lacked the signal for the mass of the peptide corazonin. All moths have the nonapeptide Manse-AKH expressed in their CC. In addition, whereas the silk moth has the decapeptide Bommo-AKH as a second peptide, all other moths (all noctuids) express the decapeptide Helze-HrTH. In *M. brassicae* and *L. oleracea* a novel amidated Gly-extended Manse-AKH is found as a possible third AKH. The noctuid moth species also all express the same FLRF amide-I, corazonin, and a group-specific isoform of a γ-PGN-(=γ-SGNP) peptide. In *L. oleracea*, however, the latter peptide has a novel sequence which is reported for the first time, and the peptide is code-named Lacol-PK.

1. Introduction

Neuropeptides represent the largest single class of regulatory compounds in invertebrates, as well as in vertebrates. In insects there are various neuropeptide families, the members of which mostly occur in multiple forms and display pleiotropic actions [12]. One of the largest such family is the adipokinetic hormone (AKH) peptide family, of which a

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plethora of information has been gathered during the last 30 years [11,14]. These neuropeptides are synthesized in neurosecretory cells of the insect's corpus cardiacum (CC). They are from 8 to 10 amino acids long, and are structurally characterized by a blocked N-terminus (pGlu) and C-terminus (carboxyamide), by the occurrence of aromatic amino acids at positions 4 (Phe or Tyr) and 8 (Trp), and by a Gly residue at position 9 [11,14]. The majority of AKHs are not charged under physiological conditions.

The major function of these AKHs is to mobilize stored products (fat, glycogen or proline) by the activation of phosphorylases or lipases, but many other functions such as myostimulation, inhibition of the synthesis of RNA, fatty acids and protein in the fat body, and stimulation of substrate oxidation in the flight muscles are known to be regulated by AKH. They may also be involved in the insect immune response [14]. Many isoforms of AKHs have been identified and these peptides are present in all major insect orders [19,20].

Recently, the genome of certain insect species has been elucidated and the data are available, e.g. for *Tribolium castaneum* [http://hgsc.bcm.tmc.edu/projects/tribolium/] and for *Bombyx mori* [http://silkworm.genomics.org.cn/index.jsp; http://sgp.dna.affrc.go.jp/; http://www.ncbi.nlm.nih.gov/]. The search for structural homologs of adipokinetic peptides in these databases reveals the presence of such peptides, but this information does not indicate that the peptides are, indeed, expressed in these species. Our knowledge on AKHs in the honey bee *Apis mellifera* may support this idea that genomic presence does not guarantee expression of the encoded protein/peptide. With conventional protein chemical methods it was shown that the Italian race of the honey bee, *A. mellifera ligustica*, contains a peptide with hypertrehalosemic activity and which is structurally identical to the adipokinetic peptide of the tobacco hornworm moth, *Manduca sexta*, i.e. Manse-AKH, a nonapeptide (pELTFTSSWG amide) [40,55]. The genome of the honey bee, which was sequenced from DH4 strain of *A. mellifera*, did not, however, reveal the presence of a precursor for Manse-AKH. In contrast, an octapeptide with the sequence pELNFSTGW amide, code-named Schgr-AKH-II and first sequenced from locusts [18,49], was found to be encoded in the honey bee genome [27]. The DH4 strain of honey bee was principally *A. mellifera ligustica* and a mixed product that included the SMR (suppression of mite resistance) trait (D. Weaver, B. Weaver Apiaries, personal communication).

By protein chemical methods, Schgr-AKH-II had been found in the CC of a number of other hymenopteran species [16,41]. Surprisingly, when honey bee tissues (including CC) from adult workers of the dark European race, *A. mellifera carnica* [4] or whole heads from a mixture of the races *A. mellifera ligustica* and *A. mellifera carnica* [27] were screened by mass spectrometric techniques, neither Schgr-AKH-II nor Manse-AKH were detected. Thus, it appears that the peptide Schgr-AKH-II encoded in the honey bee genome may not be expressed (at least in the tissues investigated), or is expressed in amounts too small to be detected by mass spectrometry, and it is unclear whether Manse-AKH is indeed synthesized in the CC of honey bees.

In the present study, we sought to determine whether the AKHs that are predicted from the genome of *T. castaneum* and *B. mori* can be identified by mass spectrometric analysis (i.e. by mass data and sequencing information) in extracts prepared from the CC of these insects. Furthermore, we have expanded the group of insects under investigation to include some related species to investigate whether AKHs are structurally conserved in the same genus or insect family. For these purposes, the beetle *Tribolium brevicornis*, thought to be closely related to *T. castaneum* [43], and also a perhaps more distantly related tenebrionid, *Tenebrio molitor*, were included in the study. Many neuropeptides from the CC of *T. molitor* are known [21,53] and serve as confirmation of the methods employed. Similarly, we analyze not only the AKH and some other peptides of the CC–CA of *B. mori*, but also from additional lepidopteran species, viz. *Spodoptera frugiperda*, *Spodoptera littoralis*, *Mamestra brassicae* and *Lacanobia oleracea*, with the aims of: (i) looking for three different classical AKHs in *S. frugiperda* CCs which may be expressed, based on a cDNA study [1]; (ii) to check the hypothesis that closely related species contain the same (or similar) AKH peptides, hence the inclusion of *S. littoralis*; and (iii) to study the neuropeptide complement from a comparative point of view in species where some information on AKHs already exists (*M. brassicae*; [7]) or is known from larvae but not adults (*L. oleracea*; [2]).

To achieve these aims, CCs are first screened by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to detect the presence of AKHs. Sequence information is gathered from electrospray—or MALDI-TOF MS/MS data. We also report on masses of other prominent ions and use that information together with the data on AKHs to speculate on the relationship of certain species.

2. Materials and methods

2.1. Insects

CCs were dissected from adult specimens of indeterminate age. All species under investigation, except *B. mori* and *S. frugiperda*, were reared in the insectaries of the Animal Services and Invertebrate Supply Team, Central Science

Abbreviations: AKH, adipokinetic hormone; Anoga, *Anopheles gambiae*; Bommo, *Bombyx mori*; CA, corpora allata; CAP_{2b}, cadioactive peptide 2b; CC, corpora cardiaca; CID, collision-induced dissociation; DH, diapause hormone; ESI, electrospray ionization; FXPRL amides, pyrokinins; γ-PGN, γ-PBAN-encoding gene neuropeptide; γ-SGNP, γ-suboesophageal ganglion neuropeptide; Helze, *Heliothis zea*; HrTH, hypertrehalosemic hormone; Lacol, *Lacanobia oleracea*; LC, liquid chromatography; Leuma, *Leucophaea maderae*; Locmi, *Locusta migratoria*; MALDI, matrix-assisted laser desorption/ionization; Mambr, *Mamestra brassicae*; Manse, *Manduca sexta*; Melcin, *Melittea cinxia*; Melme, *Melolontha melolontha*; MRCH, melanization and reddish-coloration hormone; Ms, myosuppressin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MT, myotropin; NVP, NVP-motif containing peptide; PBAN, pheromone-biosynthesis-activating neuropeptide; Peram, *Periplaneta americana*; Phote, *Phormia terraenovae*; PK, pyrokinin; PSD, post-source decay; Pyrap, *Pyrrhocoris apterus*; RT, retention time; Scade, *Scarabaeus deludens*; Schgr, *Schistocerca gregaria*; Spofr, *Spodoptera frugiperda*; Spoli, *Spodoptera littoralis*; tBME, *t*-butylmethyl ether; Tenmo, *Tenebrio molitor*; TFA, trifluoroacetic acid; TOF, time-of-flight; Trica, *Tribolium castaneum*.

Laboratory, Sand Hutton, York under standardized rearing conditions. Adult lepidopterans were maintained at either 20 °C (*B. mori*, *M. brassicae*, *L. oleracea*) or 25 °C (*S. littoralis*, *S. frugiperda*) under a 16 h light:8 h dark regime. Adult coleopterans (*T. castaneum*, *T. brevicornis*, *T. molitor*) were maintained at 25 °C under constant darkness. Pupae of *B. mori* were obtained from Dr. Bernard Mauchamp, Unité Nationale Séricole/INRA, La Mulatière, France, and pupae of *S. frugiperda* were supplied, under licence, by Dr. Philippe Fournier and Dr. Emmanuelle d'Alençon, Laboratoire de Biologie Intégrative et Virologie des Insectes, INRA, Université Montpellier II, France.

2.2. Preparation of CC extract, liquid chromatography and mass spectrometry

In most cases, single CC or CC–CA pairs were dissected under saline and placed directly on a MALDI-TOF plate, rinsed with a drop of ice-cold distilled water, which was blotted off after 30 s, then covered with 0.2 μl of matrix solution diluted 50:50 (v/v) with methanol and allowed to dry. The undiluted matrix consisted of α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich) prepared at a concentration of 10 mg/ml in 50% acetonitrile, 0.05% (v/v) trifluoroacetic acid (TFA). In some cases, a perfusate of several glands in 80% methanol was concentrated to a small volume by vacuum evaporation. This was mixed with matrix 50:50 (v/v) and 1 μl dried onto a MALDI target plate prior to MALDI-TOF reflectron or MALDI-TOF MS/MS analysis. Mass spectra were acquired using either a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK) or Bruker UltraFlex TOF/TOF mass spectrometer (Bruker Daltronics GmbH, Bremen, Germany). Analyses by post-source decay (PSD), using the Voyager DE STR with angiotensin as standard, were the accumulation of seven to eight spectral segments stitched together using the Applied Biosystems Data Explorer software. The MS/MS analysis of selected peptides by Bruker UltraFlex used LIFT™ technology [51], and data analysis by FlexAnalysis 2.4 software (Bruker Daltronics GmbH).

The measured monoisotopic masses $[M + H]^+$, $[M + Na]^+$ or $[M + K]^+$ of known or conceptually derived peptides were compared to the calculated masses using Protein Prospector (University of California, San Francisco). Peptide sequences based on PSD or MS/MS fragments were either determined manually or by using Data Explorer 4.0 software (Applied Biosystems).

In a few instances, CC–CA from several individuals were dissected directly into 80% methanol and extracted by approved methods [17]. The dried material was then taken up in 50 μl of aqueous 0.1% formic acid, and 5–10 μl (containing about 1–1.5 gland equivalent) were injected onto a 150 mm × 1 mm i.d., 3 μm RP-C18 Jupiter Proteo HPLC column (Phenomenex Inc., Torrance, CA, USA). A mobile phase with solvent A:0.1% formic acid in water and solvent B:0.1% formic acid in acetonitrile was used with a gradient of 20–60% B within 12 min and a flow rate of 50 μl min^{-1}. The HPLC column was directly coupled to an LTQXL mass spectrometer (Thermo Fischer, San Jose, CA, USA) equipped with an electrospray ion source operated at 4 kV, a capillary temperature at 300 °C and a positive ion detection. The primary sequence of each peptide was deduced from the electrospray MS^N spectra obtained by the collision-induced dissociation (CID) of the detected $[M + H]^+$ ion and its product fragment ions.

2.3. Synthetic peptides

The novel peptides named Trica-AKH (pELNFSTDW amide) and Bommo-AKH (pELTFTPGWGQ amide) were synthesized on an Applied Biosystems 433 synthesizer using standard F-moc chemistry; each peptide resin was cleaved and deprotected for 4 h in reagent K, a cocktail containing 5% phenol, 5% water, 5% thioanisole, and 2.5% dithioethane in TFA [33]. After removing the resin from the reaction mixture by filtration, each peptide was precipitated in cold *t*-butylmethyl ether (tBME), followed by repeated tBME washes and air-drying. Peptides were purified by HPLC on a preparatory column (10 μm particle size, 21.2 mm × 25 cm, Jupiter C18, Phenomenex Inc., Torrance, CA) using HPLC-grade water and a linear gradient of acetonitrile (10–80% within 0–70 min) at 5 ml min^{-1}. Both the water and acetonitrile contained 0.05% TFA. Peaks were identified by MALDI-TOF mass spectrometry. After numerous purification runs, peaks of interest were pooled and lyophilized.

All other synthetic AKH peptides had been custom-synthesized before or were purchased previously from Peninsula Laboratories (Belmont, CA, USA).

3. Results

3.1. Adipokinetic peptides of beetles and mass information on some other abundant peptides

When an aliquot of five glands of *T. castaneum* was analyzed directly by MALDI-TOF MS (Applied Biosystems Voyager), two clear pairs of ions with a difference of 16 mass units could easily be identified at *m/z* 1014.2/1030.2 and at 1023.3/1039.3 (Fig. 1). Such pairs are indicative of the sodiated and potassiated forms of members of the AKH family [37]. A prominent ion was observed at *m/z* 952.3, together with lower intensity ions at *m/z* 883.4 and 974.2. There were no other readily discernible peptide ions within the mass range examined (*m/z* 500–1500).

An aliquot of a more concentrated extract of CC–CA from *T. castaneum* was analyzed using the MALDI-TOF–TOF MS (Ultraflex), whereupon a much larger number of peaks was observed beyond the mass range recorded previously but these will be dealt with elsewhere. Using MS–MS analysis, the mass ion at *m/z* 883.4 produced insufficient fragment ions for sequence determination, but is likely to be the same as that in *T. brevicornis* and *T. molitor* (see below). Fragmentation of the peak at *m/z* 952.3 produced daughter ions sufficient to identify the peptide as NVP-like peptide 4 GRWGGFADS-OH ($[M + H]^+ = m/z$ 952.43). The fragment ions observed included, y8-NH_3, y6, y6-H_2O, y5-H_2O, y3, y3-H_2O, and y2, b8, b6-NH_3, b5, b5-NH_3, b4, b3, b3-NH_3, and b2, a7, a7-NH_3, a6-NH_3 and a3, internal fragments WGG, GGF, FAD, WGGF-28 and GGFAD, and immonium ions F, R and W (data not shown). There are at least three candidates for the peak at *m/z* 974.2, which, as with the mass ion at *m/z* 883.5, was too weak for sequence determination using the material available. These possibilities are dealt with in Section 4.

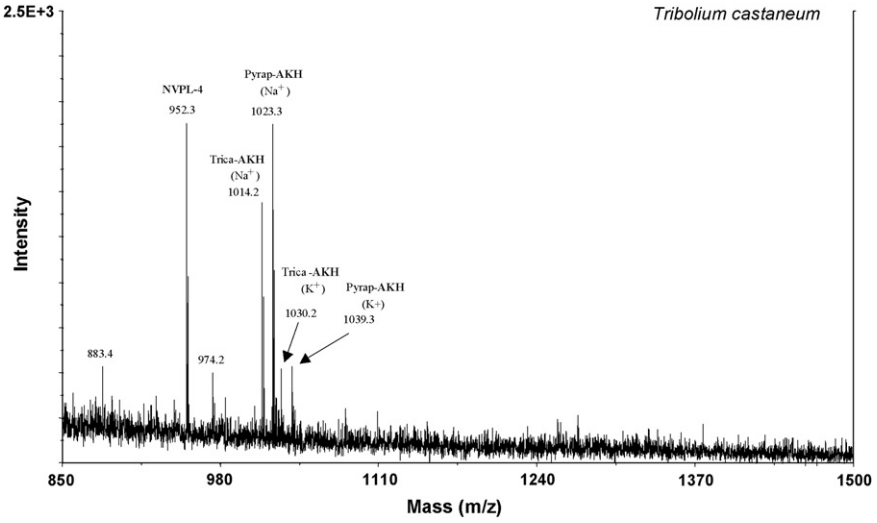

Fig. 1 – Annotated MALDI-TOF mass spectrum (positive ion reflector mode on a Voyager DE-STR) of five CC–CA complexes from adult *Tribolium castaneum* after peptide extraction by sonication in 80% methanol. The annotated peptide sequences were confirmed by MS/MS analyses (see text). Trica-AKH, pELNFSTDW amide; Pyrap-AKH, pELNFTPNW amide; NVP-like peptide 4, GRWGGFADS-OH.

When an aliquot of a crude methanolic extract from the CC of *T. castaneum* was separated via LC and analyzed by electrospray tandem mass spectrometry, one major peak at a retention time of 7.76 min with a shoulder peak at 7.68 min was clearly detected in the total ion chromatogram (Fig. 2A); these peaks corresponded to *m/z* values of 1001.5 ($[M + H]^+$ for peak with RT of 7.68 min) and 992.5 ($[M + H]^+$ for the peak with RT of 7.76 min), respectively (Fig. 2B). The complete primary sequence of the two peptides was deduced by CID MS^2 spectra of the respective $[M + H]^+$ ions (Fig. 3). The characteristic y- and b-type product ions, in conjunction with diagnostic y-NH_3 and b-H_2O ions characterized these peptides unequivocally as members of the AKH family (see insets in Fig. 3). In both cases the ambiguity at the N-terminus, Leu or Ile at position 2, could easily be solved: the synthetic peptides with a Leu at position 2 were available and had exactly the retention time as predicted (an Ile containing compound would have a RT of 0.2 min earlier than the same peptide with a Leu at position 2; G. Gäde and P. Šimek, unpublished results) and the MS^2 mass spectra of the Leu^2 peptide were identical to the ones achieved with crude extract.

A MALDI-TOF mass spectrum from CC of a species from the same genus, the beetle *T. brevicornis*, did not show ion peaks identical to the AKHs of *T. castaneum*. In contrast, clear peaks at *m/z* 1009.5 and 1025.5 were present (Fig. 4), which correspond to the $[M + Na]^+$ and $[M + K]^+$ forms of Tenmo-AKH identified in the yellow mealworm beetle *T. molitor* and other species of Tenebrionidae (Table 1). Likewise, Tenmo-AKH was detected in single CC–CA from *T. molitor* in the present study by its characteristic peaks at *m/z* 1009.5 and 1025.5, the spectrum of which is shown here for comparative purposes (see inset, Fig. 4). Besides the ions for AKHs, *T. brevicornis* CC–CAs showed distinct peaks at *m/z* 883.5, together with possible sodium (*m/z* 905.5) and potassium (*m/z* 921.5) adducts. The peptide at *m/z* 883.5 was sequenced by MALDI-MS/MS and revealed the structure as the pyrokinin/PRL amide Peram-PK-2; SPSFAPRL amide ($[M + H]^+$ calc. = 883.5). Fragment ions observed included, y2, y3, y4, y5, y6 and y7 (with accompanying y-NH_3 ions, except y5), b2, b3, b4 and b5, together with a3, a4, a5 and b2-H_2O, b5-H_2O and b6-H_2O (data not shown). Other distinctive mass peaks at *m/z* 1052.6, 1081.7 and 1257.7 in the mass spectrum of *T. brevicornis* (Fig. 4) were also identified by MS/MS spectra as representing other pyrokinins, namely Tenmo-PK-3 and PK-1, and the myosuppressin Leuma-Ms. In each case, an almost complete set of y- and a- or b-ions was observed (results not shown). The same mass ion peaks at *m/z* 883.6, 1052.6, 1081.7 and 1257.8 were confirmed in the MALDI

Table 1 – Primary sequences of adipokinetic peptides of insects

(A) Adipokinetic peptides of tenebrionid beetles	
Pyrap-AKH	pELNFTPNW amide (this study)
Tenmo-AKH	pELNFSPNW amide [21]
Trica-AKH	pELNFSTDW amide (this study)
(B) Charged adipokinetic peptides	
Trica-AKH	pELNFSTDW amide
Melme-CC	pELNYSPDW amide [8]
Phote-HrTH	pELTFSPDW amide [22,47]
Scade-CC	pEFNYSPDW amide [13]
(C) Adipokinetic peptides of Lepidoptera	
Helze-HrTH	pELTFSSGWGN amide [31]
Melcin-AKH	pELTFSSGW– amide [G. Gäde et al. unpublished]
Manse-AKH	pELTFTSSWG- amide [55]
Bommo-AKH	pELTFTPGWGQ amide (this study)
(D) Brain-derived adipokinetic decapeptides	
Trica-AKH-II	pEVTFSRDWNP amide (predicted)
Bommo-AKH-II	pEITFSRDWSG amide (predicted)
Anoga-AKH-II	pEVTFSRDWNA amide [32]
Locmi-HrTH	pEVTFSRDWSP amide [48]

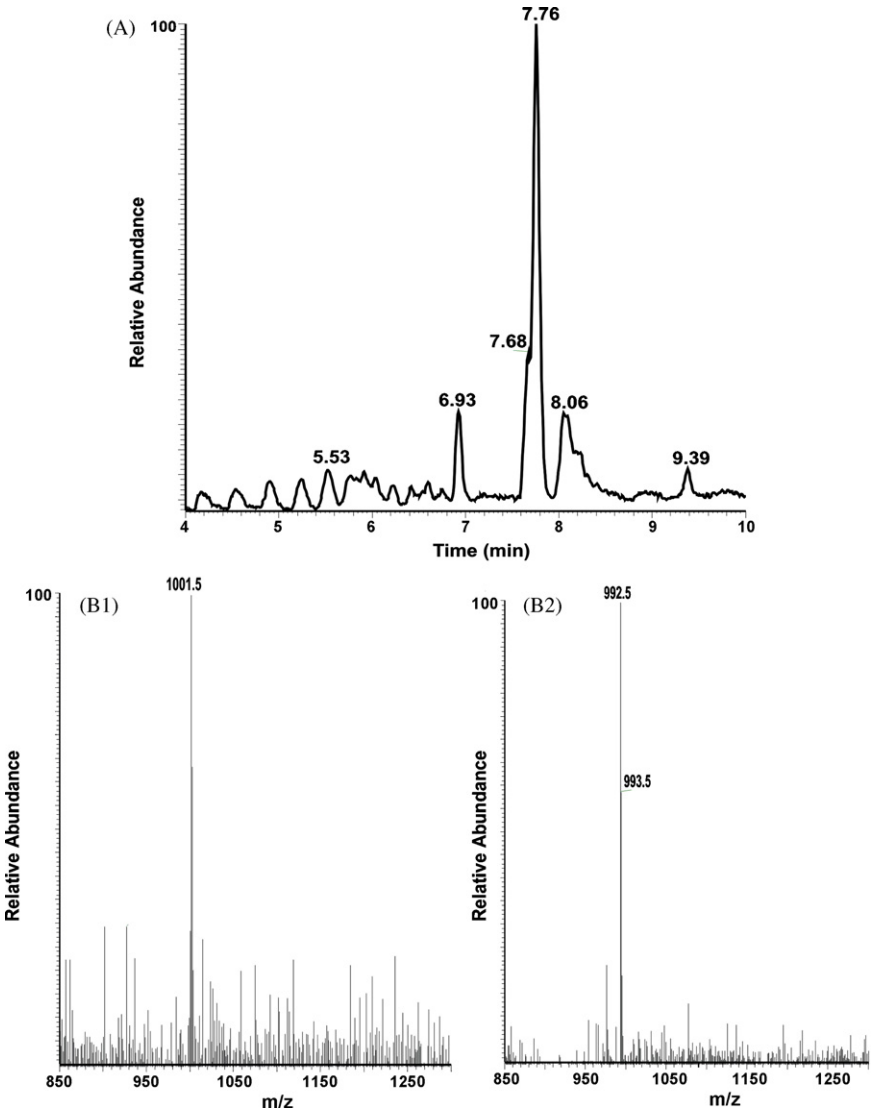

Fig. 2 – LC/MS analysis of CC–CA complexes from *T. castaneum*. (A) The total ion chromatogram is shown. (B) A full scan electrospray ionization (ESI) mass spectrum of the peak with the retention time of 7.68 min showing $[M + H]^+$ at *m/z* 1001.5 and the peak at 7.76 min with $[M + H]^+$ at *m/z* 992.5.

spectrum of *T. molitor* (inset Fig. 4). In addition to the identifiable and fully confirmed mass ion peaks, there were two additional unidentified peaks at *m/z* 1064.7 and 1093.7 in the MALDI-TOF spectra of both *T. brevicornis* and *T. molitor* (Fig. 4 and inset).

3.2. *Adipokinetic peptides of moths and mass information on some other abundant peptides*

Direct MALDI-TOF analysis of one CC from the silkmoth *B. mori* was sufficient for the detection of two distinct pairs of ion masses with a difference of 16 mass units which characterizes the underlying peptides as members of the AKH family (Fig. 5A). The masses were found at *m/z* 1030.6/1046.5, representing the sodiated and potassiated masses of Manse-AKH, and at *m/z* 1138.6/1154.6, representing the same adducts for the peptide Bommo-AKH, both of which are predicted from the *B. mori* genome (Fig. 6A and B). The fragmentation pattern of the two Na-precursor ions (1030.6 and 1138.6, respectively) by MALDI-MS/MS resulted in b-, a- and y-ions that confirmed the peptides as predicted (Fig. 5B and C).

The genome sequence of the silkmoth *B. mori* also includes a coding sequence for a third 'AKH-like' peptide precursor (predicted peptide pEITFSRDWSG amide; Fig. 6C), the respective sodiated and potassiated ion pairs of which were not detected in this analysis of the expressed CC peptides. A more detailed analysis (data not shown), using an extract of five pairs of *B. mori* CC in 80% methanol, also failed to show any of the additional predicted ions (whether protonated, sodiated or potassiated) for any potential, partially processed products assuming absence of C-terminal amidation and/or N-terminal cyclization to pE.

Besides these AKHs a number of other identifiable mass peaks can be seen in the mass spectrum of *B. mori* CC (Fig. 5A), which are assigned either by similarity in mass to known

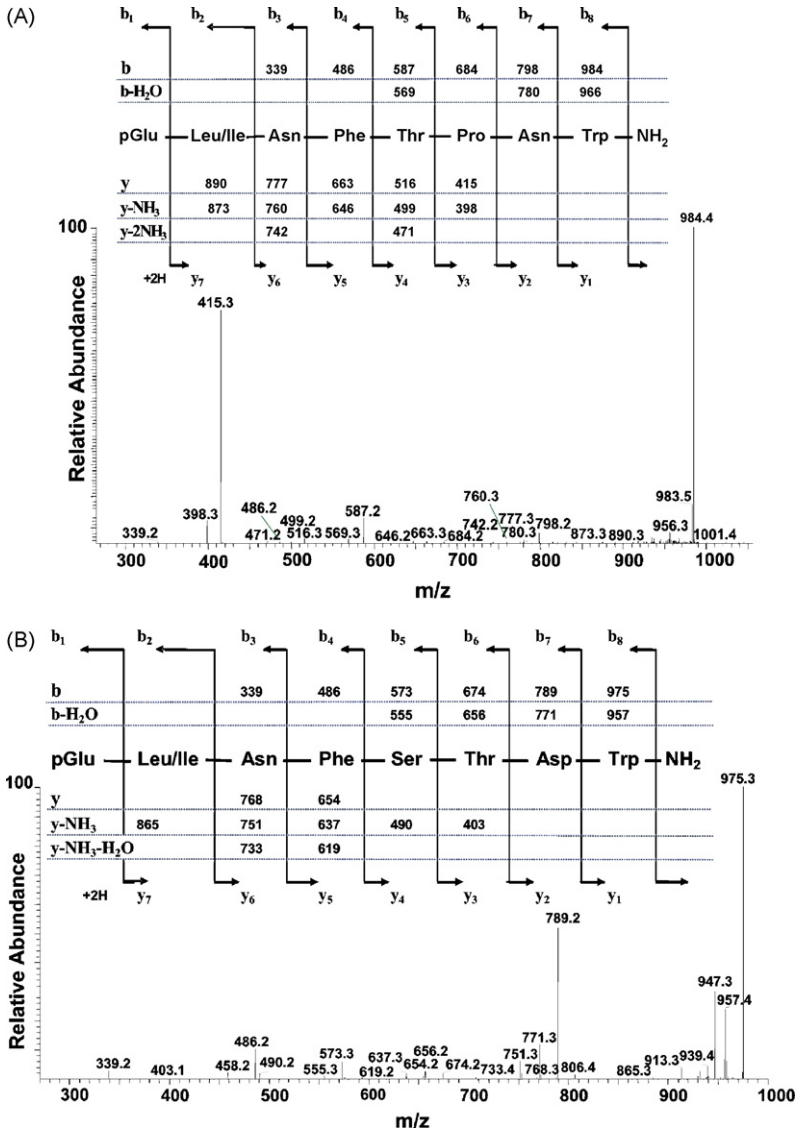

Fig. 3 – The CID MS^2-ESI spectra of (A) the ion [M + H]$^+$ *m/z* 1001.5 and (B) 992.5 (see Fig. 2). The respective inset shows the sequence of the assigned peptide, together with the theoretical calculated masses for b-type and y-type fragment ions which are observed in the MS^2 mass spectrum.

lepidopteran peptides, or by direct MS/MS sequencing. The peaks at *m/z* 937.6 and 975.5 can be interpreted as γ-SGNP and CAP_{2b}, respectively, though these interpretations have not been confirmed by MS/MS. The signals at *m/z* 1229.8 and 1246.8 represent the pGlu and Gln form of FLRF amide-I. The sequence of the former is fully confirmed by MS/MS: fragmentation of the precursor ion produced a full series of C-terminal y-ions (y1, y2, y3, y4, y5, y6, y7 and y8) and prominent b-ions corresponding to b5 and b6, together with a5 and b6-H_2O counterparts, and numerous other masses in confirmation (data not shown). The peak at *m/z* 1369.8 corresponds to Arg^7-corazonin plus its sodiated form at *m/z* 1391.8. The identity of the pE form was fully confirmed by MS/MS analysis, which again showed a full series of y-ions (y1–y9), plus an almost complete series of b-ions (b3–b9), and several other identifiable fragments (data not shown). In addition, the mass spectrum shows an unidentified peak at *m/z* 1407.7.

By cDNA cloning, it was shown that the fall armyworm moth, *S. frugiperda*, contains precursors for three classical AKHs (Fig. 7A–C) and a fourth, somewhat unusually elongated form (Fig. 7D). In the current study, direct MALDI MS of single pairs of CC of this noctuid moth revealed mass pairs at *m/z* 1030.6/1046.6 and 1100.6/1116.6, which are indicative for the Na^+ and K^+ adducts of Manse-AKH and Helze-HrTH, but no mass pair (at *m/z* 929/945) for the third AKH (predicted as pELTFSSGW amide) (Fig. 8A). The spectrum of *S. frugiperda* also lacked a mass pair at *m/z* 1392/1408, which would have been expected for the fourth, longer 'AKH-like' peptide (pELTFSSGWGNCTS-OH). Fragmentation of the mass ion at *m/z* 1030.6 and 1100.6 was used to confirm these as sodiated forms of the predicted Manse-AKH and Helze-HrTH, respectively, with b- and y-ions to assign the peptide sequences. For Manse-AKH ([M + Na]$^+$) the observed fragment ions included y2–y7, b3, b4 and b7, a2–a7 (all sodiated), while for Helze-HrTH

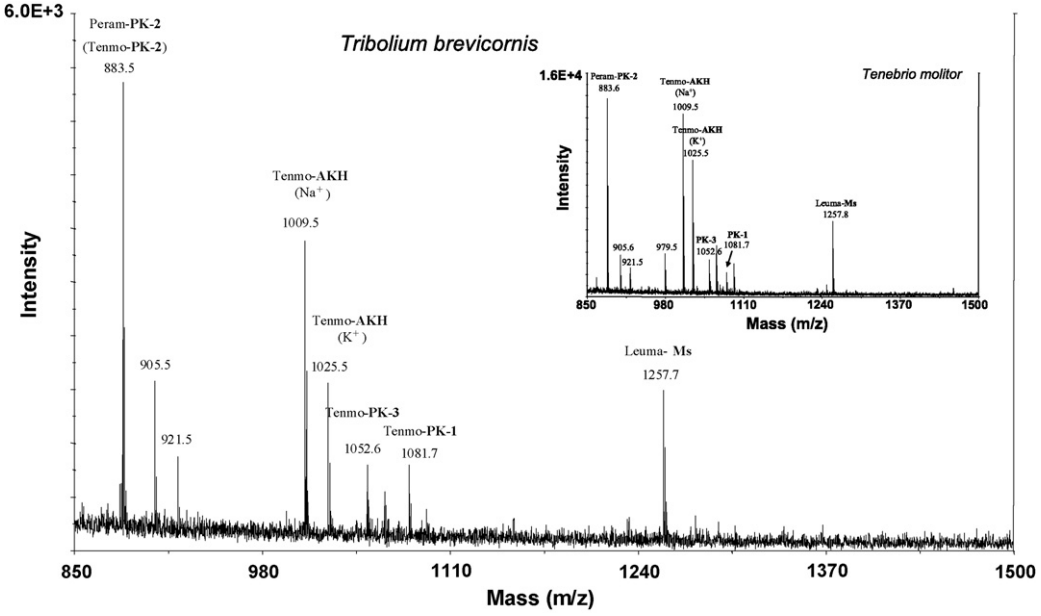

Fig. 4 – Annotated MALDI-TOF mass spectrum of three CC–CA complexes from adult *Tribolium brevicornis* and (inset) from adult *Tenebrio molitor*. The same conditions were employed as in Fig. 1. The annotated peptide sequences were confirmed by MS/MS analyses (see text). Peram-PK-2, SPPFAPRL amide; Tenmo-AKH, pELNFSPNW amide; Tenmo-PK-3, HL(I)SPFSPRL amide; Tenmo-PK-1, HVVNFTPRL amide; Leuma-Ms, pEDVDHVFLRF amide.

the ions observed included y2–y8, b2, a1 and a3–a8 (again all sodiated; data not shown).

In addition to the AKH peptide masses, the mass ions at *m*/z 964.6, 1229.8 and 1369.8 (see Fig. 8A) represent the known lepidopteran peptide Helze-PGN-8, together with FLRF amide-I and corazonin, as described for *B. mori*. The sequence of all three of these peptides was confirmed in *S. frugiperda* by MS/MS fragmentation (data not shown). Also seen in the mass spectrum of single CC of *S. frugiperda* are prominent mass ions at *m*/z 877.6, 1194.7, 1291.9, 1376.9 and 1467.0 but these were not analyzed further.

Preparations of a single CC of the closely related Egyptian cotton leaf worm moth, *S. littoralis*, revealed exactly the same pattern in masses as for *S. frugiperda* AKHs (*m*/z 1030.6/1046.6 and 1100.6/1116.6), as well as for other peptides such as Helze-PGN-8 (*m*/z 964.6, with potential adduct forms at *m*/z 986.6 and 1002.6) (Fig. 8B). Also seen in the mass spectrum of *S. littoralis* were peaks corresponding to FLRF amide-I (*m*/z 1229.9) and Arg^7-corazonin (*m*/z 1369.9) (Fig. 8B). The sequence of Arg^7-corazonin was confirmed by MS/MS, but the other peptides were not investigated further. Again, there were no mass ions that matched any of the predicted *m*/z values for other AKHs predicted for *S. frugiperda* (see Fig. 7C and D).

The MALDI spectrum from a single CC of the noctuid *M. brassicae* showed only a relatively small signal for the Na^+ form of Manse-AKH at *m*/z 1030.6 but the K^+ adduct at *m*/z 1046.6 was very prominent (Fig. 9A). Also evident were the sodiated (at *m*/z 1100.6) and potassiated adducts (at *m*/z 1116.6) of Helze-HrTH. The spectrum was dominated however (in the mass range 1000–1200), by an apparent additional mass pairing at *m*/z 1087.6 and 1103.6, each of which are, respectively, 57 mass units greater than the equivalent Manse-AKH Na^+/K^+ adducts. The nature of the peptide(s) giving rise to these ions at *m*/z 1087.6 and 1103.6 was investigated in *L. oleracea* (see below).

Additional peaks in the spectrum of *M. brassicae* (Fig. 9A), at *m*/z 964.6, 1229.8 and 1369.8 correspond to Helze-PGN-8, FLRF amide-I and Arg^7-corazonin, as seen in the other noctuids, *S. frugiperda* and *S. littoralis*. Other peaks in the spectrum of *M. brassicae* are less intense, but are similar to those observed in *S. frugiperda* or *S. littoralis*, except for a peak at *m*/z 1407.8.

Single CC of adult tomato moth, *L. oleracea* gave MALDI spectra (Fig. 9B) that showed similarity to those observed for the *Spodoptera* species and *M. brassicae*. Clear masses were seen at *m*/z 1030.5/1046.5, corresponding to the Na^+/K^+ adducts of Manse-AKH, and at *m*/z 1100.6/1116.6, which are consistent with the Na^+ and K^+ forms of Helze-HrTH. Moreover, the same additional mass pair, at *m*/z 1087.5 and 1103.5, as observed in *M. brassicae* was also evident in *L. oleracea* (Fig. 9B). Analysis by MALDI-TOF MS/MS of the mass ion at 1103.5 on an extract of 15 CC pairs from *L. oleracea* yielded only partial sequence information that was inconclusive. Subsequent analysis of a methanolic extract from 40 pairs of CC–CA by LC-ESI-MS gave three distinct peaks in the total ion chromatogram at 6.58, 7.01 and 7.28 min which corresponded to mass ions at *m*/z 1078.5, 1065.5 and 1008.4, respectively (data not shown). Analysis by MS/MS of these ions revealed all the expected b, y, b-H_2O, b-$2H_2O$, y-NH_3, y-$2NH_3$ and y-$CONH_2$ masses for $[M + H]^+$ 1078.5 and 1008.4 to assign the sequences for Helze-HrTH and Manse-AKH, respectively (data not shown). MS/MS analysis of the mass ion at *m*/z 1065.5 showed the necessary b, y, b-H_2O, b-$2H_2O$, y-NH_3 and other ions to assign this peak to a C-terminally amidated Gly-extended form of Manse-AKH (Fig. 9C).

An additional mass ion observed in the MALDI-TOF mass spectrum of *L. oleracea* (Fig. 9B) was a very prominent peak at

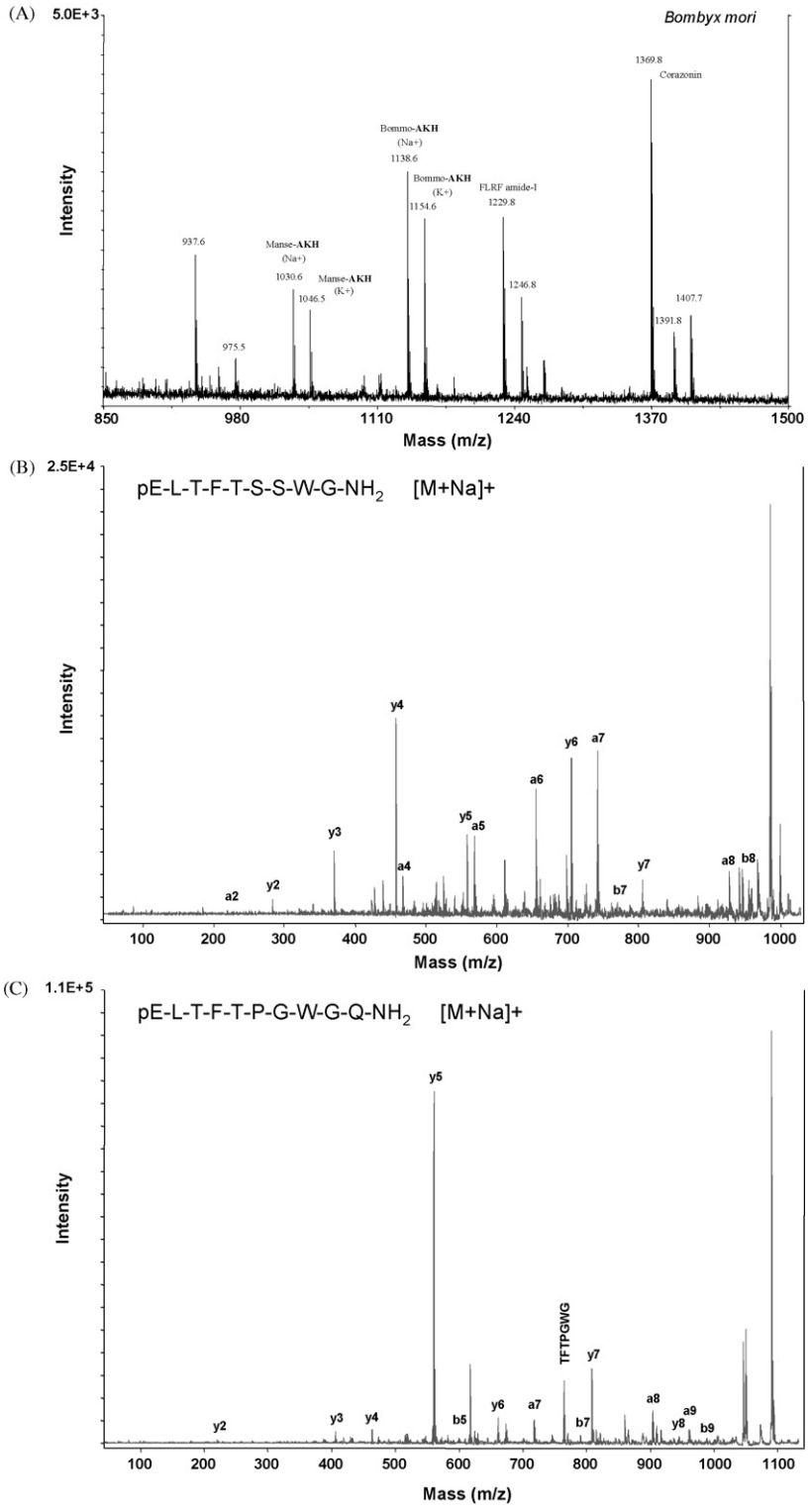

Fig. 5 – Annotated MALDI-TOF mass spectrum of (A) single CC–CA complex from adult *Bomby mori*; the same conditions were employed as in Fig. 1. The annotated peptides were (mostly) confirmed by MS/MS analyses (see text). Manse-AKH, pELTFTSSWG amide; Bommo-AKH, pELTFTPGWGQ amide; FLRF amide-I, pEDVVHSFLRF amide; corazonin, pETFQYSRGWTN amide. Tandem collision-induced dissociation mass spectra (MS–MS) of the ions at (B) *m/z* 1030.5 and (C) *m/z* 1138.5 from an extract from *B. mori* CC–CA. In each case, a number of y-, a- and b-ions are observed, plus internal fragments. The fragment ions in (B) are consistent with the sodiated adduct, $[M + Na]^+$ of Manse-AKH, pELTFTSSWG amide, while the fragment ions in (C) confirm the sequence of the sodiated adduct of Bommo-AKH, pELTFTPGWGQ amide.

(A) **AKH-I prohormone** (Bommo-AKH I = Manse-AKH)
BAAB01005426 *Bombyx mori* DNA, whole genome shotgun sequence (wgss) contig 112192
AADK01011161 *Bombyx mori* Dazao strain DNA, wgss contig 011161

*MMYKFTILFLVLACFIMAEA*QLTFTSSWGGKRAAIAGTVSCRNDESLASIYKLIQVI
LYFIYFGFNGPIRRADSPGYQNPKMQQS

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QLTFTSSWGG-OH	1082.5	1083.5	1105.5	1121.5
pELTFTSSWGG-OH	1065.5	1066.5	1088.5	1104.5
QLTFTSSWGamide	1024.5	1025.5	1047.5	1063.5
pELTFTSSWGamide	1007.5	1008.5	**1030.5**	**1046.5**

(B) **AKH-II prohormone** (Bommo-AKH)
BAAB01184896 *Bombyx mori* DNA, wgss contig 688235
AADK01005451 *Bombyx mori* Dazao strain DNA,wgss contig 005451

*MGRALVLVLILSAALLVCEA*QLTFTPGWGQGKRSEATDYRNDGCSSEDSVYTIYKLI
KVCKYY

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QLTFTPGWGQG-OH	1190.6	1191.6	1213.6	1229.6
pELTFTPGWGQG-OH	1173.6	1174.6	1096.6	1212.6
QLTFTPGWGQamide	1132.6	1133.6	1155.6	1171.6
pELTFTPGWGQamide	1115.6	1116.6	**1138.6**	**1154.6**

(C) **AKH-Like prohormone** (Bommo brain-AKH-L)
BAAB01189542 *Bombyx mori* DNA, wgss contig 704406
AADK01007419 *Bombyx mori* Dazao strain DNA, wgss contig 007419

*MKVKIFRPYFSSFFVWAACALAAVAA*QITFSRDWSGGKRSVAEAPVDCRQFTRFCRH
FVVSMQPHHSICSSGPRTQLGSR

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QITFSRDWSGG-OH	1252.6	1253.6	1275.6	1291.6
pEITFSRDWSGG-OH	1235.6	1236.6	1258.6	1274.6
QITFSRDWSGamide	1194.6	1195.6	1217.6	1233.6
pEITFSRDWSGamide	1177.6	1178.6	**1200.6**	**1216.6**

Fig. 6 – Deduced amino acid sequences of the precursors for AKHs of the silk moth. Sequences encoding potential *Bombyx mori* (A–C) AKHs are highlighted in bold on a shaded background. Potential signal sequences are shown in italics, and dibasic cleavage sites are underlined. The calculated masses and predicted mass ions (protonated, [M + H]$^+$, sodiated, [M + Na]$^+$ and potassiated [M + K]$^+$) for peptides potentially processed from each precursor are shown below each sequence. Precursor sequences for the silkmoth, *B. mori* were derived from genomic databases [http://silkworm.genomics.org.cn/index.jsp; http://sgp.dna.affrc.go.jp/; http://www.ncbi.nlm.nih.gov/].

(A) **Spofr-AKH 1 (= Manse-AKH)**

*MLLLIFSLYSINTTKSF*SIGSYLTNTSSVSSA**ELTFTSSWGG**RRPVCVRACLCLRRA
LVSAVLASRTFPIHFNVNFFQV

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
ELTFTSSWGG-OH	1083.5	1084.5	1106.5	1122.5
pELTFTSSWGG-OH	1065.5	1066.5	1088.5	1104.5
ELTFTSSWGamide	1025.5	1026.5	1048.5	1064.5
pELTFTSSWGamide	1007.5	1008.5	**1030.5**	**1046.5**

(B) **Spofr-AKH 2 (= Helze-HrTH)**

*MNECNAALVLMSLSCSPNSAAA*EQSLDTTRHHTAPQRTHHYLCIHDYADLHHQHWEI
VSSHIYYYVTQSPYCYVMPYLDLRTLQVDRTFTYDEYLILRIYTRICKIQSIHNFNL
IVIIADLT**QLTFSSGWGNG**KRRILLYTSIYLGVWHTHVKNSGLVFIHFAKQIDISIE
SLSKKKNIHLFI

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QLTFSSGWGNG-OH	1152.5	1153.5	1175.5	1191.5
pELTFSSGWGNG-OH	1135.5	1136.5	1158.5	1174.5
QLTFSSGWGNamide	1094.5	1095.5	1117.5	1133.5
pELTFSSGWGNamide	1077.5	1078.5	**1100.5**	**1116.5**

(C) **Spofr-AKH 3 (isoform A)**

*MISTSRWKLTCISAALMAHTGSTCA*VHGRSWCWLPVRWSPLRT**QLTFSSGWG**KRSDS
IQVYIPCRTAIVYARVD

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QLTFSSGWG-OH	981.5	982.5	1004.5	1020.5
pELTFSSGWG-OH	964.4	965.4	987.4	1003.4
QLTFSSGWamide	923.4	924.4	946.4	962.4
pELTFSSGWamide	906.4	907.4	929.4	945.4

(D) **Spofr-AKH 4**

*MNAEYAGLLLIPFSTFIET*KNLY**QLTFSSGWGNCTS**KRNY

Potential peptides	Mass	M/z [M+H$^+$]	M/z [M+Na$^+$]	M/z [M+K$^+$]
QLTFSSGWGNCTS-OH	1386.6	1387.6	1409.6	1425.6
pELTFSSGWGNCTS-OH	1369.6	1370.6	1392.6	1408.6

Fig. 7 – Deduced amino acid sequences of the precursors for AKHs and adipokinetic hormone-like peptides (AKH-L) of the fall armyworm. Sequences (A–D) encoding potential *Spodoptera frugiperda* AKH (A–C) and AKH-L (D) peptides are highlighted in bold on a shaded background. Potential signal sequences are shown in italics, and dibasic cleavage sites are underlined. The calculated masses and predicted mass ions (protonated, [M + H]$^+$, sodiated, [M + Na]$^+$ and potassiated [M + K]$^+$) for peptides potentially processed from each precursor are shown below each sequence. Precursor sequences for the fall armyworm are from data in [1].

m/z 977.5. The sequence of this peptide was revealed by MS/MS analysis as NMNFSPRL amide or perhaps (less likely) GGMNFSPRL amide. Both of these peptides have the theoretical protonated mass of 977.499, and would thus only be distinguishable in MALDI-TOF MS/MS by the y8 ion (920.48), b1 ion (58.03) and glycine immonium ion (30) of the GG peptide, none of which were clearly observed in the obtained spectra (data not shown). Additional ion peaks in the spectrum (Fig. 9B) at *m*/z 1229.7, 1369.7 and 1407.7 were detected as in the sample of CC from *M. brassicae* and are indicative of FLRF amide-I, Arg^7-corazonin and an unknown peptide, respectively.

4. Discussion

4.1. *Adipokinetic peptides*

This paper has a multifaceted approach. One objective was to detect AKHs in those species where genomic information existed but no peptide purification had taken place. It is clear from our data that both *T. castaneum* and *B. mori*, each contain and express two AKHs, which were predicted from the genomic information.

Despite the small size of the beetle, it was possible to dissect the retrocerebral glands, (corpora cardiaca and corpora allata) and perform MALDI-TOF-MS on a sample containing 1–3 gland pairs. The resulting spectra showed clearly the characteristic Na$^+$ and K$^+$ adducts for two AKHs. Employing HPLC coupled to ion trap electrospray MS, the complete sequence (including the ambiguity of the Leu^2/Ile^2 residue) was assigned and each peptide was confirmed as having the sequences predicted in the genome. The two AKHs in *T. castaneum* are both octapeptides, one (Trica-AKH) has a novel sequence (pELNFSTDW amide), whereas the other (pELNFTPNW amide) has the same sequence as a peptide found in the CC of the fire bug *Pyrrhocoris apterus* [35] and the grasshopper *Lamarckiana sparrmani* [15] and is therefore denoted Pyrap-AKH (Table 1). In contrast, Li et al. [39] report only one expressed AKH in *T. castaneum* by mass spectrometry, which they call *Tribolium* AKH 2, but which corresponds to Pyrap-AKH.

From a comparative point of view, both Pyrap-AKH and Trica-AKH are different from the AKH denoted Tenmo-AKH,

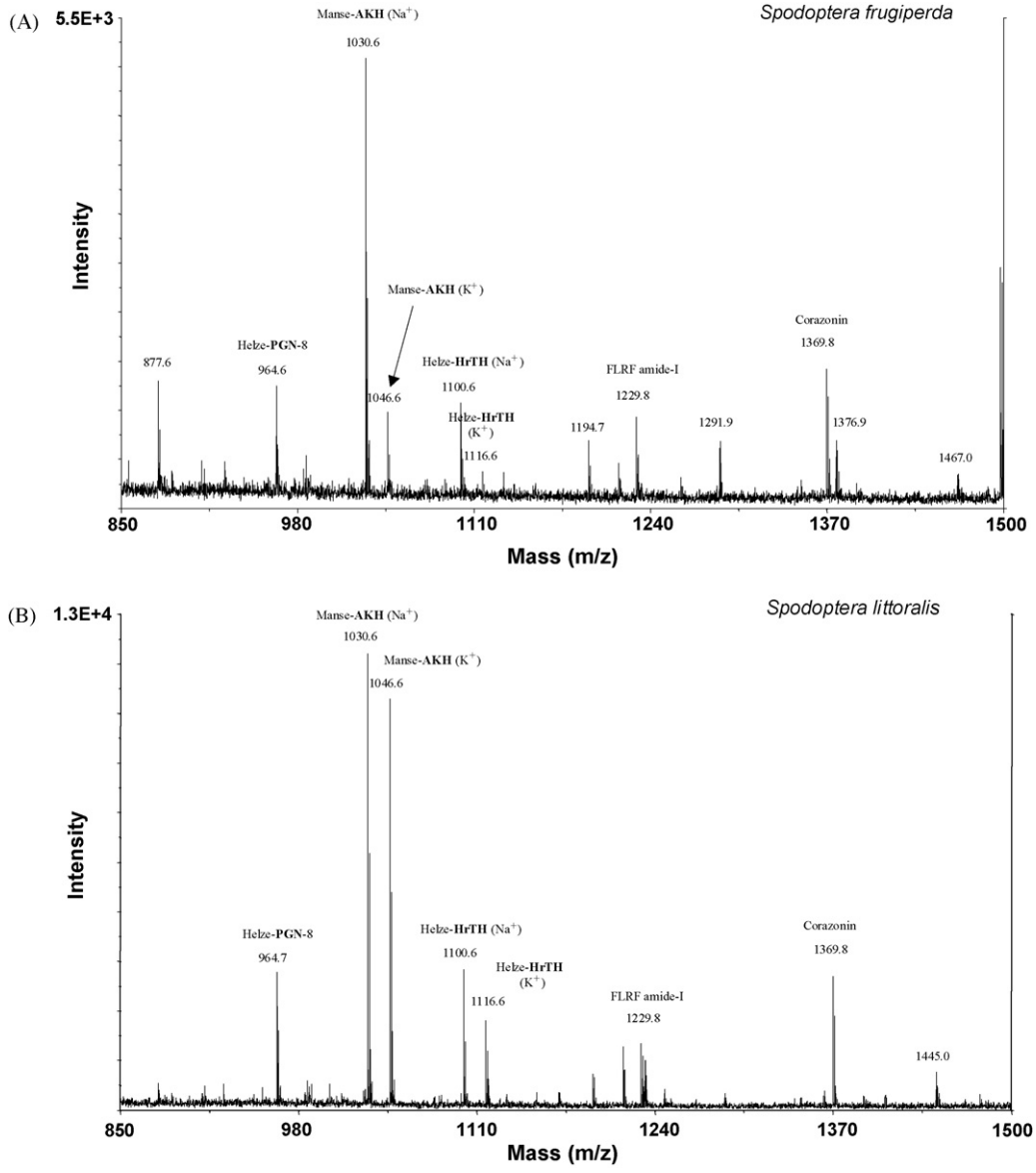

Fig. 8 – Annotated MALDI-TOF mass spectrum of single CC–CA complex from adult (A) *Spodoptera frugiperda* and (B) *Spodoptera littoralis*; the same conditions were employed as in Fig. 1. The annotated peptides were (mostly) confirmed by MS/MS analyses (see text). Manse-AKH, pELTFTSSWG amide; Helze-HrTH, pELTFSSGWGN amide; FLRF amide-I, pEDVVHSFLRF amide; corazonin, pETFQYSRGWTN amide; Helze-PGN-8, TMNFSPRL amide.

which is found in closely related tenebrionid beetles such as *T. molitor*, *Zophobas rugipes*, *Onymacris plana*, *O. rugatipennis* and *Physadesmia globosa* [10,21,53]. Tenmo-AKH has only one conservative amino acid exchange compared with Pyrap-AKH (S^5 vs. T^5), which can be explained by point mutation; thus only one base pair change (see Table 1). In contrast, there are two (albeit also point mutational) exchanges between Tenmo-AKH and Trica-AKH (P^6 vs. T^6, and N^7 vs. D^7; Table 1). The latter exchange is noteworthy because Trica-AKH with a D^7 is one of few AKHs that has a charged amino acid (for other examples see Table 1), whereas the majority of the ca. 45 known AKHs are not charged [12,20]. Structure–activity studies have suggested that certain AKH receptors (e.g. in *Locusta migratoria* and *Periplaneta americana*) do not recognize such charged molecules too well [9] and that, conversely, the AKH receptors in the fruit beetle *Pachnoda sinuata* (which has the peptide denoted Melme-CC) apparently show preference for the charged AKH [5]. Such data suggest that the two AKHs of *T. castaneum*, Pyrap-AKH and Trica-AKH, will very likely act via different receptors. At present, only one AKH receptor from *T. castaneum* is known [38] but it is not clear which of the two AKHs is the preferred ligand.

By comparison, *T. brevicornis* contains only one AKH and not the two that were found in its close relative, *T. castaneum*. In contrast, the AKH of *T. brevicornis* is identical to the one present in *T. molitor*. Using morphological characters, *T. brevicornis* was assigned the most primitive status in the *Tribolium* group [24]. More recently, by examining the

phylogenetic relationships of this group using molecular markers (sequencing parts of the mitochondrial DNA of cytochrome oxidase I and mitochondrial 16S rDNA), a very similar relationship has been suggested [43]. According to Meštrović et al. [43], the strict consensus and most parsimonious tree shows *T. brevicornis* close to the outgroup, which was *T. molitor*, but more distant from *T. castaneum*. This is congruent with our data on the AKH sequences of these three tenebrionid species. As shown below, mass data of other peptides from the CC of *T. brevicornis* are also indicative that this species is more closely related to the genus *Tenebrio* than to the genus *Tribolium*.

The second objective was to compare the AKHs of *B. mori* and *S. frugiperda* with those of other moth species. Our results reveal a number of trends for the AKHs in moths, which are: (1) in the species investigated two classical members of the AKH family are identified, although in *M. brassicae* and *L. oleracea* an additional, amidated Gly-extended peptide is also found; (2) one of the two peptides is always the nonapeptide Manse-AKH, and; (3) the silkmoth *B. mori* (Bombycidae) has a different second AKH, viz. Bommo-AKH, than the Noctuidae, which instead have Helze-HrTH (see Table 1 for structures). The structural similarity between Bommo-AKH and Helze-HrTH is that they are both decapeptides, however, they differ at three positions (5, 6 and 10; see Table 1). Interestingly, when Chinese Hamster Ovary cells which express the promiscuous G protein G16, aequorin and the AKH receptor of *B. mori* were tested with various peptides, Helze-HrTH and not Manse-AKH, which occurs in *B. mori* and has only two amino acid exchanges (positions 5 and 7; see Table 1) but is a nonapeptide, was the most potent [50]. From these results, Staubli et al. [50] concluded that there may be a second AKH present in the silk moth. It seems likely then that the characterized receptor is the one that binds Bommo-AKH and that the receptor for Manse-AKH has still to be identified.

Although we have detected the second AKH in *B. mori* which was predicted from the genome, we did not find the third predicted AKH expressed in *S. frugiperda*. The same peptide (pELTFSSGW amide), however, has been shown to occur in the CC of the nymphalid butterfly *Melittea cinxia*, has been sequenced by ESI-MS and named Melcin-AKH (G. Gäde, H. Fescemeyer and P. Šimek, unpublished results). This peptide shares the same eight amino acids with Helze-AKH and is possibly produced by gene duplication and deletion of two amino acids at the C-terminus.

If we consider data from all lepidopteran species that have been investigated to date, it is clear that Manse-AKH and a second AKH (Helze-AKH, Bommo-AKH, Melcin-AKH) are found in each species except in the tobacco hawkmoth, *Manduca sexta*, which was the first species in which Manse-AKH was completely structurally assigned [55]. It would be worthwhile to check other members of the family Sphingidae to which *M. sexta* belongs to clarify if this is characteristic for this particular lepidopteran family. However, *M. sexta*, which does not have a second AKH, contains a non-amidated C-terminally extended Manse-AKH [3] which is thought to be a result of incomplete processing of the Manse-AKH precursor molecule. Such extended forms deriving from the Manse-AKH precursor occur in a number of Lepidoptera [2,36].

In the current study, evidence is supplied for a novel form of Manse-AKH which should be considered as a novel AKH and, in time, will be given an appropriate designation; at present we refer to it as an amidated Gly-extended Manse-AKH. The rationale for this is the following: in *M. brassicae* and *L. oleracea* which are closely related (Sub family Hadeninae; Tribe Hadenini), mass ions at *m/z* 1087 and 1103 were prominent, and these mass peaks seemed always to have the same relative intensity as the Na^+ and K^+ adducts of the corresponding Manse-AKH, suggesting that they could also be Na^+ and K^+ adducts, but of a peptide of mass 1064.5. Analysis by ESI-MS also showed a corresponding $[M + H]^+$ peak at *m/z* 1065.5 and, hence, we first interpreted these mass peaks as adducts of a Gly-extended Manse-AKH. However, this interpretation entails some problems: the precursor of Manse-AKH in *M. sexta* is encoded as ...QLTFTSSWGGKRA... [6] or, in *S. frugiperda*, as ...ELTFTSSWGGRRP... [1], and the second Gly is used for amidation of the final AKH product of this precursor. If, in the case of *L. oleracea*, the precursor looks similar, then one would expect a non-amidated Gly-extended Manse-AKH as found and described in other species (see above); however, this is not supported by the mass data (pELTFTSSWGG-OH = *m/z* calc. 1066.48 protonated, 1088.48 sodiated, and 1104.48 potassiated). The peptide therefore seems to be C-terminally amidated (1 mass unit less), and this is confirmed by MS/MS sequencing. One could speculate that another enzymatic mechanism is taking place to achieve the amidation but such a mechanism would be novel and is not supported by any experimental data. The second possible interpretation of the current data is that there is a second precursor for Manse-AKH present in both *L. oleracea* and the cabbage moth, *M. brassicae*, which contains a third Gly residue (i.e. ...QLTFTSSWGGGKR...), and it is this that is used for amidation. Although this is the more plausible theory, further experiments to identify the AKH precursors in *L. oleracea* and *M. brassicae* are needed. The same mass ions for such an amidated Gly-extended Manse-AKH were observed in the CC of *M. brassicae*, but these were not investigated further (no MS/MS data) and were not interpreted in this way [7].

The current data on *S. frugiperda* lend support to a study by Garden et al. [23] on pGlu neuropeptides from the seahare *Aplysia*, in which the authors showed that pGlu can be produced from either Glu or Gln at the N-terminus. Previously it was shown that the precursor for Manse-AKH in *S. frugiperda* contains a Glu at the first position for the putatively processed AKH [1], instead of the more usual Gln as in all other known AKH precursor molecules. That this Glu is converted to a pGlu is confirmed by our MALDI results, which clearly demonstrate that the processed and expressed peptide is Manse-AKH, i.e. corresponding to a pGlu N-terminus.

Besides the three "classical" adipokinetic peptides which are produced in neurosecretory cells of the CC of *Locusta migratoria*, a fourth peptide which fitted somewhat into the characteristics of the AKH peptide family was first found in this species [48] and was more recently also shown to occur in the Malaria mosquito [32] (Table 1). By scanning the genomic information of *T. castaneum* and *B. mori*, a precursor coding sequence for structurally very similar peptides could be identified, one in each species (Table 1). However, none of the predicted masses from either peptide (Na^+/K^+ adducts,

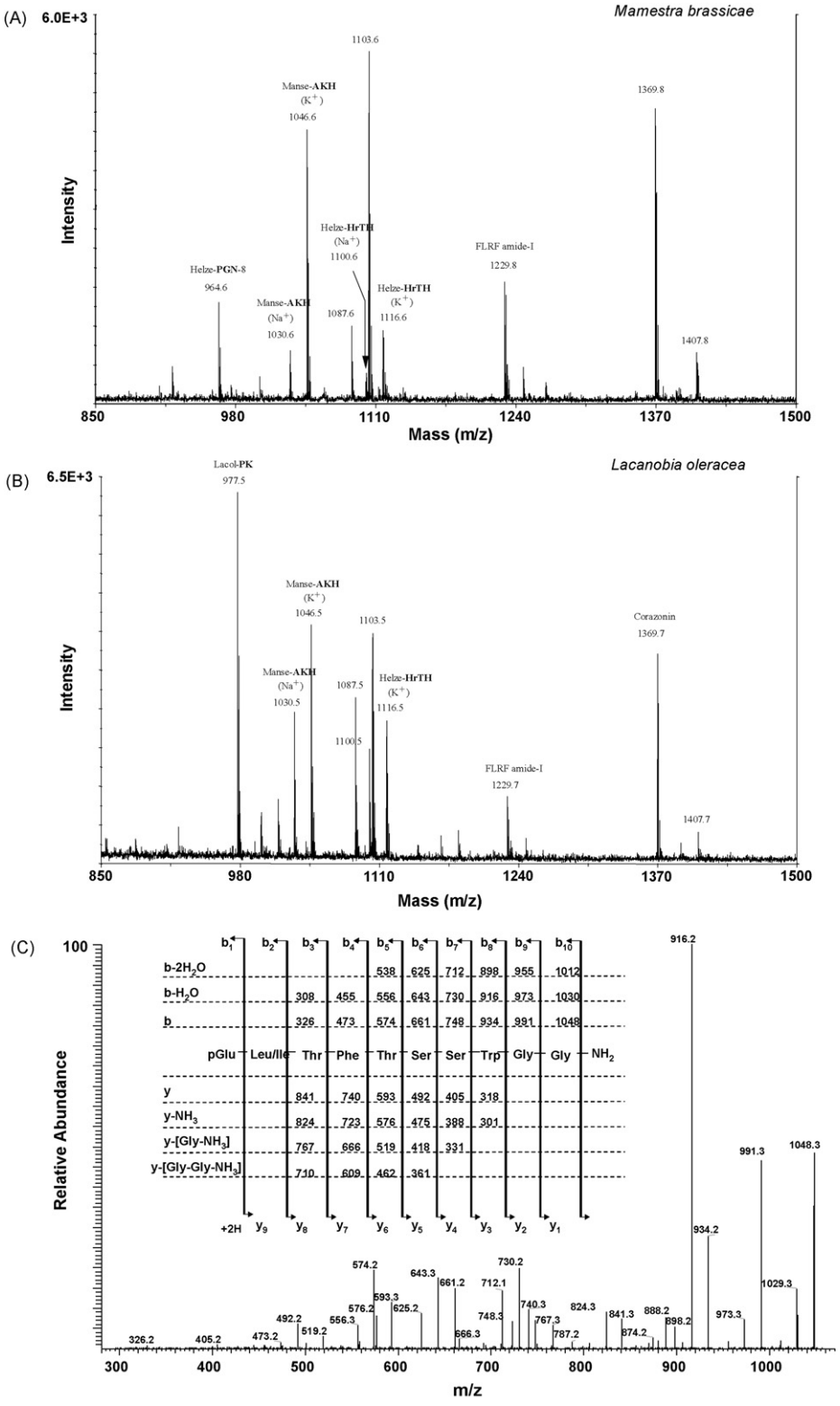

Fig. 9 – Annotated MALDI-TOF mass spectrum of single CC–CA complex from adult (A) *Mamestra brassicae* and (B) *Lacanobia oleracea*; the same conditions were employed as in Fig. 1. The annotated peptides were (mostly) confirmed by MS/MS analyses (see text). Manse-AKH, pELTFTSSWG amide; Helze-HrTH, pELTFSSGWGN amide; FLRF amide-I, pEDVVHSFLRF amide; corazonin, pETFQYSRGWTN amide; Lacol-PK, NMNFSPRL amide. (C) The CID-MS^2-ESI spectrum of the ion [M + H]$^+$ 1065.5 from *Lacanobia oleracea* CC–CA. The inset shows the sequence of the assigned peptide, together with the theoretical calculated masses for b-type and y-type fragment ions which are observed in the MS^2 mass spectrum.

protonated ion, or partially processed products) were detected in our study. The reason may be simple. In the migratory locust, *L. migratoria*, it is believed that this AKH-like peptide is synthesized in the neurosecretory cells of the brain, rather than the CC, since it is only present in the clearly distinguishable storage lobes of the CC of this insect which contain the axon endings of cells originating in the brain [48]. In the present study we did not analyze brain tissue only CC/CA. Again, Li et al. [39] have also not found this peptide in their study on *T. castaneum* with mass spectrometric techniques.

4.2. Other peptides

In general, the following is clear from the present study: (1) in beetles and moths, many of the peptide masses identified in the CC belong to the so-called pyrokinin (PK)/myotropin (MT) peptide family; these peptides are mainly characterized by the carboxyl-terminal sequence F/YXPRL amide and, in some cases, by pGlu at the N-terminus (see reviews by [12,20]). Peptides that are derived from a common precursor also belong to this group of neuropeptides, and are called variously pheromone-biosynthesis-activating neuropeptide (PBAN), diapause hormone (DH), puparium accelerating factor, and melanization and reddish-coloration hormones (MRCH) – which are all named according to the major biological effect they exert; (2) the mass spectra for tenebrionid beetles are different from those of the moths investigated in this study; none of the major masses identified occur in the other group. Hence, we discuss these two orders separately.

In *T. castaneum*, but not in the other two tenebrionid beetles, we found mass ions at *m/z* 952.3 and 974.2. Fragmentation of the peak at *m/z* 952.3 produced fragment ions that sufficed to identify the peptide as the *T. castaneum* NVP-like peptide 4 (GRWGGFADS-OH), which can be deduced from the NVP-like putative transcript [39; online supplementary data]. In the honey bee, the NVP-peptide precursor was named because one of the derived peptides possesses an ''NVP'' motif [27]. If the NVP-like peptide precursor of *T. castaneum* is homologous to the NVP-containing peptide precursor of *A. mellifera*, then we expect that other peptides from this precursor would also be expressed. Other peptides predicted from this precursor would have monoisotopic masses $[M + H]^+$ 1516.8, 2167.2 and 2563.3, plus 739.3, 750.4, 1360.7 and 1940.0, including truncated or partial sequences. Although the present paper focuses only on peptides smaller than 1500 Da, we can report that we observed mass peaks in *T. castaneum* that correspond to NVP-l (IPASLVEEIKTNELRNNKH-OH; *m/z* 2167.2), NVP-$l^{(1–17)}$ (IPASLVEEIKTNELRNN-OH; *m/z* 1940.1), and NVP-2 (AHPQLNVGEHGREVPYYSKPTAI-OH; *m/z* 2563.3) in spectra obtained using the Bruker Ultraflex (data not shown). Together with the observed and sequenced NVP-like 4, it seems highly plausible that the NVP-like peptide precursor is, indeed, expressed in the CC–CA of adult *T. castaneum*.

As mentioned previously, there are at least three possible identities for the peak at *m/z* 974.2, in which peak intensity was low and did not yield sufficient fragment ions for sequence determination. The peak may represent the Na^+ adduct of GRWGGFADS-OH, or similar adduct of the *T. castaneum* tachykinin, APSGFMGMR amide ($[M + H]^+ = m/z$ 952.45), of which three copies occur in the genomically derived precursor sequence [39]. An alternative suggestion is that the peak represents the short neuropeptide F (SPSLRLRF amide) of *T. castaneum* in its protonated form ($[M + H]^+ = m/z$ 974.59 calc.).

In all three of the beetle species examined in the current study, we find the peptide with a mass ion 883.5 and structure SPPFAPRL amide, which was first sequenced from the CC–CA complexes of *P. americana* and was denoted Peram-PK-2 although it is not strictly a pyrokinin as it lacks a pGlu residue [44]. As stated above, PKs belong to the F/YXPRL amide family, and are known for a number of quite diverse functions, such as regulation of pheromone and pigment synthesis, muscle activity and induction of diapause [12]. In *T. brevicornis* (this report) and *T. molitor* [53] two more PKs were identified and are named Tenmo-PK-1 and -3 according to previous research [53]. One of these peptides, Tenmo-PK-1, is genomically predicted to occur in *T. castaneum*; in the current study the deduced peptide could not be confirmed, but was observed in the work by Li et al. [39]. The *T. castaneum* equivalent of Tenmo-PK-3 is a single amino acid variant (Trica-PK-3; HSSPFSPRL amide) that would yield a mass ion at *m/z* 1026.5 $[M + H]^+$. Again this could not be clearly differentiated in our present study, but has been reported by Li et al. [39]. Both *T. brevicornis* and *T. molitor* contain the peptide leucomyosuppressin (Leuma-Ms), which was first detected in the cockroach *Leucophaea maderae* where it inhibits the spontaneous contraction of the hindgut [25], but was also shown to occur in CC–CA complexes of *A. mellifera* [4]. The apparent absence of Leuma-Ms from the CC of *T. castaneum*, as revealed in the present study may reflect a further difference between the tenebrionid species, alternatively it could be due to temporal specificity and relatively limited sample size. In conclusion then, it appears that the complement of identifiable masses in *T. brevicornis* is in agreement with those found in *T. molitor* rather than in *T. castaneum* which, again, points to a more distant relatedness between the two species of the same genus as discussed above (see Section 4.1). From the current study, it is also clear that certain neuropeptides, such as corazonin and FLRF amides, are not expressed in beetles, an observation that has also been noted in other reports [39,53].

In the lepidopteran CC–CA, two peptides are always found: these are FLRF amide-I (pEDVVHSFLRF amide) and Arg^7-corazonin (pETFQYSRGWTN amide). FLRF amide-I was first isolated from nerve cord of *M. sexta* [34], it is similar in sequence to the above mentioned leucomyosuppressin, can be found in its modified (pGlu) or unmodified (Gln) form and has been shown to occur in most insects and specifically in all lepidopteran CC and brain preparations previously investigated, including a brain preparation from day 0 pupae of *B. mori* [3,7,20,28,54]. In the silk moth this peptide is structurally identical to the FLRF amide-I molecule, yet it was misguidedly re-named Bommo-myosuppressin [54]. Functionally, however, FLRF amide-I of *B. mori* has been shown to have a novel inhibitory action, viz. on ecdysteroidogenesis [54].

Arg^7-corazonin was first characterized as a cardioactive peptide from the CC of *P. americana* [52] but later shown to regulate the dark coloration in a number of insects, reduce the rate of spinning silk in the silk moth and to affect initiation events during ecdysis; other isoforms have been identified but none of these are present in lepidopteran CCs [45].

In all lepidopteran species investigated in the present study, we found a peptide that is part of a large preprohormone which encodes mainly for the diapause hormone (DH) and the pheromone biosynthesis activating neuropeptide (PBAN). In three out of the five species investigated we detect the mass of 964.6 (and in one instance its additional sodiated and potassiated forms in *S. littoralis*) and identified this peptide as TMNFSPRL amide which was named Helze-PGN-8, Mambr-γ-NP or Spoli-γ-NP and identified as part of the gene encoding Helze-, Mambr- or Spoli-PBAN [29,30,42]. A bioanalog of this peptide with an N^3 to S^3 substitution was assigned in *B. mori* (ion signal at *m*/z 937.6), has the structure TMSFSPRL amide, is code-named Bommo-γ-SNP and is located on the precursor that encodes for DH as well as PBAN in the silk moth [46]. In *L. oleracea* the mass ion peak of 977.9 is with all likelihood interpreted as NMFSPRL amide. This represents only a single amino acid substitution from the analog Helze-PGN-8 (see above) from T^1 to N^1 which is a conservative substitution requiring only a single nucleotide change (ACU or ACC to AAU or AAC). This novel sequence is named Lacol-PK and is presumed to be derived from the PBAN/DH preprohormone of *L. oleracea*, which has not been identified yet. Finally, the signal at *m*/z 975.5 in *B. mori* was assigned as the peptide CAP_{2b} which was first characterized in *M. sexta* as a cardioactive peptide [26] and has been found in the brain of *M. sexta* and *L. oleracea* as well [2].

In conclusion, this study of the expressed AKHs and other small peptides in three species of beetles and five species of moths has confirmed the presence of certain predicted peptides in the CC–CA, and shown the likely absence of others. It has revealed both commonalities and differences between members of the same order, and in some cases significant differences between apparently closely related species. Several new peptides have been identified, although all except the NVP-like 4 of *T. castaneum* belong to previously well-described peptide families. Two of the beetle species have only one AKH, while *T. castaneum* and the Lepidoptera have at least two each. In the case of the tomato moth, *L. oleracea*, and cabbage moth, *M. brassicae*, a new amidated form of Gly-extended peptide has been confirmed as a possible third AKH.

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