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New insect order Mantophasmatodea: species differentiation by mass fingerprints of peptide hormones?

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Abstract

We analysed peptide hormones of South African species of the recently described insect order Mantophasmatodea. Mass spectrometric techniques made it possible to analyse minute amounts of material stored in different neurohemal organs of single specimens. The methodological approach underlying this application is described and resulted in the first completely sequenced peptide hormones of the Mantophasmatodea, namely adipokinetic hormone, leucomyosuppressin, and a novel member of the periviscerokinins. The extensive peptide screening revealed a surprisingly clear differentation of species by peptide mass fingerprints. This is the first successful attempt to catalogue neuropeptides of insects for systematic/taxonomic purposes. In total, 14 of 32 peptides differed across species. Moreover, the data presented here show a hierarchy in the tendency for modifications among the different peptide hormones.

Key words: insects – Mantophasmatodea – neuropeptides – mass spectrometry

Introduction

The discovery of the new insect order Mantophasmatodea (Klass et al. 2002) immediately raised questions about its phylogenetic relationships. Based on morphological and DNA sequence studies, Mantophasmatodea ordinal status and monophyly was confirmed, and phylogenetic affinities with some of the lower Neoptera [Dictyoptera, Phasmatodea, Notoptera (=Grylloblattodea)] (Klass et al. 2002, 2003a,b) determined. Notoptera, a small relict group with a preference for low temperatures, are secondarily wingless like the Mantophasmatodea. Very recently, the Notoptera have been postulated to be the sister group of the Mantophasmatodea, on the basis of DNA sequence data (Jarvis and Whiting 2003).

We started a comprehensive study of peptide hormones from Mantophasmatodea in order to compare the obtained sequences with those of related insects groups. Such an approach can contribute to a better understanding of neuropeptide evolution. In this specific context, however, we focussed on the potential of peptide sequences for taxonomic and systematic purposes within the Mantophasmatodea. Neuropeptides are produced in the nervous system, and form the most diverse group of messenger molecules in animals. In contrast to many morphological characters, sequences of peptide hormones are less influenced by ecological factors. Their role as ligands, which usually act via specific receptors, limits their ability to undergo drastic sequence modifications without loss of function. A much slower co-evolution of receptors and the respective ligands, however, occurs. In invertebrates, a number of neuropeptide families contain multiple forms, which are often expressed from a single precursor gene. This multiplicity obviously enables single peptide forms to undergo sequence modifications without hampering the general functions of the neuropeptide family to which they belong. Its evolutionary advantage is still under debate, nevertheless the multiplicity of peptide forms produces a wealth of group specific sequences, that can possibly be used for phylogenetically oriented research.

The shortness of sequences of most neuropeptides makes identification of peptide-encoding DNA a very complicated task, and often fails even if the complete sequences of encoded peptides are known, such as in well studied cockroach and locust species (Schoofs et al. 1997; Predel and Eckert 2000a). Thus, direct analysis of expressed peptides is often preferable over gene sequencing. As shown in this study, mass spectrometric techniques make it possible to analyse minute amounts of material stored in different neurohemal organs. These hormone release sites accumulate a majority of neuropeptides produced in the central nervous system.

This paper deals with the South African species of Mantophasmatodea. While three species are known from Namibia and one from Tanzania (Klass et al. 2003a,b) the majority of species are known from the winter-rainfall area of the Western and Northern Cape Province, which is likely to be the distribution centre of these insects; with eight described and numerous additional undescribed species (Picker et al. 2002; Klass et al. 2003a,b). The extensive peptide screening in Mantophasmatodea revealed a clear differentiation of species by peptide mass fingerprints. In addition, the peptide hormones show a very different tendency to sequence modifications. Thus, sequences of more conserved peptides can be used for group recognition, whereas other peptides are typical of individual species or species groups only. This is the first attempt to analyse neuropeptides for taxonomic purposes. Underlying sequences, necessary for phylogenetic reconstructions, will be analysed in a next step. The methodological approach, however, is described in this manuscript and resulted in the first completely sequenced peptide hormones of the Mantophasmatodea.

Materials and methods

Insects

In September 2003, living Mantophasmatodea were collected from 10 locations in the Western and Northern Cape Provinces of South Africa (Fig. 1). Most (9) of the localities fell within the succulent karoo biome. Of the karoo localities, Driefontein (Northern Cape, 32.02S 19.22E) represented a described species (*Karoophasma biedouwensis*; Klass et al. 2003a,b). Additional material obtained from the succulent karoo was collected from: Steinkopf, Northern Cape, 29.266S 17.714E

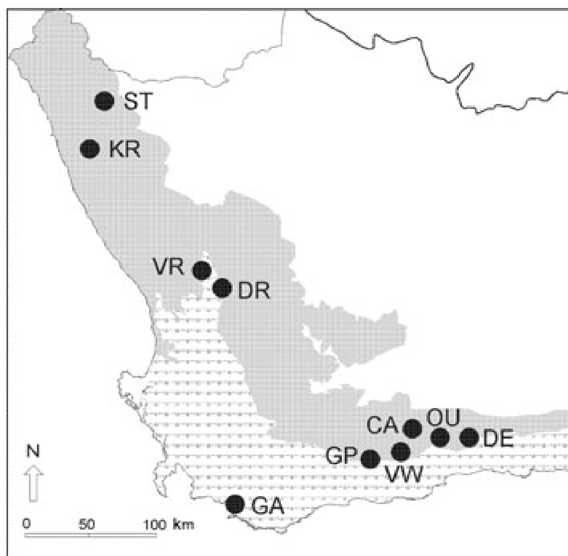

Fig. 1. Localities for Mantophasmatodea material sampled. All material was collected from either the succulent karoo biome (fine stippling) or fynbos biome (dispersed stippling). ST, Steinkopf; KR, Karootjie; VR, VanRhynsdorp; DR, Driefontein; GA, Gansbaai; GP, Garcia Pass summit; VW, VanWyksvlei; CA, Calitzdorp; OU, Oudtshoorn; DE, De Rust

(*Namaquaphasma cf. ookiepensis*); Karootjie, Northern Cape, 29.808S 17.355E (*N. cf. ookiepensis*); Vanrhynsdorp, Northern Cape, 31.666S 18.893E (*Karoophasma* spec. 2); De Rust, Western Cape, 33.50S 22.527E; Garcia Pass, Western Cape, 33.80S 21.133E; VanWyksvlei, Western Cape, 33.729S 21.6E; Calitzdorp, Western Cape, 33.519S 21.844E; and Oudtshoorn, Western Cape, 33.593S 22.181E. The last four localities are close geographically, and likely represent populations of a single species, *Hemilobophasma cf. montaguensis* (Klass et al. 2003a,b). Specimens from De Rust belong to an undescribed species of *Hemilobophasma* or *Austrophasma* (K.D. Klass, pers. comm.) and will be treated as *Hemilobophasma* spec. 2 throughout the text. Material of *Austrophasma gansbaaiensis* was obtained from the fynbos biome of the Western Cape (Gansbaai, 34.54S 19.41E). Living specimens were subsequently placed in separate plastic containers and fed with *Drosophila*. Some Northern Cape material (Steinkopf, Karootjie, Vanrhynsdorp) was found as second/third larval instars and kept in the laboratory until they reached adulthood; mainly to make dissection of neurohemal organs easier. After dissection of neurohemal organs, remains of the insects were kept in 70% ethanol and sent together with intact specimens from the same location to K.D. Klass (Dresden, Germany) for determination. The designations of the different species used in this study result from his preliminary investigation of the material, whereas KDK was blind to our results. A revision of the South African Mantophasmatodea, which also includes the specimens investigated herein, is in preparation (K.D. Klass, pers. comm.).

Dissection of neurohemal organs and sample preparation for mass spectrometry

Body cavities of decapitated insects were opened under a stereomicroscope, parts of the nervous system and adjacent neurohemal release sites were made visible by removal of other tissues, and flushed with insect saline. Neurohemal organs were dissected rapidly using fine scissors and transferred to a sample plate for matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. On the sample plate, the dried organs were rinsed with a drop of pure water which was removed using cellulose paper. Subsequently, a limited amount of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water) was pumped onto the dried preparations over a period of approximately 10 s using a Nanoliter injector (World Precision Instruments, Berlin, Germany). This method resulted in sufficiently small spots covered with matrix solution and prevented a dilution of the peptides by spreading over the sample plate. Each preparation was allowed to dry and then covered with pure water for 30 s, which was than again removed by cellulose paper.

Aqueous extracts (5 μl) of corpora cardiaca/abdominal PSOs for tandem mass spectrometry on an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer were shortly sonicated, centrifuged, and the supernatant loaded onto a home-made micro column (purification capillary for electrospray mass spectrometry).

Mass spectrometry

MALDI-TOF mass spectrometry

Mass spectra were obtained using a Voyager DE biospectrometry workstation (Perseptive Biosystems, Framingham, MA, USA). Positive-ion mass spectra (800–3000 Da) were measured in the reflectron mode with a delay time of 150 ns, 20 kV accelerating voltage, 75% grid voltage and 0.1% guide wire voltage. Spectra are the unsmoothed average of about 100 scans. An external mass spectrum calibration was performed using the cockroach peptides *Periplaneta*-pyrokinin-2 and -5 [monoisotopic $(M + H)+$: 883.4; 1651.8]. Identified *Mantophasmatodea* peptides were later used for internal mass calibration.

MALDI-TOF-TOF mass spectrometry

Mass spectra were obtained using a 4700 Proteomics Analyzer with TOF/TOF optics (Perseptive Biosystems). Identified peptides were selected and subsequently fragmented, which resulted in collision induced dissociation (CID) fragment ion series.

ESI-Q-TOF mass spectrometry

Nanoelectrospray mass spectra were acquired in the positive-ion mode using the API Qstar Pulsar (Applied Biosystems, Applera Deutschland GmbH, Darmstadt, Germany) fitted with a Protana (Odense, Denmark) nanoelectrospray source. Typically 950–1000 V was applied as an ionspray voltage. Samples were purified using a homemade spin column. Approximately 1–2 mm of Poros R2 (PerSeptive Biosystems) was loaded into a 2 cm capillary column with a needle tip. Liquids are passed through the column by securing the capillary column to a purification needle holder (Proxeon Biosystems A/S, Odense, Denmark) and centrifugation. After the column was equilibrated in 5% formic acid, the samples were loaded and rinsed with 5% formic acid. Peptides were eluted from the column with 30% methanol (5% formic acid) and collected into a metal coated nanoelectrospray capillary. The purified samples were then loaded onto the source and analysed. After determining the m/z of the peptides in MS mode, a collision energy (10–40 V) was applied. The m/z of interest was isolated and fragmented with the instrument in 'enhance all' mode. MS/MS data were acquired over 3–10 min and manually analysed.

Immunocytochemistry (peroxidase antiperoxidase technique)

The central nervous system with the attached PSOs was removed and fixed for 6 h at 4°C in 0.1 M phosphate buffered saline (pH 7.4) containing 4% formaldehyde. The fixed tissue was dehydrated in ascending alcohol series (each step 15 min), cleared in xylene and blocked for endogenous peroxidase in a mixture of 1% H_2O_2 in methanol. After rehydration in a descending alcohol series, the nervous system was placed in 0.1 M TRIS-HCl buffer (pH 7.6) containing 3% NaCl and 1% Triton X-100 (Tris-NaCl-TX) and digested in 0.01% collagenase–hyaluronidase–$CaCl_2$ (Sigma-Aldrich, Steinheim, Germany) dissolved in 0.1 M Tris-HCl buffer (pH 7.6) for 6 min at 37°C. Digestion was stopped with 0.01 N HCl in 0.9% NaCl (2 h) and the tissue was washed in Tris-NaCl-TX for 1 h. Anti-Pea-PVK-2 serum (Predel et al. 1998) was applied for 48 h at room temperature in a dilution of 1:2000 in phosphate buffered saline, containing 0.25% bovine serum albumin. After washing in TRIS-NaCl-TX for 48 h at 4°C, the tissue was incubated with goat anti-rabbit IgG (diluted 1 : 20 in

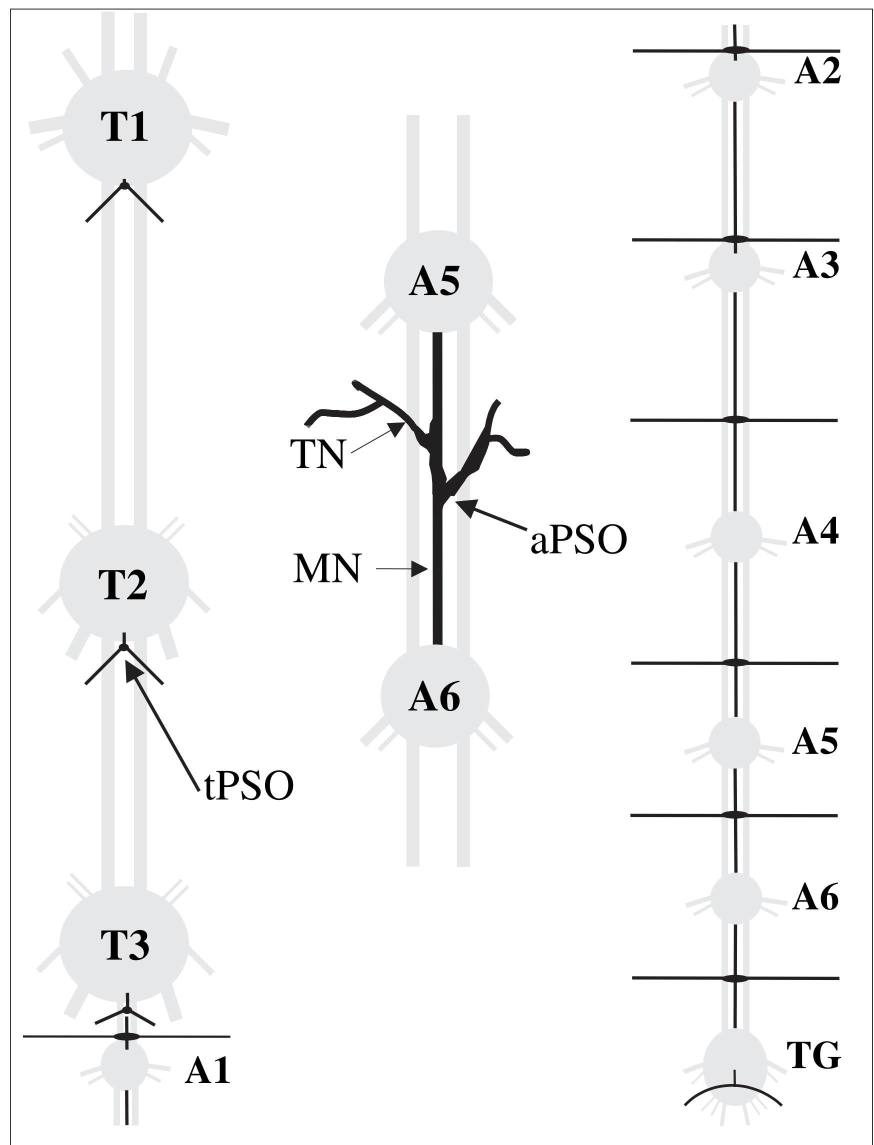

Fig. 2. Semi-schematic drawing of the ventral nerve cord of adult Mantophasmatodea (*Hemilobophasma cf. montaguensis*); size and distances between the ganglia are given in correct proportions. Abdominal perisympathetic organs are mainly located at the junctions of median nerves and transverse nerves (TN). Transverse nerves leave the median nerve somewhat displaced in these insects (see enlarged section). A, abdominal ganglion; MN, median nerve; T, thoracic ganglion; TN, transverse nerve; TG, terminal ganglion

phosphate buffered saline-bovin serum albumin, for 24 h). After washing (Tris-NaCl-TX, 12 h), the ganglia were placed in peroxidase antiperoxidase for 24 h (diluted 1:400 in Tris-HCl-TX). After another washing procedure, the tissue was incubated with 3.3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. Finally, the preparation was embedded in glycerol.

Statistical analysis

Similarity patterns of species according to the presence–absence-data of the distinct mass signals of peptides were explored using principal component analysis (PCA), which was performed with CANOCO 4.5 (ter Braak and Smilauer 2002).

Results

Neuropeptide inventory of neurohemal organs (*Hemilobophasma cf. montaguensis*)

Major neurohemal organs of insects are the corpora cardiaca (CCs), which store neurosecretions of the brain, and segmentally arrayed perisympathetic organs (PSOs). Most insects possess three thoracic PSOs and a variable number of abdominal PSOs. The total number of abdominal PSOs in an insect usually depends on the degree of fusion of abdominal neuromers in the ventral nerve cord.

All species of Mantophasmatodea used in this study possess six unfused abdominal ganglia and a terminal ganglion which is the fusion product of abdominal neuromeres 7–11. The exact location of the three thoracic and eight abdominal PSOs is shown in Fig. 2. With the exception of adipokinetic hormones, which are produced in the glandular portion of the CCs, all abundant neurohormones stored in the different neurohemal organs are synthesized in neurosecretory cells of the central nervous system.

Specimens of *Hemilobophasma cf. montaguensis* from Oudtshoorn (OU; see Fig. 1) were chosen for the initial study of neuropeptides. The data obtained from these insects formed the basis for a subsequent comparison of peptides between the different populations. *H. cf. montaguensis* from the Oudtshoorn area were selected because of the large sample available for initial studies. MALDI-TOF mass spectra of single abdominal PSOs revealed the appearance of a number of putative

peptides in the mass range of 800–3000 Da (Fig. 3). Abundant signals, which were consecutively numbered as putative abdominal PSO-peptides 1–8, were found in each of the eight abdominal PSOs. Completely different substances were detected in thoracic PSOs (Fig. 4). Again, each of the three thoracic PSOs contained, however, an identical set of putative peptides, named thoracic PSO-peptides 1–14. Finally, the CCs were screened for neuropeptides. In contrast to PSO-preparations, mass spectra showed somewhat different relative abundances of ion signals between the individuals. Putative peptides, clearly recognizable in all mass spectra from CC-preparations, are designated as CC-peptides 1–10 (Fig. 5). A comparison of males and females revealed no sex-specific substances among the designated putative peptides.

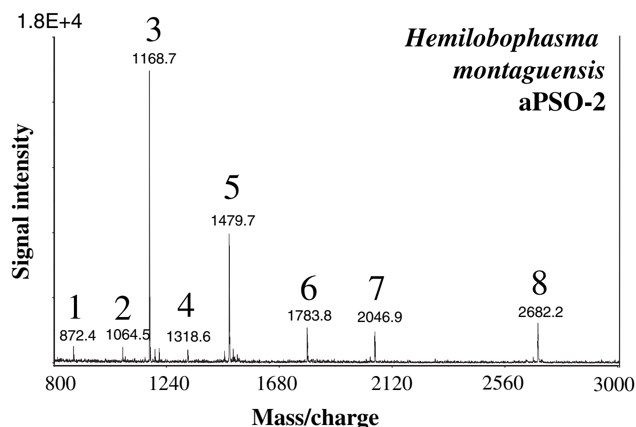

Fig. 3. MALDI-TOF mass spectrum from a preparation of a single abdominal PSO (aPSO-2) of an adult *H. cf. montaguensis* female (OU). The peptides repeatedly observed in the different abdominal PSOs are numbered. Signal intensity not only depends on the amount of a given peptide but is influenced by the presence of basic amino acids (particularly arginine; see Predel 2001) as well as the length of the peptide sequences. Thus, correct interpretation of relative abundances of the different designated peptides is impossible without knowing the sequences

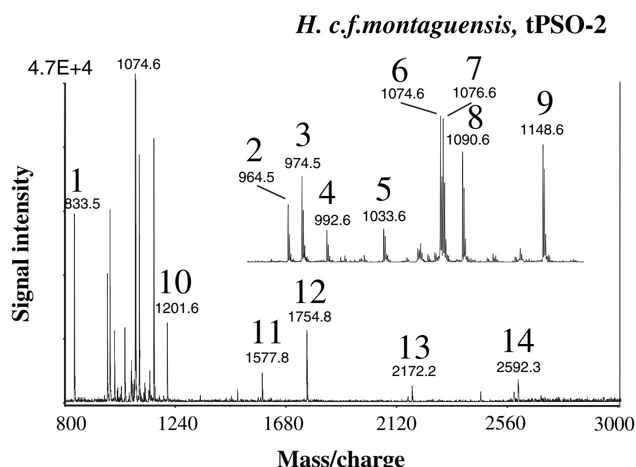

Fig. 4. MALDI-TOF mass spectrum from a preparation of a single thoracic PSO (tPSO-2) of an adult *H. cf. montaguensis* female (OU) (inset: mass range of 930–1170). The peptides repeatedly observed in the different thoracic PSOs are numbered. Note that not a single mass peak is identical with a mass peak observed in abdominal PSO-preparations (Fig. 2). Moreover, not a single of the 22 designated peptides from abdominal and thoracic PSOs is mass-identical with an already known insect neuropeptide

De-novo sequencing of the most abundant neuropeptides from the CCs and abdominal PSOs

Extracts of six CCs and 16 abdominal PSOs of *H. cf. montaguensis* (OU) were prepared for ESI-QTOF mass spectrometry, which operates with liquid samples. In the CC-extract, the double charged ion signals of the putative CC-peptide 5, as well as a single charged ion signal of CC-peptide 1 were detected and subsequently fragmented by tandem mass spectrometry (Fig. 6). Analysis of these fragment series confirmed the identity of CC-peptide five with leucomyosuppressin (pQDVDHVFLRF-NH_2; Holman et al. 1986). CC-peptide 1 turned out to be a member of the adipokinetic hormones (Psi-AKH., Gäde 1997) with the sequence of pQVNFTPGW-NH_2.

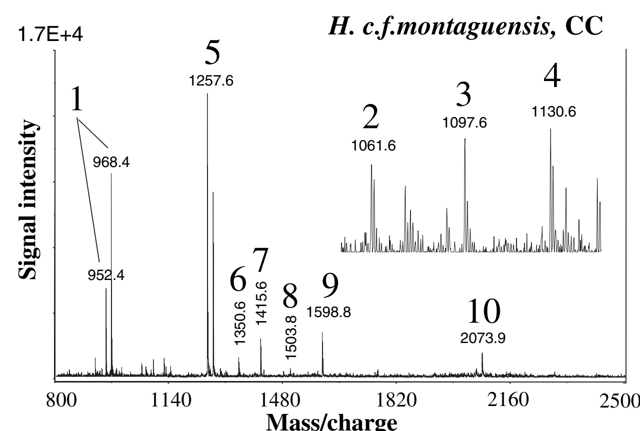

Fig. 5. MALDI-TOF mass spectrum from a corpora cardiaca preparation of an adult *H. cf. montaguensis* female (OU). Among the less abundant ion signals some variations occurred in different specimens; only those peptides regularly occurring are designated. The two ion signals at 952.4 and 968.4 (1) represent the Na^+- and K^+-adduct ions of a neutral peptide (Fig. 6). All remaining peptides, including those of the PSOs, are represented by their $[M+H]^+$-ions

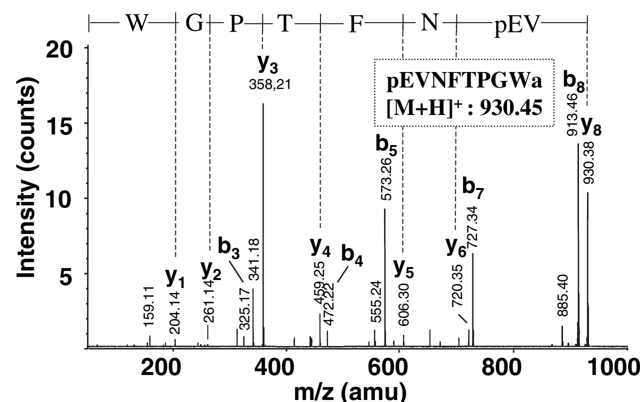

Fig. 6. Collision induced dissociation spectrum of the CC-peptide 1 (Fig. 4) following electrospray ionization. The *y*- and *b*-type fragment ions (C-terminal and N-terminal fragment series, respectively) are labelled. The fragments were analysed manually and the resulting sequence is given in the inset. The peptide represents a member of the adipokinetic hormones

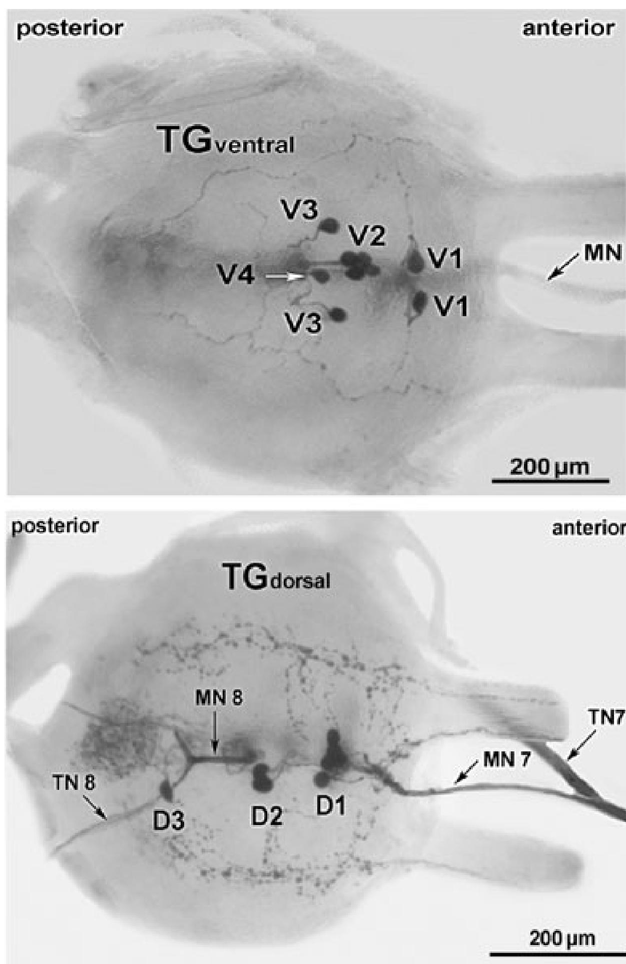

Fig. 7. Microphotograph of periviscerokinin-like immunoreactive cells that project to the PSO-7 and -8 of the terminal ganglion (*H. cf. montaguensis*, OU). The immunostained axons leave the ganglion via the median nerves, and supply the PSO with neurosecretions. V, ventral neurosecretory cells; D, dorsal neurosecretory cells

The same procedure was repeated with the extract of abdominal PSO. From the extract of abdominal PSOs; only abdominal PSO-peptide-5 was detected and subsequently fragmented. Resulting fragment series revealed a novel peptide of the periviscerokinin-family (Wegener et al. 2001) with the sequence of EAAGLIPFPRV-NH_2. Leucin/isoleucin cannot be distinguished by this method due to nearly identical masses. Remaining substances earlier obtained in MALDI-TOF mass spectra were not detected with that method. By means of an antiserum raised against a cockroach periviscerokinin (Predel et al. 1998), we could specifically stain the median neurosecretory cells in abdominal ganglia with their neurites into PSOs (Fig. 7). These immunoreactive neurons are likely the source of most if not all of the neuropeptides which were obtained in mass spectra from abdominal PSOs.

Another approach to analyse sequences of the putative peptides was performed on a *state of art* MALDI-TOF-TOF mass spectrometer using the same sample preparation (single organs) as described above for MALDI-TOF mass spectrometry. With that method we were able to confirm, that all designated substances from the different neurohemal organs are indeed peptides. The complete analysis of the fragments, however, is even more difficult than that of ESI-Q-TOF fragment series and was beyond the scope of this paper.

Comparison of neuropeptides from different species/populations of South African Mantophasmatodea

Although only a few specimens could be collected from most localities, mass spectrometric analysis of neurohemal organs was successful in nearly all cases. A summary of the measured peptides of Mantophasmatodea from the 10 localities is given in Table 1. Attempts to screen the neuropeptides from larvae did not reveal a peptide inventory different from that of adults (reared from larvae) from the same locality.

Most Mantophasmatodea collected from the different localities turned out to be easily distinguishable by peptide mass fingerprints. A wide range of differences between the masses of the respective peptide hormones could be detected. *Namaquaphasma cf. ookiepensis* from two different locations in the northern parts of the succulent karoo (ST, KR) could be separated by a single different peptide form only. *N. cf. ookiepensis* (ST), however, differed from *H. cf. montaguensis* (OU) by not less than 14 of 32 peptides (Fig. 8). Completely identical peptides were found in *H. cf. montaguensis* specimens from the four geographically close Little Karoo localities (GP, CA, OU, VW) although the VanWyksvlei site lies at a higher altitude, and is characterized by fynbos as opposed to karooid vegetation. This indicates that the same species may occur in more than one vegetation type. As shown in Table 1, variations among peptides are not uniformly distributed among the 32 peptides but are focused on certain peptide forms with an obvious tendency to distinct sequence variations. To a certain degree, peptide variations can be grouped according to the geographical distribution of the Mantophasmatodea populations sampled (Fig. 9). However, the specimens of *Hemilobophasma* spec. 2 collected at the most eastern point of the current known distribution of South African Mantophasmatodea (DE) expressed a peptide inventory more closely related to that of *Karoophasma biedouwensis* than to the neighbouring *H. cf. montaguensis* populations of the Little Karroo.

Discussion

This study describes the first successful attempt to catalogue neuropeptides of insects for systematic/taxonomic purposes. The data from peptide hormones which could be repeatedly observed in mass spectra from neurohemal organs of Mantophasmatodea made it evident that the different species expressed species–specific combinations of peptides. In addition, a number of peptides were identical in all species studied, but not known from other insects such as well studied cockroaches and locusts. Possibly these peptides are unique to Mantophasmatodea and autapomorphic for the order, or possibly synapomorphic for the South African species. In most cases there was good general agreement between neuropeptide mass fingerprints, and the taxonomy and cladistic relationships between the species examined. For example, the mass fingerprint of *K. biedouwensis* most closely resembled that of *H. montaguensis*, sister taxa on DNA sequencing (Klass et al. 2003a,b). The greatest fingerprint differences are observed between *N. ookiepensis* and the other species. Unfortunately, the systematics of this and other populations is not yet resolved, but it is expected that *Namaquaphasma* will be distantly related to other South African Austrophasmatidae. The apparent anomaly where *K. biedouwensis* and *K.* spec. 2 have quite different mass fingerprints awaits the systematic study of the Vanrhynsdorp material; its is possible that these

Table 1. Summary of distinct mass signals of peptides repeatedly detected in preparations from major neurohemal organs of different Mantophasmatodea species

	OU *H. cf. montaguensis*	GP dto.	CA dto.	VW dto.	DE *H. spec.* 2	VR *K. spec.* 2	DR *K. biedouw.*	GA *A. gansb.*	ST *N. okiep.*	KR *N. okiep.*
aPSO										
1	872.42	+	+	+	+	+	+	+	+	+
2	1064.49	+	+	+	+	+	+	+	+	+
3	1168.68	+	+	+	+	+	+	1182.69	1184.70	1184.71
4	1318.61	+	+	+	+	+	1304.62	1317.63	1288.60	1288.61
5	1479.66	+	+	+	1493.71	1493.66	+	1493.69	1493.69	1493.69
6	1783.79	+	+	+	+	1809.80	1995.04	+	+	+
7	2046.89	+	+	+	+	+	2037.15	+	+	+
8	2682.24	+	+	+	+	+	2672.48	+	2664.37	2664.65
tPSO										
1	833.51	+	+	+	+	+	+	+	+	+
2	964.53	+	+	+	+	+	+	1046.58	+	+
3	974.54	+	+	+	+	+	1004.51	1004.56	+	+
4	992.57	+	+	+	+	+	+	+	+	+
5	1033.58	+	+	+	+	+	+	+	+	+
6	1074.63	+	+	+	+	+	+	+	+	+
7	1076.61	+	+	+	+	+	+	+	+	+
8	1090.61	+	+	+	+	+	+	+	+	+
9	1148.63	+	+	+	+	+	+	+	+	+
10	1201.58	+	+	+	1228.61	1215.62	+	1272.64	1228.60	1228.65
11	1577.85	+	+	+	+	+	+	+	1590.81	1590.88
12	1754.81	+	+	+	+	+	+	+	1789.92	1770.91
13	2172.21	+	+	+	2199.16	2185.74	+	2242.76	2199.13	2199.18
14	2592.31	+	+	+	+	+	+	+	2605.29	+
CC										
1	952.43	+	+	+	+	+	+	+	+	+
2	1051.60			+		+	+	+	+	+
3	1097.60			+		+	+	1111.58	1111.59	1111.56
4	1130.62			+		+	+	+	1104.61	1104.54
5	1257.64	+	+	+	+	+	+	+	+	+
6	1350.64	+	+	+	+	+	+	+	+	+
7	1415.80			+		+	+	1429.76	1517.81	1517.81
8	1503.81			+		+	1497.88	+	?	?
9	1598.80			+		1612.82	1612.90	1616.72	1612.84	1612.82
10	2073.86			+	+	+	+	+	+	+

For clarity, all peptide species, which were mass identical with those of specimens from OU, are marked with a plus (+) only. All specimens of *H. cf. montaguensis* collected on different locations, expressed identical peptides. The remaining Mantophasmatodea populations are each clearly separable from each other by the peptide mass fingerprints. CC-preparations of specimens from GA, CA and DE were performed on dead (frozen) material which resulted in poor mass spectra (only more abundant peptides could be detected). aPSO, abdominal perisympathetic organ; tPSO, thoracic perisympathetic organ; CC, corpora cardiaca.

represent different genera, as the assignment of these new populations to currently known taxa was tentative.

A few peptides occurred which are known to have a wider appearance in hemimetabolous insects, namely leucomyosuppressin and His-corazonin. Both peptides were, for instance, also found in the phasmid *Carausius morosus* (Predel et al. 1999), a group discussed as a possible sister group of the Manthophasmatodea (Klass et al. 2002).

A mass spectrometric approach was used for the first time in this context and therefore this method deserves some specific notes. Recent developments in mass spectrometry increased the resolution and sensitivity considerably, which make such techniques suitable for screening of neurosecretions from minute amounts of biological tissue. Direct analysis of peptides from single neurosecretory cells was first performed on large molluscan neurons in 1994 (Li et al. 1994); very recently even neuropeptides from much smaller *Drosophila*-neurons could be analysed by means of MALDI-TOF mass spectrometry (Predel et al. 2004). Thus, successful screening of insect neurohemal release sites, which accumulate peptidergic neurohormones, rather depends on preparation skills than on limitations set by the mass spectrometer. Therefore, a detailed morphological description of the tiny PSOs was included in the paper. Further development of mass spectrometry, particularly MALDI-TOF and ESI-QTOF mass spectrometry, is forced by the huge demands of proteomics-research (Aebersold and Mann 2003), that operates with expressed peptides/proteins in a physiological context. The medical importance of proteomics cannot be over-estimated and a side-effect of this development is the increasing availability of mass spectrometer which can be used for projects like this one.

In our research group, neuropeptides from neurohemal organs of insects were extensively studied over the last few years, mainly focussing on cockroaches (Predel and Eckert 2000b; Predel et al. 2000; Predel 2001). In the present study, on Mantophasmatodea, we were able to demonstrate that the methods initially developed for studying larger insects, are suitable for a relatively fast screening of neuropeptides from medium-sized insects as well. In average, dissection of the three different neurohemal organs (corpora cardiaca, thoracic and abdominal PSOs), sample preparation and mass spectrometric analysis took 2 h for a single specimen. Appropriate dissections, however, need some experience. On the other hand, the repetitive occurrence of PSOs (Fig. 2) allows a certain number

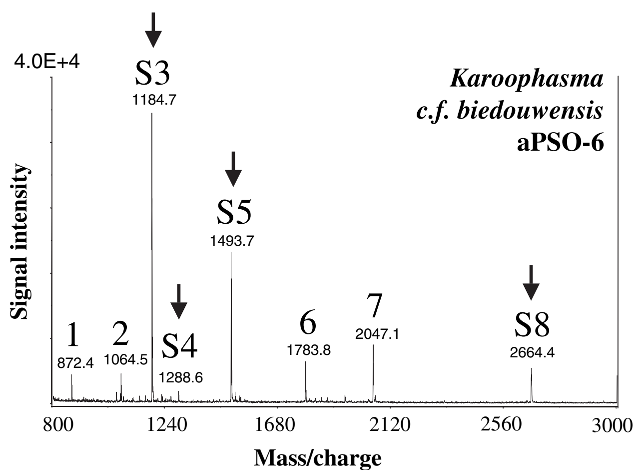

Fig. 8. MALDI-TOF mass spectrum from a preparation of a single abdominal PSO (aPSO-6) of an adult *N. cf. ookiepensis* female from Steinkopf (ST). Not less than four of eight abundant peptides are different from those of *H. cf. montaguensis* collected in OU. These peptides are marked with an arrow, their designations are in accordance with designations created for the peptides of the Oudtshoorn species/population but the suffix S (for Steinkopf) was added

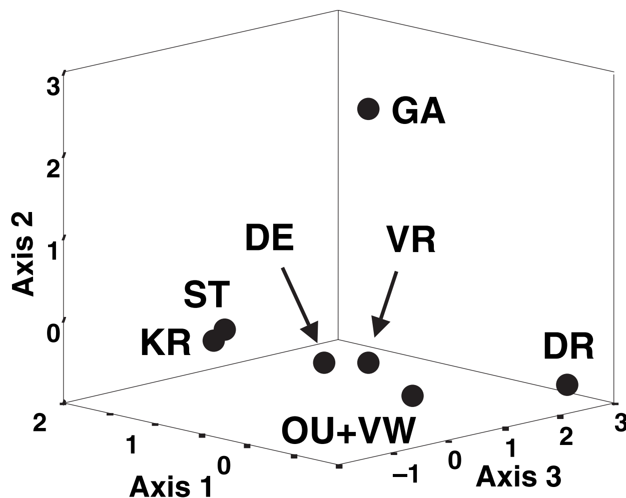

Fig. 9. Similarity of species according to the PCA by using the presence–absence-data of ion signals of peptides (Table 1). Mass signals from the corpora cardiaca of *Hemilobophasma spec. 2* from DE were uncomplete, and (artificially) completed with those from OU specimens (neighboring sample spot). The first, second and third PCA axis are presented. The eigenvalues (multiplied by 100) of the PCA are: axis1 = 44.6/axis2 = 19.6/axis3 = 17.2/axis4 = 9.40

of failures; the results described here confirm, that the method is suitable also for the investigation of single specimens. Altogether, 32 characters (masses of different peptide hormones) were included in the study; the respective peptides were selected after studying the mass spectra of several insects collected from the same location (OU). The only criterion for selecting a peptide was its reproducible appearance in all spectra from a specific neurohemal organ.

The specific peptide inventory from individuals from a certain location cannot be used simplistically to establish species. On theory, different species can express identical peptides, and a single species may have isolated populations with different peptide forms. Peptide hormone sequences, however, are generally not that variable, and increasing differences accumulate with increased phylogenetic distance. In the current study on Mantophasmatodea, we did not observe any difference in peptides from specimens of a local population. An example of peptide uniformity between geographically distant populations of a species is demonstrated by neuropeptides from the cockroach *Periplaneta americana*, where not a single peptide modification could be obtained over the years of analysis, independent of the continent the cockroaches were caught (R. Predel, unpublished data). It thus appears that peptide signatures have much potential, not only as taxonomic characters for species recognition in morphologically uniform taxa, but also for phylogenetic insights into relationships between species. In this context it seems particularly important, that the studied neuropeptide sequences are identical in males, females, and larvae.

A PCA analysis using the obtained mass data of peptides revealed a reasonable correlation between the geographical distance of the sample sites and the differentiation of peptide sequences as detected by mass fingerprints. The most southern and northern sample sites (GA, ST/KR) appear well separated on the ordination. On the other hand, specimens from distant locations like DE and DR did not follow that simple rule and further collecting on an even finer scale will be necessary to resolve this affinity. The tendency to modifications among peptide hormones can be used for rapid identification, not only of species within Mantophasmatodea but also for separation of this insect order from other insect orders. The phylogenetic relationships between the various insect orders, however, can only be estimated by using the complete sequence information of suitable peptides. This complex question will be targeted in our next experiments and will complement morphological as well as molecular biological data acquisitions of other research groups.

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Zusammenfassung

Die neue Insektenordnung Mantophasmatodea: Artunterscheidung durch Massenspektrometrie von Peptidhormonen?

Die vorliegende Arbeit beschreibt eine umfassende Analyse von Peptidhormonen der kürzlich beschriebenen Insektenordnung Mantophasmatodea. Mittels moderner massenspektrometrischer Methoden ist es gelungen, die Neurohaemalorgane von einzelnen Individuen verschiedener Arten zu untersuchen. Der methodische Ansatz hierzu wird vorgestellt und die ersten komplett sequenzierten Neurohormone der Mantophasmatodea beschrieben. Die Ergebnisse belegen eine überraschend klare Differenzierbarkeit der Arten anhand der ermittelten Massendaten, und ermöglichen es erstmals, Neuropeptide für taxonomisch/systematische Zielstellungen zu katalogisieren. Die vorliegenden Daten bestätigen zudem eine unterschiedliche Tendenz zu Sequenzmodifizierungen zwischen den verschiedenen Peptidfamilien.

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