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Comparative peptidomics of four related hemipteran species: Pyrokinins, myosuppressin, corazonin, adipokinetic hormone, sNPF, and periviscerokinins

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ABSTRACT

We performed the first comprehensive peptidomic analysis of neurohormones from hemipteran insects by analyzing the neuropeptides of two major neurohemal organs, namely the corpora cardiaca and abdominal perisympathetic organs. For the experiments we selected four related species of polyphagous stinkbugs (Pentatomidae), three of which are known to attack several important food crops. Peptide sequences were identified by MALDI-TOF mass spectrometry; tandem fragmentation of myosuppressin, sNPF, CAPA-periviscerokinins and pyrokinins revealed novel sequences not known from other insects so far. Most Leu/Ile and Glu/Lys ambiguities could be solved by either specific side-chain fragmentations or on-plate acetylation experiments. The identification of the specific sequences provides a solid basis for forthcoming pharmacological tests to study the neuroendocrine system of these pest insects. However, it should be mentioned in this context that the sequences of the peptides from different stinkbugs are likely not representative of Hemiptera in general. The forthcoming release of the genome from the reduviid *Rhodnius prolixus* will provide sufficient data to clear this point.

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1. Introduction

In insects, the majority of neuropeptides have been initially purified from large hemimetabolous insects such as cockroaches [4,16] and locusts [17]. In contrast, the recent elucidation of insect genomes focused entirely on holometabolous insects, including Diptera [*Drosophila* spp., *Anopheles gambiae*, *Aedes aegypti* (L.)], Hymenoptera [*Apis mellifera* (L.)], Lepidoptera [*Bombyx mori* (L.)], and Coleoptera [*Tribolium castaneum* (Herbst)]. However, the neuropeptides of the largest group of hemimetabolous insects, the Hemiptera, which includes species-rich taxa such as aphids, cicadas and true bugs, have been largely ignored. This is surprising, since neuropeptides occupy a key position in the modulation of physiological events, and the aforementioned taxa contain numerous economically important pest species as well as vectors of diseases. In this study, we performed the first comprehensive peptidomic analysis of neurohormones from

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four related species of stink bugs (Pentatomidae). The green stink bug (*Acrosternum hilare* (Say)), brown stink bug (*Euschistus servus* (Say)), and the southern green stink bug (*Nezara viridula* (L.)) are polyphagous economically important pentatomids affecting product quality and yield in cotton and other row, fruit and nut crops [11]. In most crop-growing areas of the world, control relies mainly on chemical insecticides, thus there is a great need for more sustainable control methods [19]. More recently, adult southern green stink bugs have been shown to vector plant pathogens in cotton [5].

Two major neurohemal organs were selected for a detailed analysis, the corpora cardiaca (CC) and abdominal perisympathetic organs (aPSOs). Sequences of the following peptides were identified in our experiments: pyrokinins, myosuppressin, corazonin, adipokinetic hormone, sNPFs, and periviscerokinins. MALDI-TOF/TOF tandem mass spectrometry allowed de-novo sequencing of the respective peptides via direct analysis of the tissues, including a correct assignment of internal Leu/Ile positions via collision-induced side-chain fragmentation [8]. The results revealed nearly identical peptidomes for the three stinkbug species *N. viridula*, *A. hilare*, and *Banasa dimiata*. The fourth species, *E. servus*, expresses two pyrokinins that are very different from those of the other species and have unique modifications at the C-terminus.

2. Materials and methods

2.1. Insects

Adult stink bugs (*N. viridula*, *E. servus*, *A. hilare*, *B. dimiata*) were captured in 40 W light traps (with live insect canisters) located adjacent to fields cultivated in corn, cotton, sorghum and soybeans in Burleson County, Texas.

2.2. Dissection and sample preparation for mass spectrometry

For CC-preparations, the pronotum of adult insects was removed, and the neck region of the head opened with scissors. The CC together with an adjoining portion of the aorta was removed and transferred to a stainless steel sample plate for mass spectrometry. For the identification of CAPA-peptides, the ventral nerve cord of adult stinkbugs was transferred onto a separate chamber filled with insect saline. The fused ventral nerve cord of Heteroptera (true bugs) does not develop median nerves with abdominal perisympathetic organs typical of many insects. Instead, three pairs of CAPA-peptide expressing neurons in the abdominal neuromeres project in the second, third and fourth abdominal nerves (ABN) where they develop neurohemal sites [10,14]. For mass spectrometric analyses, we dissected the most posterior part of the dorsal ganglionic sheath together with the aforementioned nerves and prepared these tissues directly for MALDI-TOF mass spectrometry. The respective nerves as well as the CC-preparations were rinsed in a drop of water before being moved to the final position on the sample plate. A limited amount of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water) was pumped on the dried preparations. Each preparation was allowed to dry again and then covered with pure water for a few seconds, which was removed by cellulose paper.

2.3. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

Mass spectrometric analysis was performed on the ABI 4800 proteomics analyzer (Applied Biosystems, Framingham, MA). Due to the nature of the samples all acquisitions were taken in manual mode. Initially the instrument was operated in reflectron mode, in order to determine the parent masses. For the tandem MS experiments, the CID (collision-induced dissociation) acceleration was 2 kV in all cases. In order to

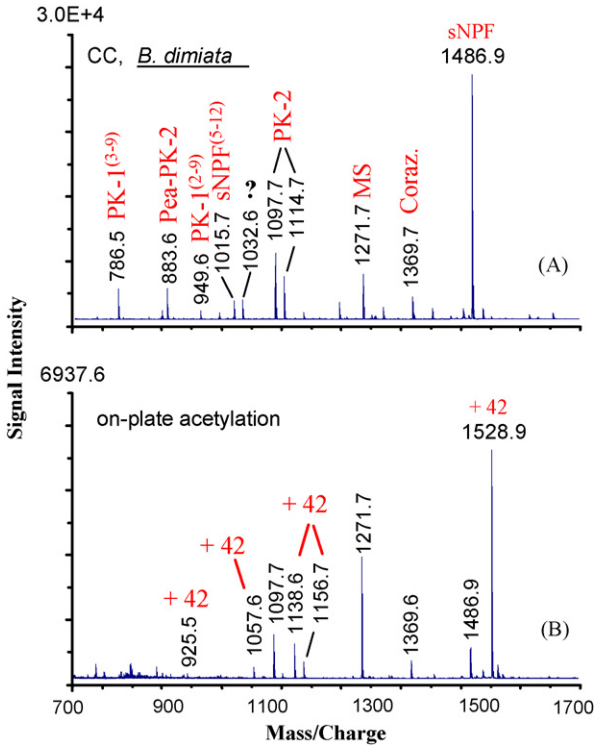

Fig. 1 – MALDI-TOF mass spectra of a preparation of the posterior CC and adjoining aortal tissue of *B. dimiata* before (A) and after (B) on-plate acetylation. (A) With the exception of the peptide at *m/z* 1032.6, all prominent ions which are marked could be structurally elucidated in this study. Note the abundance of the truncated PK-1 forms; the full length PK-1 (1096.7 Da) is not specifically marked due to the mass similarity with the more prominent PK-2 signal (1097.7 Da). (B) Mass spectrum of the preparation shown in (A) after on-plate acetylation. Masses of the peptides with a C-terminal pyroglutamate (PK-2, corazonin, MS) remained unchanged; all other peptides show a mass shift of +42 Da due to the formation of anhydrides with the N-terminal amino groups. For sNPF (1486.9 Da), the fragmentation data did not solve the Gln/Lys ambiguity at position 6 of the C-terminus, because no additional shift of +42 Da due to acetylation of a primary amine group of putative Lys was detectable, the sequence of stinkbug sNPF is determined to be FAPRSPQLRLRFamide.

change the net amount of activation energy imparted to the primary ions, the collision gas (atmospheric air) pressure was increased. Two gas pressures were employed by selecting the following two instrument settings: 'none' and 'high'. The fragmentation patterns from these different settings were used to determine the sequences of the peptides. An unambiguous assignment of internal Leu/Ile was achieved by means of CID under high gas pressure that revealed unique and distinct patterns for the side chains of Leu and Ile [8]. The fragmentation data obtained in these experiments were analyzed using the Data Explorer™ software package. Samples with peptides that contained Lys/Gln ambiguities were analyzed again after dissolving the respective preparations in acetic anhydride (2:1 methanol/acetic anhydride) which results in selective acetylation of the ε-amino group of Lys.

3. Results

3.1. Peptidomics of the CC

Abundant peptides from the CC of stinkbugs were first elucidated using the Red-backed Stinkbug, *B. dimiata* (Fig. 1A). MALDI-TOF mass spectra obtained on preparations of CCs from single specimens were acquired and the abundant ions in the mass range of 800–1600 Da were subsequently fragmented. Altogether, 12 peptides whose sequences could be assigned to known peptide families were found (see Table 1). The most prominent ion signal at *m/z* 1486.8 turned out to be a novel small Neuropeptide F (sNPF) sequence containing a Gln substitution of Ser within the conserved C-terminal octapeptide. A truncated form of this peptide (sNPF$^{5-12}$) was also found, although at a much lower signal intensity. The occurrence of the Gln instead of the mass-related Lys in the sNPF sequence was verified by on-plate acetylation of the original CC preparation and subsequent mass spectrometric analysis (Fig. 1B). This procedure led to the acetylation of the N-terminal amino group, but no additional shift of +42 Da, which would have indicated an acetylation of the primary amine group of a putative Lys. A second distinct ion signal at *m/z* 1271.7 turned out to be a novel myosuppressin; with Val replaced by Leu at position 3 of the N-terminus (Fig. 2A). In addition to the myosuppressin containing a pyroglutamate, a less abundant non-blocked form of this peptide (*m/z* of 1288.7) was detectable in all preparations. The fragmentation of two additional peptides yielded sequences that were originally described from the American cockroach, namely [Arg^7]-corazonin (1369.6 Da) and Peram-pyrokinin-2 (883.5 Da) [12,18]. In addition, the recently described adipokinetic hormone of *N. viridula* (Panbo-RPCH; [2]) was detected as well ($M + Na^+$: 952.4 Da). The remaining peptides, which were successfully sequenced, turned out to be pyrokinins. One of these pyrokinins (PK-1, 1096.7 Da) was accompanied by two

Table 1 – List of neuropeptides that were identified from different stinkbug species

Peptide	Southern green stinkbug *Nezara viridula*	Green stinkbug *Acrosternum hilare*	Red-backed stinkbug *Banasa dimiata*	Brown stinkbug *Euschistus servus*
aPSO-peptides				
CAPA-PVK-1	DQLFPFPRVa[a]	+	DQLIPFPRVa	+
CAPA-PVK-2	EQLIPFPRVa[a]	+	+	EQLIPFPRIa
CAPA-PK	NGSAGNGGLWFGPRLa[a]	+	+	+
CC-peptides				
corazonin	pQTFQYSRGWTNa	+	+	+
myosuppressin	pQDLDHVFLRFa	+	+	+
unblocked MS	QDLDHVFLRFa	+	+	
sNPF	FAPRSPQLRLRFa	+	+	+
sNPF$^{(5-12)}$	SPQLRLRFa	+	+	+
AKH (Panbo-RPCH)	pQLNFSPGWa[b]	+	+	+
Pyrokinins				
Peram-PK-2	SPPFAPRLa	+	+	+
PK-1	FYAPFSPRLa	+	+	LYTHFSTRLa
PK-1$^{(2-9)}$	YAPFSPRLa	+	+	YTHFSTRLa
PK-1$^{(3-9)}$	APFSPRLa	+	+	THFSTRLa
PK-2	pQLVSFRPRLa	+	+	pQLAFRPMLa
unblocked PK-2	QLVSFRPRLa	+	+	QLAFRPMLa

The C- and N-terminal Leu/Ile ambiguities (grey shaded) could not be solved by mass spectrometry.
[a]Predel et al., 2007; [b]Gäde et al. [2].

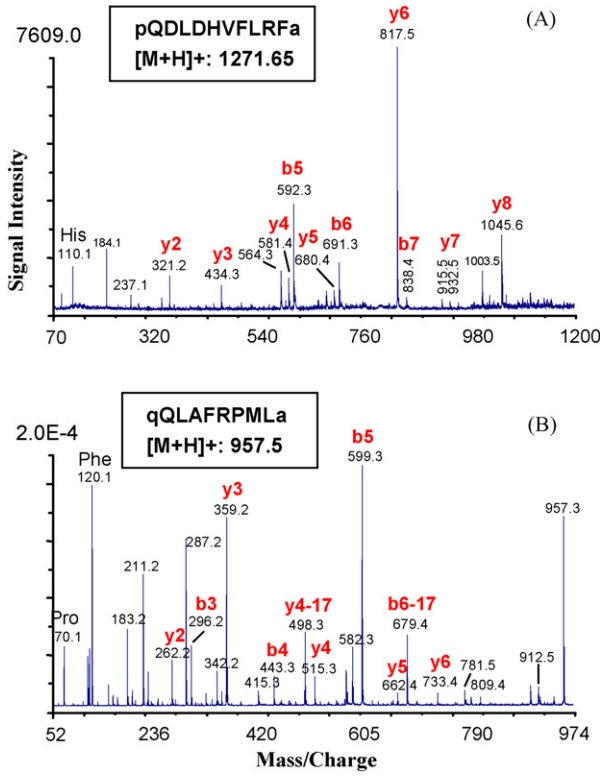

Fig. 2 – MALDI-TOF/TOF tandem mass spectra of the peptides at (A) *m/z* 1271.7, and (B) *m/z* 957.3. Prominent y- and b-type fragments are labeled. Fragments were analyzed manually and the resulting sequences (see insets) verified the expression of a novel myosuppressin (A) and pyrokinin (B), respectively. Assignments of the internal Leu/Ile ambiguities was possible after collision-induced fragmentation under high gas pressure that revealed unique patterns for the side chains of Leu and Ile (not shown).

truncated forms. Among these forms, PK-1[3–9], was particularly abundant (see Fig. 1A). The second pyrokinin, PK-2 (1097.7 Da), was identified in two forms, namely N-terminally blocked by pyroglutamate and with the non-blocked form of the peptide. Since the masses of PK-1 and PK-2 are nearly identical, the fragmentation of these peptides was performed following the on-plate acetylation, which separated the non-blocked PK-1 from PK-2. With the exception of the conserved C-terminal Leu of pyrokinins [15], all Leu/Ile ambiguities of the novel neuropeptides from the CC of *B. dimiata* could be solved by high-energy collision-induced dissociation of parent ions that revealed distinct side-chain fragments (Fig. 3). However, it should be noted that from the many known pyrokinins of insects, no forms with a C-terminal Ile are known.

The peptides that were structurally elucidated from the CC of *B. dimiata* were subsequently also identified in the two stinkbugs *A. hilare* and *N. viridula*. In all cases, sequence identity was confirmed by fragmentation, side-chain fragmentation, and acetylation experiments. Only an incompletely sequenced *B. dimiata* peptide at *m/z* 1032.6 was found to be modified in *A. hilare* and *N. viridula*. Analysis of partial sequences revealed a related peptide at *m/z* 1006.6 typical of these species. The brown

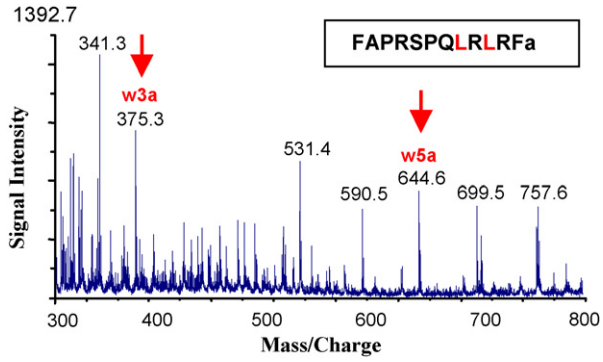

Fig. 3 – MALDI-TOF/TOF tandem mass spectrum of stinkbug sNPF under conditions of high collision energy. The confirmation of side-chain fragments typically of Leu (arrows) allowed the assignment of the following sequence: FAPRSPQLRLRFamide.

stinkbug *E. servus*, however, expresses two highly modified pyrokinins (Fig. 4; Table 1). The orthologue of Nezvi-PK-1 (Eusse-PK-1; 1136.6 Da) contains not less than four amino acid replacements. One of these modifications concerns the highly conserved C-terminal FXPRLamide and contains a Thr substituted for Pro. As was found for Nezvi-PK-1, several truncated forms of the PK-1 of *E. servus* were observed in the mass spectra. Interestingly, the second pyrokinin, Eusse-PK-2 (957.5 Da; see Fig. 2B), also contains a unique modification at the C-terminus. In this peptide, the penultimate, positively charged Arg is replaced by a neutral Met. This is a highly significant modification, as the Arg residue in the C-terminal core pentapeptide is highly critical for biological activity in pupariation [9], pheromone biosynthesis [5], and other pyrokinin bioassays [6] (Nachman RJ, unpublished data).

3.2. *Peptidomics of the aPSOs (CAPA-peptides)*

Earlier experiments on *N. viridula* [14] revealed the occurrence of two CAPA-periviscerokinins (PVKs) and a CAPA-PK. In this

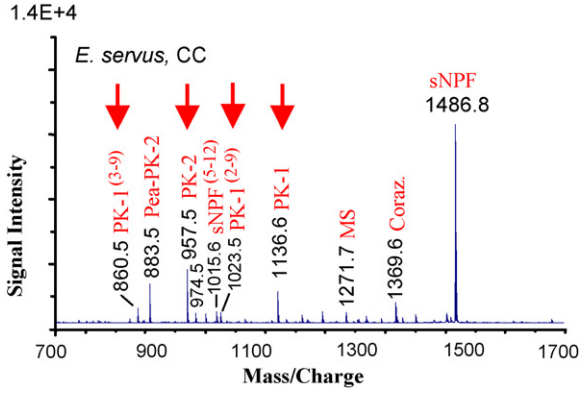

Fig. 4 – MALDI-TOF mass spectrum of a preparation of the posterior CC (without the glandular part) of *E. servus*. The two modified pyrokinins (PK-1, PK-2) that are different from those of the other stinkbug species as well as the truncated forms of PK-1 are marked by arrows.

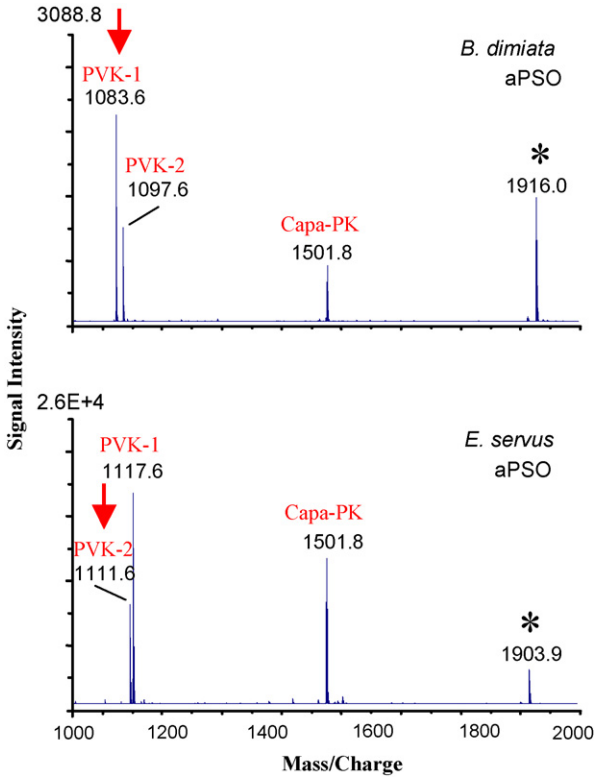

Fig. 5 – MALDI-TOF mass spectra of preparations of the posterior dorsal ganglionic sheath of the ventral nerve cord with attached abdominal nerves 2–4 of *B. dimiata* and *E. servus*. All prominent ion signals represent CAPA-peptides which were fragmented in subsequent experiments. The peptides marked with an arrow represent novel PVKs. Ion signals marked by asterisks are C-terminal extended versions of the respective CAPA-pyrokinins (1916.0 Da (*B. dimiata*, see also *N. viridula* in Predel et al. [14]) and 1903.9 Da (*E. servus*)); the exact sequence of these extensions could not be solved by fragment analysis.

study, these CAPA-peptides were also found in *A. hilare*, whereas *B. dimiata* and *E. servus* each express slightly modified CAPA-PVKs (Fig. 5; Table 1).

4. Discussion

Among the insects that do not show a complete metamorphosis, Hemiptera belong to the most diverse and successful taxa. The development of a proboscis which is specialized to suck the juices from plants (in some groups the proboscis may also be specialized to suck blood or hemolymph from animals) made it possible to use nutritional resources largely not available for other insects. An excessive supply of liquid nutrition of uniform composition makes it necessary to develop a highly efficient control of water and ion balance, which is in part regulated by neuropeptides. In sharp contrast to the diversity and economic importance of these insects, the identification of neuropeptides from Hemiptera, however, has been largely neglected. Only a few adipokinetic hormones from cicadas and true bugs [1,3], as well as CAPA-peptides of *N. viridula* [14] are known, and these data do not provide sufficient information for an analysis of the evolution of hemipteran neuropeptides.

For our comprehensive peptidomic approach we have chosen four different species of true bugs (Heteroptera) belonging to a single taxon, the Pentatomidae. Although these species are placed in different genera, the peptide sequences show a high degree of identity between the species. Only the pyrokinins of the brown stinkbug, *E. servus*, strongly differ from those of the other stinkbugs. The most prominent ion signal in mass spectra of the CC was assigned to stinkbug sNPF, and the signal intensity of that peptide became even more distinct in the aortal tissue posterior of the CC (not shown). In *D. melanogaster*, sNPF peptide is also very abundant in the retrocerebral complex. Interestingly, in the fruitfly a truncated form of sNPF (Drome-sNPF-1^{4-11}) is much more abundant than sNPF itself [13], whereas in the four stinkbug species sNPF is much more abundant than the truncated form (Nezvi-sNPF$^{5-12}$). The cleavage site (Arg-Ser) to generate the truncated sNPFs is identical in both peptides. The stinkbug sNPF shows a replacement of Ser with Gln in the highly conserved C-terminal octapeptide, and is the first identified sNPF of insects with a modification within this motif. In addition, stinkbugs also express a novel myosuppressin sequence not known from any other insect. Thus, most neuropeptide sequences which were identified in this or earlier studies (*N. viridula*: Panbo-RPCH [2]; CAPA-peptides [14]) are typical of stinkbugs and not known from other insects.

The pyrokinin sequences which were identified in this study deserve some specific remarks. First of all, three stinkbugs (*N. viridula*, *B. dimiata*, *A. hilare*) express identical pyrokinins, but the fourth species (*E. servus*) has two highly modified pyrokinin sequences. This is indicative of a very close evolutionary relationship between *N. viridula*, *B. dimiata*, *A. hilare*, and separates *E. servus* from this group. More importantly, the *E. servus* pyrokinins have unique modifications in the C-terminal FXPRLamide sequence which are known to be important for biological activity [5–7,9]. These modifications include substitutions of Pro with Thr (Eusse-PK-1) and of Arg with Met (Eusse-PK-2). The two modifications in the highly conserved C-terminal FXPRLamide region observed for Eusse-PK-2 may be indicative of a pyrokinin receptor structure that is divergent from the other three stink bug species, as well as that of other insect taxa. Future experiments will show if these peptides remain biologically active or if the only conserved pyrokinin which was found in all stinkbugs (Peram-PK-2) is primarily responsible for pyrokinin-related biological activity. A survey of the pyrokinin-sequences in insects revealed that most insects express a pyrokinin identical or very similar in sequence to the cockroach pyrokinin Peram-PK-2 whereas the orthologue relationship of the other pyrokinins (with the exception of the CAPA-pyrokinin) is less clear.

This peptidomic approach revealed a number of novel peptide sequences from true bugs. Most Leu/Ile and Gln/Lys ambiguities were solved by either specific side-chain fragmentations or on-plate acetylation experiments. Only the C-terminal Leu/Ile of the pyrokinins, the N-terminal Leu/Ile of *E. servus* PK-1, and the C-terminal Ile/Leu of CAPA-PVK-2 of *E.*

servus could not be assigned by mass spectrometry. Not a single pyrokinin, however, is known to contain a C-terminal Ile. Thus, the identification of the stinkbug-specific sequences provides a solid basis for forthcoming pharmacological tests to study the neuroendocrine system of these pest insects. However, it should be mentioned in this context that the sequences of the peptides from different stinkbugs are likely not representative of Hemiptera in general. The forthcoming release of the genome from the reduviid *Rhodnius prolixus* will provide sufficient data to clear this point.

Acknowledgments

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