

Neuropeptidomics of the Mosquito *Aedes aegypti*

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Neuropeptidomic data were collected on the mosquito *Ae. aegypti*, which is considered the most tractable mosquito species for physiological and endocrine studies. The data were solely obtained by direct mass spectrometric profiling, including tandem fragmentation, of selected tissues from single specimens, which yielded a largely complete accounting of the putative bioactive neuropeptides; truncated neuropeptides with low abundance were not counted as mature peptides. Differential processing within the CNS was detected for the CAPA-precursor, and differential post-translational processing (pyroglutamate formation) was detected for AST-C and CAPA-PVK-2. For the first time in insects, we succeeded in the direct mass spectrometric profiling of midgut tissue which yielded a comprehensive and immediate overview of the peptides involved in the endocrine system of the gut. Head peptides which were earlier identified as the most abundant RFamides of *Ae. aegypti*, were not detected in any part of the CNS or midgut. This study provides a framework for future investigations on mosquito endocrinology and neurobiology. Given the high sequence similarity of neuropeptide precursors identified in other medically important mosquitoes, conclusions regarding the peptidome of *Ae. aegypti* likely are applicable to these mosquitoes.

Keywords: *Aedes aegypti* • Culicidae • peptidomics • insect neuropeptides • midgut • MALDI-TOF • CAPA-peptides

Introduction

Mosquitoes require blood for egg maturation and thereby transmit disease-causing viruses and parasites by subsequent feeding on human and animal hosts. Mosquito–pathogen associations are generally species specific: *Anopheles spp.*–*Plasmodium spp.* (malaria), *Culex spp.*–encephalitis viruses and nematodes (lymphatic filariasis) and *Aedes spp.*–yellow fever and dengue viruses and nematodes (heartworm). There is an urgent need to improve the control of these diseases and their vectors, and the availability of the genome and EST databases for *Anopheles gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus* at a central site (<http://www.vectorbase.org/index.php>) has accelerated research to develop new mosquito control strategies. In this context, it is noteworthy that no

peptidomic study of neuropeptide expression in mosquitoes has been reported. This is surprising since neuropeptides regulate many key processes in the physiology and behavior of mosquitoes.¹

Neuropeptides and protein hormones are produced by endocrine cells or neurons as larger precursors (prepropeptides). Subsequently, these prepropeptides are processed, stored and released within the nervous system as neurotransmitters or neuromodulators and from the midgut endocrine system, neurosecretory cells of the central nervous system (CNS), and peripheral neurosecretory cells as circulating hormones. These peptide messengers exert their action by binding to membrane receptors, most often to G-protein coupled receptors (GPCRs) and, to a lesser extent, to receptor tyrosine kinases. With bioinformatic approaches, 35 genes encoding putative neuropeptides and protein hormones were annotated from the *An. gambiae* genome.¹ Cloned products confirmed the transcription and expression of only a few genes in different life stages and tissues of *An. gambiae*: neuropeptide F (NPF) and its GPCR,² short NPF (sNPF) and its GPCR,³ adipokinetic hormone 1 (AKH-1), AKH-2, and the AKH-1 GPCR,^{4,5} and seven insulin-like peptide (ILP) genes.⁶

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This is somewhat different for *Ae. aegypti*, which is considered the most tractable mosquito species for physiological and endocrine studies. Many neuropeptides and protein hormones in this species are characterized at the transcript, processed peptide, and function levels: head peptides,^{7–9} insect kinins,¹⁰ allatostatins (AST-A),¹¹ ovary ecdysteroidogenic hormone (OEH),¹² NPF,¹³ allatotropin (AT),¹⁴ AST-C,¹⁵ eight different ILPs,^{16,17} ecdysis triggering hormones (ETHs),¹⁸ and AKHs.¹⁹

In this study, a comprehensive peptidomic analysis of processed mature neuropeptides from *Ae. aegypti* was performed using mass spectrometry (MS). In earlier investigations of other insect species with complete genome information, predominantly LC–ESI–QTOF MS analysis of extracts from brain or other parts of the CNS was used to reveal the peptidome as a list of expressed neuropeptides.^{20–22} In the current study neuropeptidomic data were collected on the mosquito *Ae. aegypti* entirely via direct profiling of portions of the nervous system or of midgut tissue by MALDI-TOF MS. In insects, this type of approach (termed “mass spectrometric morphology”) was first introduced using the nervous system of the American cockroach *Periplaneta americana*.²³ Such strategy made it possible to focus, step by step, on processed products of specific neuropeptide genes that are expressed in well-defined compartments. The proper dissection of these tissues may yield mass spectra that predominantly contain products of specific neuropeptide genes. It also enhanced the completeness of the collected data set and enabled an estimation of the relative abundances of the mature peptides from single neuropeptide genes. Owing to the rapid development of sensitive MALDI-TOF mass spectrometers with TOF/TOF optics, mass fingerprints of *Ae. aegypti* tissues could be corroborated by the fragmentation data that were obtained from the same samples. This effort resulted in a final list of detected neuropeptides that covers almost all of the predictable neuropeptides and a number of unexpected processing products.

Experimental Section

Animals. Adult *Ae. aegypti* (strain “Liverpool”) were kindly provided by Prof. Craig Coates from a colony maintained at the Department of Entomology, Texas A&M University. In addition, eggs (strain “Monheim”) were obtained from Bayer CropScience (Monheim, Germany). Final fragmentation data were obtained using the strain “Monheim”. Hatched larvae were reared in small pans containing 2 L of water until adulthood.

Gene and Transcript Identification. To identify genomic sequences encoding *Ae. aegypti* neuropeptides and peptide hormones, trace DNA sequences generated by TIGR and the Broad Institute were first searched by TBLASTN²⁴ using amino acid sequences of candidate peptides from *An. gambiae*, *D. melanogaster*, other insects, invertebrates, and vertebrates. Nucleotide sequences were downloaded, assembled and analyzed as previously described.¹ TBLASTN searches, using the amino acid sequences of putative *Ae. aegypti* neuropeptide and peptide hormone orthologs identified in the trace data, were performed on the assembled *Ae. aegypti* genome and transcript DNA sequences deposited in the GenBank database.²⁵ Analyses of the putative prepropeptides included signal peptide identification using both the Neural networks and Hidden Markov models contained in the SignalP program (<http://www.cbs.dtu.dk/>)²⁶ and determination of proteolytic processing sites.

Dissection and Sample Preparation for Mass Spectrometry. Males or females (nonblood fed) were fixed with needles, submerged in insect saline (pH 7.25) of the following composi-

tion: NaCl (7.50 g/L), KCl (0.20 g/L), CaCl₂ (0.20 g/L), and NaHCO₃ (0.10 g/L), and the body cavity opened with scissors. The different parts of the CNS, corpora cardiaca (CC; see for terminology²⁷), and midgut were dissected and transferred with a stainless steel insect pin or a glass capillary into a drop of distilled water on the sample plate for MALDI-TOF MS. The water was subsequently removed using a glass capillary. Approximately 50 nL of matrix solution (saturated α -cyano-4-hydroxycinnamic acid dissolved in methanol/water [1:1]) was pumped onto the dried tissue over a period of about 5 s using a Nanoliter injector (World Precision Instruments, Berlin, Germany). Each preparation was air-dried and covered with pure water for a few seconds, which was removed by cellulose paper. At least 10 preparations of each tissue sample were prepared. For the analysis of ETHs, tracheal trunks of larvae were dissected in an analogous manner.

MALDI-TOF MS. MALDI-TOF analyses were performed on an ABI 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA). Due to the nature of the samples all acquisitions were taken manually. Initially the instrument was operated in reflectron mode, in order to determine the parent masses. A laser intensity of 3800 was typically employed for ionizing the neuropeptides. For the tandem MS experiments (performed in gas on and gas off mode), a CID acceleration of 1 kV was used in all cases. The number of laser shots used to obtain a spectrum varied from 800–4000, depending on signal quality. The fragmentation patterns were used to manually determine the sequence of the peptides by using the Data ExplorerT software package.

Results and Discussion

Homology-based searches of the *Ae. aegypti* genome and EST databases resulted in the identification of 43 neuropeptide and protein hormone genes listed in Table 1 along with the homologue genes of *Drosophila melanogaster*^{28,29} and *An. gambiae*.¹ For practical reasons, processed peptides are classified as neuropeptides if their mass is <3000 Da and protein hormones, if >3000 Da. Calcitonin-like diuretic hormone (DH 31), with a mass just above 3000 Da, was included in the list of neuropeptides. Accordingly, 22 neuropeptide genes exist in *Ae. aegypti*, and the purpose of this study was to identify the products of these genes in the nervous system and midgut of adults.

As in other insects, the predicted amino acid sequences for prepropeptides encoded by neuropeptide genes in *Ae. aegypti* often contain multiple repeated peptide sequences that begin and end with protease cleavage sites (Supplemental Table 1, Supporting Information). Genes with this feature include AST-A, myoinhibitory peptides (MIPs), CAPA-peptides, ETHs, extended FMRFamides, head peptides, kinins, neuropeptide-like precursor 1 (NPLP-1) peptides, orcokinin, pyrokinins (PKs), sNPF, sulfakinins (SKs), and tachykinin-related peptides (TKRPs).

Profiling of the Central Nervous System (CNS). For an overview of neuropeptides present in the *Ae. aegypti* CNS, the following distinct regions were dissected from single specimens, prepared and directly profiled using MALDI-TOF MS: abdominal ganglia with attached perisymphatic organs (PSOs), postero-lateral edges of thoracic ganglia, medio-ventral parts of the subesophageal ganglion (SEG), pars lateralis and pars intercerebralis (protocerebrum). As with other insects, these compartments contain neurosecretory neurons which store expressed neuropeptides that are easily identified by MS.³⁰ This concept is best illustrated by the detection and sequence

Table 1. Neuropeptides and Protein Hormones Identified in Genome Databases for the Mosquitoes, *Aedes aegypti* and *Anopheles gambiae*, and the Fly *Drosophila melanogaster*

peptide name	<i>Ae. aegypti</i> ¹	<i>Ae. aegypti</i> transcript	<i>D. melanogaster</i> ²	<i>An. gambiae</i> ³
Neuropeptides				
Adipokinetic hormone 1 (AKH)	gblAAGE02022019.1l	gblAY431300.1l	CG1171	XP_001238167
AKH-2	gblAAGE02019657.1l	gblXM_001661147.1l		
Allatostatin-A	gblAAGE02032617.1l	gblU66841.1l	CG13633	XP_313511
Allatostatin-B (MIP)	gblAAGE02022351.1l	gblXM_001655823.1l	CG6456	XP_316799
Allatostatin-C	gblAAGE02009205.1	gblXM_001651331.1l	CG14919	XP_001238143
Allatotropin	gblAAGE02016767.1l	gblU65314.1l	ND	XM_320402
Calcitonin-like diuretic hormone (DH-31)	gblAAGE02013728.1l	gblXM_001658818.1l	CG13094	XP_321755.2
CAPA	gblAAGE02008523.1l	gblXM_001650839.1l	CG15520	XP_566030
Crustacean cardioactive peptide (CCAP)	gblAAGE02000952.1l	gblXM_001649143.1l	CG4910	XP_318812
Corazonin	gblAAGE02008171.1l	gblXM_001650517.1l	CG3302	XP_001238800
Diuretic hormone (DH-44)	gblAAGE02030775.l	ND	CG8348	XP_001230569
	gblAAGE02017550.1			XM_001230568
Ecdysis triggering hormone (ETH)	gblAAGE02002869.1l	gblDQ864499.1l	CG18105	XP_308702
Extended FMRFamide	gblAAGE02026154.1l	gblXM_001663782.1l	CG2346	XP_556154
Insect kinin	gblAAGE02018083.1l	gblU66832.1l	CG13480	Leucokinins
Myosuppressin (MS)	gblAAGE02012008.1l	gblXM_001652600.1l	CG6440	XP_321650
Neuropeptide F (NPF)	gblAAGE02004205.1l	gblAF474405.1l	CG13968	XP_315165
	gblAAGE02017729.1l			
Proctolin	ND	ND	CG7105	AgPT
Pyrokinin (PK)	gblAAGE02022205.1l	gblXM_001662162.1l	CG6371	XP_307885
Short Neuropeptide F (sNPF)	gblAAGE02023256.1l	gblDQ459411.1l	CG13968	ABD96048
	gblAAGE02023254.1l			
SIFamide	gblAAGE02017476.1l	gblXM_001654001.1l	CG4681	XP_308708
Sulfakinin (SK)	gblAAGE02010444.1l	gblDV383998.1l	CG18090	AAR03495
Tachykinin related peptide (TKRP)	gblAAGE02010775.1l	Not found	CG14734	XP_319161
Orcokinin	gblAAGE02012230.1l	Not found	—	XP_320317
Pigment dispersing hormone (PDH)	gblAAGE02002858.1l	gblXM_001653921.1l	CG6496	XP_315791
Neuropeptide-like precursor-1 (NPLP)	gblAAGE02023487.1l	gblXM_001649319.1l	CG3441	XP_311578
	gblAAGE02030041.1l			
Protein hormones (>3000 Da)				
Bursicon-alpha subunit	gblAAGE02007773.1l	ND	CG13419	AY735443
	gblAAGE02011233.1l			
Bursicon-beta subunit	gblAAGE02026399.1l	gblXM_001663860.1l	CG15284	XP_313804
Eclosion hormone 1 (EH)	gblAAGE02020363.1l	gblXM_001661458.1l	CG5400	XP_001230805
Eclosion hormone 2	gblAAGE02020363.1l	gblXM_001661457.1l		
Eclosion hormone 3	gblAAGE02034655.1l	gblXM_001647603.1l		
Eclosion hormone 4	gblAAGE02028822.1l	gblXM_001648672.1l		
Eclosion hormone 5	gblAAGE02010971.1l	gblXM_001652153.1l		
Glycoprotein hormone alpha 2 (GPA2)	gblAAGE02002350.1l	gblBN001241.1l	AAX38184.1	XP_317164
Glycoprotein hormone beta-5 (GPB5)	gblAAGE02002350.1l	gblXM_001653331.1l	CG40041	XP_555160
Insulin-like peptide 1	gblAAGE02001358.1l	gblDQ845750.1l	CG14173	AY324307
Insulin-like peptide 2	gblAAGE02001358.1l	gblDQ845751.1l	CG8167	AY324308
Insulin-like peptide 3	gblAAGE02001358.1l	gblDQ845752.1l	CG14167	AY324309
Insulin-like peptide 4	gblAAGE02001358.1l	gblDQ845753.1l	CG6736	AY324310
Insulin-like peptide 5	gblAAGE02004597.1l	gblDQ845758.1l	CG33273	AY324311–12
	gblAAGE02004598.1l			
Insulin-like peptide 6a	gblAAGE02007124.1l	gblDQ845755.1l	CG14049	AY324313
Insulin-like peptide 6b	gblAAGE02007124.1l	gblDQ845756.1l		
Insulin-like peptide 7	gblAAGE02001358.1l	gblDQ845757.1l	CG13317	AY324314–15
Insulin-like peptide 8	gblAAGE02001358.1l	gblDQ845754.1l		
Ion transport peptide (ITP)	gblAAGE02002845.1l	gblAY950506.1l	CG13586	XP_313928
	gblAAGE02002848.1l			
Ovary Ecdysteroidogenic Hormone (OEH; Neuroparsin A homologue)	gblAAGE02006435.1l	gblU69542.1l	ND	XP_311039
Prothoracicotropic hormone (PTTH)	gblAAGE02026723.1l	gblDV370510.1l	CG13687	XP_555854
	gblAAGE02034932.1l			

¹ *Aedes aegypti* neuropeptide and protein hormone names are assigned to encoding GenBank genes and ESTs as identified. ² *Drosophila melanogaster* names are assigned to CG numbers or GenBank accession numbers. ³ *Anopheles gambiae* names are assigned to encoding GenBank genes based on Riehle.¹

elucidation of CAPA peptides, which are typically found in such cells in the abdominal ganglia.³¹ In mosquitoes, two neurosecretory neurons in each abdominal ganglion express CAPA

peptides which are transported via the median nerve to neurohemal release sites along the body wall (abdominal PSOs, see inset Figure 1A and OEH I³²). Six short neuropeptides were

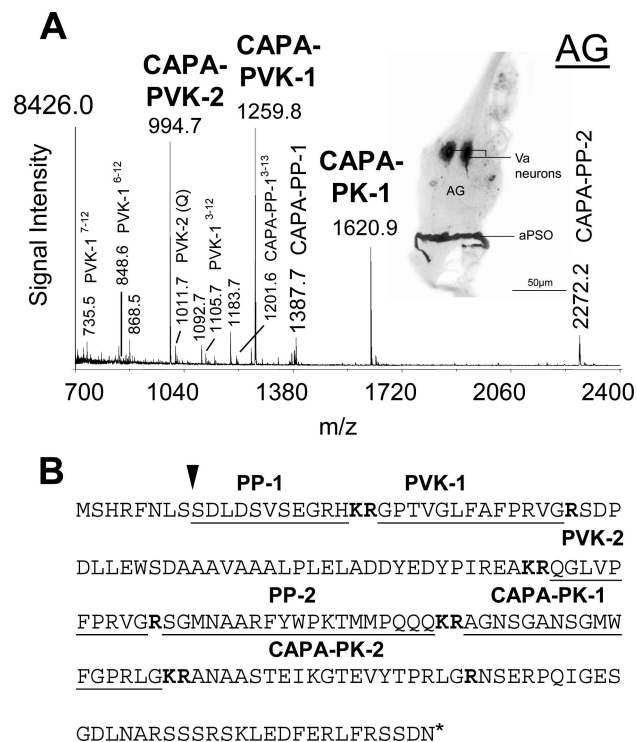


Figure 1. Detection of putative CAPA-peptides from *Ae. aegypti* by means of MALDI-TOF MS. (A) MALDI-TOF mass spectrum typical of an abdominal ganglion preparation with attached perisymphetic organ (aPSO). Only CAPA-peptides are labeled, and the most prominent mature products are given in bold. Note the complete absence of the ion signal of putative CAPA-PK-2 ([M + H]: 1920.0). The inset shows an abdominal ganglion immunostained with an anti-CAPA-PVK serum (Neupert, unpublished). (B) Sequence of the CAPA-precursor with the putative cleavage sites are labeled in bold. No signal peptide was predicted. Neuropeptides with mass match in (A) are underlined.

predicted to be processed from the CAPA-prepropeptide, namely two CAPA-periviscerokinins (PVKs), two CAPA-PKs, and two precursor peptides (PPs), which precede the PVK-1 and CAPA-PK-1 sequences. Long precursor peptides (>3000 Da), such as those located between PVK-1 and PVK-2 sequences, as well as between the CAPA-PK-2 sequence and the stop codon, were generally not detected in this study. For *D. melanogaster*, it was confirmed that CAPA-PVKs and CAPA-PK interact with their own specific receptor.^{33–35} The physiological relevance of the CAPA-PPs is not known.

Mass spectra of abdominal ganglia with attached abdominal PSOs ($n = 10$) revealed prominent ion signals that were assigned, by mass-match, to putative cleavage products of the CAPA-precursor (Figure 1). Altogether, the mass fingerprints provided evidence for five of the six predicted CAPA-peptides, although the ion signal of CAPA-PP-1 had very low intensity. CAPA-PP-1 has an uncertain N-terminal extension because no signal peptide was identified for the CAPA-precursor. By using the same preparations, the putative CAPA-peptides were subsequently fragmented to confirm their sequences (see Figure 2 for CAPA-PVK-2). Remarkably, no signal was observed for CAPA-PK-2, although this putative neuropeptide is flanked by a dibasic and a monobasic cleavage site common to other PVKs. A predicted CAPA-PK ortholog also was not detected in *Tribolium castaneum*,²² and a homologous sequence is missing in the *capa* gene of *An. gambiae* and *D. melanogaster*.

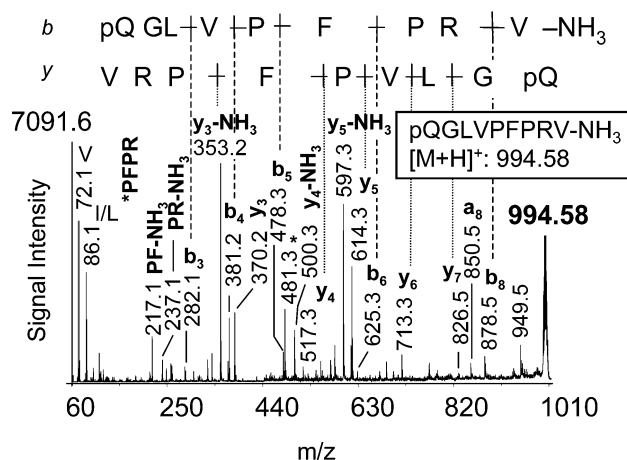


Figure 2. CID mass spectrum of the CAPA-peptide at $[M + H]^+$: 994.58 Da. The fragments were analyzed manually and the resulting sequence is given in the inset. A number of fragment ions are labeled which confirmed the amino acid sequence of N-terminally blocked (pGlu) CAPA-PVK-2.

In a final experimental step, all remaining ion signals from preparations of abdominal ganglia that had sufficient intensity for tandem MS analyses were screened for other processed peptides in the CAPA prepropeptide. This effort resulted in the identification of the N-terminally blocked (pGlu) form of CAPA-PVK-2. The blocked form showed higher signal intensity than the nonblocked form. In addition, truncated forms of CAPA-PVK-1 with low signal intensity and an extended form of CAPA-PP-1 with high signal intensity were found (Table 2). Signal intensity of the truncated forms was increased by using a higher laser power during the acquisition of the fingerprints. For that reason, these peptides and other truncated peptides with low abundance were classified as artifacts in this study and generally not considered to be mature peptides. The extended form of CAPA-PP-1 did not match with the annotated N-terminus of the CAPA-precursor, differing in the first two amino acids of the N-terminus. In summary, peptidomic analysis of CAPA processing in abdominal ganglia identified five predicted peptides, confirmed a posttranslationally modified form of CAPA-PVK-2, and detected an inaccurately annotated N-terminus of the precursor sequence. Mass spectra also led to the conclusion that CAPA-PK-2 is not processed as expected.

The same approach was performed with other neural compartments of the CNS which contain neurosecretory cell clusters (see above) and the following gene products were identified: extended FMRFamides (postero-lateral edges of thoracic ganglia), PKs (medio-ventral part of SEG), corazonin (pars lateralis), SIFamide (pars intercerebralis), and myosuppressin (pars intercerebralis). Most of these peptides, except for corazonin, were also found in other parts of the CNS but with lower signal intensity. The signal intensity of extended FMRFamides was generally low in mass spectra, even in samples from postero-lateral edges of thoracic ganglia, where neurosecretory FMRFamide producing cells are present.³⁶ This resulted in a limited number of fragments obtained during tandem fragmentation of these peptides. The co-occurrence of multiple forms of extended FMRFamides in single mass spectra was therefore taken as an additional confirmation for their presence. The other neuropeptide precursors were also processed as expected, including the N-terminal pyroglutamate in corazonin (a nonblocked form was not detected). In the pars intercerebralis cells of 3 out of 12 mosquitoes, an

Table 2. List of Mature Neuropeptides of *Ae. aegypti* that were Identified by Mass Spectrometry^a

peptide name	peptide sequence	[M + H] ⁺ , <i>m/z</i>	peptide name	peptide sequence	[M + H] ⁺ , <i>m/z</i>
Adipokinetic hormones			Myoinhibitory peptide		
AKH-1	pQLTFTPSWa	961.48	MIP-1	TWKNLQGGWa	1088.56
Allatostatin-A			MIP-2	AWNKGWGa	1044.54
AST-1	SPKYNFGLa	924.49	MIP-3	VNAGPAQWNKFRGSWa	1716.87
AST-2	LPHYNFGLa	959.51	MIP-4 ^b	EPGWNNLKGLWa	1312.68
AST-3	ASAYRYHFGLa	1183.60	MIP-5	SEKWNKLSSSWa	1350.68
AST-4	RVYDFGLa	868.47	Myosuppressin		
AST-5	LPNRYNFGLa	1092.59	MS	TDVDHVFLRFa	1247.65
ext. AST-5	VYEDKRLPNRYNFGLa	1882.99	Short Neuropeptide F		
PP-1 ^a	RYIIEDVPGA–OH	1132.59	sNPF-1	KAVRSPSLRLRFa	1428.89
Allatostatin-C			sNPF-1 ^{4–11}	SPSLRLRFa	974.59
AST-C	QIRYRQCYFNPISCF–OH	1935.91	sNPF-2 + 4	APQLRLRFa	999.62
	pQIRYRQCYFNPISCF–OH	1918.91	sNPF-3	APSQRLRWa	1012.58
Allatotropin			PP-2 ^b	SDPSVPVEPEDDDMDVQRSI–OH	2229.98
AT	APFRNSEMMTARGFa	1613.77	PP-4 ^b	SGGGMFSTNDVMQQA–OH	1770.81
CAPA			Neuropeptide-like precursor 1		
CAPA-PVK-1	GPTVGLFAFPRVa	1259.73	NPLP-1–1	SYRSLLRDGFa	1384.73
CAPA-PVK-2	pQGLVPFPRVa	994.58	NPLP-1–2	NLGSLARAGLLRTPSTDYL–OH	2018.10
	QGLVPFPRVa	1011.61	NPLP-1–4	NLASARASGYMLNa	1366.69
CAPA-PK	AGNSGANSWMWFGPRLa	1620.77	NPLP-1–5	NIASLARKYELPa	1373.79
PP-1	SDLDVSEGRH–OH	1201.54	NPLP-1–6	NIQSLLRGTGMLPSIAP–OH	1710.96
ext. PP-1	DASDLDSVSEGRH–OH	1387.61	ext. NPLP-1–6	NIQSLLRGTGMLPSIAPK–OH	1839.05
PP-2	SGMNAARFYWPKTMMPPQQ–OH	2272.05	NPLP-1–7	NMQSLARDNSLPHFAGAAAQES–OH	2315.08
Corazonin			NPLP-1–8	NIQTLVRDWNLPQQSMAADNE–OH	2599.27
Cor	pQTFQYSRGWTNa	1369.63	NPLP-1–9	NIQSLKNAQGGGSSSGa	1688.83
Diuretic hormones			Pigment-dispersing hormone		
DH31	TVDFGLSRGYSGAQEAKHRMA MAVANFAGGPa	3195.56	PDH	NSELINSLSLPKKLNDAA	1968.11
Ecdysis triggering hormones			Pyrokinins		
ETH-1 ^b	DETPGFFIKLSKSVPRiA	1933.09	PK-1	AAAMWFGPRLa	1118.59
ETH-2 ^b	GDFENFFLKQSKSVPRiA	2011.08	PK-2	DASSNENNSRPPFAPRLa	1957.95
Extended FMRFamides			PK-3	NLPFSPRLa	942.55
FMRFa-1	SALDKNFMRFa	1227.63	PP-1	GEVPDATEQKINNFLASGKDSIEDLS	2664.25
FMRFa-2	ASKQANLMRFa	1164.63	PP-2A ^b	TIASELHDEMMDIENNPLIYSa	2600.12
FMRFa-3	AGQGFMRFa	912.45	Sulfakinins		
FMRFa-4	DSPKNLMRFa	1106.58	SK-1	FDDY(SO ₃)GHMRFa	1186.51 ^d
FMRFa-5	DDTNKFLRLS–OH	1208.64	SK-2	GGGGEQFDDY(SO ₃)GHMRFa	1857.76 ^d
FMRFa-6	ANLMRFa	750.41	Tachykinin-related peptides		
FMRFa-7	AGSEAGGNLQRTNFLRFa	1836.95	TKRP-1 and 4	APSGFLGLRa	916.54
FMRFa-8	GSGNLMRFa	880.45	TKRP-2	VPSGFTGMRa	950.49
FMRFa-9	AKGNLMRFa	935.52	TKRP-3	APSGFLGMRa	934.49
FMRFa-10	SDPFLRLV–OH	1102.64	TKRP-5	VPNGFLGVRa	957.56
FMRFa-11	MDNNFMRFa	1073.43	PP-1	NIPFYPLRLYP–OH	1392.77
SIFamide peptide			Orcokinins		
SIFa	GYRKPPFNGSIFa	1381.74	Orc-1	NFDEIDRYSTFGa	1462.66
ext. SIFa	GYRKPPFNGSIFG–OH	1439.74	PP-1	NYEFMTPQERHSTI–OH	1752.80
Kinins			Orc. not assigned	NFDEIDRYXXXX	1517.69
Kin-1 ^c	NSKYVSKQKFYSWGa	1720.88			
Kin-2 ^c	NNPNVFYPWGa	1206.57			
Kin-3 ^c	NTGRVHRQPKVIRNPFHAWGa	2468.37			

^a PP, precursor peptide. ^b Mass match only. ^c Identified in Veenstra (1994).¹⁰ ^d Sulfation only detectable in the negative mode.

increased amount of a C-terminal extended SIFamide peptide (GYRKPPFNGSIFG–OH) was observed. In these mosquitoes, this peptide was present throughout the CNS, although it was always less abundant than the mature peptide.

The following processed peptides from other neuropeptide precursors were mainly identified in preparations of neuropil regions, such as optic lobes, antennal lobes, and dorso-caudal neuropil (DCN) of the terminal ganglion and in samples of brain tissue: AST-A, AST-C, AT, DH-31, NPLP-1 peptides, SKs,

sNPFs, MIPs, pigment dispersing hormone (PDH), TKRPs, and orckinins. With the exception of the putative orckinin-2 (NFDEIDQWAamide), all predicted neuropeptides with homologues in other insects were identified from these precursors. The two SKs of *Ae. aegypti*, identified only in brain tissue, were confirmed to be sulfated by reanalyzing samples in the negative mode (Figure 3).³⁷ Due to low signal intensity, one of the MIPs (MIP-4) could not be fragmented successfully, though a correct mass match together with the co-occurrence of the other

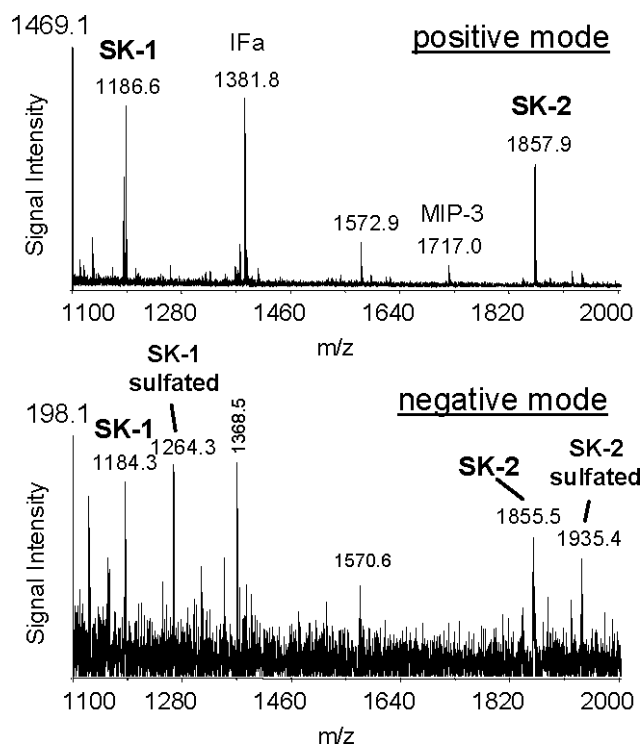


Figure 3. MALDI-TOF mass spectra of a preparation of brain tissue with fairly high concentrations of sulfakinins. The comparison of mass spectra in the positive and negative mode indicates the occurrence of the sulfate groups (mass difference of 80 Da). As already shown for other sulfakinins,³⁷ ions with sulfation are detectable in the negative mode only. The generally low amount of sulfakinins in the brain of *Ae. aegypti* limited the quality (signal-to-noise) of the mass spectra in the negative mode.

peptides from the MIP-precursor suggested that this peptide is cleaved as expected.

A number of additional peptides with at least moderate signal intensity could be fragmented from samples of the nervous system, and subsequent fragment analysis yielded other peptides not initially predicted. These neuropeptides are listed in Table 2 together with the other identified products of the respective neuropeptide precursors. One of these peptides was an orckinin (monoisotopic mass $[M + H]^+$ 1517.6; NFDEIDRY. . .) with high sequence similarity to orckinin-1, but it could not be assigned to the annotated orckinin-precursor. An unusual peptide identified was a C-terminally extended form of NPLP-1-6 which contains the Lys from the common Lys-Arg cleavage site and was always much more abundant than the predicted NPLP-1-6 (see Figure 4). No trace of a similarly extended form of the other NPLPs or any other neuropeptide from *Ae. aegypti* was found. To our knowledge, only two similarly extended peptides were identified from insects so far; namely an intermediate product of AKH-processing that was found in *D. melanogaster*³⁰ and a product of the NPLP-1 precursor of *D. melanogaster*.²⁰ The complete processing of all other NPLP-1 peptides of *Ae. aegypti* supports the idea that the C-terminally extended form of NPLP-6 is the mature peptide rather than an intermediate product. A single peptide from the AST-A precursor (AST-5) was identified as an N-terminally extended form that incorporates a predicted Lys-Arg cleavage site, but the extended form was much less abundant (approximately 10%) than the completely processed AST-5. Similarly extended forms of ASTs were also reported from the honey bee, *A. mellifera*.³⁸

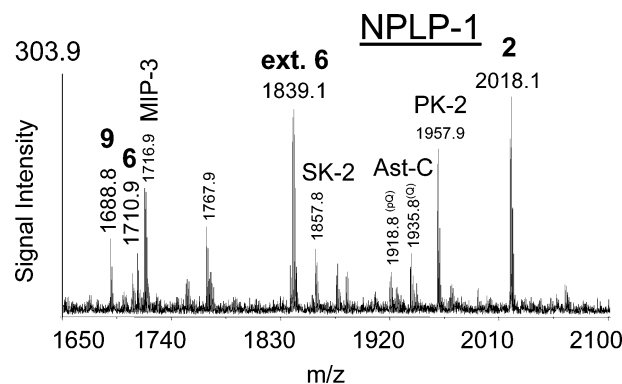


Figure 4. MALDI-TOF mass spectrum of a preparation of the tritocerebrum demonstrating the abundance of the C-terminal extended NPLP-1-6 in comparison to the predicted NPLP-1-6 sequence (for better visibility only a limited mass range of 1650–2100 is shown). The NPLP-1 peptides are designated with numbers. SK, sulfakinin; PK, pyrokinin; AST, allatostatin.

The peptidomic analysis of the CNS resulted in the unambiguous and nearly complete identification of processed peptides encoded by 17 neuropeptide genes. *Aedes* kinins were not sequenced in this study but were in an earlier study.¹⁰ Of the identified neuropeptide genes listed in Table 1, no peptides encoded by the *akh*, *eth*, *ccap* (crustacean cardioactive peptide) genes were detected in the CNS, but AKH-1 and ETHs were detected in other tissues (see below). Thus, CCAP was the only neuropeptide not detected in any tissues from single adults, most likely because of its low abundance. For all peptides with a mass-match ($[M + H]^+$: 956.5) predicted for CCAP, subsequent MS/MS analyses revealed either fragments from sodium adduct ions of TKRP-3 ($[M + Na]^+$: 956.5), extended FMRFa-9 ($[M + Na]^+$: 957.5), or fragments of mass-related TKRP-5 ($[M + H]^+$: 957.5). Analysis of CNS extracts from other holometabolous insects by MS (MALDI-TOF or LC-ESI-QTOF) also failed to identify a CCAP (*A. mellifera*;^{21,38} *D. melanogaster*;^{20,30} *T. castaneum*²²).

Differential Distribution of Neuropeptide-Precursor Products in the CNS. In addition to the detection of *Aedes* neuropeptides, comprehensive profiling of the CNS revealed a different processing of products for the CAPA-precursor and the AST-C precursor in different parts of the CNS. In abdominal ganglia as well as in the brain, two CAPA-PVKs, CAPA-PK-1 and the CAPA-PP-1 and 2 were identified, but mass spectra of the SEG yielded only signals of CAPA-PK-1 and CAPA-PP-2. An identical situation was reported for *D. melanogaster*^{30,39} and suggests a different processing of the CAPA precursor in the SEG and abdominal ganglia/brain of these insects. CAPA-PVKs are known to stimulate diuresis by the Malpighian tubules in the abdomen of Diptera^{40,41} and other insects.³¹ A recent study confirmed that the CAPA-PVK-receptor of *D. melanogaster*⁴² is predominantly expressed in Malpighian tubules. CAPA-PVKs of *Ae. aegypti* and *D. melanogaster* are seemingly not expressed in the CAPA cells of the SEG. Instead, the CAPA-PK is processed, which activates a specific receptor with an unknown function. In addition to the differences in the synthesis of CAPA-PVKs and PK in different ganglia, differential posttranslational processing of CAPA-PVK-2 in *Ae. aegypti* tissues was observed. The N-terminally blocked form (pGlu) of CAPA-PVK-2 was abundant in the abdominal ganglia (at a ratio blocked/nonblocked form of about 5:1), but in the brain, the nonblocked form (at a ratio blocked/nonblocked form of about 1:4) was much more prominent.

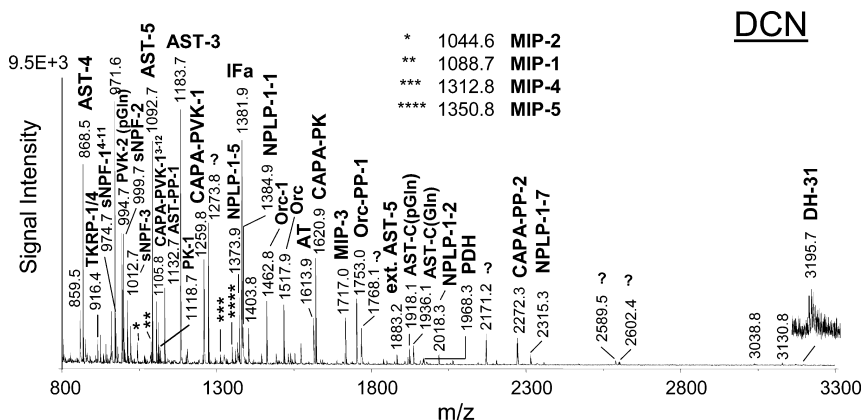


Figure 5. MALDI-TOF mass spectrum of a preparation of the dorso-caudal neuropil (DCN) of the terminal ganglion. The number of different neuropeptides in the DCN of *Ae. aegypti* was not surpassed by any other distinct region of the CNS; in this spectrum, mature products of 12 neuropeptide precursors are detectable (due to shortage of space, not all neuropeptide signals are designated). The question marks designate putative peptides which could not be assigned to the neuropeptide precursors of *Ae. aegypti*. PK, pyrokinin; AST, allatostatin; TKRP, tachykinin-related peptide; sNPF, short neuro peptide F, PVK-periviscerokin; NPLP, neuro peptide-like precursor; Orc, orcokinin; AT, allatotropin; MIP, myoinhibitory peptide; PDH, pigment dispersing hormone; DH, diuretic hormone.

A nearly identical situation was found for the N-terminally blocked form (pGlu) of AST-C that was more abundant in the abdominal and thoracic ganglia (3:1) than the nonblocked form. This ratio was reversed (1:2) in the brain and dorso-caudal neuropil of the terminal ganglion. This differential post-translational processing has not been reported in insects so far, and it may be related to their roles as hormones or neuromodulators within the CNS. When CAPA-PVK-2 or AST-C are likely to be released as hormones into the hemolymph, they are predominantly blocked and therefore less suitable to degradation by hemolymph enzymes.

A third important finding of the peptidomic analysis of the CNS was the astonishing number of different neuropeptides in the DCN of the terminal ganglion of *Ae. aegypti* (Figure 5). The number was not surpassed by any other region of the CNS analyzed in this study. The DCN is a distinct neuropil region that links neurons distributed all over the CNS with the proctodeal nerve (Neupert, unpublished). The peptidome of the DCN of *Ae. aegypti* was very different from that of other parts of the terminal ganglion or unfused abdominal ganglia. It accordingly should be regarded as part of a “posterior brain”.

Profiling of the Corpora Cardiac, Inka-Cells, and Midgut. The direct mass spectrometric profiling of the CNS was complemented by the analysis of the peptidome of the CC, Inka-cells, and midgut. The CC of adult mosquitoes is a neurohemal storage area fused around the aorta from which peptide hormones produced in brain neurosecretory cells are released into the hemolymph.^{27,43} A cluster of neurosecretory cells (X cells) is separate and posterior to the CC, and axons from these cells extend to the CC. In female *An. gambiae* and *Ae. aegypti*, these cells and other brain cells with axons to the CC and along the anterior midgut contain AKH-immunoreactivity.^{4,19} In this study, mass spectra of the CC from *Ae. aegypti* yielded distinct signals of the predicted AKH-1 with an N-terminus pGlu (Figure 6), and its sequence was confirmed by tandem MS.

Expression of a gene encoding AKH-2 was characterized in *Ae. aegypti*.¹⁹ Native AKH-2 was resolved by HPLC from head extracts of female *An. gambiae* in parallel with synthetic AKH-2, as detected by radioimmunoassay with the same AKH antiserum as used for immunocytochemistry.⁴ In this study, we did not detect AKH-2 in the brain or in the CC. Due to the

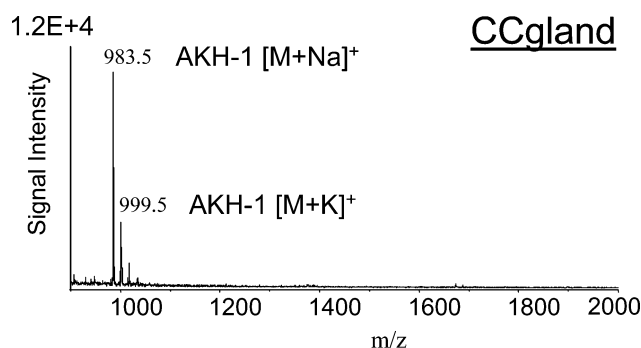


Figure 6. MALDI-TOF mass spectrum from the glandular portions of the corpora cardiaca. Only AKH-1 is detectable, ion signals of the putative AKH-2 are completely missing. AKH, adipokinetic hormone; CCgland, glandular portions of the corpora cardiaca.

lack of basic amino acids, AKHs are generally not easily ionized and yield low signal intensities in MALDI-TOF mass spectra and therefore low amounts may become undetectable.

In preparations of the CC, corazonin, orcokinins, sNPFs, myosuppressin, and two CAPA-peptides (CAPA-PK-1, CAPA-PP-2) were identified and their sequences confirmed by MS/MS analyses. The occurrence of these peptides in the CC indicates a role as classical neurohormones. In addition to these neuropeptides, two distinct signals at *m/z* 1161.7 and 1752.8 were observed. These substances were partially sequenced but could not be assigned to any of the annotated precursor sequences.

Inka-cells are peripheral endocrine cells attached to the tracheal system of insects that produce and release ecdysis triggering hormones (ETH) at each ecdysis.⁴⁴ Peptide synthesis in these cells is restricted to larval stages. In *Ae. aegypti*, single Inka-cells are associated with epitracheal glands located along the lateral tracheal trunks.^{45,18} Pieces of the tracheal trunks of *Ae. aegypti* larva revealed the presence of the two ETHs (Figure 7).

The midgut of all mosquito life stages contains tens to hundreds of dispersed endocrine cells.^{46,36} It was supposed that these cells may produce many of the same regulatory peptides found in the CNS.⁴⁷ This feature resembles the brain-gut system of peptides found in vertebrates. In addition to the endocrine cells, peptidergic neurons from the CNS innervate the muscles

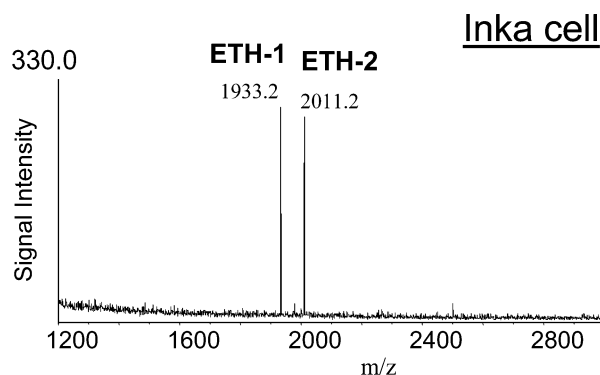


Figure 7. MALDI-TOF mass spectrum of a preparation of a lateral tracheal trunk (with Inka-cells) of an *Ae. aegypti* larvae. Two distinct ion signals representing the ETHs are marked. The synthesis and release of these (neuro)peptides is restricted to Inka-cells of larval stages.

of the gut, either via the stomatogastric nervous system or the proctodeal nerves. To our knowledge, direct mass spectrometric profiling of midgut endocrine peptides has not been accomplished for any insect to date. Previously, specific peptides were purified by HPLC from gut extracts, based on bioactivity or immunoreactivity, for only a few insects.^{48–52} It was therefore a surprise that experiments with midgut tissue of freshly emerged male mosquitoes succeeded in the detection of many neuropeptides, including sNPFs, TKRPs, MIPs, ASTs, and CAPA-peptides. As expected from earlier immunocytochemistry studies,^{53–56} the gut peptides were not evenly distributed along the midgut, which can be divided into a narrow anterior part and expanded posterior part. Pieces from the most anterior section of the midgut contained very abundant ion signals of sNPFs and, with a lower signal intensity, TKRPs, MIPs, and CAPA-peptides (Figure 8A). As in preparations from SEG and CC, only CAPA-PK-1 and CAPA-PP-2 were detected from the CAPA-prepropeptide. Mass signals of sNPFs and the CAPA-peptides disappeared in preparations of the posterior midgut. Instead, AST-A peptides were abundant (Figure 8B). Occurrence of AST-A-like immunoreactivity in the most posterior section of the midgut was suggested based on immunocytochemistry.⁵⁶ Presence of sNPFs in the anterior midgut of adult *Ae. aegypti* was not detected by antisera specific to RFamide or NPF/pancreatic polypeptide,^{53,56,57} but such staining was present in hundreds of endocrine cells in the posterior midgut. The occurrence of CAPA-peptides in the midgut has not been previously reported. The peptidome of the hindgut of male *Ae. aegypti* consisted mainly of AST-A and AST-C peptides and other substances that could not be assigned to the annotated neuropeptide precursors. Some of the above peptides were tested for effects on ion transport and peristalsis of midguts from *Ae. aegypti* larvae, including ASTs, allatotropin, head peptide, sNPFs, and NPF.⁵⁸ Since the hindgut contains no endocrine cells, the allatostatins are likely produced in the CNS and transported via the proctodeal nerve to the surface of the hindgut.

A final comment is needed for the head peptides of *Ae. aegypti* that were initially isolated based on their RF-immunoreactivity from extracts of adult heads⁷ and other body regions.⁵⁹ Synthetic head peptides were shown to inhibit host-seeking by female mosquitoes,⁸ and their hemolymph titer was profiled in females post blood meal. Molecular characterization of the *head peptides* gene revealed a precursor with three copies of head peptide and its expression in many female and male

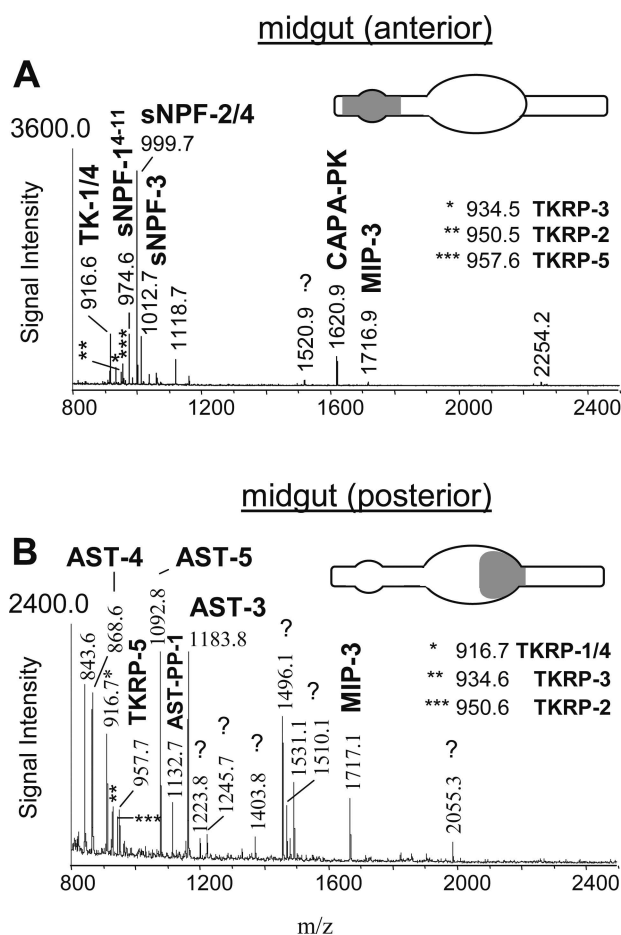


Figure 8. MALDI-TOF mass spectra of preparations of midgut tissue from freshly hatched male *Ae. aegypti*; which demonstrate the uneven distribution of a number of neuropeptides in the midgut. (A) Preparation of an anterior portion of the midgut. (B) Preparation of a posterior portion of the midgut. AST, allatostatin; TKRP, tachykinin-related peptide; sNPF, short neuropeptide F, MIP, myoinhibitory peptide; PK, pyrokinin.

tissues.⁹ We were unable to identify the *head peptide* gene in the completed *Ae. aegypti* genome by bioinformatics, but it was found in trace sequences obtained from the Broad Institute excluded reads database (identifier: G719P616810RC2.T0). Interestingly, our mass spectrometric analyses failed to detect head peptides in any tissue, even though large amounts were reported to be present in adult extracts.^{7,59} No *head peptide* gene homologues are known for *An. gambiae*, *D. melanogaster*, or any other insect, but it does share limited similarity to the *snpf* gene identified in *Ae. aegypti* and many other insects. In this study, the products of the *snpf* gene of *Ae. aegypti* were found to be abundant in different parts of the CNS and also in the gut.

Conclusions

The first comprehensive peptidomic analysis of a mosquito species resulted in an almost complete list of mature neuropeptides of *Ae. aegypti*. The data were solely obtained by direct mass spectrometric profiling of selected tissues from single specimens which yielded a largely complete accounting of the putative bioactive neuropeptides. Other methods such as ESI-QTOF MS, which consume more material, may provide more complete fragmentation spectra, but the time for con-

secutive fragmentations is sometimes not sufficient to obtain all peptides with an intensity to identify the neuropeptides. In this study, the main focus was not the counting of observed peptides (including breakdown products) but the generation of data that can be used for future peptidomic studies. As shown, single specimens are sufficient to obtain the complete neuropeptidome on a tissue-specific level once the identity of neuropeptides has been confirmed by fragmentation data. Hence, only fingerprints are necessary to obtain differences in the time- or site-specific expression/processing of certain neuropeptides during development or following genetic manipulation. Particularly important in this context is the finding that the midgut tissue can be included in such an approach. Altogether, this study provides a framework for future investigations on mosquito endocrinology and neurobiology. Given the high sequence similarity of neuropeptide precursors identified in the other medically important mosquitoes, *An. gambiae* and *C. quinquefasciatus*, conclusions regarding the peptidome of *Ae. aegypti* likely are applicable to these mosquitoes.

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Supporting Information Available: Fasta file for putative transcriptions of annotated neuropeptide and protein hormone genes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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