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Structural Diversity of Adipokinetic Hormones in the Coleopteran Suborder Polyphaga (Excluding Cucujiformia)

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ABSTRACT

Beetles are the largest animal group, in general. Phylogenetically, beetles belong to the order Coleoptera, the most species-rich of the Insecta. Coleoptera is divided into four suborders: Polyphaga, Adephaga, Archostemata, and Myxophaga. Specimens from the latter two are difficult to obtain, hence, we have focused our research into the adipokinetic hormone (AKH) peptide family on the former two suborders. Data on the Adephaga were concluded in 2017. The "core Polyphaga" consists of three series: Elateriformia, Staphyliniformia, and Cucujiformia; the latter was concluded in 2019. Here, we report on the AKH sequence(s) of 23 species of beetles from 4 families of Elateriformia, namely, the Buprestidae, Cantharidae, Elateridae, and Lampyridae; and 4 families of Staphyliniformia, namely, the Hydrophilidae, Silphidae, Lucanidae, and Scarabaeidae. Sequence elucidation by mass spectrometry or Edman degradation revealed 13 octapeptides: 5 are novel, 12 are beetle-specific and Schgr-AKH-II is produced in the basal Polyphaga (Elateriformia, in Hydrophilididae and Silphidae). Since Schgr-AKH-II is also found in Adephaga, this confirms the ancestral AKH of Coleoptera. The first change in sequence is recorded in Staphylinoidea with two different residues, notably, the switch from Phe^4 to Tyr^4. Duplication of AKH peptides is first seen in Lucanidae, as well as the appearance of atypical AKH sequences, such as Phe^2, Met^4, Leu^4, or Phe^7 encountered in the Scarabaeoidea. The vast majority of the pest beetles do not have beetle-specific AKHs or share the same AKH as nonharmful beetles. Ideas for finding a lead compound for green insecticides are discussed.

1 | Introduction

The adipokinetic hormone (AKH) family is one of the better-known neuropeptide members belonging to the gonadotropin-releasing hormone (GnRH) superfamily (see Zandawala et al. 2018; Tsai 2018). The superfamily is named after the vertebrate GnRH, and other members include corazonin and the AKH/corazonin-related peptide of the invertebrates. Genes, precursors, receptors, and metabolic functions of the three invertebrate neuropeptide families in the Pancrustacea (Hexapoda and Crustacea) were reviewed very recently (Marco et al. 2024). In the current work, we concentrate on structural aspects of the ever-increasing AKH family in a subset of Coleoptera.

In insects, AKHs are solely produced in neurosecretory cells of the neurohaemal corpus cardiacum organ and have a chain length of

Gerd Gäde and Simone König contributed equally to this work.

Summary

- The majority of basal families of Polyphaga synthesize octapeptide Schgr-AKH-II as do Adephaga.
- Schgr-AKH-II is assumed as ancestor of AKH family in Coleoptera.
- Radiation of beetle AKH diversity occurred during the evolution of the Scarabaeoidea.

8–10 amino acids with blocked termini: a modified glutamine/glutamate residue at the N-terminus, i.e., pyroglutamate, and the C-terminus is carboxyamidated. From the more than 100 mature AKH confirmed peptide sequences commonly, residue 3 is either Asn or Thr, residue 4 the aromatics Phe or Tyr, residue 5 Ser or Thr, residue 8 Trp, and residue 9 is Gly; at position 2 aliphatic (Leu, Ile, Val) or aromatic (Phe, Tyr) amino acids are located; at position 6 mostly (but not exclusively) Pro and its oxidized derivative hydroxyproline (Hyp); at position 7 at least 10 different amino acids have been found in the various insects studied with Gly as the most frequently occurring residue; and in position 10, Gln is mostly detected from amongst 10 amino acids (for primary structures, see Gäde 2009; Gäde et al. 2020, 2021; Marco et al. 2020, 2023; Gäde and Marco 2022; König et al. 2023a; Jiang et al. 2023).

The major function of AKHs in insects is the control of intermediary metabolism, that is, they mobilize lipid and carbohydrate fuels stored in the fat body to be released into the hemolymph for use to contract the respective muscles during flight or swimming episodes (see reviews by Gäde and Marco 2006; Marco and Gäde 2020). Renewed interest in the AKH neuropeptide family came more than a decade ago when research efforts concentrated to study the neuropeptide ligand and its cognate G-protein-coupled receptor (GPCR) system to discover biorational and biodegradable insecticides (Gäde and Goldsworthy 2003; Audsley and Down 2015; Davies 2017) using the same intellectual approach as the pharmaceutical industry exploits for drug discovery, as in the case of the well-known beta-blockers (Black 2010; Baker et al. 2011). One of the major and prerequisite tasks for such ligand/receptor work is the knowledge of the primary structure of these ligands (and thereafter of the receptors), to successfully find lead chemical compounds from which peptide mimetics can be developed that are not deleterious to beneficial insects. Of course, identification and structural information of the requisite GPCRs go hand-in-hand with the discovery process of the so-called "green insecticide."

The order Coleoptera, the beetles, is not only the most species-rich of the Insecta with ~386,000 described extant species (Ślipiński et al. 2011) but also the largest animal group, in general. Phylogenetically, Coleoptera is divided into four suborders (see Figure 1) with the Polyphaga as sister group to the Adephaga which are sister to Archostemata and Myxophaga: (Polyphaga (Adephaga (Myxophaga, Archostemata))) (McKenna et al. 2015, 2019; Sharkey et al. 2017; Zhang et al. 2018). AKHs of the Adephaga have been elucidated (Gäde and Marco 2017). The "core Polyphaga" (McKenna et al. 2015; Zhang et al. 2018) consist of three series: Elateriformia, Staphyliniformia, and Cucujiformia; AKHs from 17 species of Cucujiformia have been identified (Gäde et al. 2019). In the current study, we investigate the complement of AKHs from 4 of the 20 families of Elateriformia, namely, the Buprestidae, Cantharidae, Elateridae, and Lampyridae; Lycidae was previously done (Gäde et al. 2015). Staphyliniformia have also around 20 families, of which we here study the AKH structures from four families, namely, the Hydrophilidae, Silphidae, Lucanidae, and Scarabaeidae (some species were previously investigated: Gäde 1997; Gäde et al. 2016); Geotrupidae was also done (Gäde 1991; Gäde et al. 2016). It stands to reason that there is still much structural information and distribution patterns of AKHs to glean from this speciose suborder of the Coleoptera; information that is interesting and useful alike for comparative endocrinologists, peptide chemists and the pharmacologists involved in developing novel pesticides.

The questions we want to answer in this study are: do the more basal Polyphaga families also have the AKH octapeptide called Schgr-AKH-II produced in their corpora cardiaca (CC), as we have found in all investigated members of the Adephaga, from whence we denoted this peptide the ancestral coleopteran AKH (Gäde and Marco 2017)? At which phylogenetic point do changes in the structure of the AKH occur? What are the changes and are there sufficient structural differences to Schgr-AKH-II to warrant further investigations and use such forms as lead substances to develop a suitable mimetic agonist or antagonist? And finally, are such modified AKHs especially found in those families that have "pest" status? For AKH identification, we used high-resolution mass spectrometry (HRMS) as a tool to detect and validate the peptides in the methanolic extracts of corpora cardiaca of the five superfamilies Buprestoidea, Elateroidea, Hydrophiloidea, Staphylinoidea, and Scarabaeoidea. In one instance, sequencing proceeded via Edman degradation.

2 | Materials and Methods

2.1 | Insects

Throughout the entire study, we investigated adult specimens of unknown age, and both sexes were used. Live beetles were collected in the field or were purchased from breeders. In total 23 species of Polyphaga were studied: 20 for the first time in respect of AKH complement. Details of the beetle species and the taxonomic affiliations are given below and following the phylogenetic outline of McKenna et al. (2019). For illustration, an abbreviated phylogenetic tree is supplied (Figure 1) which mainly shows those clades that are investigated in the current study.

2.1.1 | Superfamily Buprestoidea

Two species of jewel beetles (Family: Buprestidae) are included. Specimens of the genus *Julodis* were collected in a woody bushland in the Cedarberg mountains, Western Cape Province of South Africa. The species *Sternocera orissa*, (giant jewel beetle) came from the Kgalagadi Transfrontier National Park in the Northern Province of South Africa. Specimens love hot temperatures, are mostly sitting quietly eating the pollen of certain plants and can be easily plucked from the plants in the heat of the day. They are a sought after protein food for indigenous people of poor rural communities (Shadung 2012).

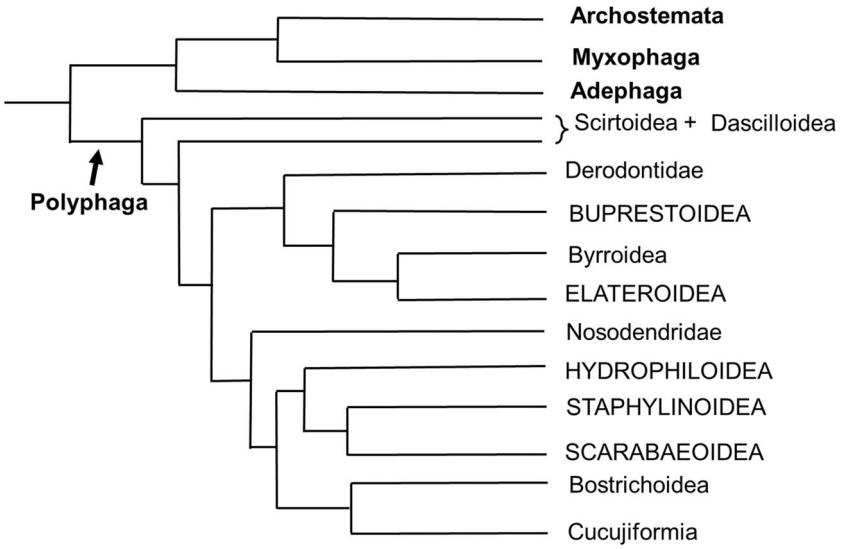


FIGURE 1 | Phylogenetic tree of Coleoptera (adapted and simplified after McKenna et al. 2019) showing the four suborders (in bold) and the major superfamilies. In capital letters are the superfamilies investigated in the current study.

2.1.2 | Superfamily Elateroidea

Specimens of the mouse grey click beetle (*Adelocera murina* = *Agrypnus murinus*) of the family Elateridae were collected in forests around Schwaan in Mecklenburg-Vorpommern Province of Germany. Adults were eating from the flowers of *Apiaceae*. Specimens of the red–brown click beetle (*Athous haemorrhoidalis*) were collected from leaves of the stinging nettle in the Lower Saxony Province in Germany around Bad Iburg. The species *Cantharis pellucida* of the soldier beetles (Family: Cantharidae) was also collected in this region. One species of the fireflies or lightning bugs (Family: Lampyridae) *Lamprohiza splendidula* was netted at dusk in the forest around Jena in the Thuringia Province of Germany. Only males were collected, since they are actively flying and glowing, whereas females are sedentary without functional wings but able to send lightning signals to attract the active males (Münch et al. 2010).

2.1.3 | Superfamily Hydrophiloidea

One species of the water scavenger beetles was investigated. The lesser silver water beetle (*Hydrochara caraboides*, Family: Hydrophilidae) came from a small forest pond near Schwaan/Mecklenburg-Vorpommern/Germany. This species is fully aquatic as adults and can stay below the water surface for extended times due to a specific mechanism to trap air between the numerous fine body hairs and under their elytra (Short and McIntosh 2014).

2.1.4 | Superfamily Staphylinoidea

Six species of carrion beetles (Family: Silphidae) were investigated. Specimens of *Phosphuga atrata* were found in a rotten tree stump close to Burg Strechau an der Muhr in Austria; this species is well known for devouring snails. The five different species of the genus *Nicrophorus* were a gift from Prof. Sandra Steiger (University of Bayreuth, Germany); the species were *Nicrophorus vespilloides*, *Nicrophorus pustulatus*, *Nicrophorus orbicollis*, *Nicrophorus quadripunctatus*, *Nicrophorus defodicus*, all of which display various stages of parental care (Capodeanu-Nägler et al. 2016).

2.1.5 | Superfamily Scarabaeoidea

Ten species of this large clade were studied. Three species of the Family Lucanidae included the stag beetles *Lucanus cervus*, *Serrognathus typhon*, both of which came from commercial breeders, and *Dorcus parallelipipedus*, which was caught in Nordrhein Westfalia close to Aachen, Germany. Seven members of the species-rich family Scarabaeidae were included, namely, specimens of the European June beetle *Aphimallon solstitiale* (Subfamily: Melolonthinae) were collected in a private garden in Bad Iburg during flight in the summer solstice at about 22h00 in the Northern hemisphere. Three species in total belong to the subfamily Cetoniinae: two species were collected in Hungary, namely, *Tropinota hirta* from flowers close to a vineyard in Villany and *Cetonia aurata* in a private garden of a Budapest suburb, while the third cetoniid species, the Eurasian bee beetle *Trichius fasciatus*, was collected when foraging thistle pollen in a private garden in Bad Iburg. Three species belong to the subfamily Scarabaeinae: the specimens of the species *Xinidium dentilabris* and *Copris caelatus* were caught at the foot of the Drakensberg near Winterton in KwaZulu-Natal/South Africa, while *Ontophagus binodis* came from the region around Ficksburg in the Free State Province of South Africa.

2.2 | Dissection of CC, Peptide Extraction, Mass Spectrometry, and Sequence Analysis

2.2.1 | Dissection

Corpora cardiaca (CC) were dissected from the head of adult beetles from each species under a stereomicroscope at 20-fold magnification and placed into 80% v/v methanol. Extracts were prepared as outlined previously (Gäde et al. 1984) and dried in a vacuum concentrator.

2.2.2 | Mass Analysis of AKHs

The vacuum-centrifuged dried extracts were dissolved in 10 μL methanol followed by 10 μL 0.1% formic acid containing 5% acetonitrile. For liquid chromatography (LC)-HRMS, Synapt G2 Si coupled to M-Class nanoUPLC (Waters Corp., Manchester, UK) was employed using C18 μPAC columns (trapping and 50 cm analytical; PharmaFluidics, Ghent, Belgium) with a 30 min gradient (10%–60%; solvent system 100% water vs. 100% acetonitrile, both containing 0.1% formic acid; 1 μL injection volume). AKH candidates were identified by target-MS (MS/MS on preselected *m/z* values) for eligible known peptide masses from related insect species using their singly and doubly charged ions, as well as by screening with low/high collision energy switching for the gas phase loss of the tryptophan immonium ion in data-independent runs. Moreover, AKH candidates were obtained by manual interrogation of data-dependent runs and the use of marker fragment ions discovered for proline-containing AKHs (König et al. 2023b).

Sequence ion assignment was used as calculated by the MassLynx spectrometer software, which treats pyroglutamate (Pyr) as terminal modification rather than a modified amino acid thus creating a label shift for ion assignment by one in comparison to the amino acid number. The fragment ion tables for the spectra shown here are available in the Supporting Information for clarification. Peptide sequences were validated by comparison to the performance of the respective synthetic peptides; in the case of the hydroxyproline forms, where synthetic peptides were not available, the sequences are considered putative. For validation, both the endogenous and the synthetic samples were spiked with bradykinin 1–7 (Sigma, 1 pmol/μL stock solution) for control of the retention time (RT); bradykinin 1–7 eluted about 6 min earlier than the AKHs. This allowed for correction of the LC arrival time following heavy unrelated use of the instrumentation, when necessary. The peptides were run with identical parameters separated by blank runs. Glu-fibrinopeptide was measured as lockmass in each run and used for internal correction if needed. Pacsi-AKH was validated with the synthetic peptide using LCMS-8050 (Shimadzu) and an InfinityLab Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 micron, Agilent) column.

Note that the AKHs are code-named according to the species in which the AKH was first structurally characterized, using the nomenclature for naming insect neuropeptide families as proposed by Coast and Schooley (2011). A five-letter code is used where the first three letters from the genus are combined with the first two letters of the species name.

2.2.3 | Enzymatic Deblocking of N Terminus for Edman Degradation Sequencing

The AKH from the buprestid beetle *S. orissa* was analyzed via classical amino acid sequencing. Thus, the methanolic CC extract was first separated on reversed-phase high-performance liquid chromatography (RP-HPLC) as outlined previously (Gäde 1985). The peak fraction containing the AKH was collected, dried, and subjected to enzymatic deblocking of the N-terminal pyroglutamate residue using L-pyroglutamate aminopeptidase as described (Gäde et al. 1988). The deblocked peptide was purified on RP-HPLC, manually collected, dried, and subjected to the automated Edman degradation procedure with a pulsed-liquid phase sequencer (Model 477 A; Applied Biosystems) as described (Gäde and Kellner 1992).

2.3 | Synthetic Peptides

The following octapeptides (see Table 1 for amino acid sequences) were purchased: Schgr-AKH-II and Pacsi-AKH from Synpeptide Co. Ltd. (Shanghai, China); Nicve-AKH, Dorpa-AKH, Cirba-AKH, Ontbi-AKH, Ampso-AKH-I, Ampso-AKH-II, and Lucce-AKH from Pepmic Co. Ltd. (Suzhou, China); Melme-CC from Novabiochem GmbH (Sandhausen, Germany). Scade-CC-I and Scade-CC-II were previously custom-synthesized by Dr. R. Kellner (Merck KGaA, Darmstadt, Germany).

3 | Results

3.1 | Mass Spectral Analyses of AKHs

All methanolic CC extracts, except one (see Section 3.1.2), were analyzed by RP-LC coupled to HRMS. The MS analysis procedure was the same for all peptide species and the sequences were all validated using the relevant synthetic peptide. For the sake of conciseness, exemplary data are shown: we do not repeat the information when the same peptide was detected in different insect samples; RT in LC and the gas phase fragment ion data generated by collision-induced dissociation (CID) in the mass spectrometer matched between samples containing the identical peptide.

3.1.1 | Superfamilies Hydrophiloidea and Elateroidea

In the CC extract from *H. caraboides* (superfamily Hydrophiloidea, family Hydrophilidae) a peptide with RT of 34.8 min was identified as a member of the AKH family. A peptide with the same RT was measured and assigned as an AKH in each of the four species from three different families of the Elateroidea, the Elateridae, Cantharidae, and Lampyridae (Table 1); the peptides yielded identical sequencing data. For this peptide identification, analyses and validation using the synthetic peptide standard, exemplary data from the CC extract of *H. caraboides* are presented in Figure 2. In the MS overview scan, the singly charged peptide ion (*m/z* 934.44) was in agreement with the AKH peptide code-named Schgr-AKH-II (first sequenced from the neuroendocrine glands of locust species [Siegert et al. 1985; Gäde et al. 1986]). This was corroborated and proven by gas phase fragmentation for sequence analysis of this peptide. All expected fragment ions of the amino acid residue losses from either end of the peptide were detected (for major ions, see Figure 1C; for all calculated ions, see Figure S1); and, thus, the sequence was assigned as pGlu-Leu/Ile-Asn-Phe-Ser-Thr-Gly-Trp amide. Moreover, the CID spectrum of the endogenous peptide versus that of the synthetic standard peptide (Schgr-AKH-II) was identical (Figure 2A, B). The agreement of both sequences was finally confirmed by their identical RT

TABLE 1 | Coleopteran AKH peptides identified in this study.

Superfamily, family, subfamily	Species	Amino acid sequence	Molecular weight	Peptide name
Buprestoidea, Buprestidae	*Julodis spec*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
	Sternocera orissa	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
Elateroidea, Elateridae	*Adelocera murina*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
	Athous haemorrhoidalis	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
Elateroidea, Cantharidae	*Cantharis pellucida*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
Elateroidea, Lampyridae	*Lamprohiza splendidula*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
Hydrophiloidea, Hydrophilidae	*Hydrochara caraboides*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
Staphylinoidea, Silphidae	*Phosphuga atrata*	pQ LNFSTGW amide	933.4345	Schgr-AKH-II
	Nicrophorus vespilloides	pQ LTYSTGW amide	936.4342	Nicve-AKH
	Nicrophorus pustulatus	pQ LTYSTGW amide	936.4342	Nicve-AKH
	Nicrophorus orbicollis	pQ LTYSTGW amide	936.4342	Nicve-AKH
	Nicrophorus quadripunctatus	pQ LTYSTGW amide	936.4342	Nicve-AKH
	Nicrophorus defodicus	pQ LTYSTGW amide	936.4342	Nicve-AKH
Scarabaeoidea, Lucanidae	*Dorcus parallelipipedus*	pQ VNYSPVW amide	973.4667	Dorpa-AKH
		pQ VNYSHypVW amide	989.4607	Dorpa-AKH-Hyp
		pQ LNYSPDW amide	1003.4400	Melme-CC
		pQ LNYSHypDW amide	1019.4349	Melme-CC-Hyp
	Serrognathus typhon	pQ LNYSPDW amide	1003.4400	Melme-CC
		pQ VNYSPVW amide	973.4667	Dorpa-AKH
		pQ VNYSHypVW amide	989.4607	Dorpa-AKH-Hyp
	Lucanus cervus	pQ VNYSPVW amide	973.4667	Dorpa-AKH
		pQ VNYSHypVW amide	989.4607	Dorpa-AKH-Hyp
		pQ FNYSPQW amide	1050.456	**Lucce-AKH**
Scarabaeoidea, Scarabaeidae, Melolonthinae	*Amphimallon solstitiale*	pQ LNMSTGW amide	917.4066	**Ampso-AKH-II**
		pQ VNYSPDW amide	989.4243	**Ampso-AKH-I**
Scarabaeoidea, Scarabaeidae, Cetoniinae	*Trichius fasciatus*	pQ INLTTGW amide	913.4658	**Pacsi-AKH**
		pQ LNYSPDW amide	1003.4400	Melme-CC
	Cetonia aurata	pQ LNYSPDW amide	1003.4400	Melme-CC
		pQ LNYSHypDW amide	1019.4349	Melme-CC-Hyp

(Continues)

TABLE 1 | (Continued)

Superfamily, family, subfamily	Species	Amino acid sequence	Molecular weight	Peptide name
	Tropinata hirta	pQ LNYSPDW amide	1003.4400	Melme-CC
		pQ INLTTGW amide	913.4658	**Pacsi-AKH**
		pQ INLTTGW amide (modified)	993.4226	Pacsi-AKH, modifed
Scarabaeoidea, Scarabaeidae, Scarabaeinae	*Copris caelatus*	pQ FNYSPDW amide	1037.4243	Scade-CC-I
		pQ FNYSPVW amide	1021.4658	Scade-CC-II
	Xinidium dentilabris	pQ FNYSPDW amide	1037.4243	Scade-CC-I
		pQ FNFSAGW amide	937.4083	Cirba-AKH
	Ontophagus binodis	pQ FNYSPDW amide	1037.4243	Scade-CC-I
		pQ VNYSPFW amide	1021.4658	**Ontbi-AKH**
		pQ VNYSHypFW amide	1037.4607	Ontbi-AKH-Hyp

Note: In bold text: novel AKH sequences and the names given to these peptides. Posttranslational modifications: HYP, hydroxyproline—this is considered "putative" since the equivalent synthetic structure was not available for confirmation; "modified," this PTM needs further corroboration.

(34.8 min) in LC. Hence, Leu at position 2 was confirmed, since a peptide having the isobaric Ile would have a different RT as shown several times previously (e.g., Gäde, Auerswald et al. 2003; Gäde et al. 2016).

3.1.2 | Superfamily Buprestoidea

Schgr-AKH-II was detected and validated in the CC extract of the jewel beetle of the genus *Julodis* in the same way as in Section 3.1.1. The same peptide sequence was also found in *S. orissa* but via a different methodology: after LC purification of the material, the N-terminal pyroglutamate was enzymatically cleaved off and the purified remaining peptide was analyzed via Edman sequencing where the amino acid Leu was detected in Cycle 1 and the next cycles resulted in Asn, Phe, Ser, Thr, Gly and a small amount of Trp, respectively. The amino acid sequence of Schgr-AKH-II was, thus, confirmed, especially the Leu and not Ile at position 2.

3.1.3 | Superfamily Staphylinoidea

MS analyses of CC extracts from the carrion beetle *P. atrata* revealed all data of the sequence Schgr-AKH-II. A different picture arose, however, in the analyses of five different species of the genus *Nicrophorus*: each showed an extracted chromatogram with a peak at an earlier RT than Schgr-AKH-II, i.e. at 31.4 min, with an MH^+ ion at *m/z* 937.44; MS/MS sequencing assigned the sequence pGlu-Leu-Thr-Tyr-Ser-Thr-Gly-Trp amide, a peptide that is known from previous work on the species *N. vespilloides* (Gäde et al. 2015) and is code-named Nicve-AKH. Validation was achieved again by processing the synthetic peptide under the same conditions as the natural CC extract: RT and MS fragmentation data matched. For the native Nicve-AKH peptide, we observed a number of tryptophan oxidation products during MS; this phenomenon was previously described along with the MS data for *N. vespilloides* (König et al. 2023a).

3.1.4 | Superfamily Scarabaeoidea

From the large superfamily Scarabaeoidea members of the two families Lucanidae (stag beetles) and Scarabaeidae (scarab beetles) were analyzed for their complement of AKHs. For the Lucanidae, 3 species were examined. First, we identified the AKHs of the smaller stag beetle *D. parallelipipedus*—this is a repeat of an earlier analysis (Gäde et al. 2016), and served as a control for the collection, extraction, processing and prolonged storage of other beetle extracts that were subjected to the same treatment on a specific collecting trip. True to expectation, MS analysis of the ions at MH^+ 1004.45 and 974.47, respectively, resulted in the identification and subsequent validation of the AKH peptides code-named Melme-CC (pGlu-Leu-Asn-Tyr-Ser-Pro-Asp-Trp amide) and Dorpa-AKH (pGlu-Val-Asn-Tyr-Ser-Pro-Val-Trp amide). We do not repeat evidence of this here, since both peptides were also detected previously and their fragmentation shown and discussed in detail (see Gäde et al. 2016). In the present analyses, a hydroxyproline modification at position 6 of both Dorpa-AKH and Melme-CC were

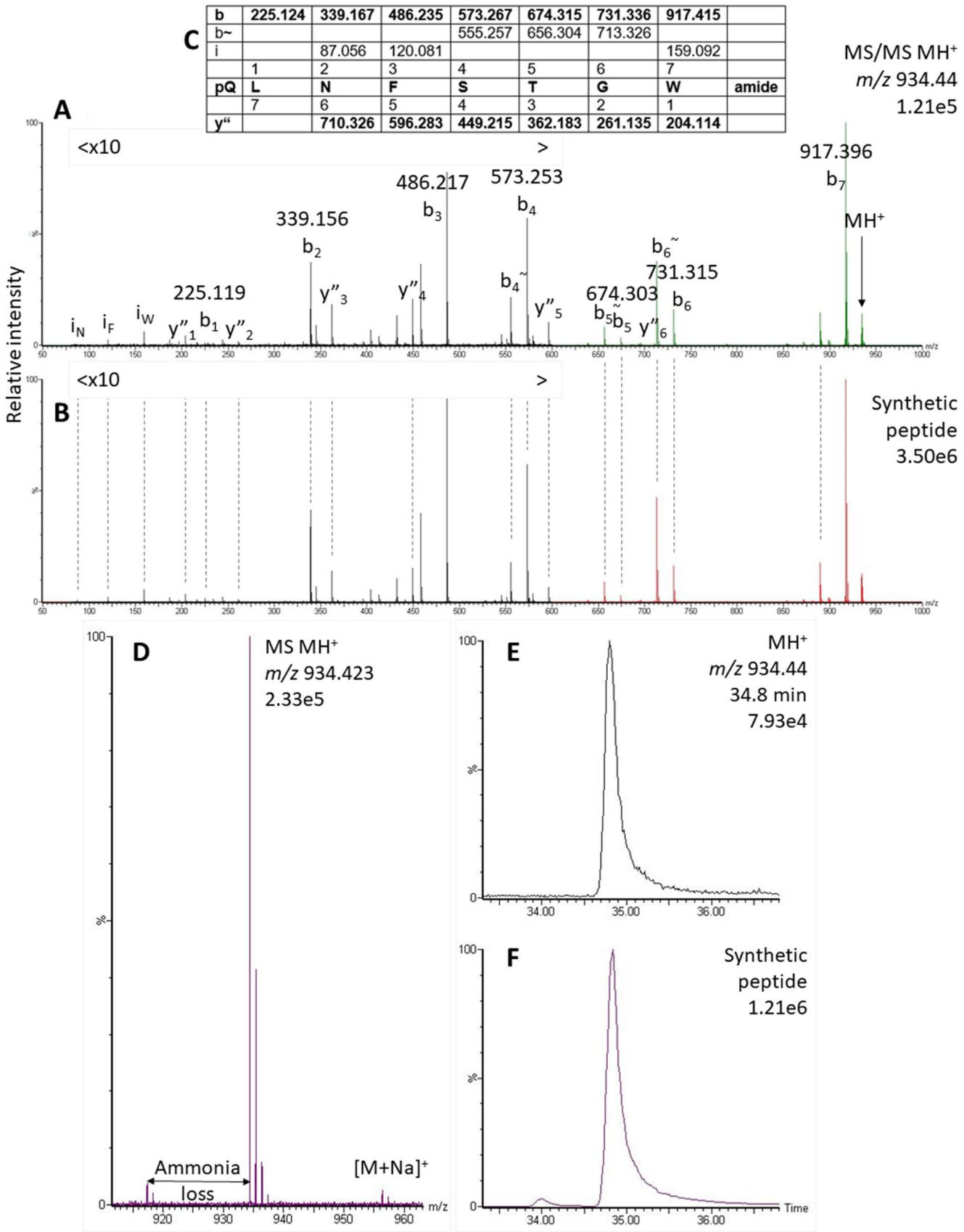
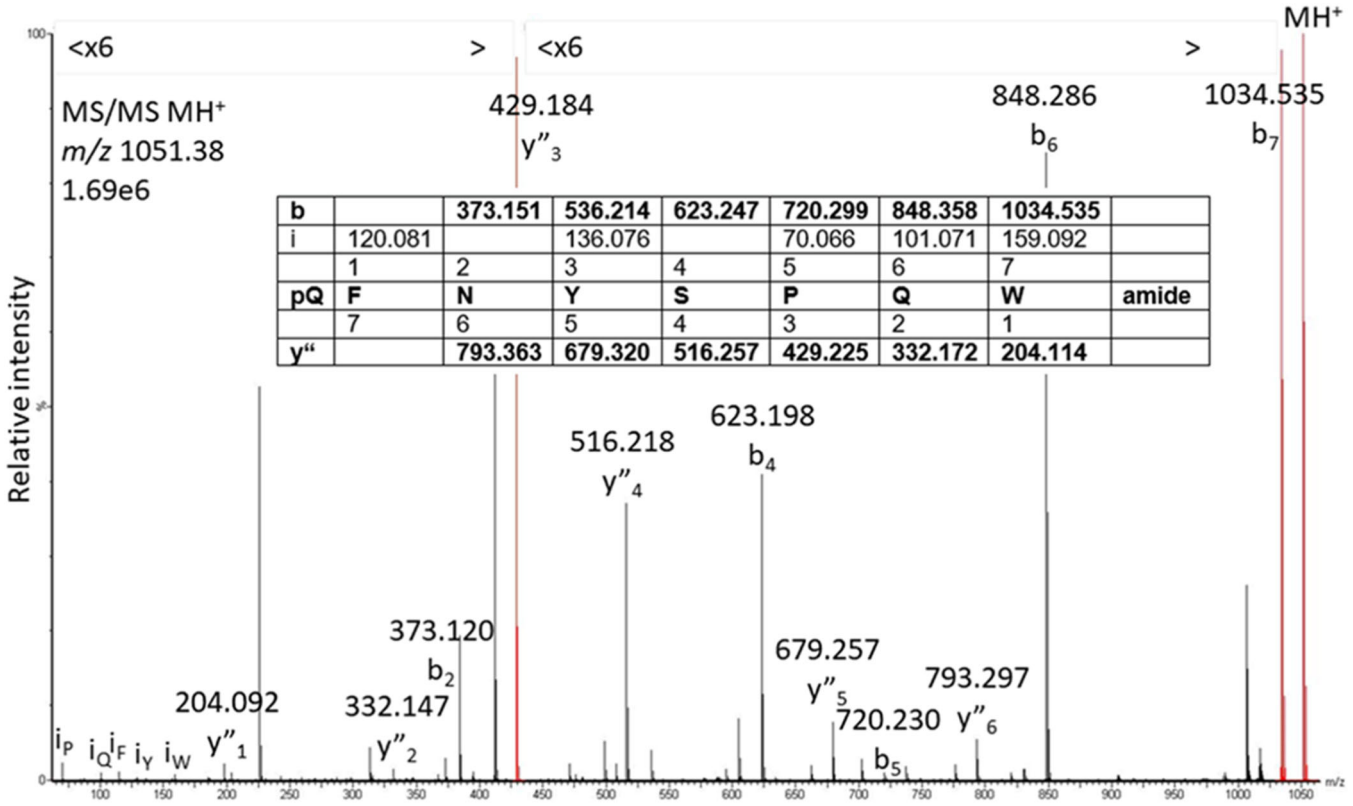


FIGURE 2 | Legend on next page.



b		373.151	536.214	623.247	720.299	848.358	1034.535	
i	120.081		136.076		70.066	101.071	159.092	
	1	2	3	4	5	6	7	
pQ	F	N	Y	S	P	Q	W	amide
	7	6	5	4	3	2	1	
y"		793.363	679.320	516.257	429.225	332.172	204.114	

FIGURE 3 | MS/MS analysis for Lucce-AKH-II in *L. cervus* using the singly charged peptide ion (*m/z* 1051.38). Peaks were labeled according to the b- and y-ion series as shown for the major ions in the inset and calculated for all expected ions in Figure S3.

also detected in the CC extracts of *D. parallelipipedus*. Such modifications have been known to occur in a number of AKHs (Gäde et al. 2011; König et al. 2023a; Marco et al. 2023).

In the CC extract of the second lucanid, *S. typhon*, Dorpa-AKH, Melme-CC, and Dorpa-AKH-Hyp were identified and sequenced; the hydroxyproline form of Melme-CC could, however, not be detected.

The third lucanid investigated, the greater stag beetle *L. cervus*, gave a slightly different result: whereas one peptide was easily identified as Dorpa-AKH (MH^+ 974.47) based on its characteristic MS/MS pattern (for spectrum, see König et al. 2023b, Supporting Information); the other peptide had a singly charged ion species at *m/z* 1051.38; gas phase fragmentation thereof assigned the peptide sequence as pGlu-Phe-Asn-Tyr-Ser-Pro-Gln/Lys-Trp amide (Figure 3). The ambiguity at position 7, Gln or Lys, was solved by exact mass measurement with HRMS using internal lockmass correction. We measured *m/z* 1051.465 for the singly charged ion species (for spectrum, see Figure S4), which was only 0.002 mass units different from the expected value of 1051.463 for the Gln-containing peptide (the Lys-containing MH^+ species would calculate to 1051.500). This is a novel peptide and is code-named Lucce-AKH. A small ion was noted for the Hyp-form of Lucce-AKH but was not sequenced at this time.

Of the family Scarabaeidae, we analyzed AKHs from three subfamilies: Melolonthinae, Cetoniinae, and Scarabaeinae. The melolonthid European June beetle, *A. solstitiale*, displayed an interesting and novel complement of AKHs with molecular weights of 989.4 and 917.4 Da. MS analysis assigned the sequences pGlu-Val-Asn-Tyr-Ser-Pro-Asp-Trp amide and pGlu-Leu-Asn-Met-Ser-Thr-Gly-Trp amide, respectively; the spectral data and RT of the endogenous peptides matched those of the synthetic peptides (Figure 4). Both peptides represent novel sequences and are accordingly named Ampso-AKH-I and -II.

Three members of the subfamily Cetoniinae were studied: *T. fasciatus*, *C. aurata*, and *T. hirta*. All three species had in common an AKH with the ion MH^+ 1004.44 which we know already from the lucanids (see Section 3.1.4 above), and which was identified and validated again as Melme-CC. In *C. aurata* this peptide had been sequenced before (Gäde et al. 2016) but in the current study the hydroxyprolinated form was also found, however only in the sample of *C. aurata*. In the Eurasian bee beetle, *T. fasciatus*, a second AKH peptide occurred at MH^+

FIGURE 2 | Target MS/MS (A) and MS (D) analysis for Schgr-AKH-II in *H. caraboides* versus the synthetic peptide (B) using the singly charged peptide ion (*m/z* 934.44) demonstrating the same gas phase fragmentation behavior for both species. In A/B, major peaks were labeled for the b- and y-ion series indicating amino acid residue losses from either end of the peptide as given in (C) for the visible ions (for all expected ions, see Figure S1; more ions can be visualized by zooming into the spectrum). Note zoom factor 10 for the left part of the spectra. For original spectra, see Figure S2. In the MS scan (D), the peptide ion easily loses ammonia, and it is also present as sodiated ion. In LC, both the insect peptide (E) and the synthetic standard (F) were eluted at the same RT (34.8 min) confirming the sequence assignment.

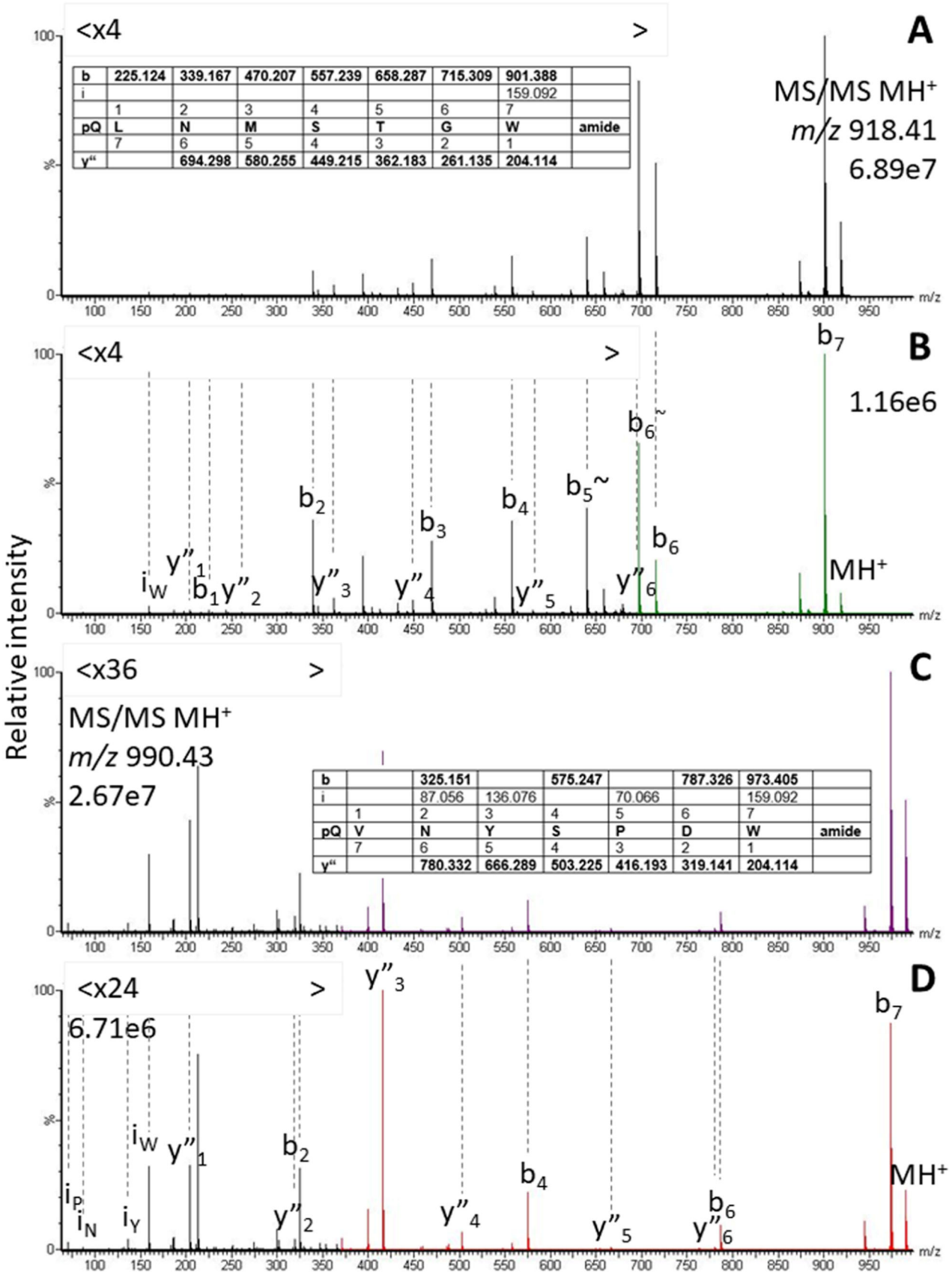


FIGURE 4 | CID spectra for Ampso-AKH-I (B) and -II (D) measured in CC extract from *A. solstitiale* versus the respective synthetic peptide (A/C) using the singly charged peptide ions (*m/z* 990.43 and 918.41). Peaks were labeled according to the b- and y-ion series as calculated in Figure S5.

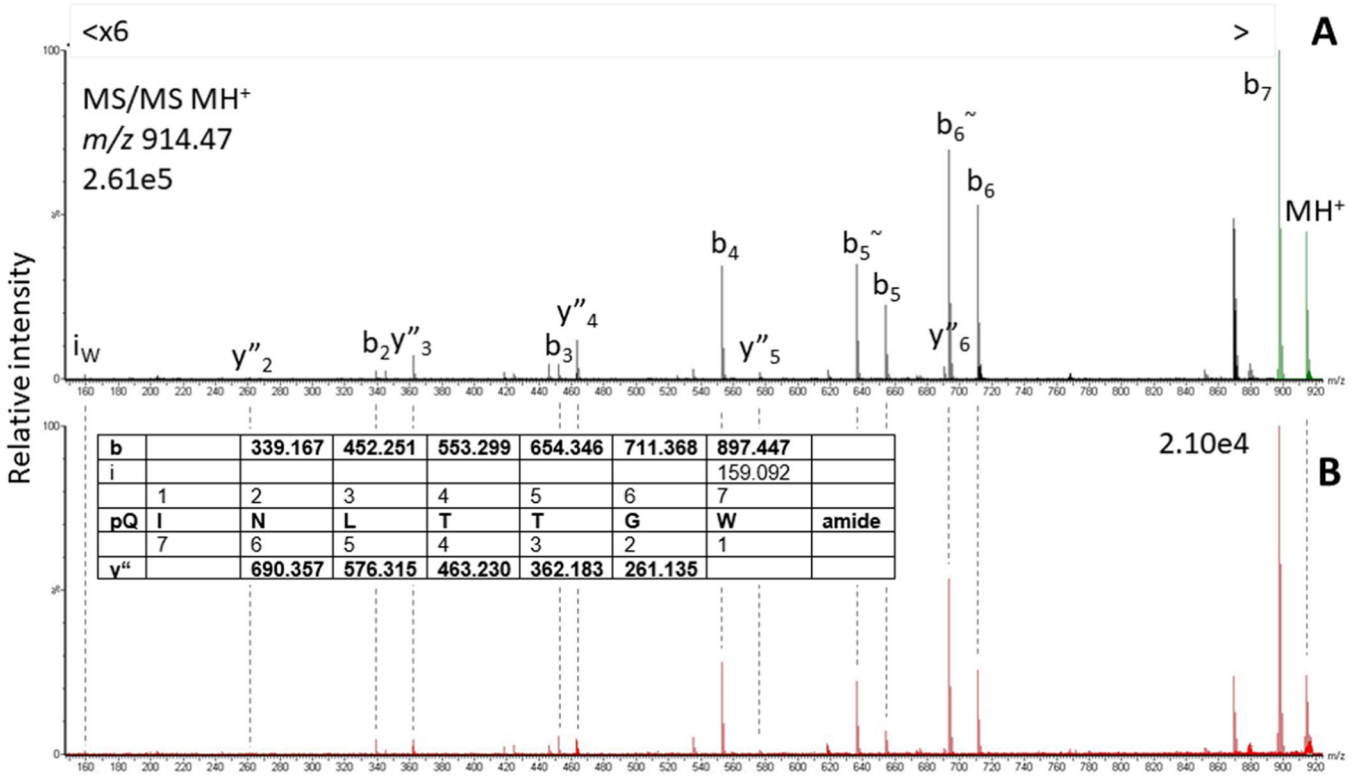


FIGURE 5 | CID spectra for a novel AKH peptide detected in CC extract from *Trichius fasciatus* (A) and *T. hirta* (B) using the singly charged peptide ion *m/z* 914.47. Peaks were labeled according to the b- and y-ion series as calculated in Figure S6 for a sequence pQINLTTGW amide. The sequence was validated using the synthetic peptide as shown in Figure S7. LC RT and CID matched.

914.47 in addition to Melme-CC (Figure 5). The CID spectrum was interpreted, and a novel AKH assigned as pGlu-Ile/Leu-Asn-Ile/Leu-Thr-Thr-Gly-Trp amide. The double isobaric ambiguities (at positions 2 and 4) could be solved. Earlier work on the cetoniid fruit beetle *Pachnoda sinuata* had shown a second Trp-fluorescence peak in RP-HPLC with a longer RT than Melme-CC, adipokinetic activity in locusts and hyperprolinaemic and small hypertrehalosaemic activity in the fruit beetle itself (Gäde 1989; Auerswald 1997). Edman sequencing of the fruit beetle peak clearly resulted in Ile at position 2 and Leu at position 4 (Auerswald 1997). Based on this peptide sequence information from *P. sinuata*, we purchased the synthetic peptide with the sequence pGlu-Ile-Asn-Leu-Thr-Thr-Gly-Trp amide and could show that RT and CID was identical to the natural peptide from *T. fasciatus* CC extract (Figure S7). We propose to give this octapeptide the code-name Pacsi-AKH after the first sequencing in the fruit beetle *P. sinuata*.

In the third cetoniid species, *T. hirta*, the AKH pattern became more complicated. In addition to Melme-CC, a second peptide ion was detected at *m/z* 994.431 but which, curiously, lost 80 mass units easily during MS analysis—even when no collision energy was applied (Figure 6). The resulting peptide "product" at *m/z* 914.47 (Figure 5B) was reminiscent of Pacsi-AKH that is present in *T. fasciatus* (above). MS/MS of both peaks (*m/z* 994.431 and *m/z* 914.47) revealed that these peaks are related: apart from the neutral loss, the CID spectra were essentially identical. This novel "plastic" peptide at *m/z* 994.431 is not code-named here as it awaits further investigation and validation. Three members of the subfamily Scarabaeinae were examined. All three species *C. caelatus*, *X. dentilabris*, and *O. binodis* had a different complement of AKHs, but each had one AKH in common, namely, Scade-CC-I (pGlu-Phe-Asn-Tyr-Ser-Pro-Asp-Trp amide). It was previously shown to occur in certain dung beetles of this subfamily (see Table 1 in Gäde et al. 2016).

The second peptide in each species was different. In *C. caelatus*, Scade-CC-II (pGlu-Phe-Asn-Tyr-Ser-Pro-Val-Trp amide) was detected, a peptide known from earlier work (see Gäde et al. 2016). In *X. dentilabris*, the second peptide was Cirba-AKH (pGlu-Phe-Asn-Phe-Ser-Ala-Gly-Trp amide), which had been previously identified in the flightless dung beetle *Circellium bacchus* (Gäde et al. 2016). In the CC extract of the last beetle *O. binodis*, an ion at the same *m/z* 1022.47 as Scade-CC-II in *C. caelatus* was detected, which, however, presented with a different CID spectrum (Figure 7A). In this AKH, Val and Phe changed positions (pGlu-Val-Asn-Tyr-Ser-Pro-Phe-Trp amide), hence, a novel peptide of this family was found, which was code-named Ontbi-AKH. Its correct assignment was validated by processing the synthetic peptide under identical conditions, for both peptides the RT was 39.9 min (Figure 7B). The Hyp-form of this peptide was also seen at low intensity.

4 | Discussion

The present study closes the gap in knowledge of the primary structure of the metabolic hormone AKH in beetles; formerly we had investigated the Adephaga (Gäde and Marco 2017) and, from the Polyphaga, the large series of Cucujiformia (Gäde et al. 2019). The data of the current study on most families in between these groups shed some new light on structural and

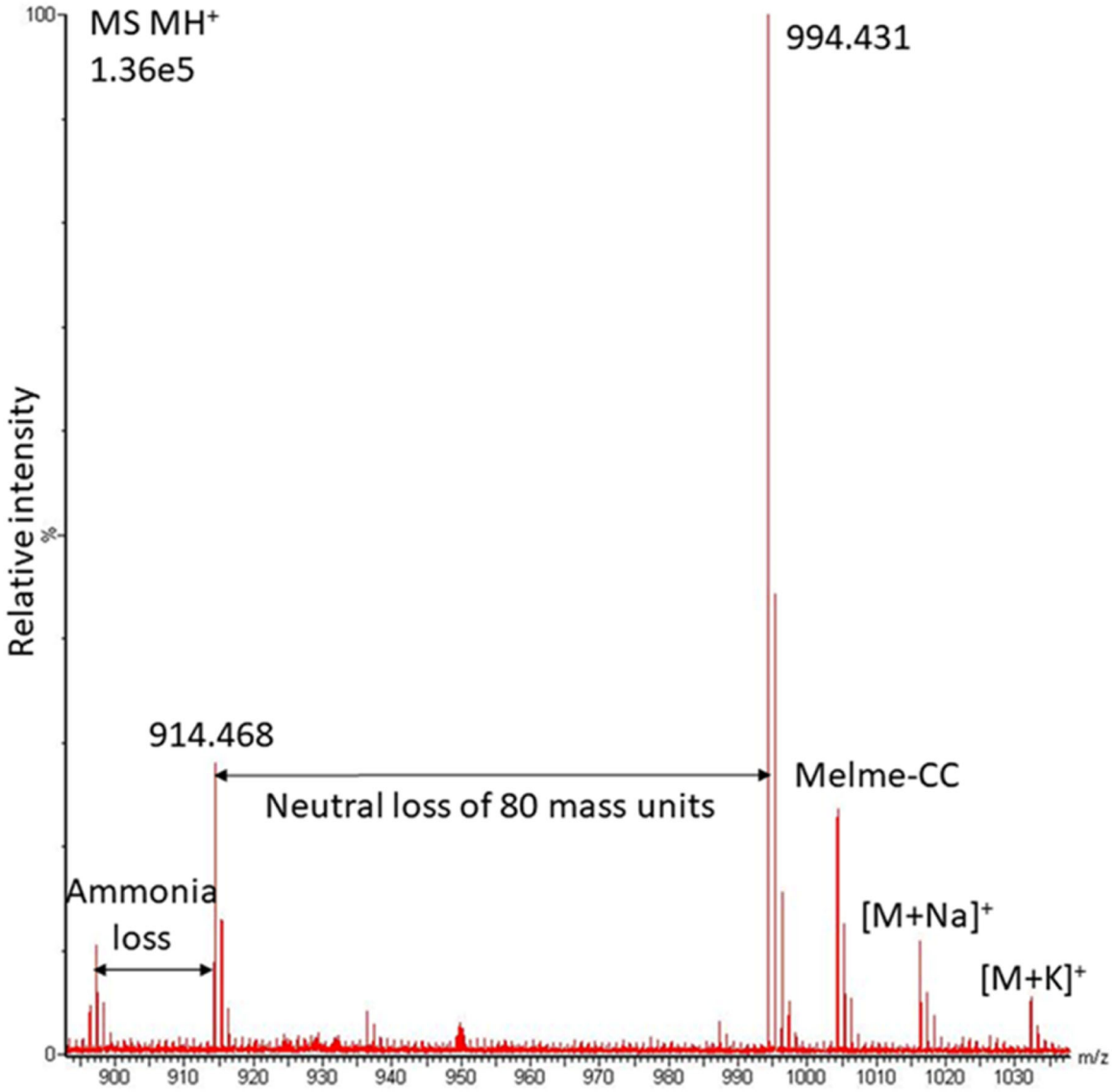


FIGURE 6 | MS spectrum for a novel peptide found in *T. hirta*. It lost 80 mass units quickly during mass analysis even when no collision energy was applied. Visible is residual Melme-CC, the second AKH found in this species. Other peaks represent the commonly observed sodiated and potassiated ion species and the neutral loss of the elements of ammonia.

evolutionary trends of the peptide family and warrant discussion of these aspects.

4.1 | Certain Features Help in the Assignment of the Correct AKH Sequence

A Pro residue at position 6 in peptides of the AKH family causes a specific fragmentation pattern during MS/MS sequencing and results in characteristic CID spectra which helps the interpretation enormously (see also König et al. 2023a). Careful observation of the MS data also shows for many Pro-containing AKHs the hydroxylated form. This form is mostly present in low intensity, and it is suggested that the concentration is lower than the detection limit in those cases where such Hyp AKHs are not seen. It is clear from previous work that such modified AKHs are authentic and not artefactually formed during sample handling and that they are biologically active, thus may play a functional role which is not clear yet (Gäde et al. 2011; König et al. 2023a).

Many AKHs contain isobaric Leu or Ile residues. RT in LC can solve this conundrum quite easily: the Ile-containing AKH is less hydrophobic and elutes earlier than the Leu-containing form (see Gäde, Auerswald et al. 2003). In the case of Pacsi-AKH, a newly found AKH of this study which contains Ile at position 2 and Leu at position 4 Edman degradation sequencing gave the unambiguous result. Finally, a synthetic peptide is always needed to confirm CID pattern and RT.

Another isobaric case, Gln versus Lys, is best solved as shown in this work by HRMS.

4.2 | All Examined Beetle AKHs of This Study Are Octapeptides: Comparison With All Beetle AKH Sequences and Other Holometabolic Orders

All 12 AKHs identified here are octapeptides. This is remarkable since previously also only one decapeptide AKH has been

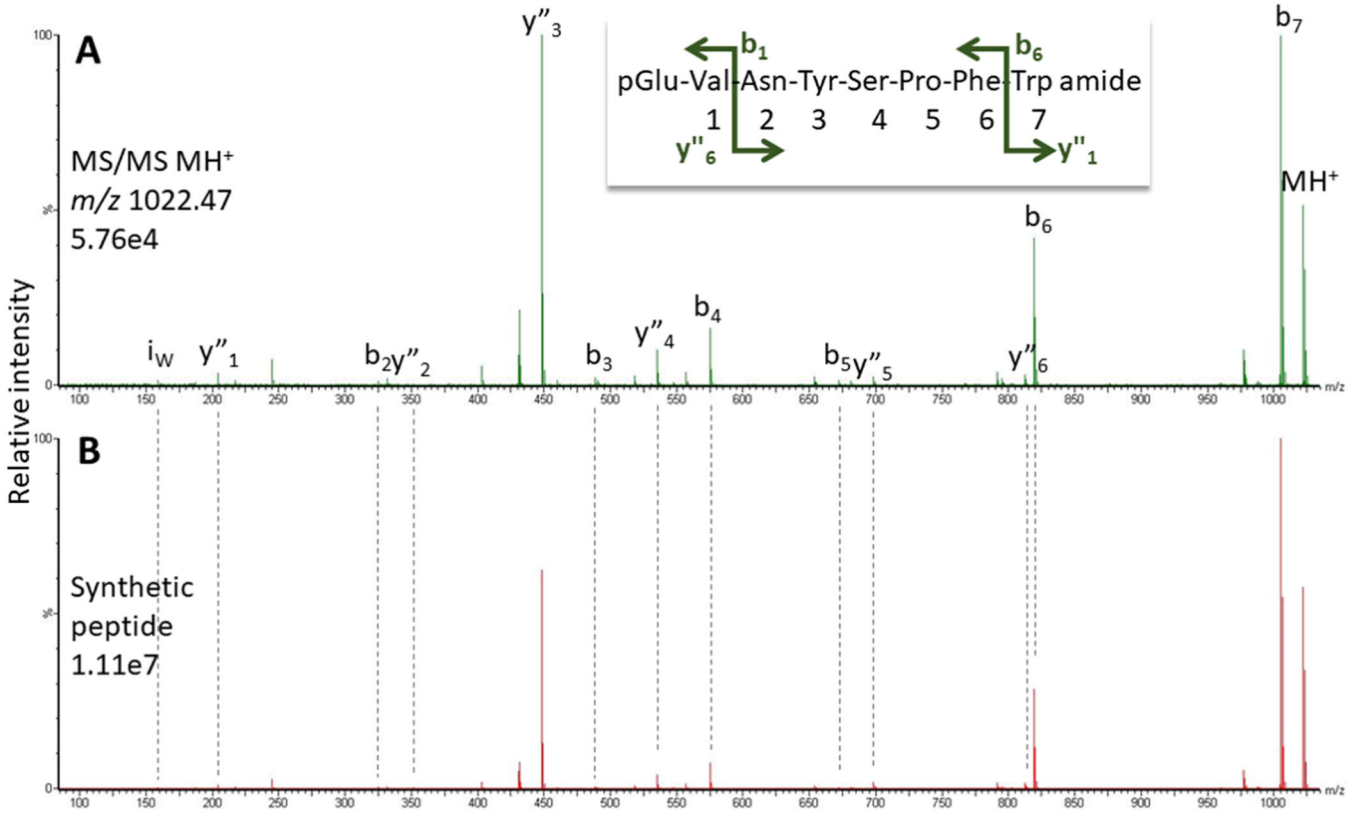


FIGURE 7 | CID spectra for a novel AKH peptide detected in (A) CC extract from *O. binodis* and in (B) the synthetic peptide at 39.9 min using the singly charged peptide ion at *m/z* 1022.47. Peaks were labeled according to the b- and y-ion series as calculated in Figure S8 for sequence pQVNYSPFW amide.

chemically identified in Coleoptera (in the family Meloidae by Gäde 1995) and another one predicted from genomic/transcriptomic mining (in the same family and genus by Veenstra 2019). This size distribution is reminiscent of the situation in the order Diptera which is currently also characterized by only one decapeptide AKH member out of 14 chemically analyzed AKHs (Gäde et al. 2020). The other large holometabolic order, the Lepidoptera, however, displays almost equal numbers of octa-, nona-, and decapeptides (Marco et al. 2020).

4.3 | The Newly Identified AKHs Are All Found in the Scarabaeoidea

Apart from the Scarabaeoidea, we also analyzed AKHs from four other Polyphaga superfamilies (spanning six families). Yet, only in the Scarabaeoidea were novel AKHs identified. The Lucanidae, for example, all have two main AKHs (plus maybe Hyp modified forms), of which one is always Dorpa-AKH. The second AKH is Melme-CC in *D. parallelipipedus* and *S. typhon*, whereas the novel Lucce-AKH is present in *L. cervus*. Lucce-AKH differs from Melme-CC at positions 2 (Phe instead of Leu) and 7 (Gln instead of Asp). Whether this distribution is related to taxonomic distribution is unknown; all three species belong to the subfamily Lucaninae but different tribes. The former two species belong to the Dorcini whereas the latter is a Lucanini.

Lucce-AKH is most closely related in structure to the peptides Scade-CC-I and II (only position 7 varies; Table 1) which are found in a few tribes of Scarabaeinae in this study and earlier work (Gäde et al. 2016).

Prior to the current study, only one species of the Melolonthinae had been examined in regard to AKHs, the cockchafer *Melolontha melolontha* produces Melme-CC (Gäde 1991). Here, we present *A. solstitiale* with two novel AKHs. Ampso-AKH-I is identical to Melme-CC except for a Val at position 2 instead of the Leu residue, an exchange quite often found in AKHs. The sequence of Ampso-AKH-II is nearly identical to that of Schgr-AKH-II which is found in all evolutionary lower superfamilies (see Section 4.4) but it is most unusual in that a Met residue is at position 4. This place in the AKH peptide chain is characteristically taken up by an aromatic amino acid, mostly Phe but also Tyr, especially in Scarabaeoidea. It would be an interesting project to perform activation studies of the *A. solstitiale* AKH receptor to find out whether both endogenous peptides bind equally well despite the quite different properties of the two amino acids at position 4, which is vital for activity in other AKH systems (see, e.g., Gäde and Hayes 1995; Marco and Gäde 2015).

The fourth novel AKH of this study is found in a cetoniid beetle. All of the cetoniid beetles studied here and previously (Gäde et al. 2016) contain Melme-CC but in *T. fasciatus* a second octapeptide was found code-named Pacsi-AKH, as explained in Section 3.1.4. Pacsi-AKH is almost identical to the peptide code-named Euoin-AKH found in the scarabaeid beetle *Euoniticellus intermedius* (Gäde et al. 2016), except for the uncharacteristic occurrence of a Leu at position 4 instead of the characteristic

Phe or Tyr of hitherto typical AKHs. This is the second such "AKH" peptide of Coleoptera that does not conform to the accepted features of the peptide family. While Ampso-AKH-II (Met^4) has not been tested in biological assays, the peptide we have now code-named Pacsi-AKH was earlier detected in the fruit beetle *P. sinuata* and shown to function as an AKH in *Locusta migratoria*, and in the fruit beetle it significantly mobilized proline and to a lesser extent, carbohydrates (Gäde 1989; Auerswald 1997). These results indicate that this atypical AKH can bind and activate the AKH receptor of an insect in which typical AKHs are found. Whether this locust AKHR is the same one as previously characterized (Zheng et al. 2020), or whether a completely different receptor is involved, remains an open question.

The fifth and last novel AKH is exhibited in *O. binodis*, a member of the subfamily Scarabaeinae. Its peptide, Ontbi-AKH, is unusual as well, having the aromatic Phe residue at position 7; this has never been shown before in any AKH and one wonders how the cognate receptor deals with this switch, for it certainly would result in a different peptide conformation. Overall, the peptide is most similar to Ampso-AKH-I and Dorpa-AKH differing only at position 7 (either Asp or Val; Table 1).

One can surmise that the study of further members of the superfamily Scarabaeoidea will very likely contribute to the finding of additional novel AKHs and, very likely, the identification of those structures that are predicted in the evolutionary scheme (see Section 4.5).

4.4 | Comparison of the Occurrence of Schgr-AKH-II in Beetles and Other Clades

The first time Schgr-AKH-II was sequenced was in the order Orthoptera from the CC of the locusts *Schistocerca gregaria*, *S. nitens*, and *L. migratoria* (Siegert et al. 1985; Gäde et al. 1986) and later in other caeliferan and ensiferan Orthoptera (Gäde 2009; Gäde, Marco et al. 2003). It has never been found in the basal orders of the class Insecta, i.e. Archaeognatha, Zygentoma, Odonata, Ephemeroptera, and not in other orders of the Polyneoptera than Orthoptera. It is present in a few species of heteropteran Hemiptera (Gäde and Marco 2022) and in certain Hymenoptera (Lorenz et al. 2001). In the order Coleoptera, Schgr-AKH-II is known from the Adephaga (Gäde and Marco 2017) and now in this study from most of the more basal families of Polyphaga whereas the more advanced families, including the Phytophaga are devoid of Schgr-AKH-II (Gäde et al. 2019). Putative AKH precursor sequences predicted from the genomes of 16 beetle species (Veenstra 2019) provide additional evidence for the claim that Schgr-AKH-II is present only in Adephaga and basal families of the Polyphaga.

Interestingly Schgr-AKH-II is not present in the CC of five species of the genus *Nicrophorus* but is present in the genus *Phosphuga*, although both belong to the same family, the Silphidae (Superfamily Staphylinoidea). They are, however, members of different subfamilies, the Nicrophorinae and Silphinae, respectively. These phylogenetic differences may be reflected in the AKH peptides. To corroborate such a statement the genera *Silpha* and *Thanatophilus* of the Silphinae should be studied in the future. From the genome of *Aleochara* spp. (Family Staphylinidae) Schgr-AKH-II is predicted (Veenstra 2019); although this needs to be chemically proven, it indicates that additional species in the Staphylinoidea superfamily express the "ancestral" beetle AKH.

4.5 | Assuming Schgr-AKH-II as the Ancestral AKH of Coleoptera; The Molecular Evolution of AKHs in Beetles

The peptide Schgr-AKH-II is the only AKH in the Adephaga (Gäde and Marco 2017) and in most of the basal families of Polyphaga (Table 1), indicating that this is the ancestral AKH of Coleoptera. If we accept this assumption, the question arises whether a molecular evolution of the AKHs currently found in the various beetle clades can be constructed taking (mostly) single point mutations into account? Figure 8 presents one of a few possible scenarios with only three hypothetical peptides included that have not been shown to exist. Yet, one may interject because out of the more than 360,000 known extant beetle species (and possibly another few 100,000 extant species waiting to be described) only a small number has been studied for its complement of AKH peptides. Out of the known 23 different beetle AKHs fully chemically characterized (Gäde et al. 2016, 2019; Gäde and Marco 2017), more than 50% stem from the Scarabaeoidea and more AKHs are anticipated from this superfamily in which, apparently, an explosive radiation of primary structures of AKHs have occurred. Interestingly, almost all AKHs found in the more advanced species of Polyphaga, the Cucujiformia series, cluster together at the left-hand side of the scheme in Figure 8, lending credibility to the data set. As more structures will be elucidated, this molecular evolution scheme will be updated and refined.

4.6 | Some Remarks to the Possible Use of Schgr-AKH-II as Lead Compound for a Green Insecticide

The term "green insecticide" refers to an environmentally friendly alternative to conventional pesticides, specifically based on manipulating bioactive peptides and/or their cognate GPCR binding to disrupt important physiological processes in so-called pest insects, with minimal harmful effects on other animals (Altstein and Nässel 2010; Gäde et al. 2017). If we want to examine the merit of a lead AKH for developing a mimetic to specifically combat beetle pests by interfering with the insect metabolic pathways, we need an "identikit" of the pests and their complement of AKHs, as well as those for the beneficial insects. The most iconic beneficial insect is the honeybee *Apis mellifera*; the AKH in the honeybee and several other species of bees (Hymenoptera), is Schgr-AKH-II. Section 4.4 lists the distribution of Schgr-AKH-II in other insect orders, such as Orthoptera (Caelifera and Ensifera), and a few species of Hemiptera. The distribution list of Schgr-AKH-II includes the Adephaga (one of the basal suborders of Coleoptera; Gäde and Marco 2017) and now extends further into Polyphaga (Table 1), namely, the basal superfamilies Buprestoidea, Elateroidea, Hydrophilioidea and thus far, chemically confirmed in only one species of the Staphylinoidea (Subfamily Silphinae), although it

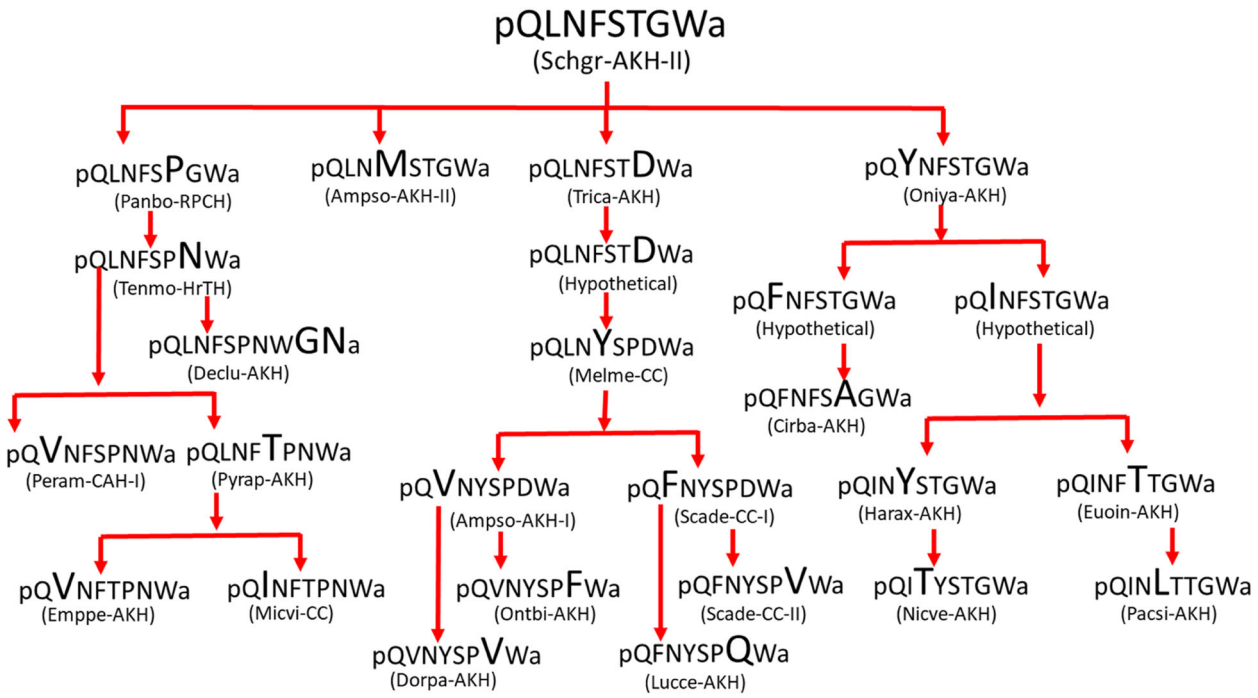


FIGURE 8 | Proposed sequence of amino acid changes to account for AKH biodiversity in Coleoptera assuming Schgr-AKH-II as the ancestral peptide. Note that (1) almost all substitutions are point mutations, and (2) three hypothetical peptides are included that have not (yet) been found in Coleoptera. To date, AKHs are distributed as follows in the coleopteran superfamilies: Schgr-AKH-II in Adephaga, as well as in the Polyphaga superfamilies Buprestoidea, Elateroidea (also Panbo-RPCH in Family Lycidae), Hydrophyloidea and Staphylinoidea (although Nicve-AKH predominates in Family Silphidae). Tenmo-HrTH, Peram-CAH-I, Pyrap-AKH, Emppe-AKH, Trica-AKH, Harax-AKH, and Declu-AKH are found in the Cucujiformia. Melme-CC, Scade-CC-I & –II, Dorpa-AKH, Oniay-CC, Euoin-AKH, Cirba-AKH, Trifa-CC, Lucce-AKH, Ampso-AKH-I & -II, Pacsi-AKH and Ontbi-AKH are synthesized in Scarabaeoidea (Gäde et al. 2016, 2019; Gäde and Marco 2017).

is predicted to occur in a second species (Family Staphylinidae, Subfamily Aleocharinae) (Veenstra 2019).

In the case of Coleoptera, it is undisputed that the "pests" are predominant in the Cucujiformia series of Polyphaga (see Gäde et al. 2019), including pests of stored food products (Superfamily Tenebrionoidea; Family Tenebrionidae), blister beetles that are notoriously poisonous to man and other vertebrates (Superfamily Tenebrionoidea; Family Meloidae), a multitude of agricultural and forestry pests (Superfamilies Chrysomeloidea and Curculionoidea), and beehive pests (Superfamily of Cucujoidea). None of these beetle pest insects produce Schgr-AKH-II; many of the Cucujiformia AKHs are not pest-insect specific and are also present in termites, cockroaches, Hemiptera, and Archaeognatha (Marco et al. 2014; Gäde and Marco 2022; Jiang et al. 2023). Other serious pests among Coleoptera are found in the Superfamily Scarabaeoidea: subfamilies Melolonthinae and Dynastinae. The larval stages of chafer beetles (Melolonthinae) feed on grass roots, consequently killing the grass and making chafer beetles a serious pest of lawns and sports fields, as well as of certain commercial root crops (Held and Potter 2004; Yang et al. 2019). In the subfamily Dynastinae, the rhinoceros beetle larvae feed on rotten wood and the adults feed on nectar, plant sap, and fruit; not all species are beneficial, and some species are considered pests to palm trees and their commercial crops, like coconuts, palm oil, palm hearts, or date fruit (Bedford et al. 2015). None of these beetles synthesize Schgr-AKH-II; instead, Melme-CC is found in the dynastid *Oryctes rhinoceros* (Ajaykumar and Gokuldas 2011), and in a variety of melolonthid beetles (Gäde et al. 2016), but the latter also display novel AKHs (current study). Melme-CC would not be a good lead for a mimetic as potential insecticide since this AKH is produced in beneficial beetles too, such as the stag beetles (Family Lucanidae) that aid with the removal and recycling of nutrients from decaying wood, dung beetles from the family Geotrupidae, and cetoniid beetles that feed on nectar and pollen (current study and Gäde et al. 2016).

A surprising addition to the beetle pests are the jewel beetles (Superfamily Buprestoidea)—usually only when they become invasive to an area do the beetles reach significant numbers; their larvae bore into tree stems and roots thereby disrupting the flow of nutrients and water, and so affect tree stands and plantations, for example, the Emerald ash borer, *Agrilus planipennis*, affects ash trees in Europe and North America (Poland and McCullough 2006; Valenta et al. 2017). So-called "nuisance" beetles are a few species from the Superfamily Elateroidea, Family Elateridae (click beetles) that pose serious agricultural damage to crops by feeding on root systems (Parker and Howard 2001; Vernon et al. 2008). The invasive jewel beetle pests and the nuisance species all synthesize Schgr-AKH-II, but so do the harmless Adephaga beetles, the diving beetles (Hydrophilioidea) and at least one species of the Staphylinoidea, as mentioned above. The collateral damage, especially considering the honeybee could be too great should Schgr-AKH-II be chosen as lead peptide for designing a green insecticide. Nonetheless, there are other options to consider and tests

to be carried out pertaining the receptor binding pocket before summarily discarding a potential lead.

Whichever AKH is selected for peptide mimetic development, computational and experimental approaches to quantify protein binding interactions between the AKH ligand and/or its mimetic and the cognate AKHR should be carried out. The AKHR of the honeybee was tested in this manner with a cyclic mimetic that was based on the primary structure of one of the three AKHs produced in the CC of the desert locust, namely, Locmi-AKH-I (Abdulganiyyu et al. 2020): while full dose–response binding curves were achieved with the locust AKHR and peptides Locmi-AKH-I and Schgr-AKH-II, as well as with the cyclic AKH mimetic (albeit it at considerably higher concentrations than the endogenous locust peptides), the honeybee AKHR also demonstrated full dose–response binding curves with Schgr-AKH-II and Locmi-AKH-I but the cyclic analog exhibited only trace activity at 10^{-4} M on the honeybee receptor and no EC_{50} could be determined. In other words, the cyclic AKH analog selectively activated only the AKH receptor of the pest insect (desert locust) and not that of the beneficial insect (honeybee); this is likely related to the less accessible binding pocket of the honeybee receptor and how restrictive this pocket is, compared with the binding pocket of the desert locust AKHR as shown by computational docking and molecular dynamics. The docking results showed that the cyclic AKH analog was not able to enter the receptor binding site and instead bound to the extracellular surface of the honeybee receptor (Abdulganiyyu et al. 2020). This raises the important point that ultimately, knowledge of AKHR sequences is imperative in taking the concept of green insecticides further. Marchal et al. (2018) cloned the AKHR of several insect species, including the honeybee, the desert locust, and two Cucujiformia representatives—a tenebrionid beetle *Tribolium castaneum* (Superfamily Tenebrionoidea) and a pine weevil *Hylobius abietis* (Superfamily Curculionoidea) and carried out receptor expression studies in vitro, with various AKHs. The results showed that the AKHRs had the strongest affinity for their cognate ligand in the nM range, and that the beetle and honeybee receptors were more selective in binding other AKHs than was the *S. gregaria* AKHR. Unfortunately, no further such research on beetle AKHRs has been published. A useful next step would be to use computational chemistry and molecular dynamics to understand the molecular interactions of ligand-receptor binding in beetle AKH signaling and then to use this information to perform virtual screenings of compound libraries in the quest for finding an AKHR agonist for further investigations. Such a strategy had been employed successfully with knowledge from the AKH signaling system in *S. gregaria* to find a competitive antagonist (Jackson et al. 2022).

Author Contributions

Gerd Gäde: conceptualization, investigation, writing – original draft, project administration, funding acquisition, writing – review and editing, validation, data curation, resources, supervision. **Simone König:** investigation, methodology, funding acquisition, writing – original draft, writing – review and editing, formal analysis, data curation, validation, resources, visualization. **Heather G. Marco:** conceptualization, investigation, methodology, funding acquisition, writing – review and editing, resources, visualization.

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Ethics Statement

Animal ethics approval is not required for research on insects at our research institutes. Nonetheless, insects were not mistreated during our study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data not enclosed in the manuscript and Supporting File can be obtained from the authors on reasonable request.

References

Abdulganiyyu, I. A., K. Kaczmarek, J. Zabrocki, et al. 2020. "Conformational Analysis of a Cyclic AKH Neuropeptide Analog That Elicits Selective Activity on Locust Versus Honeybee Receptor." *Insect Biochemistry and Molecular Biology* 125: 103362.

Ajaykumar, A. P., and M. Gokuldas. 2011. "Primary Structure of an Adipokinetic Neuropeptide From the Rhinoceros Beetle, *Oryctes rhinoceros* L (Coleoptera: Dynastidae)." *Annals of Neuroscience* 18: 100–104.

Altstein, M., and D. R. Nässel. 2010. "Neuropeptide Signalling in Insects." *Advances in Experimental Medicine and Biology* 692: 155–165.

Audsley, N., and R. E. Down. 2015. "G Protein Coupled Receptors as Targets for Next Generation Pesticides." *Insect Biochemistry and Molecular Biology* 67: 27–37.

Auerswald, L. 1997. "Fuels for Flight in the Fruit Beetle, *Pachnoda sinuata*, and Control of Flight Metabolism." PhD thesis, University of Cape Town, RSA.

Baker, J. G., S. J. Hill, and R. J. Summers. 2011. "Evolution of β-Blockers: From Anti-Anginal Drugs to Ligand-Directed Signalling." *Trends In Pharmacological Sciences* 32: 227–234. https://doi.org/10.1016/j.tips.2011.02.010.

Bedford, G. O., M. A. Al-Deeb, M. Z. Khalaf, K. Mohammadpour, and R. Soltani. 2015. "Dynastid Beetle Pests." In *Sustainable Pest Management in Date Palm: Current Status and Emerging Challenges. Sustainability in Plant and Crop Protection*, edited by W. Wakil, J. Romeno Faleiro, and T. Miller. Springer. https://doi.org/10.1007/978-3-319-24397-9_5.

Black, J. 2010. "A Life in New Drug Research." *British Journal of Pharmacology* 160, no. S1: S15–S25. https://doi.org/10.1111/j.1476-5381.2010.00848.x.

Capodeanu-Nägler, A., E. M. Keppner, H. Vogel, et al. 2016. "From Facultative to Obligatory Parental Care: Interspecific Variation in Offspring Dependency on Post-Hatching Care in Burying Beetles." *Scientific Reports* 6: 29323.

Coast, G. M., and D. A. Schooley. 2011. "Toward a Consensus Nomenclature for Insect Neuropeptides and Peptide Hormones." *Peptides* 32: 620–631.

Davies, S. A. 2017. "nEUROSTRESSPEP: Novel Biocontrol Agents for Insect Pests From Neuroendocrinology." *International Pest Control* 59: 164–165.

Gäde, G. 1985. "Isolation of the Hypertrehalosaemic Factors I and II From the Corpus Cardiacum of the Indian Stick Insect, *Carausius morosus*, by Reversed-Phase High-Performance Liquid Chromatography, and Amino-Acid Composition of Factor II." *Biological Chemistry Hoppe-Seyler* 366: 195–200.

Gäde, G. 1989. "Characterisation of Neuropeptides of the AKH/RPCH-Family From Corpora Cardiaca of Coleoptera." *Journal of Comparative Physiology B* 159: 589–596.

Gäde, G. 1991. "A Unique Charged Tyrosine-Containing Member of the Adipokinetic Hormone/Red Pigment-Concentrating Hormone Peptide Family Isolated and Sequenced From Two Beetle Species." *Biochemical Journal* 275: 671–677.

Gäde, G. 1995. "Isolation and Identification of AKH/RPCH Family Peptides in Blister Beetles (Meloidae)." *Physiological Entomology* 20: 45–51.

Gäde, G. 1997. "Distinct Sequences of AKH/RPCH Family Members in Beetle (*Scarabaeus* Species) Corpus Cardiacum Contain Three Aromatic Amino Acid Residues." *Biochemical and Biophysical Research Communications* 230: 16–21.

Gäde, G. 2009. "Peptides of the Adipokinetic Hormone/Red Pigment-Concentrating Hormone Family: A New Take on Biodiversity." *Annals of the New York Academy of Sciences* 1163: 125–136.

Gäde, G., L. Auerswald, P. Šimek, H. G. Marco, and D. Kodrík. 2003. "Red Pigment-Concentrating Hormone Is Not Limited to Crustaceans." *Biochemical and Biophysical Research Communications* 309: 967–973.

Gäde, G., and G. J. Goldsworthy. 2003. "Insect Peptide Hormones: A Selective Review of Their Physiology and Potential Application for Pest Control." *Pest Management Science* 59: 1063–1075.

Gäde, G., G. J. Goldsworthy, G. Kegel, and R. Keller. 1984. "Single Step Purification of Locust Adipokinetic Hormones I and II by Reversed-Phase High-Performance Liquid-Chromatography, and Amino Acid Composition of the Hormone II." *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 365: 393–398.

Gäde, G., G. J. Goldsworthy, M. H. Schaffer, J. C. Cook, and K. L. Rinehart. 1986. "Sequence Analyses of Adipokinetic Hormones II From Corpora Cardiaca of *Schistocerca nitans*, *Schistocerca gregaria* and *Locusta migratoria* by Fast Atom Bombardment Mass Spectrometry." *Biochemical and Biophysical Research Communications* 134: 723–730.

Gäde, G., and T. K. Hayes. 1995. "Structure-Activity Relationships for *Periplaneta americana* Hypertrehalosemic Hormone I: The Importance of Side Chains and Termini." *Peptides* 16: 1173–1180.

Gäde, G., C. Hilbich, K. Beyreuther, and K. L. Rinehart. 1988. "Sequence Analyses of Two Neuropeptides of the AKH/RPCH-Family From the Lubber Grasshopper, *Romalea microptera*." *Peptides* 9: 681–688.

Gäde, G., and R. Kellner. 1992. "Primary Structures of the Hypertrehalosemic Peptides From Corpora Cardiaca of the Primitive Cockroach *Polyphaga aegyptiaca*." *General and Comparative Endocrinology* 86: 119–127.

Gäde, G., and H. G. Marco. 2006. "Structure, Function and Mode of Action of Select Arthropod Neuropeptides." In *Studies in Natural Product Chemistry*, edited by Atta-ur-Rahman, 33, 69–139. Elsevier.

Gäde, G., and H. G. Marco. 2015. "The Decapod Red Pigment-Concentrating Hormone (Panbo-RPCH) Is the First Identified Neuropeptide of the Order Plecoptera and Is Interpreted as Homoplastic Character State." *General and Comparative Endocrinology* 221: 228–235.

Gäde, G., and H. G. Marco. 2017. "The Adipokinetic Hormone of the Coleopteran Suborder Adephaga: Structure, Function, and Comparison of Distribution in Other Insects." *Archives of Insect Biochemistry and Physiology* 95: e21399.

Gäde, G., and H. G. Marco. 2022. "The Adipokinetic Peptides of Hemiptera: Structure, Function, and Evolutionary Trends." *Frontiers in Insect Science* 2: 891615.

Gäde, G., H. G. Marco, and L. Desutter-Grandcolas. 2003. "A Phylogenetic Analysis of the Adipokinetic Neuropeptides of Ensifera." *Physiological Entomology* 28: 283–289.

Gäde, G., P. Šimek, and H. G. Marco. 2011. "An Invertebrate [Hydroxyproline]-Modified Neuropeptide: Further Evidence for a Close Evolutionary Relationship Between Insect Adipokinetic Hormone and Mammalian Gonadotropin Hormone Family." *Biochemical and Biophysical Research Communications* 414: 592–597.

Gäde, G., P. Šimek, and H. G. Marco. 2015. "Two Novel Tyrosine-Containing Peptides (Tyr^4) of the Adipokinetic Hormone Family in Beetles of the Families Coccinellidae and Silphidae." *Amino Acids* 47: 2323–2333.

Gäde, G., P. Šimek, and H. G. Marco. 2016. "Novel Members of the Adipokinetic Hormone Family in Beetles of the Superfamily Scarabaeoidea." *Amino Acids* 48: 2785–2798.

Gäde, G., P. Šimek, and H. G. Marco. 2017. "The African Froghopper *Ptyelus flavescens* (Suborder: Cicadomorpha) Contains Two Novel and One Known Peptides of the Adipokinetic Hormone (AKH) Family: Structure, Function and Comparison With Aphid AKH (Suborder: Sternorrhyncha)." *Amino Acids* 49: 1679–1690.

Gäde, G., P. Šimek, and H. G. Marco. 2019. "Structural Diversity of Adipokinetic Hormones in the Hyperdiverse Coleopteran Cucujiformia." *Archives of Insect Biochemistry and Physiology* 102: e21611.

Gäde, G., P. Šimek, and H. G. Marco. 2020. "The Adipokinetic Peptides in Diptera: Structure, Function, and Evolutionary Trends." *Frontiers in Endocrinology* 11: 153.

Gäde, G., P. Šimek, and H. G. Marco. 2021. "Biochemically Identified Neuropeptides in a Caddisfly (Trichoptera) and a pygmy Mole Cricket (Orthoptera: Caelifera: Tridactyloidea)." *Archives of Insect Biochemistry and Physiology* 106: e21778.

Held, D. W., and D. A. Potter. 2004. "June Beetles, *Phyllophaga* spp. (Coleoptera: Scarabaeidae: Melolonthinae: Melolonthini)." In *Encyclopedia of Entomology*. Springer. https://doi.org/10.1007/0-306-48380-7_2257.

Jackson, G. E., G. Gäde, and H. G. Marco. 2022. "In Silico Screening for Pesticide Candidates Against the Desert Locust *Schistocerca gregaria*." *Life* 12: 387.

Jiang, S., H. G. Marco, N. Scheich, et al. 2023. "Comparative Analysis of Adipokinetic Hormones and Their Receptors in Blattodea Reveals Novel Patterns of Gene Evolution." *Insect Molecular Biology* 32: 615–633.

König, S., H. G. Marco, and G. Gäde. 2023a. "Oxidation Products of Tryptophan and Proline in Adipokinetic Hormones - Artefacts or Post-Translational Modifications?" *Life* 13: 2315.

König, S., H. G. Marco, and G. Gäde. 2023b. "The Proline Effect and the Tryptophan Immonium Ion Assist in De Novo Sequencing of Adipokinetic Hormones." *Scientific Reports* 13: 10894. https://doi.org/10.1038/s41598-023-38056-2.

Lorenz, M. W., R. Kellner, W. Völkl, K. H. Hoffmann, and J. Woodring. 2001. "A Comparative Study on Hypertrehalosaemic Hormones in the Hymenoptera: Sequence Determination, Physiological Actions and Biological Significance." *Journal of Insect Physiology* 47: 563–571.

Marchal, E., S. Schellens, E. Monjon, et al. 2018. “Analysis of Peptide Ligand Specificity of Different Insect Adipokinetic Hormone Receptors.” *International Journal of Molecular Sciences* 19: 542.

Marco, H. G., and G. Gäde. 2015. “Structure-Activity Relationship of Adipokinetic Hormone Analogs in the Striped Hawk Moth, *Hippotion eson*.” *Peptides* 68: 205–210.

Marco, H. G., and G. Gäde. 2020. “Chapter 3 ‘Adipokinetic Hormone: A Hormone for All Seasons?’.” In *Advances in Invertebrate (Neuro)endocrinology: A Collection of Reviews in the Post-Genomic Era* 2, edited by S. Saleuddin, A. B. Lange, and I. Orchard, 129–176. Apple Academic Press.

Marco, H. G., S. Glendinning, T. Ventura, and G. Gäde. 2024. “The Gonadotropin-Releasing Hormone (GnRH) Superfamily Across Pancrustacea/Tetraconata: A Role in Metabolism?” *Molecular and Cellular Endocrinology* 590: 112238. https://doi.org/10.1016/j.mce.2024.112238.

Marco, H. G., S. König, and G. Gäde. 2023. “Predicted Novel Hypertrehalosaemic Peptides of Cockroaches Are Verified by Mass Spectrometry.” *Amino Acids* 55: 1641–1654.

Marco, H. G., P. Šimek, and G. Gäde. 2014. “Adipokinetic Hormones of the Two Extant Apterygotan Insect Orders, Archaeognatha and Zygentoma.” *Journal of Insect Physiology* 60: 17–24.

Marco, H. G., P. Šimek, and G. Gäde. 2020. “Unique Members of the Adipokinetic Hormone Family in Butterflies and Moths (Insecta, Lepidoptera).” *Frontiers in Physiology* 11: 614552.

McKenna, D. D., S. Shin, D. Ahrens, et al. 2019. “The Evolution and Genomic Basis of Beetle Diversity.” *Proceedings of the National Academy of Sciences* 116: 24729–24737.

McKenna, D. D., A. L. Wild, K. Kanda, et al. 2015. “The Beetle Tree of Life Reveals That Coleoptera Survived End-Permian Mass Extinction to Diversify During the Cretaceous Terrestrial Revolution.” *Systematic Entomology* 40: 835–880.

Münch, M., M. Nuss, and J. Seidel. 2010. “Das Glühwüermchen (*Lamprohiza splendidula* (Linnaeus, 1767)) in Sachsen- Ergebnisse der sächsischen Suchaktion. Wo tanzt das Glühwürmchen? Aus dem Jahre 2009 (Coleoptera: Lampyridae) [The Glowworm (Lamprohiza Splendidula (Linnaeus, 1767)) in Saxony - Results of a Search Expedition in Saxony. Where Does the Glowworm Dance? From the Year 2009 (Coleoptera: Lampyridae).].” *Sächsische Entomologische Zeitschrift* 5: 40–48.

Parker, W. E., and J. J. Howard. 2001. “The Biology and Management of Wireworms (*Agriotes* Spp.) on Potato With Particular Reference to the U.K.” *Agricultural and Forest Entomology* 3: 85–98.

Poland, T. M., and D. G. McCullough. 2006. “Emerald Ash Borer: Invasion of the Urban Forest and the Threat to North America's Ash Resource.” *Journal of Forestry* 104: 118–124.

Shadung, K. G. 2012. “Improving Attractiveness of an Insect Pest Through Value-Addition: A Possible Insect Management Strategy.” MSc dissertation, University of Limpopo, Sovenga, South Africa.

Sharkey, C. R., M. S. Fujimoto, N. P. Lord, et al. 2017. “Overcoming the Loss of Blue Sensitivity Through Opsin Duplication in the Largest Animal Group, Beetles.” *Scientific Reports* 7: 8.

Short, A. E. Z., and C. E. McIntosh, IV. 2014. “Review of the Giant Water Scavenger Beetle Genus *Hydrophilus* Geoffroy (Coleoptera: Hydrophilidae) of the United States and Canada.” *Coleopterists Bulletin* 68: 187–198.

Siegert, K., P. Morgan, and W. Mordue. 1985. “Primary Structures of Locust Adipokinetic Hormones II.” *Biological Chemistry Hoppe-Seyler* 366: 723–728.

Ślipiński, S. A., R. A. B. Leschen, and J. F. Lawrence. 2011. “Order Coleoptera Linnaeus, 1758.” In *Animal Biodiversity: An Outline of Higher-Level Classification and Survey of Taxonomic Richness*, edited by Z.-Q. Zhang, 3148, 203–208. Magnolia Press.

Tsai, P.-S. 2018. “Gonadotropin-Releasing Hormone by Any Other Name Would Smell as Sweet.” *General and Comparative Endocrinology* 264: 58–63.

Valenta, V., D. Moser, S. Kapeller, and F. Essl. 2017. “A New Forest Pest in Europe: A Review of Emerald Ash Borer (*Agrilus planipennis*) Invasion.” *Journal of Applied Entomology* 141: 507–526.

Veenstra, J. A. 2019. “Coleoptera Genome and Transcriptome Sequences Reveal Numerous Differences in Neuropeptide Signaling Between Species.” *PeerJ* 7: e7144.

Vernon, R. S., W. van Herk, J. Tolman, H. Ortiz Saavedra, M. Clodius, and B. Gage. 2008. “Transitional Sublethal and Lethal Effects of Insecticides After Dermal Exposures to Five Economic Species of Wireworms (Coleoptera: Elateridae).” *Journal of Economic Entomology* 101: 365–374.

Yang, X.-Z., L. Zhang, R.-Q. Feng, L.-J. Zhang, F.-Z. Luo, and M.-L. Yuan. 2019. “Mitochondrial Genome of *Amphimallon solstitiale* (Coleoptera: Scarabaeidae: Melolonthinae) and Phylogenetic Analysis.” *Mitochondrial DNA Part B* 4: 110–111.

Zandawala, M., S. Tian, and M. R. Elphick. 2018. “The Evolution and Nomenclature of GnRH-Type and Corazonin-Type Neuropeptide Signaling Systems.” *General and Comparative Endocrinology* 264: 64–77.

Zhang, S.-Q., L.-H. Che, Y. Li, et al. 2018. “Evolutionary History of Coleoptera Revealed by Extensive Sampling of Genes and Species.” *Nature Communications* 9: 205.

Zheng, H., C. Chen, C. Liu, Q. Song, and S. Zhou. 2020. “Rhythmic Change of Adipokinetic Hormones Diurnally Regulates Locust Vitellogenesis and Egg Development.” *Insect Molecular Biology* 29: 283–292.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.