The Evolution of Neuropeptides in Blattodea

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I hereby declare that I alone am responsible for the content of my doctoral dissertation and that I have only used the sources or references cited in the dissertation.

Shixiong Jiang

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List of abbreviations

General

Aa	Amino acid
AKHR	Adipokinetic hormone receptor
CC	*Corpora cardiaca*
C_ter	C-terminus
ECL	Extracellular loop
GPCR	G-protein-coupled receptor
JH	Juvenile hormone
MS	Mass spectrometry
N_ter	N-terminus
ICL	Intracellular loop
ORF	Open reading frame
PTM	Post-translational modifications
TM	Transmembrane domain

Neuropeptides

AKH	Adipokinetic hormone
ALP	Agatoxin-like peptide
ACP	AKH/corazonin-related peptide
AstA	Allatostatin A
AstB	Myoinhibitory peptide
AstCC	Allatostatin CC
AstCCC	Allatostatin CCC
AT	Allatotropin
Burα	Bursicon alpha
Burβ	Bursicon beita
CAPA	Periviscerokinin
CCAP	Crustacean cardioactive peptide
CCH	CCHamide
CCRF	CCRFamide
CNM	CNMamide
CNP	Carausius neuropeptide-like precursor
Crz	Corazonin
CTA	Calcitonin A
CTB	Calcitonin B
CTDH	Calcitonin-like diuretic hormone
CRFDH	CRF-like diuretic hormone
EH	Eclosion hormone
ETH	Ecdysis triggering hormone
Evn	Elevenin
Flik	Fliktin

FMRF	FMRFamide
GPA2	Glycoprotein hormone alpha 2
GPB5	Glycoprotein hormone beta 5
Gon	Gonadulin
Han	HanSolin
ILP	Insulin-like peptide
IPTH	Invertebrate parathyroid hormone
K	Kinin
LMS	Leucomyosuppressin
MIP	Myoinhibitory peptide
Nat	Natalisin
NP	Neuroparsin
NPF	Neuropeptide F
NPLP	Neuropeptide-like peptide
NVP	NVP-like peptide
OK	Orcokinin
PDF	Pigment dispersing factor
PT	Proctolin
PTTH	Prothoracicotropic hormone
PK	Pyrokinin
RFL	RFLamide
RLP	Relaxin-like peptide
RY	RYamide
sNPF	Short Neuropeptide F
SIF	SIFamide
SMY	SMYamide
Sul	Sulfakinin
TK	Tachykinin
Tri	Trissin
TPK	Tryptopyrokinin
VP	Vasopressin

Summary

This thesis presents a detailed analysis of neuropeptide evolution, gene regulation, and function in *Blattella germanica* and other species of Blattodea. Combining genomic, transcriptomic, and peptidomic methods, I examined neuropeptide diversity and evolutionary origins with a focus on adipokinetic hormones (AKHs) and their receptors, as well as exploring their regulatory functions in cockroach physiology and their potential applications in pest management.

In **Chapter I**, I conducted a comprehensive comparative genomic analysis of neuropeptide precursors across 49 Blattodea species, encompassing a diverse taxonomic range of termites and four cockroach species. The study revealed significant gene loss, duplication, and conservation patterns across different lineages. Notably, I observed the absence of specific neuropeptide genes such as ACP and Gonadulin in several termite families, suggesting potential associations with changes in reproductive strategies or ecological adaptations. In contrast, cockroaches exhibited gene duplications, including duplicates of the AKH gene, indicating diversification of neuropeptide functions within cockroach lineages. Additionally, phylogenetic analyses based on 32 neuropeptide precursors closely aligned with established evolutionary relationships within Blattodea, underscoring the value of neuropeptide genes as molecular markers in evolutionary studies.

In **Chapter II**, I delved into the evolution of AKH ligands and their receptors in Blattodea, uncovering new gene duplication and diversification patterns. Phylogenetic analyses of AKH precursor sequences suggest an ancient AKH gene duplication event in the common ancestor of Blaberoidea, leading to a new set of putative decapeptides specific to this clade. I identified 16 different AKH peptides from 90 species, including the prediction of seven novel decapeptides for the first time. Analysis of AKHR sequences from 18 species reveals highly conserved transmembrane regions characteristic of GPCRs. Phylogenetic analyses based on AKHR sequences support established relationships among termite and cockroach lineages. Additionally, the study investigates predicted post-translational modification sites in AKHRs and finds no significant differences between solitary cockroaches and social termites.

In **Chapter III**, I utilized transcriptomic and peptidomic analyses to carry out a comprehensive analysis of the neuropeptidome of *B. germanica*. I discovered 69 neuropeptide or neurohormone precursor transcripts in the brain transcriptome, encompassing most of the known insect neuropeptide families. Mass spectrometry confirmed 79 likely bioactive mature neuropeptides and precursor sequences, with many being reported in this species for the first time. Moreover, the bioassay demonstrated that two AKH peptides, including the recently identified novel decapeptide (AKH2), increase carbohydrate levels in both adult male and female *B. germanica*. Interestingly, females exhibited greater hemolymph

carbohydrate mobilization than males when treated with an equal dosage of the AKH peptides, indicating sex-specific metabolic responses.

In **Chapter IV**, I further examined the impact of two distinct AKH peptides on *B. germanica* at the transcriptome level following the injection of two adipokinetic hormone peptides. RNA sequencing at 3 and 18 hours post-peptide injection revealed significant alterations in metabolic pathways, including enhanced glycolysis, increased tricarboxylic acid cycle activity, and biosynthetic process shifts. I observed distinct transcriptional responses between males and females, indicating potentially differential hormonal regulation, and therefore sexual dimorphism in key physiological traits. Furthermore, I investigated RNA interference-mediated knockdown of *AKHR* on the host's response to pathogen infection. I found that knockdown of *AKHR* led to reduced survival rates upon bacterial infection with *Pseudomonas entomophila*, underscoring the potential role of AKH signaling in immune defense.

In conclusion, this thesis provides a comprehensive exploration of neuropeptide evolution, function, and regulation in Blattodea, with a particular focus on the metabolic roles of AKHs in *B. germanica*. By integrating genomic, transcriptomic, and peptidomic methodologies, this study enhances our understanding of how neuropeptides contribute to the physiological adaptations of cockroaches and termites. The findings highlight the evolutionary significance of neuropeptide signaling and its potential applications in pest management strategies. This work lays the foundation for future studies on the molecular mechanisms underlying neuropeptide function and their implications for insect ecology and control. Targeting neuropeptide pathways, such as AKH signaling, may offer innovative and sustainable approaches for managing pest populations, thereby mitigating the health risks associated with species like *B. germanica*.

Zusammenfassung

Diese Dissertation präsentiert eine detaillierte Analyse der Neuropeptid-Evolution, Genregulation und Funktion in *Blattella germanica* und anderen Arten der Blattodea. Durch die Kombination von genomischen, transkriptomischen und peptidomischen Methoden habe ich die Vielfalt und evolutionären Ursprünge von Neuropeptiden untersucht, mit einem Schwerpunkt auf Adipokinetischen Hormonen (AKHs) und ihren Rezeptoren. Zudem wurden ihre regulatorischen Funktionen in der Physiologie von Schaben erforscht und potenzielle Anwendungen im Schädlingsmanagement aufgezeigt.

In **Kapitel I** führte ich eine umfassende vergleichende genomische Analyse von Neuropeptid-Vorläufern über 49 Blattodea-Arten durch, die ein breites taxonomisches Spektrum von Termiten und vier Schabenarten abdecken. Die Studie enthüllte signifikante Muster von Genverlust, Duplikation und Konservierung in verschiedenen Abstammungslinien. Bemerkenswert ist das Fehlen spezifischer Neuropeptid-Gene wie ACP und Gonadulin in mehreren Termitenfamilien, was auf potenzielle Zusammenhänge mit Veränderungen in Reproduktionsstrategien oder ökologischen Anpassungen hindeutet. Im Gegensatz dazu zeigten Schaben Gen-Duplikationen, einschließlich Duplikaten des AKH-Gens, was auf eine Diversifizierung der Neuropeptid-Funktionen innerhalb der Schabenlinien hinweist. Darüber hinaus stimmten phylogenetische Analysen basierend auf 32 Neuropeptid-Vorläufern eng mit den etablierten evolutionären Beziehungen innerhalb der Blattodea überein und unterstreichen den Wert von Neuropeptid-Genen als molekulare Marker in Evolutionsstudien.

In **Kapitel II** vertiefte ich mich in die Evolution von AKH-Liganden und ihren Rezeptoren in Blattodea und entdeckte neue Muster von Gen-Duplikation und Diversifikation. Phylogenetische Analysen der AKH-Vorläufersequenzen deuten auf ein uraltes AKH-Gen-Duplikationsereignis im gemeinsamen Vorfahren der Blaberoidea hin, was zur Entstehung einer neuen Reihe mutmaßlicher Decapeptide führte, die spezifisch für diese Klade sind. Ich identifizierte 16 verschiedene AKH-Peptide aus 90 Arten, einschließlich der erstmaligen Vorhersage von sieben neuartigen Decapeptiden. Die Analyse der AKHR-Sequenzen aus 18 Arten zeigt hochkonservierte Transmembranregionen, die charakteristisch für GPCRs sind. Phylogenetische Analysen basierend auf AKHR-Sequenzen unterstützen die etablierten Beziehungen zwischen Termiten- und Schabenlinien. Zudem untersucht die Studie vorhergesagte posttranslationale Modifikationsstellen in AKHRs und findet keine signifikanten Unterschiede zwischen solitären Schaben und sozialen Termiten.

In **Kapitel III** nutzte ich transkriptomische und peptidomische Analysen, um eine umfassende Untersuchung des Neuropeptidoms von *B. germanica* durchzuführen. Ich entdeckte 69 Neuropeptid-

oder Neurohormon-Vorläufertranskripte im Gehirntranskriptom, die die meisten der bekannten Insekten-Neuropeptid-Familien umfassen. Die Massenspektrometrie bestätigte 79 vermutlich bioaktive reife Neuropeptide und Vorläufersequenzen, von denen viele erstmals in dieser Art berichtet wurden. Darüber hinaus zeigte der Bioassay, dass zwei AKH-Peptide, einschließlich des kürzlich identifizierten neuartigen Decapeptids (AKH2), die Kohlenhydratspiegel sowohl bei männlichen als auch weiblichen adulten *B. germanica* erhöhen. Interessanterweise zeigten Weibchen eine stärkere Mobilisierung von Kohlenhydraten im Hämolymph als Männchen bei gleicher Dosierung der AKH-Peptide, was auf geschlechtsspezifische metabolische Reaktionen hinweist.

In **Kapitel IV** untersuchte ich weiter die Auswirkungen von zwei verschiedenen AKH-Peptiden auf *B. germanica* auf Transkriptomebene nach Injektion. Die RNA-Sequenzierung 3 und 18 Stunden nach Peptid-Injektion zeigte signifikante Veränderungen in Stoffwechselwegen, einschließlich verstärkter Glykolyse, erhöhter Aktivität des Tricarbonsäurezyklus und Verschiebungen in biosynthetischen Prozessen. Ich beobachtete unterschiedliche transkriptionelle Reaktionen zwischen Männchen und Weibchen, was auf eine potenziell differenzielle hormonelle Regulation und somit auf sexuellen Dimorphismus in wichtigen physiologischen Merkmalen hindeutet. Zudem untersuchte ich die RNA-Interferenz-vermittelte Herunterregulierung von *AKHR* auf die Wirtsreaktion bei einer Pathogeninfektion. Ich stellte fest, dass die Herunterregulierung von *AKHR* zu reduzierten Überlebensraten bei bakterieller Infektion mit *Pseudomonas entomophila* führte, was die potenzielle Rolle der AKH-Signalübertragung in der Immunabwehr unterstreicht.

Abschließend bietet diese Dissertation eine umfassende Erforschung der Neuropeptid-Evolution, -Funktion und -Regulation in Blattodea, mit besonderem Fokus auf die metabolischen Rollen von AKHs in *B. germanica*. Durch die Integration von genomischen, transkriptomischen und peptidomischen Methoden vertieft diese Studie unser Verständnis dafür, wie Neuropeptide zu den physiologischen Anpassungen von Schaben und Termiten beitragen. Die Ergebnisse heben die evolutionäre Bedeutung der Neuropeptid-Signalübertragung und ihre potenziellen Anwendungen in Schädlingsmanagementstrategien hervor. Diese Arbeit legt den Grundstein für zukünftige Studien über die molekularen Mechanismen der Neuropeptid-Funktion und ihre Implikationen für Insektenökologie und -kontrolle. Die Zielgerichtetheit auf Neuropeptid-Wege, wie die AKH-Signalübertragung, könnte innovative und nachhaltige Ansätze für das Management von Schädlingspopulationen bieten und somit die Gesundheitsrisiken im Zusammenhang mit Arten wie *B. germanica* mindern.

General introduction

1.1 Neuropeptides in insects

1.1.1 Neuropeptide overview

Neuropeptides are small, protein-like molecules made up of around 5-80 amino acids, which perform essential roles in the nervous systems of animals (Grimmelikhuijzen and Hauser 2012; Nässel and Winther 2010; Schoofs et al. 2017). These neuropeptides are produced from larger precursor proteins called prepropeptides in neuronal cell bodies. They undergo various enzymatic post-translational modifications, such as cleavage, amidation, and phosphorylation, to become mature, bioactive peptides (Nässel and Larhammar 2013; Van Den Pol 2012; Veenstra 2000; Yeoh et al. 2017). These modifications are necessary for the functionality of neuropeptides as they influence the peptides' stability, receptor binding affinity, and biological activity (Fricker 2005; Gäde et al. 2006; Scherkenbeck and Zdobinsky 2009; Velentza et al. 2000) (Figure 1).

The Database for Insect Neuropeptide Research (DINeR) has documented the presence of over 50 distinct neuropeptides in insects, spanning across over 400 species (Yeoh et al. 2017). This diversity reflects the evolutionary adaptation of neuropeptide systems to cater to the specific physiological and ecological needs of different insect species (Jékely 2013; Nässel et al. 2019; Nässel and Zandawala 2019; Paluzzi et al. 2013).

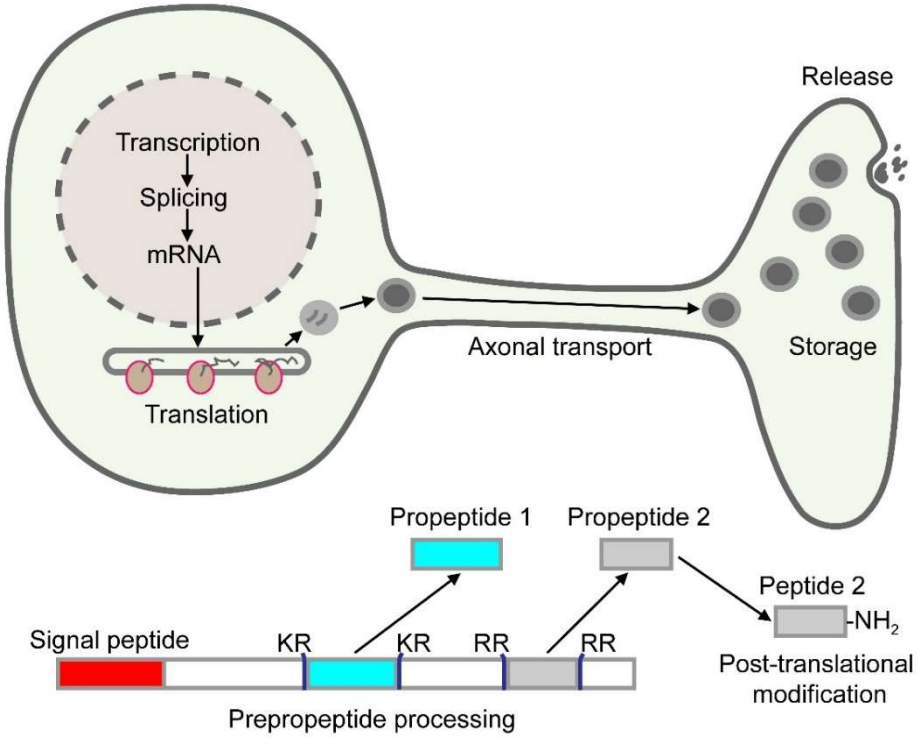

Figure 1. Schematic of insect neuropeptide biosynthesis and secretion. Neuropeptide genes undergo transcription and splicing to form mRNA, which is subsequently translated into prepropeptide precursors. At the terminals, peptidases cleave the precursors at specific sites, typically containing basic amino acid sequences like KR, RR, or RXXR, following translation (from Nässel and Zandawala 2019).

Neuropeptides significantly influence a broad spectrum of physiological and behavioral processes in insects, acting as neurotransmitters, neuromodulators, or neurohormones. These molecules are key players in virtually every aspect of insect biology, including metabolism, growth, reproduction, stress responses, and immune function. Their diverse roles highlight the significance of neuropeptides in coordinating complex physiological responses, enabling insects to adapt to their environments (Gäde 2004; Gáliková et al. 2017; Kaufmann and Brown 2008; Kubrak et al. 2016; Urbański et al. 2022; Veenstra 2023; Veenstra et al. 2021). For instance, proctolin (PT) was one of the first neuropeptides to be discovered in the American cockroach *Periplaneta americana.* Functional studies revealed its essential role in muscle contraction (Starratt and Brown 1975). Later, it was found to perform similar functions in muscle contraction and the regulation of motor activities in several other insects (Orchard et al. 1989; Ormerod et al. 2016; Schoofs et al. 2017; Starratt and Brown 1979; Wegener and Nässel 2000). Another example is pheromone biosynthesis activating neuropeptide (PBAN), which regulates the production of pheromones that are vital for mating and species-specific interactions (Altstein and Nässel 2010; Rafaeli 2009; Ragionieri et al. 2017; Raina and Kempe 1990). Neuropeptides like neuropeptide F (NPF) and pigment-dispersing factor (PDF) are involved in regulating circadian rhythms and feeding behaviors in several species, influencing daily activity patterns and nutrient intake (Colizzi et al. 2023; Li et al. 2023; Nässel and Wegener 2011; Veenstra 2021).

Moreover, neuropeptides have a systemic activity, affecting both central and peripheral systems within the insect body. Neuropeptides are present in specific regions of the central nervous system (CNS), such as the accessory medulla, central complex, antennal lobe, and optic lobe, as well as in peripheral areas like the gut and Malpighian tubules (Coast and Schooley 2011; Nässel 2002; Nässel and Homberg 2006; Nässel and Zandawala 2019; Schoofs et al. 2017). In *Drosophila*, peptidergic neurons project to various sensory and neuromodulatory pathways, playing critical roles in regulating behavior and homeostasis (Ly et al. 2019; Nässel and Winther 2010; Nässel and Zandawala 2019; Predel et al. 2018). This widespread distribution allows neuropeptides to integrate signals from multiple sources, coordinating functions across different physiological pathways.

1.1.2 Distribution and classification

Neuropeptides distribution

Neuropeptides are primarily localized in the accessory medulla (AMe) and central complex of the insect brain, while they are less abundant in antennal lobes, optic lobes, and mushroom bodies. *In situ* hybridization and immunohistochemistry have been used to detect neuropeptides in a majority of brain interneurons and ventral nerve cords (VNC), but their distribution varies significantly among distinct neuronal populations (Nässel and Homberg 2006).

The distribution of neuropeptides has been investigated in detail in *Drosophila melanogaster*, where several neuropeptides are expressed in diverse principal cell types, including central nervous system interneurons, neuroendocrine cells, enteroendocrine cells, peripheral sensory neurons, and adipose tissue cells. Figure 2 depicts the distribution of neuropeptides across the hormonal system, central nervous system (CNS), and the gut. Most neuropeptides belong to one or more of these systems, whereas others are exclusively associated with the hormonal system or the central CNS (Nässel and Zandawala 2019).

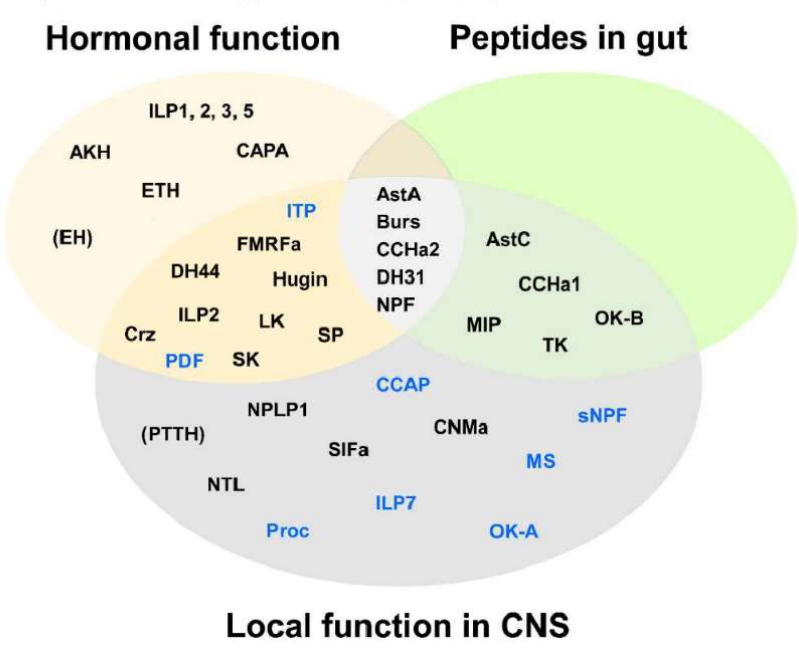

Figure 2. Venn diagram showing the distribution of neuropeptides in hormonal systems, interneurons, and the intestine of *Drosophila*. Multiple systems share most neuropeptides. Highlighted in blue are peptides produced by efferent CNS neurons that innervate the intestine, particularly in neurohemal areas of the anterior intestine (e.g., AKH, CRZ, ILPs). Neuropeptides in brackets have not been confirmed in adult structures. Some peptides are omitted due to insufficient data (from Nässel et al. 2019).

Neuropeptides classification

Insect neuropeptides exhibit a diverse range of types and functions and are evolutionarily conserved. Despite being classified into different families, their naming conventions lack standardization and remain somewhat disorganized. Various naming conventions exist, including those based on biological activity, homology to vertebrate peptides, or chemical structure (Chowanski et al. 2017; Coast and Schooley 2011; Veenstra 2014).

Neuropeptides named based on biological activity. Numerous neuropeptides are named based on their biological activity, a convention that mirrors their physiological functions and the circumstances of their initial discovery. For instance, Adipokinetic hormones (AKHs) are necessary for mobilizing energy substances by regulating lipid and carbohydrate metabolism during periods of high metabolic demand or stress; Neuropeptide Allatostatins (Asts) and Allatotropins (ATs) play important roles in

regulating the synthesis of juvenile hormone (JH), in turn affecting growth, development, and reproduction. Additionally, corazonin (Crz), named for its cardioacceleratory effects in the cockroach *P. americana*, is involved in regulating stress responses, pigmentation, and reproduction. Diuretic hormones (DHs), including diuretic hormone 31 (DH31) and diuretic hormone 44 (DH44), are essential for regulating water and ion balance in varying environmental conditions. Proctolin, for its significant involvement in muscle contraction effects in hindgut tissues; Bursicon regulates cuticle tanning, sclerotization, and wing elongation following eclosion; Eclosion Hormone (EH) and Ecdysis-Triggering Hormone (ETH) regulate the molting process, initiating ecdysis during insect development; Diapause Hormone (DH) regulates diapause, a critical survival strategy during adverse environmental conditions; PBAN regulates pheromone synthesis and release, thus directly affecting mating behavior and reproductive success in moths. A brief overview of insect neuropeptide diversity and function categorized according to biological activity is given in Table 1.

Table 1. List of some neuropeptides are named for their physiological functions

Neuropeptide	Functions	Studied Species	References
Adipokinetic hormones	Regulate lipid and carbohydrate metabolism	Various insects	Gäde (2004); Gäde & Auerswald (2003); Goldsworthy et al. (2003); Van der Horst et al. (2001)
Allatostatins and Allatotropins	Affect growth, development, and reproduction by regulating JH synthesis	*Manduca sexta*, *Diploptera punctata*	Audsley & Weaver (2009); Skinner et al. (1997)
Corazonin	Regulates stress responses, pigmentation, and reproduction	*L. migratoria*, *D. melanogaster* *Harpegnathos saltator*	Boerjan et al. (2010); Tawfik et al. (1999); Veenstra (1989)
Diuretic hormones	Regulate water and ion balance	*Rhodnius prolixus*, *Aedes aegypti*	Coast & Schooley (2011); Paluzzi et al. (2014)
Proctolin	Induces muscle contractions, especially in the hindgut muscles	*P. americana* *Rhodnius prolixus*	Ormerod et al. (2016); Starratt & Brown (1975); Wegener & Nässel (2000)
Bursicon	Controls cuticle tanning, sclerotization, and wing expansion post-eclosion	*D. melanogaster*, *Tribolium castaneum*, *Anopheles gambiae*	Arakane et al. (2008); Honegger et al. (2011); Loveall & Deitcher (2010)
Eclosion hormone and Ecdysis-triggering hormone	Initiate ecdysis during molting	Various insects	Ewer et al. (1997); Horodyski (1996); Kim et al. (2006); Park et al. (1999); Žitňan et al. (2007)
Diapause hormone	Controls diapause during adverse conditions	*Helicoverpa zea*, *Bombyx mori*	Denlinger (2002); Hasegawa (1957); Zhang & Denlinger (2012)
Pheromone hiosynthesis activating neuropeptide	Regulates pheromone synthesis and release	*Heliothis peltigera*, *H. armigera*	Nachman et al. (2009); Rafaeli (2009); Raina & Kempe (1990)

Neuropeptides named based on structural features. Certain neuropeptides are given their names based on their specific structural features. These structural motifs often serve as defining characteristics for different peptide families, offering valuable insights into their functions across various species (Grimmelikhuijzen and Hauser 2012; Veenstra 2000). For instance, CCHamides (CCHs), is characterized by two conserved C-terminal cysteines and an amidated histidine. FMRFamide-Related Peptides (FMRFs) are characterized by their C-terminal FMRFamide sequences. Likewise, Short Neuropeptide F (sNPF) is characterized by the conserved C-terminal RLRFamide sequence; Natalisins (Nat) are characterized by the C-terminal motifs: FxPxRamide or FWxxRamide. Furthermore, Tachykinin-Related Peptides (TKs) are characterized by a conserved C-terminal motif Fx_1Gx_2Ramide. A brief overview of insect neuropeptide diversity and function categorized according to structural features is given in Table 2.

Table 2. List of some neuropeptides characterized by specific structural motifs

Neuropeptide	Structural Feature	Function(s)	Studied Species	References
CCHamides	Two conserved C-terminal cysteines and an amidated histidine	Role in feeding and growth	*D. melanogaster*, *B. mori*	Ida et al. (2012); Roller et al. (2008)
FMRFamide-related Peptides	C-terminal sequence FMRFamide	Regulate muscle contraction, heart rate, and neurotransmission	*M. sexta*, *D. melanogaster* *P. americana*	Nichols (2003); Orchard et al. (2001)
Short neuropeptide F	Conserved C-terminal sequence RLRFamide	Regulates feeding, growth, and stress responses	Various insects	Amir et al. (2022); Li et al. (2023); Medla et al. (2023)
Natalisins	C-terminal motifs FxPxRamide or FWxxRamide	Regulate mating and reproductive behaviors	*T. castaneum*, *B. mori*, *Bactrocera dorsalis*	Gui et al. (2018); Jiang et al. (2013)
Tachykinin-related peptides	Conserved C-terminal motif Fx_1Gx_2Ramide	Involved in muscle contraction, nociception, and stress responses	*L. migratoria*, *D. melanogaster* *Tenebrio molitor*	Nässel & Zandawala (2019); Song et al. (2014); Urbański & Rosinski (2018)

Neuropeptides named based on homology to vertebrate peptides. Other neuropeptide descriptions are derived from homology with other animal groups, typically reflecting ancient evolutionary relationships and structural resemblances to vertebrate peptides. Insulin-Like Peptides (ILPs), homologous to vertebrate insulin, are essential for growth, metabolism, and reproduction (Ament et al. 2008; Castro-Arnau et al. 2019; Domínguez et al. 2022; Géminard et al. 2006; Veenstra 2020; Veenstra 2023). Sulfakinins (Sul) are pleiotropic neuropeptides with the homology to vertebrate gastrin/cholecystokinin peptide family, regulating feeding and digestive processes in several insect

species (Dickinson et al. 2007; Downer et al. 2007; East et al. 1997; Marciniak et al. 2011). Pigment-Dispersing Factor (PDF), due to its high sequence similarity to crustacean β-pigment-dispersing hormone (PDH), regulates circadian rhythms and locomotor activity in insects like *D. melanogaster* and cockroaches such as *L. maderae* and *B. germanica* (Helfrich-Förster 2009; Rao and Riehm 1993; Lee et al. 2009; Umezaki et al. 2012).

1.1.3 Neuropeptide functions in biology

Neuropeptides are essential regulators of various physiological and behavioral processes in insects, as described above, and these diverse roles of neuropeptides highlight their importance in maintaining homeostasis and enabling adaptive responses to environmental change. Historically, certain cockroach species, such as *P. americana* and *Diploptera punctata*, have been instrumental in the discovery and understanding of insect neuropeptides. For example, the first allatostatin A (AstA) was identified in *D. punctata* in 1997 (Gäde, 1997) and the first Calcitonin-Like Diuretic Hormone (CT-DH) peptide was isolated from the same species (Furuya et al. 2000). Additionally, a recently discovered neuropeptide Flik, was first identified in *P. americana* (Zeng et al. 2021), and the first sNPF was isolated from the midgut of *P. americana* (Veenstra and Lambrou 1995). These foundational discoveries of neuropeptides in cockroaches have paved the way for the later numerous reports of neuropeptides and their biological functions across a wide range of insects. Given the high degree of functional and sequence conservation among insect neuropeptides, the general functions described below are broadly applicable across insects, including within Blattodea. More detailed examinations of neuropeptides in Blattodea are provided in subsequent chapters, particularly **Chapter III**. These neuropeptides, according to their specific roles in insect physiology, are described in the following section:

Development and metamorphosis. In insects, prothoracicotropic hormone (PTTH) and eclosion hormone (EH) have been widely reported to play important roles in development by regulating molting and metamorphosis through the control of ecdysteroid synthesis and release (Gäde and Hoffmann 2005; Horodyski 1996; Malhotra and Basu 2023; McBrayer et al. 2007; Myers 2003; Okamoto and Watanabe 2022; Sauman and Reppert 1996). ETH coordinates the sequence of behaviors associated with molting and reproduction, highlighting the intricate regulation of developmental processes by ETH (Areiza et al. 2014; Asuncion-Uchi et al. 2010; Dai and Adams 2009; Ewer et al. 1997; Malhotra and Basu 2023; Park et al. 1999; Roller et al. 2010; Zitnan et al. 2002). ILPs, another neuropeptide family associated with insect development and metamorphosis, regulates growth and coordinating energy balance during development (de Azevedo and Hartfelder 2008; Géminard et al. 2006; Iga and Smagghe 2011; Oldham et al. 2000; Slaidina et al. 2009; Smith et al. 2014; Veenstra 2023). In *Locusta migratoria*, gene expression patterns reveal that multiple neuropeptide genes are both developmentally and phase-related (Figure 3).

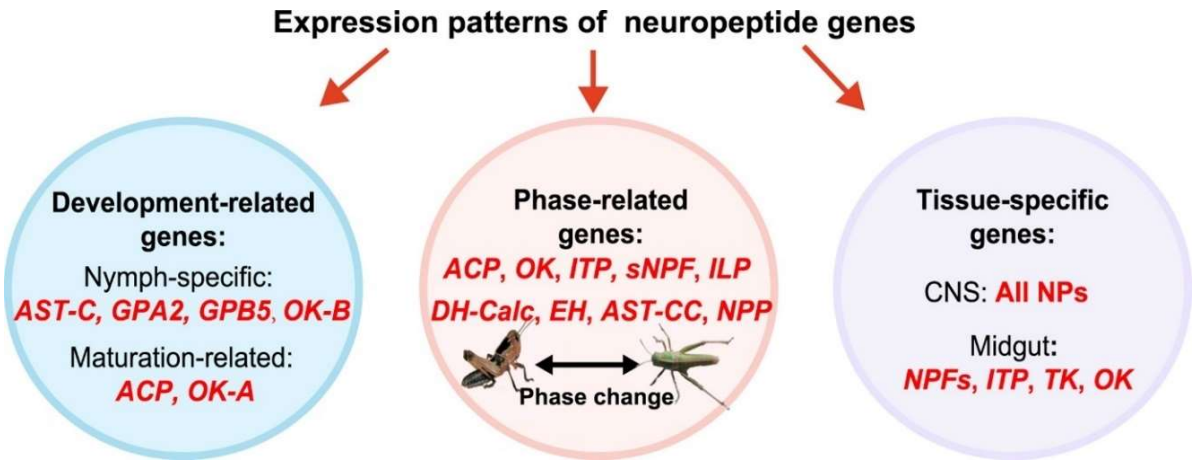

Figure 3. Expression patterns of neuropeptide genes in the migratory locust, *Locusta migratoria* (from Hou et al. 2015).

Metabolism and energy homeostasis. Adipokinetic hormones (AKHs) and neuropeptides such as sNPF and neuropeptide F (NPF) are essential metabolic regulators that mobilize energy reserves during periods of high energy demand. By stimulating lipid and carbohydrate metabolism, AKHs enable insects to convert stored nutrients into usable forms, such as trehalose and diacylglycerol, supporting essential functions like locomotion and thermoregulation (Auerswald et al. 2005; Chino et al. 1989; Gäde and Adrianus 1977; Gäde and Marco 2017; Gäde et al. 2005; Gáliková et al. 2015; Goldsworthy et al. 2003; Jedlicka et al. 2012; Lee and Park 2004; Stone et al. 1978). sNPF and NPF modulate feeding behavior and nutrient intake, highlighting their roles in nutrient metabolism and energy homeostasis (Amir et al. 2022; Carlsson et al. 2013; Lee et al. 2004; Yu et al. 2004).

Reproduction and reproductive behaviors. Neuropeptides influence reproductive processes and behaviors by modulating hormonal and neural pathways. PBAN is critical in pheromone production, directly affecting mating and reproductive success (Rafaeli 2009; Ragionieri et al. 2017; Raina and Kempe 1990). The sex peptide (SP) in *Drosophila* interacts with multiple G protein-coupled receptors (GPCRs) to regulate post-mating behaviors and fecundity, illustrating the complex signaling networks involving neuropeptides in reproductive regulation (Chen et al. 1988; Tsuda and Aigaki 2016; White et al. 2021). Additionally, allatotropin (AT) influences JH levels, which are necessary for reproductive maturation and vitellogenesis (Hassanien et al. 2014; Lee and Horodyski 2006; Teal 2002).

Stress responses and immune function. Neuropeptides play central roles in the insect body's response to stress by facilitating physiological adjustments to environmental stressors (Bednarova et al. 2015; Broughton et al. 2005; Kahsai et al. 2010; Kodrík et al. 2015; Nässel and Winther 2010). Tachykinin-related peptides (TKs) are pivotal in managing feeding behavior under stressful conditions and also influence the production of antimicrobial peptides, thereby modulating immune functions in the mealworm beetle *Tenebrio molitor* (Urbański et al. 2022; Urbański and Rosinski 2018). CAPAs are essential for maintaining fluid balance during stress events like dehydration by modulating the diuretic

and antidiuretic activities in the Malpighian tubules (Coast and Schooley 2011; Davies et al. 2012; Halberg et al. 2015).

Other regulatory functions. In addition to the fundamental functions mentioned above, neuropeptides also regulate other essential physiological and behavioral processes. Allatostatin-C (AstC) regulates the activity of clock neurons in *D. melanogaster*, impacting sleep duration and the circadian rhythm: disruption of AstC signaling affects the sleep cycle and induces arrhythmic locomotion, suggesting its function in circadian regulation (Dubowy and Sehgal 2017; Hamasaka et al. 2007). Pigment-dispersing factor (PDF) also coordinates the function of circadian clock neurons in *D. melanogaster*. Mutants lacking PDF exhibit altered sleep-wake cycles and impaired circadian rhythms (Choi et al. 2009; Helfrich-Förster 2005). In the ant *Harpegnathos saltator*, corazonin (Crz) has been reported to regulate social hierarchy and caste differentiation by regulating hormone signaling pathways, consequently connecting neuropeptide signaling to social behaviors (Gospocic et al. 2017). ILPs regulate social consumption and cooperative behaviors in the honeybee *Apis mellifera*, regulating worker interactions and colony dynamics (Ament et al. 2008). Inotocin, an oxytocin/vasopressin-like peptide, has been discovered in the ant *Camponotus fellah*, where it regulates social communication and hydrocarbon synthesis, essential for nestmate recognition (Koto et al. 2019).

PTMs and regulatory mechanisms

Neuropeptides undergo various post-translational modifications (PTMs), such as amidation, phosphorylation, and glycosylation, which are necessary for their stability, receptor binding, and biological activity (Veenstra, 2000; Fricker, 2005; Hummon et al., 2006). Amidation, for instance, is essential for the activation of many neuropeptides, enhancing their affinity for receptors and prolonging their half-life, thereby ensuring effective signaling (Fricker 2005; Gäde 1997a; Scherkenbeck and Zdobinsky 2009; Veenstra 2000). These modifications contribute to the diversity and specificity of neuropeptide functions, allowing insects to adapt their physiological responses to varying environmental conditions.

Neuropeptides and their receptors, primarily GPCRs, form complex regulatory networks that control a wide range of physiological and behavioral activities (Caers et al. 2012; Hauser et al. 2006; Hauser et al. 2008; Latorraca et al. 2017; Pandit et al. 2018; Tanaka et al. 2014; Zhang et al. 2020). The diversity of GPCRs involved in neuropeptide signaling reflects the complexity and specificity of neuropeptide functions (Rios et al. 2001; Veenstra et al. 2012; Venkatakrishnan et al. 2013).

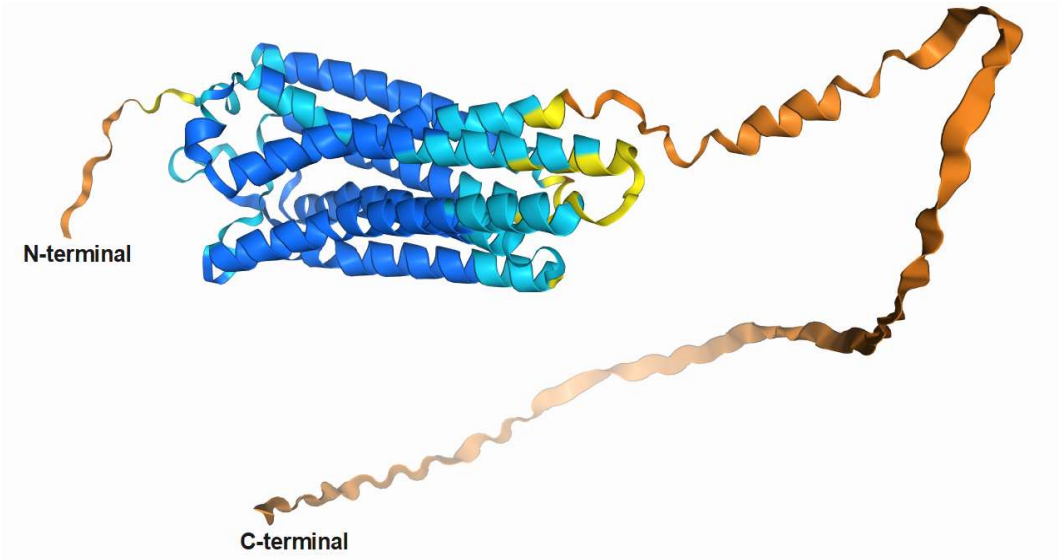

Figure 4. Predicted structure of AKHR protein of *B . germanica* (GenBank: ADL60118.1) by AlphaFold3 (Abramson et al. 2024). The N- and C-terminals are depicted. AKHR belongs to the typical GPCR A family, the largest and most diverse and widely studied GPCR subfamily.

The evolutionary interplay between neuropeptides and their receptors has led to the diversification of signaling pathways, enabling insects to adjust their responses to changing environmental and physiological demands (Jékely 2013; Möller et al. 2001; Staubli et al. 2002; Veenstra 2014). In *Drosophila*, over 40 G-protein-coupled peptide receptors have been identified and for most of these the ligands have been identified (Grimmelikhuijzen and Hauser 2012; Hauser et al. 2006; Nässel and Winther 2010). The extensive diversity of GPCRs allows for specific and tuned neuropeptide signaling, in turn enabling insects to finely regulate feeding, reproduction, and stress responses. Upon binding to their receptors, neuropeptides activate intracellular signaling pathways involving secondary messengers such as cyclic AMP (cAMP), calcium ions (Ca^{2+}), and inositol triphosphate (IP_3), leading to a cascade of intracellular responses, including changes in gene expression, enzyme activation, and alterations in cellular metabolism (Altstein and Nässel 2010; Jékely 2013; Nässel and Winther 2010).

1.2 Adipokinetic hormones (AKH) and AKH receptors (AKHR)

AKH is one of the most extensively researched neuropeptides in insects, due to its very important role in energy mobilization and metabolism (Gäde 1997b; Gäde and Auerswald 2003; Gäde and Goldsworthy 2003; Gäde and Marco 2011; Gäde and Marco 2012; Isabel et al. 2005). Following its discovery in the 1960s, initial studies focused on the ability of AKH to mobilize lipid reserves during flight (Gäde 1997b; Goldsworthy et al. 1975; Stone et al. 1976). The subsequent determination of the primary structures of various AKHs revealed a family of related peptides with conserved functions across insect species (Gäde 1997a; Gäde 2004; Gäde and Marco 2011; Gäde and Marco 2022; Marco et al. 2014; Marco et al. 2020). The cloning and characterization of adipokinetic hormone receptor (AKHR) genes further provided significant insights into the molecular mechanisms underlying AKH signaling (Caers et al. 2016; Hansen et al. 2006; Huang et al. 2012; Iyison et al. 2020; Konuma et al. 2012; Staubli et al. 2002; Yang et al. 2018; Zhu et al. 2009).

Studies on AKH diversity have demonstrated that although its fundamental roles are preserved across species, there is considerable variability in peptide sequences and receptor affinities, reflecting adaptations to specific ecological niches and physiological demands (Gäde and Marco 2011; Gäde et al. 2009; Hansen et al. 2010; Li et al. 2016; Marciniak et al. 2022; Marco and Gade 2015). AKHs and their receptors are widely distributed across various insect orders, as reported in Diptera, Lepidoptera, Coleoptera, Blattodea, and other orders (Gäde et al. 2019; Jiang et al. 2023; Kaufmann et al. 2009; Marco et al. 2020). AKHs serve as key regulators of energy metabolism by manipulating lipid and carbohydrate mobilization, feeding behavior, stress responses, and reproductive functions. During periods of elevated energy requirements, such as flight or starvation, AKHs are released into the hemolymph and bind to AKHRs on target tissues like the fat body (Gäde and Auerswald 2003; Gäde and Kellner 1992; Isabel et al. 2005; Oguri and Steele 2003; Tomčala et al. 2010; Van der Horst 2003; Veenstra and Camps 1990). In *Drosophila*, AKH deficiency leads to increased lipid storage and obesity-like phenotypes, emphasizing its role in maintaining energy balance (Bharucha et al. 2008; Gáliková et al. 2015; Isabel et al. 2005). In addition, AKH signaling modulates oxidative stress responses and feeding behavior (Bednarova et al. 2015; Hou et al. 2017; Kodrík et al. 2015; Zandawala et al. 2015). AKH also plays a role in reproductive processes in certain species by interacting with other hormonal pathways, highlighting its diverse range of functions (Hou et al. 2017; Tang et al. 2020).

Notably, certain species, such as locusts and cockroaches, have multiple AKH peptides, indicating complex regulation of energy metabolism suited to their life histories (Auerswald et al. 2005; Gäde et al. 2013; Jackson et al. 2019; Marco and Gäde 2019). However, despite the discovery of multiple AKH isoforms in some species, previous studies have not investigated the biological functional differences

between sexes in response to these peptides. This gap highlights the need for studies focusing on sex-specific responses to AKHs to fully understand their physiological and ecological significance.

1.3 Blattodea overview

Blattodea is an insect order encompassing cockroaches and termites, constituting a diverse group with over 7,500 described species. In traditional classification, termites were described as a separate order: Isoptera, but recent studies show that termites are nested within the cockroach clade, leading to the reclassification of termites as a clade within Blattodea (Bell et al. 2007; Bourguignon et al. 2014; Ewart et al. 2024; Hellemans et al. 2022; Inward et al. 2007).

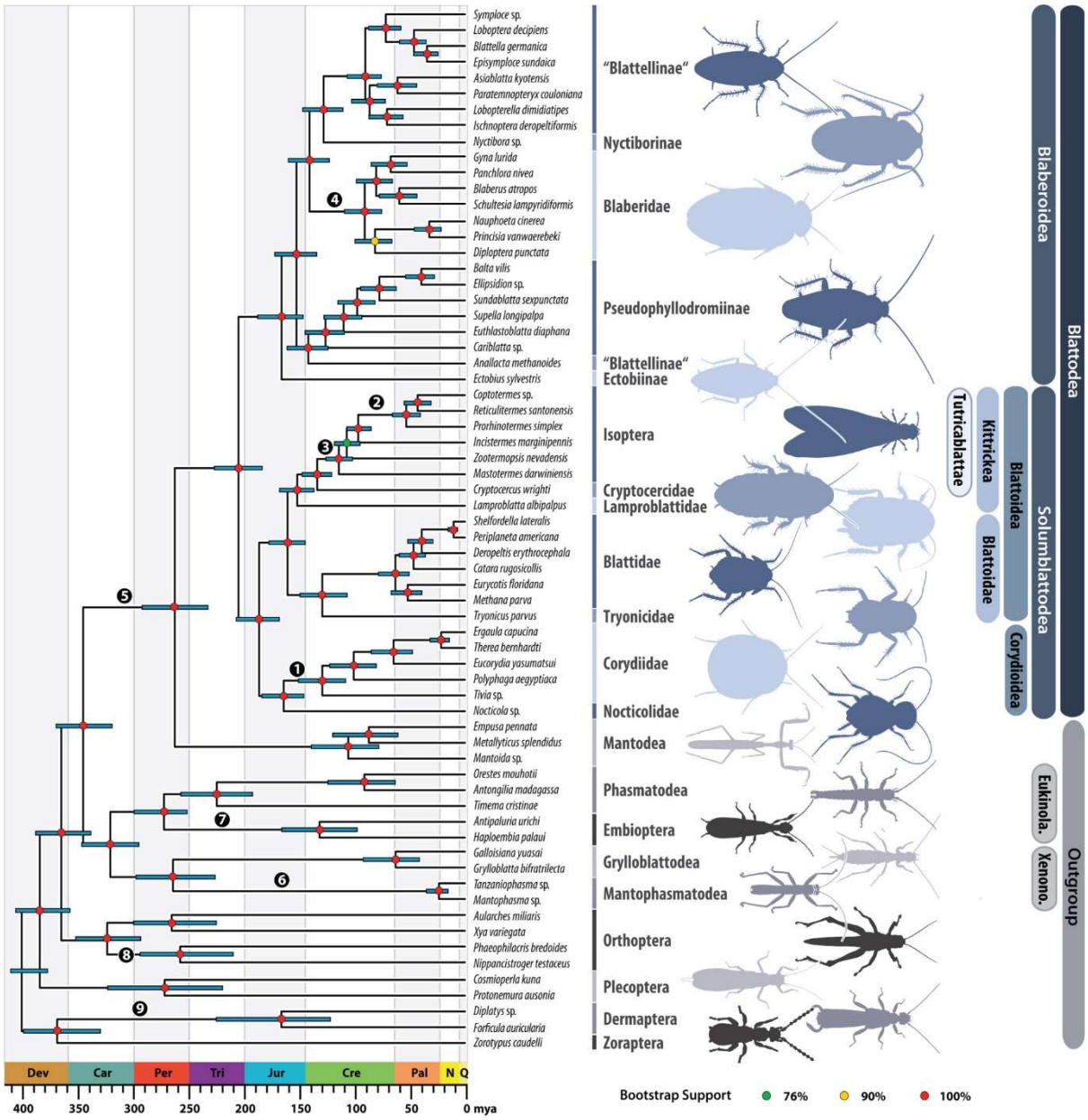

Figure 5. Time-calibrated phylogeny of Blattodea. Analyses of the phylogenetic relationships within Blattodea confirm previously uncertain hypotheses, such as the sister-group relationship between Blaberoidea and the rest of Blattodea. It also indicates that Lamproblatta is the closest relative to the social and wood-feeding *Cryptocercus* and termites (from Evangelista et al. 2019).

Recent molecular and morphological analyses have reshaped our understanding of Blattodea phylogeny. A major revelation of systematic entomology revealed that termites (Isoptera) belong inside the cockroach lineage and are most closely related to wood-feeding cockroaches of the genus *Cryptocercus* (Evangelista et al. 2019; Inward et al. 2007). *Cryptocercus* and termites share traits such as gut symbionts and xylophagy (Bourguignon et al. 2014; Bucek et al. 2019; Murienne 2009; Nalepa 2015; Thompson et al. 2000). Phylogenomic studies utilizing transcriptome data have offered an improved resolution of relationships within Blattodea, although uncertainties persist due to limited taxon sampling and complex evolutionary histories (Berger et al. 2022; Evangelista et al. 2024; Evangelista et al. 2019; Wang et al. 2017). Based on molecular clock analyses and fossil records, termites are estimated to have diverged from other cockroaches approximately 150 million years ago during the Jurassic period (Bourguignon et al. 2014; Ware et al. 2008). Eusociality is defined by cooperative brood care, overlapping generations within a colony, and a division of labor into reproductive and non-reproductive castes (Boomsma 2009; Harrison et al. 2018; Nalepa 2015; Oster and Wilson 1978). Fossil evidence, including the earliest known termite from the Cretaceous period, supports the ancient origin of eusociality in termites (Engel et al. 2009; Krishna et al. 2013; Vršanský and Aristov 2014).

Termites demonstrate one of the most sophisticated forms of eusociality among insects, with intricate colony structures comprising workers, soldiers, and reproductive individuals (Harrison et al. 2018; Noirot and Pasteels 1987; Thorne 1997). This social structure facilitates efficient resource utilization, improved defence mechanisms, and adaptive colony responses to environmental challenges (Bagnères and Hanus 2015; Hartke and Baer 2011; Lo and Eggleton 2011; Pervez 2018). JH plays a central role in caste determination, with varying levels guiding individuals toward distinct developmental pathways (Hartfelder 2000; Miura et al. 2003; Nijhout and Wheeler 1982; Scharf et al. 2003). Environmental factors such as colony density, pheromonal signals, and nutritional status also influence caste differentiation, ensuring plastic allocation of resources (Chouvenc 2020; Noirot 1985; Oster and Wilson 1978; Watanabe et al. 2014). By interacting with hormones such as JH, neuropeptides such as Asts and ATs, regulate gene expression and physiological processes during development (Veenstra 2014; Veenstra 2023; Weaver and Audsley 2009; Yagi et al. 2008). A comprehensive understanding of these regulatory mechanisms is essential for unravelling the developmental plasticity and social organization of termites.

In contrast, most cockroach species are solitary or exhibit only rudimentary social behaviors, lacking the complex caste systems and cooperative brood care observed in termites (Bell et al. 2007; Evangelista et al. 2019; Wang et al. 2021). Comparing the solitary lifestyle of cockroaches with the eusocial structure of termites provides valuable insights into the evolutionary mechanisms underlying

social complexity and highlights the role of both environment and genetic factors in the regulation and emergence of eusociality (Harrison et al. 2018; Inward et al. 2007; Legendre et al. 2015; Terrapon et al. 2014).

From an ecological perspective, termites play a critical role in decomposition and nutrient cycling, impacting carbon and nitrogen cycles, which ultimately impact ecosystem productivity and biodiversity (Bell et al. 2007; Bignell and Eggleton 2000; Freymann et al. 2008; Jouquet et al. 2016). Moreover, some cockroaches, while not as prominent, also contribute to decomposition processes and are an important food source for various predators, thus playing a role in food webs and energy flow (Bell et al. 2007; Carlson et al. 2017; Evangelista et al. 2019).

Termites possess a wide variety of feeding behavior, some other termite species are highly destructive pests that cause substantial economic damage to wooden structures, crops, and forestry products (Chouvenc et al. 2016; Khan and Ahmad 2018; Su and Scheffrahn 2000). Species such as *Reticulitermes flavipes*, *Mastotermes darwiniensis* and *Coptotermes formosanus* are particularly notorious for their invasive potential and destructive impact on urban infrastructures (Evans et al. 2019; Lenz et al. 2013). Cockroaches, especially in urban areas, are commonly regarded as pests because of their tendency to contaminate food, cause damage to various materials, and serve as carriers of various harmful microorganisms, including bacteria, viruses, fungi, and parasites, which are theorized to pose public health risks. Pathogens such as *Salmonella*, *Escherichia coli*, and *Staphylococcus aureus* can be carried by cockroaches, potentially leading to foodborne illness and infection (Fathpour et al. 2003; Gore and Schal 2007; Kleine-Tebbe et al. 2019; Tang et al. 2024). Furthermore, cockroach allergens are major contributors to asthma and allergic reactions, particularly in urban areas, affecting millions of people worldwide (Arruda et al. 2001; Cohn et al. 2006; Gore and Schal 2007; Wang et al. 2008).

1.4 Aims of this thesis

This thesis seeks to advance understanding of neuropeptide evolution, diversity, and function within Blattodea, including both cockroaches and termites. I integrate genomic, transcriptomic, and peptidomic analyses to achieve this overarching goal. Specifically, the objectives of this thesis are to:

1. Investigate the conservation, loss, and duplication patterns of neuropeptide genes across different Blattodea species to gain insight into how social complexity and ecological adaptations have influenced their neuropeptide profiles.
2. Examine the evolution of AKH peptides and their receptors in Blattodea and elucidate the evolutionary origins and relationships of AKH gene duplications within this order.

3. Establish a comprehensive profile of the neuropeptidome in the German cockroach, *Blattella germanica*, by integrating transcriptomic and peptidomic approaches to identify both conserved and novel neuropeptides. By conducting a comparative analysis of the peptide sequences with closely related species in Blattodea, I seek to broaden knowledge of their potential roles in regulating critical physiological and behavioral processes.

4. Test the hypothesis that the two AKH decapeptides have sex-specific roles in cockroach metabolism. I investigate their regulatory effects in male and female cockroaches by conducting bioassays and transcriptomic analyses. Additionally, to determine if AKH signaling interacts with immune defense, I explore whether *AKHR* knockdown-mediated disruption of metabolism can negatively impact host survival following pathogen exposure.

1.5 Description of the project

This project is structured into four interrelated chapters to elucidate the evolutionary dynamics and functional roles of neuropeptides in Blattodea.

The **first chapter** aims to conduct a comprehensive comparative genomic analysis of neuropeptide precursor genes across 49 species in Blattodea, encompassing a diverse range of termites and cockroaches. Firstly, by investigating neuropeptide genes, I seek to uncover significant patterns of gene loss, duplication, and conservation across different lineages. Specifically, I uncover patterns of loss of certain neuropeptide genes, such as adipokinetic hormone-corazonin-like peptide (ACP) and Gonadulin, and examine gene duplications, including the adipokinetic hormone (AKH) gene and insulin-like peptides (ILPs). These patterns are analyzed in the context of sociality and ecology, providing insights into how evolutionary pressures may have shaped neuropeptide gene repertoires in eusocial versus solitary species. Moreover, to further understand the evolutionary trajectories of neuropeptide genes, I perform phylogenetic analyses based on neuropeptide precursor sequences, which allow assessment of their utility as molecular markers in evolutionary studies, following comparison with established phylogenies within Blattodea. Integrating molecular data with known phylogenetic relationships can also facilitate insights into interesting gene family divergence patterns across termite or cockroach lineages, thereby enhancing our understanding of their evolutionary history and possible novel functions.

Building upon the comparative genomic insights from the first chapter, the **second chapter** delves deeper into the evolutionary patterns and diversification of AKH peptides and their receptors across Blattodea. Firstly, I tackle AKH ligand and receptor gene evolution and the evolutionary origins of AKH gene paralogues from the order Blattodea. Followed by phylogenetic analyses of AKH precursor sequences from 90 species, I aim to uncover ancient AKH gene duplication events, particularly in the

common ancestor of the Blaberoidea clade. This includes the identification of 16 different AKH peptides, with the prediction of seven novel decapeptides for the first time. By mapping these duplications and novel peptides, I seek to understand how AKH diversity has evolved and its potential functional implications in different species. Furthermore, by analyzing AKHR sequences and assessing structural conservation from 18 species, I evaluate the conservation of transmembrane regions characteristic of G protein-coupled receptors (GPCRs). The investigation focuses on predicted post-translational modification sites to understand structural variations and their potential functional consequences. This analysis aims to reveal how receptor evolution corresponds with ligand diversification, providing insights into hormone-receptor co-evolution.

Transitioning from a broad evolutionary perspective to a species-specific focus, the **third chapter** aims to create a comprehensive profile of the neuropeptidome of the German cockroach, *B. germanica*, by applying both transcriptomic and peptidomic approaches. Firstly, I characterize neuropeptide precursors and mature neuropeptides by analyzing the brain transcriptome and conducting mass spectrometry-based peptidomics. This detailed neuropeptide profiling expands our knowledge of the neuropeptide repertoire in *B. germanica*, providing a foundation for functional studies. Secondly, I conduct comparative analyses with closely related Blattodea species to understand the identified peptides' evolutionary relationships and potential functions. This can help to understand how neuropeptide sequences may have diverged or been conserved, shedding light on their roles in regulating key physiological and behavioral processes. Finally, through bioassays, I examine the metabolic effects of two AKH peptides, including a recently identified novel peptide (AKH2), on carbohydrate mobilization in both male and female *B. germanica*. This reveals sex-specific metabolic responses and enhances our understanding of AKH function in energy metabolism.

Based on the findings from the neuropeptidomic profiling and functional assays in the previous chapters, the **fourth chapter** investigates the regulatory roles of the two distinct AKH peptides in *B. germanica* at the transcriptomic level, with an emphasis on their potential sex-specific roles in metabolism. RNA sequencing is employed to examine differential gene expression at 3 and 18 hours post-AKH injection. Through gene enrichment and pathway analysis, we explore the sex-specific effects of the two neuropeptides in male and female cockroaches to understand the hormonal regulation of sexual dimorphism in physiological traits. Moreover, to assess the impact of AKHR knockdown on host immune defence, RNA interference (RNAi) is used to experimentally suppress *AKHR* expression via injection of synthesized dsRNA, and to investigate the impact of knockdown on survival following infection with the bacterium *Pseudomonas entomophila*. This provides an additional perspective on the role of AKH signaling in insect defense, and highlighting its potential importance in pathogen resistance.

Together, this project aims to provide a comprehensive understanding of neuropeptide evolution, diversity, and function in Blattodea, focusing on the roles of AKHs in metabolism, sex-specific differences, and potential interactions with immune defense. The findings broaden our knowledge of blattodean physiology and evolution. Moreover, this research has potential applications in developing targeted pest management strategies by exploiting neuropeptide signaling pathways; offering innovative approaches to controlling pest species such as cockroaches and termites.

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Chapter I

Genomic exploration of neuropeptides in Blattodea: divergent profiles in termites and cockroaches

Genomic exploration of neuropeptides in Blattodea: divergent profiles in termites and cockroaches

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JS and DPM conceived the overall idea. AC, LC, and BT collected samples and assembled the genomes. CA, JS and LC devised the methodology and analyzed the data. JS and DPM wrote the manuscript. All authors contributed critically to the drafts.

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2.1 Abstract

Neuropeptides play a vital role in regulating various physiological and behavioral processes in insects, acting as essential signaling molecules that influence numerous functions. This study provides the most extensive analysis to date of neuropeptide precursors across 49 species within the order Blattodea, covering a broad taxonomic range of termites and four cockroach species. By leveraging newly sequenced genomes, we explored the evolutionary dynamics of neuropeptide genes and uncovered significant patterns of gene loss, duplication, and conservation across different lineages. Notably, we found the loss of AKH/corazonin-related peptide gene in several termite families, such as Termitidae and Heterotermitidae, along with the loss of genes like Gonadulin and Tryptopyrokinin in specific families. In contrast, we observed gene duplications, such as Adipokinetic hormone genes in solitary cockroaches and relatively conserved CCHamide duplications across all species. Additionally, cockroaches exhibited more Birpin (Insulin-like peptide) genes compared to their termite relatives. Our phylogenetic analysis, incorporating 32 neuropeptide precursors, closely aligns with established evolutionary relationships within Blattodea, underscoring the robustness of these markers in evolutionary studies. Our findings of both conserved and novel neuropeptide gene diversification and loss not only deepen our understanding of neuropeptide evolution within Blattodea genomes, which may be linked to changes in social structure and/or ecology, but also lay a strong foundation for future research, including the development of targeted pest control strategies using peptide mimic “green pesticides”.

Keywords

Termite genomics; neuropeptides; Blattodea; evolutionary biology; pest control

2.2 Introduction

In insects, neuropeptides constitute a complex signaling system that is crucial for the regulation of numerous physiological and behavioral processes (Altstein and Nässel 2010, Mykles et al. 2010, Schoofs, De Loof and Van Hiel 2017). These small molecules interact by binding to specific receptors and initiating pathways that regulate cellular activities (Gäde 1997, Strand 1999, Nässel 2000, Salio et al. 2006, Wegener and Gorbashov 2008, Nässel and Winther 2010, Nässel and Zandawala 2019).

The order Blattodea, comprising termites and cockroaches, includes approximately 7,600 recognized species, reflecting both its remarkable diversity and ecological significance (Inward, Beccaloni and Eggleton 2007). Molecular phylogenetic studies have reclassified termites as eusocial cockroaches, placing them within Blattodea, and revealing their close evolutionary relationship with wood-feeding cockroaches of the genus *Cryptocercus* (Inward, Vogler and Eggleton 2007, Legendre et al. 2015, Evangelista et al. 2019). This reclassification has significant implications for understanding evolution of Blattodea, and the emergence of eusociality in termites, with neuropeptide signaling likely playing a key role in these divergent evolutionary trajectories. Termites are eusocial insects characterized by a complex social structure, where castes such as workers, soldiers, and reproductives perform specialized tasks critical for the colony's survival and reproduction (Husseneder, Vargo and Grace 2003, Nalepa 2015, Chouvenc et al. 2021). On the other hand, cockroaches, though lacking advanced sociality, have successfully adapted to various ecological niches, demonstrating significant versatility and resilience in various environments. Species like *P. americana* and *B. germanica*, have become particularly notorious for their close association with human habitats, where they pose public health risks as potential vectors of disease and allergens (Gore and Schal 2007, Li et al. 2018, Wang, Lee and Rust 2021, Tang et al. 2024).

Technological advances in genomics, transcriptomics, and proteomics, particularly through mass spectrometry techniques like MALDI-TOF and ESI-Q-TOF, have significantly enhanced our ability to study insect neuropeptides. These techniques allow for the precise identification of neuropeptides from small or otherwise problematic samples, which is especially beneficial for studying otherwise inaccessible species. Consequently, they are essential for comprehensive neuropeptidome analyses across diverse insect taxa (Li et al. 2008, Weaver and Audsley 2010, Dircksen et al. 2011, Predel et al. 2012, Rahman, Neupert and Predel 2013, Liessem et al. 2018, Predel et al. 2018, Ly et al. 2019, Bläser and Predel 2020, Ragionieri and Predel 2020, Habenstein et al. 2021, Shi et al. 2021, Thiel et al. 2021, Zeng et al. 2021, Ragionieri et al. 2022, Ragionieri et al. 2023). In Blattodea, however, despite their remarkable diversity and ecological significance, the neuropeptidomic profiles of these insects have been fully investigated in only a handful of species (Veenstra 2014, Christie 2015, Zeng et al. 2021).

Despite the critical roles of neuropeptides in insect physiology, our knowledge of these molecules within Blattodea is still limited, particularly concerning how neuropeptide diversity contributes to the wide range of social behaviors and ecological adaptations observed across species. To address this knowledge gap in the neuropeptidomics of Blattodea, we conducted a comprehensive genomic analysis by characterizing the diversity and evolutionary patterns of neuropeptides from a panel of 47 newly sequenced genomes from Blattodea, encompassing a broad taxonomic diversity but with a focus on termites. These species include 11 recognized termite families and 12 subfamilies within the Termitidae, spanning both wood- and soil-feeding groups, and the two cockroach species, *Blatta orientalis* and *Cryptocercus meridianus*. Two further published cockroach genomes *P. americana* (Zeng et al. 2021) and *B. germanica* (see **Chapter III**), were included, bringing the total number of species analyzed to 49.

The present study aims to conduct an extensive investigation of the neuropeptide families across a broad range of termites and cockroaches, with an emphasis on their sequence evolution. By examining the evolutionary patterns of neuropeptide genes, including gene loss and duplication, we aim to better understand the evolutionary dynamics of neuropeptide genes and their implications for phylogenetic relationships. Through the analysis of genomic data from these species, we expect to reveal molecular features that correlate with ecological success, as well as potential associations with advanced social living in termites.

2.3 Material and methods

Genome datasets mining

We analyzed 47 genomes to obtain a comprehensive understanding of neuropeptides in Blattodea. At date of submission, the genomes were as yet unpublished. In this chapter, we provide a brief overview of the methods used to generate these genomic resources. This includes information on sample origin, transcriptome sequencing, genome assembly, and annotation.

Samples and DNA sequencing

Forty-five termite species and two cockroach species (*B. orientalis* and *C. meridianus*) were selected for this study, representing 11 of the 13 recognized families and 12 of the 18 subfamilies of Termitidae. Samples were collected from various geographical locations worldwide. For most species, the heads of one to twelve worker specimens from the same colony were pooled to obtain sufficient DNA. Short-read sequences were generated for genome polishing using the Illumina HiSeq X platform, while long-read libraries were prepared and sequenced on the Oxford Nanopore PromethION platform.

Transcriptome sequencing, genome assembly, and annotation

For each of the genomes sequenced, transcriptomes from various life stages, castes, and body parts were generated. Total RNA was extracted using the Invitrogen™ PureLink™ RNA Mini Kit and the RNeasy® Plus Mini Kit (Qiagen, Germany). Messenger RNA (mRNA) enrichment was performed using the NEBNext® Poly(A) mRNA Magnetic Isolation Module, and cDNA libraries were prepared using the NEBNext® Ultra™ II RNA Library Preparation Kit for Illumina®. Paired-end 150 bp sequencing was performed on the Illumina HiSeq X or NovaSeq 6000 platforms. Long reads were assembled into contigs using Flye, and short reads were used for polishing the assemblies. Contaminations were checked, and genomes were scaffolded using Omni-C and Hi-C data with the YAHs tool. Genome completeness was assessed using BUSCO v5.0. Repeat elements were detected and masked using RepeatModeler and RepeatMasker. Protein-coding genes were identified using a combination of protein-to-genome alignments, transcript-to-genome alignments, and *ab initio* gene predictions. Gene functions were inferred using eggNOG-mapper, InterProScan, and KOfamScan, with the best hits from the nr database used for annotation.

Neuropeptide precursors searching

We first compiled sequences of known insect neuropeptide precursors as reference queries, using the recently published datasets from Blattodea: two termite species: *Mastotermes darwiniensis* and *Zootermopsis nevadensis* (Veenstra 2014, Christie 2015) and two cockroach species: *P. americana* (Zeng et al. 2021) and *B. germanica* as described in **Chapter III** (*de novo* assembly). These sets of neuropeptide precursors were used to build HMM (Hidden Markov Model) profiles for further analyses.

Predicted neuropeptide precursors were primarily derived from annotated genomes, where representative transcripts were used for identification. However, this approach was not always feasible, as reported by Veenstra (2019) when investigating neuropeptides in Coleoptera utilizing genome and transcriptome datasets. Several reasons contribute to this, including insufficient sequencing depth of some transcriptome datasets, which often fails to detect neuropeptide genes expressed at a low level, leading to incomplete or inaccurate annotations. Additionally, many transcripts exhibit multiple isoforms, some of which may be incorrect.

To address these challenges, sequence prediction was also conducted using assembled transcripts from transcriptomes and by searching generated 6-frame translations of the assembled genomes, which captured all possible transcripts from the genomes (minimum peptide length: 10 amino acids) as described by Aumont et al. (unpublished data). Incomplete or ambiguous neuropeptide precursors were then manually corrected based on homology with known proteins.

Sequence alignment and phylogenetic analysis

To ensure a more comprehensive understanding of neuropeptide sequences in Blattodea, previously reported and analyzed neuropeptide precursor sequences from two cockroach species, *B. germanica* and *P. americana*, were included. These sequences, along with data from 47 additional species from genomic datasets, were used for sequence alignment and phylogenetic analysis.

The signal peptide cleavage site for each candidate precursor sequence was determined either by comparing homologous sequences or by using the SignalP 5.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) for prediction (Almagro Armenteros et al. 2019). The multiple sequence alignments for amino acid sequences were performed using the MAFFT with the E-INS-I algorithm (Katoh and Standley 2013).

We performed two separate phylogenetic analyses. The first analysis focused on various types of Insulin-like peptides (ILPs) using amino acid (Aa) alignments, while the second analysis targeted a dataset of 32 neuropeptide precursors. For both analyses, aligned sequences were trimmed using trimAl v1.2 (Capella-Gutiérrez, Silla-Martínez and Gabaldón 2009). In the case of the neuropeptide precursor datasets, the "-gappyout" option was employed, whereas for ILPs, the trimming was done with the options “-gt 0.9 -cons 60”. We then reconstructed phylogenetic trees using a maximum likelihood (ML) approach in RAxML v8.2.12 (Stamatakis 2014) performing 1000 rapid bootstrap (BP) replicates with the PROTGAMMAAUTO model for both analyses.

Sequence alignments were visualized using Jalview (Waterhouse et al. 2009), while sequence logos were generated with TBtools software (Chen et al. 2023). Phylogenetic trees were visualized and annotated using the web application tvBOT (Xie et al. 2023).

2.4. Results

2.4.1 Taxon description and species classification

After adding two cockroach species, we revised the phylogenetic tree, now comprising 45 termites and 4 cockroach species. Based on their relationships with other species, we grouped *P. americana* within the same clade as *Blatta orientalis* (family Blattidae), while positioning *B. germanica* (family Ectobiidae)

as an independent outgroup due to its more distant relationship with the other species. Consequently, the tree now includes 49 species from 14 distinct families (Figure 1, Supplementary Table 1).

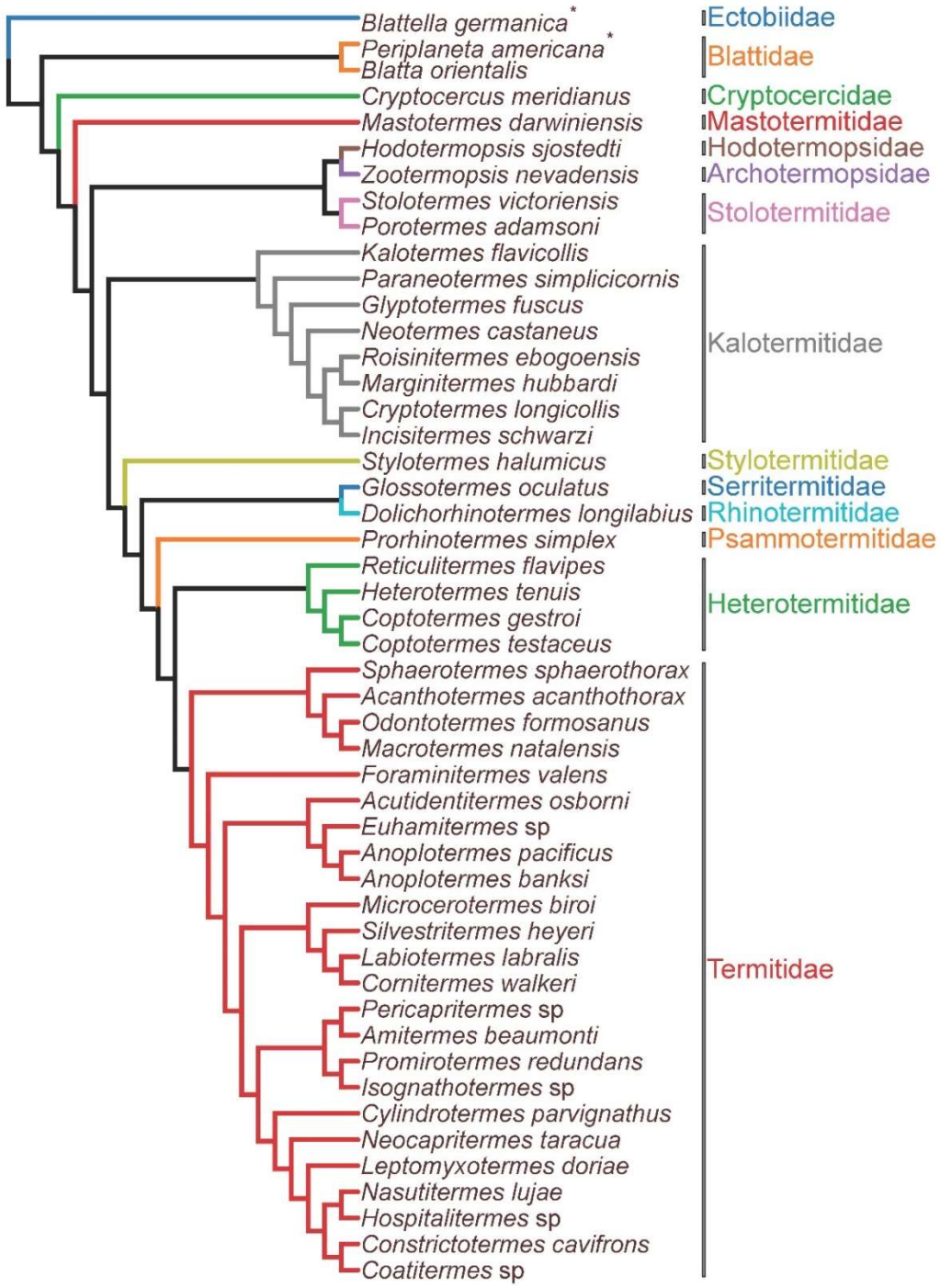

Figure 1. The phylogenetic tree represents the 49 termite and cockroach species investigated in this study, based on the phylogenetic relationships from Evangelista et al. (2019) and Hellemans et al. (2024). Two species, *B. germanica* and *P. americana*, denoted with an asterisk (*), were included based on their phylogenetic relationships with the other species. Different colors of branches are marked to differentiate distinct families.

We retrieved 69 genes encoding neuropeptides and neuropeptide-like sequences as queries from homologous species, as described in **Chapter II** (Table 1). The genes were employed to identify neuropeptide precursor sequences in the targeted genomes of termites and cockroaches. The species included in this investigation are documented in Supplementary Table 2, which includes a detailed

overview of the neuropeptide precursors that were identified, as well as those that were not found (but were expected), in the 49 distinct termite and cockroach genomes.

In brief, almost all gene families could be detected in these species, including: AKH, AllatostatinA (AstA), AllatostatinCC, AstCCC, Allatotropin (AT), Bursicon, Crustacean cardioactive peptide (CCAP), Calcitonin-like diuretic hormone (CRF-DH), Eclosion hormone (EH), Ecdysis triggering hormone (ETH), Fliktin (Flik), Insulin-like peptide (ILP), Ion transport peptide (ITP), Kinin (K), Myosuppressin (Myo), Neuroparsin (NP), Neuropeptide-like precursor (NPLP), NVP-containing peptide (NVP), Orcokinin (OK), Pigment dispersing factor (PDF), Pyrokinin (PK), Prothoracicotropic hormone (PTTH), Periviscerokinin (CAPA), Sulfakinin (Sul), Short neuropeptide F (sNPF). However, the number of genes presented in the table includes only those for which we have identified either complete or partial sequences (datasets of these gene families are available on: https://github.com/RoachRanger). The occurrence of incomplete sequences may be influenced by genome sequencing or annotation incompleteness. Even after extracting data from the genome and manually curating them using transcriptome datasets, a proportion of aligned gene sequences could only be partially retrieved.

2.4.2 Absence of neuropeptide genes

We found gene losses to be common, as has also been seen in other insect groups, such as the absence of neuroparsin in the *D. melanogaster* subgroup and the loss of Leucokinin in Coleoptera (Veenstra 2010, 2019). Our analysis revealed some interesting patterns of gene loss across various groups within the Blattodea. We found that the AKH/corazonin-related peptide (ACP) gene has been lost in all species within the families of Termitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, Psammotermitidae, as well as in some species from the Heterotermitidae family, such as *Heterotermes tenuis* and *Reticulitermes flavipes* (Figure 2a).

Table 1. Query neuropeptide and neuropeptide-like genes.

No.	Neuropeptide	Abbreviation	No.	Neuropeptide	Abbreviation
1	Adipokinetic hormone 1	AKH1	36	Insulin-like peptide 4	ILP4
2	Adipokinetic hormone 2	AKH2	37	Insulin-like peptide 5	ILP5
3	Agatoxin-like peptide	ALP	38	Insulin-like peptide 6	ILP6
4	AKH/corazonin-related peptide	ACP	39	Invertebrate parathyroid hormone	IPTH
5	Allatostatin A	AstA	40	ITP transcript A	ITPa
6	Allatostatin CC	AstCC	41	ITP transcript B	ITPb
7	Allatostatin CCC	AstCCC	42	Kinin	K
8	Allatotropin	AT	43	Leucomyosuppressin	LMS
9	Bursicon alpha	Burα	44	Myoinhibitory peptide	MIP, AstB
10	Bursicon beita	Burβ	45	Natalisin	Nat
11	Calcitonin A	CTA	46	Neuroparsin	NP
12	Calcitonin B	CTB	47	Neuropeptide F1 transcript A	NPF1a
13	Calcitonin-like diuretic hormone	CTDH	48	Neuropeptide F1 transcript B	NPF1b
14	Carausius neuropeptide-like precursor	CNP	49	Neuropeptide F2	NPF2
15	CCHamide 1	CCH1	50	Neuropeptide-like peptide	NPLP
16	CCHamide2	CCH2	51	NVP-like	NVP
17	CCRFamide	CCRF	52	Orcokinin 1	OK1
18	CNMamide 1	CNM1	53	Orcokinin 2	OK2
19	CNMamide 2	CNM2	54	Periviscerokinin	CAPA
20	Corazonin	Crz	55	Pigment dispersing factor	PDF
21	CRF-like diuretic hormone	CRFDH	56	Proctolin	PT
22	Crustacean cardioactive peptide	CCAP	57	Prothoracicotropic hormone	PTTH
23	Ecdysis triggering hormone	ETH	58	Pyrokinin	PK
24	Eclosion hormone 1	EH1	59	Relaxin-like peptide	RLP
25	Eclosion hormone 2	EH2	60	RFLamide	RFL
26	Elevenin	Evn	61	RYamide	RY
27	Fliktin	Flik	62	Short Neuropeptide F	sNPF
28	FMRFamide	FMRF	63	SIFamide	SIF
29	Glycoprotein hormone alpha2	GPA2	64	SMYamide	SMY
30	Glycoprotein hormone beta5	GPB5	65	Sulfakinin	Sul
31	Gonadulin	Gon	66	Tachykinin	TK
32	HanSolin	Han	67	Trissin	Tri
33	Insulin-like peptide 1	ILP1	68	Tryptopyrokinin	TPK
34	Insulin-like peptide 2	ILP2	69	vasopressin	VP
35	Insulin-like peptide 3	ILP3			

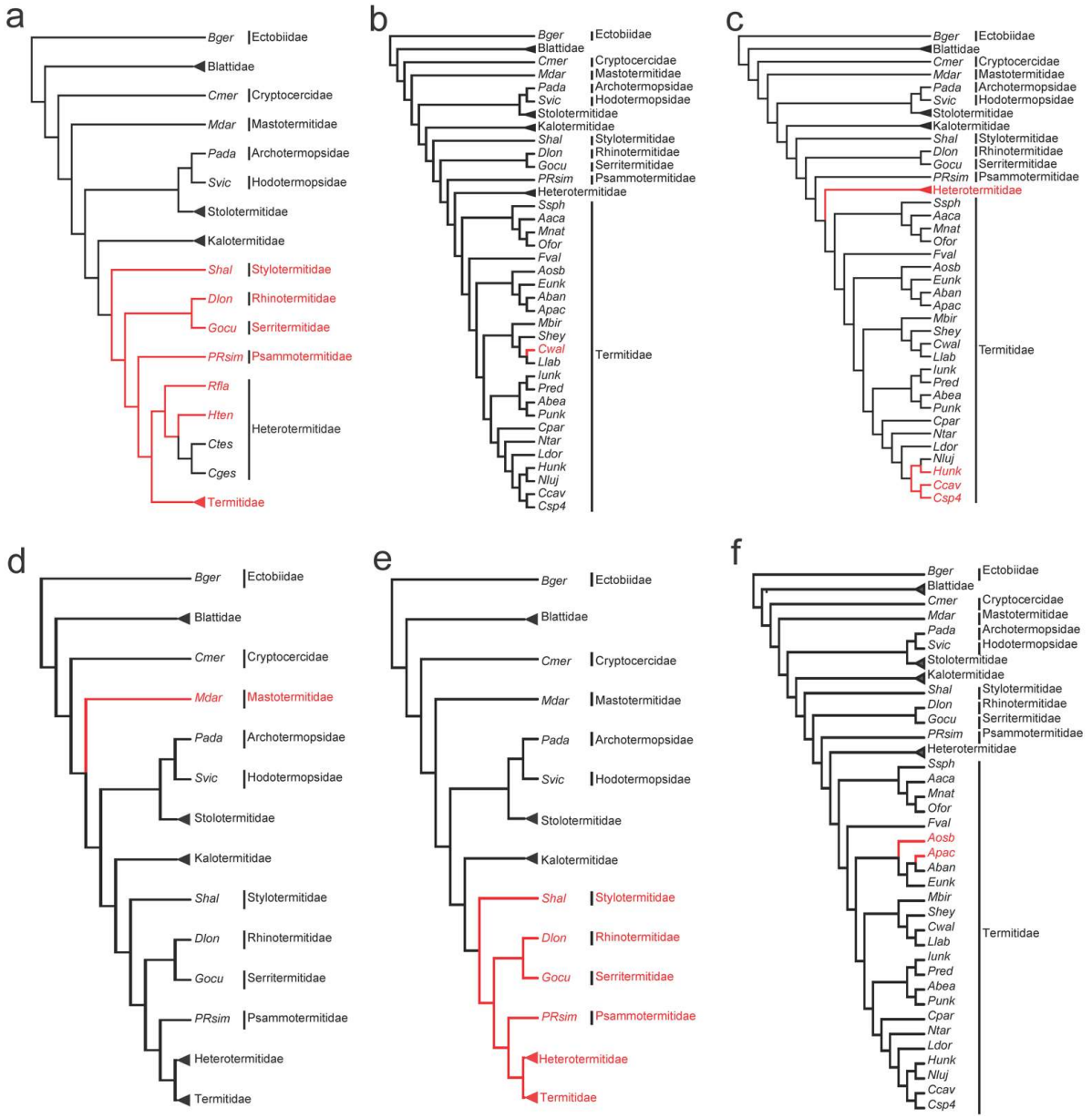

Figure 2. Absent genes were observed in various species of termites and cockroaches. The species names and families highlighted in red indicate the absence of the respective genes. Each panel represents the loss of a specific gene: (a) ACP, (b) Crz, (c) Gon, (d) RFL, (e) TPK, and (f) Tri.

Moreover, evidence from the precursor sequence alignment indicates that in the two species where ACP has been detected in Heterotermitidae, it appears to be undergoing pseudogenization, as it appears to have undergone excessive amino acid substitution as well as truncation, not only in the mature peptide region but also in the N- and C-terminal terminals, consistent with a previous findings in Blattodea by Veenstra (2023). Given the significantly diverged sequence of the potential bioactive ACP peptide (compared to those of the other 17 species where ACP was detected), we conducted further BLASTp searches using the partial sequences from two *Coptotermes* species against the Nr database to look for possibly related sequences in other insects, to examine the possibility that this could represent a novel neuropeptide, rather than a modified ACP. However, we were only able to identify similarity to sequences belonging to ACP genes from blattodean species such as *Cryptotermes*

secundus, *Z. nevadensis*, and *B. germanica*. This pattern indicates a pattern of evolutionary redundancy or functional loss, as indicated by its absence in all other Neoisoptera.

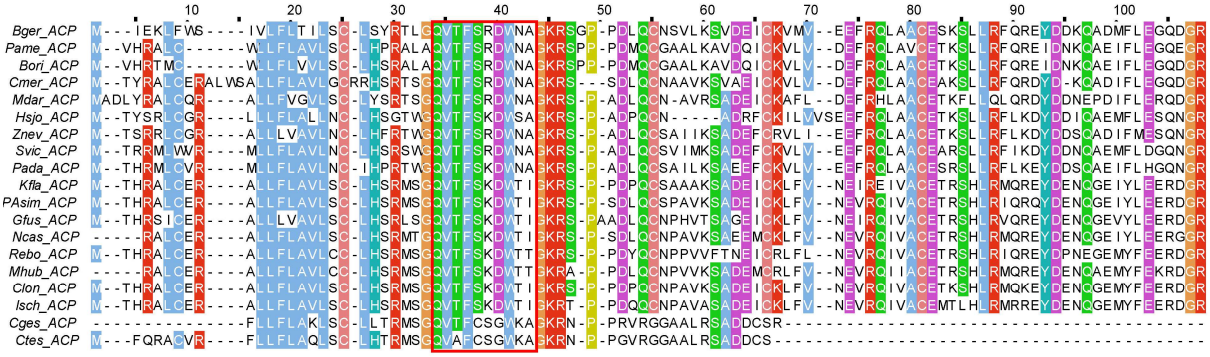

Figure 3. Comparative analysis of ACP precursor sequences shows that genes are lost in certain termite species, with 19 ACP sequences showing while 30 are missing. The alignment reveals conserved regions within the sequences, with the red square indicating bioactive ACP. For the two *Coptotermes* species, pseudogenization is supported by the excessive number of amino acid substitutions in the bioactive region as well as gene truncation.

In addition, our research indicates that the Gonadulin (Gon) gene, which is a recently identified insulin-like peptide, is absent in species belonging to the Heterotermitidae family. It is also missing in specific members of the Termitidae family, such as *Coatitermes* sp., *Constrictotermes cavifrons*, and *Nasutitermes lujae*, which are members of the Nasutitermitinae subfamily (Figure 2c). The sequence alignment of Gon precursors exhibits lower conservation among the families Rhinotermitidae, Serritermitidae, Psammotermitidae, Heterotermitidae, and Termitidae, especially the poorly conserved N and C terminals (Supplementary Figure 1).

Furthermore, we could not identify the Corazonin (Crz), RFLamide (RFL), Tryptopyrokinin (TPK) and Trissin Tri genes in certain species. Specifically, Crz is only absent in the *Cornitermes walkeri* within the Termitidae family (Figure 2b); RFL is only missing in the Mastotermitidae family (Figure 2d); TPK is missing across several families, including Stylotermitidae, Rhinotermitidae, Serritermitidae, Psammotermitidae, Heterotermitidae, and Termitidae (Figure 2e); Tri gene is absent in multiple species in the subfamily Apicotermitinae. We can be confident in our patterns of gene loss when the absence of candidate genes is shared across several related species. However, the lack of genes in the transcriptomes or genomes of single species does not conclusively indicate their loss (Veenstra 2019), due to potential incompleteness in sequencing or annotation.

2.4.3 Duplication of neuropeptide genes

All termite species and wood-roach *C. meridianus* (Cryptocercidae) have one AKH gene. However, cockroaches in the Ectobiidae and Blattidae families have 2 AKH genes. Furthermore, Ectobiidae have bioactive AKH sequences of 10 amino acid in length whereas all other blattodean species mature AKH peptides of 8 amino acids in length (Figure 4).

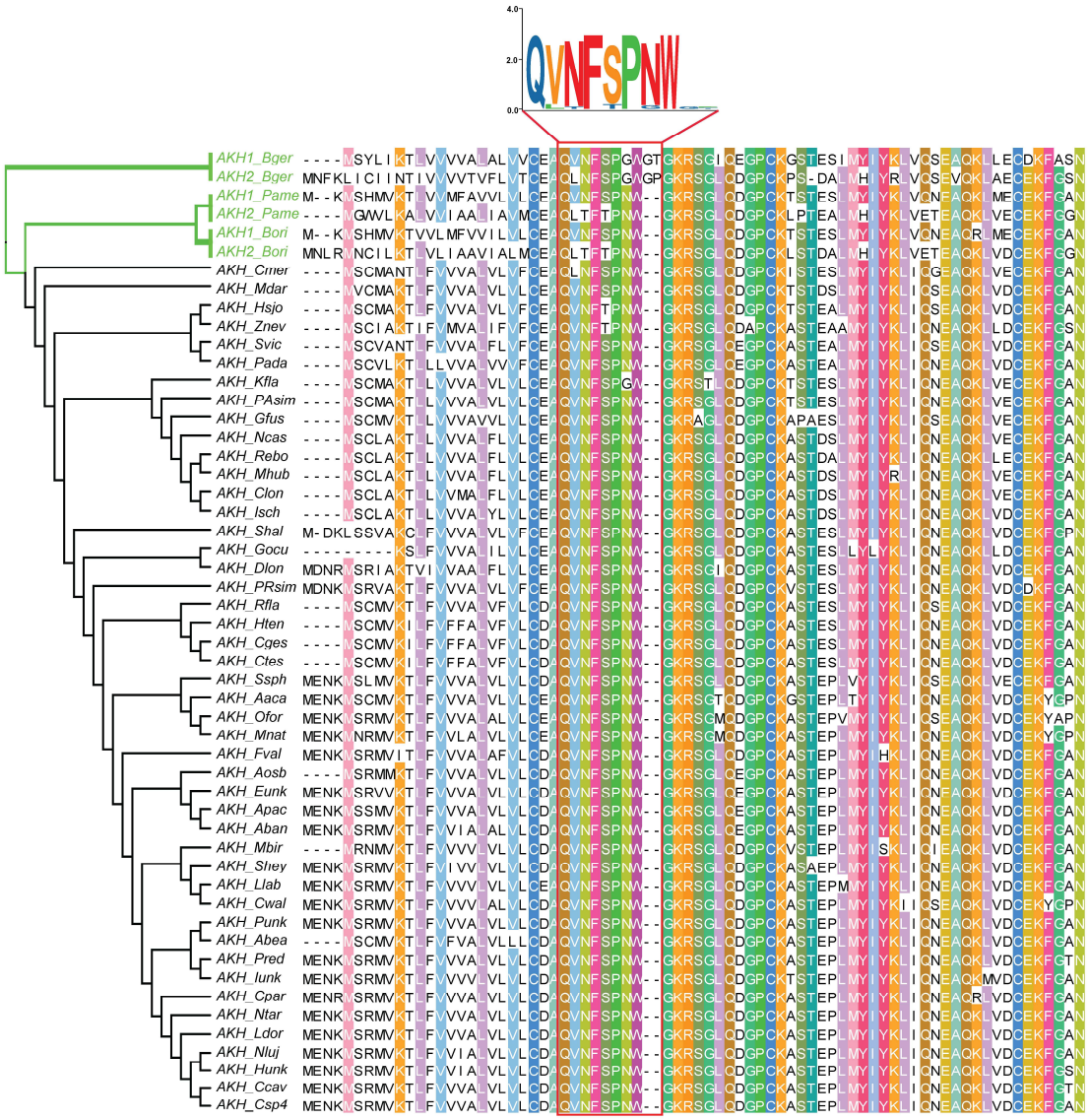

Figure 4. Comparative analysis of AKH precursor sequences reveals gene duplications in three cockroach species: *B. germanica*, *P. american*, and *B. orientalis*. The species names and branches highlighted in green indicate the presence of duplicate AKH genes. The alignment reveals conserved regions within the sequences, whereas the sequence logo shown above highlights the conserved bioactive AKH peptide. The height of the letters in the logo reflects to the relative occurrence of each amino acid at that specific position in the alignment.

Unlike the AKH gene duplication events, which are confined to certain cockroach species, several other genes are duplicated across all cockroach and termite species. For instance, all species possess two CCHamide genes (CCH1 and CCH2), which encode CCHamide peptides of different lengths (Figure 5). Additionally, there are two Bursicon genes (Bursicon alpha and Bursicon beta), two Calcitonin genes (Calcitonin A and Calcitonin B), and two Eclosion hormone genes (EH1 and EH2) in these species.

Interestingly, all 49 species were found to have two CNMamide (CNM) genes, except that CNM1 is absent in the Kalotermitidae family.

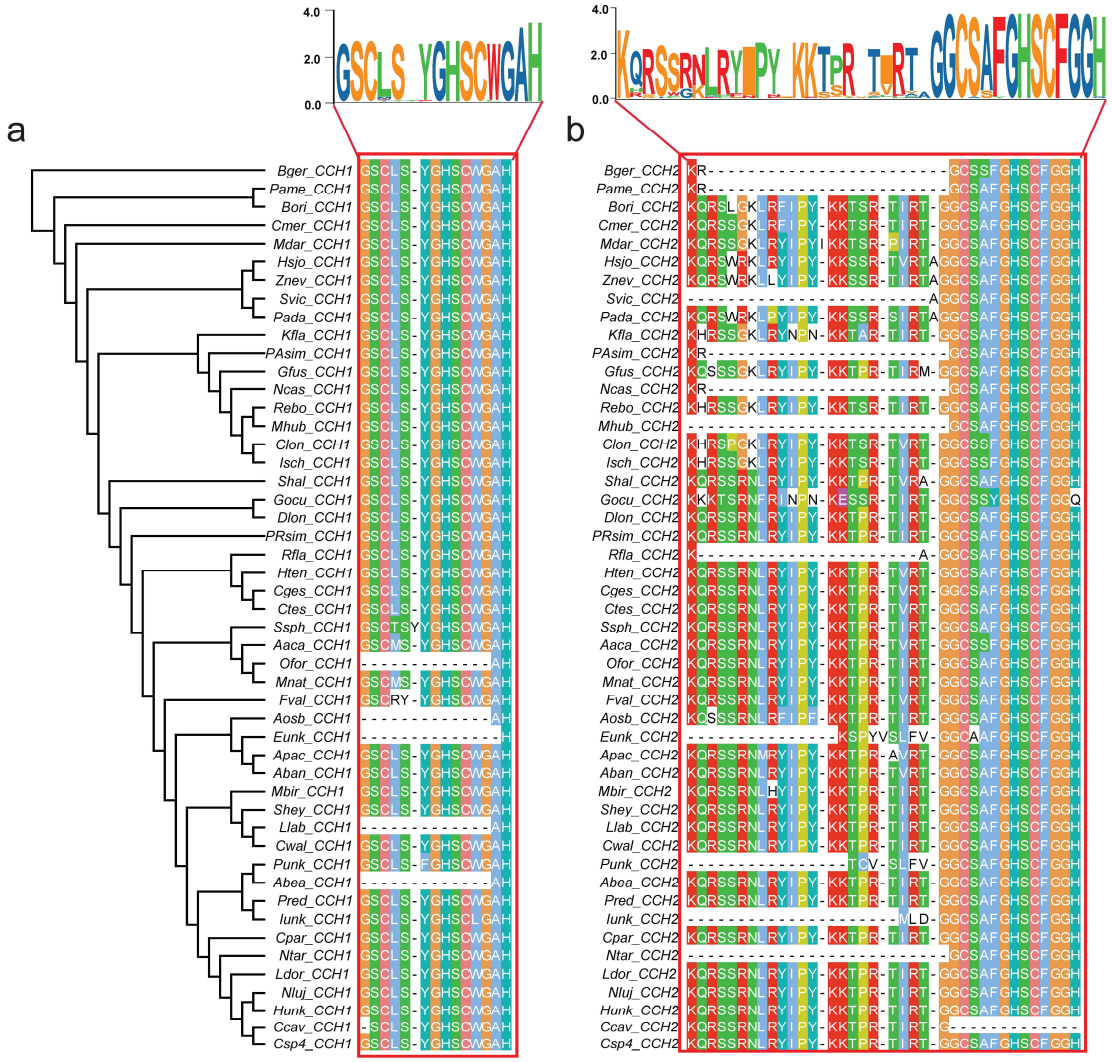

Figure 5. Comparative bioactive peptide sequences for two CCH genes, CCH1 (a) and CCH2 (b), among all 49 species. The sequence logos displayed above each alignment depict conserved motifs found within the bioactive peptide regions of CCH1 and CCH2. The height of each letter in the logos corresponds to the relative frequency of the amino acid at that specific position, the dash in the alignment indicates incomplete sequences at those positions.

Termites possess a total of seven distinct insulin-like peptides (ILPs), which consist of three original ILPs (Gonadulin, IGF, and the Dilp7 ortholog) and four short IGF-related peptides (sIrps), that have individually been called atirpin, birpin, cirpin and brovirpin. Nevertheless, Gonadulin is thought to be absent in higher termites (Veenstra 2023). First, we aligned all ILP sequences from 49 species with those reported by Veenstra (2023), such as *Macrotermes natalensis* and *Labritermes buttelreepeni*, being assigned the corresponding gene names, and then used as sequences for phylogenetic gene tree analysis. Our gene trees using these retrieved sequences from genomic datasets clearly identify six clades of ILPs (excluding Gonadulin due to its absence in higher termites) (refer to Figure. S1). In addition, we found that basal cockroaches, such as *P. americana* and *B. germanica*, have two or three Birpin genes in comparison to wood-roach and termite species, which have one Birpin gene. As an

illustration, *B. germanica* possesses 3 Birpin clades in addition to other ILP2 variants, which confirms the recent discoveries reported by Veenstra (2023).

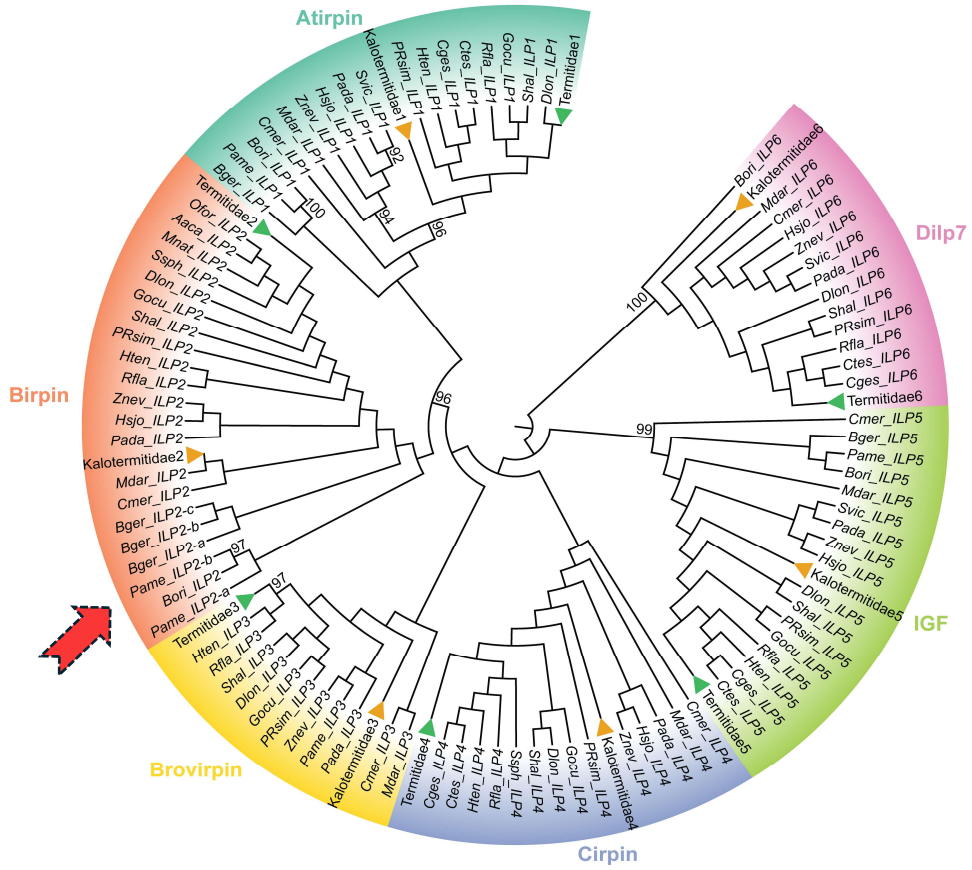

Figure 6. Phylogenetic analysis of insulin-like peptide precursors in termites and cockroaches, revealing six distinct clades, labeled Atirpin, Birpin, Brovirpin, Cirpin, IGF, and Dilp7. Each clade has been assigned a different color. The branches representing the Termitidae and Kalotermitidae families have been collapsed for clarity. The tree only displays bootstrap values that exceed 90, indicating robust support for a subset of internal branches separating clades. The red dashed arrow indicates the duplication of Birpin in *B. germanica* and *P. americana*.

In *P. americana*, nine genes encoding ILPs have been identified, with seven of them exhibiting significant expression in the central nervous system (CNS) (Veenstra, 2023). Additionally, basal cockroach species tend to possess a greater number of short insulin-like related peptides (sirps) (Veenstra, 2023). The variation in distribution and the presence of different paralogs across species may be linked to specific evolutionary pressures or functional requirements. Our analysis confirms the conservation of all ILP clades across the major termite lineages, except the loss of Gonadulin in higher termites. The duplication of Birpin in cockroaches is not conserved in termites.

2.4.4 Neuropeptide sequence conservation

Neuropeptides are usually produced from larger precursor molecules that undergo posttranslational processing and occasionally modifications to produce mature peptides (Mains et al. 1983, Schmutzler et al. 1992). A single neuropeptide precursor molecule can generate either a single neuropeptide, multiple distinct neuropeptides, multiple copies of a single neuropeptide, or any combination of these

variations (Strand 1999, Salio et al. 2006, Bläser, Misof and Predel 2020). Studies have shown that some neuropeptide sequences are highly conserved across species, indicating their functional importance in various biological processes (Stemmler et al. 2007, Wegener and Gorbashov 2008, Bläser, Misof and Predel 2020). This conservation extends to both peptide sequences and their precursors, as well as their receptors, suggesting long-term coevolution of ligand-receptor pairs (Jékely 2013).

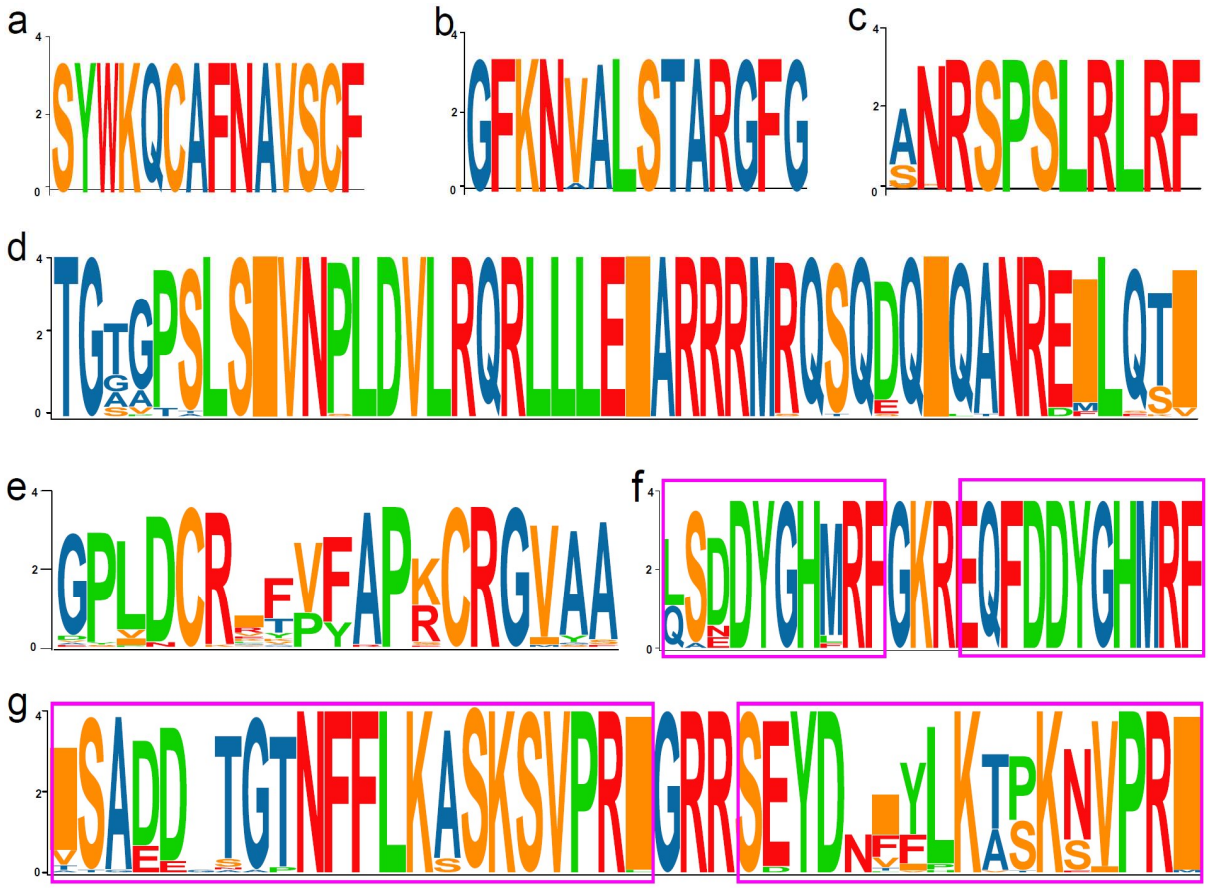

Figure 7. Logo plots for neuropeptide sequence conservation across 49 Blattodea species, illustrated for the following peptides: (a) AstCCC, (b) AT, (c) sNPF, (d) CRFLDT, and (e) ETH, which are single copy peptides. (f) Sul and (g) Evn, are multiple copy neuropeptides, with each bioactive region being marked by a colored square. The height of each letter represents the relative frequency of that amino acid across the alignment.

Neuropeptides in cockroaches and termites are largely conserved, especially those that exist as single-copy peptides. Some examples of these include Allatostatin CCC (Ast CCC), Allatotropin (AT), Short Neuropeptide F (sNPF), CRF-like diuretic hormone (CRFLDT), and Ecdysis-triggering hormone (ETH) (Figure 7a-e). On the other hand, while certain multi-copy neuropeptides like Sulfakinin (Sul) and Elevenin (Evn) are conserved in some specific positions (Figure 7f, 7g), it is often the single-copy peptides that exhibit a higher amount of conservation (Wegener and Gorbashov 2008). Multiple gene copies can result in functional redundancy, wherein one copy can compensate for mutations in another. This duplication enables a wider range of genetic diversity and adaptability, resulting in less strict conservation measures than peptides existing as only one copy.

2.4.5 Phylogenetic reconstruction using neuropeptide precursors

Neuropeptide sequences, knowing that they have evolved alongside their receptors, are highly conserved and therefore ideal for establishing evolutionary relationships within insect orders (Veenstra 2019). Recent investigations on Mantophasmatodea, Blattodea, and Coleoptera have shown that these precursor sequences are effective molecular markers for phylogenetic analysis (Roth et al. 2009, Predel et al. 2012, Bläser, Misof and Predel 2020, Ragionieri et al. 2023).

Given an extensive range of species and the comprehensive sequences retrieved from the genomic dataset, we were able to reconstruct the phylogenetic relationships from these species. This reconstruction involved the selection of 32 neuropeptide precursor sequences, comprising single-copy and multiple-copy neuropeptide precursors (Table 2).

Table 2. Neuropeptide precursors used in the phylogeny reconstruction

No.	Gene	Length (aa)	No.	Gene	Length (aa)
1	Agatoxin-like peptide	93-105	17	Glycoprotein hormone beta5	149-156
2	Allatostatin A	320-373	18	HanSolin	105-118
3	Allatostatin CCC	54-92	19	ITP transcript A	111-111
4	Allatotropin	77-122	20	ITP transcript B	115-119
5	Bursicon alpha	148-149	21	Neuroparsin	86-105
6	Bursicon beita	138-141	22	Neuropeptide F2	69-122
7	Calcitonin A	111-113	23	NVP-like	355-398
8	Calcitonin-like diuretic hormone	114-114	24	Periviscerokinin	198-210
9	Carausius neuropeptide-like precursor	487-661	25	Proctolin	83-84
10	CRF-like diuretic hormone	188-194	26	Pyrokinin	156-177
11	Crustacean cardioactive peptide	154-172	27	Relaxin-like peptide	145-147
12	Ecdysis triggering hormone	167-186	28	Short Neuropeptide F	83-99
13	Eclosion hormone 1	62-80	29	SIFamide	61-72
14	Eclosion hormone 2	64-78	30	SMYamide	75-78
15	Elevenin	135-143	31	Sulfakinin	118-124
16	Glycoprotein hormone alpha2	117-117	32	Vasopressin	114-170

These sequences correspond to genes that are found in all 49 species examined, except for those with notable sequence incompleteness. Following sequence alignment and gap trimming, an average sequence length of 5086 amino acids could be utilized for phylogenetic analysis, representing the most

extensive sequence dataset used to date for exploring phylogenetic relationships using neuropeptide precursors.

The phylogenetic tree, derived from the analysis of 32 neuropeptide precursors, offers good evidence for currently accepted evolutionary relationships between various species of cockroaches and termites. The tree is robust, with high bootstrap values supporting the evolutionary positions of the Ectobiidae, Blattidae, and Cryptocercidae families within the cockroach clade (Figure 7). In addition, the lower termites exhibit substantial support for the families Mastotermitidae, Archotermopsidae, Hodotermitidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, Psammotermitidae, and Heterotermitidae, which indicates distinct evolutionary clades.

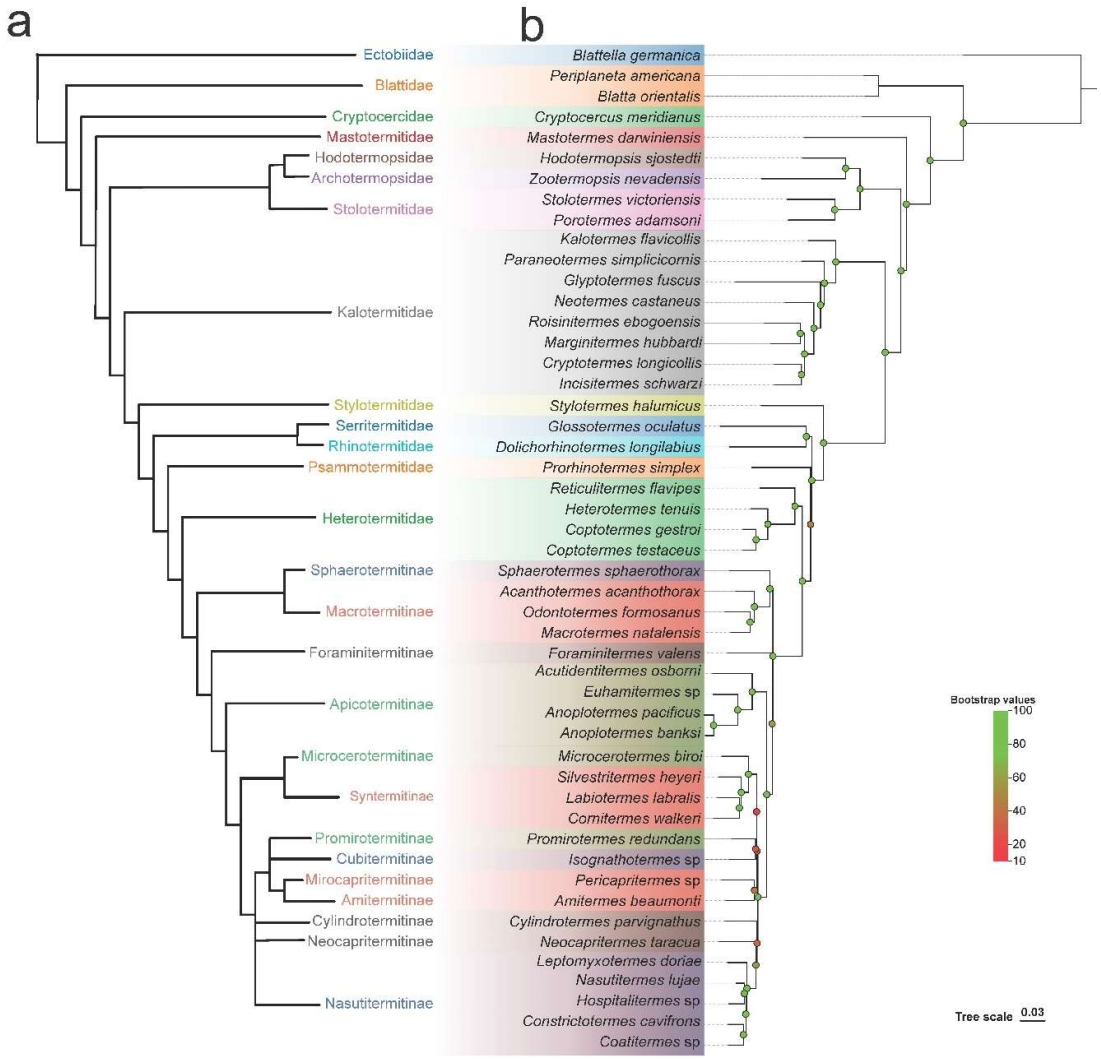

Figure 7. ML phylogenetic trees constructed from 32 neuropeptide precursors from 49 termites and cockroaches largely support accepted relationships among termite and cockroach lineages. (a) Simplified phylogenetic scheme of Blattodea adapted from Evangelista et al. (2019) and Hellemans et al. (2024); (b) ML phylogenetic trees constructed using 32 neuropeptide precursors. Trees were reconstructed from alignments of the whole ORF amino acids sequence (average Length: 5086 Aa).

In addition, the phylogenetic analysis supports associations among numerous subfamilies within the higher termite family Termitidae, namely Sphaerotermitinae, Macrotermitinae, Foraminitermitinae,

Apicotermitinae, Microcerotermitinae, and Syntermitinae (see Supplementary Figure 2 for a closer view of the phylogeny without branch lengths). The utilization of neuropeptide precursor sequences in this study indicates that they are dependable molecular indicators for reconstructing phylogenetic relationships, if a sufficient number of them can be sequenced. These molecular markers are effective at both the family and subfamily levels, offering insights into the evolutionary history of these species.

2.5 Discussion

Neuropeptides represent the largest and most diverse group of signaling molecules in multicellular organisms, playing a central and ancient role in regulating a wide array of physiological processes (Strand 1999, Nässel 2000, Salio et al. 2006, Nässel and Winther 2010, Schoofs, De Loof and Van Hiel 2017). The comparative analysis conducted in this study examines neuropeptides across 49 termites and cockroach genomes provides valuable insights into the evolutionary processes of these signaling molecules within Blattodea. These findings notably reveal distinct neuropeptide genes being lost or duplicated in various families. Additionally, the phylogenetic associations deduced from neuropeptide precursor sequences support those derived from larger-scale phylogenomic analyses, thereby confirming the reliability of these sequences as markers for evolutionary analysis.

A key finding was the loss of neuropeptide genes such as ACP and Gon in some termite families. For example sequence alignments revealed a lack of conservation in ACP genes from two *Coptotermes* species from within a clade of termites (Neoisoptera) which had otherwise apparently lost the ACP gene. This suggests a gradual process of pseudogenization and eventual loss of ACP in Neoisoptera (Veenstra 2019). Consistent with previous reports, the absence of ACP is not unique to termites but is also observed in other taxa, including *Drosophila*, honeybees (*Apis mellifera*), and various beetle species (Hansen et al. 2010, Veenstra 2019). In the migratory locust, *L. migratoria*, Gon has been identified as a significant player in reproductive processes, particularly in the synthesis of vitellogenin, a precursor to egg yolk proteins (Veenstra et al. 2021). However, our analysis of Gon sequences provides more evidence supporting its absence in higher termites, which is consistent with the prior findings reported in a recent study, which suggests that this loss may be linked to changes in reproductive strategies or environmental adaptations in higher termites (Veenstra 2023).

Gene duplication events play a significant role in the evolutionary diversification of neuropeptides in insects, which likely facilitates the emergence of new functions or the refinement of existing ones, allowing species to better adapt to specific or novel exogenous or endogenous challenges (Veenstra 2014, Derst et al. 2016, Veenstra 2020, Shigenobu et al. 2022). In a previous study, we reported an ancient AKH gene duplication event in the common ancestor of Blaberoidea by phylogenetic analyses of AKH precursor sequences, yielding a new group of putative decapeptides (Jiang et al. 2023). In the

cockroaches *B. germanica and P. americana*, the identification of multiple Birpin genes suggests that gene duplication events have contributed to the diversification of other neuropeptide functions in these species. The consequences of neuropeptide duplications are significant, as they can help fine-tune metabolic processes and regulate growth in response to changing environmental conditions (Castro-Arnau et al. 2019, Domínguez, Pagone and Maestro 2022). For example, a recent finding indicates that in termites, ILP expression varies significantly between castes, implying that these neuropeptides play a critical role in regulating the physiological differences observed among workers, soldiers, and reproductive individuals (Veenstra 2023).

Neuropeptide precursors, particularly those encoding single-copy neuropeptides, are frequently conserved because of their essential role in intercellular signaling and their necessity of maintaining functional compatibility with their corresponding receptors. This conservation is particularly noticeable in phylogenetic studies, where neuropeptide precursor sequences have proven to be highly effective molecular markers for reconstructing evolutionary relationships within Blattodea (Bläser, Misof and Predel 2020). In this study, we conducted an extensive analysis of 32 neuropeptide precursors across 49 Blattodea species, providing additional corroborative evidence for the evolutionary relationships among major lineages within the order. Recent research involving darkling beetles (Coleoptera: Tenebrionidae) additionally used neuropeptide precursors for phylogenetic analysis, demonstrating how these markers are broadly applicable and reliable in resolving evolutionary relationships across diverse taxa (Ragionieri et al. 2023). This reinforces the value of neuropeptide precursors in phylogenetic investigations.

This study is the most comprehensive investigation to date of neuropeptide precursors across 49 genomes of termites and cockroaches within the order Blattodea. By utilizing newly sequenced genomes and employing phylogenomic approaches, we revealed evidence of gene loss, duplication, and highlighted the potential consequences for functional diversification or loss, which can be used to guide future experimental research. Despite our efforts to manually curate numerous neuropeptide precursor sequences using transcriptomic data, full-length sequences could not be reconstructed in many cases. Improved transcriptomic sequencing should in future help to obtain complete gene sequences for these species. Furthermore, an exhaustive curation of 69 neuropeptide gene families from 47 species is beyond the scope of the current work. This study significantly enhances our knowledge of neuropeptide diversity in termites and cockroaches, offering a comprehensive framework for further investigation into the diversity and function of neuropeptides in Blattodea. Additionally, it contributes to both scientific knowledge and potentially also practical applications in pest control and biotechnology.

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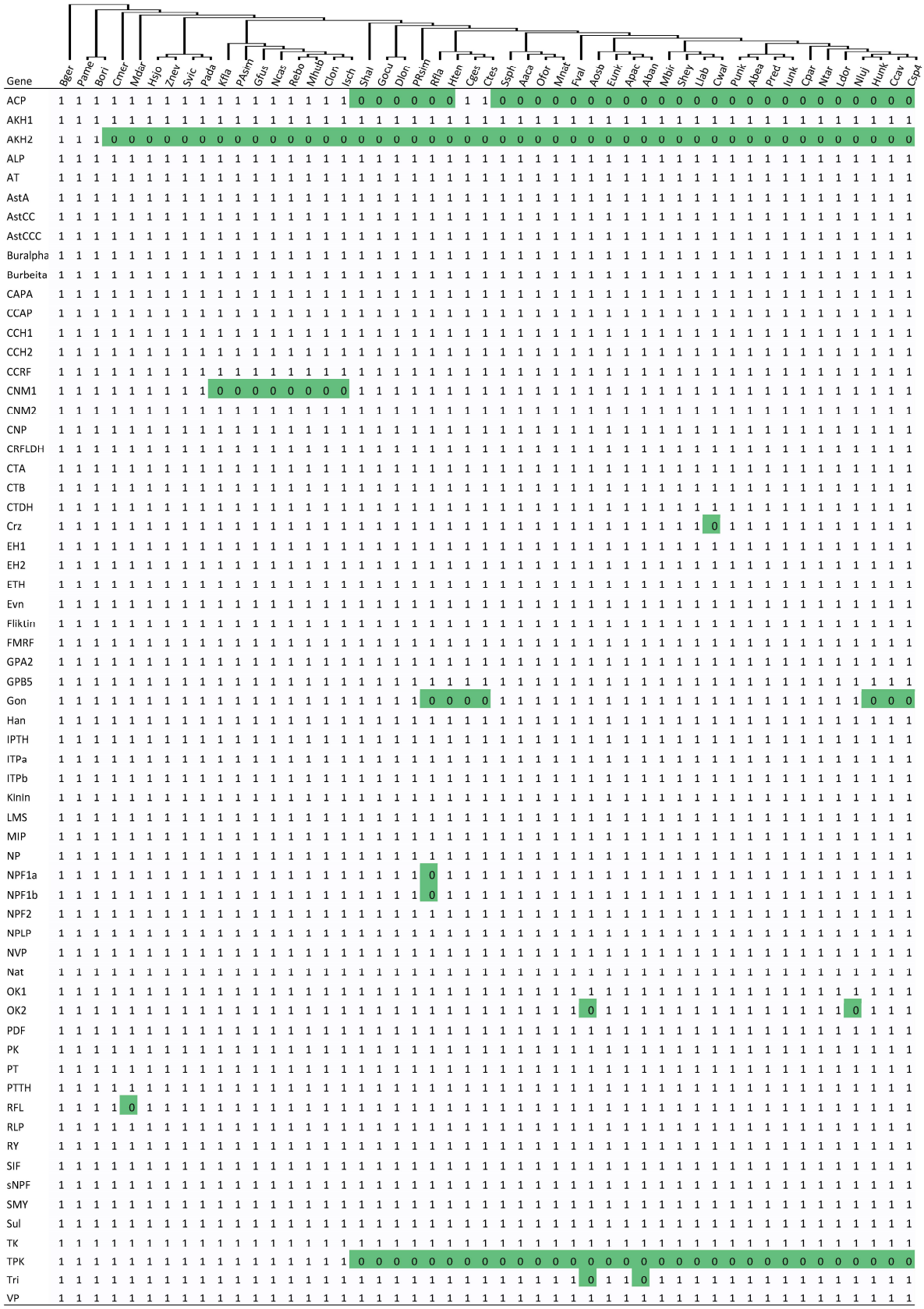
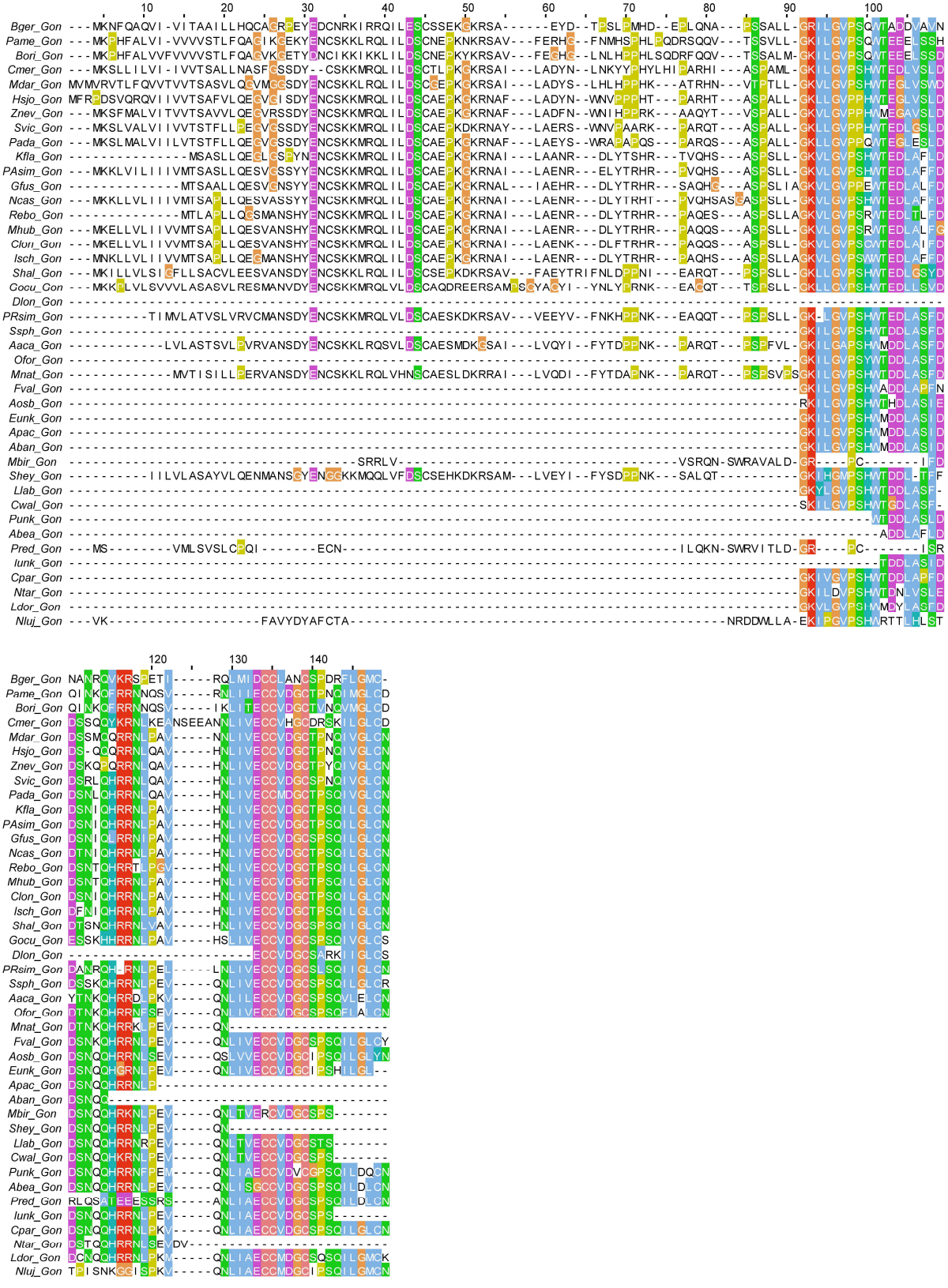
Supplementary Table1. List of studied 49 termite and cockroach species in this study.

No.	Species	Abbreviation	No.	Species	Abbreviation
1	*Acanthotermes acanthothorax*	*Aaca*	26	*Kalotermes flavicollis*	*Kfla*
2	*Acutidentitermes osborni*	*Aosb*	27	*Labiotermes labralis*	*Llab*
3	*Amitermes beaumonti*	*Abea*	28	*Leptomyxotermes doriae*	*Ldor*
4	*Anoplotermes banksi*	*Aban*	29	*Macrotermes natalensis*	*Mnat*
5	*Anoplotermes pacificus*	*Apac*	30	*Marginitermes* sp	*Mhub*
6	*Blatta orientalis*	*Bori*	31	*Mastotermes darwiniensis*	*Mdar*
7	*Blattella germanica*	*Bger*	32	*Microcerotermes biroi*	*Mbir*
8	*Coatitermes* sp	*Csp4*	33	*Nasutitermes lujae*	*Nluj*
9	*Constrictotermes cavifrons*	*Ccav*	34	*Neocapritermes taracua*	*Ntar*
10	*Coptotermes gestroi*	*Cges*	35	*Neotermes castaneus*	*Ncas*
11	*Coptotermes testaceus*	*Ctes*	36	*Odontotermes formosanus*	*Ofor*
12	*Cornitermes walkeri*	*Cwal*	37	*Paraneotermes simplicicornis*	*PAsim*
13	*Cryptocercus meridianus*	*Cmer*	38	*Pericapritermes* sp	*Punk*
14	*Cryptotermes longicollis*	*Clon*	39	*Periplaneta americana*	*Pame*
15	*Cylindrotermes parvignathus*	*Cpar*	40	*Porotermes adamsoni*	*Pada*
16	*Dolichorhinotermes longilabius*	*Dlon*	41	*Promirotermes redundans*	*Pred*
17	*Euhamitermes* sp	*Eunk*	42	*Prorhinotermes simplex*	*PRsim*
18	*Foraminitermes valens*	*Fval*	43	*Reticulitermes flavipes*	*Rfla*
19	*Glossotermes oculatus*	*Gocu*	44	*Roisinitermes ebogoensis*	*Rebo*
20	*Glyptotermes fuscus*	*Gfus*	45	*Silvestritermes heyeri*	*Shey*
21	*Heterotermes tenuis*	*Hten*	46	*Sphaerotermes sphaerothorax*	*Ssph*
22	*Hodotermopsis sjostedti*	*Hsjo*	47	*Stolotermes victoriensis*	*Svic*
23	*Hospitalitermes* sp	*Hunk*	48	*Stylotermes halumicus*	*Shal*
24	*Incisitermes schwarzi*	*Isch*	49	*Zootermopsis nevadensis*	*Znev*
25	*Isognathotermes* sp	*Iunk*			

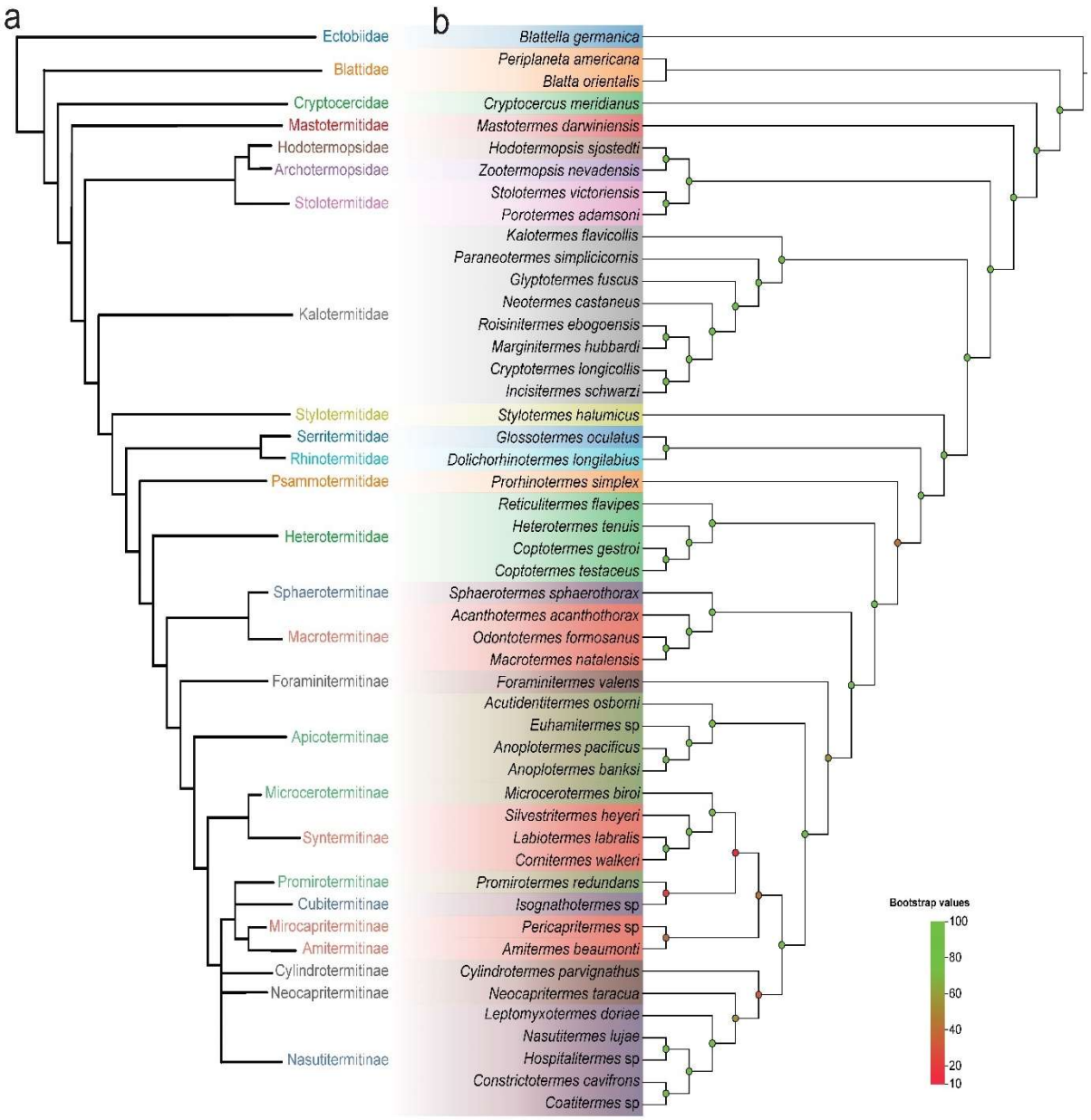
Supplementary Table2. Comparative presence and absence of neuropeptide precursors across 49 termite and cockroach species. **Note**: insulin-like peptides (ILPs) are not listed in this table.



Note: The gene count in this table includes partial gene fragments or putatively pseudogenized sequences (as evidenced by excessive substutions or indel occurrence in highly conserved regions such as ACP and Gon).



Supplementary Figure 1. Comparative analysis of Gon precursor sequences reveals gene losses in certain termite species, with 42 Gon sequences showing while 7 are missing.



Supplementary Figure 2. ML phylogenetic trees constructed from 32 neuropeptide precursors from 49 termites and cockroaches largely support accepted relationships among termite and cockroach lineages. (a) Simplified phylogenetic scheme of Blattodea modified from Evangelista et al. (2019) and Hellemans et al. (2024); (b) ML phylogenetic trees constructed using 32 neuropeptide precursors. Trees were reconstructed from alignments of the whole ORF amino acids sequence. Branch lengths are not depicted in order to provide a clearer view of internal branching patterns.

Chapter II

Comparative analysis of adipokinetic hormones (AKHs) and their receptors (AKHRs) in Blattodea reveals novel patterns of gene evolution

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Comparative analysis of adipokinetic hormones (AKHs) and their receptors (AKHRs) in Blattodea reveals novel patterns of gene evolution

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Author Contributions

SJ and DPM conceived the overall idea. SJ, HGM, and NS conducted investigations. SJ, HGM, SH, GG and DPM devised the methodology and analyzed the data. SJ, DPM, HGM, and GG wrote the manuscript. All authors contributed critically to the drafts.

3.1 Abstract

Adipokinetic hormone (AKH) is a neuropeptide produced in the insect corpora cardiaca that plays an essential role in mobilising carbohydrates and lipids from the fat body to the haemolymph. AKH acts by binding to a rhodopsin-like G protein-coupled receptor (GPCR), the adipokinetic hormone receptor (AKHR). In this study, we tackle AKH ligand and receptor gene evolution as well as the evolutionary origins of AKH gene paralogues from the order Blattodea (termites and cockroaches). Phylogenetic analyses of AKH precursor sequences point to an ancient AKH gene duplication event in the common ancestor of Blaberoidea, yielding a new group of putative decapeptides. In total, 16 different AKH peptides from 90 species were obtained. Two octapeptides and seven putatively novel decapeptides are predicted for the first time. AKH receptor sequences from 18 species, spanning solitary cockroaches and subsocial wood roaches as well as lower and higher termites, were subsequently acquired using classical molecular methods and in silico approaches employing transcriptomic data. Aligned AKHR open reading frames revealed 7 highly conserved transmembrane regions, a typical arrangement for GPCRs. Phylogenetic analyses based on AKHR sequences support accepted relationships among termite, subsocial (*Cryptocercus* spp.) and solitary cockroach lineages to a large extent, while putative post-translational modification sites do not greatly differ between solitary and subsocial roaches and social termites. Our study provides important information not only for AKH and AKHR functional research but also for further analyses interested in their development as potential candidates for biorational pest control agents against invasive termites and cockroaches.

Keywords

Adipokinetic hormone; adipokinetic hormone receptor; ‘green’ pesticide; neuropeptide; termite

3.2 Introduction

The adipokinetic hormones (AKHs) of insects are well-investigated neuropeptides, synthesized and released from the *corpora cardiaca* (CC). The biologically active peptides belong to the well know AKH/RPCH (red pigment-concentrating hormone) family of peptides, of which more than 90 different mature AKHs have been identified by primary sequence or predicted from arthropods (Gäde, 1997; Gäde et al., 1997b; Gäde, 2004; Gäde and Marco, 2009; Gäde et al., 2013; Gäde and Marco, 2022). In the biosynthesis and processing pathways in the CC, each AKH is derived from an individual preprohormone that is composed of a signal peptide and two potential peptides: the precursor of the bioactive AKH and a structurally-unrelated peptide known as the AKH precursor-associated peptide, to which no biological activity has been assigned to date. Following cleavage from the preprohormone and post-translational modification, the mature AKH and the unrelated peptide are ready for release from storage/secretory granules (Van der Horst et al., 2001). Peptides of the AKH/RPCH family share typical features: at least 8 but no more than 10 amino acids in length, a blocked pyroGlu (pQ) N-terminus, and a C-terminus blocked by amidation. The aromatic residues phenylalanine (Phe) or tyrosine (Tyr) occur at position 4 and tryptophan (Trp) at position 8 (Gäde et al., 1997b; Gäde, 2004; Li et al., 2016). Investigated insect species may synthesize only one AKH peptide, whereas others produce from two to as many as five different AKH peptides in their CC (Gäde, 2009; Gäde et al., 2013; Veenstra, 2014; Marco and Gäde, 2019; Marciniak et al., 2022). However, the evolutionary origins and relationships between AKH gene paralogues in insects are poorly characterized.

It is a well-established fact that in insects, AKHs play a critical role in the control of energy mobilization in various physiological processes, such as in locomotory activity, starvation, and stress response (Goldsworthy et al., 1975; Stone et al., 1976; A.Holwerda et al., 1977; Gäde and Beenakkers, 1977; Robinson and Goldsworthy, 1977; Chino et al., 1989; Kodrik et al., 2000; Gäde, 2004; Auerswald et al., 2005; Isabel et al., 2005; Kodrík, 2008; Gäde and Marco, 2013; Sajwan et al., 2015; Marco and Gäde, 2017; Tang et al., 2020). These physiological functions are mediated by adipokinetic hormone receptors (AKHRs), specifically, GPCRs related to vertebrate gonadotropin-releasing hormone (GnRH) receptors. During energy-demanding circumstances, AKHs are released from the CC into the hemolymph to reach the AKHRs expressed on fat body cells. AKHR binding and activation initiates a signal transduction cascade that causes the activation of either glycogen phosphorylase or triacylglycerol lipase, which in turn activates specific cellular pathways to release trehalose or diacylglycerol and free fatty acids from the fat body (Gäde and Auerswald, 2003; Gáliková et al., 2015). In 1998 an insect GPCR was cloned from *D. melanogaster*, and four years later it was confirmed to be an AKH receptor (Hauser et al., 1998; Park et al., 2002; Staubli et al., 2002). The AKHR from the silkworm *Bombyx mori* was also identified and described in 2002 (Staubli et al., 2002). Since then,

AKHRs have been described and some have been functionally studied from a wide variety of insect species and orders, such as *Anopheles gambiae, Sarcophaga crassipalpis*, *Glossina morsitans morsitans* and *Bactrocera dorsalis* in Diptera (Belmont et al., 2006; Kaufmann and Brown, 2006; Bil et al., 2016; Caers et al., 2016; Hou et al., 2017), *M. sexta* (Lepidoptera) (Ziegler et al., 2011), *Tribolium castaneum* (Coleoptera) (Li et al., 2008), *Periplaneta americana* and *Blattella germanica* (Blattodea) (Hansen et al., 2006; Wicher et al., 2006; Huang et al., 2012), *Schistocerca gregaria* and *Gryllus bimaculatus* (Orthoptera) (Konuma et al., 2012; Jackson et al., 2019), *Acyrthosiphon pisum* and *Rhodnius prolixus* (Hemiptera) (Jedlička et al., 2012; Zandawala et al., 2015; Alves-Bezerra et al., 2016) and *Carausius morosus* in Phasmatodea (Birgul Iyison et al., 2020).

Termites are a relatively small group of social insects consisting of more than 3000 species (Krishna et al., 2013), which were traditionally classified as the order Isoptera. More recent research demonstrates that termites appear as the sister clade to the *Cryptocercus* lineage of woodroaches, nesting within cockroaches and, thus, belong to the order Blattodea (Inward et al., 2007; Klass et al., 2008; Krishna et al., 2013; Bourguignon et al., 2015; Evangelista et al., 2019). Blattodea together with Mantodea (mantids) comprise the superorder Dictyoptera, which share a common recent ancestor (Ware et al., 2008).

The most-recent study on structural diversity of AKHs in 13 termite species (Jedlickova et al., 2016), used molecular methods (7 species) and *in silico* bioinformatic searches (6 species) from 5 families of Isoptera for phylogenetic analyses between termites and their closest cockroach relatives. These analyses were, however, carried out without sequence data of representatives from all termite families, and in the absence of data from woodroaches (*Cryptocercus*) since the AKH preprohormone sequence was not known at the time. In fact, at that time, only the mature AKH sequences were known from 3 termite species (Liebrich et al., 1995) and over 30 cockroach species (Gäde, 2009; Roth et al., 2009). Despite the short peptide sequences of AKHs, these have been useful in some insect orders, including cockroaches, to investigate phylogenetic trends and evolutionary relationships between insect lineages (Gäde, 1989; Gäde and Marco, 2005; Roth et al., 2009; Marco et al., 2020). However, the occurrence of multiple AKH genes, including within Blattodea, can hamper the interpretation of phylogenetic approaches (Bläser et al., 2020; Bläser and Predel, 2020), particularly when dealing with incomplete or missing data and insufficient taxon sampling. For example, while termites possess just one AKH gene, several cockroach species have 2 AKH peptides (Gäde and Rinehart, 1990; Gäde, 2009; Jedlickova et al., 2016).

In contrast to the peptide ligands, only a few AKH receptor sequences are known from Blattodea, to date. The AKHR of *P. americana* was the first to be identified from a hemimetabolous insect (Hansen

et al., 2006). The AKHR of *Blattella germanica* was cloned in 2012 (Hansen et al., 2006; Huang et al., 2012), while there are two putative AKHR sequences from termites available on NCBI: *Zootermopsis nevadensis* (XM_022082426.1) and *Cryptotermes secundus* (XM_033755069.1). There is, thus, a paucity of information regarding AKHR sequences of Blattodea. Receptor sequences are fundamentally important for further molecular and pharmacological characterization of receptor-ligand systems, especially in the identification of potential methods to combat pest insect species.

In addition to representing a number of global pest species, cockroaches and termites play a key role in ecosystem services, including in the decomposition of deadwood (Bignell and Eggleton, 2000; Wilson et al., 2007). Currently, researchers are considering the use of peptide mimetics (as so-called “green” insecticides), based on the interaction of neuropeptide ligands with receptors, to negatively influence the physiology and/or behavior of specific pest insects without harming beneficial insects (Altstein and Nässel, 2010; Gäde et al., 2017; Marco et al., 2018). RNAi represents an alternative potential strategy for pest control, enabling downregulation of specific gene targets via the host's RNAi pathway, whereby dsRNA is converted to small interfering RNA (siRNA), in turn binding to the RNA-induced silencing complex (RISC), which then efficiently locates and eliminates target mRNA, and reducing its translation into protein (Arakane et al., 2008; Rewitz et al., 2009; Lee et al., 2011; Kapan et al., 2012; Burand and Hunter, 2013; Park et al., 2014; Yu et al., 2023). For such applications, it is imperative to know peptide and receptor sequences and structures in as many pest species as possible, as well as in non-pest species to evaluate the potential collateral damage or success of sequence-based green insecticides.

With the current study we employ bioinformatic and molecular approaches to characterize AKH precursor sequences and cognate AKHR sequences from a wide diversity of termite and cockroach species, including wood-roaches. We combine recently sequenced transcriptomic data with publicly available Sequence Read Archive (SRA) data and classical molecular approaches to conduct a comprehensive sequence analysis of AKH and AKHR in termites and their nearest cockroach relatives. Our aims are to investigate the evolution of AKH peptides and their receptors in Blattodea, as well as resolve the evolutionary origins and relationships of AKH gene duplications in this group. Additionally, we enhance termite AKH and AKHR datasets for comparative analysis, carry out an assessment of patterns of post-translational modification during the evolution of termite sociality, and establish a data framework for research aiming to exploit AKH and AKHRs as potential targets for “green” pesticide development against invasive termites and cockroaches, or for possible RNAi interventions.

3.3 Material and methods

Insect samples

The termite species *Coptotermes formosanus*, *Kalotermes flavicollis*, *Mastotermes darwiniensis*, *Cryptotermes sp.*, *Prorhinotermes inopinatus* and *Reticulitermes flavipes*, as well as cockroach species, *Blattella germanica* were bred from laboratory colonies of the Federal Institute of Materials Research and Testing (BAM), Berlin, Germany. One species of subsocial woodroaches, *Cryptocercus meridianus* was collected in Yunshanping (27'14'N, 100'23'E), Yulongxueshan, Lijiang, Yunnan, China, and one higher social termite, *Indotermes* sp. was collected in Mengla County, Xishuangbanna Dai Autonomous Prefecture, Yunnan, China (N21.61799°, E101.58134°).

Sequence retrieval

AKH preprohormone sequences were retrieved from published literature (Jedlickova et al., 2016) and accessible SRA datasets for *Cryptotermes domesticus* (SRR2039534) and *Reticulitermes grassei* (SRR13251[02-10]). The following AKHR nucleotide sequences were retrieved from SRA datasets and the nucleotide collection from GenBank: *Periplaneta americana* (DQ217786.1), *Blattella germanica* (GU591493.1), *Zootermopsis nevadensis* (XM_022082426.1) and *Cryptotermes secundus* (XM_033755069.1).

In addition, AKHs and AKHRs were searched from assemblies of 18 cockroach and termite transcriptomes (He et al., 2021) and transcriptome sequence assemblies (TSA) from "The 1KITE project: evolution of insects" (BioProject: PRJNA183205). We adopted a hidden Markov model (HMM) approach, as described by He et al. (2020) with slight modifications (Supplementary Data 2). Briefly, candidate AKH and AKHR sequences were searched in the transcriptome assemblies by querying against a reference dataset of diverse AKH precursor and AKHR sequences compiled from NCBI (Supplementary Table 1). The online tool SignalP 5.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0) was then used to predict the signal peptide cleavage site from each candidate AKH precursor sequence (Almagro Armenteros et al., 2019).

Sequencing the AKH precursor gene and AKH receptor gene

For some species, the complete open reading frame (ORF) of AKHR could be obtained from assembled transcriptome data alone, including *C. wrighti*, *Globitermes* sp., *B. orientalis*, *P. simplex*, *N. castaneus*, *R. grassei*, *Symploce* sp., *Asiablatta kyotensis*, *Paratemnopteryx couloniana*, *Ischnoptera deropeltiformis*. However, none or only partial AKHR fragment(s) were retrieved from *Indotermes* sp., *C. formosanus*, *C. meridianus*, *K. flavicollis*, *M. darwiniensis*, *P. inopinatus, Indotermes* sp., and *R. flavipes*. Additionally, to verify the presence of the novel AKH peptide type in *Blattella germanica*. We therefore employed classical molecular methods to attain the full length AKH/AKHR sequences of

these remaining species. ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to determine ORFs in the sequences.

Apart from *C. meridianus*, whose abdomens were cut with sterile scissors, the whole body of other termite species were used for total RNA isolation. Pre-cooled Trizol reagent (Thermo Fisher Scientific) was used to preserve insect tissues and then homogenized with a FastPrep®-24 homogenizer (MP Biomedicals). Recovery of RNA was followed according to manufacturer's recommendation for Trizol (Thermo Fisher Scientific), with chloroform extraction and isopropanol precipitation, followed by re-dissolving RNA in Nuclease-free water, and subsequent incubation with TurboDNase for 30 min at 37 °C to remove remaining DNA (TURBO DNA-free Kit, Ambion). cDNA synthesis was performed with oligo-dT primers using the M-MLV Reverse Transcriptase (Promega). PCR was performed using Taq DNA Polymerase (Red Load Taq Master (5x), NEB) with specific or degenerate forward and reverse primers (Supplementary Table 2).

Primers were manually selected based on conserved homologous nucleotide sequences or designed online: https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Ye et al., 2012) using the assembled transcripts and available full-length gene sequences from the species *B. germanica*, *P. americana*, *C. wrighti* and *C. secundus* to guide primer sequence design. PCR products were either cleaned directly with a Monarch® PCR & DNA Cleanup Kit (NEB) (for a specific PCR product) or separated on a 1.5% agarose gel (Agarose NEEO ultra-quality Roth®, Karlsruhe, Germany) and the expected size band excised and extracted using the QIAquick Gel Extraction Kit (Qiagen). Cleaned-up products were then sent for Sanger sequencing (Eurofins Genomic).

Sequence alignment and phylogenetic analysis

The multiple sequence alignment program MAFFT (Katoh and Standley, 2013) with E-INS-I algorithm was used to perform multiple sequence alignments for AKH precursor sequences and AKHRs. Alignments were visualized using Jalview (Waterhouse et al., 2009), and the transmembrane domains of the AKHR receptors were predicted with the TMHMM server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Pairwise sequence comparisons for AKHR amino acid identity and similarity were computed using the online tool: SIAS (http://imed.med.ucm.es/Tools/sias.html) using the "length of smallest sequence" option.

To explore the evolutionary relationships of AKH precursor sequences and AKHR sequences, we carried out phylogenetic reconstruction on amino acid or nucleotide-based alignments. Aligned sequences were trimmed using trimAl v1.2 (Capella-Gutiérrez et al., 2009) with the function -gappyout prior to use in phylogenetic tree reconstruction. We employed a maximum likelihood (ML) approach in RAxML

v8.2.12 (Stamatakis, 2014) and a Bayesian inference (BI) approach in MrBayes v3.2.7a (Huelsenbeck and Ronquist, 2001) to reconstruct phylogenies.

For ML, we used 1000 rapid bootstrap (BP) replicates with the PROTGAMMAAUTO model (amino acid alignment) or the GTRGAMMAI model (nucleotide alignment). For BI, we employed a model-jumping approach with the command "prset aamodelpr=mixed" (amino acid alignment), or "lset nst =mixed" (nucleotide alignment). Four chains of the Markov Chain Monte Carto (MCMC) with two independent runs of 15 million generations were conducted, sampling every 500 generations. Average standard deviation of split frequencies was inspected to ensure these were less than 0.01. Posterior probabilities (PB) were calculated from the posterior distribution of trees after discarding 25% as burn-in.

Prediction of AKHR post-translational modifications (PTMs)

Putative glycosylation and myristoylation sites of AKHRs were detected using MotifScan (https://myhits.sib.swiss/cgi-bin/motif_scan) using default settings (Sigrist et al., 2010) and phosphorylation sites of the intracellular domains of AKHRs were predicted using the NetPhos server (https://services.healthtech.dtu.dk/service.php?NetPhos-3.1), displaying only scores higher than 0.5 threshold (Blom et al., 1999; Blom et al., 2004).

3.4 Results

3.4.1 AKHs in termites and cockroaches

From the complete list of AKH mature peptides derived from 90 species (Table 1) we were able to obtain sequence information for 85 partial or complete AKH precursors from a total of 62 species spanning diverse families across the order Blattodea. The information was collated from preexisting (published) data as well as from transcriptome and SRA data and bioinformatic searches of databases, as well as via Sanger sequencing in selected cases to confirm the *in silico* findings. The aligned AKH preprohormone sequences of these species demonstrate that both signal peptides and AKH-associated peptides are conserved at the amino acid level (Figure 1). Precursor sequences in cockroaches, especially for those species in the same family (Table 1), show high levels of sequence conservation, such as only two substitutions between AKH1 of *Shelfordella lateralis* and AKH1 of *P. americana*, or five substitutions between AKH2 of *A. kyotensis* and AKH2 of *S. sexpunctata*.

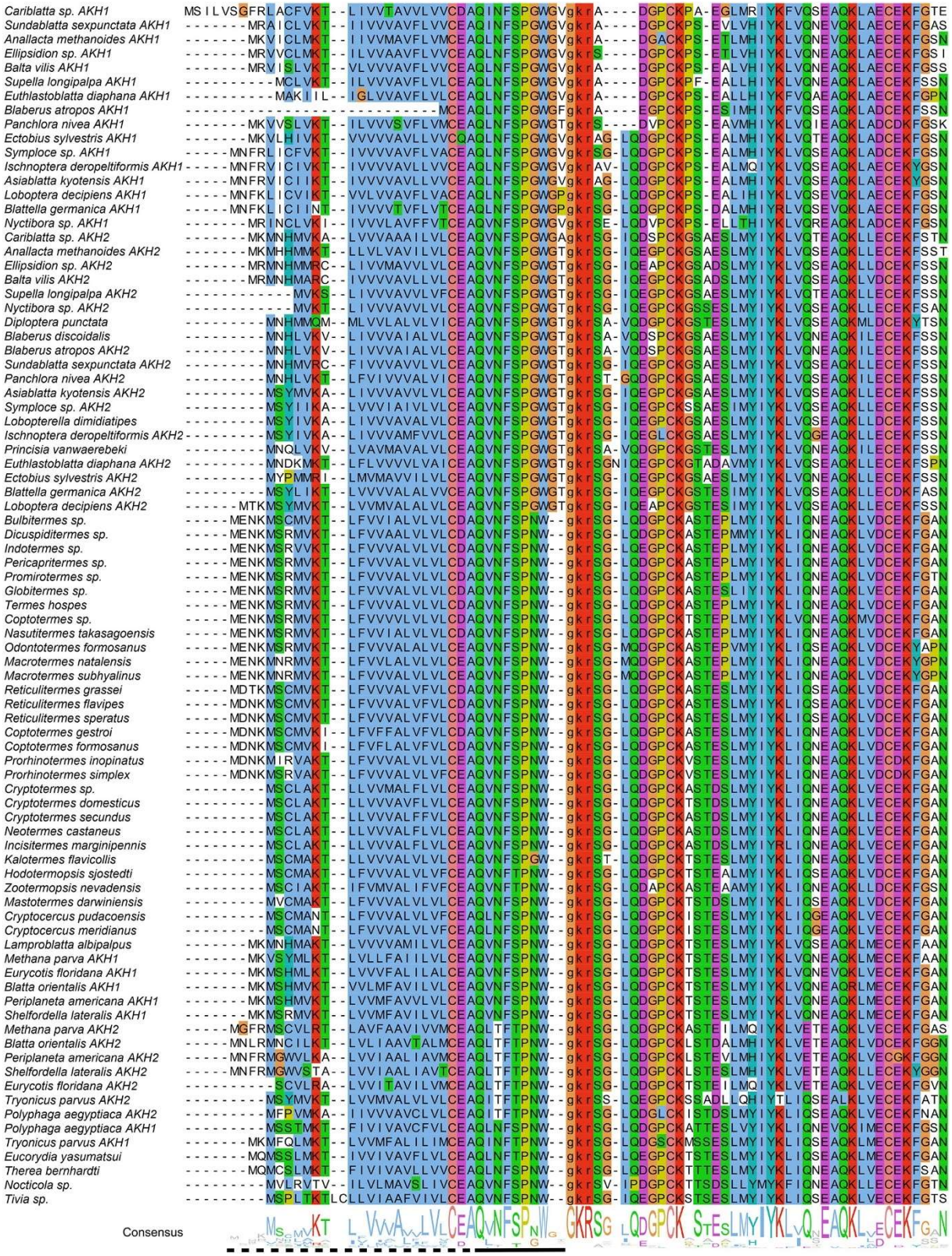


Figure 1. Multiple sequence alignment of 85 termite and cockroach AKH preprohormones from 62 species. The sequence logo beneath depicts the consensus sequence and the solid line under the sequence logo identifies the AKH bioactive peptide. The sequence before the bioactive peptide is referred to as the signal peptide (dashed line), while the sequence following is the AKH associated peptide. Putative amidation and dibasic cleavage sites are indicated in lower case based on known homologous precursors in other species. Available sequence accession codes are listed in Supplementary Data 1.

The mature peptide for each species was classified based on amino-acid sequence identity to known AKHs and categorized based on taxonomy. Our new sequence data are compared alongside previously published findings in Table 1. A total of 16 AKH primary sequences from 90 species were identified. Table 1 shows that all predicted bioactive mature peptides consist of 8 or 10 amino acids. In Blaberoidea, only decapeptides were found, with Bladi-HrTH being the most frequently detected peptide (Table 1, Figure 2), which was found in all species except *Cariblatta sp.* (where two putative novel decapeptides are identified). Further to this, we identified seven putative novel decapeptides in Blaberoidea via *in silico* characterization. The presence of the putative novel 2 type in *Blattella germanica* was confirmed via Sanger sequencing.

By contrast, species in Solumblattodea (Blattoidea + Corydioidea) contained only octapeptides. The most frequently detected AKH in Solumblattodea was Peram-CAH-I (Figure 2), which occurs not only in most termite families such as Termitidae, Rhinotermitidae, Mastotermitidae and Kalotermitidae, but also in the cockroach family Lamproblattidae, Blattidae, Corydiidae and Nocticolidae. Two AKH octapeptides were recorded in some cockroaches: Peram-CAH-I and Peram-CAH-II in Blattidae, Tenmo-HrTH and Polae-HrTH in Corydiidae (*Polyphaga aegyptiaca*) and Emppe-AKH + Polae-HrTH in Tryonicidae (*Tryonicus parvus*). Micvi-CC is exclusively found in the Hodotermitidae (Table 1).

The sister group to the termites, the woodroaches (genus *Cryptocercus*) possess a Tenmo-HrTH peptide, which differs only in the occurrence of leucine at position 2 in comparison to valine in Peram-CAH-I. Interestingly, a unique AKH peptide, Manto-CC, in *K. flavicollis* (Kalotermitidae) was identified using Sanger sequencing in the current study, and further confirmed and validated by mass spectrometry (Marco et al., 2022) for the first time in cockroaches and termites, with a structure that differs from Peram-CAH-I at position 7 by a glycine/asparagine substitution. The other unique octapeptide peptide, Pyrap-AKH in *Eucorydia yasumatsui* (Corydiidae), was found for the first time in Blattodea, with a structure that differs from Tenmo-HrTH at position 5 by a serine/threonine substitution (Table 1).

Table 1. AKH peptides of termites and cockroaches. The taxonomic classification is per Evangelista et al. (2019) and Hellemans et al. (2022). Adipokinetic hormone nomenclature: Peram-CAH: *Periplaneta americana* cardioacceleratory hormone; Bladi-HrTH: *Blaberus discoidalis* hypertreahlosemic hormone; Manto-CC: Mantophasmatodea CC; Emppe-AKH: *Empusa pennata* AKH; Micvi-CC: *Microhodotermes viator* CC; Tenmo-HrTH: *Tenebrio molitor* hypertreahlosemic hormone; Pyrap-AKH: *Pyrrhocoris apterus* AKH; Polae-HrTH: *Polyphaga aegyptiaca* hypertreahlosemic hormone. For peptide source details see Supplementary Table 1.

Taxonomy sub-/family			Species	AKH name	AKH sequence	References
Blaberoidea	Blattellinae		*Lobopterella dimidiatipes*	Bladi-HrTH	pQVNFSPGWGTa	This study (source: 1KITE)
			Loboptera decipiens			Roth et al. (2009)
			Symploce sp., Asiablatta kyotensis, Ischnoptera deropeltiformis, Anallacta methanoides	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)
			Loboptera decipiens	Bladi-HrTH putative novel 2	pQVNFSPGWGTa pQLNFSPGWGPa	This study (source: 1KITE)
			Blattella germanica			Gäde and Rinehart (1990); Veenstra and Camps (1990); This study (source: Sanger sequencing)
	Nyctiborinae		*Nyctibora sp.*	Bladi-HrTH putative novel 3	pQVNFSPGWGTa pQVNFSPGWGVa	This study (source: 1KITE)
	Blaberidae		*Aptera fusca, Archimandrita tessellate, Bantua robusta, Blaptica dubia, Blepharodera discoidalis, Diploptera punctata, Elliptorhina sp., Eublaberus distanti, Gyna caffrorum, Lucihormetica grossei, Panaesthia sp., Panchlora viridis, Princisia vanwaerebeki*	Bladi-HrTH	pQVNFSPGWGTa	Roth et al. (2009)
			Blaberus discoidalis			Hayes et al. (1986)
			Gromphadorhina portentosa, Leucophaea maderae			Gäde and Rinehart (1990)
			Nauphoeta cinerea			Gäde and Rinehart (1986)
			Panchlora nivea	Bladi-HrTH putative novel 4	pQVNFSPGWGTa pQLNFSPGWGTa	This study (source: 1KITE)
			Blaberus atropos	Bladi-HrTH putative novel 5	pQVNFSPGWGTa pQLNFSPGWGFa	This study (source: 1KITE)
			Princisia vanwaerebeki, Diploptera punctata	Bladi-HrTH	pQVNFSPGWGTa	This study (source: 1KITE)
	Pseudophyllodromiinae		*Balta vilis, Ellipsidion sp., Supella longipalpa, Euthlastoblatta diaphana,*	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)
			Sundablatta sexpunctata	Bladi-HrTH putative novel 6	pQVNFSPGWGTa pQINFSPGWGVa	This study (source: 1KITE)
			Cariblatta sp.	putative novel 6 putative novel 7	pQINFSPGWGVa pQVNFSPGWGAa	This study (source: 1KITE)
	Ectobiinae		*Ectobius sylvestris*	Bladi-HrTH putative novel 1	pQVNFSPGWGTa pQLNFSPGWGVa	This study (source: 1KITE)
Solumblattodea	Isoptera	Termitidae	*Odontotermes formosanus, Macrotermes natalensis, Nasutitermes takasagoensis, Termes hospes*	Peram-CAH-I	pQVNFSPNWa	Jedlickova et al. (2016)
			Trinervitermes trinervoides			Liebrich et al. (1995)
			Bulbitermes sp., Dicuspiditermes sp., Globitermes sp., Indotermes sp., Macrotermes subhyalinus, Pericapritermes sp., Promirotermes sp.			This study (source: He et al., 2021)
		Rhinotermitidae	*Coptotermes formosanus, Coptotermes gestroi, Prorhinotermes simplex, Reticulitermes flavipes, Reticulitermes speratus*			Jedlickova et al. (2016)
			Reticulitermes grassei, Prorhinotermes inopinatus			This study (source: He et al., 2021)
		Kalotermitidae	*Neotermes castaneus*			Jedlickova et al. (2016)
			Cryptotermes sp., Cryptotermes domesticus Incisitermes marginipennis			This study (source: *I. marginipennis:* 1KITE; *C. domesticus*: He et al., 2021, *Cryptotermes sp.*: Sanger sequencing)
			Kalotermes flavicollis	Manto-CC	pQVNFSPGWa	This study (source: Sanger sequencing)
		Archotermopsidae	*Hodotermopsis sjöstedti, Zootermopsis nevadensis*	Emppe-AKH	pQVNFTPNWa	Jedlickova et al. (2016)
		Hodotermitidae	*Microhodotermes viator*	Micvi-CC	pQINFTPNWa	Liebrich et al. (1995)
		Mastotermitidae	*Mastotermes darwiniensis*	Peram-CAH-I	pQVNFSPNWa	Liebrich et al. (1995); Jedlickova et al. (2016)
	Cryptocercidae		*Cryptocercus darwini*	Tenmo-HrTH	pQLNFSPNWa	Roth et al. (2009)
			Cryptocercus punctulatus			Gäde et al. (1997a)
			Cryptocercus pudacoensis, Cryptocercus meridianus			This study (source: He et al., 2021)
	Lamproblattidae		*Lamproblatta albipalpus*	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)
	Blattidae		*Blatta orientalis*	Peram-CAH-I Peram-CAH-II	pQVNFSPNWa pQLTFTPNWa	Gäde and Rinehart (1990)
			Brinckia hanstroemi, Celatoblatta sp., Deropeltis erythrocephala, Eurycotis floridana, Neostylopyga rhombifolia, Pseudoderopeltis foveolate, Shelfordella lateralis			Predel and Gäde (2005)
			Periplaneta americana			Scarborough et al. (1984); Witten et al. (1984); Zeng et al. (2021)
			Shelfordella lateralis, Eurycotis floridana, Methana parva			This study (source: 1KITE)
	Tryonicidae		*Tryonicus parvus*	Emppe-AKH Polae-HrTH	pQINFTPNWa pQITFTPNWa	This study (source: 1KITE)
	Corydiidae		*Polyphaga aegyptiaca*	Tenmo-HrTH Polae-HrTH	pQLNFSPNWa pQITFTPNWa	Gäde and Kellner (1992)
			Ergaula capucina	Tenmo-HrTH	pQLNFSPNWa	König et al. (2005)
			Therea petiveriana			Gäde et al. (1997a)
			Eucorydia yasumatsui	Pyrap-AKH	pQLNFTPNWa	This study (source: 1KITE)
			Therea bernhardti	Tenmo-HrTH	pQLNFSPNWa	This study (source: 1KITE)
			Tivia sp.	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)
	Nocticolidae		*Nocticola sp.*	Peram-CAH-I	pQVNFSPNWa	This study (source: 1KITE)

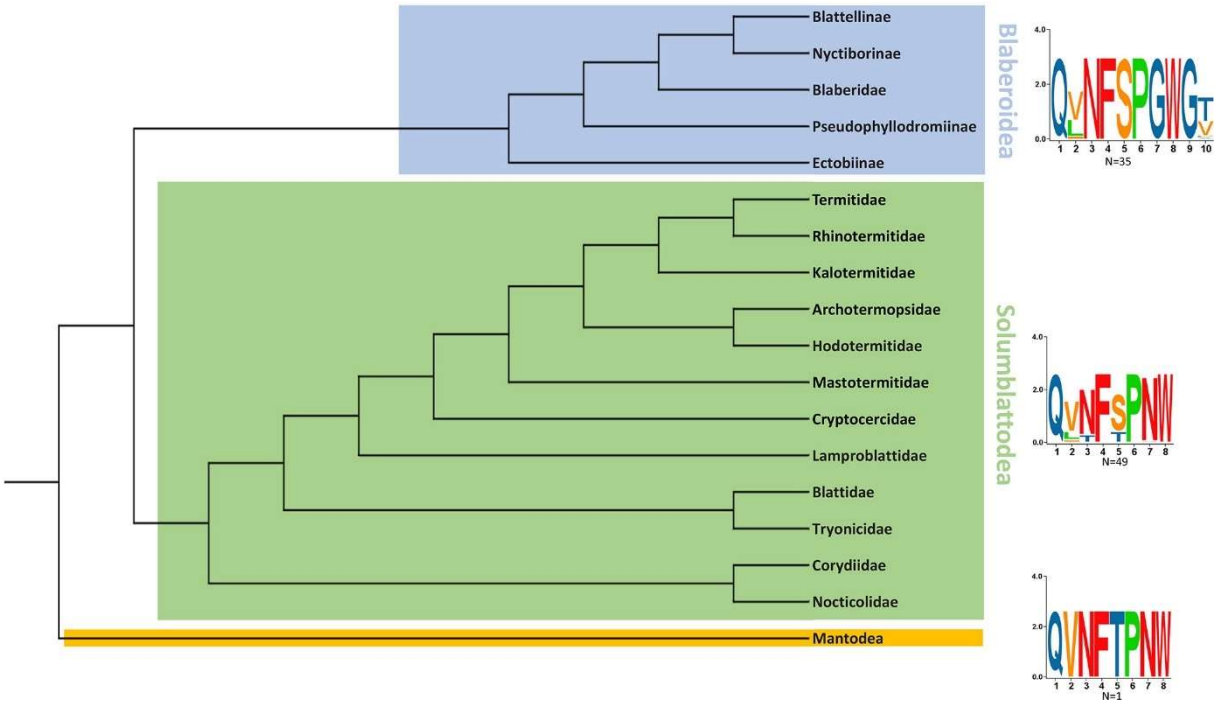


Figure 2. Simplified phylogenetic scheme of Blattodea modified from Evangelista et al. (2019) and Hellemans et al. (2022). The sequence logo represents the degree of conservation of amino acids in AKH neuropeptides in Blaberoidea and Solumblattodea. N: number of AKH neuropeptide sequences. Mantodea AKH (Emppe-AKH; Gäde and Marco (2017)) is used as an outgroup.

3.4.2 Phylogenetic analysis of AKH in termites and cockroaches

Phylogenetic trees obtained from two different methods, RAxML and MrBayes, rooted between the major blattodean clades: Blaberoidea and Solumblattodea (Blattoidea + Corydioidea) (Figure 3, Supplementary Figure 1) support an AKH duplication event in the ancestor of Blaberoidea; leading to a Bladi-HrTH decapeptide clade (except *Cariblatta* sp., which possesses a novel decapeptide, although this still groups with high support in the Bladi-HrTH clade) and a clade of novel putative decapeptides. Node support for this hypothetical duplication event is high when using nucleotide data, and slightly less so when employing amino acid sequences. Termite AKHs nested paraphyletically within Solumblattodea sequences, with limited bootstrap support at deeper nodes in this clade for both nucleotide and amino acid trees. In terms of sequence evolution, termitid plus *Reticulitermes* and *Coptotermes* AKHs appear to diverge from the remaining lower termite and cockroach sequences.

The relationships between Corydiidae + Nocticolidea (Corydioidea), Blattidae and other lineages within Solumblattodea are generally equivocal. The nucleotide phylogeny suggests that a parallel AKH

duplication is likely to have taken place in Blattidae, although this receives limited statistical support in both ML and BI approaches, and so should be interpreted with a degree of caution.

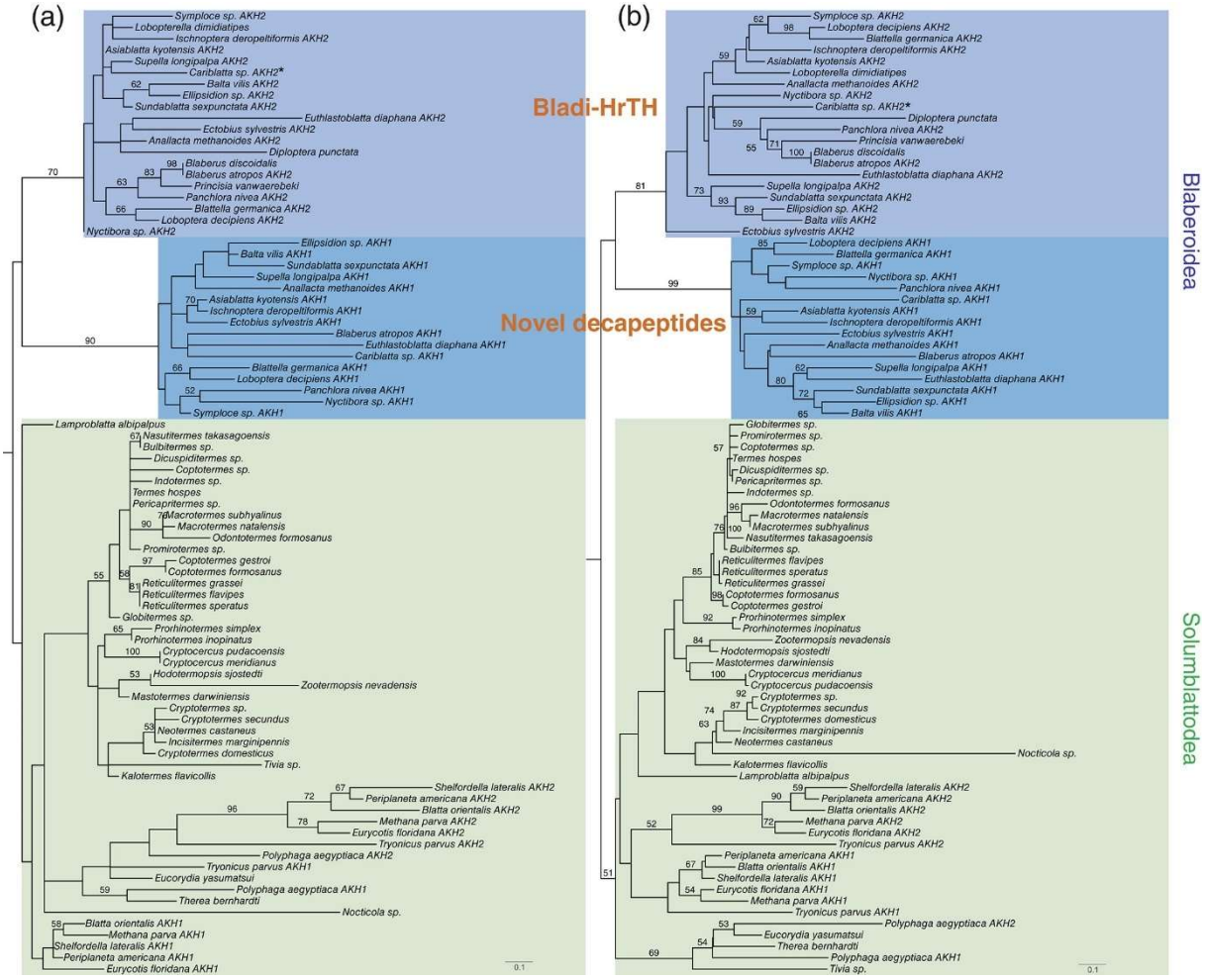


Figure 3. ML phylogenetic trees constructed from AKH preprohormones from termites and cockroaches. Trees were reconstructed from alignments of the whole ORF sequence, using amino acids (a) or nucleotides (b). Numbers at each node represent bootstrap support values (in percent), with only values above 50 being shown. The putative novel 7 decapeptide of *Cariblatta* sp. AKH2 (*) grouped with Bladi-HrTH. For comparison, equivalent BI trees are shown in Supplementary Figure 1.

3.4.3 AKHRs in termites and cockroaches

A total of 18 new AKH receptor sequences were retrieved bioinformatically and/or sequenced using a classical molecular approach. We aligned the sequences for these 18 species and for the other 4 available species to obtain a better understanding of amino acid variation among blattid and termite AKHR sequences (Figure 4). The ORF (open reading frame) of all termite and cockroach AKHRs contains sequences of between 405 to 467 amino acid residues in length. Except for *Cryptotermes sp.* and *C. secundus*, the shortest amino acid sequences belong to the Blattellinae subfamily: *B. germanica*, *Symploce sp.*, *I. deropeltiformis*, *A. kyotensis* and *P. couloniana.* The results derived from the aligned sequences demonstrate that termite and cockroach AKH receptors share highly conserved sequence elements, particularly in the seven transmembrane domains (TMs), which corresponds to the typical arrangement found in G-protein-coupled receptors (TM1-TM7 highlighted in Figure 4). Sequence

conservation is especially high in TM2, TM6 and TM7, although moderate levels of sequence conservation could be detected across most of the ORF, except close to the N- and particularly the C-termini.

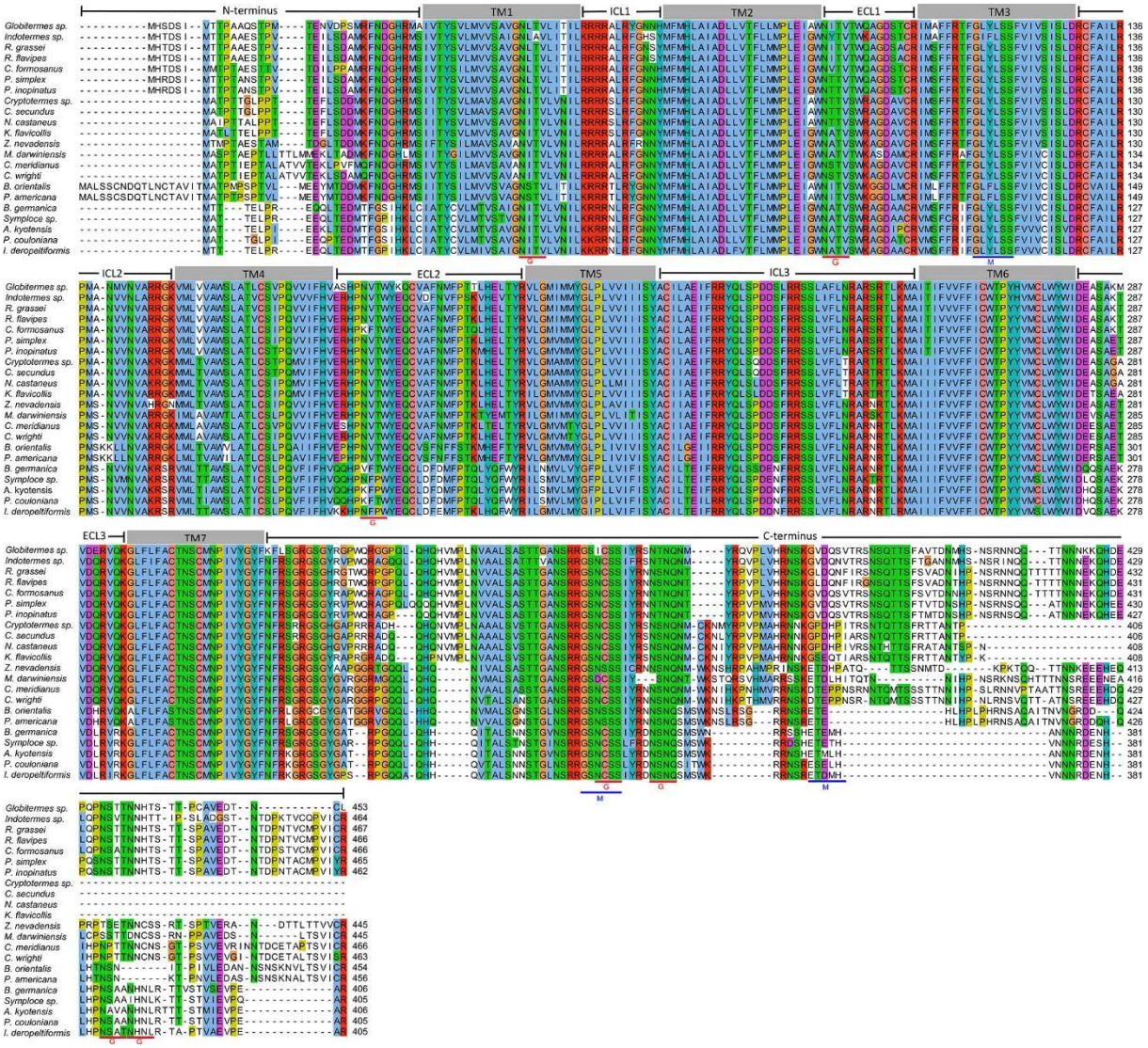


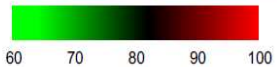
Figure 4. Comparative amino acid sequence alignment of termite and cockroach AKHR sequences. Intracellular (ICL) and extracellular (ECL) loops, N-terminus and C-terminus are labeled above the alignment. Transmembrane domains 1-7 are highlighted in grey. Blue and red lines under the alignment show putatively conserved myristoylation and glycosylation sites, respectively.

We also compared pairwise identity and similarity at the amino acid level of the AKHRs identified to date of the full 22 Blattodea species (Table 2). Identities and similarities range from 64.0% to 99.6% and from 68.4% to 99.6%, respectively. Notable levels of identity and similarity include comparisons between solitary cockroaches (Blattidae + Blattellinae) and wood-feeding cockroaches (*Cryptocercus*): 71.6-81.5%, whereas between solitary cockroaches and higher termites (Termitidae), identity and similarity values range from 64.0-76.0%. Termitidae and *Cryptocercus* are separated by intermediate levels (71.3-79.0%) of amino acid identity and similarity. AKHR identity and similarity within kalotermitids and rhinotermitids range from 93.6-99.3% and 92.2-99.6%, respectively, whereas

between these families, values range from 81.1-88.2%. All other pairwise comparisons are shown in Table 2.

Table 2. Pairwise comparison (%) of amino acid identity (lower triangular) and similarity (upper triangular) from 22 AKHRs of termites and cockroaches.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Globitermes sp.(1)	100	89.0	89.6	89.4	90.5	89.8	88.7	82.0	81.8	81.6	82.6	77.1	76.2	76.8	76.4	68.4	69.1	71.9	71.6	71.9	73.1	73.3
Indotermes sp.(2)	85.0	100	92.0	91.8	90.9	90.3	89.8	84.5	84.5	84.6	85.8	80.2	79.6	78.2	79.0	71.8	71.5	74.4	73.8	74.9	75.3	76.0
R.grassei(3)	87.9	88.1	100	99.6	95.1	94.2	93.7	84.7	84.7	85.0	85.8	82.2	82.0	80.3	81.9	73.6	73.9	76.1	75.6	76.4	77.3	77.5
R.flavipes(4)	87.6	87.9	99.6	100	94.8	94.2	93.7	84.7	84.7	85.0	85.8	82.2	81.6	79.8	81.4	73.3	73.7	76.1	75.6	76.4	77.3	77.5
C.formosanus(5)	89.0	86.6	93.6	93.3	100	94.8	94.4	85.0	84.7	84.6	86.5	82.5	81.8	80.3	81.2	72.7	73.2	75.9	75.6	76.6	78.0	76.3
P.simplex(6)	88.3	86.4	92.9	92.7	93.5	100	99.4	86.7	86.5	86.8	88.0	83.1	81.6	80.2	82.5	72.9	73.9	75.6	75.1	76.1	76.8	76.8
P.inopinatus(7)	87.4	86.4	92.4	92.2	93.1	99.4	100	86.7	86.5	86.3	87.5	82.5	80.9	79.9	81.8	72.5	73.5	74.6	74.1	75.1	75.8	76.0
Cryptotermes sp.(8)	78.8	78.3	82.5	82.5	81.8	84.0	84.0	100	99.3	96.6	94.3	83.3	80.0	84.5	84.7	75.4	75.9	71.2	71.6	71.7	72.1	72.6
C.secundus(9)	78.6	78.3	82.5	82.5	81.5	83.7	83.7	99.3	100	97.0	95.1	83.3	80.0	84.7	85.0	75.6	76.1	71.4	71.9	71.9	72.3	72.8
N.castaneus(10)	77.9	77.9	82.4	82.4	81.1	83.8	83.3	96.3	96.6	100	94.4	82.6	80.6	83.6	84.6	75.5	76.0	71.2	71.6	71.7	72.1	73.1
K.flavicollis(11)	78.4	78.9	83.1	83.1	82.6	84.8	84.3	93.6	94.1	93.4	100	84.1	82.1	85.5	86.3	76.7	77.2	73.4	73.8	73.9	74.1	75.1
Z.nevadensis(12)	73.0	73.3	78.7	78.7	79.3	80.0	79.3	81.5	81.5	80.9	81.4	100	82.7	87.2	87.6	76.2	77.3	77.3	77.0	77.6	78.0	78.5
M.darwiniensis(13)	71.2	71.9	77.5	77.3	77.1	76.9	76.4	76.8	76.8	77.5	78.4	78.7	100	85.2	85.8	77.3	77.8	76.8	77.8	77.8	78.3	78.3
C.meridianus(14)	73.3	71.3	76.8	76.4	76.6	76.3	76.2	82.3	82.5	81.1	83.1	83.4	81.1	100	96.3	76.4	77.0	78.8	79.0	79.8	80.2	79.8
C.wrighti(15)	72.6	72.1	78.6	78.2	77.8	78.8	78.1	82.5	82.8	82.4	84.1	83.8	81.6	96.1	100	78.0	78.5	80.0	80.0	81.0	81.2	81.5
B.orientalis(16)	64.0	65.0	67.8	67.8	67.0	67.4	67.2	70.4	70.7	70.3	71.6	70.8	71.7	71.6	72.9	100	98.2	75.4	76.0	76.8	78.3	77.0
P.americana(17)	64.9	65.1	68.4	68.4	67.8	68.6	68.4	71.2	71.4	71.1	72.3	71.9	72.1	72.4	73.7	97.8	100	76.1	76.3	77.6	79.0	77.8
B.germanica(18)	65.0	65.8	69.7	69.7	69.2	69.0	68.2	66.5	66.7	66.5	68.2	71.7	70.4	73.4	74.1	69.2	69.7	100	94.1	94.3	94.6	93.6
Symploce sp.(19)	64.2	64.7	68.4	68.4	68.1	67.9	67.2	66.7	66.9	66.7	68.4	70.9	70.6	73.1	73.6	69.1	69.6	92.6	100	93.6	93.6	93.3
A.kyotensis(20)	64.8	65.3	69.2	69.2	69.0	68.7	68.0	66.7	67.0	66.7	68.2	71.2	70.7	73.4	74.1	69.2	69.7	92.6	91.4	100	96.3	92.6
P.couloniana(21)	65.9	65.7	69.9	69.9	70.6	69.6	68.9	67.2	67.4	67.2	69.1	71.1	71.1	74.1	74.6	70.9	71.4	91.9	91.1	94.3	100	93.1
I.deropeltiformis(22)	65.9	66.7	69.9	69.9	68.6	69.4	68.9	66.9	67.2	67.2	68.4	71.9	71.4	73.8	74.6	70.4	70.9	90.1	89.1	89.6	90.4	100



3.4.4 Phylogenetic analysis of AKHRs in termites

Phylogenetic reconstructions of 22 AKHR sequences are depicted in Fig. 5. The evolution of AKHR largely reflects the accepted view of termites and cockroach lineage diversification (Figure 5).

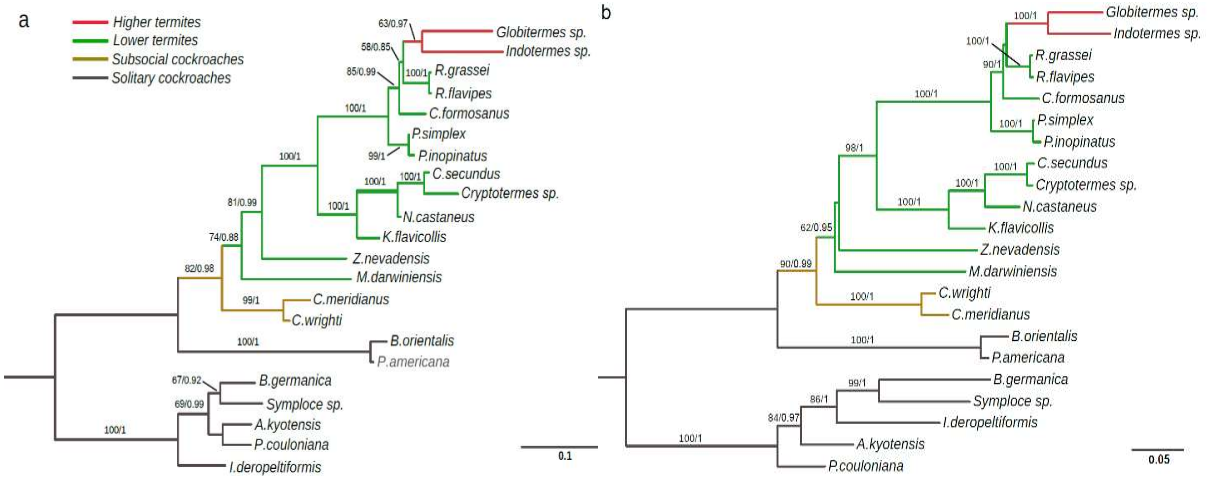


Figure 5. Phylogenetic reconstruction of adipokinetic hormone receptors (AKHRs) in termites and cockroaches using amino acids (a) or nucleotides (b). Numbers at each node represent bootstrap support values/posterior probabilities (ML/BI respectively). Different colors indicate traditional classifications of termites and cockroaches (Evangelista et al., 2019). Bootstrap support/Posterior probability values above 50/0.8 are displayed.

The topologies of the ML and BI phylogenetic trees are identical. Five AKHRs from Blaberidae cluster together as a monophyletic group. *B. orientalis* and *P. americana* in the Blattidae family are closely clustered with high support and *Cryptocercus* occurs as the nearest lineage to the termites. Within the termites, *Mastotermes*, *Zootermopsis* and Kalotermitidae occur in expected positions in the phylogenies, as do the AKHR sequences belonging to the Neoisoptera (Rhinotermitidae + Termitidae). The rhinotermitids *Reticulitermes* and *Coptotermes* are paraphyletic with respect to Termitidae (instead of appearing as a monophyletic clade). But overall the evolution of AKHR in termites and cockroaches mostly reflects the accepted view of termite lineage diversification and the majority of ancestral nodes receive unequivocal support.

3.4.5 Predicted AKHR post-translational modifications

Post-translational modifications (PTMs) and their potential interactions contribute significantly to the biological functions of proteins, and GPCRs are an important class of proteins that are regulated by PTMs. To investigate the putative post-translational modifications of AKHRs in termites, three modifications, glycosylation, myristoylation and phosphorylation were explored. The results reveal that all 22 species contain putative glycosylation sites in at least two domains (in the ECL1 and C-terminus, Figure 4). Among them, cockroaches in the Blattidae family (*B. orientalis* and *P. americana*) have the most widely distributed glycosylation sites, being found in the following domains: N-terminus, TM1, ECL1, ECL2, ICL3 and the C-terminus.

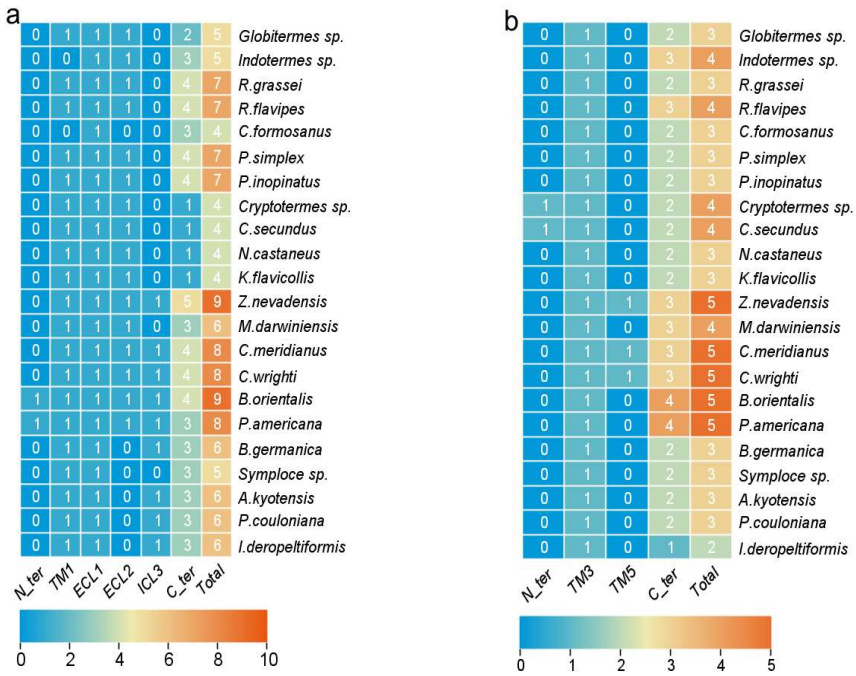


Figure 6. Distribution of putative glycosylation (a) and myristoylation sites (b) from AKHRs in 22 blattodean species (13 termite and 9 cockroach species). ICL: Intracellular loop; ECL: Extracellular loop; N_ter: N-terminus; C_ter: C-terminus.

Two subsocial cockroaches in the genus *Cryptocercus* (*C. pudacoensis* and *C. meridianus*) and in Archotermopsidae (*Z. nevadensis*) have five glycosylation domains in the TM1, ECL1, ECL2, ICL3 and C-

terminus. By contrast, *C. formosanus* contain two (ECL1 and C-terminus) and *Indotermes sp.* only three (ECL1, ECL2 and C- terminus). All 22 species contain one glycosylation site in the ECL1. In addition, all cockroach species, except *Symploce sp.,* and *Z. nevadensis* contain one glycosylation site in ICL3 (Figure 6a).

In terms of myristoylation sites, all AKHR sequences contained at least two putative domains in TM3 and the C-terminus. In addition, *Cryptotermes sp.* and *C. secundus* (Kalotermitidae) contained an additional site localized in the N-terminus, while *C. pudacoensis*, *C. meridianus* and *Z. nevadensis* (*Cryptocercus* + *Archotermopsidae*) contained a further myristoylation site in the TM5 (Figure 6b).

Three putative phosphorylation sites (serine, threonine and tyrosine residues) were quantified in the intracellular domains. In ICL1, two sites in *B. orientalis* and *P. americana* (Blattidae), *N. castaneus*, *C. secundus, Cryptotermes sp.* and *K. flavicollis* (Kalotermitidae) were detected. One site was found in *R. grassei* and *R. flavipes* (Rhinotermitidae) and *Globitermes sp.* (Termitidae). In ICL2, two sites were found for *A. kyotensis* and *P. couloniana*, and one site was detected in *B. orientalis*, *P. americana*, *B. germanica*, *I. deropeltiformis, C. pudacoensis*, *C. meridianus*, *M. darwiniensis* and *Z. nevadensis*. In ICL3, four or five sites in all roach species were recovered, as well as in *Z. nevadensis* and all kalotermitid species, while other termites possessed six sites. Regarding the C-terminus, *P. simplex* contained the most sites, followed by *P. inopinatus*. With respect to putative kinase phosphorylation site varieties, *M. darwiniensis* contained the highest number of phosphorylation sites in ICL and in the C-terminus (Figure 7a, b).

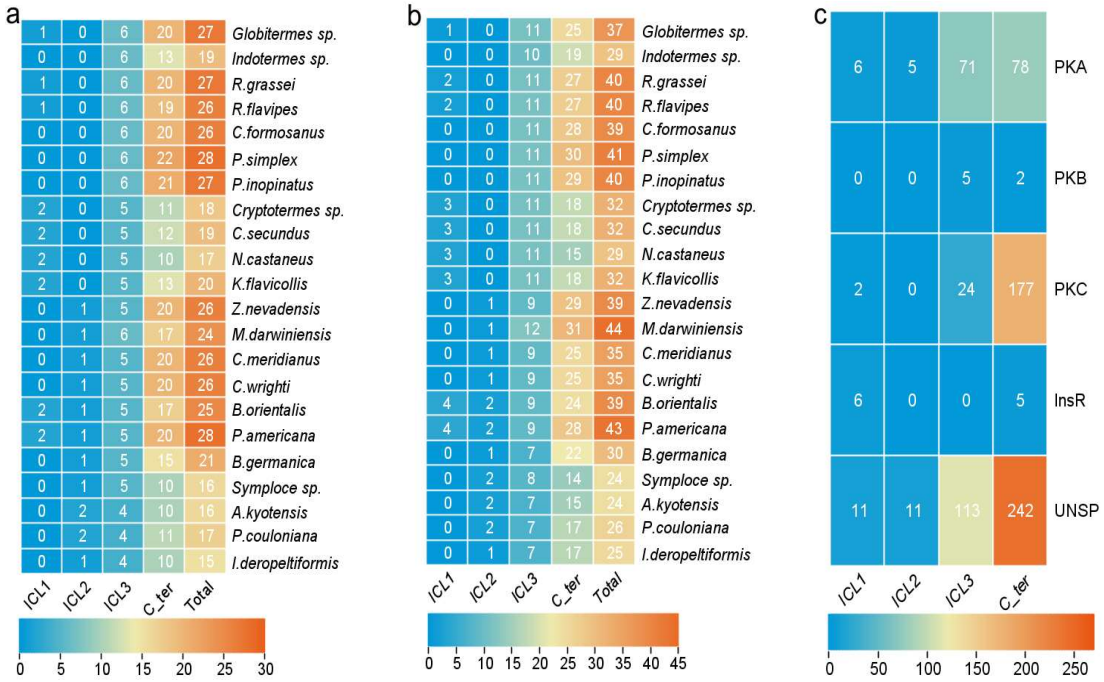


Figure 7. Phosphorylation features of cockroach and termite AKHR intracellular domains. (a) distribution of phosphorylation sites (Ser, Thr and Tyr); (b) varieties of putative protein kinase phosphorylation sites and (c) their distribution. PKA: Protein kinase A; PKB: Protein kinase B; PKC: Protein kinase C; InsR: Insulin receptor; UNSP: Unspecified kinase; ICL: Intracellular lo op; C_ter: C-terminus.

Further analysis of specific kinases indicates that putative PKA and UNSP sites are distributed among three ICL domains and the C-terminus. Most protein kinase phosphorylation sites were found in the C-terminus, while PKB, PKC, and InsR were restricted to two or three ICL domains. Termite species possess up to 4 additional protein kinase phosphorylation sites (except *Z. nevadensis*) in the ICL3 compared with wood and solitary cockroaches (Figure 7c, Supplementary Table 3), while they have lost 1 such site in the ICL 2 (except *M. darwiniensis* and *Z. nevadensis*) compared to wood and solitary cockroaches. Otherwise, PKA, PKC, and UNSP sites were regularly predicted to occur in both the ICL3 and the C-terminus of all 22 species, indicating their potentially conserved role in intracellular signaling among blattid AKHRs.

3.5 Discussion

We present sixteen structurally different mature AKH peptides from 90 species distributed across a wide range of cockroach and termite families. Evolutionary trees based on phylogenetic analyses using AKH precursor sequences reveal a family of novel putative decapeptides unique for Blaberoidea. Phylogenetic trees indicate that an ancient duplication of AKH genes occurred in the ancestor of this group, yielding the previously described Bladi-HrTH on the one hand, and a group of novel decapeptides on the other. The decapeptide AKH, Bladi-HrTH, was until now the only decapeptide to have been reported from Blattodea (Gäde and Rinehart, 1986; Hayes et al., 1986). Our study identifies seven putative novel decapeptides, significantly increasing the known diversity of decapeptides in Blattodea. The remaining AKHs of Solumblattodea are found to be solely octapeptides in nature, with Peram-CAH-I (pQVNFSPNWamide) accounting for the largest proportion in this clade. Further species with two AKH peptides were also identified in Blattidae, Tryonicidae and Corydiidae. Phylogenies suggest that at least one parallel duplication event also took place at some point during the evolution of the Solumblattodea, although statistical support is low and inconsistently retrieved between nucleotide and amino acid-based analyses.

The presence of more than one mature AKH in a single species may indicate that each AKH plays a role in different regulatory processes (Goldsworthy et al., 1997; Kaufmann and Brown, 2008; Bártů et al., 2010). Indeed, the retention of both AKH genes in many extant species found across Blaberoidea suggests that both peptides serve an important adaptive purpose, potentially serving different physiological functions in these insects. While the functional significance of AKH peptide duplicates in Blaberoidea, and potentially also Solumblattodea, remain under investigation, we hypothesize that these may be related to the capacity of cockroaches to live in diverse and challenging niches, where adaptive responses to diverse pathogens, toxins or fluctuating environments may have necessitated important alterations to AKH-mediated energy, stress or starvation responses.

Multiple AKHs have been reported in other insects, such as one decapeptide and two octapeptides in the migratory locust, *Locusta migratoria* (Stone et al., 1976; Siegert et al., 1985; Oudejans et al., 1991). One octapeptide and two decapeptides are also described from the African froghopper, *Ptyelus flavescens* (Gäde et al., 2017), while certain grasshoppers have three octapeptides (Gäde, 2006) and even five mature peptides (octa-, nona- and decapeptides) have been isolated and sequenced from two species of the lepidopteran genus *Hippotion* (Gäde et al., 2013) . We also confirm the presence of Manto-CC in termites, being previously only recorded from the order Mantophasmatodea (Gäde et al., 2005), and also recently verified by mass spectrometry in the termite *K. flavicollis* (Marco et al., 2022). Another surprising discovery from the current work is that Pyrap-AKH is also present in cockroaches, where previously it had been found in Hemiptera (Kodrik et al., 2000; Gäde and Marco, 2022), caeliferan Orthoptera (Gäde, 2006) and in some beetle species (Gäde et al., 2019).

A study by Evangelista et al. (2019) provides a recent phylogenomic analysis of Blattodea, while a further study analyzing 17 neuropeptide precursor sequences (AKH precursor sequences were not included) revealed consistent topologies (Bläser et al., 2020). Our evolutionary trees based on AKHR genes were broadly in agreement with accepted topologies. An interesting question to ponder here is how to interpret divergent versus conserved patterns of ligand and receptor gene evolution, respectively. Multiple ligand binding may be permitted by the availability of large AKHR binding pockets which could facilitate promiscuous receptor-ligand interactions (Schwartz, 1994; Rios et al., 2001; Zhu et al., 2009; Venkatakrishnan et al., 2013; Stank et al., 2016; Marchal et al., 2018; Marco and Gäde, 2019). A recent study by Jackson et al. (2019) determined the structure of three AKHs in the desert locust, *Schistocerca gregaria* using NMR (Nuclear magnetic resonance) techniques, finding that they interact with the same receptor residues despite having varying chain lengths and sequences. Such flexibility could depend on the receptor's ligand affinity properties, the intrinsic stability of receptor states, and long-range allosteric coupling dynamics between the binding pocket and receptor regions residing on the cytoplasmic side (Möller et al., 2001; Chen et al., 2020; Cong et al., 2022; Xia et al., 2022).

We next chose to examine predicted posttranslational modification signatures in AKHR. We explored this by characterizing putative glycosylation, myristoylation and phosphorylation sites in corresponding TM regions, as well as intra- and extracellular domains of the AKHR amino acid sequence. A principle aim in this analysis was to explore whether patterns of posttranslational modification differed between social termites and their solitary cockroach relatives. There are some patterns of note, such as 1 fewer and up to 4 additional protein kinase phosphorylation sites in the ICL2 and ICL3 of termites, compared with cockroaches. Neoisopteran sequences also appear to possess an additional predicted PKA site and an additional UNSP site in ICL3 compared with other termites and

cockroaches. Kalotermitids (dry wood termites) appear to have fewer putative sites of modifications in general, which may reflect a truncated C-terminus in these species. A study by Yang et al. (2018) implied potential differences in posttranslational modification of AKHRs between solitary and social bees. Aside from the minor changes outlined above, our study did not detect obvious phosphorylation site pattern differences between social termites and subsocial or solitary cockroaches. This is perhaps surprising, given the important role of posttranslational modification in diverse functions including protein interaction and stability, signaling, β-arrestin recruitment and receptor trafficking (Withers and Dong, 2017; Chou, 2020).

Bioactive neuropeptides and their GPCRs in insects are under investigation for their potential use as a more environmentally friendly alternative to conventional pesticides. This concept of a "green insecticide" is based on the potentially disruptive consequences of interference at the level of neuropeptide-cognate receptor binding, as discussed elsewhere (Gäde and Goldsworthy, 2003; Whetstone and Hammock, 2007; Gäde et al., 2017). One of the first steps towards identifying such a lead for the chemical development of an AKH peptide mimetic with which to disrupt normal endocrine signaling in pivotal metabolic pathways in so-called pest insects, is to ascertain the complement of AKHs in insects and to establish whether cross-activity of a ligand is possible also in other insects.

In the case of Blattodea, cockroaches and termites are ecologically important detritivores, especially in the subtropical and tropical regions where they recycle wood and plant matter and represent a major driver of carbon cycles (Ulyshen, 2016; Bignell, 2019). Nevertheless, approximately 10 % of termite species are considered severe pest insects where they infect and feed on dry timber in man-made structures and are very costly to combat world-wide (Su and Scheffrahn, 2000; Khan and Ahmad, 2018). Furthermore, less than 1% of cockroach species are recognized as omnivorous pest insects, when they come into close contact with human habitation (Cochran, 1999), presenting potentially serious health threats (Rosenstreich et al., 1997). Table 1 reveals that the AKH octapeptide, Peram-CAH-I, is not pest-insect specific, being shared by 36 cockroach and termite species. In fact, Peram-CAH-I is prevalent also in other insect orders, such as Hemiptera (Gäde and Marco, 2022), Coleoptera (Gäde et al., 2019) and Archaeognatha insects (Marco et al., 2014), and is thus, not a suitable lead compound as cross-activity is likely to occur across a wide spectrum of insects. The remaining 6 octapeptide AKHs so far detected in the Blattodea are also not unique (Gäde, 2009), except for Polae-HrTH, which is synthesized in the CC of only 2 cockroach species (Table 1). However, our current study has uncovered a potential wealth in decapeptide AKHs, characterized by both *in silico* approaches as well as Sanger sequencing in the case of *B. germanica*: Table 1 lists a total of 8 decapeptides, 7 of which are novel structures, and all are present only in the Blaberoidea. These AKHs may be sufficiently different to warrant such a decapeptide as a lead peptide. The strategy is clear and partially shown for

the locust pair of AKHs and the cognate AKH receptor where a non-peptide (mimetic) has already been found to act as a competitive substance on the receptor (Jackson et al., 2022), and a case study in which a mimetic could bind the locust AKH receptor but not that of the honeybee (Abdulganiyyu et al., 2020).

Co-application of AKH with pathogens could yet further enhance these effects by interfering with the immune response in some insect species (Adamo et al., 2008; Ibrahim et al., 2017; Ibrahim et al., 2018; Gautam et al., 2020), potentially making them more susceptible to pathogen-mediated control, which in termites has proven difficult to implement, in part due to their social immune defense traits, which prevent the spread of infectious disease (He et al., 2018; Davis et al., 2018 Sep 26; Liu et al., 2019; Hassan et al., 2021).

In conclusion, our study provides valuable comparative data, not only for further research exploring the interactions between AKH and AKHR in termites and cockroaches, but also in providing necessary sequence information for functional research in an economically and scientifically important group of non-model insects, with potential applications in the development of mimetic or RNAi-based approaches to pest control. The data also constitute an important molecular framework for future studies seeking to exploit neuropeptides as a sustainable means of controlling globally important termite and cockroach pest organisms.

3.6 Acknowledgements

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Supplementary Data S1. List of AKH precursor and AKHR transcripts

- **Main retrieved two databases:**
- **1KITE:** The 1K Insect Transcriptome Evolution project (Accession: PRJNA183205).
- **He et al., 2021 :** Evidence for reduced immune gene diversity and activity during the evolution of termites. *Proc. Royal. Soc. B*. All raw data were retrieved under BioProject PRJNA635910 (doi: 10.1098/rspb.2020.3168).

List of AKH precursor transcripts

1KITE

AKH precursor	Accession
Anallacta methanoides AKH1	GEAA01034852
Anallacta methanoides AKH2	GEAA01033466
Asiablatta kyotensis AKH1	GDXG01041686
Asiablatta kyotensis AKH2	GDWW01026549
Balta vilis AKH1	GDYJ01059856
Balta vilis AKH2	GDYJ01061754
Blaberus atropos AKH1	GAYD02019017
Blaberus atropos AKH2	GAYD02026214
Blaberus discoidalis	GAYD02026214
Cariblatta sp. *AKH1*	GDZD01016829
Cariblatta sp. *AKH2*	GDZD01021355
Coptotermes sp.	GDUG01021295
Diploptera punctata	GDYG01003308
Ectobius sylvestris AKH1	GDYP01020277
Ectobius sylvestris AKH2	GDYP01009813
Ellipsidion sp. *AKH1*	GDYE01029971
Ellipsidion sp. *AKH2*	GDYE01050121
Eucorydia yasumatsui	GDZF01058490
Eurycotis floridana AKH1	GDYS01020692
Eurycotis floridana AKH2	GDYS01015750
Euthlastoblatta diaphana AKH1	GDZY01056148
Euthlastoblatta diaphana AKH2	GDZY01037853
Incisitermes marginipennis	GDBO01039354
Ischnoptera deropeltiformis AKH1	GDEC01005355
Ischnoptera deropeltiformis AKH2	GDEC01005651
Lamproblatta albipalpus	GCPS01030658
Loboptera decipiens AKH1	GDYK01028596
Loboptera decipiens AKH2	GDYK01039830
Lobopterella dimidiatipes	GDZZ01010562
Methana parva AKH1	GDWT01019620

Methana parva AKH2	GDWU01009321
Nocticola sp.	GDYB01030609
Nyctibora sp. *AKH1*	GDZE01036511
Nyctibora sp. *AKH2*	GDZE01037773
Panchlora nivea AKH1	GDWT01017866
Panchlora nivea AKH2	GDWQ01030735
Polyphaga aegyptiaca AKH1	GDWQ01027869
Polyphaga aegyptiaca AKH2	GDWJ01041318
Princisia vanwaerebeki	GDXP01038928
Prorhinotermes simplex	GASE02008684
Shelfordella lateralis AKH1	GDXP01063135
Shelfordella lateralis AKH2	GDYB01025373
Sundablatta sexpunctata AKH1	GDCJ01021870
Sundablatta sexpunctata AKH2	GDCJ01023743
Supella longipalpa AKH1	GDWU01040712
Supella longipalpa AKH2	GDWV01020684
Symploce sp. *AKH1*	GDCG01029502
Symploce sp. *AKH2*	GDCG01032358
Therea bernhardti	GDWJ01037881
Tivia sp.	GDYD01055356
Tryonicus parvus AKH1	GDWV01021169
Tryonicus parvus AKH2	GDWW01024887

He et al., 2021

AKH precursor	Subcomponent ID
Blatta orientalis AKH1	TRINITY_DN151893_c0_g1_i1
Blatta orientalis AKH2	TRINITY_DN270262_c0_g1_i1
Bulbitermes sp.	TRINITY_DN32992_c0_g2_i2
Cryptocercus meridianus	TRINITY_DN14030_c0_g1_i1
Cryptocercus pudacoensis	TRINITY_DN22634_c0_g1_i1
Dicuspiditermes sp.	TRINITY_DN30601_c0_g1_i2
Globitermes sp.	TRINITY_DN40897_c9_g1_i1
Indotermes sp.	TRINITY_DN32911_c0_g1_i1
Macrotermes subhyalinus	TRINITY_DN36462_c0_g2_i1
Mastotermes darwiniensis	TRINITY_DN47840_c0_g1_i1
Pericapritermes sp.	TRINITY_DN16240_c0_g1_i1
Periplaneta americana AKH1	TRINITY_DN36869_c0_g1_i2
Promirotermes sp.	TRINITY_DN23589_c0_g1_i2
Prorhinotermes inopinatus	TRINITY_DN65934_c0_g4_i3
Zootermopsis nevadensis	TRINITY_DN62966_c0_g1_i1

NCBI

AKH precursor	Accession
Blattella germanica	FJ943774.1
Periplaneta americana AKH2	AAV41425.1

AKH precursor sequences generated as part of this study

AKH precursor	Accession
Blattella germanica AKH2	OR134621
Cryptotermes sp.	OR134622
Kalotermes flavicollis	OR134623

Blattella germanica AKH precursor 2

Sanger reads (this study, GenBank accession: OR134621)

ATGAATTTCAAGTTGATCTGCATAATAAACACCATCGTCGTGGTGGTGACAGTGTTTTTGGTAACATGTGAAGCT
CAACTCAATTTTTCTCCGGGTTGGGGTCCTGGGAAGCGATCAGGACTTCAAGATGGACCATGCAAGCCTTCTG
ATGCTCTTATGCACATCTACAGACTAGTTCAGAGTGAAGTACAGAAATTGGCAGAGTGTGAGAAATTCGGGTCA
AATTGA

Amino acid sequence

MNFKLICIINTIVVVVTVFLVTCEAQLNFSPGWGPGKRSGLQDGPCKPSDALMHIYRLVQSEVQKLAECEKFGSN

Cryptotermes sp. AKH precursor

Sanger reads (this study, GenBank accession: OR134622)

ATGAGTTGTTTGGCGAAGACCCTGCTTGTAGTCGTGGCTTTGTTCCTCGTGTTCTGTGAAGCCCAGGTTAACTTT
TCACCCAACTGGGGCAAGAGGTCAGGTCTTCAGGATGGGCCATGCAAAGCGTCTACAGATTCCCTCATGTAC
ATATACAAACTCATCCAGAACGAAGCTCAGAAACTGCTAGAATGTGAAAAATTTGGAGCAAATTAA

Amino acid sequence

MSCLAKTLLVVVALFLVFCEAQVNFSPNWGKRSGLQDGPCKASTDSLMYIYKLIQNEAQKLLECEKFGAN

Kalotermes flavicollis AKH precursor

Sanger reads (this study, GenBank accession: OR134623)

ATGAGCTGCATGGCTAAGACCCTCCTTGTTGTCGTAGCATTGGTCCTTGTGCTCTGTGAGGCCCAGGTGAACT
TCTCACCCGGGTGGGGCAAGAGATCAACTCTCCAGGACGGGCCATGCAAGACATCTACTGAATCCCTCATGT
ACATCTATAAACTGATCCAGAATGAAGCACAGAAACTGGTGGAATGTGAGAAATTTGGAGCAAATTAA

Amino acid sequence

MSCMAKTLLVVVALVLVLCEAQVNFSPGWGKRSTLQDGPCKTSTESLMYIYKLIQNEAQKLVECEKFGAN

List of AKHR transcripts

1KITE

AKHR	Accession
Asiablatta kyotensis	GDWW01040593.1
Ischnoptera deropeltiformis	GDEC01019903.1
Paratemnopteryx couloniana	GDZI01047139.1
Symploce sp.	GDCG01067335.1

He et al., 2021

AKHR	Subcomponent ID
Blatta orientalis	TRINITY_DN190211_c8_g1_i1
Cryptocercus wrighti	TRINITY_DN12829_c0_g1_i1
Globitermes sp.	TRINITY_DN40470_c9_g10_i1 TRINITY_DN40470_c9_g2_i1
Neotermes castaneus	TRINITY_DN278844_c6_g2_i1
Reticulitermes grassei	TRINITY_DN15269_c3_g1_i1

AKHR sequences generated as part of this chapter

AKHR	Accession
Coptotermes formosanus partial cds	OR134611
Kalotermes flavicollis partial 1 cds	OR134612
Kalotermes flavicollis partial 2 cds	OR134613
Mastotermes darwiniensis partial 1 cds	OR134614
Mastotermes darwiniensis partial 2 cds	OR134615
Cryptotermes sp. *complete cds*	OR134616
Prorhinotermes inopinatus partial cds	OR134617
Reticulitermes flavipes partial cds	OR134618
Cryptocercus meridianus partial cds	OR134619
Indotermes sp. *partial cds*	OR134620

Supplementary Data S2. Query steps for searching reference datasets for AKH and AKHR sequences.

Code adapted from He et al. (2020). (doi: 10.1007/978-1-0716-0259-1_2)

#The code in “Database_build.sh”:

```
    #align sequences by AKH and AKHR gene family in clustal output format, and cat all alignment into one file
    for i in {"AKH","AKHR"};\
    do clustalo -i $i.fasta -o $i.clustalo.alin --outfmt=st;\
    sed "1a\#=GF ID $i" $i.clustalo.alin >> receptordb.sto;\
    done
    #build a hmm profile for hmm search
    hmmbuild receptordb.hmm receptordb.sto
    #build a corresponding database for all AKH and AKHR family sequences
    cat .fasta > receptordb.fa
    makeblastdb -in receptordb.fa -dbtype prot
    #get sequence-id to gene family-id.
    for i in {"AKH","AKHR"}; do awk -v "a=$i" '$1~/^>/{gsub(">","",$1); print $1"\t"a}'
                                $i.fasta >> seq2family;done
```

#The code in “Predict_script.sh”:

```
    #hmmsearch, blastp hmmdatabase, combine files, blastp uniprot database, combine all search output together
    for fasf in {"SPECIESNAME"}; do
    hmmsearch --tblout $fasf.target_out.tab --noali --notextw -E 1e-5 --domE 1e-3 --incE
                                0.001 --cpu 4 receptordb.hmm
                                "$fasf"_Trinity.fasta.transdecoder.pep;\
    blastp -query "$fasf"_Trinity.fasta.transdecoder.pep -db receptordb.fa -num_threads 4 -
                                evalue 1e-5 -max_target_seqs 1 -outfmt 6 -out
                                "$fasf"_receptordb.blastp.outfmt6;\
    sed '/^#/d' $fasf.target_out.tab |sed 's/  */ /g'|cut -f1,3,5,8 -d " " |sed 's/ /\t/g' >
                                $fasf.hmm.out;\
    awk 'NR==FNR{a[$1]=$2;next}{print $1 "\t" a[$2]}' seq2family
                                "$fasf"_receptordb.blastp.outfmt6 |sort -u| awk
                                'NR==FNR{a[$1]=$2;next}$2==a[$1]{print $0"\t" a[$1]}' -
                                $fasf.hmm.out > $fasf.hmm.blastp.out;\
    done
```

#Run "Database_build.sh"

```
$ ./Database_build.sh
```

#Run "Predict_script.sh"

```
$ ./Predict_script.sh
```

The subcomponent ID of predicted proteins are in the "SPECIESNAME.hmm.out" file.

Supplementary Table S1. GenBank accession IDs for AKH and AKHR datasets used to search assembled transcriptome data

Species	Accession IDs	Note
Apis mellifera	AEW68342.1	AKH
Aptera fusca	P85533	
Blatta orientalis	P84261	
Blattella germanica	P84220	
Bombus lantschouensis	QGN75352.1	
Cinara cedri	VVC28311.1	
Drosophila hydei	XP_023165811.1	
Eublaberus distanti	P85618	
Eurycotis floridana	P85634	
Frankliniella occidentalis	KAE8738746.1	
Gyna caffrorum	P85849	
Hodotermopsis sjostedti	AML80823.1	
Locusta migratoria	CAA60494.1	
Locusta migratoria	CAA60495.1	
Locusta migratoria	CAA60496.1	
Lucilia cuprina	KNC31651.1	
Lygus hesperus	QQW38901.1	
Lygus hesperus	QQW38902.1	
Mastotermes darwiniensis	AML80824.1	
Nilaparvata lugens	AFN26934.1	
Odontotermes formosanus	AML80833.1	
Reticulitermes flavipes	AML80831.1	
Rhodnius prolixus	ACY09071.1	
Schistocerca gregaria	1917247A	
Schistocerca gregaria	1917247B	
Schistocerca gregaria	1906177A	
Schistocerca nitens	AAA73932.1	
Schistocerca nitens	AAA73931.1	
Teleopsis dalmanni	XP_037941785.1	
Zootermopsis nevadensis	AML80834.1	
Anopheles gambiae	ABD60146.1	AKHR
Apis mellifera	NP_001035354.1	
Bactrocera dorsalis	AQX83416.1	
Blattella germanica	ADL60118.1	
Bombyx mori	NP_001037049.1	
Carausius morosus	QRN45460.1	
Chilo suppressalis	ALM88332.1	
Drosophila melanogaster	NP_001260149.1	
Frankliniella occidentalis	KAE8753142.1	
Glossina morsitans	AEH25943.1	
Gryllus bimaculatus	ADZ17179.1	

Hylobius abietis	AVI00624.1	AKHR
Locusta migratoria	ANW09575.1	
Manduca sexta	XP_030030231.1	
Nasonia vitripennis	XP_032454667.1	
Nilaparvata lugens	AZP54622.1	
Ostrinia furnacalis	AXF67446.1	
Panstrongylus lignarius	JAW11038.1	
Periplaneta americana	ABB20590.1	
Polyrhachis vicina	ADK55068.1	
Pyrrhocoris apterus	ARV86499.1	
Rhodnius neglectus	JAI53178.1	
Rhodnius prolixus	AIJ49751.1	
Rhynchophorus ferrugineus	QGA72493.1	
Sarcophaga crassipalpis	AOC38019.1	
Schistocerca gregaria	AVG47955.1	
Tribolium castaneum	ABN79650.1	

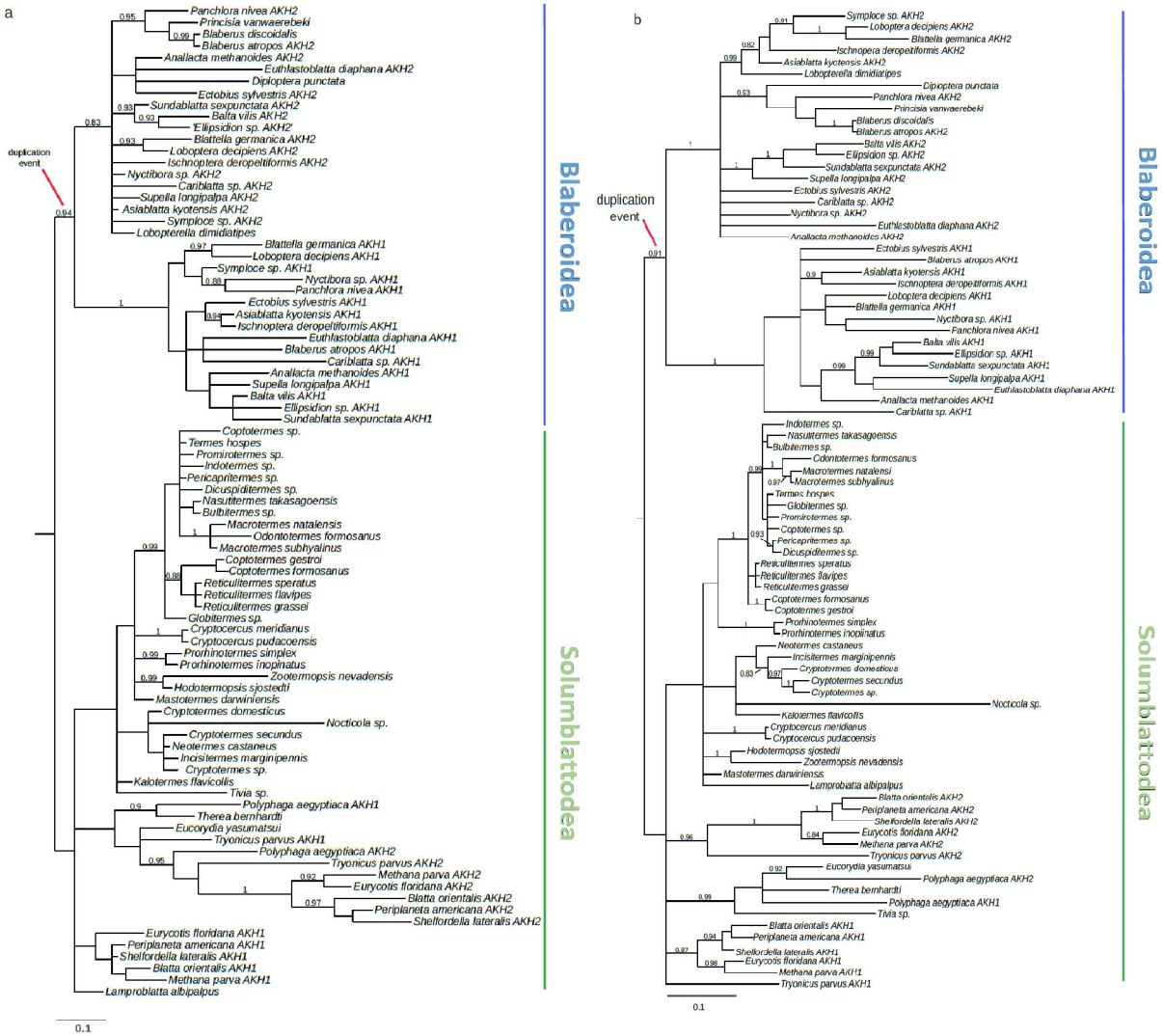
Supplementary Table S2. Primer sequences used in this chapter

Name	Direction	Sequence 5'-3'	Note
Coptotermes formosanus	Forward	TCATKGACGGYCAYTTCCG	
	Reverse	GGTTATTGCCGAAGCGAAGG	
Kalotermes flavicollis	Forward	CAAACAAATTCCTCGCTCAGG	
	Reverse	TCCCGAATTACGTTGAGTTGG	
Mastotermes darwiniensis	Forward	ATGTGTCTGTGGTACTGGAT	
	Reverse	TGGTGCCTCAGCAAAGTCGT	
Cryptotermes sp.	Forward	GCATGGCGACCCCGACAACAG	
	Reverse	CAVATTCACCTGYAGATRACAG	
Prorhinotermes inopinatus	Forward	TCATKGACGGYCAYTTCCG	AKHR
	Reverse	CGTAGTADGGBGTCCARCAGAT	
Reticulitermes flavipes	Forward	CGTGGTCTGCAGTGAAGGCAT	
	Reverse	CTATTGCCGAAGCGAAGAGC	
Cryptocercus meridianus	Forward	ATTAGTCGGAAGAAACCTTG	
	Reverse	CTCCTCGTCGATCCAATAC	
Indotermes sp.	Forward	AASTTGCCAGWTCATKGACG	
	Reverse	CAVATTCACCTGYAGATRACAG	
Kalotermes flavicollis	Forward	GGAAAGGCTTGGGCATTGAG	
	Reverse	TCAGAGCAACAGAACGTACT	
Cryptotermes sp.	Forward	CACACTCGACAGTCACTGGAA	AKH
	Reverse	CAGAAATGGACGCACAAAGAT	
Blattella germanica	Forward	AGCGCCACAGTGAACTAATTG	AKH1
	Reverse	GGCCCAAGCACAGTTATTTCT	

Supplementary Table S3. Phosphorylation sites by Protein kinase A (PKA), Protein kinase B (Akt/PKB), Protein kinase C (PKC), Insulin receptor (InsR) and unspecified kinases (UNSP) in termites and cockroaches.

Species	ICL1					ICL2					ICL3					C_ter				
	PKA	**PKB**	**PKC**	**InsR**	**UNSP**	**PKA**	**PKB**	**PKC**	**InsR**	**UNSP**	**PKA**	**PKB**	**PKC**	**InsR**	**UNSP**	**PKA**	**PKB**	**PKC**	**InsR**	**UNSP**
Globitermes sp.	0	0	0	0	1	0	0	0	0	0	4	0	1	0	6	4	0	10	0	11
Indotermes sp.	0	0	0	0	0	0	0	0	0	0	4	0	1	0	5	4	0	10	0	11
R. grassei	0	0	0	1	1	0	0	0	0	0	4	0	1	0	6	5	0	10	0	12
R. flavipes	0	0	0	1	1	0	0	0	0	0	4	0	1	0	6	5	0	10	0	12
C. formosanus	0	0	0	0	0	0	0	0	0	0	4	0	1	0	6	3	0	10	0	15
P. simplex	0	0	0	0	0	0	0	0	0	0	4	0	1	0	6	5	0	11	0	14
P. inopinatus	0	0	0	0	0	0	0	0	0	0	4	0	1	0	6	5	0	10	0	14
Cryptotermes sp.	1	0	0	1	1	0	0	0	0	0	3	1	2	0	5	3	1	7	0	6
C. secundus	1	0	0	1	1	0	0	0	0	0	3	1	2	0	5	2	1	6	0	8
N. castaneus	1	0	0	1	1	0	0	0	0	0	3	1	2	0	5	1	0	6	0	8
K.flavicollis	1	0	0	1	1	0	0	0	0	0	3	1	2	0	5	1	0	9	0	8
Z. nevadensis	0	0	0	0	0	0	0	0	0	1	3	0	1	0	5	5	0	10	0	13
M. darwiniensis	0	0	0	0	0	0	0	0	0	1	3	1	2	0	6	5	0	11	1	14
C. meridianus	0	0	0	0	0	0	0	0	0	1	3	0	1	0	5	3	0	8	1	13
C. wrighti	0	0	0	0	0	0	0	0	0	1	3	0	1	0	5	4	0	6	1	14
B. orientalis	1	0	1	0	2	1	0	0	0	1	3	0	1	0	5	3	0	10	0	11
P. americana	1	0	1	0	2	1	0	0	0	1	3	0	1	0	5	4	0	11	0	13
B. germanica	0	0	0	0	0	0	0	0	0	1	2	0	1	0	4	4	0	5	1	12
Symploce sp.	0	0	0	0	0	1	0	0	0	1	2	0	1	0	5	2	0	4	1	7
A. kyotensis	0	0	0	0	0	1	0	0	0	1	3	0	0	0	4	3	0	5	0	7
P. couloniana	0	0	0	0	0	1	0	0	0	1	3	0	0	0	4	4	0	4	0	9
I. deropeltiformis	0	0	0	0	0	0	0	0	0	1	3	0	0	0	4	3	0	4	0	10

Supplementary Figure S1. BI phylogenetic trees of AKH preprohormones in termites and cockroaches.



Trees were reconstructed from alignments of the whole ORF sequence, using amino acids (a) or nucleotides (b). Numbers at each node represent posterior probability (PP) values with only values above 0.8 being shown.

Chapter III

Transcriptomic and peptidomic analysis of the German cockroach (*Blattella germanica*) neuropeptidome

Transcriptomic and peptidomic analysis of the German cockroach (*Blattella germanica*) neuropeptidome

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Author Contributions

JS and DPM conceived the overall idea. SJ, AC, and PR conducted transcriptomic and peptidomic analysis. JS, AC, PR drafted the methodology and analyzed the data. JS and DPM wrote the manuscript. All authors contributed critically to the drafts.

Unpublished manuscript.

4.1 Abstract

Insect neuropeptides are messenger molecules with a range of roles in physiology and behavior. Knowledge of the diversity as well as the functions of the neuropeptidome of the German cockroach (*Blattella germanica*), a major insect model organism and global pest species is incomplete, representing a significant gap in our understanding. This study aims to bridge this gap through a comprehensive analysis of the *B. germanica* neuropeptidome, combining transcriptomic and peptidomic imaging with mass spectrometry techniques. Here, *B. germanica* transcriptomes were generated and mined for sequences encoding putative peptide precursors. We were able to identify 69 neuropeptide or neurohormone precursor transcripts, including most of the known neuropeptide families. Using MALDI-TOF mass spectrometry, 79 likely bioactive mature neuropeptides and precursor sequences were confirmed, many reported for the first time in this species. In a bioassay, we further found that a recently identified novel adipokinetic hormone (AKH2), which is similar in identity to AKH1, elevates the levels of carbohydrates in *B. germanica*. Comparisons indicated carbohydrate content of females increased more than that of males when treated with the same AKH peptides, suggesting sex-specific metabolic responses. This study represents a comprehensive analysis of neuropeptide precursors and peptidomically-confirmed mature peptides from *B. germanica*, paving the way for insights into the evolutionary dynamics as well as functions of newly identified neuropeptide genes in insect physiology and behavior.

Keywords

Neuropeptides; Blattodea; *Blattella germanica*; transcriptome analyses; neuropeptidomes; Mass spectrometry; carbohydrate; insect physiology

4.2 Introduction

Neuropeptides are some of the most ancient and varied signaling molecules in multicellular animals, and are primarily produced in neurons, interneurons, and neurosecretory cells within the central nervous system (CNS), as well as in the peripheral nervous system and the endocrine cells of the intestine (Nässel 2002, Nässel and Homberg 2006, Williams 2020). Neuropeptides play pivotal roles in a wide range of behavioral and physiological processes such as feeding, learning, reproduction, stress responses, social behaviors, energy homeostasis regulation, circadian rhythm and metabolism (Grimmelikhuijzen, Leviev and Carstensen 1996, Bargmann 1998, Nässel 2000, Grimmelikhuijzen and Hauser 2012, Van Wielendaele, Badisco and Vanden Broeck 2013, Takahashi and Takeda 2015, Schoofs, De Loof and Van Hiel 2017, Nässel and Zandawala 2019). Following synthesis as prohormones, neuropeptides undergo a series of post-translational modifications (PTMs), including cleavage and PTMs such as: amidation, phosphorylation, and glycosylation, one or multiple copies of mature peptides are secreted and stored through axonal pathways into the circulatory system and in the CNS (Nässel 2002), which further regulate downstream activities by interactions with specific receptors like GPCRs (Iversen et al. 2002, Rosenkilde et al. 2003, Cazzamali et al. 2005, Caers et al. 2012), and receptor tyrosine kinases (Claeys et al. 2005, Zhang et al. 2021).

Over the last decade, identification of neuropeptide precursors has progressed significantly due to the increasing availability of transcriptomic and genomic data (Dircksen et al. 2011, Veenstra 2014, Christie 2015, Veenstra 2019, Li et al. 2020, Ragionieri and Predel 2020, Yu, Han and Liu 2020, Zhang et al. 2020, Kong et al. 2021, Zeng et al. 2021, Marciniak, Pacholska-Bogalska and Ragionieri 2022, Waldman et al. 2022, Gao et al. 2023, Li et al. 2023, Wang, Wang and Nie 2023, Alamouti et al. 2024, Huang et al. 2024). Currently, over 50 genes in insects are known to encode neuropeptides, putative neuropeptides, and protein hormones (Nässel and Zandawala 2019). The main forms of PTMs in insects include sulfation, the formation of disulfide bonds between thiol groups in two cysteine residues, N-terminal cyclization of glutamine and aspartate to pyroglutamate, and C-terminal amidation of the glycine hydroxyl group. To analyze mature peptides in tissues and accurately determine the set of processed neuroactive compounds, including PTMs, advanced instruments such as Matrix-assisted laser desorption ionization time-of-flight coupled with mass spectrometry (MALDI-TOF MS) and Q-Exactive Orbitrap MS are widely employed. In recent years, the combination of transcriptomic/genomic and MS analyses has gained popularity for the simultaneous identification of expressed neuropeptide genes as well as the examination of respective putative bioactive neuropeptide processing. Such an approach has been successfully employed in many insect species, including the beetles *Carabus violaceus*, *Carabus problematicus*, *Hylobius abietis, Tenebrio molitor* and

Zophobas atratus (Coleoptera) (Pandit et al. 2018, Ragionieri and Predel 2020, Marciniak, Pacholska-Bogalska and Ragionieri 2022), the desert locust *Schistocerca gregaria* (Orthoptera) (Ragionieri et al. 2022), the ant *Cataglyphis nodus* (Hymenoptera) (Habenstein et al. 2021), the bed bug *Cimex lectularius* (Hemiptera) (Predel et al. 2018) and the stick insect, *Carausius morosus* (Phasmatodea) (Liessem et al. 2018).

Among cockroaches, the German cockroach, *Blattella germanica*, is the most common and pervasive cockroach in urban areas, posing significant sanitary risks in hospitals and residential areas worldwide (Gore and Schal 2007, Kleine-Tebbe, Hamilton and Goodman 2019, Wang, Lee and Rust 2021, Tang et al. 2024). Additionally, it is used as a model organism in a wide range of studies, including the assessment of toxicological impacts of environmental pollutants (Adedara et al. 2022), the study of symbiotic interactions (Latorre et al. 2022) and exploring RNA interference (RNAi) due to its susceptibility to delivered dsRNA (Garbutt et al. 2013). While *B. germanica* plays a significant role in both fundamental and applied research, only a limited number of neuropeptides or their precursors, including Hypertrehalosemic neuropeptide (belonging to the Adipokinetic hormone family), Allatostatins, Orcokinins (Oks), Insulin-like peptides (ILPs), Leucomyosuppressin (LMS), Sulfakinin (SK), and Tachykinin-related peptides (TKs), have been identified or functionally investigated (Veenstra and Camps 1990, Bellés et al. 1994, Aguilar et al. 2004, Vilaplana, Castresana and Bellés 2004, Pascual et al. 2008, Huang and Lee 2011, Ons, Belles and Maestro 2015, Castro-Arnau et al. 2019, Domínguez, Pagone and Maestro 2022).

It is well known that certain species possess multiple AKH peptides, which play major roles in regulating energy metabolism. Surprisingly, despite the prevalence of multiple AKH peptides in these species, there is scarcely any research focusing on sex-specific responses to these peptides.

In this study, we combined transcriptomic and MALDI-TOF MS analyses with a functional assay in order to carry out a comprehensive analysis of the *B. germanica* neuropeptidome. Additionally, we employed predicted neuropeptidomes from one cockroach species, *P. americana* (Zeng et al. 2021), two termite species, *M. darwiniensis* (Christie 2015) and *Z. nevadensis* (Veenstra 2014) for comparative analysis within Blattodea. To address the gap in knowledge regarding sex-specific responses to AKHs, we further carried out the carbohydrate measurement bioassay. This work aims to enhance our investigation of the types and functions of neuropeptides in the German cockroach as well as lay the foundation for future research into the potential of these peptides to guide green pesticide development for use in public health.

4.3 Materials and methods

Insect samples

Blattella germanica were bred from laboratory colonies maintained at the Federal Institute of Materials Research and Testing (BAM), Berlin, Germany. *B. germanica* were housed at a constant temperature of 28 °C and subjected to a 12 h:12 h dark/light cycle, the diet consisted of a mixture containing 77.0% dog biscuit powder, 19.2% oat flakes and 3.8% brewer's yeast and supplied with water. *B. germanica* adults were used for *de novo* RNA sequencing assembly from whole brain tissue and mass spectrometry analysis.

Brain dissection

Cockroaches were anaesthetized with CO_2 for a few seconds and beheaded. The head was placed in ice-cold Phosphate Buffer Solution (PBS, composition: Sodium Chloride (mw: 58.44 g/mol) : 8 g, 0.137 M; Potassium Chloride (mw: 74.55 g/mol): 0.2 g, 0.0027 M; Sodium Phosphate Dibasic (mw: 141.96 g/mol): 1.44 g, 0.01 M; Potassium Phosphate Monobasic (mw: 136.09 g/mol): 0.245 g, 0.0018 M) and dissected under a stereoscope (Olympus BRAND). The cuticle of the head capsule was punctured above the brain region and removed to access the brain. Adipous tissue surrounding the brain was removed and the brain was gently pulled out of the cephalic capsule. Brains were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. All dissection were performed below 4°C and within 10 minutes after beheading. A total of 30 cockroach brains were dissected, each RNA sample contained a pool of 5 brains from cockroaches of the same sex and same husbandry population, resulting in 6 RNA samples.

Library construction and transcriptome sequencing

Total RNA from brain tissue was extracted using the RNeasy Plus Mini Kit (Qiagen, Germany) including a gDNA eliminator column in the manufacturer's protocol, which provided higher yields with this type of tissue. RNA was quantified on a Qubit (Invitrogen) and RNA integrity was assessed on an Agilent 2100 Bioanalyzer. RNA was then stored at -80°C until library preparation. To gain a more comprehensive neuropeptides investigation, 6 brain transcriptomic libraries were prepared. Messenger RNA (mRNA) enrichment was performed using the NEBNext® Poly(A) mRNA Magnetic Isolation Module, cDNA synthesis and further library steps were carried out with the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®, and paired-end 2x100 bp sequencing was performed on the NovaSeq 6000 with a S4 XP v1.5 Flowcell at the DRESDEN-concept Genome Center (DcGC).

***De novo* assembly and neuropeptide gene mining**

Raw sequence reads were processed to remove adapters and barcodes. Short reads (<36 bp) and low quality bases (Phred score < 33) were filtered out using Trimmomatic, as incorporated in Trinity (Bolger, Lohse and Usadel 2014). The retained reads were then used for *de novo* transcriptome assembly using Trinity (v 2.10.0) (Grabherr et al. 2011, Haas et al. 2013) with default parameters. An assembly generated from the merged six *de novo* transcriptomes was further optimized to reduce transcript redundancy and generate unique gene clusters by maintaining a 98% similarity threshold using the tool CD-hit (v4.8.1) (Fu et al. 2012). In addition, TransDecoder (v5.7.0) (https://github.com/TransDecoder/TransDecoder) was used to identify open reading frames (ORFs) of the transcripts (Minimum Aa: 45). Basic assembly metrics was evaluated with the trinity perl script TrinityStats.pl, and assembly completeness was assessed by Benchmarking Universal Single-Copy Orthologs (BUSCO v 5.1.2) (Manni et al. 2021) with the Arthropod BUSCO gene set from orthoDB resources (v10) (Kriventseva et al. 2019).

For the compiling of precursor sequences, we performed a search with the BLAST+ command line tool (Camacho et al. 2009), using sequences of known insect neuropeptide precursors as reference queries, using the recently published datasets from Blattodea: two termite species: *M. darwiniensis* and *Z. nevadensis* (Veenstra 2014, Christie 2015) and one cockroach species: *P. americana* (Zeng et al. 2021), as well as from another polyneopteran insect, *Carausius morosus* (Phasmida) (Liessem et al. 2018). The structures of mature neuropeptides and cleavage sites were initially assigned based on known cleavage sites in homologous precursors from other species or predicted following a published workflow (Veenstra 2015, Christie 2016). Specifically, each candidate precursor sequence was assessed for the signal peptide cleavage site using the online tool SignalP 6.0 Server (https://services.healthtech.dtu.dk/service.php?SignalP-6.0). Possible cysteine-cysteine disulfide bridges were predicted by homology to known peptide isoforms and/or with the online server (http://disulfind.dsi.unifi.it).

Sample preparation for Mass spectrometry

We carefully dissected the nervous tissues from the cockroach brain for direct tissue profiling. Single *B. germanica* were pinned with microneedles and submerged in insect saline (pH = 7.4; Composition: 126 mM NaCl, 5.4 mM KCl, 0.17 mM NaH_2PO_4, 0.22 mM KH_2PO_4). The head cavity was opened using fine forceps and ultrafine scissors, the brain was dissected and cut into smaller pieces, which were then placed on a sample plate, rinsed with water to minimize salt contamination and left to air dry. *Corpora cardiaca* (CC) tissue, the known site of synthesis and storage of AKH peptides, was also prepared. Afterwards, the tissues were covered with less than 0.4 µL of different matrices employed

in this investigation, namely CHCA (α-cyano-4-hydroxycinnamic acid) and DHB (2,5-dihydroxybenzoic acid) (Marciniak, Pacholska-Bogalska and Ragionieri 2022). An ultrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Germany) was used to acquire mass fingerprint (MS1).

Biological assay

Males and females were collected from breeds and housed in separate containers, with the bioassay being conducted on 5-day-old cockroaches to minimize variations across individuals. Two adipokinetic peptides, AKH1 (Bladi-HrTH: pQVNFSPGWGTa, we used the term "AKH1" instead of "Bladi-HrTH" for coherence in this study) and AKH2 (pQLNFSPGWGPa), were purchased from Pepmic Co., Ltd. (Suzhou, China) with a purity exceeding 95%, were dissolved in Ringer's solution to a final concentration of 20 pmol/µL. Each cockroach was injected with 0.5 µL of this solution using the Nanoject III™ injector (Drummond Scientific Company, USA).

Carbohydrate levels were measured by following the detailed methodology described previously (Gäde 1980, Katali, Marco and Gäde 2020). Both male and female cockroaches were selected in the study. Briefly, treatment groups were injected with either of the two AKH peptides, while the control group was injected with an equivalent volume of Ringer's solution. Following injection, cockroaches were incubated in the dark for 3 hours. Hemolymph was then collected using the two-tube centrifugation procedure described previously (Lu et al. 2018). Here, each cockroach was carefully wounded by leg amputation, placed into a 0.5 mL centrifuge tube with a small incision opening, and placed into a 1.5 mL Eppendorf tube. Centrifugation was carried out at 4000*g* at 4°C for 3 minutes. Each cockroach constituted a single biological replicate, with 17-20 replicates per treatment group. Next, 1 µL of hemolymph was collected using microcapillaries (Drummond Microcaps) and blown into test tubes containing 100 µL of sulfuric acid, followed by adding 2 mL of anthrone solution. The test tube was vortexed and heated in a water bath at 100°C for 8 minutes, then cooled in a cold-water bath for 6 minutes, before being kept in the dark for 30 minutes. Absorbances were then measured at 585 nm using a spectrophotometer (Novaspec Plus, Amersham Biosciences). Detailed preparation of anthrone solution and calibration curves for carbohydrates using known amounts of glucose are described in previous studies (Supplementary Figure S10) (Holwerda, Doorn and Beenakkers 1977, Marco, König and Gäde 2023).

Data analysis

Alignments were visualized using Jalview (Waterhouse et al. 2009), and sequence logos were generated using TBtools software (Chen et al. 2023). Spectra data was analyzed using flexAnalysis 3.4 (Bruker Daltonics, Germany). The resulting ion signals were compared with the theoretical masses of

the predicted peptides using Protein the online Prospector MS-product program in UCSF (https://prospector.ucsf.edu/prospector/mshome.htm).

We examined the effects of AKH peptide injection on carbohydrate levels on cockroaches of both sexes, fitting a Gaussian Generalized Linear Model (GLM) to the data. The response variable was carbohydrate concentration (µg/µL), and the explanatory variables were sex (male/female), treatment (Ringer, AKH1, AKH2 injection) and their two-way interaction. We applied an F-test to test the significance of the model variables. Tukey post-hoc analyses, which included a correction for multiple comparisons to control the family-wise error rate, were performed to compare treatments. Statistical analyses and plots were produced using R (v4.3.3) and RStudio (v2023.12.1).

4.4 Results and discussion

4.4.1 Transcriptome assembly and completeness

After eliminating adaptors and conducting quality control, we obtained a combined total of 346.5 million clean RNA-sequencing reads from all libraries. The obtained reads were subsequently used for *de novo* assembly, followed by redundancy reduction by CD-HIT, yielding a total of 54,529 'genes' with a GC percentage of 41.48% (Table 1). Assembly completeness, using a BUSCO score, was assessed to be 97.2%. A detailed breakdown of both single-copy and duplicated BUSCOs can be found in Table 1.

Table 1. Summary statistics of sequencing and *de novo* transcriptome assembly data of *B. germanica*

de novo Trinity Assembly	
Total trinity genes	54529
Total trinity transcripts	101749
Percent GC	41.48
Contig N50	954
Median contig length	231
Average contig	481.81
Total assembled bases	49023240
BUSCO assessment	
BUSCO	97.2%
Single-copy BUSCOs	88.3%
Duplicated BUSCOs	8.9%
Missing BUSCOs	1.4%
Total BUSCO groups searched	1367

4.4.2 Neuropeptide precursors from transcriptome

We employed BLASTp to search for homologous genes in our *de novo* transcriptome assembly of *B. germanica*, which yielded an extensive representation of precursor genes, yielding 69 putative neuropeptide and neurohormone precursor-encoding transcripts in total, as outlined in Table 2.

These transcripts varied in length from 72 to 988 bp, with most containing complete (ORFs with exceptions only to the Relaxin-like peptide (RLP) and Tryptopyrokinin (TPK). Sequences and potential cleavage sites of mature neuropeptides are given in Table S1, the latter were estimated using known or predicted cleavage sites in orthologous precursors of related insects , the majority of precursors contained a single neuropeptide, while others possessed multiple neuropeptide sequences (Supplementary Table S1).

Table 2. Neuropeptide and neuropeptide-like precursors identified in *B. germanica*

Neuropeptide	Acronym	Detected	ORF length (Aa)	Detected in other blattodean species		
				Z. nevadensis[a]	*M. darwiniensis*[b]	*P. americana*[c]
AKH/corazonin-related peptide	ACP	+	97	+	+	+
Agatoxin-like peptide	ALP	+	98	-	-	+
Adipokinetic hormone 1	AKH1	+	72	+	+	+
Adipokinetic hormone 2	AKH2	+	75	-	-	+
Allatostatin A	AstA	+	378	+	+	+
Allatostatin CC	AstCC	+	150	+	-	+
Allatostatin CCC	AstCCC	+	96	-	-	+
Allatotropin	AT	+	125	+	+	+
Calcitonin A	CTA	+	112	+	-	+
Calcitonin B	CTB	-	-	+	-	+
Calcitonin-like diuretic hormone	CT-DH	+	116	+	+	+
Crustacean cardioactive peptide	CCAP	+	154	+	+	+
Periviscerokinin	CAPA	+	239	+	-	+
CCHamide 1	CCH1	+	202	+	+	+
CCHamide2	CCH2	+	127	+	-	+
CCRFamide	CCRF	+	105	-	-	+
ITP transcript A	ITP A	+	115	+	+	+
ITP transcript B	ITP B	+	118	+	-	+
CNMamide 1	CNM1	+	163	+	-	+
CNMamide 2	CNM2	+	157	+	-	+
Carausius neuropeptide-like precursor	CNP	+	508	-	-	+
Corazonin	Crz	+	127	+	+	+
CRF-like diuretic hormone	CRF-DH	+	198	+	+	+
Ecdysis triggering hormone	ETH	+	183	+	+	+
Elevenin	Evn	+	138	+	-	+
FMRFamide related peptide	FMRF	+	493	+	+	+
Gonadulin	Gon	+	125**	-	-	+
HanSolin	Han	+	128	-	-	+
Pyrokinin	PK	+	195	+	-	+
SIFamide	SIF	+	73	+	+	+

SMYamide	SMY	+	78	+	-	+
Invertebrate parathyroid hormone	IPTH	+	133	-	-	+
Kinin	K	+	779	+	+	+
Leucomyosuppressin	LMS	+	96	+	+	+
Myoinhibitory peptide	MIP, AstB	+	278	-	+	+
Natalisin	Nat	+	395	+	-	+
Neuropeptide F1 transcript A	NPF1a	+	91	+	+	+
Neuropeptide F1 transcript B	NPF1b	+	128	+	-	+
Neuropeptide F2	NPF2	+	123	+	-	+
Neuropeptide-like peptide	NPLP	+	432	+	-	+
NVP-like	NVP	+	358	-	-	+
Orcokinin A	OKa	+	167	+	+	+
Orcokinin B	OKb	+	404	+	+	+
Fliktin	Flik	+	988	-	-	+
Pigment dispersing factor	PDF	+	87	+	+	+
Proctolin	PT	+	85	+	-	+
RFLamide	RFL	+	187	-	-	+
RYamide	RY	+	148	+	+	+
Short Neuropeptide F	sNPF	+	101	+	+	+
Sulfakinin	SK	+	122	+	+	+
Trissin	Tri	+	113	+	-	+
Tachykinin	TK	+	357	+	+	+
Tryptopyrokinin	TPK	+	106*	+	-	+
Inotocin	Inotocin	-	-	+	-	+
Bursicon alpha	Burα	+	151	+	+	+
Bursicon β	Burβ	+	139	+	+	+
Eclosion hormone 1	EH1	+	84	+	+	+
Eclosion hormone 2	EH2	+	78	+	-	+
Glycoprotein hormone alpha 2	GPA2	+	130	+	-	+
Glycoprotein hormone beta 5	GPB5	+	156	+	-	+
Insulin-like peptide	ILP1-7	+	-	ILP1-5	ILP1-4	ILP1-6
Relaxin-like peptide	RLP	+	146	+	-	+
Ortholog of Apis ITGQGNRIF	ITGQGNRIF	+	220	+	-	-
Neuroparsin 1	NP1	+	110	+	-	+
Neuroparsin 2	NP2	+	108	-	-	+
Prothoracicotropic hormone	PTTH	+	214	+	-	+

Note: a,b,c: three species reported, from either genomic or transcriptomic data investigation; *: incomplete sequence. **: sequence from NCBI (PSN45462.1). Deduced protein type. -: No value. For Insulin-like peptides (ILPs), only numbers are shown for different species.

The list of identified neuropeptide precursors encompasses several that have been previously described in *B. germanica*, including AKH1 (Huang and Lee 2011), two OKs (Ons, Belles and Maestro

2015), seven ILPs (Castro-Arnau et al. 2019), and the precursor sequence of LMS (Vilaplana, Castresana and Bellés 2004). Additionally, almost all previously identified insect neuropeptide gene families, including those already described from other Blattodea species were identified, and are presented in Table 2. However, the neuropeptide precursors for Allatostatin C (AstC), Calcitonin B (CTB), Diuretic hormone 31 (DH31) and Inotocin, were not found from the transcriptomic dataset.

4.4.3 Neuropeptides confirmed by MALDI-TOF MS

We followed an established approach to identify neuropeptides in *B. germanica* (Weaver and Audsley 2010). Briefly, the presence of predicted peptides was supported by comparison of the theoretical monoisotopic protonated masses ($[M+H]^+$) with masses (m/z) observed in tissue samples directly profiled by MALDI-TOF MS. We also examined potential PTMs for peptides, considering N-terminal pyroglutamate formation, C-terminal amidation, disulfide bonds, and tyrosine sulfation, based on information from other insect taxa already analyzed.

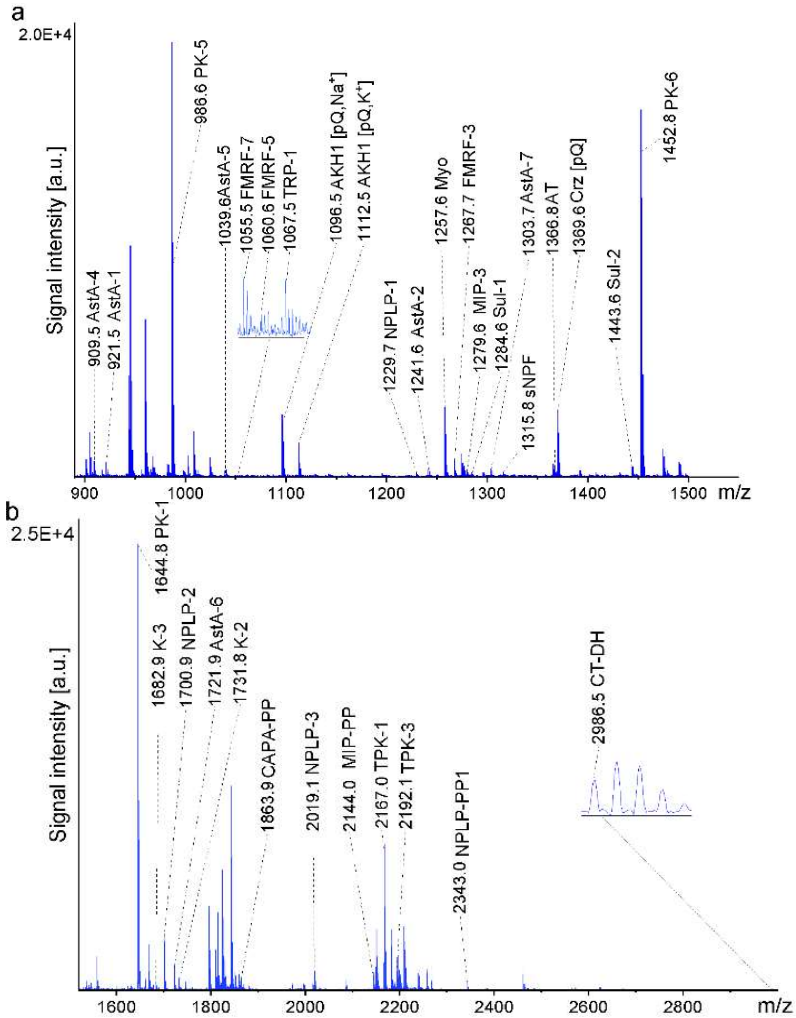

Figure 1. Direct tissue profiling by MALDI-TOF MS, tissue profiling MS spectra showing the dominated prominent ion signals of neuropeptides obtained from a preparation of *B. germanica* brain tissue. All marked ion signals represent single charged peptides ($[M+H]^+$) aside from sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts for AKH1, due to the lack of basic amino acids in this peptide. (a) m/z: 880−1500; (b) m/z: 1500−3000.

Overall, we observed ion signals identical to the predicted masses of 79 neuropeptides, neuropeptide-like peptides, and several precursor peptides (PPs) in MS spectra of brain tissue of *B. germanica*, most prominent signals likely represented AKH1, AstAs, Crz, FMRFs, LMS, NPLPs, PKs, and TPKs (Figure 1, Table 3).

Table 3. MALDI-TOF detected neuropeptides, neuropeptide-like, protein hormones, and precursor peptides (PP) of *B. germanica* brain. Note: (+): detected with Na^+/K^+ adducts; * HrTH, hypertreahlosemic hormone; underlined, one half of a disulfide bridge.

Designation	Peptide sequence	Calculated $[M+H]^+$	MALDI-TOF MS
AKH/corazonin-related peptide			
ACP	pQVTFSRDWNA-NH_2	1205.6	+
Adipokinetic hormone			
AKH 1 (*HrTH)	pQVNFSPGWGT-NH_2	1074.5	(+)
AKH 1-PP	SGIQEGPCKGSTESIMYIYKLVQSEAQKLLECDKFASN-OH	4195.0	+
AKH 2	pQLNFSPGWGP-NH_2	1084.5	(+)
AKH 2 PP	SGLQDGPCKPSDALMHIYRLVQSEVQKLAECEKFGSN-OH	4078	+
Allatostatin A			
AstA 1	LPVYNFGL-NH2	921.5	+
AstA 2	AGSDGRLYSFGL-NH2	1241.6	+
AstA 3	DRLYSFGL-NH2	969.5	+
AstA 4	ARPYSFGL-NH2	909.5	+
AstA 5	AGGRLYSFGL-NH2	1039.6	+
AstA 6	PVNSGRQSGSRFNFGL-NH2	1721.9	+
AstA 7	SPQEHRFSFGL-NH2	1303.7	+
AstA 8	SLHYPFGF-NH2	966.5	+
Allatotropin			
AT	GFKNVALSTARGF-NH2	1366.8	+
Crustacean cardioactive peptide			
CCAP 1	QVDPAEMERLLDP-OH	1512.7	+
CCAP 2	PFCNAFTGC-NH2	956.4	+
Carausius neuropeptide-like precursor			
CNP 1	GFHESVFDGFGDYYPPW-NH2	2018.9	+
CNP 2	SNTRHLSDKAEEILNKILSSSQ-OH	2470.3	+
Corazonin			
Crz	pQTFQYSRGWTN-NH2	1369.6	+
Crz PP	AGPLLVPSAASGLLQDADESNPCSQLQRIKFLLGARNPQQIFFPCDTW REVSESASDNTSERF	6905.4	+

Corticotropin-releasing factor-like Diuretic hormone			
CRF-DH	TGTGPSLSIVNPLDVLRQRLLLEIARRRMRQTQSQIQANRDILESI-NH2	5254.9	+
CRF-DH PP1	YYESPLLEALTAPSPDHETSSYLLPRLAAKYRPHGDWESAPDPRFYVLTE LDRDSSQAARRV-OH	7103.5	+
CRF-DH PP2	EVNLSQQHNIDQDDADELDADLLIASSEDKSGHNSSANNRSPESSDW STASNSRWNNEYTSQHHS-OH	7240.2	+
Calcitonin-like diuretic hormone			
CT-DH	GLDLGLSRGFSGSQAAKHLMGLAAANYAGGP-NH2	2986.5	+
Fliktin			
Flik	VAVKKEKAEEKKDDDKE-OH	1989.0	+
FMRFamide related peptide			
FMRF 1	RCSNQNFIRF-NH2	1283.6	+
FMRF 2	QNKDSNFIRF-NH2	1267.7	+
FMRF 3	DKSDSFIRF-NH2	1113.6	+
FMRF 4	GKVDNFIRL-NH2	1060.6	+
FMRF 5	GKQDNFIRFG-NH2	1180.6	+
FMRF 6	MKDNFVRF-NH2	1055.5	+
FMRF 7	GGNSDSNFIRF-NH2	1212.6	+
FMRF 8	GGSNFIRL-NH2	862.5	+
Insulin-like peptide			
ILP1	ETPHRYCGRHLVSILQLLCGSNYNGDIEK-OH	3312.7	+
ILP2	NTNKYCGRNLANMLQLVCNGNYYPMF-OH	3038.4	+
ILP3	pQSTHRYCGPHLVSALRLLCNGRYYTPDEDEDDTTTE-OH	4137.9	+
ILP4	HASRKYCGHNLVLVMQLVCDSRYNSPRPSNPS-OH	3628.8	+
Kinin (K)			
K 1	AFTSTGSSRSPAFSSWG-NH2	1731.8	+
K 2	LDRSEGRVSKALFSSWG-NH2	1894.0	+
K 3	SDKGNNLRKISPQNL-NH2	1682.9	+
Myoinhibitory peptide			
MIP 1	AWDELRPMW-NH2	1202.6	+
MIP 2	PWDKFHGAW-NH2	1142.6	+
MIP 3	AADWANFRGSW-NH2	1279.6	+
MIP 4	DPGWNNLKGLW-NH2	1298.7	+
MIP 5	ADSNWNRLSAAW-NH2	1389.7	+
MIP PP	SIGGETGIKEDQPRAPGSSEE-OH	2144.0	+
Leucomyosuppressin			
LMS	pQDVDHVFLRF-NH2	1257.6	+
Neuropeptide-like peptide			

NPLP 1	NLASLARTGGLSA-NH2	1229.7	+
NPLP 2	NVASLMRNGISPFAQP-NH2	1700.9	+
NPLP 3	NIGAMARNWHLPDHLKF-NH2	2019.1	+
NPLP 4	YVAALLRH-NH2	941.6	+
NPLP PP1	GDESEDDELIRELWHELEE-OH	2343.0	+
NPLP PP2	NIGALARNGYL-OH	1161.6	+
NPLP PP3	YLGSIMRNQ-OH	1081.5	+
NVP-like peptide			
NVP 1	SYRPELPFVMPPDDLTSSRSRL-OH	2563.3	+
NVP 2	DSSQWGGFAKD-OH	1197.5	+
Proctolin			
Proct	RYLPT-OH	649.4	+
Proct PP	EVPMMASEQQQHAPLMAAQQ-OH	2225.0	+
RYamide			
RY	pQQFYASGRY-NH2	1101.5	+
Pyrokinin			
PK 1	SQPAETSGLWFGPRL-NH2	1644.8	+
PK 2	SIDDTLDGGDSSKEEEIVELLRETPWALVPLKG-NH2	3610.8	+
PK 3	pQTSFIPRL-NH2	832.5	+
PK 4	SPPFAPRL-NH2	883.5	+
PK 5	LVPFKPRM-NH2	986.6	+
PK 6	DHIPQDIYSPRL-NH2	1452.8	+
PK PP	SIPAPHLQEQKNNPKQH-OH	1966.0	+
Short Neuropeptide F			
sNPF	ANRSPSLRLRF-NH2	1315.8	+
Sulfakinin			
Sul 1	QSEDYGHFRF-NH2	1284.6	+
Sul 2	EQFDDYGHMRF-NH2	1443.6	+
Tachykinin related peptide			
TK 1	GPSVGFFAMR-NH2	1067.5	+
TK 2	APSGFMGMR-NH2	952.4	+
Tryptopyrokinin			
TPK 1	DKKSAEMQESAGIWFGPRM-NH2	2167.0	+
TPK 2	DKKSVEMQESAGMWFGPRM-NH2	2213.0	+
TPK 3	DKKLAQIKESAGMWFGPRM-NH2	2192.1	+
TPK 4	EEQSTQMQENAGMWFGPRM-NH2	2256.0	+

We then investigated further details of some of the major peptide and neuropeptide-like families in *B. germanica* and related species. We focused on comparative neuropeptide characteristics in Blattodea, with emphasis on species that have been extensively studied in terms of their neuropeptidomes. These species were: *Z. nevadensis* (Veenstra 2014), *M. darwiniensis* (Christie 2015) and the American cockroach, *P. americana* (Zeng et al. 2021).

Adipokinetic Hormone (AKH). In 1990, the AKH1 peptide (Hypertrehalosemic hormone) from *corpora cardiaca* (CC) was first identified in *B. germanica* (Veenstra and Camps 1990). Recently, a second AKH gene (AKH2) was reported in this species, with evidence of an ancient duplication event of decapeptide AKH in the common ancestor of Blaberoidea (Jiang et al. 2023). Using MADLI-TOF we were able to verify two AKH peptides with Sodium [Na+] adducts in the tissue of the CC. These peptides have a blocked pyroGlu (pQ) N-terminus and their C-terminus is blocked by amidation. We validated the PP sequences for both AKH1 and AKH2 and were able to show that AKH1 is observed at a higher intensity than AKH2 in the CC, as shown in Figure 2a. AKHs in *B. germanica* are decapeptides instead of octapeptides, the latter of which being typical for species belonging to the Solumblattodea (Figure 2b) (Jiang et al. 2023), with some having been further verified by HPLC MS (Marco, König and Gäde 2023).

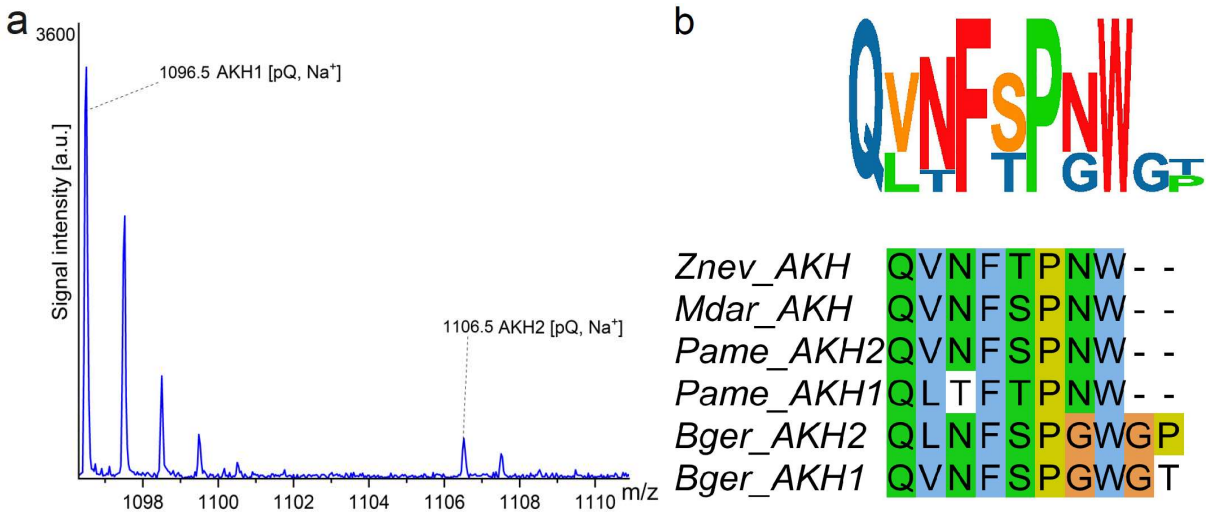

Figure 2. (a) MALDI TOF direct tissue profiling of a dissected *corpora cardiaca* of *B. germanica* showing ion signals with mass identity to two AKHs. AKH-1 ([M+Na]$^+$, m/z: 1096.5) and AKH-2 ([M+Na]$^+$, m/z: 1106.5); (b) Multiple sequence alignment of bioactive AKH. The sequence logo above depicts the consensus sequence. Abbreviation: *Bger*: *Blattella germanica*; *Znev*: *Zootermopsis nevadensis*; *Mdar*: *Mastotermes darwiniensis*; *Pame*: *Periplaneta americana*.

AKH/Corazonin-Related Peptide (ACP). ACP, as its name implies, is structurally intermediate between Corazonin and AKH and exhibits similarity to the vertebrate gonadotropin-releasing hormone (GnRH). It has critical roles in energy mobilization and physiological regulation (Hansen et al. 2010, Marco et al. 2024). ACP was reported to have been lost in certain termite species (Veenstra 2023). We identified

an ACP signal from preparations of brain tissue in *B. germanica*. The detected ACP peptide sequence, a decapeptide: pQVTFSRDWNAa, with blocked pyroGlu (pQ) N-terminus and C-terminus blocked by amidation, is identical to that reported in *M. darwiniensis*, *Z. nevadensis* and *P. americana* (Supplementary Figure S1).

Allatostatin A (AstA). AstA peptides are characterized by a highly conserved sequence motif Y/FXFGLamide and are expressed mainly in the nervous system and mid-gut in insects. The first AstA was discovered in the cockroach *Diploptera punctata* in 1997 (Gäde 1997). In a previous study, four Ast neuropeptides were isolated from extracts of the brain of *B. germanica* (Bellés et al. 1994). We identified 8 AstA ion signals from 13 putative AstAs derived from transcriptomic data, having the common characteristic of a conserved C-terminal FGL-amide, with masses ranging from 921.5-1721.9 (m/z) (Figure 1a, Supplementary Table S1, Supplementary Figure S2).

Allatotropin (AT). AT is a neuropeptide found in several invertebrates, playing a multifunctional role by indirectly regulating vitellogenesis through the stimulation of juvenile hormone production, and influencing visceral muscle activity, heart rate regulation, and digestive processes (Kataoka et al. 1989, Duve, East and Thorpe 1999, Rudwall, Sliwowska and Nässel 2000, Petri et al. 2002, Li et al. 2003, Sterkel, Riccillo and Ronderos 2010, Fukumura 2021, Mamtha et al. 2021). In MALDI-TOF spectra, we found the corresponding ion signal of AT, albeit at relatively low abundance (Figure 1a). In *B. germanica*, as well as in other species within Blattodea, it is composed of 13 Aa, the consensus C-terminus sequence of AT, which includes a typical GFKNV(A/G)A(Y)LSTARGFamide sequence, is conserved across Blattodea.

Calcitonin-Like Diuretic Hormone (CT-DH). CT-DHs are part of the family of diuretic hormones similar to mammalian calcitonin, essential for controlling water balance and ion homeostasis (Coast et al. 2001, Zandawala et al. 2013). The first CT-DH peptide in Blattodea was isolated and characterized in the cockroach *D. punctata* (Furuya et al. 2000). In this study, we identified the ion signal of CT-DH in MALDI-TOF for *B. germanica* (Figure 1b), which consists of a 31 Aa sequences GLDLGLSRGFSGSQAAKHLMGLAAANYAGGPamide, identical to that found in two termites: *M. darwiniensis*, *Z. nevadensis*, and two cockroaches: *D. punctata* and *P. americana*.

Corticotropin-Releasing Factor-Like Diuretic Hormone (CRF-DH). CRF-DHs are named due to their structural and functional similarities to the vertebrate corticotropin-releasing factor (CRF). Other important functions have been revealed such as modulation of desiccation tolerance (Furuya et al. 2000, Schooley, Horodyski and Coast 2012, Cannell et al. 2016). In this study, we detected the ion

signal of CRF-DH as well as the two PPs for *B. germanica* in the MALDI-TOF MS (Figure 3a). CRF-DH consists of 46 Aa in *B. germanica*, exhibiting high similarity to CRF-DHs from other Blattodea species (Figure 3b).

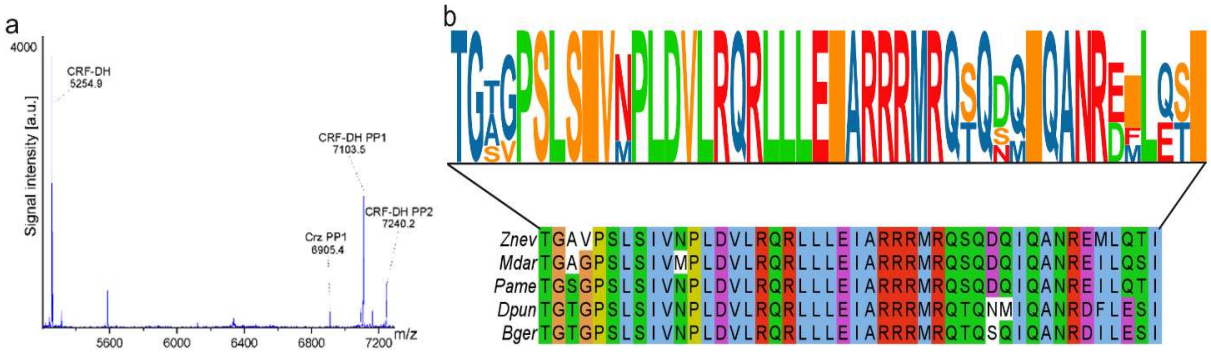

Figure 3. MALDI TOF direct tissue profiling of a dissected brain of *B. germanica* showing ion signals with mass identity to CRF-DT and its 2 PPs (a) and (b). alignment of CRF-DT mature sequences of 5 cockroach and termite species. The sequence logo above depicts the consensus sequence. Abbreviation: Crz: Corazonin; *Dpun*: *Diploptera punctata*; PP: precursor peptides. The same for the below.

Crustacean cardioactive peptide (CCAP). CCAPs in insects primarily regulate ecdysis and serve as both a hormone released into the haemolymph and a neuromodulator or neurotransmitter in the nervous system. A previous study examined the distribution of CCAP in the cephalic ganglia of two species of cockroaches, *P. americana* and *Gromphadorhina portentosa* (Gładysz et al. 2015). Within the MALDI-TOF MS spectra, one CCAP signal and one of its corresponding PP signals were detected. The CCAP in *B. germanica* is composed of a 9 amino acid sequence: PFCNAFTGCamide, which is the same as found in *M. darwiniensis*, *Z. nevadensis* and *P. americana* (Supplementary Figure S3).

Carausius Neuropeptide-Like Precursor (CNP). CNPs are a novel class of neuropeptides first identified in *Carausius morosus* in 2018 (Liessem et al. 2018), with subsequent studies having identified CNPs in other insects, including *P. americana* (Zeng et al. 2021), *Schistocerca gregaria* (Ragionieri et al. 2022) and *Picromerus lewisi* (Li et al. 2023). In this study, the ion signals of two putative CNPs were identified in MALDI-TOF MS spectra for *B. germanica*. CNPs were not reported in two termite species: *M. darwiniensis* and *Z. nevadensis*.

Corazonin (Crz). Crz is a highly conserved undecapeptide that performs diverse activities across different organisms (Veenstra 1989, Tanaka et al. 2002, Predel et al. 2007, Kubrak et al. 2016). The initial Crz in insects was isolated from the CC of *P. americana* (Veenstra 1989). Here, we identified the Crz and its PP ion signals in *B. germanica* (Figure 1a, Figure 3a). The Aa sequence of Crz in *B. germanica* is pQTFQYSRGWTN, which is similar to those found in *M. darwiniensis*, *Z. nevadensis*, and *P. americana*.

Fliktin (Flik). Fliks is a newly discovered neuropeptide that was first identified in *P. americana* (Zeng et al. 2021), and subsequently named Fliktin, following its description in the ant *Cataglyphis nadus*

(Habenstein et al. 2021). In this study, one neuropeptide-like ion signal was identified in MALDI-TOF MS spectra for *B. germanica*.

FMRFamide-Related Peptides (FMRFs). FMRFs directly modulate muscle contractions in *Drosophila* through the activation of its cognate G protein-coupled receptors (Clark et al. 2008). Twenty-three FMRFs were identified in *P. americana*, which is the highest number identified for any mature FMRF (Predel et al. 2004). Eight out of 18 putative FMRFs were confirmed here via MALDI-TOF MS spectra in *B .germanica* (Figure 1). *B. germanica* shows a significant degree of similarity to sequences in other Blattodea (Supplementary Figure S4).

Insulin-like peptides (ILPs). A previous study demonstrated that *B. germanica* has 7 ILPs, which are differentially expressed across various tissues and in response to distinct physiological conditions (Castro-Arnau et al. 2019, Domínguez, Pagone and Maestro 2022). In this study, four mature ILPs were confirmed via MALDI-TOF MS. These sequences contain the typical two cysteine forms disulfides (Supplementary Table 1).

Kinin (K). Kinin was first isolated from head extracts of the Madeira cockroach *L. maderae* (Holman, Cook and Nachman 1986b), with eight kinins being isolated from the retrocerebral complex of *P. americana* (Predel et al. 1997). The kinin family and the lengths of the mature peptides are divergent among species (Tanaka 2016). Three Ks were identified in *B. germanica* via MALDI-TOF MS (Figure. 1b), with the kinin sequence being characterized by an amidated C-terminal motif: FXSWGamide in *B. germanica*, which is conserved across Blattodea species (Supplementary Table 1).

Myoinhibitory peptide (MIP, AstB). MIPs are characterized by a conserved C-terminal sequence W(X6)Wamide. It was initially identified based on its ability to inhibit visceral muscle contractions in insects (Predel, Rapus and Eckert 2001, Liessem et al. 2021). Here, we identified 5 out of 10 putative MIPs and confirmed one PP sequence from the MALDI-TOF spectra. A previous study in adult female *B. germanica* found that three galanin-related MIP peptides strongly inhibited foregut and hindgut contractions, and two of them significantly reduced food intake (Aguilar, Maestro and Bellés 2006). MIPs have been reported in *Z. nevadensis*, and *P. americana*, but not in *M. darwiniensis* (Supplementary Figure S5).

Myosuppressin (Myo). The first Myo was isolated and structurally characterized from CNS tissues of the cockroach *Leucophaea maderae* in 1986 (Holman, Cook and Nachman 1986a), Subsequently, Myos were found in the cockroaches *P. americana* (Holman et al. 1991) and *B. germanica* (Aguilar et al. 2004). The mature Myo neuropeptide sequence has a high degree of conservation among insects. We

identified the Myo ion signal in *B. germanica* in the MALDI-TOF spectra, with the sequence: pQDVDHVFLRFamide being identical to sequences found in *M. darwiniensis*, *Z. nevadensis, L. maderae,* and *P. americana*.

Neuropeptide-Like Peptide (NPLP). We identified four NPLPs and 3 PPs in *B. germanica* via MALDI-TOF MS (Fig. 1B). These sequences show similarities to NPLPs in other Blattodea, including conserved motifs found in related species like *Z. nevadensis, L. maderae* and *P. americana* (Supplementary Figure S6).

NVP-Like Peptide (NVP). Hummon et al. (2006) reported the first neuropeptide from the NVPL family in the honey bee brain. It is reported that injecting NVP into *Zophobas atratus* larvae may cause an increase in the level of free sugars in the haemolymph (Marciniak, Kuczer and Rosinski 2011). We identified 2 NVP-like ion signals in *B. germanica* through MALDI-TOF MS. NVPs have been reported in *P. americana*, but not in *M. darwiniensis* and *Z. nevadensis*.

Proctolin (PT). PT is a well-known neuropeptide that stimulates muscle contraction and modulates neurotransmission (Bishop, O'Shea and Miller 1981, Orchard, Belanger and Lange 1989, Ormerod et al. 2016), it was first identified in *P. americana* (Starratt and Brown 1975). In *B. germanica*, we identified the PT peptide and one PP sequence via MALDI-TOF MS. The PT peptide, with a highly conserved pentapeptide RYLPT, is identical with those reported in other termite and cockroach species.

RYamide. Rys are neuropeptides found in arthropods, characterized by their arginine-tyrosine amidated C-terminus. However, their specific roles are currently not known. We identified one RY peptide from three putative RY sequences. RY peptides in *B. germanica* are characterized by the C-terminal sequence FXXXXRYamide, consistent with RY peptides reported in the species *M. darwiniensis, Z. nevadensis*, and *P. americana* (Supplementary Figure S7).

Pyrokinin (PK/PBAN). The first member of the PK/PBAN family was isolated from brain extracts of the cockroach *Leucophaea maderae* in 1986 and was identified as a peptide hormone that controls hindgut contraction (Holman, Cook and Nachman 1986b). Notably, this study identified six ion signals in *B. germanica* corresponding to PBAN/PKs and one PP, with some being dominantly abundant in the MALDI-TOF MS (Figure 1). Comparison of the primary sequences of the PKs shows that most PKs in Blattodea share a conserved C-terminal sequence: PR(L/M)amide (Supplementary Figure S8).

Short Neuropeptide F (sNPF). The first sNPF was isolated from the midgut of the American cockroach, *P. americana* (Veenstra and Lambrou 1995). Since then, sNPF has been implicated in regulating a diverse array of biological processes in many insect species, including learning, feeding, and growth (Johard et al. 2008, Nässel and Wegener 2011, Zeng et al. 2021). In *B. germanica*, we identified a peptide with the sequence ANRSPSLRLRFamide using MALDI-TOF MS, which is identical to those reported in other species, such as *C. secundus*, *M. darwiniensis*, *Z. nevadensis*, *Diploptera punctata* and *P. americana* (Figure 4a).

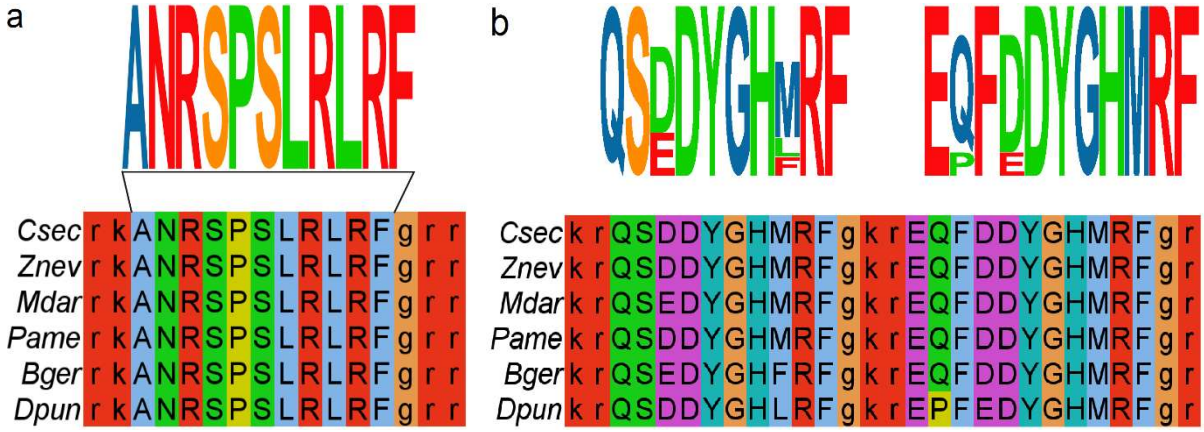

Figure 4. Sequence alignment of sNPF (a) and sulfakinin (b) peptides in some Blattodea species. The sequence logo above depicts the consensus sequence, putative amidation and dibasic cleavage sites are indicated in lowercase. Abbreviation: *Csec*: *Cryptotermes secundus*; *Dpun*: *Diploptera punctata*. sNPF (XP_023709025.1) and sulfakinin (XP_023701497.1) sequences for *C. secundus* and sNPF (KAJ9582741.1) and sulfakinin (KAJ9587735.1) sequences for *D. punctata* were retrieved from NCBI.

Sulfakinin (Sul). Suls have been identified in specific brain cells and are widely distributed throughout the nervous system of *P. americana* (East, Hales and Cooper 1997). One Sul peptide: EQFDDYGHMRFa was isolated from brain extracts by HPLC fractionation and subsequently evaluated for its ability to inhibit food intake in *B. germanica* (Maestro et al. 2001). In the present study, we employed MALDI-TOF MS to identify two Suls in *B. germanica* (Figure 1a). These Sul sequences exhibit a high degree of conservation compared to those reported in other blattodean species (Figure 4b).

Tachykinin-Related Peptide (TK). In 2008, a tachykinin peptide: APSGFLGVR-NH2 was discovered in *B. germanica*, subsequent experiments showed that peptide injection could significantly increase the food consumption of adult female cockroaches (Pascual et al. 2008). In this study, MALDI-TOF MS identified two TK peptides from 13 putative precursor TK sequences. The identified TK sequences align with known TKs from other species of Blattodea (Supplementary Figure S9).

Tryptopyrokinin (TPK). As Veenstra (2014) described, TPKs have not been detected in non-insect arthropods, suggesting that they are evolutionarily derived in insects. We identified 4 TPKs in *B.*

germanica through MALDI-TOF MS. *B. germanica* has C-terminal consensus sequences: GPRMamide. However, in *Z. nevadensis* and *P. americana,* they have the typical C-terminal motif: GPRLamide.

MALDI-TOF direct tissue profiling did not detect all neuropeptides

Several neuropeptides could not be detected by MALDI-TOF direct brain tissue profiling. Some protein hormones, including Ion transport peptides (ITPs), Bursicons (Bur*α* and Bur*β*), Glycoprotein hormones (GPA2 and GPB5), Gonadulin (Gon), Neuroparsins (NPs), and Prothoracicotropic hormone (PTTH), were not identified. This could be due to their masses being beyond the detection range of the detector or because some of them are not expressed in the brain. In addition, MALDI-TOF was unable to detect the neuropeptides Allatostatin CC, Allatostatin CCC, periviscerokinin (CAPA), CCHamide, Pigment dispersing factor (PDF), SIFamide, Ecdysis triggering hormone (ETH), Elevenin (Evn), Invertebrate parathyroid hormone (IPTH), RFLamide, and Trissin (Tri). This may be a result of their low abundance in brain tissue or due to the possibility that some of them exist in forms with extended N-termini, as previously observed in two *Carabus* beetle species (Ragionieri and Predel 2020).

Previous studies have also shown that some neuropeptides including multiple FMRFs and MIPs in *Cimex lectularius* (Predel et al. 2018) and CCHs, NPFs, NVPs, OKs, Suls in *Carabus violaceus* (Ragionieri and Predel 2020) could be detected using Quadrupole Orbitrap MS but not MALDI-TOF. The use of such a follow-up technique may be useful for enhancing neuropeptide identification in *B. germanica* in the future.

4.4.4 Biological activity of two AKH peptides

AKHs play a very important role in the control of energy mobilization in various physiological processes. A key role of AKH1 in *B. germanica* is the regulation of cyclic fluctuation of trehalose in the hemolymph and oviposition in virgin females (Huang and Lee 2011). A second AKH precursor sequence (AKH2) was recently characterized by sequencing (Jiang et al. 2023), and now verified here using MALDI-TOF MS (Figure. 2a). We examined whether this peptide has the same metabolic function in carbohydrate mobilization as the AKH1 peptide previously reported in *B. germanica* (Gäde and Rinehart 1990, Huang and Lee 2011). We synthesized both AKHs and evaluated their capacity to mobilize carbohydrates.

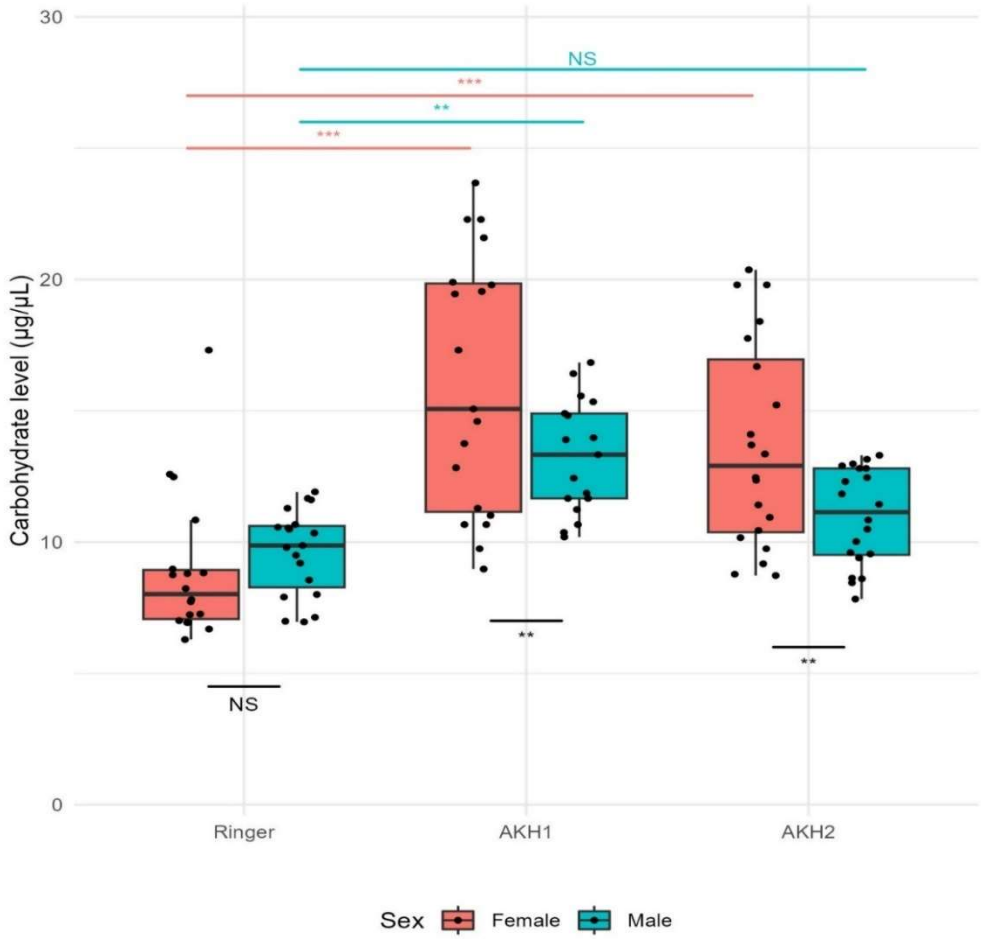

Figure 5. Carbohydrate content comparison following 3h of AKH peptides injection in male and female cockroaches represented as violin plots. Comparison between the sexes for equivalent treatments and between treatments for each sex were performed following Tukey post-hoc test. NS.: no statistically significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Bars represent mean + SEM.

We found variations of carbohydrate concentrations 3 hours after injection across treatments between male and female cockroaches (GLM; interaction term: $F_{2,107}=3.76$, $p=0.0264$). More specifically, compared to Ringer injection, males showed an elevation of carbohydrate levels when injected with AKH1 but not AKH2 (Tukey post-hoc test; AKH1-Ringer: $t_{1,107} = 3.46$, $p = 0.002$, AKH2-Ringer: $t_{1,107} = 1.34$, $p = 0.379$) while females carbohydrate levels rose in both AKH1 and AKH2 peptide injection treatments (Tukey post-hoc test; AKH1-Ringer: $t_{1,107} = 6.90$, $p < 0.001$, AKH2-Ringer: $t_{1,107} = 4.67$, $p < 0.001$; Figure 5). Comparing male and female cockroach response, female carbohydrate levels were significantly higher compare to males when injected either with AKH1 (Tukey post-hoc test; female-male: $t_{1,107} = 2.66$, $p = 0.009$) or AKH2 (Tukey post-hoc test; female-male: $t_{1,107} = 2.73$, $p = 0.008$) but not Ringer (Tukey post-hoc test; female-male: $t_{1,107} = -0.68$, $p = 0.495$) (Figure 5). These data demonstrate the significant impact of AKH1 peptide on stimulating carbohydrate levels in both males and females, while also revealing sex-specific variations in metabolic responses to the AKH2 peptide treatment.

4.5 Conclusion

This study combines transcriptomic analysis with MALDI-TOF mass spectrometry to investigate the neuropeptidome of the German cockroach, *B. germanica*. We identified 69 transcripts of neuropeptide precursors, encompassing a large majority of known insect neuropeptide families, with MS analysis further confirming the presence of 79 neuropeptides and neuropeptide hormones. Most of these precursor transcripts and neuropeptides are reported for the first time in this species. Bioassays further reveal that two AKH peptides, one of which only being recently described, elevate carbohydrate levels in both male and female *B. germanica*. Interestingly, females displayed increased hemolymph mobilization compared with males when treated with equal concentrations of the both AKH peptides, while males responded more sensitively to AKH1 than to AKH2 injection. These results are indicative of a sex-specific metabolic response to AKH signaling in *B. germanica*.

Neuropeptides are essential for various behavioral and physiological processes in insects. Our study for the first time provides information on the wide diversity of precursor sequences in *B. germanica,* a notorious global pest. These data could potentially be harnessed to disrupt neuropeptide genes involved in metabolic pathways through RNA interference (RNAi). In addition to being interesting avenues for future research into insect physiology and behavior, unique neuropeptides from *B. germanica* could be harnessed as targets for species-tailored mimetic compound design, which if successfully developed, could help to mitigate against the negative effects of treatment on unintended beneficial insect species.

Acknowledgments

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Supplementary Table S1. Amino acid sequences of deduced neuropeptide precursor transcripts of *Blattella germanica*. Predicted neuropeptides including cleavage and amidation sites are indicated.

Signal peptide

Predicted bioactive peptide

Predicted C-terminal glycine amidation site

Predicted dibasic cleavage site

Predicted C-bridge site

Underlined sequence: neuropeptide sequence confirmed by MALDI-TOF

?: incomplete precursor sequence

>AKH/corazonin-related peptide (ACP)

MIEKLFWSIVLFLTILSCLSYRTLGQVTFSRDWNAGKRSGPPDLQCNSVLKSVDEICKVMVEEFRQLA
ACESKSLLRFQREYDDKQADMFLEGQDGR*

>Agatoxin-like (ALP)

MRTFLIFLACGLLLLGQIVLPAMAGPYLVERDEGLEDYSDNNLERLLQDRAEKRACIRRGGNCDHRPK
DCCYNSSCRCNLWGTNCRCQRMGLFQKWGK*

>Adipokinetic hormone 1 (AKH1)

MSYLIKTLVVVVALALVVCEAQVNFSPGWGTGKRSGIQEGPCKGSTESIMYIYKLVQSEAQKLLECDK
FASN*

>Adipokinetic hormone 2 (AKH2)

MNFKLICIINTIVVVVTVFLVTCEAQLNFSPGWGPGKRSGLQDGPCKPSDALMHIYRLVQSEVQKLAE
CEKFGSN*

>Allatostatin A (AstA)

MPGPRTCYSLQAALVLSLLLKLSSSAFATTTSAGTHAVQEESSAGGGAEILPRLEELADNSELDLVKR
LYDFGLGKRAYSYVSEYKRLPVYNFGLGKRSKMYGFGLGKRAGSDGRLYSFGLGKRDYDDYYGDD
DEEDHQTSADEDIEDADSVDLMDKRDRLYSFGLGKRARPYSFGLGKRAPSSAQRLYGFGLGKRALY
SFGLGKRAGGRLYSFGLGKRPVNSGRQSGSRFNFGLGKRSDDFDIRELEGKFAEEDKRSPQEHRFS
FGLGKREVAPSELEAVKNEEKDSVSNQEKKNNTNDAYIHNGERVKRSLHYPFGFGKQDSGFDLHSS
SLSSEENDDIGPEEFARMVRRPFEYARQKQVPMYDFGIGKRSER*

>Allatostatin CC (AstCC)

MGHRPSQHHPCTILHPSPISSTTLASFLWLLVLALVTLFSLAGTTDAAPSSVSQHQIYKRSVTEGNMG
AADYPDYQSGVRYDEYPVVVPKRTALLLDRIMVALQKAVEEEKGGGRNYAPDLAESKMDLQRRGQT
KGRVYWRCYFNAVTCF*

>Allatostatin CCC (AstCCC)

MSAITTTKLMFVMLVGLLTLSWAVGKTLGQPGDKERLLSELDLVDDDGSVETALINYLFAKQVVNRLR
SQMDVSDLQRKRSYWKQCAFNAVSCFGK*

>Allatotropin (AT)

MRQSLTVYSVIAITVIVMLVLCGTVSAGSYQTSRNKPRTIRGFKNVALSTARGFGKRDGALEYLTGNA
NNAAEQNPDRMPESLPVEWFVEELRTNPELARIIVHKFVDADQDGELSAEELLRPMY*

>Calcitonin A (CTA)

MEWKREVTLALLVVMATAAWASTKEIAQELLDSQIKSLQDHRKTVHLLKNLLNELDTNMEAVQKRTTC
WINAGLSHACDNRDYLAALEENRYWSSLDSPGKRRRRNSEKQQR*

>Calcitonin-like diuretic hormone (CT-DH)

MNSYALLLTSALLVGAILMFSVGHASESVPLSSSHRNSYITDMDSEPDSEYVLEMLARLGQSIIRANDM
ENSKRGLDLGLSRGFSGSQAAKHLMGLAAANYAGGPGRRRRSSDESS*

>Periviscerokinin (CAPA)

MKENLLCCSAAVYLILLLVVSAVHCDGENVGSSSSVVKTRRGSSGLIPMGRVGRGGIPWTFQPSDED
VGPGTSLTKFRRGSSGLISMPRVGRGNIPWTFQISGVEDEKSKRGSSGMIPFPRVGRSDFLAHFPIAD
SFLEDGEAFVSVPGKRSEGGSGEANGMWFGPRLGKRSKRAADYPWAVVTVKEFPGNRRYGGFTP
RLGRESSENEEEDEGFLEEDDSQNGKNNLPAAGAKTHNYN*

>CCHamide 1 (CHH1)

MILSTSATRTSVVGAAARIAVLLFIFGLAECAAGSCLSYGHSCWGAHGKRSGNAASPPEEVPVPDDG
TEGLATPEDTRWFFSKLVQADPATKNKLWQRFGSVHAMDRRHKEQPWKGNGLEEDGEGAPGILRE
NDAFPRSRGLRLEGSDESAPGIFIPASGEFPGEESQDADVLVMADDQPIRRVPKKLRVFKIMNPERKL
DK*

>CCHamide 2 (CHH2)

MALLRCQSLLIVAITIVVLMVQIDQSSAKRGCSSFGHSCFGGHGKRADEDVLLLPGSDAVFPPSSSGV
AAQEDGGDDVMMQTAGFGGARAPSSSSSLVSPPQQYNLSPFLRQWLQSYRRSTGDLEVK*

>CCRFamide

MSCPRSLLPAALVLVILLQWGSPLEVAAVVDDIPEDCAPRQLRCELLCHVVELSLQCAKCRSRAPVRF
GKRTPESAETPLQAHDQLCCGNLLTVLLRKAAAHVDK*

>CNMamide 1 (CNM1)

MPSSRTVRSRTVLMWVLMLIVTFSCGVQAAPEAYHHRQGDPTLVIPAPALNDIEELGINENIDDPKLR
EQLTEMLAILQMYKNKVQQGNEEDPSGLDSGAMVAALEGLSNMPLPASLQAKLFRSQEGVNKEEMK
RGSYMSLCHFKICNMGRKRNLRWNPWIRR*

>CNMamide 2 (CNM2)

MPSSRTVRSRTVLMWVLMLIVTFSCGVQAAPEAYHHRQGDPTLVIPAPALNDIEELGINENIDDPKLR
EQLTEMLAILQMYKNKVQQGNEEDPSGLDSGAMVAALEGLSNMPLPASLQAKLFRSQEGVNKEEMK
RGSYQPSLCYFKICNMGRKRNVR*

>Carausius neuropeptide-like precursor (CNP)

MTAHLLLMFMVLRLGTTLPDLQNNMVPTDEQILRELLEQEKNARTADQGQPEVEDSLGLPTDEDSYN
ELRNLFALGLPGSSGTHHFVSSSFPEVDARGFHESVFDGFGDYYPPWGRHKRDPLGINSRGFHDDV
FNRDFGSFHTVKRSNTRHLSDKAEEILNKILSSSQRKKRDTTESLSESNDEHKQNEDETGKQSVSEK
QNEHTSDSTKYRRDVSRAEMEESADKRRPEMDGAGFHGDTFNSGFGDFWTMKKKEALKREGSNS
TDHNYWSLHKRRLGMGPSGFHGDTFTSGFGDFSTMKRTEMGSTSELGSDDMYKSEDGKRKPEMG
SSGFYGDTFSNGFGDFWTMKRMNDASKSEGRLEHKRRPEMDSSGFHGDTFRNGFGDFWTMKKRR
PEMDSSGFHGDTFKGGFGDFWTMKKRKPEMDSSGFHGDTFNSGFGDFWTMKKRRPEMDSSGFH
GDTFKGGFGDFWTMKKRSVSSENSSNDQKEQPCTSCTQSSVDSLDKVGSPNGH*

>Corazonin (Crz)

MQSHNRCRSHKFFRILLILSCLTGAVLAQTFQYSRGWTNGRKRAGPLLVPSAASGLLQDADESNPCS
QLQRIKFLLGARNPQQIFFPCDTWREVSESASDNTSERFKRRAARDAATLNASDEMNHEN*

>CRF-like diuretic hormone (CRF-DH)

MMVAVVPSLLLAALVSCSMAYYESPLLEALTAPSPDHETSSYLLPRLAAKYRPHGDWESAPDPRFYV
LTELDRDSSQAARRVKRTGTGPSLSIVNPLDVLRQRLLLEIARRRMRQTQSQIQANRDILESIGKREVN
LSQQHNIDQDDADELDADLLIASSEDKSGHNSSANNRSPESSDWSTASNSRWNNEYTSQHHS*

>Crustacean cardioactive peptide (CCAP)

MQMCHIIIGCSLAALLMILHLPAISCDDVIVQKRQVDPAEMERLLDPKRKRPFCNAFTGCGKKRSDES
MGTLVEMNSEPAVEELSRQILSEAKLWEAIQEARAELFRRRQEQFQQSNNAMERPLPLPIAGYRKKR
FAAADRTSQVEENAKPWNR*

>Ecdysis triggering hormone (ETH)

MGKYCLSLGLCARIAVAVLVVVSTLAVGATGEDGAGTNFFLKASKSVPRIGRRSEYDNFFLKASKSVP
RIGRRRELSPLTEGRDWGNVPWFRTSDNIPGPSRRADYYIHEGGPPAHPLSWNDVEKTMEESPELW
KPDLWRKNNENFPLRDEFDIQHIVRRSTGPFNKDNTKRETNDEQNLTEI*

>Elevenin (Evn)

MSGGCVHVVSTMMLLLVFISTQVAPEPIDCRQFVFAPKCRGVAAKRNFQSLNGPGYILDTNRKLDSG
LEEVLGMYVTPQPIAMPQQSENRGQIGRGHASRDRAWNSAPDQQGLKTDFLYDWYLSNKKRTRES
DVAYDY*

>Fliktin (Flik)

MKSWTAPLWCMLILLSLTQGQDDQTSDSLRTAIEAVSRRQRDLATAGPKYYSNAGLSQYRYPERDS
AAPEELAFLATPRDFTGDGQPENIGYGYQKTIASPSGMFPQPPLDGPGPISPEHVPKSKMLEKMLVD
YLEEEMADEKYGDEGDDNEAYYHKRSAFRERTDDGRHNYDGVKKRGRFRSIVPSAFRERVHGSSL
VNDMEEQKRKVITEALLRKMEQEEDERKDKQRGRIFEDEDAEEEYLDVLKNVWEKYRKNNPQVIDIE
DISEGDVGEILNYFGNSGFLDDDDIEGIKDEVSKRQYGNYDFNTHNAAMGGWGGHGFKKRWNQRLD
GEENQKGNFLYSLKFVSPAINREAIESLKNEDDLELPDERDEDVLRLTSDVRREPDPWFPAFERGEA
PEELFGNPGEEEYQRLLLAQQNDRQPSMKRIVAIRPHYSIRESSPPEVFLSPEKKYMYDTAIMKKRFP
VAKRSSNFYTSPPLLHHKNFAFMDSTDARKKKDALGNSVATTDPKVAREINQIFSSPIAGEHVHDDLH
AKDISKDSSKSSSEPLQVTTTHAPVVVSSTAVTKTKENSTEKSDKDSKSIQKKPGSVEQTVGQPITMS
RSETPLDIKKKSINWSEYFGIDKRRKKTELDGSAADSNSHPVDNEWLLNQYKTFAMTTNPDKKKSILH
SHDQAKSKKTALQQPFDTRVFDTDIFARSAQRDYNPAKKSDQEPGQNEEIRIDNMDAKLRNIEDLIVN
GAVKYTGAHEGTTDSKEIQKVKDKVMARLAAAYSLEKMRQALGEFKSSLMAQKMSKYNPENIQSAN
VDEKKKRVAVKKEKAEEKKDDDKEKRGSDQPDADEDEEFLDGPIAVQPISEGDMGRQDLNDDDDMK
CPILDQIINKCRSAGNIVGDHDQLFLPLCSLHQICHMCAPELGVPSRAACDVMFITEAESLCSEDQRCE
LAARRNIALLRSWQDQMGEGECWRSTCITHHFLHSPLPAPLPASSMR*

>FMRFamide

MLWATLFLASATLSAMAYPTDSPISEPPNIVLASPDDMDNAIGALDISETSQEDGDCEPEVETTAFRVK
RSDEQEQQQPRRCSNQNFIRFGRASAGVTSELDSPGNKEGNFIRLGRGGKSNDNFIRLGRQNKDSN
FIRFGRDKSDSFIRFGRGKTDNFIRFGRGRTDNFIRLGKGKVDNFIRLGKGKPDNFIRFGRGKQDNFIR
FGGRMKDNFVRFGRDGLSNVDDSYLDSDFVPNDNTLRVSRGGNSDSNFIRFGRGGSNFIRLGRGN
DAEITEREERSRANNFVRFGRNYDDEDFLRLSRSGNSNDLRRGKLTDRNFIRLGRSESQYETQDTDE
NSVRSSRSNTNRNFIRLGKRTDQSLQNHLLRFGRDVEQIDEMPVLSSTESNQSDLENKTDKEEYRHS
RNKRSLSFPNEEDTTEDSSDYPIIIGSNNYGEKQTSGDPKTPFGYYSPLTSGIPNYILGPELAVLAPLSN
GAESKRAKARDHNRNYIRLG*

>HanSolin (Han)

MLWPLLILSYVVLVTSRPPPSTSDDILDDVTWRDLPPEMLRQQSRQLISLYNNPESPGPGKIDISRPEK
RALSVLSRWKPFSMGFSNLVGRYPPRAPLLSMVPELDFVSAETRGTLRPIGQPLRWGRR*

>Invertebrate parathyroid hormone (IPTH)

MNTRALLICSTAAVLFLAALAHARPHRQKRVSDQRLAELETYIALRNLAGKIVTVPVGFGQVDPAKIGR
RRRRSAELLLQELLNSPTAHEDAIAEAADAILSDNNVESEEELREAHRPTQQQWLPEWSRRVQV*

> Ion transport peptide A (ITP A)

MEQQQLSRVLTCSLLVSIMLASLVAVPASGRVLGHSVNKRSFFELQCKGVYDKSIFARLDRICEDCYN
LFREPHLHTLCRSNCFSSPYFDGCIEALLLDKEKENFSQMIEFLGKK*

> Ion transport peptide B (ITP B)

MEQQQLSRVLTCSLLVSIMLASLVAVPASGRVLGHSVNKRSFFELQCKGVYDKSIFARLDRICEDCYN
LFREPHLHTLCRKDCFTTEYFKGCMEVLLEDDIEKYQSWIKQLHGADPGF*

>Kinin (K)

MRVLLLLAVVTSARSQVLGWSVHNAPDNDGIIPADRESLLTPRSKVLSLSRLLFSTRGDIYQTLPELAQ
LYRDSEGATVDWRSRVDPTTDTEPWSRTDIGSDAELEPEPLCKVGSTLWSPCHRSSDTSLDYDSSS
SLSLGLSPTDDNDEMKPVIRPSQLKRKQQSTKNRYVGRAKREAVPMETDTQEEAEKRSSAFNSWGG
KRGSGFNSWGGKRNPPFSVLGTVRRRAFTSTGSSRSPAFSSWGGKRSQSFSIVGERRMNSWGGN

SNPSLTMSTSDDIPAFSILGSKRPASFSSWGGKRDAGFSSWGGKRDPAFTILGGHHNQAYREPAFRII
GGGINEPAFTIVNDNREPAFKILGNHDFPAFSILGNSYEPSFRVLSSKRSSGFSSWGGKRDPAFNSW

GGKRDPVFKSWGGKRDAAFSSWGGKRGSGFSSWGGKRDAAFSSWGGKRDDDSDVSESGSKFSS
WGGKRDLDVEEKRSFSSWGGKRDISDSPKRGFSSWGGKRRVATQTDFENHHENGTGSNDVVGNG
LSDADKEKEETEMLEKEKAEEYQDMDNEADESGRKHASTQTSSDFQQFLDEFDNKMQLEDNQEKE
DENKEVNGENEGQAEEIEHKSEDENKADINMDQEPLATEDKSVGTTHTEEKSIGTSDSLSISKRLDRS
EGRVSKALFSSWGGKRVSHSPSLFSILGSMHKGLGRSDGILSDFLYKRGSTRHSALGSKKWGQSSV
GAVFSSWGGKRSDKGNNLRKISPQNLGRQYRRGAEFYSWGGKR*

> Leucomyosuppressin (LMS)

MKYVSVVLISVLAVLLACMPHMASAVPPPQCSPNILDDVPPRVRKVCAALSTIYELSNAMEAYLDDKV
VRENTPLVDTGVKRQDVDHVFLRFGRRR*

>Myoinhibitory peptide (MIP, AstB)

MQYAVLTGAVLWLLALVSPSSQGDPPAPPGAVASGEAQETPTQVQGPEEDKRAWRDLQGGWGKR
GWQDLQGGWGKRGWQDLQGGWGKRGWQDLQGGWGKRGWQDLQGGWGKRGWQDLQGGWG
KRGWQDLQSGWGKRAWSDLQGGWGKRAWDELRPMWGKRPWDKFHGAWGKRDSDFEIEGGNM
EEDLVPEDLAEEDDEDVKRAWSSLKGGWGKRAADWANFRGSWGKRDPGWNNLKGLWGKRADSN
WNRLSAAWGKRSIGGETGIKEDQPRAPGSSEE*

>Natalisin (Nat)

MPPASIIAILILTLAWSVAVFSNPEETNSTNASAVDEVKHRVTRSDVRAALGEKLDPGFWPSRGRRSN
SEEVAPPFWANRGRSLKLSDQEMSVLEELMKFWKDGYANVESQRLRREEPLYADEPHWLLLGRRD
EPEDVYMENRGESILNQNDPFWVARGRRRYEAPFQTKTATGEVDSSWASGNRRSLKRGLQELISSE
EPFWAARGKRSQSAEEPFWAARGKKGSPRMVEDYRNRRGLLNSAEEPFWAARGKKSNSGRRFLE
SLSSEEPFWAARGRRTARLEALSSEEPFWAARGKKGLLESLSAEEPFWAARGRRGLLESLSAEEPF
WAARGKKNSQPMEDTLSQMRTRETGTNIDPWWPVRGKRVVEEEANPEDERFWRVLESRAQNNSR
TS*

>Neuropeptide F1 transcript A (NPF1a)

MQSSLCWLLVVGCTVVLIPYLTPGVWGKSADPDQLAAMADTLRYLQELDRYYSQVARPRFGKRAEL
RPIPEQESAPDDSSDKLWRRFASRR*

>Neuropeptide F1 transcript B (NPF1b)

MQSSLCWLLVVGCTVVLIPYLTPGVWGKSADPDQLAAMADTLRYLQELDRYYSQVARPSPRSGSGR
AHELTKVENALKMLQLQELDRFYSPRTRPRFGKRAELRPIPEQESAPDDSSDKLWRRFASRR*

>Neuropeptide F2 (NPF2)

MQSPMSLMMAACLCGVVISMAMPCYSDPVAASAEIASRPTRPKVFTSPDQLRTYLQELGNYYAIEGR
PRFGKRVPAPGFRPGSSALGFAASPAAAESSNYLLRFPAQSNARSDVYQMLFPYEE*

>Neuropeptide-like peptide (NPLP)

MWPAVLLLVAALATLPQTHGDEDKRSFSSLARNGDLPLYARTWNKKMHPMMSNGKRYVGALAKTG
GLPYGKRGDESEDDELIRELWHELEEKRNLASLARTGGLSAGKRSVEALARAGYLPQPKQPQDSEE
YSHESSENNDDIKRNIGALARNGYLKRDGDELDELMEELYEKRNVASLMRNGISPFAQPGKRYLGSI
MRNQRNIGAMARNWHLPDHLKFGKRQDDEEPAEEDDTEEDLEEVAKRYVAALLRHGRLPVGGSSG
NDISEDKRHIGSLAAKSSFQVHKKSVRSAGSEDSAYNSTAKTEENKRSKRQATYLANSDEYPMPVLQ
NTDLFDYEDLADVLNGEGAPEKRFLGSVARSGWFRENSGNRMLHSSTMTKRHIGSLARLGWLPAFR
STRYSRSGRASPAPPDDEDEEDEEEEHSRSAHVPYH*

>NVP-like (NVP)

MMMAGVATAGVLVALLVAAATGLPSTLLEDTKQAAQVAANSEATAFNKASTKQEKEEELPTALPIAIS
STSKSWEPHGNAGKSSGRSNVQYPGGSRAQFHQELGSEQHQDQGHGKTVSQYEKGYQYGVGKA
ALDKHVENALLKSELYGDPSAVNQYRYYGGASERKRNQHLTYQPPSKRSYRPELPFVMPPDDLTSS
RSRLKRDLELDPEDVLTVLSLWEAEHRAKSENNPSIDPSWFSYYGLDTPDPFQEEELENEEDDDSSQ
IDGGWLEGPVAHPSSSSHRYRLERRGGYYYPLQYPYPTQKRDSSQWGGFAKDKRFMVTRKRQVST
PRDEVQTLAQLLNHPYRDPGVPLYRRVVL*

>Orcokinin A (OKA)

MKLLALLVVTIAATSVPSSASPIQSDALRESAFRDYRADSGDEENVVRHLDSIGGGHLLRELDGLSHF
PRRTRSGLDSLSGASFGGNKRFDTLSGISFGNQKRNFDEIDRSGFNSFVKKNFDEIDRSGFDSFVKR
NFDEIDRVGFGSFVKRNAPLFLTRYYDKQENH*

>Orcokinin B (OKB)

MKLLALLVVTIAATSVPSSASPIQSDALRESAFRDYRADSGDEENVVRHLDSIGELSKKEDGPKDREE
ELEEHKIKNLKKFLTHGQHSRLDSIGGGNIVRGIHPFNRELLKELESLRSGHIVTRNLESIGGGNIVGRS
LDSIGGNIVGRSLDPIGGGNIVGRSIDPIGGGGIVGRRIESIGGGNIVRAIDSIGGNILGRSLDSIGGGNL
VRALDSIGGGNLVGRSIDDIGGGNIVGRRIDSLGGGNLVGRKIESIGGGNIVGRSLDSIGGGNLVRALD
SIGGGNLVGRNIDGIGGGNLVRALDSIGGGNLVGRSIDDIGGGNILGRGHSRTIESIGGDGGIVRSLDSI
GGGNLLGRGPSRTIESIGSDGGIVRDNEDNFDIYEERLFQTKHRNKQSEESLEDKS*

>Pigment dispersing factor (PDF)

MKHLGTIILFLYLLRMAFTSPAIQLEDDRYMDKEFQTNAVNARELTNWIMQILMHKGEPTVCTHKRNS
ELINSLLGLPKVINDAGRK*

>Proctolin (PT)

MCCRQVLLVALLLVALYAATEARYLPTRSQDDRLDRLRELLRDLLESEIEKTNVNNYERRMIYKREVP
MMASEQQQHAPLMAAQQ*

>Pyrokinin (PK)

MRSRISARQHLLYYRCILLFVTAVLSSANGFRISSSLFREADPFEDAIMVDLDGNREALMVKRSQPAET
SGLWFGPRLGRRDKRSIDDTLDGGDSSKEEEIVELLRETPWALVPLKGGKRQTSFIPRLGRDSSEED
ELDLEQRSPPFAPRLGRRLVPFKPRMGRDHIPQDIYSPRLGRSIPAPHLQEQKNNPKQH*

>RFLamide

MHTTFLGRLLGLAFLFNYVHYSVGLATINGGNIVSETADSDTEHNALNPSANEKWDADMEYLTTYLEQ
LLRDGEVWDVPAGPLLYVEDVPTGYSSETTEEDDGLSIPWKRSRYYRRYPWKRQNGRHRAQYSDA
SRYMCNPTREDVFQLLVALHEAREGNTRRTVSFCNRKRPASTIFTNIRFLGRRK*

>RYamide

MAFTSSALLLLMLVTCSLVIIACSAQQFYASGRYGKRGSSTFWSGSRYGRSGGGIGRRQQSGGGSP
VEVSARNDRFFIGSRYGKRSDETVPSAEPGINDLGGLSEVLLPSDEDVNSQVTCLYTGVTNLYRCFN
RKDNASEQIVSPHQQ*

>Short Neuropeptide F (sNPF)

MQSFLTVKCVTVALCLLIVAAEFVTSAPSYSDYESVRDLYELLLQKEAMENRMQQQGQHEIVRKANR
SPSLRLRFGRRADPLLAGSPYSEHSSVESSVGEN*

>SIFamide

MQNRVAATCLLLLAVLLFADLAAATYRKPPFNGSIFGKRGNVVEYDGTGKALSALCEIASEACSAWFP
SADNN*

>SMYamide

MQFSQSVIFFLAILLLTLSTTCNPGVPFRRLPFNGSMYGKRASSALPMDYDNNKAFSSLCELAAEVCS
TWYPQQVENN*

>Sulfakinin (Sul)

MSNSMVATLLVTLGVYIVLQHHYVNAVHAAPSSSDAGGNNLEGAGQRSRVRPFLPASSRTSQYMRA
RLVPIEAPADVLNDFVIDDDVVDFSKRQSEDYGHFRFGKREQFDDYGHMRFGRSLD*

>Trissin (Tri)

MSGGPHFNMLVIGLVLWSVCTWSVGMSCNSCGSECQSACGTRNFRTCCFNYLRKRSAGGEEGDG
PGLRLELLVVPELAARYWEQQMEAKQAPPVAEADNSDSVPGRMQLVYNA*

>Tachykinin (TK)

MVLPRPRSRVGALVLVTLSLIAVVLCAPEESPKRAPSGFLGVRGKKDSGPDFNSDELNDVLDKRAPA
MGFQGVRGKKDQDEELGYDKRGPSMGFHGMRGKKDQQDLLEEYLDKRGPSRGFMGMRGKKDPM
DLDFYDKRAPSMGFQGMRGKKDDWEDEDEIYKRAPSMGFQGMRGKKDYFDDDEDEYVKRMGFM
GMRGKKEDFEAEDYPEEGIWGEDEETEELNKRAPAGFFGMRGKKVPAAGFFGMRGKKGPSVGFFA
MRGKKAPSAGFMGMRGKKAPSGFMGMRGKKDDTEDLDSLLQYLGAAYQHGRDKRNGERAPGSKK
APSGFLGTRGKKDWLSQQGGETGTEPETHINLSSK*

>Tryptopyrokinin (TPK)

??MQESAGMWFGPRMGKREDQSAQMQESAGIWFGPRMGKKDKKSAEMQESAGIWFGPRMGKRE
DQSAQMQESAGIWFGPRMGKRDKKSVEMQESAGMWFGPRMGKRDKKLAQIKESAGMWFGPRMG
KREEQSTQMQENAGMWFGPRMGKRDKKSAEMQESAGMWFGPRMGKRD???

List of precursor sequences containing protein hormone sequences

>Bursicon alpha (Burα)

MACKQTSTQQIVTGVLLVALVYVVLVGAMDECQVTPVIHVLQYPGCVPKPIPSFACTGRCSSYLQVS
GSKIWQMERSCMCCQESGEREASVSLFCPKAKAGERKFRKVTTKAPLECMCRPCTTVEESAVIPQEI
AGYADEGPLSNHFRKSL*

>Bursicon beta (Burβ)

MMNKVTSYVFLITVLMAIPPYVHPEEDAVCETLPSEIHIIKEEFDDLGRLQRTCNGDVGVNKCEGACNS
QVQPSVITPTGFLKECYCCRESFLRERTVTLTHCYDPDGGRLTKEGQATMDIRVREPADCKCFKCGD
FSR*

>Eclosion hormone 1 (EH1)

MESHKISGAVVMSFMILLCSFLSSEATTSYSINICIKNCAQCKKLFGPYFEGQLCADACVKFKGKMIPD
CEDINSIAPFLNKFE*

>Eclosion hormone 2 (EH2)

MVGHHPVLYLVILIVASTENGVASKLGVCIANCGQCKQMYGHYFQGQVCAEACLSTDGRLLPDCNNP
NTLLGFLKRLY*

>Gonadulin (Gon) [GenBank: PSN45462.1]

MKNFQAQVIVITAAILLHQCAGRPEYEDCNRKIRRQILESCSSEKGKRSAEYDTPSLPMHDEPLQNAP
SSALLGRILGVPSQWTADDVAVNNANRQVKRSPETIRQLMIDCCLANCSPDRFLGMC

>Glycoprotein hormone alpha2 (GPA2)

MFPRCWRVQCCSLVLVFFALLVLVSRTSARDAWERPGCHKVGHTRKISIPDCVEFHITTNACRGYCE
SWAVPSALDTLRVNPHQAITSVGQCCNIMDTEDVEVRVMCLDGTRDLVFKSAKSCSCYHCKKD*

>Glycoprotein hormone beta5 (GPB5)

MTLPFNRLCFVSICLMFVALWLGAESSSMQDTTLASTLDCHRRVYAYKVSKTDSAGRICWDVISVMS
CWGRCDSNEISDWRFPYKRSYHPVCLHDNRSVKEVTLRNCEEGVEPGTEVYEYLQAESCRCMVCK
SSEASCEGLRYRGQRSGPFLGGGR*

>Insulin-like peptide 1 (ILP1)

MVWKFCLCVMIVSIMCACALPENPSTMFQFVRKRETPHRYCGRHLVSILQLLCGSNYNGDIEKKRSS
EIRDSKPMQDADELPWLQSQPFEEGSEAEFPFRSRSVANSLRNRLFRRHSRDGGIVDECCIYKGCTT
SELAEYCLDR*

>Insulin-like peptide 2 (ILP2)

MWRICLQLVAIAALCLCTLAQAQSDLFQFADKRNTNKYCGRNLANMLQLVCNGNYYPMFKKSSQDM
DDMNDSGFWIQPSTMEEQQLQYPFRSRSSASALVSGSFRRRTRGVYDECCRKSCSIQEMASYCGK
R*

>Insulin-like peptide 3 (ILP3)

MWKVFLKLVVLMTICFSLSESQSDLIEFMEKRQSKRYCGNKLVDMVRLVCSSVYYTPSPKSTTTTTTT
QIPSLDKKSDDAGDDFWMQRLIQESEDQYYMFPFQSEARAHNILKRYPRGIANECCIYKGCTIEELMS
YCGK*

>Insulin-like peptide 4 (ILP4)

MWQAFCRLLIIVTVCVSLSESQSDVYQMMDKRQTRRYCGSNLVEIMQFVCNGSYNGMSTHLSQKKS
ETDDDFWMQLLQGEEQYKYPFRSRSSAHRIFKRYPGGIAYECCISKGCNIYELRSYCAPSSK*

>Insulin-like peptide 5 (ILP5)

MKMWKILLAIAIVGIVWSNALPKDSASKMHMIRKRQSTHRYCGPHLVSALRLLCNGRYYTPDEDEDDT
TTEKRSTTTNELEDIDNPILAKRKYSEESEKPQFPFRSREEANSLKPKFFRRKRRMIVEECCNLKGCS
VNELMEYCAD*

>Insulin-like peptide 6 (ILP6)

MKNAYLSLFLAAVTCFCLSDCQSETFQVDKRHASRKYCGHNLVLVMQLVCDSRYNSPRPSNPSKKS
DTDDFWQQLEVQSSEQEYRFPFRSLSNAFRLMKRGGGIADECCYNKGCTYDELRSYCST*

>Insulin-like peptide 7 (ILP7) (ILP7)

MLKCGIVTALVLVTTMVSGAPTIRMQMCGSQLANTLAQICSAYGYHDPFSQTRRVNSPSSGVNTTPN
RLRVRRGVADECCKTGCTLDTMEQYCSAPLTPAQRARFLQQYQSNALNRILQEVPSGSSLNAGSKIS
KDPKNDLSSKVRRQQDKKGQRGNNRCRCRRRRRRGKGDSEEIERQQNHIAPVIGTINPSYFGVPVF
LSPRLKKQETQQDRHRK*

>Relaxin-like peptide (RLP)

MLLPVTTVTTLCLLFEISRSTNSEQELEEMFKARSDNEWENVWHQERHTRCQEMLLRHLYWACEKDI
YRLSRRNGFQDLQLLDKYNPKYPFLSVVEARVFLRNRRGRRRRSAEPSITDECCHNSAGCTWEEYA
EYCPANKRLRKFV*

>Ortholog of the Apis ITGQGNRIF precursor

MRTLLTGTLALLAVLHGVTAWGGLFNRFSPEMLSNLGYGGHGGYRAQPFLQRLSPAEVFQDLQEDV
EPCYGKHCVTNEQCCPGSVCVYVDGMGSCIFAYGLKQGELCRRDNDCETGLLCTETGGEGRTCQP
LSSNRKQYSEDCTMSRECDISKGLCCQLQRRHRQAPRKACSYFKDPLICIGPVATDQVKDDNIEHTA
GEKRLTGKTASVNAYNNLRRRK*

>Neuroparsin 1 (NP1)

MNFCSLVLLVAMSTALLLHSCEARNPLCMPCIGNECNIEPENCEHGEVRDYCGRRVCAKGPGESCG
GPKAMRGICGDGMNCSCSKCTGCSLTTLECYTRHDHMIECLLNV*

>Neuroparsin 2 (NP2)

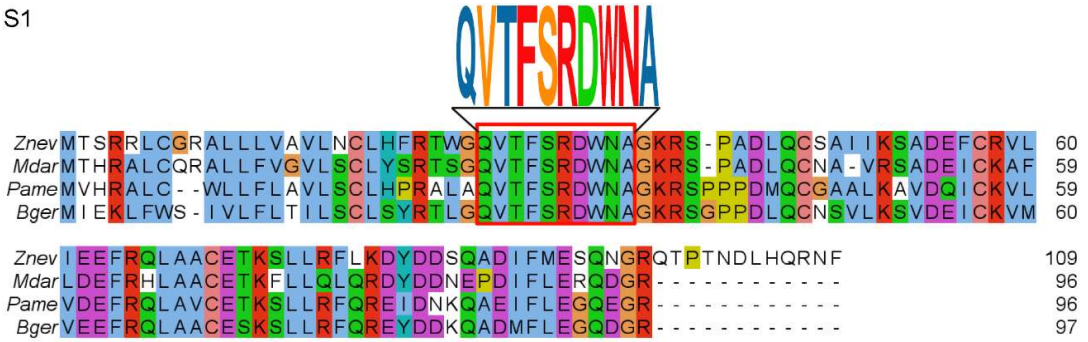
MNFCSLVLLVAMSTALLLHSCEARNPLCMPCIGNECNIEPENCEHGEVRDYCGRRVCAKGPGEFCG
GPNDVRGKCGNGMHCACSKCTGCSLATLDCYWIERDQLIDCL*

>Prothoracicotropic hormone (PTTH)

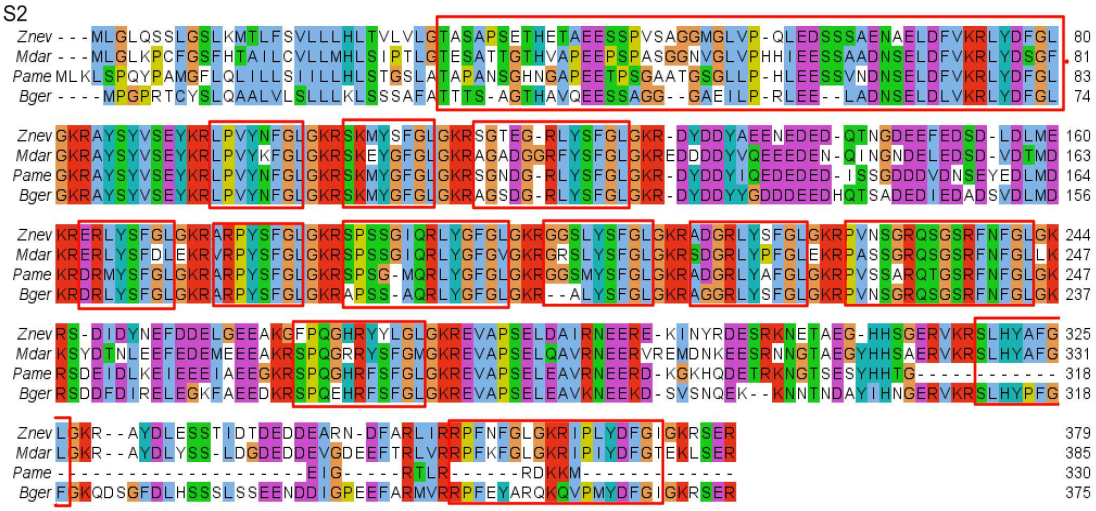
MRANHAARPSRTSYKSNITVSFLLLICNAWQSCVEASRFPVYCCSQSSPALEDYEDPDCFEDLCMSK
NIHKLDPHGMSYMLNYLHRNQDEYGINDPIDKRNPPPEAQNIDSSSGTVLFRDANPTPCSCFSDSIER
DLGRGVYPRYMKDLECNTTSCGNPLYRCHSLKREILVLTEKTQHTAPGEARLPISLRRRWTFEGVNIT
VACICQRHYSH*

Supplementary Figure S1. Alignment of full-length of ACP genes in 4 species.

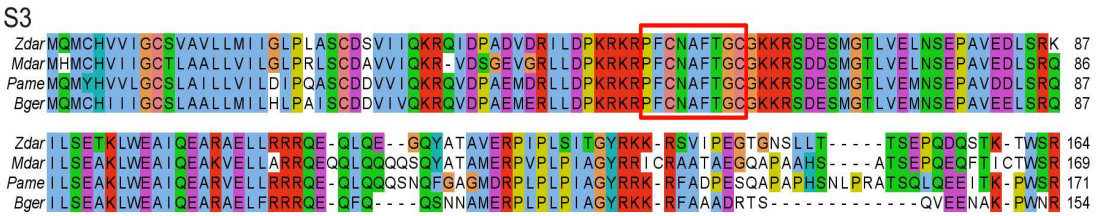
Note: the full sequence of the ACP gene in *M. darwiniensis* was used from our unpublished data. The red square indicates the bioactive peptide sequence, and the sequence logo above depicts the consensus bioactive sequences.



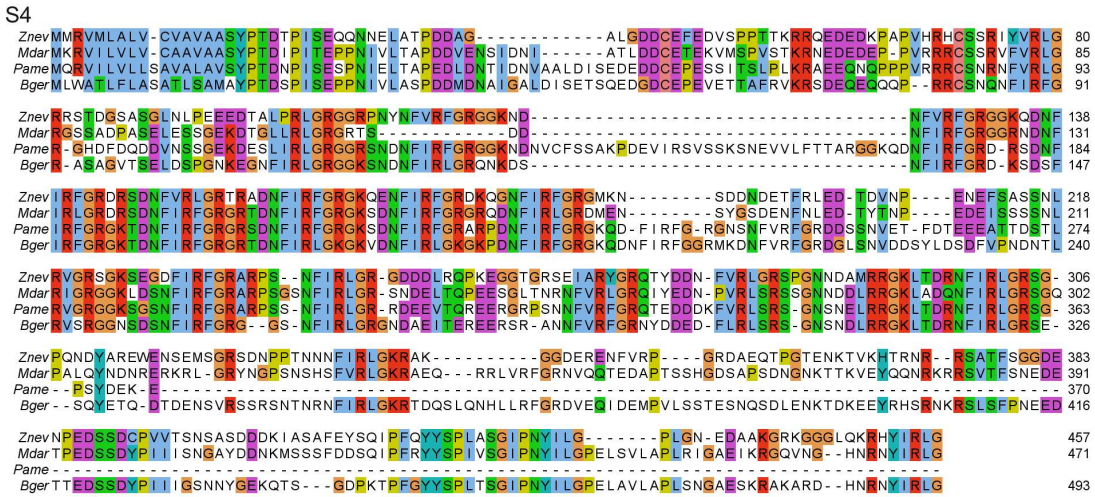
Supplementary Figure S2. Alignments of the full-length of AstA genes in 4 species.



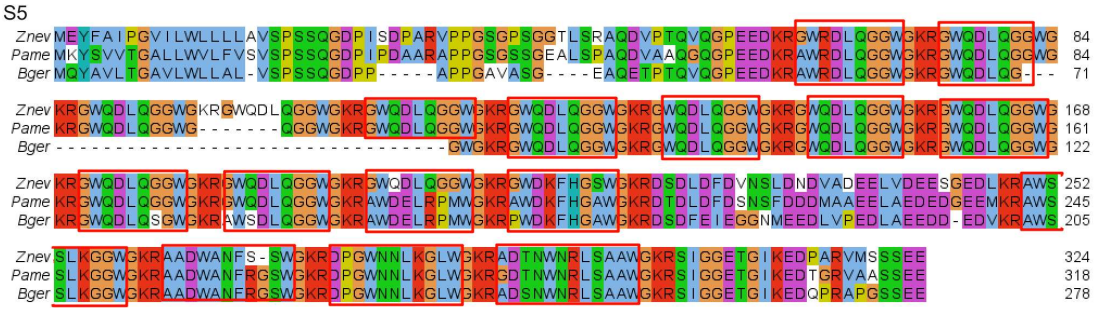
Supplementary Figure S3. Alignment of full-length CCAP genes in 4 species. The red square indicates the bioactive peptide sequences.



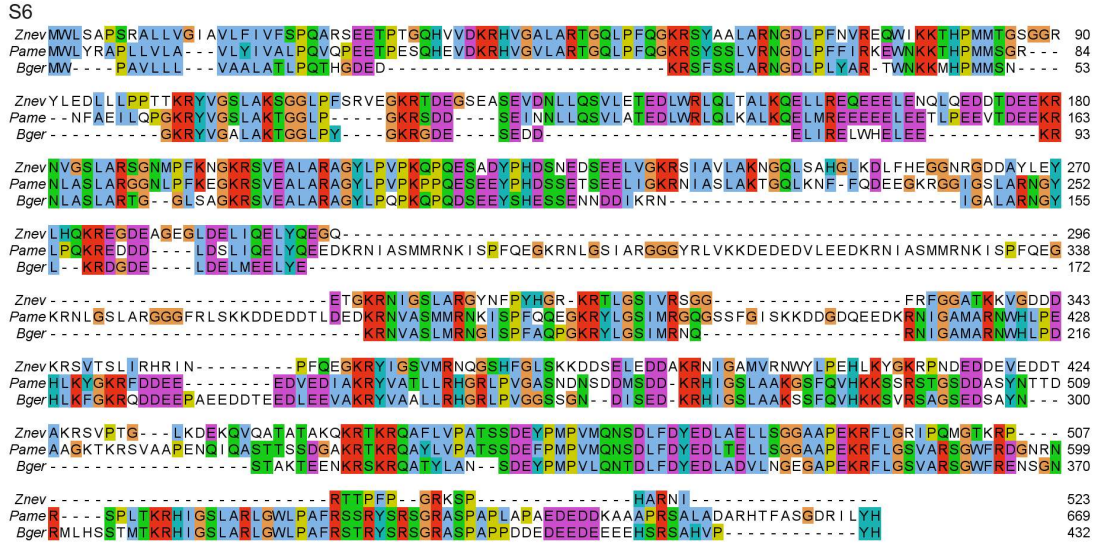
Supplementary Figure S4. Alignment of full-length of FMRF genes in 4 species.



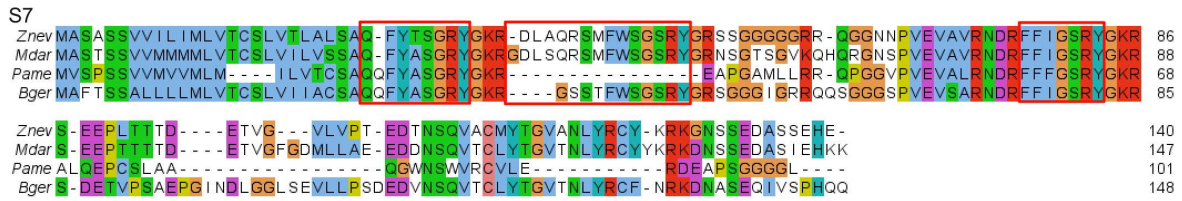
Supplementary Figure S5. Alignment of full-length of the MIP genes in 3 species.



Supplementary Figure S6. Alignment of full-length of NPLP genes in 3 species.

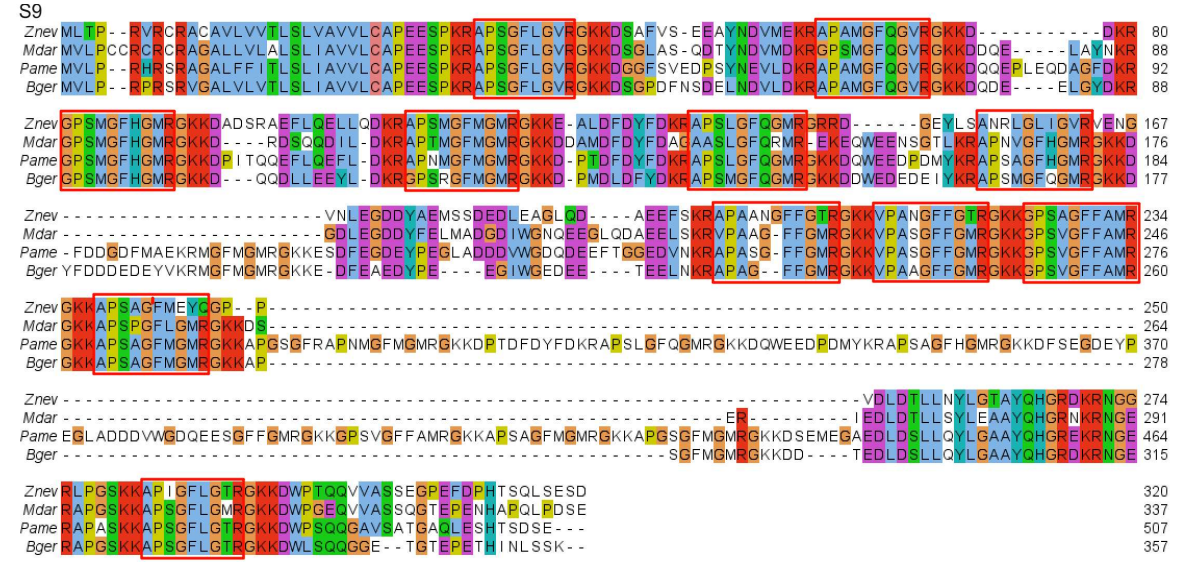


Supplementary Figure S7. Alignment of full-length of RYamide sequences in 4 species. Note: the full sequence of the RYamide of *M. darwiniensis* was used from our unpublished data; the red square indicates the bioactive peptide sequence.

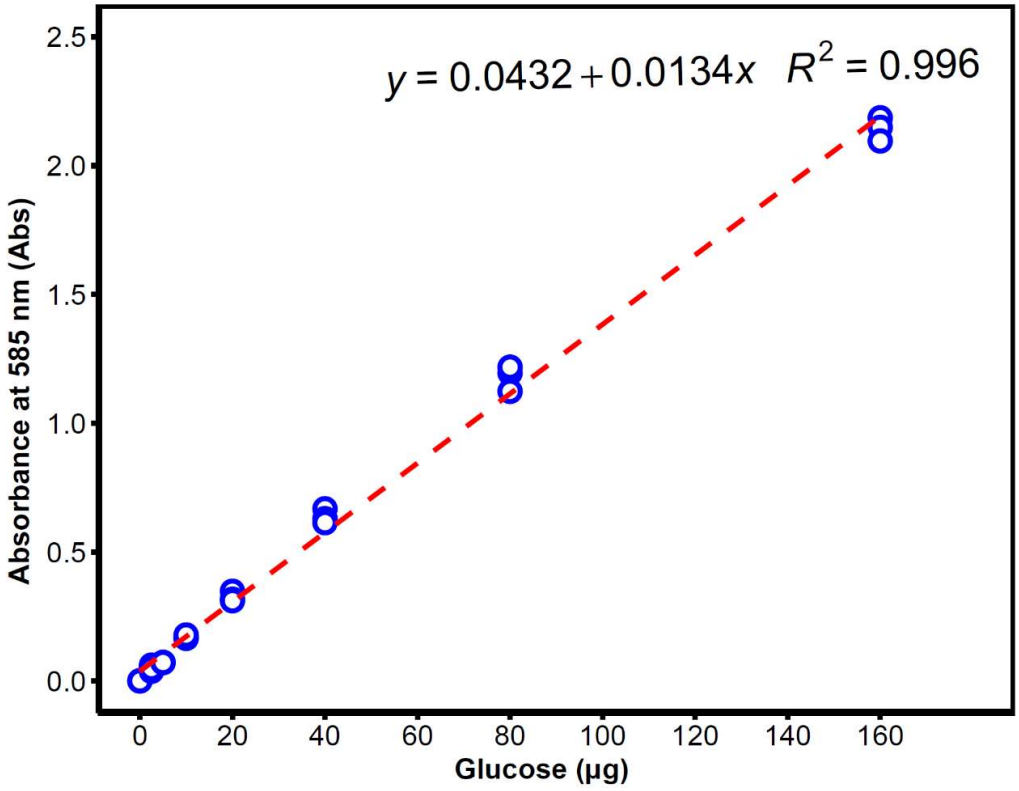


Supplementary Figure S8. Alignment of full-length of the PK/PBAN genes in 4 species.

Supplementary Figure S9. Alignment of full-length of the Tachykinin-related peptide genes in 4 termite and cockroach species.



Supplementary Figure S10. The standard curve generated from various glucose concentrations is used to measure the total carbohydrate content in cockroach hemolymph.



To establish the standard curve, 7 glucose concentrations were generated with water to obtain 160, 80, 40, 20, 10, 5, and 2.5 mg/ml concentrations. Blanks containing H_2SO_4 but no glucose were added. Each concentration consisted of three replicates, the absorbance is measured at 585 nm.

Chapter IV

Transcriptomic insights into the regulatory roles of two AKH peptides in the German cockroach, *Blattella germanica*

Transcriptomic insights into the regulatory roles of two AKH peptides in the German cockroach, *Blattella germanica*

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Author Contributions

JS and DPM conceived the overall idea. JS, ZC and LE collected and analyzed the data. JS and DPM wrote the manuscript. All authors contributed critically to the drafts.

Unpublished manuscript.

5.1 Abstract

Adipokinetic hormones (AKHs) are pivotal neuropeptides that regulate energy mobilization and play critical roles in metabolism and immunity in insects. This study investigated the transcriptomic effects of two distinct AKH peptides on the German cockroach, *Blattella germanica*, which had shown evidence of sex-specific metabolic responses in a previous bioassay (Chapter III). We also examined the impact of adipokinetic hormone receptor (AKHR) knockdown on host immune defence. Using RNA sequencing (RNA-seq), we analyzed gene expression changes at 3 and 18 hours post-injection of two AKH peptides. The results revealed significant alterations in metabolic pathways, including enhanced glycolysis, increased activity of the tricarboxylic acid cycle, and shifts in biosynthetic processes, highlighting the dynamic regulatory roles of AKHs in energy metabolism. Notably, distinct transcriptional responses were observed between males and females, consistent with the carbohydrate bioassays conducted in **Chapter III**, suggesting potential hormonal regulation of sexual dimorphism in physiological traits. Furthermore, RNA interference-mediated knockdown of *AKHR* led to reduced survival upon bacterial infection with *Pseudomonas entomophila*, underscoring the hormone's potential crosstalk with host immune defense. These findings provide valuable insights into the complex endocrine control mechanisms in *B. germanica* and suggest that AKH signaling exhibits potentially sex-specific differences in in metabolism and pathogen defense.

Keywords

Adipokinetic hormone; *Blattella germanica;* RNAi; transcriptomic analysis; energy metabolism; immunity

5.2 Introduction

Adipokinetic hormones (AKHs) are essential insect neuropeptides that regulate energy mobilization during physiological stress, locomotion, and metabolic processes. Synthesized and secreted by the *corpora cardiaca* (CC), these peptides belong to the adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family, comprising over 90 known variants identified in arthropods (Gäde 1997, Gäde, Hoffmann and Spring 1997, Gäde 2004, Gäde and Marco 2009a, Gäde et al. 2013, Gäde and Marco 2022). Following cleavage and post-translational modification, the mature AKH is released and binds to specific adipokinetic hormone receptors (AKHRs) on target tissues like fat body cells. Investigated insect species may synthesize only one AKH peptide, while others produce up to five different peptides in their CC (Gäde and Auerswald 2003, Gäde et al. 2005, Gäde 2009, Gäde and Marco 2009b, 2011, Gäde et al. 2013, Marco and Gäde 2019).

In insects, AKHs play a pivotal role in regulating energy homeostasis, particularly during periods of increased metabolic demand, such as locomotion, starvation, and stress responses (Goldsworthy, Jutsum and Robinson 1975, Stone et al. 1976, Gäde and Beenakkers 1977, Holwerda, Doorn and Beenakkers 1977, Robinson and Goldsworthy 1977b, a, Chino, Kiyomoto and Takahashi 1989, Kodrík et al. 2000, Gäde 2004, Auerswald, Siegert and Gäde 2005, Isabel et al. 2005, Kodrík 2008, Gäde and Marco 2013, Sajwan et al. 2015, Marco et al. 2017, Tang et al. 2020). They facilitate the mobilization of energy stores like glycogen and lipids by activating enzymes such as glycogen phosphorylase and triacylglycerol lipase, converting them into trehalose and diacylglycerol (Gäde and Auerswald 2003, Gáliková et al. 2015). These physiological functions are mediated by AKHRs, which are G protein-coupled receptors related to vertebrate gonadotropin-releasing hormone receptors (Hauser, Sondergaard and Grimmelikhuijzen 1998, Park, Kim and Adams 2002, Staubli et al. 2002). AKHRs have been identified and functionally studied in *P. americana* and *B. germanica* (Blattodea) (Hansen et al. 2006, Wicher et al. 2006, Huang, Belles and Lee 2012) (see **3.2, Chapter II**).

The German cockroach, *B. germanica*, is a globally pervasive urban pest that poses significant sanitary risks in hospitals and residential areas due to its role in spreading allergens and pathogens (Gore and Schal 2007, Kleine-Tebbe, Hamilton and Goodman 2019, Wang, Lee and Rust 2021, Tang et al. 2024). Additionally, it serves as a valuable model organism for studies on neuropeptide function, toxicology, symbiotic interactions, and genetic manipulation techniques like RNA interference (RNAi) due to its susceptibility to delivered double-stranded RNA (dsRNA) (Garbutt et al. 2013, Adedara et al. 2022, Latorre et al. 2022).

In **Chapter II**, we identified a second AKH from *B .germanica* and further verified its presence, as well as testing the role of both AKH peptides in energy metabolism in this species in **Chapter III**. The bioassay indicated the important role of each peptide in carbohydrate metabolism in both male and female cockroaches. However, the specific effects of each AKH peptide on gene expression and immune function in *B. germanica* remain unexplored.

This study aimed to understand the transcriptomic effects of the two AKH peptides. Specifically, we sought to characterize gene expression changes induced by AKH peptide injection at two time points, 3 hours and 18 hours post-treatment, using RNA sequencing (RNA-seq). By analyzing differential gene expression, we aimed to uncover the metabolic and biosynthetic pathways influenced by AKH peptides to enhance our understanding of their potential roles, especially in sex-specific metabolic responses. Furthermore, in a survival assay, we examined the impact of *AKHR* knockdown on pathogen defence. By employing RNA interference to knock down *AKHR* expression, we investigated whether reduced AKHR levels affected the survival rates of *B. germanica* following infection with the entomopathogenic bacterium *Pseudomonas entomophila*. This aspect of the study sought to understand the role of AKH signaling in modulating susceptibility to bacterial infection, thereby exploring the potential immunological functions of AKHs.

5.3 Materials and methods

Insects

The stock cultures of the German cockroach, *B. germanica*, were maintained as outlined in **Chapter III**. In the RNA sequencing experiment, which involved two AKH peptides and Ringer-treated groups, only 5-day-old adult males and females were used to reduce individual variation. For the AKHR gene knockdown experiment, only adult males were used.

Peptide injection and sample collection

We utilized the same peptide dosage described in the bioassay to prepare two adipokinetic hormone peptides, AKH1 (P1: pQVNFSPGWGTa) and AKH2 (P2: pQLNFSPGWGPa), as detailed in **Chapter III, Bioassay section**. In brief, adult cockroaches were split into three treatment groups: Ringer solution (control), Peptide 1, and Peptide 2. Each group was then divided into two time points: 3 hours and 18 hours post-treatment. Following injection with 10 pmol (in 0.5 µL) of the peptides or 0.5 µL of Ringer solution, the cockroach guts were removed to minimize bacterial contamination at the respective time points. Subsequently, the remaining tissue was immediately snap-frozen in liquid nitrogen and stored at -70 °C until library preparation for RNA sequencing.

RNA extraction, library preparation and sequencing

Total cockroach RNA was extracted using the RNeasy Mini Kit (Qiagen). During the extraction process, the RNase-Free DNase Set (Qiagen) was employed to eliminate residual genomic DNA following the manufacturer's protocol. The RNA was quantified using a Qubit (Invitrogen), and its integrity was assessed with an Agilent 2100 Bioanalyzer. However, the heating step at 70°C for 2 minutes was omitted. Subsequently, the RNA was stored at -80°C until library preparation. Transcriptomic libraries were prepared with the NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (PerkinElmer). Each library was barcoded with NEXTFLEX Unique Dual Index Barcodes, and libraries were pooled at equimolar concentrations before sequencing. The pooled samples were sequenced on an Illumina platform using paired-end reads. The 3-hour samples were sequenced at the Competence Centre for Genomic Analysis (CCGA) in Kiel, Germany, and the 18-hour samples were sequenced at the Max Planck Institute for Molecular Genetics in Berlin, Germany.

Quality control and preprocessing

Raw sequence reads were cleaned using the wrapper tool Trim Galore (Martin 2011), which removed adapters, barcodes, short reads (< 25 bp), and low-quality bases (Phred score below 20). Additionally, residual rRNA was filtered out using SortMeRNA with the default datasets: smr_v4.3_default_db (Kopylova, Noé and Touzet 2012). FastQC was used initially to assess sequencing quality. Genome of *Blattella germanica* [GCA_003018175.1] (Harrison et al. 2018) was downloaded from InsectBase 2.0 (Mei et al. 2021) as the reference genome for reads mapping. We apply HISAT2 to align clean reads upon the reference genome (Kim, Langmead and Salzberg 2015). For post-alignment processing, mapped reads were sorted and indexed using SAMtools (Li et al. 2009). FeatureCounts (Liao, Smyth and Shi 2014), in combination with the reference annotation file, were used to quantify gene counting.

Differential gene expression and Gene Ontology enrichment

The analysis compared gene expression in the control and peptide-treated groups using the DESeq2 package (Love, Huber and Anders 2014). Genes were considered significantly differentially expressed if they met the criteria of an adjusted p-value < 0.05 and |log2 fold-change| ≥ 1. The protein datasets were functionally annotated using the eggNOG-mapper web server (http://eggnog-mapper.embl.de) (Cantalapiedra et al. 2021) with default settings. Gene Ontology (GO) enrichment analysis was conducted to identify overrepresented biological processes, cellular components, and molecular functions among the differentially expressed genes. Additionally, enriched pathways were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. GO terms with Q values < 0.05

were considered significantly enriched, and similarly, KEGG pathways with Q values < 0.05 were regarded as significantly enriched.

AKHR knockdown and RT-PCR

The nucleotide sequence of AKHR from the German cockroach was obtained from the NCBI database (GenBank: GU591493.1). Briefly, the cDNA of *B. germanica* was prepared from extracted total RNA through reverse transcription, as described in **Chapter II**. A DNA template for dsRNA synthesis was then generated from the cDNA using PCR with a T7-tailed primer (see detailed sequence information in the Supplemental Information S1), which was designed using the online tool SnapDragon-dsRNA Design (https://www.flyrnai.org/cgibin/RNAi_find_primers.pl). The PCR product was subsequently purified using the Monarch® PCR & DNA Cleanup Kit (NEB). The cleaned product was used for dsAKHR synthesis following the manufacturer's instructions, using the HighYield T7 P&L RNA Synthesis Kit (Jena Bioscience). A noncoding sequence amplified from the pSTBlue-1 vector (Novagen) served as the control dsRNA (dsMock). 1 µL (1500 ng/ µL) of synthesized dsRNA per adult male cockroach was injected through the abdomen using the Nanoject III (Drummond Scientific Company, USA). The same dose of dsMock was used as a control. Treated cockroaches were then incubated at 28°C for 5 days. On day 5 of incubation, total RNA was extracted using the Qiagen RNeasy Mini Kit, following the manufacturer's instructions. Reverse transcription was performed by using Reverse Transcriptase (H Minus M-MLV Reverse Transcriptase, Metabion). To measure knockdown efficiency, a separate primer pair targeting the AKHR sequence, designed to avoid overlap with the dsRNA target regions, was used in quantitative real-time PCR (qPCR) with a three-step cycling protocol using the SensiFAST SYBR No-ROX Kit (Bioline). The efficiency of this primer set was validated using five serial dilutions. The *B. germanica* Actin 5c gene was used as the reference gene. All primers used in this study are listed in Table 1.

Table 1. The primer sequences used for AKHR gene knockdown in *Blattella germanica*
Note: sequences in bold showing T7 polymerase promoter

Primer	Sequence (5'-3')
dsAKHR-Fw	**TAATACGACTCACTATAGGGA**GAATCTGCAATTGGCAACATCA
dsAKHR-Rv	**TAATACGACTCACTATAGGGA**GACGAAGTCCAAACACTGCTCA
dsMock-Fw	**TAATACGACTCACTATAGGGA**AAGCTC
dsMock-Rv	**TAATACGACTCACTATAGGGA**ATACAGCGGCCGCGAG
AKHR-Fw	GTGTACGGTTTCCCACTCCTCGTCA
AKHR-Rv	TCAGGAACACAAGGCTCGATCTCCG
Actin5C-Fw	TCGTTCGTGACATCAAGGAGAAGCT
Actin5C-Rv	TGTCGGCAATTCCAGGGTACATGGT

Microorganism preparation and infection

The entomopathogenic bacterium strain, *Pseudomonas entomophila* (DSM 28517T, Gram-negative) was used to assess susceptibility in *AKHR gene* knockdown cockroaches. *P. entomophila* was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and stored at -70 °C prior to use. The preparation of *P. entomophila* followed the method described by He et al. (2021) and Esparza-Mora et al. (2023). Briefly, *P. entomophila* was activated overnight and then inoculated for growth at 28 °C and 30 °C in nutrient broth, following DSMZ instructions. All cultures were washed twice with Ringer's solution. Bacteria concentrations were determined by measuring the optical density at 600 nm, reaching a final concentration of 4.0 x 10^5 CFUs/µL.

Adult male cockroaches treated with dsRNA (dsMock and dsAKHR) injection were injected with 0.5 µL of the *P. entomophila* solution (final dosage: 2.0 x 10^5 CFUs per individual) using the Nanoject III (Drummond Scientific Company, USA). Control males, which had already been injected with Ringer solution on the same day as the dsRNA injections, were this time injected with 0.5 µL of Ringer solution. After injection, all cockroaches were provided water and food, as described in **Chapter III**. The mortality was monitored every 12 hours until no further deaths were observed by the 20th day.

Statistical analysis

Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), with ΔΔCT values normalized to the average Ct values of the dsMock control group. Normalized gene expression values were compared using Student's t-tests (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$). All qPCR reactions were performed in technical triplicates, with mean Ct values were used for analysis. Statistical analyses were conducted using R (v 4.3.3) and RStudio (v2023.12.1).

Survival data were analyzed using the Kaplan-Meier method to estimate cumulative survival curves, and comparisons between groups were performed using the log-rank (Mantel-Cox) test with Bonferroni correction, and p-values were categorized as: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Survival analysis was performed using the "survival" package (Therneau, 2024), and survival curves were visualized with the "survminer" package (Kassambara, Kosinski, and Biecek, 2021) in R (v 4.3.3).

5.4 Results

After read cleanup, we mapped the reads to the reference genome and evaluated mapping rates in combination with the FastQC report result. Replicates with a mapping rate below 80% were excluded, likely due to low RNA abundance resulting from library preparation using single individuals, which could lead to biased sequencing outcomes (Supplementary Figure 1). To investigate the changes in gene expression caused by AKH peptide injection, we conducted RNA-seq analysis at 3 and 18 hours after treatment in both male and female cockroaches.

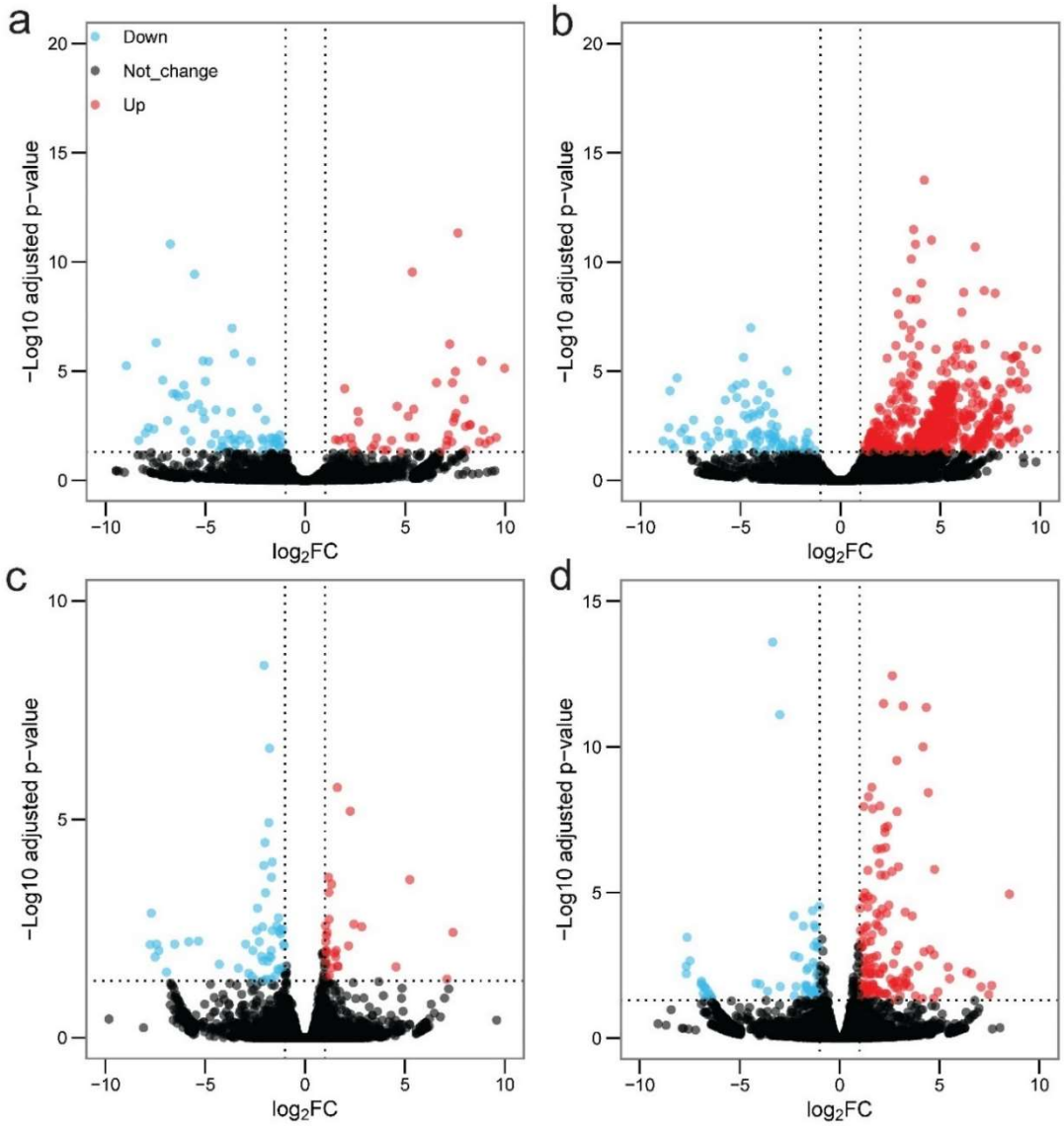

Figure 1. Volcano plot analysis of differentially expressed genes (DEGs) at 3 hours post-AKH injection in male and female cockroaches. Panels (a) through (d) show volcano plots representing upregulated and downregulated genes when comparing Ringer's solution (control) with AKH peptide treatments in both male and female cockroaches: (a) Ringer vs. P1 (male), (b) Ringer vs. P2 (male), (c) Ringer vs. P1 (female), and (d) Ringer vs. P2 (female). In the plots, red dots indicate significantly upregulated genes, while blue dots indicate significantly downregulated genes.

5.4.1 Differentially expressed genes (DEG) analysis at 3 hours

At the 3-hour time point in male cockroaches, P1 resulted in 57 up-regulated and 70 down-regulated genes. In comparison, P2 showed 823 up-regulated and 87 down-regulated genes (Figure 1a, 1b). In female cockroaches, treatment with P1 led to 32 up-regulated and 55 down-regulated genes, whereas P2 induced 144 up-regulated and 63 down-regulated genes (Figure 1c, 1d).

5.4.2 Enrichment analysis at 3 hours

To explore the biological processes influenced by AKH injection after 3 hours, we performed GO enrichment analysis on the DEGs. In male *B. germanica*, the administration of P1 resulted in a significant enrichment of GO terms related to metabolic and biosynthetic processes, such as "glycolytic process through glucose-1-phosphate" (GO:0061622), "arginine biosynthetic process" (GO:0006526), "sulfate assimilation" (GO:0000103), and "carbohydrate phosphorylation" (GO: 0046835) (Figure 2a, 2b, Supplementary Table 2). As expected, these terms indicate that there is an increased regulation of pathways involved in energy metabolism and biosynthesis, suggesting a shift towards immediate metabolic adjustments.

The administration of P2 to males resulted in a more widespread activation of key energy pathways, including "NAD metabolic process" (GO:0019674), “NADH metabolic process” (0006734), "gluconeogenesis" (GO: 0006094), and "tricarboxylic acid metabolic process" (GO:0072350), highlighting a significant increase in ATP production and energy balance mechanisms.

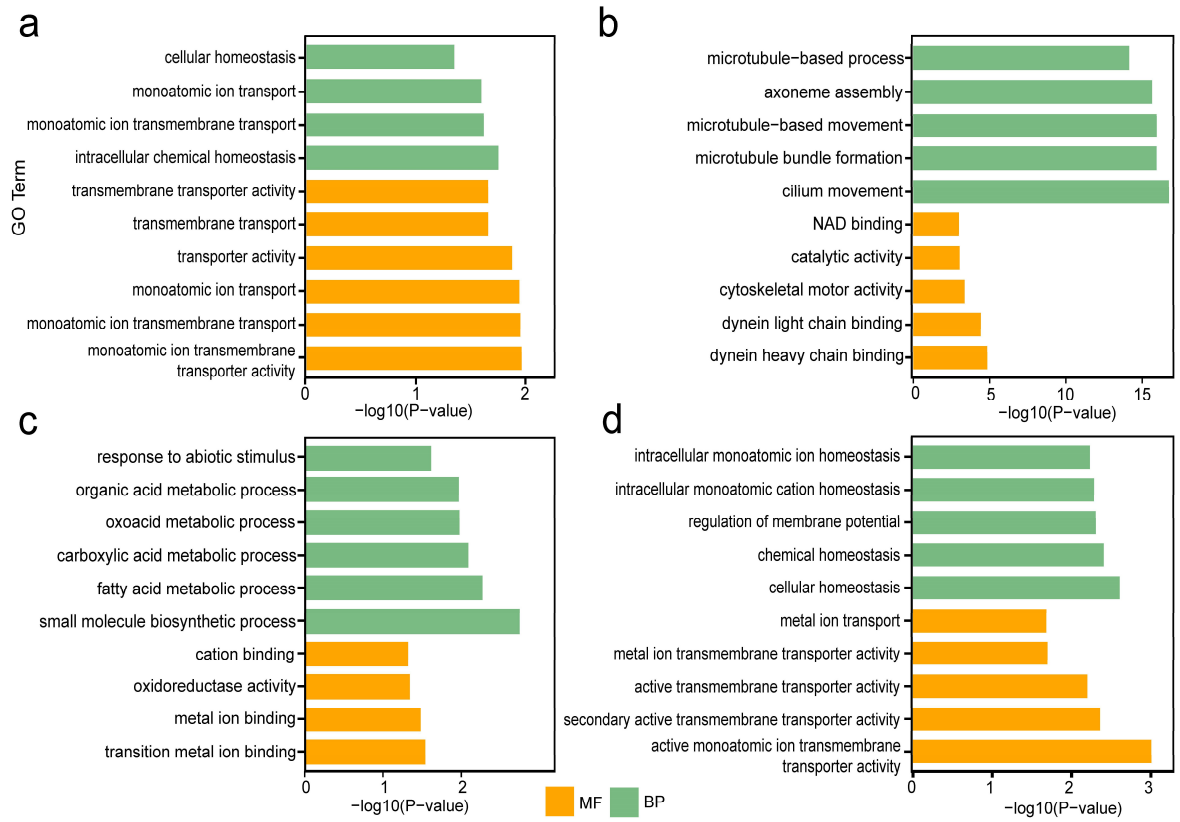

Figure 2. GO Enrichment analysis of differentially expressed genes (DEGs) at 3 hours post-AKH injection in male and female cockroaches. Panels display bar plots representing significantly enriched GO terms within the Biological Process (BP) and Molecular Function (MF) categories (the top 10 are shown). (a) Ringer vs. P1 (male); (b) Ringer vs. P2 (male); (c) Ringer vs. P1 (female), and (d) Ringer vs. P2 (female). The bar plots highlight the functional impact of AKH peptide treatments, illustrating key biological processes (BP) and molecular functions (MF) that are significantly enriched in response to the treatments.

In female cockroaches, several enriched GO terms overlapped with those observed in males (Figure 2c, 2d, Supplementary Table 1). Such as "small molecule biosynthetic process" (GO:0044283) and "carboxylic acid metabolic process" (GO:0019752) indicated similar activation of energy-related metabolic pathways. However, females also showed unique responses based on the peptide treatment. With P1, terms related to biosynthesis and metabolism were prominent, similar to the male response. On the other hand, treatment with P2 resulted in the enrichment of processes like "lipid biosynthetic process" (GO:0008610), "regulation of hormone secretion" (GO:0046883), and "monatomic ion transmembrane transporter activity" (GO:0015077), indicating a distinct regulatory adaptation involving both metabolic and hormonal pathways.

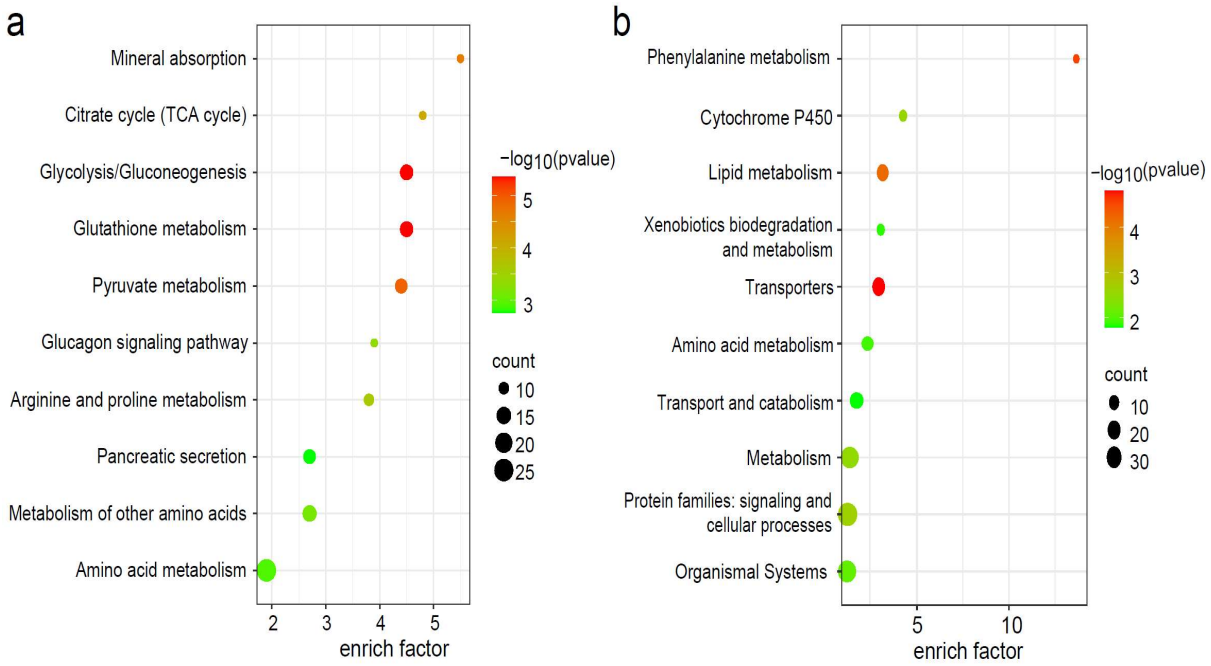

Figure 3. Dot plots of KEGG pathway enrichment analysis of up and down-regulated DEGs across both genders and two peptides at 3-hour treatment. (a) top 10 enriched pathways of up-regulated DEGs at 3 hours; (b) top 10 enriched pathways of down-regulated DEGs at 3 hours.

We also analyzed the KEGG pathway enrichment of the upregulated and downregulated genes at 3 hours post-AKH injection across both genders and two peptides (P1 and P2) revealed significant activation and suppression of key metabolic pathways. Specifically, pathways related to energy metabolism, such as "Glycolysis/Gluconeogenesis" and "Citrate cycle (TCA cycle)", were enriched, suggesting an increased demand for rapid energy production and glucose utilization in response to AKH treatment. The enrichment of "Glutathione metabolism" and "Arginine and proline metabolism" indicates heightened amino acid metabolism, supporting cellular adaptation to stress. Additional enriched pathways include "Pyruvate metabolism", which serves to link glycolysis and the TCA cycle, further showcasing the metabolic reprogramming towards energy production. The presence of pathways such as "Glucagon signaling pathway" and "HIF-1 signaling pathway" indicates involvement in glucose mobilization and hypoxic responses, aligning with AKH's metabolic role in promoting immediate energy mobilization.

The KEGG analysis of downregulated genes revealed the suppression of pathways involved in lipid metabolism, detoxification, and various signaling processes. Specifically, the "Lipid metabolism" pathway was notably downregulated, along with pathways such as "Amino acid metabolism" and "Transport and catabolism", suggesting a temporary decrease in amino acid catabolism and cellular transport activities. Additionally, pathways related to "Alcoholism" and "Substance dependence" were

downregulated, indicating a broader suppression of processes associated with cellular detoxification and stress responses.

5.4.3 Differentially expressed genes (DEG) analysis at 18 hours

At the 18-hour time point in male cockroaches, P1 resulted in 268 up-regulated and 208 down-regulated genes. Meanwhile, P2 showed 74 up-regulated and 73 down-regulated genes (Figure 4a, 4b). In female cockroaches, treatment with P1 led to 92 up-regulated and 176 down-regulated genes, whereas P2 induced 89 up-regulated and 133 down-regulated genes (Figure 4c , 4d).

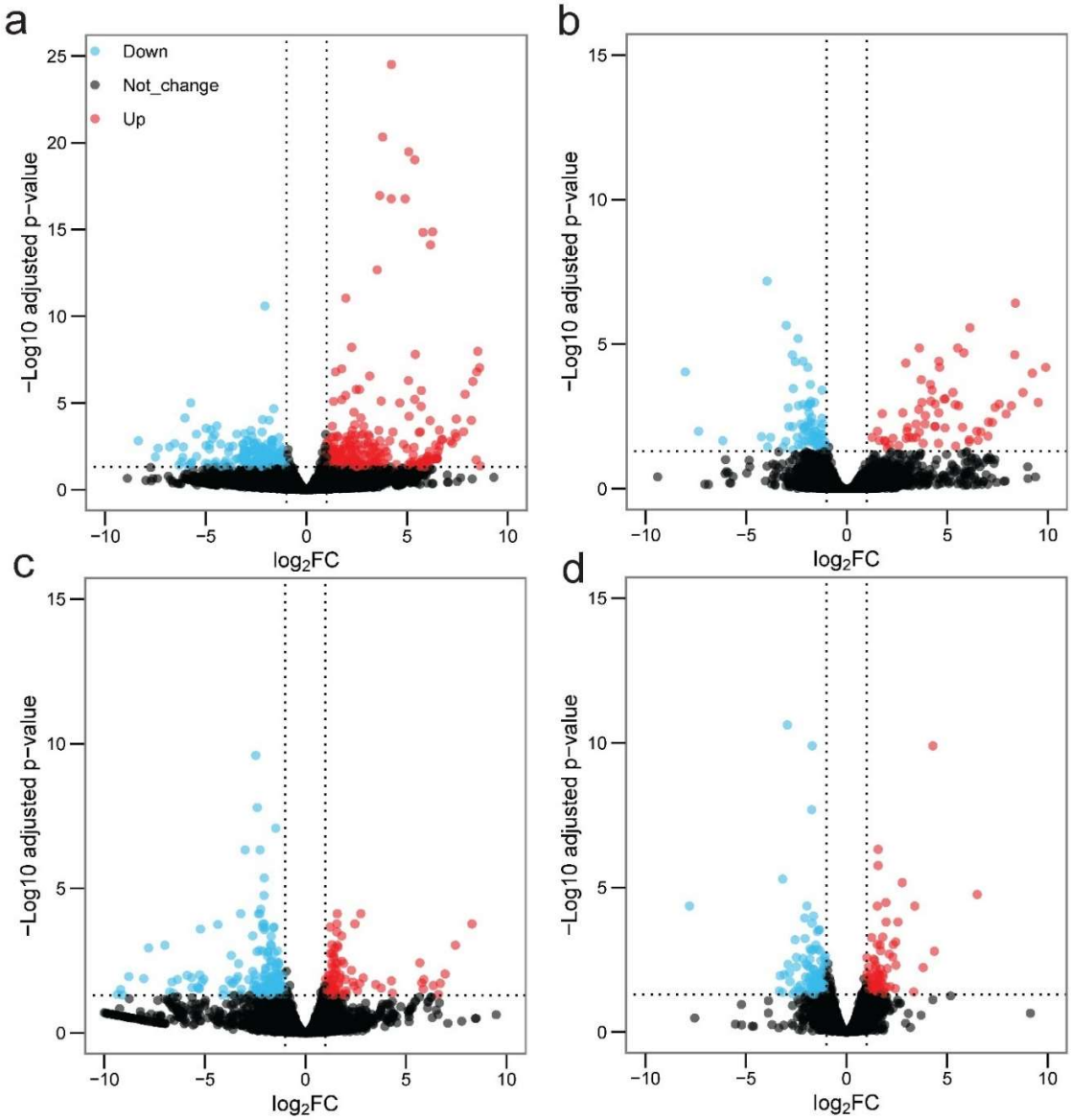

Figure 4. Volcano plot analysis of differentially expressed genes (DEGs) at 18 hours post-AKH injection in male and female cockroaches. Panels show volcano plots representing upregulated and downregulated genes when comparing Ringer's solution (control) with AKH peptide treatments in both male and female cockroaches: (a) Ringer vs. P1 (male); (b) Ringer vs. P2 (male); (c) Ringer vs. P1 (female); and (d) Ringer vs. P2 (female). In the plots, red dots indicate significantly upregulated genes, while blue dots indicate significantly downregulated genes.

5.4.4 Enrichment analysis at 18 hours

The analysis of differentially expressed genes (DEGs) at the 18-hour time point after AKH injection in *B. germanica* showed a significant change in the biological processes affected by the peptide treatments compared to the 3-hour time point.

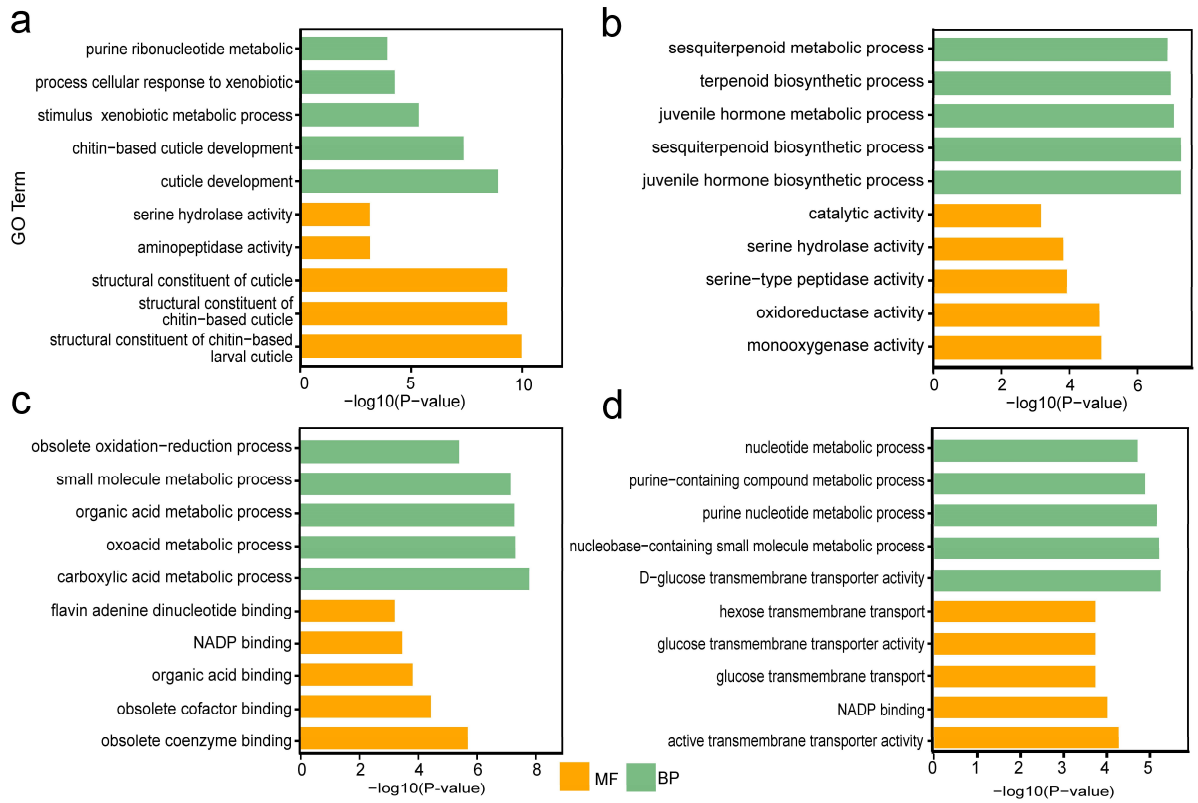

Figure 5. GO Enrichment analysis of differentially expressed genes (DEGs) at 18 hours post-AKH injection in male and female cockroaches. Panels display bar plots representing significantly enriched GO terms within the Biological Process (BP) and Molecular Function (MF) categories (the top 10 are shown). (a) Ringer vs. P1 (male); (b) Ringer vs. P2 (male); (c) Ringer vs. P1 (female); and (d) Ringer vs. P2 (female). The bar plots highlight the functional impact of AKH peptide treatments, illustrating key biological processes (BP) and molecular functions (MF) that are significantly enriched in response to the treatments.

In male cockroaches treated with P1, the enriched GO terms mainly revolved around nucleotide metabolism and energy-related processes. These included "purine nucleotide metabolic process" (GO:0006163), "ribose phosphate metabolic process" (GO:0009156), and "ATP metabolic process" (GO:0046034). These findings suggest sustained metabolic activity aimed at maintaining energy balance and nucleotide synthesis, indicating an ongoing adaptation to prolonged AKH exposure. Furthermore, terms related to cellular energy production, such as "aerobic electron transport chain" (GO:0022900) and "cellular respiration" (GO:0045333), were also significantly enriched, indicating the continued activation of mitochondrial functions (Figure 3a, 3b, Supplementary Table 1). In contrast, males treated with P2 exhibited an enrichment of biosynthetic and hormonal processes, including "juvenile hormone biosynthetic process" (GO:0006706) and "terpenoid biosynthetic process"

(GO:0016114). This suggests a regulatory adjustment towards hormone production and secondary metabolite biosynthesis, reflecting a nuanced response that differs from the energy-focused pathways observed at earlier time points.

In females, the 18-hour response to P1 showed significant enrichment of GO terms related to lipid and amino acid metabolism. These included "fatty acid metabolic process" (GO:0006631), "lipid oxidation" (GO:0034440), and "glutamine family amino acid metabolic process" (GO:0009064). These findings indicate a shift towards catabolic processes that support sustained energy release and adaptation to metabolic stress. Notably, these responses suggest a broader reliance on lipid utilization, which contrasts with the immediate energy mobilization observed at 3 hours. At 18 hours, female subjects treated with P2 showed increased enrichment of GO terms, including "glucose transmembrane transporter activity" (GO:0005355), "active transmembrane transporter activity" (GO:0022804), and "carbohydrate derivative biosynthetic process" (GO:1901135). These results suggest a heightened focus on glucose handling and carbohydrate metabolism, indicating an adaptive regulatory strategy prioritizing glucose uptake and utilization over other energy sources in response to AKH. The GO enrichment analysis at 18 hours demonstrates a shift from immediate responses to more sustained and specialized metabolic adjustments, highlighting the dynamic regulatory capacity of AKH peptides in modulating energy metabolism and adaptive processes over time. These findings differ significantly from the patterns observed at 3 hours.

Analysis of the genes upregulated and downregulated 18 hours after AKH injection in both genders and two peptides (P1 and P2) showed significant changes in pivotal metabolic and regulatory pathways. Upregulated genes were enriched in pathways related to energy metabolism, such as "Glycolysis/ Gluconeogenesis" and "Oxidative phosphorylation," indicating a sustained need for rapid energy production and increased glucose utilization in response to AKH treatment. Pathways like "Thermogenesis" and "Energy metabolism" suggested a shift toward energy-intensive processes, supporting continued metabolic activity and adaptation to prolonged AKH exposure. In contrast, downregulated genes exhibited suppression of pathways related to lipid metabolism and hormone biosynthesis, redirecting resources toward more immediate energy demands. Additionally, pathways related to "Insulin signaling" and "Insect hormone biosynthesis" were downregulated, indicating a modulation of hormonal regulatory mechanisms under AKH influence. This pattern suggests that AKH treatment induces broad metabolic reprogramming, enhancing pathways critical for immediate energy production while downregulating those associated with lipid metabolism, hormone synthesis, and detoxification processes.

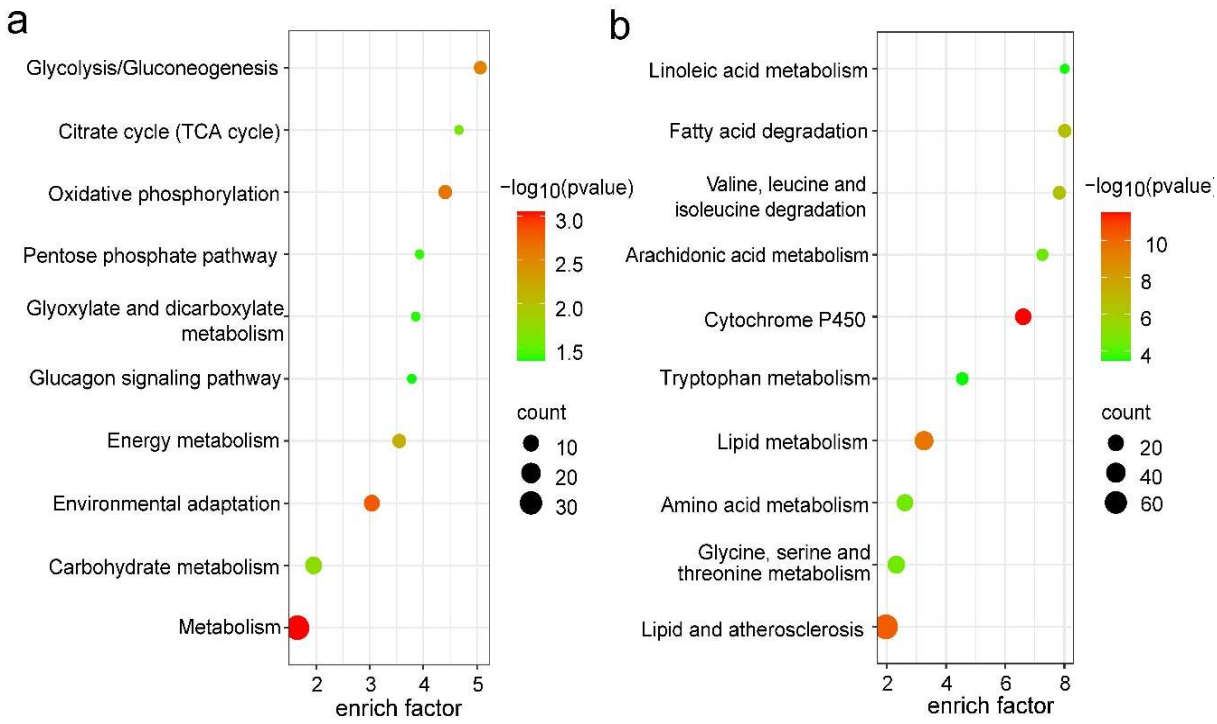

Figure 6. Dot plots of KEGG pathway enrichment analysis of up and down-regulated DEGs across both genders and two peptides at 18-hour treatment. (a) top 10 enriched pathways of up-regulated DEGs at 18 hours; (b) top 10 enriched pathways of down-regulated DEGs at 18 hours.

5.4.5 AKHR knockdown effect

In **Chapter III,** we confirmed the absence of two AKHs in *B .germanica* using MALDI-TOF tissue profiling, and further confirmed that both peptides could significantly increase carbohydrate levels in the hemolymph on male and female cockroaches through peptide injection.

In 2012, the *AKHR* gene of *B. germanica* was cloned, and its distribution in various tissues and stages was studied, further the knockdown effects were tested in both male and female cockroaches (Huang, Belles and Lee 2012). Since the effects of AKHs are mediated through their receptor, AKHR, we hypothesized that knocking down *AKHR* expression could influence cockroach energy metabolism and potentially alter their susceptibility to pathogen infection. To investigate this, we targeted 5-day-old adult male *B. germanica*, employing RNAi to suppress *AKHR* expression. While the previous study focused on RNAi-mediated knockdown effect in the fat body, our study expanded the analysis to encompass the whole body, confirming efficient knockdown, with *AKHR* expression reduced by approximately 75% five days after dsAKHR injection compared to controls (dsMock) (t-test, $P < 0.001$) (Figure 7).

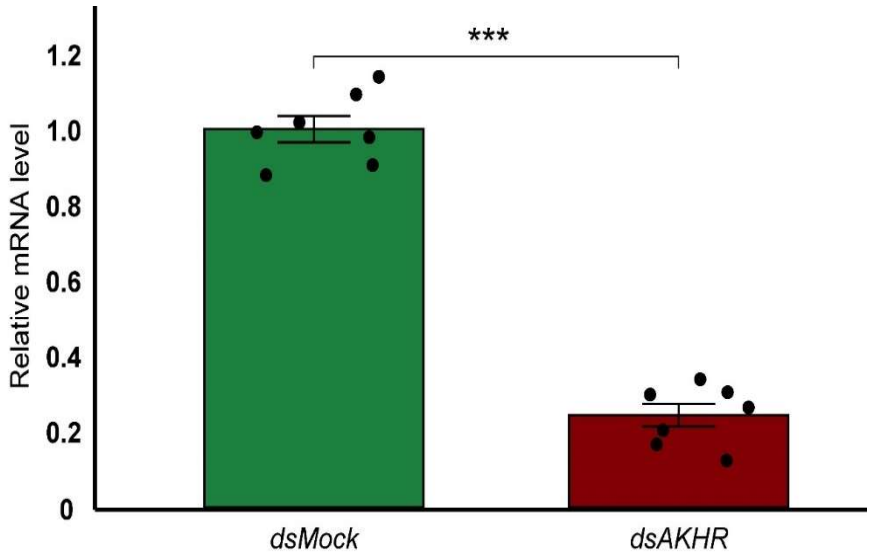

Figure 7. Effect of dsRNA-mediated *AKHR* knockdown on mRNA levels. After 5 days of treatment with synthesized dsRNA, *AKHR* levels in the whole bodies of adult male *B. germanica* were significantly reduced. The same dosage of dsMock was used as a negative control (n=7). Gene expression data were normalized, and comparisons between groups were analyzed using a Student's t-test (*: P < 0.05; **: P < 0.01; ***: P < 0.001). Bars represent mean + SEM.

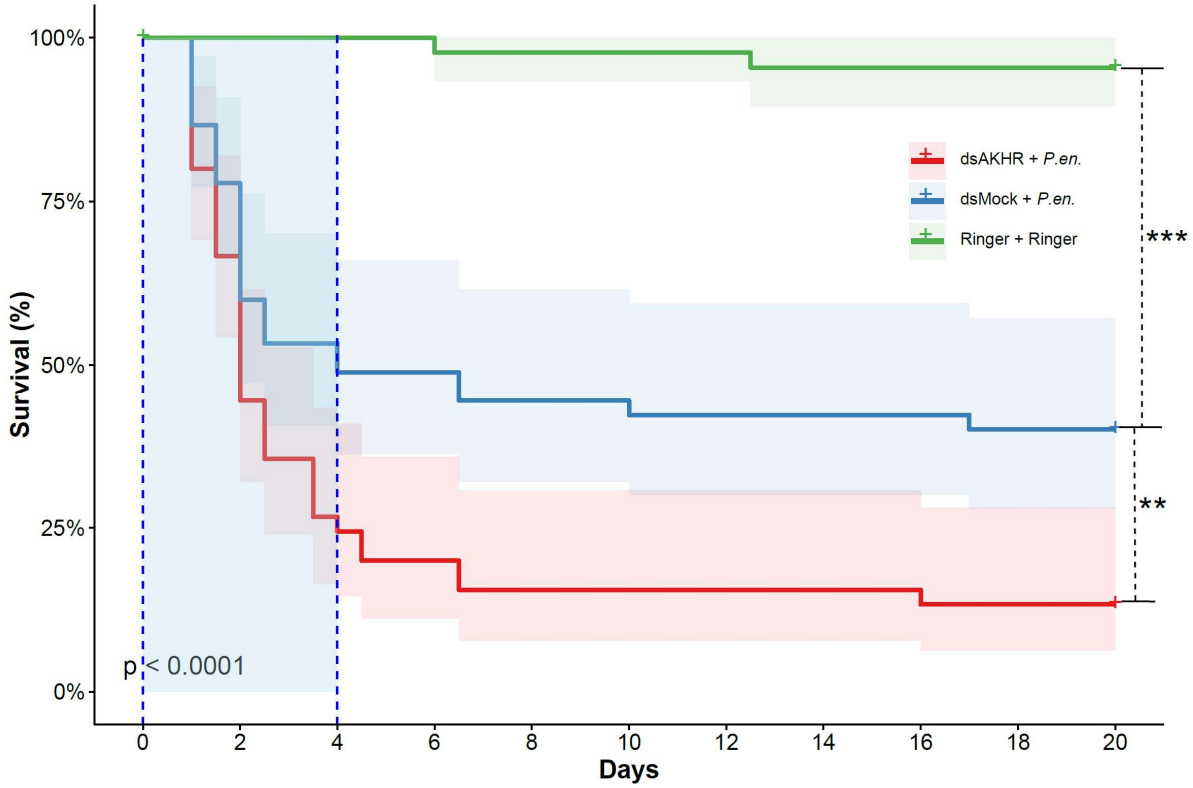

Figure 8. Kaplan-Meier survival curves were used to compare the susceptibility of cockroaches to *P. entomophila* after treatment with dsAKHR, dsMock (control), and Ringer-only controls. Adult male *B. germanica* were injected with the same dosage of *P. entomophila* following 5 days of dsRNA treatment (n=45 per treatment), the Ringer-only control group consisted of cockroaches that were injected with Ringer solution only and did not receive any dsRNA treatment.. Pairwise comparisons between groups were performed using the log-rank test (*: P < 0.05; **: P < 0.01; ***: P < 0.001). The blue dashed lines highlight the rapid mortality observed in the dsAKHR group within the first 4 days.

5.4.6 Effect of AKHR knockdown on male infection responses

Here, the objective was to explore the role of the AKH signaling pathway in influencing the susceptibility of male *B. germanica* to bacterial infections by RNAi-mediated *AKH receptor* (*AKHR*) knockdown. We employed three treatment groups: Ringer, dsMock, and dsAKHR, with 45 individuals in each group, and administered treatments over a 5-day period before subjecting them to bacterial challenge. *B. germanica* injected with Ringer solution showed minimal or no mortality throughout the observation period. However, survival analysis indicated significant differences across the groups ($p < 0.001$). Specifically, the dsAKHR-treated group displayed a notable reduction in survival compared to the Ringer group ($\chi^2 = 69.56$, $p < 0.001$), while the dsMock group also showed lower survival relative to the Ringer group ($\chi^2 = 32.89$, $p < 0.001$). Notably, the dsAKHR group demonstrated a significantly higher mortality rate compared to the dsMock group ($\chi^2 = 7.80$, $p = 0.0052$), with a more rapid decline in survival observed particularly within the first 4 days, as highlighted by the blue dashed lines (Figure 8). This result indicates increased susceptibility to infection following *AKHR* suppression.

5.5 Discussion

In this study, we investigated the transcriptomic effects of two adipokinetic hormone (AKH) peptides on *B. germanica* and examined the impact of *AKHR* knockdown on responses to pathogen infection. Our RNA sequencing (RNA-seq) analysis revealed that AKH peptides elicit distinct transcriptional responses at both short-term (3 hours) and medium (18 hours) time points. GO and KEGG enrichment analyses uncovered significant shifts in metabolic pathways. Furthermore, *AKHR* interference via synthesized dsAKHR increased the cockroaches' susceptibility to bacterial infection, underscoring the intricate regulatory roles of AKH peptides in energy metabolism and immune functions.

At the 3-hour mark, we observed a significant upregulation of genes associated with energy metabolism pathways such as glycolysis and the tricarboxylic acid (TCA) cycle. This indicates an immediate metabolic shift towards energy production, as corroborated by enriched GO terms and KEGG pathways related to ATP production and NAD metabolic processes. These findings align with the established role of AKH as a key energy metabolic regulator in insects, mobilizing energy stores to meet immediate physiological demands (Gäde and Rinehart 1990, Gäde and Auerswald 2003, Gäde and Goldsworthy 2003, Van der Horst 2003, Gäde 2004). Specifically, in male cockroaches treated with P1, we observed enrichment of GO terms associated with metabolic and biosynthetic processes, such as "small molecule biosynthetic process" and "carboxylic acid metabolic process". This suggests enhanced metabolic activity geared towards rapid energy production. Similar findings have been

reported in other insects where AKH triggers immediate metabolic responses to meet energy demands (Gäde and Rinehart 1990, Patel, Soulages and Arrese 2006, Kodrík 2008). Treatment with P2 resulted in a broader activation of key energy pathways, including the "NAD metabolic process" and the "tricarboxylic acid metabolic process. This indicates increased ATP production and mechanisms involved in maintaining energy balance (Lorenz 2003, Gäde 2004, Gáliková et al. 2015, Rajan et al. 2017). In female cockroaches, both peptides induced similar energy-related metabolic pathways at the 3-hour time point. However, P2 treatment uniquely enriched processes like "lipid biosynthetic process" and "regulation of hormone secretion", indicating a more nuanced regulatory adaptation involving both metabolic and hormonal pathways. This suggests that gender-specific metabolic needs and hormonal differences may drive differential responses to AKH peptides. Similar sexually dimorphic responses have been reported in *Aedes aegypti*, where AKH peptides are encoded by distinct genes and exhibit temporal differences in expression (Kaufmann, Merzendorfer and Gäde 2009). Moreover, studies in *D. melanogaster* have demonstrated sex-specific differences in AKH signaling pathways and metabolic regulation (Chatterjee et al. 2014, Gáliková et al. 2016).

We observed a notable difference in the number of up- and down-regulated genes between the P1 and P2 treatments at the 3-hour mark in male cockroaches, with P2 resulting in a significantly higher count of up-regulated genes compared to P1. This finding stands in contrast to our bioassay results, which showed that male cockroaches exhibited elevated carbohydrate levels after injection with P1, but not with P2 (**Chapter III**). One possible explanation for this discrepancy could be technical issues related to library preparation. Since we utilized individual cockroaches as single biological replicates during library preparation, this may have introduced biases, such as variations in RNA abundance among samples. These technical factors, along with the biological differences observed in the bioassay, may have contributed to the variation in regulated gene counts between the two treatments. Future studies with increased replication or pooled samples could help minimize these technical biases and enhance our understanding of metabolic responses.

At the medium (18-hour) time point, gene expression profiles shifted towards more sustained and specialized metabolic adjustments. In males treated with P1, continued enrichment of terms related to nucleotide metabolism and aerobic respiration was observed, indicating sustained energy production and metabolic activity. This prolonged metabolic adjustment may reflect the need for continued energy availability during extended periods of activity or stress (Gäde and Auerswald 2003, Van der Horst 2003). Conversely, P2 treatment in males led to upregulation of biosynthetic processes, including hormone biosynthesis and terpenoid biosynthesis, suggesting a regulatory role that extends

beyond immediate energy mobilization. In females, P1 treatment at 18 hours enriched GO terms related to lipid and amino acid metabolism, such as "fatty acid metabolic process" and "glutamine family amino acid metabolic process". This reflects a shift towards catabolic processes that support sustained energy release and adaptation to metabolic stress. P2 treatment enhanced glucose handling and carbohydrate metabolism, as indicated by enriched terms like "glucose transmembrane transporter activity" and "carbohydrate derivative biosynthetic process." These findings suggest an adaptive regulatory strategy prioritizing glucose uptake and utilization in response to AKH, consistent with studies showing AKH-induced upregulation of glucose transporters (Lorenz and Anand 2004, Arrese and Soulages 2010).

The differential responses between sexes and peptides highlight the complexity of AKH signaling in *B. germanica*. Gender-specific metabolic needs and hormonal differences likely contribute to these distinct regulatory adaptations. The enrichment of pathways such as "insulin secretion" and "peptidase activity" among the upregulated genes at 3 hours supports existing studies demonstrating how AKH peptides interact with other hormones and peptides to regulate metabolism and energy balance in insects (Rajan and Perrimon 2012, Kim and Neufeld 2015). AKH acts antagonistically with insulin-like peptides (ILPs) to maintain metabolic homeostasis, as ILPs promote energy storage while AKH promotes energy mobilization (Bharucha, Tarr and Zipursky 2008b, Gáliková et al. 2015). Studies in *Drosophila* showed that AKH and ILPs coordinate to regulate carbohydrate levels and lipid storage (Broughton et al. 2005, Grönke et al. 2007). These findings are further supported by studies in *Drosophila*, where the fat body serves as the primary energy storage tissue. Glycogen and triglycerides are the major forms of energy storage for carbohydrates and lipids, respectively (Arrese et al. 2001, Arrese and Soulages 2010). Mutation of the *Drosophila* AKHR, a functional analog of the mammalian glucagon receptor, leads to abnormal accumulation of both lipids and carbohydrates, resulting in obese phenotypes and marked starvation resistance (Lee and Park 2004, Bharucha, Tarr and Zipursky 2008a).

In our study, *AKHR* knockdown in male *B. germanica* resulted in a significant reduction in *AKHR* expression levels and had a notable impact on host survival rates. Specifically, reduced *AKHR* expression led to decreased survival rates following infection with the entomopathogenic bacterium *P. entomophila*. This indicates that AKH signaling may play an important role in immune regulation, either directly by modulating immune pathways or indirectly through its effects on energy metabolism. This is consistent with findings in other insects; for example, in the brown planthopper *Nilaparvata lugens*, *AKHR* knockdown disrupted trehalose metabolism and impaired reproductive function,

leading to delayed oocyte maturation and reduced fecundity (Lu et al. 2018). Similarly, the previous study demonstrated that knockdown of the *AKHR* in *B. germanica*, led to significantly lower hemolymph trehalose levels and reduced protection against oxidative stress, highlighting the important role of AKH signaling in mediating anti-oxidative responses (Huang, Belles and Lee 2012).

The interplay between energy metabolism and immunity is a critical aspect of insect physiology. As energy reserves are essential for mounting effective immune responses, and hormones like AKH that regulate energy availability can thus influence immunity (Schmid-Hempel 2005, Adamo et al. 2008). In *L. migratoria*, AKH has been shown to enhance immune responses by increasing hemocyte activity and antimicrobial peptide expression (Goldsworthy, Opoku-Ware and Mullen 2002, Goldsworthy, Chandrakant and Opoku-Ware 2003). Our findings in *B. germanica* suggest a potential role for AKH signaling via its receptor (AKHR) in maintaining metabolic homeostasis and supporting immune defenses against pathogenic challenges. This implies that AKH may influence immune responses through its effects on energy allocation, ensuring that sufficient resources are available for immune defense. Alternatively, neuropeptide signaling might directly affect the expression of immune-related genes, as has been proposed in studies on other insect species. For instance, it has been demonstrated in *Tenebrio molitor* that tachykinin-related peptides (TRPs) regulate immune responses by modulating the expression of a wide range of immune-related genes (Sadd and Schmid-Hempel 2009, Beckage 2011, Urbański et al. 2022).

5.6 Conclusion

This study provides a comprehensive view of how AKH peptides influence gene expression and pathway activation in *B. germanica*, highlighting their potentially multifaceted roles in both metabolism and immunity. Differential gene expression and enrichment analyses demonstrated that AKH treatments induce a rapid shift towards energy mobilization and biosynthesis at the 3-hour time point, while prolonged exposure at 18 hours drives more sustained metabolic adjustments involving nucleotide metabolism, lipid oxidation, and carbohydrate metabolism. The distinct regulatory adaptations observed between sexes and between the two AKH peptides underscore the complexity of AKH-mediated regulation in insects. Moreover, the knockdown of *AKHR* further emphasized the important role of AKH signaling in supporting both metabolic homeostasis and immune defense. Reduced AKHR expression led to increased susceptibility to bacterial infection, indicating that AKH signaling is integral to immune function in *B. germanica*.

From an applied perspective, our results have potential implications for pest management strategies targeting metabolic and immune pathways. Therefore, future research should focus on exploring the mechanistic aspects of AKH signaling, particularly its interactions with other hormonal pathways, such as insulin-like peptides and juvenile hormones. By investigating the downstream effectors of AKHR activation and exploring the cross-talk between metabolic and immune pathways, it will be possible to gain deeper insights into the complex regulatory networks governing cockroach physiology.

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Supplemental Information S1 : Double-stranded RNA (dsAKHR, dsMock) design

The double-stranded AKHR used in this study was amplified from synthesized cDNA templates using the designed primer sequences (shown in green). Another primer pair, designed without overlapping sequences used for dsRNA, was used to monitor the expression of the AKHR gene by qPCR (shown in pink).

>*Blattella germanica* AKH hormone receptor mRNA, complete cds [GenBank: GU591493.1]
ATGACGACTACAGAGCTGCCCCGTGAGCAGCAGCTGACTGAGGACATGACTTTTGGCTCCATACATAAGTTATGTATCGCCACGTATTG
CGTTCTCATGACTGTATCTGCAATTGGCAACATCACGGTTCTGGTCAACATACTCAAGAGACGCCGCAACCTTCGTTTTGGGAACAACTA
CATGTTCATGCATCTTGCCATAGCAGACCTATTGGTGACTTTTCTCATGATGCCGCTAGAAATCGGCTGGAATGCAACAGTGTCTTGGAG
AGCCGGTGACGCTGCTTGCAGAGTGATGTCGTTCTTCAGGATATTCGGCCTCTATCTGTCTAGCTTCGTAATTGTGTGCATCAGTCTGGA
CCGCTGTTTCGCCATTCTAAGGCCCATGTCGAACGTCGTCAACGTCGCCAAACGCAGCAGAGTAATGCTGACCACTGCCTGGTCATTGGC
CACTGTTTGTAGTTTACCTCAGGTGTTCATCTTCCACGTACAACAGCATCCCGTTTTCACATGGTATGAGCAGTGTTTGGACTTCGACATG
TTTCCGACTCAGCTGTACCAGTTCTGGTACAGGATATTAAATATGGTTCTAGTGTACGGTTTCCCACTCCTCGTCATTTTCATCTCATACGC
CTGTATCCTCACAGAGATTTTTCGCAGATATCAGCTCAGTTCAGACGAAAACTTCCGGAGATCGAGCCTTGTGTTCCTGAACAGAGCCAA
AAATAGGACGCTCAAAATGGCCATCATAATATTCGTAGTGTTCTTCATCTGCTGGACTCCCTACTATGTGATGTGTCTCTGGTACTGGATA
GACCAGCAGTCTGCTGAAAAGGTTGACTTGCGCGTGAGAAAAGGCCTGTTCCTGTTTGCTTGTACCAATTCCTGTATGAACCCGATTGTG
TACGGGTACTTCAACTTCCGTTCAGGACGGGGAAGTGGTTATGGTGCAACAAGACCAGGGCAGCAGTTACAGCATCATCAAATAACTGC
ATTGAGCAATAACTCAACGGGAGTTAACAGCCGAAGGGGAAGCAACTGCAGCAGCATCTATCGGGACAACAGCAACCAGAGCATGTCC
TGGAATCGCCGAAGCAGCCATGAAACAGAAATGCACGCCAATAACAATCGAGACGAAAATCACTTACACCCCAACTCAGCTGCGAACCA
CAATTTGCGAACTACAGTATCTACTGTGAGTGAAGTTCCTGAAGCAAGATGA

The primers used for the amplification of the construct were tagged with the T7 polymerase promoter sequence (in bold: 431bp).

dsAKHR-Fw: **TAATACGACTCACTATAGGGAGA**ATCTGCAATTGGCAACATCA
dsAKHR-Rv: **TAATACGACTCACTATAGGGAGA**CGAAGTCCAAACACTGCTCA

A noncoding sequence amplified from the pSTBlue-1 vector (Novagen) was used as control dsRNA (dsMock) with the following primers (T7 polymerase promoter sequence in bold). This sequence is commonly used as control dsRNA in *B. germanica* (citations listed below).

dsMock-Fw: **TAATACGACTCACTATAGGGA**AAGCTC
dsMock-Rv: **TAATACGACTCACTATAGGGA**ATACAGCGGCCGCGAG

Citations

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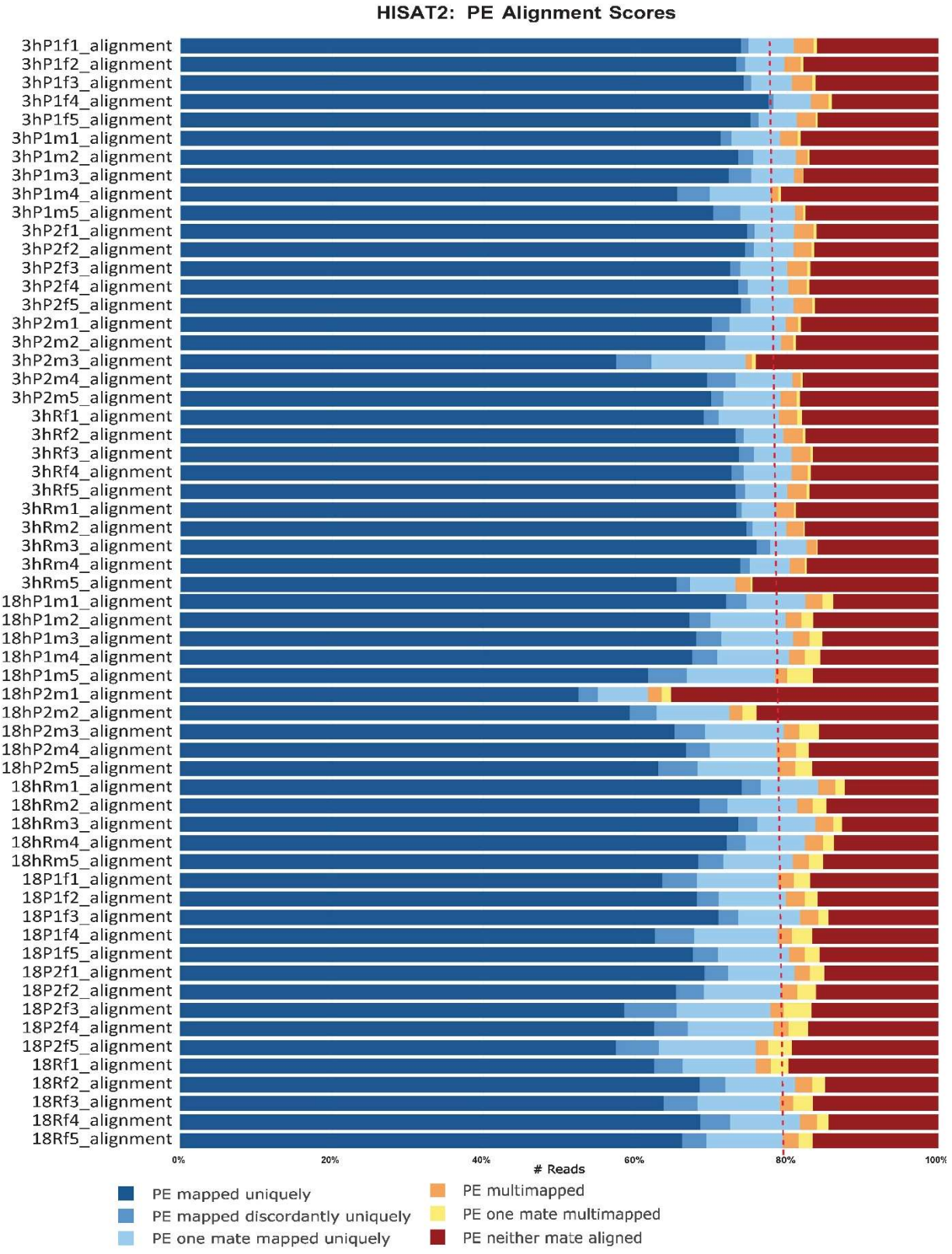
Supplementary Figure 1. Sequence alignment histogram showing the distribution of HISAT2 paired-end (PE) reads mapped to the *B. germanica* reference genome (GCA_003018175.1). Proper alignments are shown in shades of blue, while improper or incomplete alignments are indicated in yellow and red. MultiQC was used to aggregate and visualize the alignment statistics.



Supplementary Table 1. Gene Ontology (GO) enrichment analysis across different treatments, highlighting biological processes (BP), cellular components (CC), and molecular functions (MF) of differentially expressed genes. GO terms with P < 0.05 were considered significantly enriched.

Class	GO_Name	GO_ID	GO Level	P_value	HitsSet	HitsBg
Enriched GO terms in 3h_male_RvsP1						
BP	response to type II interferon	GO:0034341	6	7.20E-04	3	40
BP	sulfation	GO:0051923	5	0.002247	2	16
MF	sulfotransferase activity	GO:0008146	5	0.002469	2	17
MF	calcium ion transmembrane transporter activity	GO:0015085	7	0.00392	3	73
CC	6-phosphofructokinase complex	GO:0005945	6	0.004201	1	1
CC	sulfate adenylyltransferase complex (ATP)	GO:0009336	7	0.004201	1	1
CC	adenylyltransferase complex	GO:1902503	6	0.004201	1	1
CC	cell body fiber	GO:0070852	6	0.004201	1	1
MF	ABC-type zinc transporter activity	GO:0015633	9	0.004435	1	1
MF	6-phosphofructokinase activity	GO:0003872	8	0.004435	1	1
MF	fructose binding	GO:0070061	5	0.004435	1	1
MF	sulfate adenylyltransferase (ATP) activity	GO:0004781	8	0.004435	1	1
MF	adenylylsulfate kinase activity	GO:0004020	6	0.004435	1	1
MF	glutamate-tRNA ligase activity	GO:0004818	7	0.004435	1	1
MF	argininosuccinate synthase activity	GO:0004055	5	0.004435	1	1
BP	argininosuccinate metabolic process	GO:0000053	9	0.004488	1	1
BP	sulfate assimilation	GO:0000103	5	0.004488	1	1
BP	glycolytic process through glucose-1-phosphate	GO:0061622	16	0.004488	1	1
BP	glutamyl-tRNA aminoacylation	GO:0006424	11	0.004488	1	1
BP	arginine biosynthetic process	GO:0006526	11	0.004488	1	1
BP	smooth septate junction assembly	GO:0090528	10	0.004488	1	1
BP	glycolysis from storage polysaccharide through glucose-1-phosphate	GO:0093001	17	0.004488	1	1
BP	sequestering of zinc ion	GO:0032119	5	0.004488	1	1
BP	obsolete positive regulation of sequestering of zinc ion	GO:0061090	0	0.004488	1	1
BP	fructose 1,6-bisphosphate metabolic process	GO:0030388	6	0.004488	1	1
CC	obsolete intrinsic component of membrane	GO:0031224	0	0.005287	11	1172
CC	basolateral plasma membrane	GO:0016323	5	0.005483	4	173
CC	basal plasma membrane	GO:0009925	5	0.005483	4	173
MF	monoatomic ion transmembrane transporter activity	GO:0015075	4	0.005746	7	514
MF	transferase activity, transferring sulphur-containing groups	GO:0016782	4	0.005757	2	26
BP	response to zinc ion	GO:0010043	6	0.007332	2	29
MF	transporter activity	GO:0005215	2	0.007548	8	684

(table continued on next page)

MF	zinc efflux transmembrane transporter activity	GO:0022883	9	0.00885	1	2
MF	sulfate adenylyltransferase activity	GO:0004779	7	0.00885	1	2
MF	fructose-6-phosphate binding	GO:0070095	6	0.00885	1	2
MF	proline-tRNA ligase activity	GO:0004827	7	0.00885	1	2
MF	fructose-2,6-bisphosphate 2-phosphatase activity	GO:0004331	9	0.00885	1	2
MF	monoatomic cation efflux transmembrane transporter activity	GO:0046583	6	0.00885	1	2
MF	phosphofructokinase activity	GO:0008443	7	0.00885	1	2
BP	prolyl-tRNA aminoacylation	GO:0006433	11	0.008956	1	2
BP	protein digestion	GO:0044256	4	0.008956	1	2
BP	trypsinogen activation	GO:0032023	9	0.008956	1	2
BP	response to oleic acid	GO:0034201	7	0.008956	1	2
BP	cellular response to oleic acid	GO:0071400	8	0.008956	1	2
BP	fructose 6-phosphate metabolic process	GO:0006002	6	0.008956	1	2
CC	basal part of cell	GO:0045178	3	0.009752	4	204
CC	calcium channel complex	GO:0034704	7	0.01034	2	37
BP	cellular response to type II interferon	GO:0071346	7	0.012382	2	38
CC	GAIT complex	GO:0097452	3	0.012552	1	3
MF	ATPase-coupled monoatomic cation transmembrane transporter activity	GO:0019829	7	0.012671	2	39
MF	transmembrane transporter activity	GO:0022857	3	0.012849	7	597
BP	sequestering of metal ion	GO:0051238	4	0.013405	1	3
BP	obsolete regulation of sequestering of zinc ion	GO:0061088	0	0.013405	1	3
BP	positive regulation of insulin secretion	GO:0032024	11	0.013662	2	40
MF	metal ion transmembrane transporter activity	GO:0046873	6	0.015297	4	221
MF	sodium-independent organic anion transmembrane transporter activity	GO:0015347	6	0.017625	1	4
BP	intracellular chemical homeostasis	GO:0055082	4	0.017658	5	346
BP	citrulline metabolic process	GO:0000052	8	0.017834	1	4
BP	sodium-independent organic anion transport	GO:0043252	7	0.017834	1	4
BP	Golgi transport vesicle coating	GO:0048200	8	0.017834	1	4
BP	COPI coating of Golgi vesicle	GO:0048205	9	0.017834	1	4
BP	COPI-coated vesicle budding	GO:0035964	8	0.017834	1	4
BP	purine ribonucleoside bisphosphate biosynthetic process	GO:0034036	11	0.017834	1	4
BP	3'-phosphoadenosine 5'-phosphosulfate biosynthetic process	GO:0050428	12	0.017834	1	4
BP	monoatomic ion transmembrane transport	GO:0034220	6	0.019773	7	635
BP	monoatomic ion transport	GO:0006811	5	0.02131	8	799
BP	organic anion transport	GO:0015711	6	0.021613	4	241
MF	organic anion transmembrane transporter activity	GO:0008514	4	0.021912	3	137
Enriched GO terms in 3h_male_RvsP2						

(table continued on next page)

BP	microtubule bundle formation	GO:0001578	8	1.11E-16	25	82
BP	microtubule-based movement	GO:0007018	4	1.11E-16	46	247
BP	axoneme assembly	GO:0035082	9	2.22E-16	23	54
BP	microtubule-based process	GO:0007017	3	6.99E-15	64	571
BP	cilium assembly	GO:0060271	8	8.88E-15	40	245
BP	cilium organization	GO:0044782	7	2.12E-14	40	251
BP	cilium or flagellum-dependent cell motility	GO:0001539	4	8.50E-13	21	76
BP	cilium-dependent cell motility	GO:0060285	5	8.50E-13	21	76
BP	cilium movement involved in cell motility	GO:0060294	6	3.57E-12	19	65
BP	axonemal dynein complex assembly	GO:0070286	7	6.29E-12	13	27
CC	ciliary plasm	GO:0097014	6	1.05E-11	20	78
CC	axoneme	GO:0005930	3	1.05E-11	20	78
BP	plasma membrane bounded cell projection assembly	GO:0120031	7	1.85E-11	43	349
BP	cell projection assembly	GO:0030031	6	4.74E-11	43	359
BP	extracellular transport	GO:0006858	5	7.91E-11	12	26
BP	microtubule cytoskeleton organization	GO:0000226	7	1.22E-10	47	429
CC	obsolete ciliary part	GO:0044441	0	2.71E-10	33	243
CC	plasma membrane bounded cell projection cytoplasm	GO:0032838	5	7.32E-10	20	97
BP	NAD metabolic process	GO:0019674	10	3.33E-09	13	41
CC	obsolete axoneme part	GO:0044447	0	3.88E-09	13	42
CC	obsolete cytoskeletal part	GO:0044430	0	4.30E-09	56	638
BP	obsolete oxidoreduction coenzyme metabolic process	GO:0006733	0	1.37E-08	17	81
BP	pyridine nucleotide metabolic process	GO:0019362	8	3.55E-08	17	86
BP	nicotinamide nucleotide metabolic process	GO:0046496	9	3.55E-08	17	86
BP	NADH metabolic process	GO:0006734	11	4.41E-08	11	34
BP	organelle assembly	GO:0070925	6	9.35E-08	50	577
BP	pyridine-containing compound metabolic process	GO:0072524	4	1.19E-07	17	93
BP	inner dynein arm assembly	GO:0036159	8	4.15E-07	7	14
BP	oxaloacetate metabolic process	GO:0006107	8	4.15E-07	7	14
BP	motile cilium assembly	GO:0044458	9	1.60E-06	9	30
BP	microtubule-based transport	GO:0099111	5	4.51E-06	18	132
BP	dicarboxylic acid metabolic process	GO:0043648	7	4.85E-06	12	62
BP	tricarboxylic acid metabolic process	GO:0072350	7	1.03E-05	10	46
BP	cytoskeleton organization	GO:0007010	6	1.14E-05	55	773
MF	dynein heavy chain binding	GO:0045504	4	1.42E-05	5	10
BP	nicotinamide nucleotide biosynthetic process	GO:0019359	10	1.63E-05	8	30
BP	pyridine nucleotide biosynthetic process	GO:0019363	9	1.63E-05	8	30
BP	tricarboxylic acid cycle	GO:0006099	4	1.72E-05	9	39

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BP	outer dynein arm assembly	GO:0036158	8	2.13E-05	5	10
BP	male gamete generation	GO:0048232	5	2.25E-05	33	384
BP	citrate metabolic process	GO:0006101	8	2.65E-05	9	41
BP	pyridine-containing compound biosynthetic process	GO:0072525	5	2.73E-05	8	32
MF	dynein light chain binding	GO:0045503	4	3.77E-05	8	36
BP	obsolete movement of cell or subcellular component	GO:0006928	0	3.81E-05	61	926
BP	determination of left/right symmetry	GO:0007368	6	6.83E-05	14	105
BP	homologous chromosome pairing at meiosis	GO:0007129	8	6.85E-05	7	27
BP	hexose biosynthetic process	GO:0019319	7	7.38E-05	10	57
BP	gluconeogenesis	GO:0006094	8	7.38E-05	10	57
BP	meiosis I	GO:0007127	9	8.47E-05	13	94
Enriched GO terms in 3h_female_RvsP1						
BP	small molecule metabolic process	GO:0044281	3	0.027165	8	1113
CC	neuron projection	GO:0043005	5	0.011385	7	767
BP	response to abiotic stimulus	GO:0009628	3	0.024347	7	880
CC	obsolete neuron part	GO:0097458	0	0.029072	7	920
BP	small molecule biosynthetic process	GO:0044283	4	0.001748	6	395
BP	carboxylic acid metabolic process	GO:0019752	6	0.008083	6	537
BP	oxoacid metabolic process	GO:0043436	5	0.010475	6	567
BP	organic acid metabolic process	GO:0006082	4	0.010651	6	569
BP	transmembrane transport	GO:0055085	5	0.048513	6	799
CC	axon	GO:0030424	6	0.013722	5	433
BP	obsolete oxidation-reduction process	GO:0055114	0	0.033805	5	541
MF	metal ion binding	GO:0046872	6	0.033972	5	551
MF	cation binding	GO:0043169	5	0.049038	5	608
BP	carboxylic acid catabolic process	GO:0046395	7	0.003137	4	182
BP	organic acid catabolic process	GO:0016054	5	0.003137	4	182
BP	fatty acid metabolic process	GO:0006631	8	0.005695	4	215
BP	small molecule catabolic process	GO:0044282	4	0.008498	4	241
BP	muscle cell differentiation	GO:0042692	5	0.017519	4	298
BP	monocarboxylic acid metabolic process	GO:0032787	7	0.022409	4	321
MF	transition metal ion binding	GO:0046914	7	0.02799	4	349
MF	oxidoreductase activity	GO:0016491	3	0.049205	4	417
CC	Z disc	GO:0030018	3	0.002082	3	76
CC	I band	GO:0031674	3	0.002412	3	80
CC	sarcomere	GO:0030017	3	0.00901	3	128
CC	myofibril	GO:0030016	7	0.011504	3	140
CC	obsolete contractile fiber part	GO:0044449	0	0.012887	3	146
BP	muscle cell development	GO:0055001	5	0.015407	3	155

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CC	contractile muscle fiber	GO:0043292	6	0.016475	3	160
BP	regulation of transmembrane transport	GO:0034762	6	0.022882	3	180
BP	carboxylic acid biosynthetic process	GO:0046394	7	0.027431	3	193
BP	organic acid biosynthetic process	GO:0016053	5	0.027431	3	193
BP	response to hypoxia	GO:0001666	6	0.039217	3	222
BP	striated muscle cell differentiation	GO:0051146	6	0.040564	3	225
BP	response to decreased oxygen levels	GO:0036293	5	0.042863	3	230
BP	obsolete coenzyme metabolic process	GO:0006732	0	0.0438	3	232
BP	regulation of monoatomic ion transport	GO:0043269	6	0.045226	3	235
BP	response to temperature stimulus	GO:0009266	4	0.048645	3	242
BP	regulation of ventricular cardiac muscle cell action potential	GO:0098911	11	1.68E-04	2	6
BP	regulation of cardiac muscle cell action potential	GO:0098901	6	4.02E-04	2	9
MF	oxidoreductase activity, acting on the CH-CH group of donors, with a flavin as acceptor	GO:0052890	5	4.89E-04	2	10
MF	acyl-CoA dehydrogenase activity	GO:0003995	6	4.89E-04	2	10
BP	ventricular cardiac muscle cell action potential	GO:0086005	8	0.001005	2	14
BP	regulation of cardiac muscle cell contraction	GO:0086004	10	0.001005	2	14
BP	regulation of actin filament-based movement	GO:1903115	6	0.001157	2	15
BP	response to caffeine	GO:0031000	7	0.001676	2	18
BP	response to diuretic	GO:0036270	4	0.001676	2	18
BP	medium-chain fatty acid metabolic process	GO:0051791	9	0.002072	2	20
BP	pteridine-containing compound metabolic Process	GO:0042558	5	0.002742	2	23
CC	T-tubule	GO:0030315	3	0.003459	2	26
BP	cardiac muscle cell action potential involved in contraction	GO:0086002	7	0.0035	2	26
BP	response to cold	GO:0009409	5	0.004646	2	30
BP	L-amino acid biosynthetic process	GO:0170034	9	0.004956	2	31
BP	proteinogenic amino acid biosynthetic process	GO:0170038	8	0.004956	2	31
BP	cardiac muscle cell contraction	GO:0086003	6	0.004956	2	31
BP	regulation of action potential	GO:0098900	5	0.005275	2	32
BP	alpha-amino acid biosynthetic process	GO:1901607	8	0.005275	2	32
BP	cardiac muscle cell action potential	GO:0086001	6	0.005604	2	33
BP	regulation of cardiac muscle contraction	GO:0055117	9	0.005942	2	34
MF	oxidoreductase activity, acting on the CH-CH group of donors	GO:0016627	4	0.007221	2	38
MF	monooxygenase activity	GO:0004497	4	0.007221	2	38
BP	regulation of striated muscle contraction	GO:0006942	8	0.007384	2	38
MF	glucose transmembrane transporter activity	GO:0005355	9	0.008372	2	41
MF	D-glucose transmembrane transporter activity	GO:0055056	8	0.008372	2	41
MF	hexose transmembrane transporter activity	GO:0015149	7	0.008372	2	41

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MF	carbohydrate:proton symporter activity	GO:0005351	9	0.009181	2	43
BP	cardiac conduction	GO:0061337	8	0.009386	2	43
MF	monosaccharide transmembrane transporter activity	GO:0015145	6	0.009599	2	44
MF	sugar transmembrane transporter activity	GO:0051119	5	0.010025	2	45
MF	carbohydrate:monoatomic cation symporter activity	GO:0005402	8	0.010459	2	46
CC	extrinsic component of cytoplasmic side of plasma membrane	GO:0031234	5	0.01057	2	46
BP	amino acid biosynthetic process	GO:0008652	5	0.010691	2	46
MF	carbohydrate transmembrane transporter activity	GO:0015144	4	0.010902	2	47
MF	solute:proton symporter activity	GO:0015295	8	0.011353	2	48
BP	eye pigment biosynthetic process	GO:0006726	7	0.013034	2	51
BP	pigment metabolic process involved in developmental pigmentation	GO:0043324	5	0.014028	2	53
BP	pigment metabolic process involved in pigmentation	GO:0043474	4	0.014028	2	53
BP	eye pigment metabolic process	GO:0042441	6	0.014028	2	53
BP	fatty acid beta-oxidation	GO:0006635	10	0.014537	2	54
BP	regulation of heart rate	GO:0002027	8	0.014537	2	54
BP	cardiac muscle contraction	GO:0060048	7	0.015054	2	55
MF	obsolete protein self-association	GO:0043621	0	0.016839	2	59
CC	sarcolemma	GO:0042383	5	0.017011	2	59
MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016705	4	0.017937	2	61
Enriched GO terms in 3h_female_RvsP2						
MF	active monoatomic ion transmembrane transporter activity	GO:0022853	5	9.84E-04	7	198
MF	secondary active transmembrane transporter activity	GO:0015291	5	0.004378	5	134
MF	active transmembrane transporter activity	GO:0022804	4	0.006293	7	275
MF	metal ion transmembrane transporter activity	GO:0046873	6	0.020134	6	267
MF	metal ion transport	GO:0030001	7	0.02081	6	269
MF	inorganic molecular entity transmembrane transporter activity	GO:0015318	4	0.029583	9	546
MF	inorganic cation transmembrane transport	GO:0098662	7	0.037049	7	391
MF	monoatomic ion transmembrane transporter activity	GO:0015075	4	0.042222	9	582
MF	monoatomic ion transmembrane transport	GO:0034220	6	0.043421	9	585
MF	monoatomic ion transport	GO:0006811	5	0.044233	9	587
CC	basal part of cell	GO:0045178	3	1.77E-04	8	204
CC	basal plasma membrane	GO:0009925	5	3.86E-04	7	173
CC	basolateral plasma membrane	GO:0016323	5	3.86E-04	7	173
CC	membrane	GO:0016020	3	0.001305	34	2880

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CC	bounding membrane of organelle	GO:0098588	5	0.002833	14	797
CC	extracellular region	GO:0005576	3	0.003694	14	820
CC	obsolete membrane part	GO:0044425	0	0.004458	25	1971
CC	obsolete extracellular region part	GO:0044421	0	0.004631	11	575
CC	extracellular space	GO:0005615	3	0.017448	8	424
CC	transmembrane transporter complex	GO:1902495	4	0.019668	5	198
CC	transporter complex	GO:1990351	3	0.022491	5	205
CC	obsolete plasma membrane part	GO:0044459	0	0.023102	15	1116
CC	cell-cell junction	GO:0005911	5	0.026588	6	291
CC	anchoring junction	GO:0070161	4	0.026984	6	292
CC	obsolete intrinsic component of membrane	GO:0031224	0	0.034525	15	1172
CC	plasma membrane region	GO:0098590	4	0.037433	9	582
CC	endosome	GO:0005768	8	0.044924	7	417
BP	cellular homeostasis	GO:0019725	3	0.002475	10	442
BP	chemical homeostasis	GO:0048878	3	0.003933	11	551
BP	regulation of membrane potential	GO:0042391	4	0.00495	6	198
BP	intracellular monoatomic cation homeostasis	GO:0030003	6	0.005209	7	266
Enriched GO terms in 18h_male_RvsP1						
BP	xenobiotic metabolic process	GO:0006805	4	4.58E-06	24	436
BP	cellular response to xenobiotic stimulus	GO:0071466	5	5.58E-05	27	608
BP	purine ribonucleotide metabolic process	GO:0009150	9	1.21E-04	18	337
BP	pyruvate biosynthetic process	GO:0042866	9	1.41E-04	5	26
BP	purine nucleoside monophosphate metabolic process	GO:0009126	8	1.43E-04	11	145
BP	purine ribonucleoside monophosphate metabolic process	GO:0009167	9	1.43E-04	11	145
BP	purine nucleotide metabolic process	GO:0006163	8	1.55E-04	19	375
BP	ribonucleotide metabolic process	GO:0009259	8	1.57E-04	18	344
BP	obsolete oxidation-reduction process	GO:0055114	0	1.58E-04	24	541
BP	generation of precursor metabolites and energy	GO:0006091	4	1.61E-04	16	284
BP	ribose phosphate metabolic process	GO:0019693	6	1.95E-04	18	350
CC	respiratory chain complex	GO:0098803	4	1.96E-04	6	47
BP	ribonucleoside monophosphate metabolic process	GO:0009161	8	2.05E-04	11	151
CC	mitochondrial respirasome	GO:0005746	5	2.77E-04	6	50
BP	purine-containing compound metabolic process	GO:0072521	4	2.94E-04	19	394
CC	respirasome	GO:0070469	4	3.09E-04	6	51
BP	pyruvate metabolic process	GO:0006090	8	3.23E-04	7	66
BP	nucleoside monophosphate metabolic process	GO:0009123	7	3.40E-04	11	160
BP	aerobic electron transport chain	GO:0019646	8	4.00E-04	6	49
BP	nucleotide metabolic process	GO:0009117	7	4.42E-04	19	407
BP	nucleoside phosphate metabolic process	GO:0006753	6	4.70E-04	19	409

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BP	purine nucleoside triphosphate metabolic process	GO:0009144	8	5.16E-04	11	168
CC	oxidoreductase complex	GO:1990204	4	6.18E-04	7	80
BP	organophosphate metabolic process	GO:0019637	5	7.09E-04	25	635
MF	aminopeptidase activity	GO:0004177	6	7.27E-04	5	36
MF	serine hydrolase activity	GO:0017171	4	7.37E-04	8	97
MF	proton transmembrane transporter activity	GO:0015078	6	8.26E-04	9	123
BP	ATP synthesis coupled electron transport	GO:0042773	7	8.27E-04	6	56
BP	mitochondrial ATP synthesis coupled electron transport	GO:0042775	8	8.27E-04	6	56
BP	nucleobase-containing small molecule metabolic process	GO:0055086	5	8.36E-04	20	463
BP	nucleoside triphosphate metabolic process	GO:0009141	7	8.39E-04	11	178
MF	proton transmembrane transport	GO:1902600	8	9.83E-04	9	126
MF	oxidoreduction-driven active transmembrane transporter activity	GO:0015453	6	0.001058	5	39
BP	energy derivation by oxidation of organic compounds	GO:0015980	5	0.001104	12	213
BP	ATP metabolic process	GO:0046034	10	0.00115	10	157
BP	cellular respiration	GO:0045333	6	0.001459	10	162
MF	oxidoreductase activity	GO:0016491	3	0.001666	19	450
BP	purine ribonucleoside triphosphate metabolic process	GO:0009205	9	0.001675	10	165
MF	structural molecule activity	GO:0005198	2	0.001678	16	348
BP	ribonucleoside triphosphate metabolic process	GO:0009199	8	0.001752	10	166
CC	extracellular space	GO:0005615	3	0.001756	17	424
BP	aerobic respiration	GO:0009060	7	0.002	9	141
BP	respiratory electron transport chain	GO:0022904	6	0.002125	6	67
MF	active monoatomic ion transmembrane transporter activity	GO:0022853	5	0.002172	11	198
Enriched GO terms in 18h_male_RvsP2						
BP	juvenile hormone biosynthetic process	GO:0006718	10	5.30E-08	5	22
BP	sesquiterpenoid biosynthetic process	GO:0016106	9	5.30E-08	5	22
BP	juvenile hormone metabolic process	GO:0006716	9	8.50E-08	5	24
BP	terpenoid biosynthetic process	GO:0016114	8	1.06E-07	5	25
BP	sesquiterpenoid metabolic process	GO:0006714	8	1.31E-07	5	26
BP	isoprenoid biosynthetic process	GO:0008299	7	2.79E-07	5	30
BP	terpenoid metabolic process	GO:0006721	7	7.90E-07	6	68
BP	isoprenoid metabolic process	GO:0006720	6	3.90E-06	6	89
MF	monooxygenase activity	GO:0004497	4	1.16E-05	5	57
MF	oxidoreductase activity	GO:0016491	3	1.32E-05	11	450
BP	lipid biosynthetic process	GO:0008610	5	1.90E-05	10	414
BP	regulation of hormone levels	GO:0010817	4	2.54E-05	9	338

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BP	hormone biosynthetic process	GO:0042446	6	4.45E-05	5	82
MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016705	4	5.73E-05	5	79
MF	serine-type peptidase activity	GO:0008236	5	1.19E-04	5	92
BP	triglyceride metabolic process	GO:0006641	8	1.33E-04	5	103
MF	serine hydrolase activity	GO:0017171	4	1.53E-04	5	97
BP	hormone metabolic process	GO:0042445	5	1.60E-04	6	171
BP	neutral lipid metabolic process	GO:0006638	6	1.73E-04	5	109
BP	acylglycerol metabolic process	GO:0006639	7	1.73E-04	5	109
BP	obsolete oxidation-reduction process	GO:0055114	0	1.86E-04	10	541
BP	cellular lipid metabolic process	GO:0044255	5	2.86E-04	10	570
MF	catalytic activity	GO:0003824	2	6.80E-04	27	3312
BP	obsolete coenzyme metabolic process	GO:0006732	0	8.25E-04	6	232
BP	obsolete cofactor metabolic process	GO:0051186	0	0.001517	7	358
BP	lipid metabolic process	GO:0006629	4	0.002122	10	735
BP	fatty acid metabolic process	GO:0006631	8	0.003737	5	215
BP	glycerolipid metabolic process	GO:0046486	6	0.006067	5	241
BP	regulation of lipid metabolic process	GO:0019216	6	0.007072	5	250
BP	obsolete regulation of neurotransmitter levels	GO:0001505	0	0.008059	5	258
BP	small molecule biosynthetic process	GO:0044283	4	0.011911	6	397
BP	carboxylic acid metabolic process	GO:0019752	6	0.014224	7	538
BP	oxoacid metabolic process	GO:0043436	5	0.018744	7	568
BP	organic acid metabolic process	GO:0006082	4	0.019078	7	570
BP	monocarboxylic acid metabolic process	GO:0032787	7	0.01941	5	321
BP	obsolete organic substance biosynthetic process	GO:1901576	0	0.02072	14	1668
BP	carbohydrate metabolic process	GO:0005975	4	0.022644	5	334
MF	transmembrane transport	GO:0055085	5	0.022812	8	667
MF	transmembrane transporter activity	GO:0022857	3	0.022812	8	667
MF	inorganic molecular entity transmembrane transporter activity	GO:0015318	4	0.024292	7	546
CC	extracellular region	GO:0005576	3	0.026199	7	820
MF	peptidase activity	GO:0008233	4	0.026346	6	432
BP	ribose phosphate metabolic process	GO:0019693	6	0.027089	5	350
BP	purine nucleotide metabolic process	GO:0006163	8	0.035105	5	375
MF	transport	GO:0006810	4	0.03722	8	731
MF	establishment of localization	GO:0051234	3	0.037485	8	732
MF	localization	GO:0051179	2	0.037485	8	732
BP	small molecule metabolic process	GO:0044281	3	0.037683	10	1115
BP	purine-containing compound metabolic process	GO:0072521	4	0.042105	5	394
BP	transmembrane transport	GO:0055085	5	0.043562	8	826

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MF	transporter activity	GO:0005215	2	0.04457	8	757
BP	nucleotide metabolic process	GO:0009117	7	0.047359	5	407
BP	nucleoside phosphate metabolic process	GO:0006753	6	0.048201	5	409
Enriched GO terms in 18h_female_RvsP1						
BP	carboxylic acid metabolic process	GO:0019752	6	5.20E-09	27	538
BP	oxoacid metabolic process	GO:0043436	5	1.68E-08	27	568
BP	organic acid metabolic process	GO:0006082	4	1.81E-08	27	570
BP	small molecule metabolic process	GO:0044281	3	2.48E-08	39	1115
MF	obsolete coenzyme binding	GO:0050662	0	8.81E-07	12	134
BP	obsolete oxidation-reduction process	GO:0055114	0	1.82E-06	23	541
BP	carboxylic acid catabolic process	GO:0046395	7	1.86E-06	13	182
BP	organic acid catabolic process	GO:0016054	5	1.86E-06	13	182
BP	obsolete cellular carbohydrate metabolic process	GO:0044262	0	8.23E-06	9	95
BP	fatty acid metabolic process	GO:0006631	8	1.17E-05	13	215
BP	glutamine family amino acid metabolic process	GO:0009064	9	1.29E-05	6	37
MF	obsolete cofactor binding	GO:0048037	0	1.93E-05	12	180
BP	carbohydrate metabolic process	GO:0005975	4	2.06E-05	16	334
BP	proteinogenic amino acid metabolic process	GO:0170039	7	2.18E-05	9	107
BP	L-amino acid metabolic process	GO:0170033	8	3.14E-05	9	112
BP	response to xenobiotic stimulus	GO:0009410	4	3.83E-05	29	939
BP	small molecule catabolic process	GO:0044282	4	3.95E-05	13	241
BP	cellular lipid metabolic process	GO:0044255	5	5.03E-05	21	570
BP	oligosaccharide metabolic process	GO:0009311	5	5.31E-05	6	47
BP	fatty-acyl-CoA metabolic process	GO:0035337	11	5.31E-05	6	47
CC	peroxisomal matrix	GO:0005782	7	5.80E-05	6	50
CC	microbody lumen	GO:0031907	6	5.80E-05	6	50
BP	alpha-amino acid metabolic process	GO:1901605	7	6.19E-05	9	122
MF	organic acid binding	GO:0043177	4	8.82E-05	8	93
BP	fatty acid oxidation	GO:0019395	9	8.86E-05	7	74
BP	lipid metabolic process	GO:0006629	4	9.24E-05	24	735
BP	lipid oxidation	GO:0034440	7	9.66E-05	7	75
BP	fatty-acyl-CoA biosynthetic process	GO:0046949	12	1.33E-04	5	35
BP	carbohydrate derivative metabolic process	GO:1901135	3	1.49E-04	23	709
BP	purine-containing compound metabolic process	GO:0072521	4	1.51E-04	16	394
BP	obsolete cellular glucan metabolic process	GO:0006073	0	1.53E-04	5	36
BP	monocarboxylic acid metabolic process	GO:0032787	7	1.95E-04	14	321
MF	NADP binding	GO:0050661	8	2.12E-04	5	36
BP	peroxisomal transport	GO:0043574	6	2.13E-04	6	60
CC	microbody	GO:0042579	6	2.77E-04	8	123
CC	peroxisome	GO:0005777	7	2.77E-04	8	123

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BP	purine nucleotide metabolic process	GO:0006163	8	2.93E-04	15	375
BP	cellular lipid catabolic process	GO:0044242	6	3.01E-04	9	150
BP	nucleobase-containing small molecule metabolic process	GO:0055086	5	3.08E-04	17	463
BP	purine-containing compound biosynthetic process	GO:0072522	5	3.08E-04	10	184
BP	obsolete cellular polysaccharide metabolic process	GO:0044264	0	3.22E-04	5	42
BP	peroxisome organization	GO:0007031	6	3.31E-04	6	65
MF	flavin adenine dinucleotide binding	GO:0050660	6	3.97E-04	5	41
BP	lipid catabolic process	GO:0016042	5	3.99E-04	10	190
MF	carboxylic acid binding	GO:0031406	6	4.04E-04	7	88
BP	acyl-CoA metabolic process	GO:0006637	10	4.82E-04	7	97
BP	thioester metabolic process	GO:0035383	5	4.82E-04	7	97
MF	oxidoreductase activity	GO:0016491	3	4.88E-04	17	450
CC	obsolete microbody part	GO:0044438	0	5.18E-04	6	74
CC	obsolete peroxisomal part	GO:0044439	0	5.18E-04	6	74
BP	glycogen metabolic process	GO:0005977	7	5.49E-04	5	47
BP	glucan metabolic process	GO:0044042	6	5.49E-04	5	47
BP	obsolete cofactor metabolic process	GO:0051186	0	5.96E-04	14	358
BP	carbohydrate derivative biosynthetic process	GO:1901137	4	5.98E-04	15	401
Enriched GO terms in 18h_female_RvsP2						
BP	small molecule metabolic process	GO:0044281	3	5.64E-06	30	1115
BP	nucleobase-containing small molecule metabolic process	GO:0055086	5	6.15E-06	18	463
BP	purine nucleotide metabolic process	GO:0006163	8	6.90E-06	16	375
BP	purine-containing compound metabolic process	GO:0072521	4	1.29E-05	16	394
BP	nucleotide metabolic process	GO:0009117	7	1.94E-05	16	407
BP	nucleoside phosphate metabolic process	GO:0006753	6	2.06E-05	16	409
BP	alcohol metabolic process	GO:0006066	4	3.09E-05	11	207
BP	lipid metabolic process	GO:0006629	4	3.20E-05	22	735
BP	purine ribonucleotide metabolic process	GO:0009150	9	3.80E-05	14	337
BP	organophosphate metabolic process	GO:0019637	5	3.93E-05	20	635
BP	ribonucleotide metabolic process	GO:0009259	8	4.77E-05	14	344
MF	active transmembrane transporter activity	GO:0022804	4	5.30E-05	13	275
BP	gland development	GO:0048732	5	5.67E-05	15	396
BP	ribose phosphate metabolic process	GO:0019693	6	5.76E-05	14	350
BP	carbohydrate derivative metabolic process	GO:1901135	3	5.98E-05	21	709
BP	carbohydrate derivative biosynthetic process	GO:1901137	4	6.54E-05	15	401
CC	extracellular region	GO:0005576	3	6.77E-05	22	820
BP	glutamine family amino acid metabolic process	GO:0009064	9	6.99E-05	5	37
MF	NADP binding	GO:0050661	8	9.69E-05	5	36
BP	obsolete cellular carbohydrate metabolic process	GO:0044262	0	1.28E-04	7	95

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BP	acyl-CoA metabolic process	GO:0006637	10	1.46E-04	7	97
BP	thioester metabolic process	GO:0035383	5	1.46E-04	7	97
BP	carboxylic acid metabolic process	GO:0019752	6	1.64E-04	17	538
BP	regulation of lipid metabolic process	GO:0019216	6	1.71E-04	11	250
CC	obsolete extracellular region part	GO:0044421	0	1.80E-04	17	575
MF	glucose transmembrane transport	GO:1904659	9	1.83E-04	5	41
MF	glucose transmembrane transporter activity	GO:0005355	9	1.83E-04	5	41
MF	hexose transmembrane transport	GO:0008645	8	1.83E-04	5	41
MF	D-glucose transmembrane transporter activity	GO:0055056	8	1.83E-04	5	41
MF	hexose transmembrane transporter activity	GO:0015149	7	1.83E-04	5	41
MF	iron ion binding	GO:0005506	8	2.05E-04	5	42
BP	fatty acid metabolic process	GO:0006631	8	2.21E-04	10	215

General discussion

This thesis represents a detailed analysis of the evolution of neuropeptide genes within the order Blattodea, revealing significant differences in neuropeptide diversity between termites and cockroaches, potentially reflecting their differing sociality and ecology. Through the application of genomic and transcriptomic analyses across diverse species, this study found varying patterns of neuropeptide gene loss, duplication, and conservation. We propose that these patterns exhibit a significant correlation with the ecological contexts and behavioural traits exhibited by these species. For instance, eusocial termites showed losses of specific neuropeptide genes like ACP, potentially related to their cooperative behaviours and reduced individual stress responses. By contrast, solitary cockroaches displayed gene duplications, such as in Birpin (one of the ILPs), potentially enhancing their metabolic flexibility in variable environments. Notably, novel patterns in the evolution of AKHs and AKHRs were identified. Solitary cockroaches were found to possess multiple AKH peptides and conserved receptor motifs critical for energy regulation; phylogenetic analyses revealed an ancient duplication event in the ancestor of Blaberoidea, leading to a new group of putative decapeptides unique to this lineage. The analysis of the neuropeptidome in the German cockroach, *B. germanica*, revealed both conserved and novel neuropeptides. I provided a brief review of the neuropeptides identified in the Blattodea order, highlighting their crucial roles in physiological and behavioural activities, including stress responses, feeding, and reproduction. Additionally, transcriptomic analyses revealed distinct metabolic and immune responses to AKH peptides in different sexes, highlighting the complex relationship between neuropeptide signaling, metabolism, and immunity.

Evolutionary divergence of neuropeptide genes in Blattodea

Chapter I presents an extensive genomic analysis of neuropeptide precursors across 49 Blattodea species, revealing substantial evolutionary differences between termites and cockroaches. This broad comparative study utilized cutting edge long read sequencing technologies and bioinformatic tools to annotate neuropeptide genes, offering insights into their conservation and diversification patterns (Harrison et al. 2018; Terrapon et al. 2014). The study uncovered significant patterns of gene loss, duplication, and conservation, which could be associated with these insects' distinct ecological and social behaviors, as I discuss below.

The social termites demonstrated a significant loss of specific neuropeptide genes, such as the ACP gene, in comparison to their solitary cockroach relatives. Interestingly, similar patterns of neuropeptide gene loss have been observed in Coleoptera, where several beetle families lack key neuropeptide signaling systems, suggesting that the loss of neuropeptide genes is not uniform and may be associated with specific adaptations (Veenstra, 2019). ACP regulates energy homeostasis and stress responses in

insects by mobilizing lipids and carbohydrates during periods of high energy demand (Patel et al. 2014; Roch et al. 2011; Suwansa-ard et al. 2016). The significant loss of the ACP gene in higher termites may be an evolutionary adaptation to their advanced sociality and/or dietary transitions. In this system, the collective dynamics of the colony mitigate individual energetic demands and stressors (Korb and Heinze 2016; Roisin and Korb 2011; Shell and Rehan 2018). Furthermore, the gene loss observed in termites aligns with the concept of genomic streamlining in eusocial insects, where reduced gene repertoires are associated with highly cooperative and specialized colony life (Harrison et al. 2018; Simola et al. 2013; Terrapon et al. 2014). This streamlining could be due to the lower selection pressures on individual physiological functions by the buffering capacity of the society (Boomsma and Gawne 2018; Johnson and Tsutsui 2011).

The genetic makeup of cockroaches includes instances of gene duplication, particularly in neuropeptides like short IGF-related peptides (sirps) (Veenstra 2023). The existence of multiple sirps copies suggests diversification that facilitates the adaptable regulation of metabolic processes, growth, and reproduction, thereby providing adaptive advantages in their solitary and unpredictable habitats (Grönke et al. 2010; Lin and Smagghe 2019; Nijhout and Callier 2015). Moreover, the gene duplication and subsequent functional diversification of ILPs in cockroaches may enhance their ability to cope with fluctuating environmental conditions, such as variable food availability and habitat disturbances, which is essential for the survival and reproductive success of solitary cockroaches lacking the buffering effects of social living (Li et al. 2018; Pujal et al. 2024; Tang et al. 2024).

These findings are also consistent with evolutionary theories suggesting that eusociality and cooperative behavior can result in genomic simplification, leading to the loss of redundant or non-essential genes over time (Johnson and Tsutsui 2011; Wissler et al. 2013; Woodard et al. 2011). Similar trends have been reported in other eusocial insects, such as ants and bees, where gene loss is linked to the evolution of sociality and division of labor (Danforth et al. 2003; Kapheim et al. 2015; Simola et al. 2013). This supports the hypothesis that a communal lifestyle reduces the necessity for certain individual physiological processes, as tasks are distributed among colony members (O'Donnell and Bulova 2007; Smith et al. 2008).

However, when interpreting gene loss or replication, it is essential to exercise caution due to the possibility of incomplete genome annotations or low expression levels contributing to apparent absences (Simola et al. 2013; Veenstra 2019). Genomic studies may overlook genes that are actually present but not expressed in the sampled tissues or developmental stages or genes that are highly divergent, which makes it challenging to identify using homology-based methods. Future research

should aim to improve the accuracy of genome annotation incorporating high-quality transcriptomes from multiple life stages and castes of these termites and cockroaches (Ejigu and Jung 2020; Ekblom and Wolf 2014).

Novel patterns of AKH and AKHR gene evolution

Chapter II emphasizes the evolution of adipokinetic hormones (AKHs) and their receptors (AKHRs) across Blattodea. The study conducted a comparative analysis of sequences from AKH and AKHR gene families in solitary cockroaches, subsocial wood roaches, as well as lower and higher termites. Further phylogenetic analyses revealed an ancient duplication event in the ancestor of Blaberoidea, leading to a new group of putative decapeptides unique to this lineage. Consequently, while some cockroach species have multiple AKHs, termites generally possess one AKH. In particular, we identified seven putative novel decapeptides, significantly enhancing Blattodea's known decapeptide diversity.

The evolutionary conservation of specific AKH receptor motifs, which are critical for hormone binding and signal transduction, suggests that these receptors have been under intense selective pressure throughout Blattodea. However, our examination of predicted post-translational modification sites in AKHRs did not reveal significant differences between social termites and their subsocial or solitary cockroach relatives, while minor variations were observed, such as differences in the number of protein kinase phosphorylation sites in intracellular loops, which did not correlate clearly with social behavior patterns. This was somewhat unexpected, given the critical roles that post-translational modifications play in protein interaction, stability, signaling, *β*-arrestin recruitment, and receptor trafficking (Chou 2020; Patwardhan et al. 2021; Vu et al. 2018; Yang et al. 2018).

The identification of novel decapeptide AKHs unique to Blaberoidea has practical implications for developing biorational pest control agents. Since these peptides are specific to certain pest species and not widespread across beneficial insects, they present attractive targets for designing peptide mimetics that could disrupt normal endocrine signaling in pest insects (Gäde and Goldsworthy 2003; Goldsworthy et al. 2003; Whetstone and Hammock 2007). Indeed, the presence of more than one mature AKH in a single species may indicate that each AKH serves an important adaptive purpose, potentially fulfilling different physiological functions in these insects (Bártů et al. 2010; Goldsworthy et al. 1997; Kaufmann and Brown 2008).

Moreover, the findings also prompt questions about the co-evolution of neuropeptides and their receptors since the specificity and affinity between AKHs and AKHRs are critical for proper physiological responses, and changes in one component may require compensatory changes in the other (Altstein

and Nässel 2010; Nässel and Winther 2010; Van Hiel et al. 2010). Exploring these co-evolutionary dynamics can offer insights into the molecular mechanisms underlying hormone-receptor interactions and their evolution (Hansen et al. 2010). For instance, multiple ligand binding may be permitted by the availability of large AKHR binding pockets, facilitating promiscuous receptor-ligand interactions (Marchal et al. 2018; Marco and Gäde 2019; Rios et al. 2001; Schwartz 1994; Stank et al. 2016; Venkatakrishnan et al. 2013; Zhu et al. 2009).

These findings could contribute to the development of mimetic or RNAi-based pest control methods. Additionally, these data establish a molecular framework for future research on neuropeptides as sustainable pest control methods for termites and cockroaches.

Neuropeptidomic profiling of *B. germanica*

Chapter III represents a comprehensive neuropeptidomic profile of *B. germanica*, which significantly advances our understanding of the neuropeptide landscape in this worldwide pest. By integrating transcriptomic and peptidomic approaches, the study identified 69 neuropeptide precursor transcripts and confirmed 79 mature neuropeptides by MALDI-TOF mass spectrometry. The majority of these precursor transcripts and neuropeptides are being reported for the first time in this particular species. This comprehensive catalogue not only covers most of the recognized insect neuropeptide families but also reveals numerous new peptides that are unique to *B. germanica*.

A pivotal aspect of this study is the bioassay conducted to investigate the metabolic functions of AKHs. Functional assays of two AKH peptides, AKH1 and the recently identified AKH2, revealed that both elevate carbohydrate levels in *B. germanica*. Notably, females displayed increased hemolymph carbohydrate mobilization compared to males when treated with equal concentrations of both AKH peptides, suggesting sex-specific metabolic responses. A recent study shows that ACP exhibits sex-specific actions in modulating energy substrate levels in *Aedes aegypti*, further supporting the idea of sex-specific metabolic regulation by neuropeptides (Afifi et al. 2023). While numerous insect species exhibit AKH gene diversity, studies focusing on sex-specific responses to AKH are scarce. In this study, I report for the first time a sex-specific metabolic response to AKH signaling in *B. germanica*, highlighting the potential for such differentiation. The AKH gene duplication as an ancestral event within the Blaberoidea suggests that AKH peptides might have retained ancestral roles linked to energy regulation while also evolving to meet the specific metabolic demands of each sex. This duplication event provides a foundation for the functional diversification of AKH, allowing for fine-tuned metabolic responses under different physiological and environmental contexts. Additionally, the seven

decapeptides I reported in Blaberoidea (**Chapter II**) could undergo further bioassays to confirm the role of AKH gene duplication in metabolic regulation related to sex-specific responses within Blaberoidea.

This case study using two AKHs to investigate metabolic function highlights the complexity of neuropeptide interactions and their role in regulating physiological processes. Expanding on this approach to encompass other neuropeptides, such as utilizing mimic peptides or employing RNAi, could help to explore additional functions, offering a more comprehensive understanding of neuropeptide roles in this species (Griebler et al. 2008; Huang et al. 2012; Liu et al. 2021; Schoofs et al. 2017; Toprak 2020). On the other hand, developing specific neuropeptide antagonists or mimetics could lead to environmentally friendly insecticidal alternatives with high target species specificity (Gäde and Goldsworthy 2003; Gäde et al. 2017; Whetstone and Hammock 2007).

Regulatory roles of AKH peptides in metabolism and immunity

In **Chapter IV**, my primary focus was on the effects of AKH peptide on energy metabolism and immune responses. The transcriptomic analyses conducted at 3 and 18 hours post-AKH injection revealed significant molecular changes, highlighting the diverse roles of AKHs in maintaining metabolic balance and immune function. Additionally, the RNAi-mediated knockdown of *AKHR* further clarified the significance of AKH signaling, particularly in immune regulation and resistance to pathogen infection.

In *B. germanica*, the differential expression of genes involved in energy metabolism highlights the immediate and medium-term effects of AKH peptides. After 3 hours post-injection, both AKH1 and AKH2 treatments resulted in significantly upregulating genes associated with energy mobilization pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and biosynthetic processes. This is consistent with the established role of AKHs in activating critical metabolic enzymes such as glycogen phosphorylase and triacylglycerol lipase, which aid in the conversion of stored lipids and carbohydrates into readily available energy substrates (Auerswald et al. 2005; Chino et al. 1989; Gäde 2004; Gäde and Beenakkers 1977; Gäde and Marco 2013; Goldsworthy et al. 1975; Isabel et al. 2005; Kodrík 2008; Marco and Gäde 2017; Stone et al. 1976). The study uncovered sex-specific responses, with males exhibiting an increase in genes related to energy metabolism, while females displayed more intricate regulatory adaptations involving lipid biosynthesis and hormone secretion. This suggests that AKHs may have evolved to meet sex-specific physiological needs, with males prioritizing energy mobilization for locomotion and mating, and females enhancing metabolic pathways supporting reproduction and immune defense (Harshman and Zera 2007; Schwenke et al. 2016). The 18-hour transcriptomic

analysis revealed a transition from immediate energy mobilization to more sustained metabolic processes. In males, this involved the continued enrichment of nucleotide and aerobic respiration pathways, reflecting a prolonged need for energy production. In contrast, females showed an upregulation of glucose transport and carbohydrate metabolism genes, highlighting an adaptive strategy that prioritizes glucose utilization during extended metabolic stress. This shift in metabolic pathways at different time points demonstrates the dynamic regulatory capacity of AKH peptides in modulating energy balance over time (Gáliková et al. 2015; Lorenz 2003).

I further tested the significant role of AKHs in immune regulation and their involvement in metabolism. I found that *AKHR* silence led to a notable decrease in survival rates following bacterial infection with *P. entomophila*, indicating the important contribution of AKH signaling in the immune defense mechanisms of the German cockroach. These findings are consistent with prior research demonstrating the influence of AKHs on hemocyte activity and the expression of antimicrobial peptides in insects (Gautam et al. 2020; Goldsworthy et al. 2003).

The results of this study have important implications for the development of innovative pest management strategies. The discovery that AKH signaling plays a role in both energy metabolism and immune function in *B. germanica* suggests that targeting this pathway could effectively control cockroach populations. Disrupting AKH signaling, through the use of AKH antagonists or RNAi-based approaches, may impair the cockroach's ability to maintain metabolic balance and defend against pathogens, ultimately reducing their survival and reproductive success (Miyashita et al. 2019).

Understanding the temporal dynamics of AKH signaling could also inform the development of pest control methods that exploit the insect's natural physiological rhythms. One of the key findings of this study is the temporal nature of AKH signaling. The distinctive gene expression profiles observed at 3 and 18 hours post-treatment indicate that AKH effects are not static but evolve over time to meet the organism's changing physiological needs. This time-dependent regulation suggests that interventions targeting peptide pathways should consider the timing of treatment to achieve optimal disruption of metabolic and immune functions (Hentze et al. 2015).

This research offers a comprehensive overview of the regulatory functions of AKH peptides in *B. germanica*, emphasizing their pivotal roles in both metabolism and immunity. The distinct gene expression patterns observed over short- and medium-term post-treatment highlight the dynamic nature of AKH signaling. Additionally, the RNAi-mediated knockdown of *AKHR* demonstrates the hormone's likely role in pathogen resistance. These findings advance our comprehension of AKH-

mediated regulation in insects and provide valuable insights into potential pest control strategies targeting hormonal pathways to disrupt metabolic and immune functions in pest species. Future investigations should prioritize unravelling the mechanistic intricacies of AKH signaling, particularly its interactions with other hormonal pathways, to further explore its potential as a target for sustainable pest management solutions.

Broader implications, limitations, and future directions

The findings presented in this thesis have significant implications for understanding the evolution, function, and ecological importance of neuropeptides in insects. Understanding the roles of neuropeptides in physiology and behavior underscores their contribution to the adaptive strategies that enable termites and cockroaches to thrive in diverse environments (Grimmelikhuijzen and Hauser 2012; Jékely 2013; Nässel and Winther 2010). While highly conserved, neuropeptide signaling systems also exhibit flexibility, allowing insects to adjust their responses to internal and external cues finely (Elphick et al. 2018; Nässel and Homberg 2006; Raikhel et al. 2005).

One significant theme is the relationship between neuropeptide evolution and social complexity. The distinct patterns of gene loss and duplication in termites and cockroaches reflect different selective pressures associated with living in social groups versus living a solitary lifestyle (Kent and Zayed 2013; Rehan and Toth 2015). For example, in higher termites like *Macrotermes natalensis* and *R. speratus*, the brovirpin gene, a short insulin-like growth factor-related peptide (sirp), has undergone duplication, suggesting a specialization of function. In *M. natalensis*, brovirpin plays a critical role in vitellogenesis, supporting the intense reproductive output of termite queens (Veenstra, 2023). Neuropeptide signaling pathways may drive and reinforce behavioral adaptations, influencing social evolution by regulating behaviors such as foraging, reproduction, and aggression (Lin et al. 2017; Simola et al. 2013; Traniello et al. 2002; Vargo and Laurel 1994). In our study, while we did not explore the distribution or functional roles of these genes to assess their potential impact on specific species regarding losses (e.g., ACP, Gon) and duplications (e.g., CCHs, CNMs, OKs, EHs, NPFs), we can reasonably infer that these gene patterns likely play significant roles within the respective taxa.

The future of neuropeptide research should encompass a broader range of species, ecological contexts, and social structures. Exploring neuropeptides' impact on caste differentiation, reproductive regulation, and social behaviors in termites could offer deeper insights into the molecular foundations of eusociality (Korb et al. 2012; Terrapon et al. 2014; Weil et al. 2007). Comparative studies across

eusocial and solitary species could reveal how neuropeptide signaling networks have been adapted or altered during the evolution of complex social systems.

Neuropeptide systems are sensitive to temperature, nutrition, and stress, all of which are being influenced by global environmental change (Overgaard and MacMillan 2017; Purcell 2011). Furthermore, comprehending the environmental and ecological factors that influence neuropeptide function could offer insights into how insects will respond to changing climates and habitats (Perez and Aron 2020; Purcell 2011; Schowalter 2022). Cutting-edge genomic and functional techniques, such as single-cell RNA sequencing and CRISPR-Cas9 gene editing, will be crucial in dissecting the precise roles of neuropeptides (Ni et al. 2020; Shigenobu and Yorimoto 2022; Yoshinari et al. 2021). Moreover, gene editing tools can facilitate precise manipulation of neuropeptide genes and receptors, enabling causal studies of their roles in behavior and physiology (Ashok et al. 2023; Ling and Raikhel 2021). Understanding these dynamics could have implications for pest management and conservation efforts (Bale and Hayward 2010; Bowler and Terblanche 2008).

In conclusion, this thesis advances our understanding of neuropeptide dynamics in Blattodea, uncovering the intricate ways these signaling molecules contribute to the ecological and evolutionary success of termites and cockroaches. By integrating genomic, transcriptomic, and functional data, this research provides a comprehensive framework for studying neuropeptide evolution and highlights their potential as markers of evolutionary change and targets for innovative pest management solutions. As our knowledge of neuropeptide signaling expands, we will gain deeper insights into the molecular networks underpinning the remarkable adaptability of insects (Khalid et al. 2021; Meinertzhagen 2001; Nässel 1995). Continued interdisciplinary research will be essential to unravel the complexities of neuropeptide function and to translate these findings into practical applications in agriculture, public health, and biodiversity conservation.

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Curriculum vitae

Education

- Freie Universität Berlin, Berlin, Germany
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- Northwest A&F University, Yangling, China
  Master Agricultural Entomology and Pest Control Sep. 2016 - Jun. 2019

Scientific Meetings and Conferences

- XII European Congress of Entomology (ECE2023), Crete, Greece.*Oct.2023* Oral Presentation
- First International Conference for Blattodea Research, Münster, Germany. *Apr. 2023.*Oral Presentation
- Fifth Evo Eco PhD Meeting, Wittenberg, Germany. *Sep. 2022*.Oral Presentation
- Ecological Immunology Workshop 2022: Resistance, Tolerance & Symbionts, Blossin, Germany. *Sep. 2022*.Oral Presentation
- XXVI International Congress of Entomology (ICE2020), Helsinki, Finland. *Jul. 2022*. Abstract Contributor
- Fourth Evo Eco PhD Meeting, Wittenberg, Germany.*Mar.2020*.Oral Presentation

Publications

- **Jiang, S.**, Marco, H. G., et al. *2023*. Comparative analysis of adipokinetic hormones and their receptors in Blattodea reveals novel patterns of gene evolution. *Insect Molecular Biology*, 32(6), 615-633.
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