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Research paper

Identification and expression profiling of neuropeptides and neuropeptide receptor genes in *Atrijuglans hetaohei*

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ABSTRACT

Atrijuglans hetaohei Yang (Lepidoptera: Gelechioidea), is one of the major pests that can seriously damage the walnut fruits. Neuropeptides and their receptors regulate most physiological functions in insects and represent new targets for the development of control agents. To identify the neuropeptides and their receptors from *A. hetaohei*, we sequenced and analyzed its head transcriptomic data, identified 32 neuropeptides and 39 neuropeptide receptor genes. Sequence comparisons and phylogenetic analyses suggest that *A. hetaohei* neuropeptides and receptor genes have high homology with those in *Bombyx mori*, *Chilo suppressalis*, *Plutella xylostella* and *Helicoverpa armigera*. Moreover, gene expression patterns revealed that neuropeptide genes such as *AKH1*, *CP*, *MS* and *PTTH* were expressed specifically in male head, while *CAP3*, *DH*, *NPLP1*, *PBAN* and *SIF* showed higher expression in the female head. *Bur* showed abdomen biased expression in both male and female. Neuropeptide receptor genes such as *A8*, *A11*, *A15* and *LGR* were highly expressed in male head, whereas *A24* and *LGR2* were preferentially expressed in female head. This is the first sequencing, identification and expression analyses of neuropeptides and neuropeptide receptor genes from *A. hetaohei*. Our results could provide a powerful background that will facilitate the further investigations using transcriptomics to determine neuropeptides and their receptors presence, functions, and indicates potential targets in *A. hetaohei* for a novel pest management strategy.

1. Introduction

As a class of neuronal signal molecules, neuropeptides are produced by the neurosecretory cells that are mainly located in the brain, suboesophageal ganglion, among others. Neuropeptides act through specific receptor molecules located in the membrane of the target cell (Schoofs et al., 2017). Most neuropeptide receptors belong to the G protein-coupled receptor (GPCR) family, a smaller number are guanylyl cyclase or tyrosine kinase receptors (Nässel and Winther, 2010). In insects, neuropeptides and their receptors steer important physiological processes such as development, reproduction, behavior, and feeding (Caers et al., 2012). Since the first genome of model insects such as *Drosophila melanogaster* (Adams et al., 2000; Clark et al., 2007) have been uncovered, great progresses have been made over the last two decades in the identification of new and homologous neuropeptides and their receptors from number of important insect species in the human health, agriculture and ecological environment, such as *Anopheles gambiae* (Holt et al., 2002), *Bombyx mori* (Mita et al., 2004; Xia et al., 2004), and *Apis mellifera ligustica* (Hummon et al., 2006).

Further, a series of studies have investigated their structure and physiological functions. For instance, neuropeptide F (NPF) consist of 28–45 residues and have a conserved C-terminal RPRFamide. They play a very important role in feeding, ethanol sensitivity and stress responses of *D. melanogaster*. The responses include aggression, reproduction, circadian rhythm, and learning, by interaction with NPFRs, which are a type of GPCR (Huang et al., 2015). Several studies have reported the different functions of insect myoinhibitory peptide (MIP), including the inhibition of juvenile hormone synthesis (Lorenz et al., 1995), the repression of ecdysteroidogenesis (Yamanaka et al., 2010), and the regulation of ecdysis (Zitnan and Adams, 2012). In flesh fly, pyrokininp/pheromone biosynthesis activating neuropeptide/diuretic hormone (PK/PBAN/DH) can accelerate its pupariation (Zdarek et al., 1997),

Abbreviations: AKH1, adipokinetic hormone 1; CCHamide, CCH; GPCR, G protein-coupled receptor; MIP, myoinhibitory peptide; MS, myosuppressin; NPF, neuropeptide F; PK/PBAN/DH, pyrokinin/Pheromone biosynthesis activating neuropeptide/Diuretic hormone; RT-qPCR, Real-time quantitative polymerase chain reaction

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besides, it also induces ecdysteroidogenesis in the prothoracic glands of lepidopterans and stimulate trail pheromone synthesis in an invasive ant (Watanabe et al., 2007; Zhang et al., 2004; Choi and Vander Meer, 2012). Because of their importance in insect life cycle, neuropeptides and their receptors are interesting targets for the development of highly selective and environmentally friendly pesticides. The chitin synthesis inhibitors such as benzoylureas and diflubenzuron, which have been used thus far, can hinder the formation of new epidermis to prevent the insects ecdysis and pupation, and induce the morphological mutation of the diptera larvae and pupae giving rise to the termination of adults eclosion (Sun et al., 2015; Montaño-Reyes et al., 2019). Juvenile hormone analogues such as fenoxycarb and pyriproxyfen, can effectively inhibit the development and metamorphosis of insects' embryo, and adult formation (El-Sheikh et al., 2016).

Atrijuglans hetaohei Yang (Lepidoptera: Gelechioidea), is one of the major pests that can reduce the quality and yield of walnuts. They can also seriously damage the walnut forest leading to harvest loss (Wang et al., 2016). The morphology and physiology of *A. hetaohei* have been widely studied (Wang et al., 2007; Tian et al., 2010; Nan et al., 2017), there are, however, little is known about the physiological functions of neuropeptides and their receptors in comparison to other lepidoptera insects. This is largely due to the lack of the genomic, transcriptomic and peptidomic information in *A. hetaohei*. To gain an insight into the regulation of fundamental events in *A. hetaohei*, we set out to sequence the transcriptomes of various tissues (heads, abdomens, antennae and legs) from male and female adults using Illumina HiSeq(TM) 4000 sequencing approach. After transcriptome assembly, we applied BLAST-based analysis with known lepidoptera neuropeptides and receptor genes to identify *A. hetaohei* specific orthologs. Collectively, we identified 32 neuropeptides and 39 neuropeptide receptor genes from *A. hetaohei* head. Phylogenetic analyses and tissue expression (head, thorax and abdomen) evaluation were also performed. Our results indicated that the identification and expression of neuropeptides and neuropeptide receptor genes could provide a solid theoretical basis for postulating the functions of the identified genes and exploring the mechanism of physiological processes of *A. hetaohei*, which is essential to find selective targets for new pesticides.

2. Materials and methods

2.1. Insect rearing and tissue collection

The larvae of *A. hetaohei* were obtained from the Walnut Demonstration Garden in the Walnut Experimental Station associated with Northwest Agriculture and Forestry University in Shangluo city, Shaanxi province (33°2′30″–34°24′40″N, 108°34′20″–111°1′25″E). In late August 2016, walnut fruits that had been bored by *A. hetaohei* larvae were collected and placed on the soil surface. The pest overwintered in old larvae from the beginning of September 2016 to April 2017. The cocoons were screened out and reared in a temperature-controlled basin with soil at 25 ± 1 °C, 75%-80% relative humidity (RH) with a photoperiod cycle of 14hL/10hD. The newly-emerge adults were transferred into a plastic box (60*60*60 cm) according to sex. They were then supplied with a 10% (w/v) honey solution per day.

For transcriptome sequencing, tissues that include heads, abdomens, antennae and legs from male and female adults, respectively, were dissected and placed in a microcentrifuge tube (1.5 mL). For the tissue expression study, tissues from male and female, including heads (n = 30), thoraxes (n = 15), and abdomens (n = 10) were also collected under the same conditions. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. RNA extraction and transcriptome generation

Total RNA was extracted using the RNAisoPlus Total RNA Isolation kit (Takara, Dalian, China) according to the manufacturer's protocol for animal tissues. The RNA (about 3 μg for each sample) was then used in the construction of Illumina HiSeq (Illumina, Inc., San Diego, USA) library for sequencing following HiSeq 4000 platform-specific protocols (Breeding Biotechnologies, Shaanxi, China). Detailed methods and analysis of the transcriptome sequencing were implemented using previously standardized approaches (Zhang et al., 2015).

Table 1
Summary of *A. hetaohei* transcriptome assembly.

Statistics Project	Number
Clean reads from all samples	101.38 Gb
GC content	39.65%–51.95%
% ≥ Q30	90.67%
Total unigene	44,386
N50 of unigenes (nt)	2247
Median length of unigenes (nt)	1356.21
Unigenes with homolog in NR	23,807

2.3. Sequence analysis and phylogenetic tree analysis

The sequences of previously published neuropeptides and neuropeptide receptor genes from *B. mori*, *Helicoverpa armigera* and *C. suppressalis* were used for BLAST searching of the transcriptome dataset from *A. hetaohei* (https://blast.ncbi.nlm.nih.gov/Blast.cgi?). Similarity searches were performed using the NCBI-BLAST network server (http://blast.ncbi.nlm.nih.gov/). The open reading frames (ORFs) of putative neuropeptide and their receptors were predicted using ORFfinder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Putative N-terminal signal peptides were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The nucleotide sequences of neuropeptides and neuropeptide receptor genes from the head transcriptome are provided in the supplemental material Figs. S1 and S2.

The evolutionary relationships were analyzed based on the amino acid sequences encoded by the identified *A. hetaohei* neuropeptides and neuropeptide receptor genes based on the sequences identified from other insects. The neuropeptide datasets contained 33 sequences from *B. mori*, 32 from *C. suppressalis*, and 28 from *H. armigera*. The neuropeptide receptor datasets contained 39 sequences from *B. mori*, 39 from *C. suppressalis*, 25 from *H. armigera*, and 15 from *P. xylostella*. Their protein names and accession numbers were listed in supplementary Table S1 and S2. Protein sequence alignments were carried out using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html), and Maximum-likelihood trees were constructed by MEGA6.0 using the WAG + F model. The reliability of the relationships between the tested taxa using bootstrap analysis with 1000 replications (Felsenstein, 1985).

2.4. Tissue expression analysis of A. hetaohei neuropeptides and their receptors

Total RNA was extracted from male and female tissues (including heads, thoraxes and abdomens) using a RNAisoPlus Total RNA Isolation Kit (Takara, Dalian, China) according to the manufacturer's protocol. The integrity and purity of total RNA in all samples were monitored by agarose gel electrophoresis (1%), and RNA quantity was measured on a NanoDrop 8000 (Quawell, Beijing, China). Total RNA was then employed as template for first-strand cDNA synthesis using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions using 1 μg total RNA in a 20 μL final reaction volume. All products were stored at −20 °C until use.

Transcriptional expression of *A. hetaohei* neuropeptides and neuropeptide receptor genes in both male and female tissues were analyzed by Real-time quantitative polymerase chain reaction (RT-qPCR). Gene-specific primers were designed using Primer5.0 (Premier Biosoft, www.premierbiosoft.com) and are listed in Supplementary Table S3 and S4.

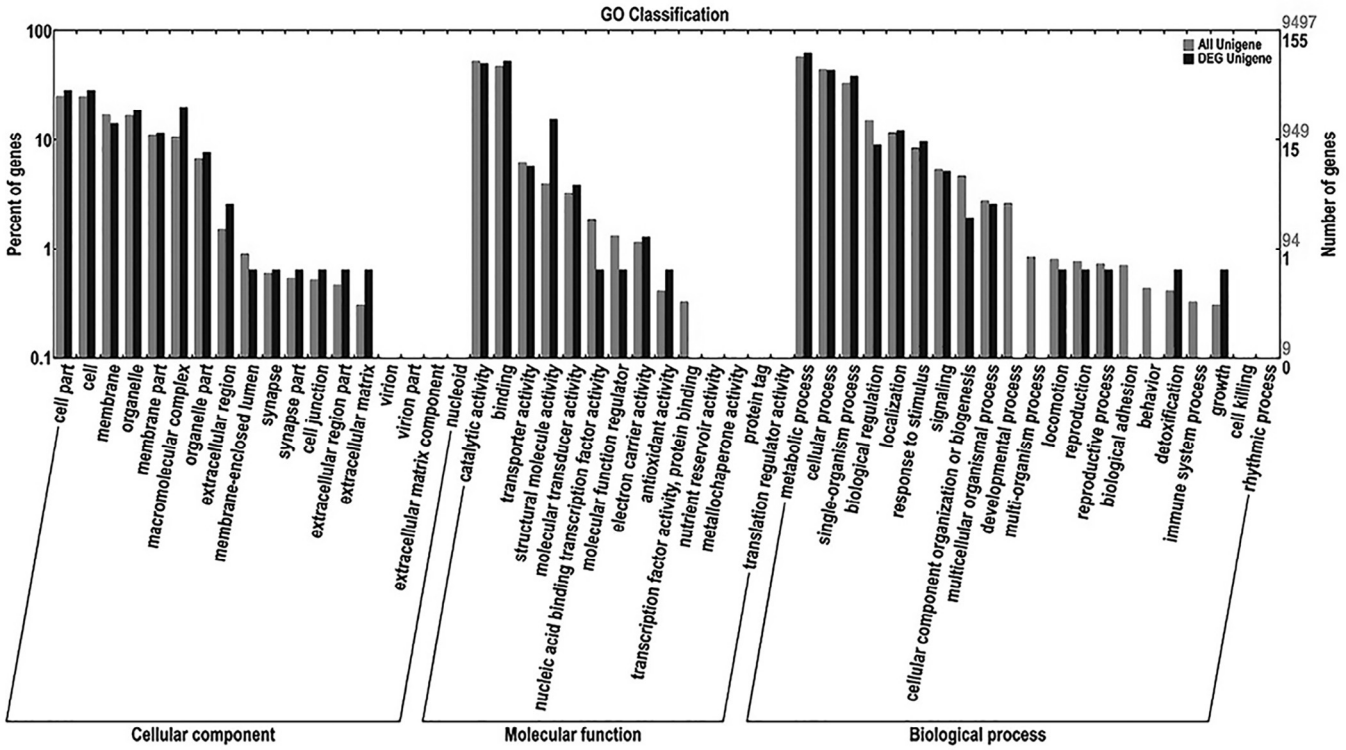

Fig. 1. Gene Ontology (GO) classification of the *A. hetaohei* transcriptome. The results were summarized in three main categories: cellular component, molecular function and biological process. The right Y-axis represents the number of genes in the category and the left Y-axis represents the percentage of a specific category of genes in that main category. The gray column indicates the annotation of all unigenes, and the black column indicates the annotation of head differentially expressed genes.

RT-qPCR was conducted on a Bio-Rad CFX96 PCR System (Hercules, CA, USA) in 20 μL reactions containing 10 μL of SYBR Premix Ex Taq II (Takara, Dalian, China), 0.8 μL of each primer (10 mM), 1 μL of sample cDNA, and 7.4 μL of ddH_2O (sterile distilled water). Cycling was performed for 30 s at 95 °C, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s, and followed by melting analysis to investigate the specificity of PCR primers: 65–95 °C held for 0.05 s for 0.5 °C. According to the methods of screening for the most suitable reference genes for gene expression studies (Ma et al., 2016; Yang et al., 2017) and our previous study about the potential reference genes for RT-qPCR analysis in *A. hetaohei* (Li, 2018), in this study, *28S* and *GAPDH* were chosen as the internal controls for normalizing the neuropeptides and the neuropeptide receptors of *A. hetaohei*. Each experiment was carried out with three technical replicates and three biological replicates for each transcript. Negative controls without template were included in each experiment to monitor reproducibility. The ΔΔCt values were obtained follow the operating instruction of CFX Manager software (3.1, Bio-Rad). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Data (mean ± standard error) of the relative expression levels from various samples were subjected to ANOVA (one-way analysis of variance), followed by Duncan's significance difference tests implemented in SPSS Statistics 17.0 (IBM SPSS Statistics, Chicago, IL, USA). OriginPro (Version 8.0) was utilized to draw the expression patterns of neuropeptides and the neuropeptide receptors in different tissues of *A. hetaohei* male and female.

3. Results

3.1. Transcriptome sequencing and sequence assembly

We carried out a next-generation sequencing analysis using cDNA libraries constructed from *A. hetaohei* tissue samples of male and female heads, abdomens, antennae and legs using Illumina HiSeq(TM) 4000 platform. The total transcriptome sequencing provided approximately 1013.8 million reads (101.38 Gb), and the clean reads of each sample reach to 4.61 Gb. After clustering and redundancy filtering, we finally acquired 44,386 unigenes with an N50 length of 2247 bp (Table 1). We named these 44,386 ones unigenes according to some recently published papers, even though each of them may not necessarily represents a unique gene (Liu et al., 2012; Li et al., 2012). Of the 44,386 unigenes, those with a sequence length of more than 1000 bp occupied for 41.92% of the total transcriptome assembly. Furthermore, annotation was used to classify the transcripts into functional groups according to NR, Swiss-Prot, KEGG, COG, KOG, GO and Pfam categories. Of the 44,386 unigenes, 27,843 (62.73%) could be annotated based on sequence homology (seen in supplementary Table S5).

As seen in Fig. 1, GO annotation illustrated that metabolic and cellular processes were the most highly represented in the biological process categories, and cell and cell part were most abundantly represented in the cellular component category. The transcript reads sequences data from *A. hetaohei* head tissue were deposited in the SRA database of the National Center for Biotechnology Information (NCBI, USA) (http://www.ncbi.nlm.nih.gov/) with the accession number of PRJNA574005.

3.2. Identification of A. hetaohei neuropeptides and their receptors

Using the reported neuropeptides and their receptors of other insect species in NCBI as queries BLAST searching was performed (Xu et al.,

Table 2
Neuropeptides identified from *A. hetaohei*.

Gene Name	Unigene ID	SP (aa)	ORF (aa)	Homology search with known protein				
				Name	Species	E-value	Accesion No.	Identity (%)
AKH1	CL31877Contig1	1-19	88	Adipokinetic hormone 1	*Chilo suppressalis*	6e-23	ALM301296.1	80%
AKH2	CL18765Contig1	1-26	89	Adipokinetic hormone 2	*Bombyx mori*	3e-08	NP_001124365.1	46%
AstB/PTSP	CL15276Contig1	NO	306	Prothoracicostatic peptide-like	*Helicoverpa armigera*	3e-109	AGH25567.1	79%
Ast	CL19098Contig1	NO	146	Allostatin	*Amyelois transitella*	3e-45	XP_013186846.1	83%
AT	CL9676Contig1	NO	173	Allatotropin precursor	*Chilo suppressalis*	5e-65	ALM30304.1	80%
Bb A2	CL11685Contig1	1-16	94	Bombyxin A-2-like	*Papilio xuthus*	2e-17	KPI98658.1	80%
Bur	CL32705Contig1	NO	196	Bursicon alpha	*Plutella xylostella*	1e-78	AJM76770.1	79%
CAP3	CL4839Contig1	NO	156	Cardio acceleratory peptide 2b-like	*Helicoverpa armigera*	7e-56	XP_021185700.1	68%
CP	CL8113Contig1	NO	154	Crustacean cardioactive peptide precursor	*Chilo suppressalis*	3e-54	ALM30313.1	76%
CCH2	CL5421Contig2	NO	133	CCHamide 2 precursor	*Chilo suppressalis*	5e-47	ALM30311.1	67%
CCH	CL5421Contig1	1-25	151	CCHamide	*Operophtera brumata*	2e-37	KOB72957.1	69%
Cor-7	CL1756Contig1	NO	69	Coronin-7 isoform X1	*Spodoptera litura*	0.0	XP_022835326.1	89%
Cor-6	CL36899Contig1	NO	498	Coronin-6 isoform X1	*Helicoverpa armigera*	0.0	XP_021186624.1	94%
CRF	CL8742Contig1	NO	376	Corticotropin-releasing factor-binding protein	*Helicoverpa armigera*	0.0	XP_021188638.1	74%
DH	CL7912Contig1	NO	130	Diuretic hormone 31 precursor	*Chilo suppressalis*	4e-42	ALM30315.1	69%
DH45	CL8969Contig1	1-25	194	Diuretic hormone 45 isoform X1	*Helicoverpa armigera*	3e-51	XP_021182113.1	81%
ETH	CL44058Contig1	NO	138	Ecdysis triggering hormone precursor	*Danaus plexippus*	7e-33	OWR51561.1	83%
FMRF	CL3517Contig1	NO	210	FMRFamide-related peptides-like	*Pieris rapae*	1e-75	XP_022116223.1	71%
GPA2	CL4469contig2	NO	101	Glycoprotein hormone alpha 2	*Operophtera brumata*	1e-50	KOB77427.1	84%
ITG	CL18Contig2	NO	235	Prohormone-3 isoform X1	*Helicoverpa armigera*	6e-120	XP_021195429.1	83%
LK	CL16030Contig1	NO	372	Leucokinin precursor	*Danaus plexippus*	2e-121	OWR49793.1	69%
MS	CL37876Contig1	1-38	130	Myosuppressin	*Papilio xuthus*	8e-40	XP_013166219.1	85%
NP	CL41044Contig1	NO	68	Neuroparsin precursor	*Danaus plexippus plexippus*	1e-20	OWR51753.1	72%
NPF	CL18971Contig1	NO	433	Neuropeptide F1 receptor	*Papilio xuthus*	0.0	XP_013165451.1	75%
NPY	CL37489Contig1	NO	93	Neuropeptide Y	*Spodoptera litura*	1e-49	AEE01342.1	82%
NPLP1	CL2155Contig1	NO	508	neuropeptide-like precursor 1	*Danaus plexippus*	0.0	KPJ13870.1	70%
OK	CL16091Contig1	1-48	171	Orcokinin precursor	*Bombyx mori*	3e-25	NP_001124366.1	79%
PBAN	CL19220Contig1	NO	205	Pheromone biosynthesis activating neuropeptide preprohormone	*Chlumetia transversa*	4e-79	AIY72749.1	73%
PTTH	CL18146Contig1	NO	140	Prothoracicotropic hormone	*Antheraea pernyi*	1e-47	AAB05259.1	72%
sNPF	CL35994Contig1	1-27	179	Short neuropeptide F	*Amyelois transitella*	5e-88	XP_013200713.1	82%
SIF	CL18517Contig1	1-24	94	SIFamide precursor	*Bombyx mori*	5e-19	NP_001124358.1	71%
TK	CL15705Contig1	1-24	261	Tachykinins	*Amyelois transitella*	3.6e-99	XP_013186067.1	77%

SP: Signal Peptide; NO: no signal peptide; 1-N: have signal peptide and its length. ORF: Open Reading Frame.

2016; Roller et al., 2008; Hewes and Taghert, 2001; Li et al., 2008; Tanaka et al., 2014; Hummon et al., 2006). We identified a total of 32 neuropeptide transcripts and 39 receptor transcripts from *A. hetaohei* head. As seen in Tables 2 and 3, unigene CL7912Contig1 is DH (Diuretic hormone) and unigene CL37861Contig1 represents its receptor; unigene CL19098Contig1 is Ast (Allostatin) and unigene CL5564Contig1 indicates its receptor; unigene CL37876Contig1 is MS (Myosuppressin) and unigene CL9158Contig1 represent its receptor; unigene CL19220Contig1 is PBAN (Pheromone biosynthesis activating neuropeptide) and unigene CL7684Contig1 represents its receptor.

Among the obtained neuropeptides, 11 have complete open reading coding frames (ORFs), including AKH1, AT, DH, DH45, FMRF, ITG, OK, PBAN, SIF, sNPF and TK; AKH2, AstB, CAPA, CCH2, PTTH, Bur, CRF, ETH, LK, and NPF have the 5′ non-coding region; CCH, MS, NP, and NPLP1 possess 3′ non-coding regions. Of the identified neuropeptide receptors, 9 have completed ORFs with A1, A4, A10, A12, A13, A15, A20, A23, and A26; LGR and SPR contain 5′ non-coding region; AstR, DHR, B3, INR, A6, A7, A8, A11, A17, A27, A29, A30; A31 and A32 comprise 3′ non-coding region. In comparison to the published sequences of *B. mori*, *D. melanogaster*, *Nilaparvata lugens*, *A. mellifera*, and *Tribolium castaneum*, we found that the number of neuropeptides in *A. hetaohei* is lower than those in other insects (seen in supplementary Table S6). Of the identified 32 neuropeptides and 39 neuropeptide receptor genes (Tables 2 and 3), their sequences are highly similar (> 60%) to other lepidopteran orthologs, whereas the sequence of neuropeptide gene *AKH2* and receptor gene *A2* found in the current study shows low similarity (< 50%) to other lepidoptera orthologs.

3.3. Phylogenetic analyses

To assign putative functions to neuropeptide genes in *A. hetaohei*, a maximum-likelihood tree was constructed based on 93 published neuropeptide sequences from lepidoptera including *B. mori*, *H. armigera*, and *C. suppressalis*, (illustrated in Fig. 2), demonstrating that *A. hetaohei* AKH2, SIF, TK, CCH2, CAP3, AT, sNPF, ETH, Ast, OK, DH45, NPLP1 and CCH were clustered together with the orthologs from other lepidoptera insects. Bur, GPA2, AKH1, NP, BbA2, PBAN, ITG, DH, FMRF, MS, CRF, LK and PTTH were clustered with the orthologs from the other lepidopteran insects in the same clade, while AstB remained in a single clade.

Phylogenetic analyses of the identified neuropeptide receptors with 118 sequences from lepidoptera including *B. mori*, *H. armigera*, *C. suppressalis*, and *P. xylostella* (shown in Fig. 3) displayed that A24 of *A. hetaohei* was clustered together with the A24 of *C. suppressalis*; A30/32/B1 were individually clustered together. A9, A12 and A13 were clustered with the orthologs from the other lepidoptera within the same clade, while A1, A4, A10, A15, A16, A27 and PBANR dispersed in different branches.

3.4. Tissue expression profile of A. hetaohei neuropeptides and their receptors

The overall relative expression profiles of 23 neuropeptides and 21 neuropeptide receptors of *A. hetaohei* in three different tissues (head, thorax, and abdomen) in both female and male adults by RT-qPCR are showed in Figs. 4 and 5 (Some neuropeptides like *AKH2*, *GPA2* and *B1*, neuropeptide receptor genes such as *A16*, *A25* and *A32* were not

Table 3
Neuropeptide receptors identified in *A. hetaohei*.

Gene Acronym	Unigene ID	Open reding frame (aa)	Homology search with known protein				
			Name	Species	E-value	Accession No.	Identity
A1	CL18320Contig1	376	Somatostatin receptor type 2-like	*Helicoverpa armigera*	0.0	XM_021339126.1	80%
A2	CL13771Contig1	298	Neuropeptide receptor A2	*Chilo suppressalis*	4e-57	ALM88297.1	39%
A4	CL17020Contig1	433	Neuropeptide F receptor	*Papilio xuthus*	0.0	XP_013165451.1	77%
A6	CL4649Contig1	479	Thyrotropin-releasing hormone receptor	*Helicoverpa armigera*	0.0	XM_021331966.1	77%
A7	CL14847Contig1	121	Prolactin-releasing peptide receptor-like	*Helicoverpa armigera*	7.00E-32	XM_021328504.1	82%
A8	CL39162Contig1	139	Neuropeptide receptor A8	*Bombyx mori*	6e-66	NP_001127743.1	96%
A9	CL7350Contig1	534	Neuropeptide receptor A9	*Chilo suppressalis*	0.0	ALM88305.1	77%
A10	CL14462Contig1	444	Prolactin-releasing peptide receptor-like	*Helicoverpa armigera*	0.0	XP_021184170.1	78%
A11	CL28008Contig1	288	prolactin-releasing peptide receptor-like	*Helicoverpa armigera*	1e-179	XP_021184179.1	92%
PRPRL	CL204Contig2	494	prolactin-releasing peptide receptor-like	*Helicoverpa armigera*	0.0	XM_021328516.1	76%
A12	CL26986Contig1	435	Neuropeptide receptor A12	*Chilo suppressalis*	0.0	ALM88308.1	87%
A13	CL7966Contig1	415	Neuropeptide receptor A13	*Chilo suppressalis*	0.0	ALM88309.1	93%
A14	CL1718Contig1	387	Neuropeptide receptor A14	*Chilo suppressalis*	0.0	ALM88310.1	84%
A15	CL8721Contig1	465	Neuropeptide CCHamide-1 receptor isoform X1	*Papilio polytes*	0.0	XP_021186063.1	85%
A16	CL28017Contig1	165	Orexin receptor	*Spodoptera litura*	4e-75	XP_022833701.1	81%
A17	CL18171Contig1	403	Galanin receptor	*Pieris rapae*	1e-146	XP_022117612.1	79%
A20	CL38467Contig1	461	Sex peptide receptor-like	*Bombyx mori*	0.0	NM_001134246.1	73%
SPR	CL7370Contig1	201	Sex peptide receptor	*Chilo suppressalis*	1e-103	ALM88340.1	78%
SPR1	CL4293Contig1	288	Sex peptide receptor	*Papilio machaon*	0.0	XP_014367912.1	94%
A23	CL5695Contig1	524	RYamide receptor-like	*Helicoverpa armigera*	0.0	XP_021201003.1	72%
A24	CL17642Contig1	450	Neuropeptide receptor A24	*Amyelois transitella*	0.0	XP_013200309.1	84%
A25	CL40551Contig1	161	Neuropeptides capa receptor-like	*Helicoverpa armigera*	7e-74	XP_021185712.1	71%
A26	CL5173Contig1	476	Cardioacceleratory peptide receptor-like	*Helicoverpa armigera*	0.0	XP_021193164.1	80%
A27	CL38066Contig1	392	Neuropeptide receptor A27	*Chilo suppressalis*	0.0	ALM88323.1	80%
A29	CL43561Contig1	174	Neuropeptide receptor A29	*Chilo suppressalis*	1.00E-126	KT031027.1	81%
A30	CL5895Contig1	136	Cardioacceleratory peptide receptor-like	*Amyelois transitella*	1.00E-92	XM_013328971.1	79%
A31	CL1513Contig1	513	Trissin receptor-like	*Spodoptera litura*	0.0	XP_022833959.1	74%
A32	CL34022Contig1	104	Neuropeptide receptor A32	*Bombyx mori*	4e-35	NP_001127748.1	62%
A33	CL15172Contig1	360	Tachykinin-like peptides receptor	*Helicoverpa armigera*	0.0	XM_011554834.1	79%
AstR	CL5564Contig1	426	Allostatin A receptor	*Spodoptera litura*	4e-71	XP_022830609.1	94%
B1	CL5589Contig1	514	Calcitonin gene-related peptide type 1 receptor	*Helicoverpa armigera*	2e-178	XP_021196683.1	84%
B3	CL36388Contig1	785	Neuropeptide receptor B3	*Chilo suppressalis*	0.0	ALM88343.1	61%
DHR	CL37861Contig1	612	Diuretic hormone receptor	*Helicoverpa armigera*	0.0	XP_021182084.1	85%
INR	CL12550Contig1	1241	Insulin receptor	*Bombyx mori*	0.0	NP_001037011.1	60%
ILR	CL37142Contig1	931	Insulin-like receptor	*Helicoverpa armigera*	1.00E-21	XM_021325388.1	66%
LGR	CL3860Contig1	644	Leucine-rich repeat G-protein coupled receptor	*Bombyx mori*	0.0	XP_012553374.1	70%
LGR2	CL11012Contig1	673	Leucine-rich repeat-containing G-protein coupled receptor	*Papilio xuthus*	0.0	KPI93132.1	61%
MSR	CL9158Contig1	102	Myosuppressin receptor	*Chilo suppressalis*	8.00E-56	KT031038.1	81%
PBANR	CL7684Contig1	496	Pheromone Biosynthesis Activating Neuropeptide Receptor	*Ostrinia nubilalis*	0.0	AGL12067.1	79%

determined due to their short sequences, or it was difficult to obtain the specific amplification target fragments). The results exhibited that neuropeptides *AKH1*, *AstB*, *Ast*, *B3*, *CCH*, *CCH2*, *CP*, *ITG*, *ETH*, *LK*, *MS*, and *OK* were expressed at much higher levels in the male head than in other tissues. *FMRF*, *NP* and *PTTH* were predominantly expressed in the male thorax. *Bur* was richly expressed in the female abdomen, *NPLP1*, *PBAN* and *SIF* were more highly expressed in the female head than that in male head.

In addition, the expression levels of neuropeptide receptor genes *A6*, *A8*, *A9*, *A11*, *A12*, *A15*, *A33* and *LGR* in male head was significantly higher than that in female head; *A24* and *LGR2* were up-regulated in female head compared with other tissues. The expression levels of *A1*, *A4*, *A10*, *DHR* and *MSR* in male abdomen were higher than that in female abdomen; while *A2* and *PBANR* displayed abdomen-biased expression in female. In both male and female, *A20* showed notably higher expression in head and abdomen than that in thorax.

4. Discussion

Neuropeptides and their receptors are key regulators of almost all insect physiological processes including development, reproduction, metamorphosis, feeding and digestion, and behavior, among others. Hence, they have been suggested as potential targets for development of safe, selective forms of pest control (Verlinden et al., 2014). With the rapid development of genomics and bioinformatics approaches, neuropeptides and their receptors have been extensively examined in insect species. So far, the whole genome of several model species such as *B. mori*, *D. melanogaster*, and *A. mellifera* have been completely sequenced

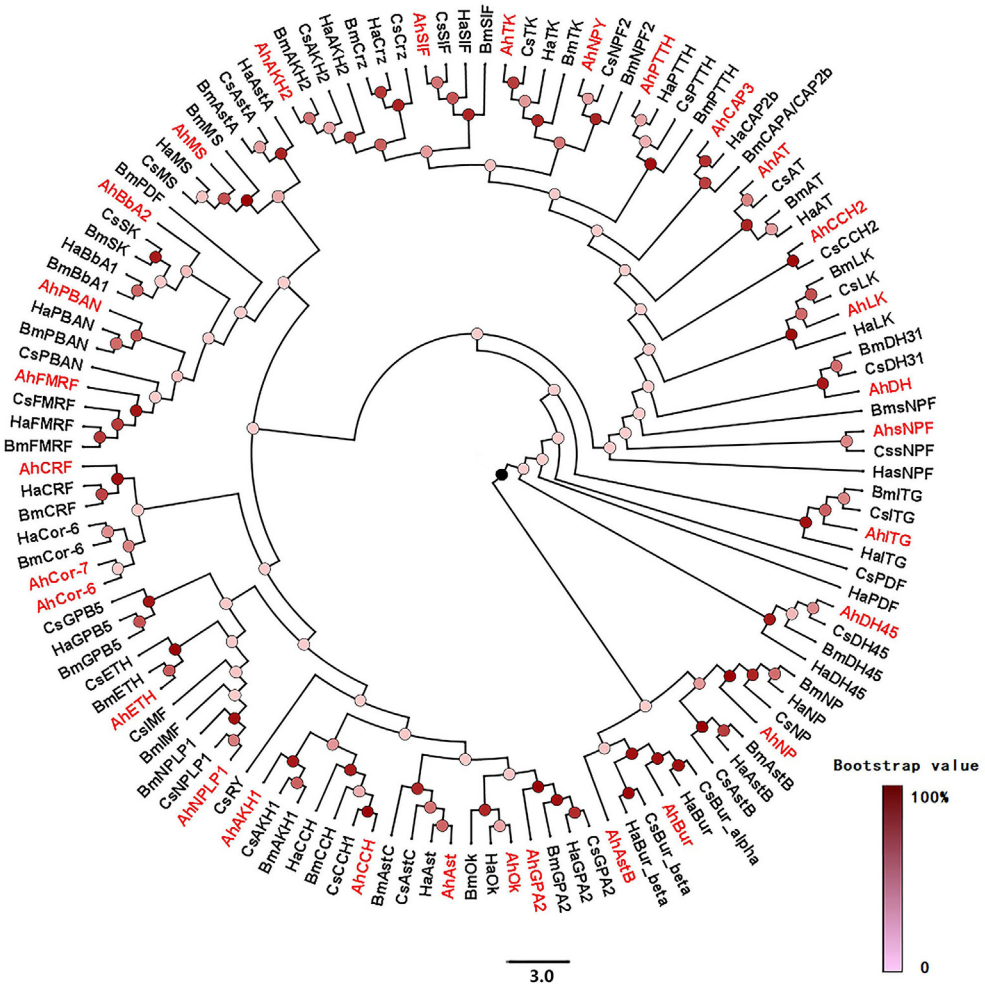

Fig. 2. Phylogenetic analysis of insect neuropeptides. Ah: *A. hetaohei*; Bm: *B. mori*; Cs: *C. suppressalis*; Ha: *H. armigera*. The *A. hetaohei* neuropeptides are highlighted in red color, accession numbers are given in supplementary Table S1. The tree was conducted with MEGA 6.0, using the Maximum-Likelihood method and the bootstrap analysis with 1000 replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and their EST library have also been edited. This has boosted the identification and characterization of novel neuropeptides and their receptors (Adams et al., 2000; Mita et al., 2004; Hummon et al. 2006). However, identification of neuropeptides and their receptors in *A. hetaohei*, elucidation of the mechanisms that mediate their physiological processes have not been reported. In the present study, sequencing and analysis of the transcriptome of *A. hetaohei* female and male adult tissues (including heads, abdomens, antennae and legs) were performed. Among the 44,386 unigenes identified by the assembly program Trinity, 62.73% could be annotated through NR, Swiss-Prot、KEGG、COG、KOG, GO and Pfam categories, indicating that several genes are either non-coding or are homologous with genes that do not have any annotation term.

We totally identified 32 neuropeptides from *A. hetaohei* head, which is less than the 41 reported for *T. castaneum*, the 49 reported for *B. mori*, the 41 reported for *D. melanogaster*, the 39 reported for *N. lugens*, and the 41 reported for *A. mellifera* (Xu et al., 2016; Roller et al., 2008; Hewes and Taghert, 2001; Li et al., 2008; Tanaka et al., 2014; Hummon et al., 2006). It is suggested that more novel neuropeptides and their receptors might be revealed by further sequencing the genome of *A. hetaohei* and the transcriptomes in its different developmental stages and tissues (Nässel and Zandawala, 2019). Bioinformatic analyses showed that 11 of the 32 neuropeptides and only 9 of the 39 identified neuropeptide receptor genes had complete ORF. Sequence comparisons revealed that most of the neuropeptides and receptors show high similarity to other lepidopteran orthologs such as *B. mori*, *C. suppressalis*, and *H. armigera*. Additionally, our phylogenetic analyses report a broad diversity of the neuropeptide receptors of *A. hetaohei* that may have differences or are new to Lepidoptera. This finding might also reflect different functions in development, reproduction, behavior, and feeding, presenting an excellent target for discovering an extremely selective environmentally friendly prevention in pest control.

Largely due to the rapid developments of powerful genetic methods, imaging techniques and innovative bioassays, a huge amount of work have been published on the functions of neuropeptides and their receptors in insect species. For instance, previous studies have reported that *myosuppressin* (*MS*) has a conserved FMRF-amide motif at C-terminal. *MSs* are braingut neuropeptides in *Rhodnius prolixus*, expressed in CNS and posterior midgut. In vitro assays proved that the *RhoprMS* inhibited heart rate (Leander et al., 2015; Lee et al., 2012) and the amplitude and frequency of spontaneous contractions from the anterior midgut and hindgut (Lee et al., 2012). One *RhoprMS* receptor was identified (Leander et al., 2015; Lee et al., 2015), being expressed in CNS, midgut, hindgut and nymph V reproductive tissue (Lee et al., 2015). In this study, the MS of *A. hetaohei* was close to the MS of *H. armigera* and *C. suppressalis*; the highest expression of *MS* and its receptor was detected in the head tissue in both sexes. It has been well reported that diuretic hormone (*DH*) is important to regulate the secretion of fluid for insect survival (Coast et al., 2002). In *B. mori*, *DH31* was predominantly expressed in the central nervous system and midgut, whereas its receptor was primarily expressed in the anterior silk gland, midgut, and ovary (Iga and Kataoka, 2015). The *DH31* receptor of *D.*

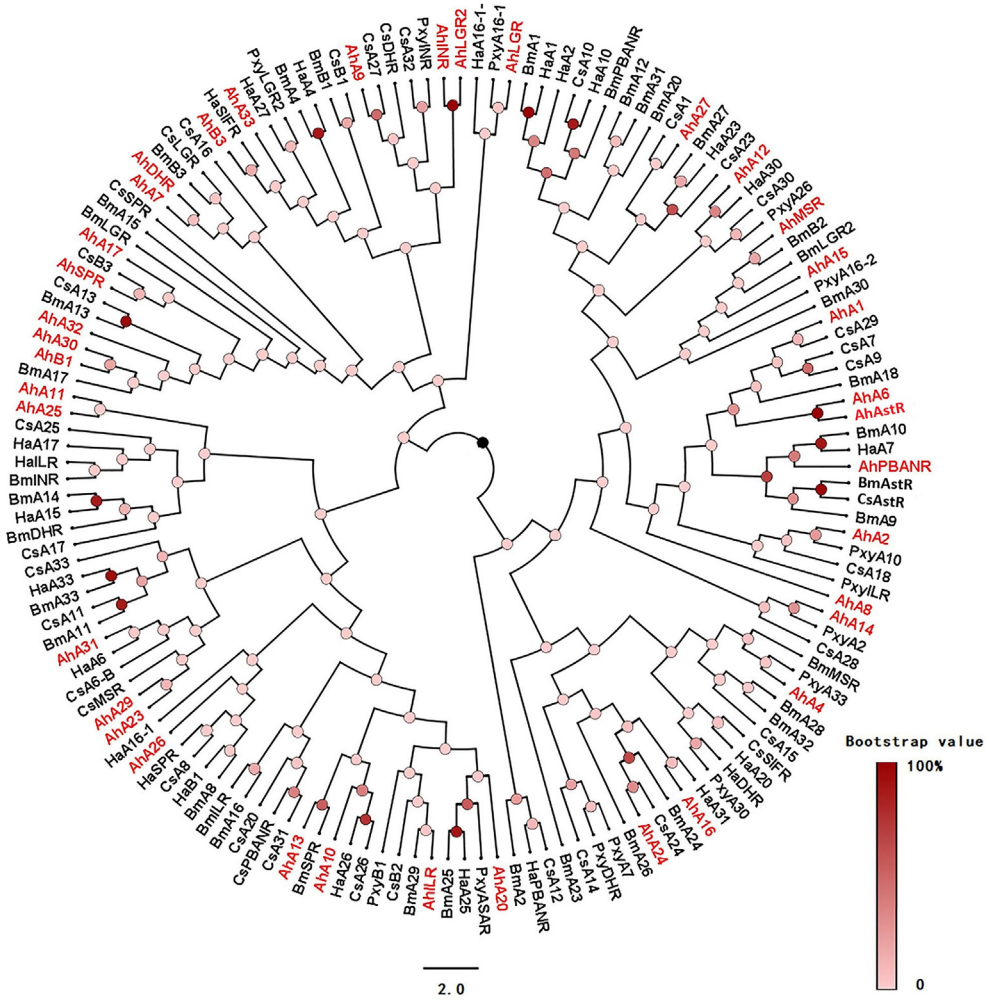

Fig. 3. A phylogenetic tree analysis of insect neuropeptide receptors. Ah: *A. hetaohei*; Bm: *B. mori*; Cs: *C. suppressalis*; Pxy: *P. xylostella*; Ha: *H. armigera*. The *A. hetaohei* neuropeptide receptors are emphasized in red color, and the accession numbers of proteins used in this analysis are listed in supplementary Table S2. Maximum-Likelihood tree of candidate neuropeptide receptor genes was constructed by MEGA 6.0 and inferred from 1000 bootstrap replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

melanogaster was expressed in the midgut visceral muscles, which would help stimulate its midgut contractions (Vanderveken and O'Donnell, 2014). *DH31* was also found to regulate the circadian activity in *D. melanogaster*, in its clock system, *DH31* was shown to be a wake-promoting neuropeptide acting before dawn (Kunst et al., 2014). In this work, the DH of *A. hetaohei* and the DH31 of *C. suppressalis* and *B. mori* clustered together in the phylogenetic tree, moreover, the *DH* of *A. hetaohei* was primarily expressed in the female head, while its receptor was widely expressed in all tested tissues. *PBAN* shares the C-terminal FXPRL-NH2 (or similar sequence), which is the most significant to activate biological function such as sex pheromone production in mating communication in moths (Raina and Kempe, 1990, Du et al., 2017). In *A. hetaohei*, phylogenetic analysis showed that PBAN was related to the PBAN of *H. armigera* and *B. mori*; in both male and female of *A. hetaohei*, *PBAN* displayed head-biased expression, while its receptor was abundantly expressed in the female abdomen.

In addition, adipokinetic hormone1 (*AKH1*) is a class of neuropeptides with pQX6Wamide structure, which releases energy-rich substrates such as sugar, lipid and proline into hemolymph by acting on glycogen phosphatase from the lipase of the fat body (Gäde and Auerswald, 2003; Sharp-Baker et al., 1996). In *D. melanogaster*, *AKHs* provide a protection effect in stress from surroundings through the inhibition of the biosynthesis of RNA fatty acids and proteins to stimulate muscle contraction (Bednářová et al., 2015). In *A. hetaohei*, AKH1 was clustered with the AKH 1 of *B. mori* and *C. suppressalis*, exhibiting exclusively high expression in both male and female head. *CCH* was first identified as *CCH2* in *B. mori* (Roller et al., 2008), which is 13 amino acid residues long, containing two cysteines and a C-terminal histidine-amide group (Hansen et al., 2011). In *C. suppressalis*, two CCHamide genes (*CCH1* and *CCH2*) have been identified. Additionally, *CCH1* has two splicing variants: *CCH1a* and *CCH1b* (Xu et al., 2016). It has been documented that CCHamide regulates feeding motivation in blowflies (Ida et al., 2012) and sensory perception and olfactory behavior in starved *D. melanogaster* (Farhan et al., 2013). In this study, phylogenetic analysis showed that the two CCH of *A. hetaohei* were closely related to the CCH of *C. suppressalis*. We also obtained the two *CCH* genes showing head-specific expression. In sum, to date, there is no report on neuropeptides and neuropeptide receptors in *A. hetaohei*, the knowledge of their expression profiles would give us clues to understand their potential physiological role in the development, reproduction, and feeding processes, however, their mechanisms which *A. hetaohei* might use to regulate its life activities still need to be further studied.

Considering the broad spectrum of physiological roles of neuropeptides and their receptors, it is important to investigate their mechanisms in the fundamental life events of *A. hetaohei* to prevent the destruction of walnut fruits. In many of the cases, larvae were used as materials to identify and functional characterize neuropeptides and their receptors (Xu et al., 2016; Sun, 2012). In the present study, we

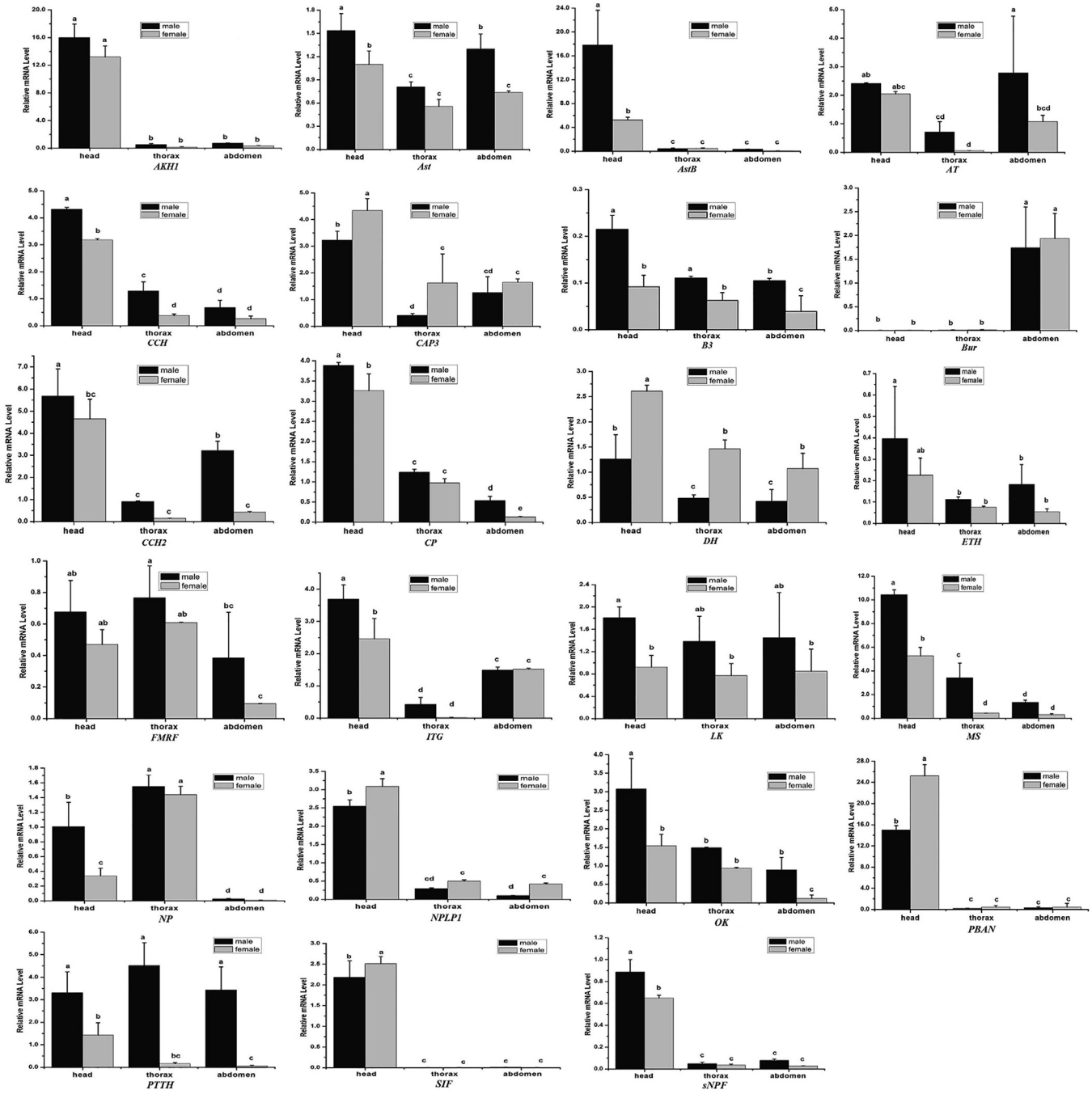

Fig. 4. RT-qPCR analysis of *A. hetaohei* neuropeptide transcripts levels in different adult tissues (head, thorax and abdomen) of male and female. Each bar represents the mean with standard error. Different letters mean significant differences ($p < 0.05$, Duncan's multiple range test).

identified 32 neuropeptides and 39 neuropeptide receptors from *A. hetaohei* head. The homology and phylogenetic relationship were analyzed and their expressions in the head, thorax, and abdominal tissues of male and female adults were also analyzed. Altogether, our findings will facilitate the functional characterization of novel neuropeptides and their receptors and further the determination on their biological role in the coordination of physiological processes of *A. hetaohei*, which will provide a theoretical support for pest management strategies.

5. Conclusion

In conclusion, through transcriptome sequencing and analyses, we obtained an extensive set of 32 neuropeptides and 39 neuropeptide receptor genes from *A. hetaohei* adult head. This is the first step towards understanding the functions of these genes. We conducted phylogenetic analyses with sequences of other insect species and examined their gene expression profiles in different tissues including the head, thorax and abdomen, in both male and female. We found several genes with tissue-biased or specific expression, implying they might associate with the development, reproduction, behavior, and feeding progresses of *A. hetaohei*. Our findings provide a foundation for further exploring the functions of these genes and screening of potential target genes, which could assist the development of novel pest control strategies.

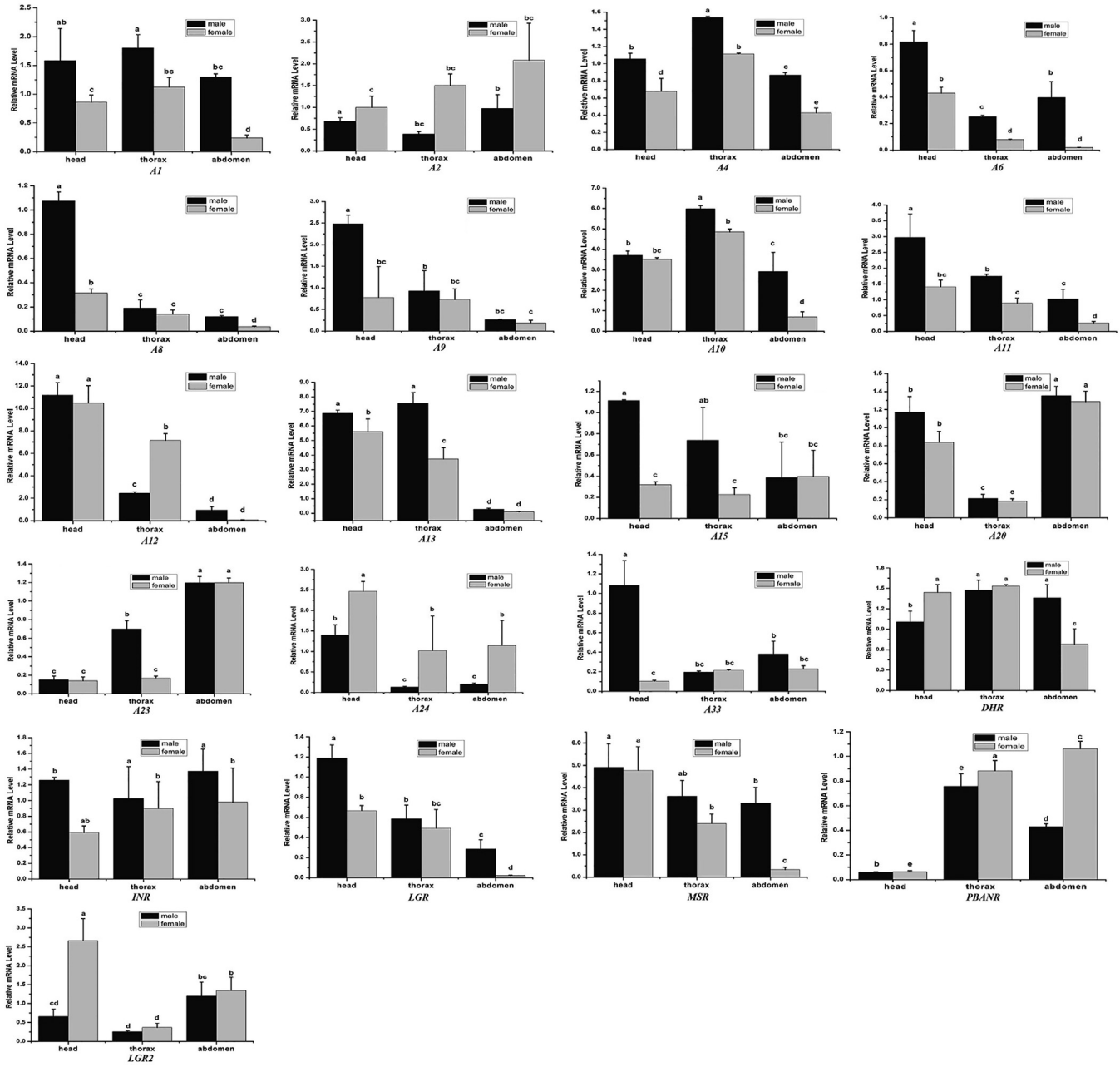

Fig. 5. Relative expression levels of *A. hetaohei* neuropeptide receptors in different adult tissues (head, thorax and abdomen) of male and female, using RT-qPCR. Each bar represents the mean with standard error; and the bar with different letters means significant differences ($p < 0.05$, Duncan's multiple range test).

CRediT authorship contribution statement

Feifei Li: Conceptualization, Software, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing. **Xing Zhao:** Conceptualization, Formal analysis, Investigation, Validation, Resources. **Shuying Zhu:** Formal analysis, Validation, Resources, Writing - original draft. **Tao Wang:** Conceptualization, Software, Writing - review & editing. **Tianfeng Li:** Data curation, Writing - original draft. **Tracy Woolfley:** Writing - review & editing. **Guanghui Tang:** Conceptualization, Supervision, Project administration, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

The conception and the design of the work was formulated by FFL, XZ, TW and GHT. Bioinformatics analyses were done by FFL, XZ, TFL and TW. The laboratory experiments were performed by FFL, XZ, TFL and SYZ. The statistic analysis was carried out by FFL, XZ, TW and SYZ. The analyses of experimental data and interpretation of data were done by FFL and XZ. The manuscript was written, validated and revised by FFL, TW, Tracy Woolfley, TFL, SYZ and GHT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2020.144605.

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