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Prediction of neuropeptide precursors and differential expression of adipokinetic hormone/corazonin-related peptide, hugin and corazonin in the brain of malaria vector *Nyssorhynchus albimanus* during a *Plasmodium berghei* infection

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ABSTRACT

Insect neuropeptides, play a central role in the control of many physiological processes. Based on an analysis of *Nyssorhynchus albimanus* brain transcriptome a neuropeptide precursor database of the mosquito was described. Also, we observed that adipokinetic hormone/corazonin-related peptide (ACP), hugin and corazonin encoding genes were differentially expressed during *Plasmodium* infection. Transcriptomic data from *Ny. albimanus* brain identified 29 pre-propeptides deduced from the sequences that allowed the prediction of at least 60 neuropeptides. The predicted peptides include isoforms of allatostatin C, orcokinin, corazonin, adipokinetic hormone (AKH), SIFamide, capa, hugin, pigment-dispersing factor, adipokinetic hormone/corazonin-related peptide (ACP), tachykinin-related peptide, trissin, neuropeptide F, diuretic hormone 31, bursicon, crustacean cardioactive peptide (CCAP), allatotropin, allatostatin A, ecdysis triggering hormone (ETH), diuretic hormone 44 (Dh44), insulin-like peptides (ILPs) and eclosion hormone (EH). The analysis of the genome of *An. albimanus* and the generated transcriptome, provided evidence for the identification of myosuppressin neuropeptide precursor. A quantitative analysis documented increased expression of precursors encoding ACP peptide, hugin and corazonin in the mosquito brain after *Plasmodium berghei* infection. This work represents an initial effort to characterize the neuropeptide precursors repertoire of *Ny. albimanus* and provides information for understanding neuroregulation of the mosquito response during *Plasmodium* infection.

1. Introduction

Neuropeptides are part of the chemical communication systems between different cells and are crucial in the regulation of a wide variety of developmental, physiological and behavioral functions throughout the life cycle of animals such as reproduction, feeding growth, development, locomotion, and metabolism (Nässel, 2002; Nässel and Homberg, 2006; Nässel and Zandawala, 2019). Neuropeptides are produced in neurons and neuroendocrine cells of the central nervous system, endocrine cells in the intestine, in sensory cells, in glial cells, muscle cells, embryonic progenitor cells and other cells (Nässel, 2002; Nässel and Homberg, 2006; Nässel and Zandawala, 2019), are synthesized by transcriptional activation of specific genes encoding precursor proteins (called pre propeptides) from which active neuropeptides can be liberated through enzymatic cleavage at specific sites (Eipper *et al.*, 1992; Nässel and Zandawala, 2019; Veenstra, 2000). Pre-propeptides consist of an N-terminal signal peptide, one to several potentially active neuropeptides flanked by cleavage sites in basic residues like lysine-

Abbreviations: NPLP1, neuropeptide-like precursor 1; sNPF, short neuropeptide F; CCAP, crustacean cardioactive peptide; PBURS, partner of bursicon; ETH, ecdysis triggering hormone, Dh44, diuretic hormone 44, ILP, insulin-like peptide; AKH, adipokinetic hormone; EH, eclosion hormone; PDH, pigment dispersing hormone; GPA2, glycoprotein 2; qPCR, quantitative polymerase chain reaction; CNS, central nervous system; K, lysine; R, arginine; GFP, green fluorescent protein; GO, Gene ontology; BLAST, Basic Local Alignment Search Tool; CDS, Coding sequence.

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arginine and many of them have a conserved glycine residue in C-terminus that provides an amide group generating an amidated motif (Veenstra, 2000). C-terminal amidation promotes peptide stability and bioactivity (Cuttitta, 1993; Eipper et al., 1992). However, in many cases the peptides are further processed to obtain N-terminal pyroglutamate cyclization, formation of disulfide bridges, sulfations and glycosylations (Nässel and Zandawala, 2019).

Currently, several putative neuropeptidome precursors of insects of agricultural, medical, and food importance have been described. There have identified and classify neuropeptides evolutionarily conserved but functionally and structurally heterogeneous (Llopis-Giménez et al., 2019; Veenstra, 2019).

In recent years, the study of the neuropeptides has been expanded in several insect orders like Diptera, Lepidoptera, Blattodea, Coleoptera, Hemiptera, and Hymenoptera (Bläser et al., 2020; Chang et al., 2018; Llopis-Giménez et al., 2019; Ragionieri and Predel, 2020; Riehle et al., 2002; Roller et al., 2008; Traverso et al., 2016).

These studies contribute to the discovery of novel neuropeptides with a better understanding of their structure, function (Hauser et al., 2006; Hewes and Taghert, 2001) and of the insect's biological plasticity and its adaptation to different environments and conditions (Hawthorne, 1997).

Mosquitoes are dipterous insects, and many species are known as vectors of pathogens that cause disease in humans and other vertebrates. Anopheline mosquitoes transmit malaria, although the genome sequence of more than 20 anopheline species is available (Neafsey et al., 2015), only the neuropeptidome of *Anopheles gambiae* has been described (Riehle et al., 2002). This information contributes to proposing new studies to understand the functional role of neuropeptides in diverse physiological and developmental processes of this mosquito (Estévez-Lao et al., 2013; Hillyer et al., 2012). Also, it has been documented that the *Plasmodium* infection, modify the expression of some neuropeptides (Marquez et al., 2011).

The subgenus *Anopheles nyssorhynchus* was recently raised to the genus status *Nyssorhynchus* (Foster et al., 2017; Harbach, 2018). Therefore, in this work, we refer to our results as *Nyssorhynchus albimanus* (*Ny. Albimanus*), but the status of *Anopheles albimanus* was maintained, as recorded, in the genome, transcriptome, proteome databases and citations consulted and referenced. *Nyssorhynchus albimanus* is an important malaria vector in México, Central America, and northern South America (Sinka et al., 2010). Some neuropeptides of *Ny. albimanus* have been described in the brain, thoracic, and ventral nerve cord (Hernández-Martínez et al., 2005); however, the information about neuropeptide precursors, neuropeptides and comprehensive description in anophelines remains limited, even less, the effect that could have the presence of parasites on the synthesis, expression, and function of this molecules in this vector. Knowledge of neuropeptides and their role in physiological processes involved in malaria transmission could contribute to the development of novel control strategies (Riehle et al., 2002). Herein we present the description of neuropeptidome precursors of *Ny. albimanus* constructed through a brain transcriptome and available genomic data analysis. Also, we analyzed the expression of several neuropeptide transcripts during *Plasmodium berghei* infection. Our results generated a list of neuropeptide precursors of a native mosquito species to México, Central America and the Caribbean and compared transcript levels of neuropeptide precursors between *Plasmodium* infected and uninfected mosquitoes brain samples and provide insights suggestive into the interactions between neuroregulation and immune response in this mosquito.

2. Experimental procedures

2.1. Insect rearing

White stripe strains *Ny. albimanus* females (Chan et al., 1994) were obtained from the insectary of the National Institute of Public Health (INSP) in Cuernavaca, Mexico. Mosquitoes were bred under a 12:12 photoperiod at 28 °C and 70–80% relative humidity, provided a cotton pad soaked with 8.0% fructose containing 0.05% para-aminobenzoic acid (PABA) and PSN 1× (5000 U of Penicillin, Streptomycin at 5 mg/ml, and Neomycin at 10 mg/ml) and gentamicin (50 μg/ml) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) ad libitum during the 72 h before infection with *P. berghei*. Cotton pads were changed daily. This antibiotic treatment eliminates almost all bacteria in the midgut of mosquitoes (Contreras-Garduño et al., 2015).

2.2. Plasmodium berghei infection

Four days post-emergence mosquitoes were fed with ookinetes of *P. berghei* ANKA strain expressing the Green Fluorescent Protein (GFP) (Franke-Fayard et al., 2004) (kindly donated by Robert E. Sinden, Imperial College, UK). Ookinetes were produced by culturing gametocyte-infected mouse blood, as described previously (Rodríguez et al., 2002). Groups of 300 female mosquitoes were fed for one h using artificial membrane feeders with: (i) mouse blood + approximately 800 per µl GFP *P. berghei* ookinetes (infected group), or (ii) uninfected mouse blood (control group). Unfed mosquitoes were removed, and the engorged ones were incubated at 21 °C to allow for parasite invasion and interaction with the mosquito midgut. At 24 h post-blood feeding, mosquitoes were sacrificed, and dissected midguts were analyzed under a 40 X fluorescence microscope (Leica DM1000) to confirm the presence (*P. berghei*-infected group) or absence (control group) of parasites. We observed ookinetes and retorts. Only midguts containing more than 300 parasites were included in the infected group, brains from these mosquitoes and all brains from the control group were collected.

2.3. Brain dissection

Mosquitoes were anesthetized by incubating them for 10 min at 4 °C. Each mosquito was placed on a sterile slide with the ventral side facing up, and then the head was separated from the body with dissecting forceps by its latero-anterior parts, while the dissecting needle was placed in a part close to the antennas, proboscis, and palps and the head was pressed so that the brain tissue left the side posterior (the part that joined the thorax). Three hundred brains were obtained from each group and collected in 200 µl of Trizol Reagent (Invitrogen Waltham, Massachusetts, USA) and stored at −70 °C until processing.

2.4. Neurotranscriptome preparation and sequencing

Total RNA from *P. berghei*-infected and control mosquito brains was obtained using Trizol Reagent following the manufacturer's instructions (Thermo Scientific). The RNA clean up kit (Zymoclean, Irvine, CA, USA) was used to eliminate the possible contamination with mosquito eye pigment. Total RNA concentration, integrity, and yield were determined using Agilent's 2100 Bioanalyzer with the RNA 6000 Pico kit, according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

Full-length cDNA libraries were synthesized using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia), according to the manufacturer's instructions. Briefly, 1 μg of RNA from each sample group (previously digested with DNase I (Invitrogen) to remove contaminating DNA) was used for first-strand cDNA synthesis with a dT oligo (CDS-*Gsu*I: 5'-AAGCAGTGGTATCAACGCAGAGTACTGGAG(T)20VN-3') and a Plug oligo adapter (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCC GGGGG-3') (Evrogen). The first-strand cDNA was used for second-strand cDNA synthesis by PCR amplification with the M1primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') (Evrogen). Later, 3 µg of each double-strand cDNA was digested with *Gsu*I (15 U) for 6 h at 30 °C. The cDNA libraries were prepared using the GS FLX Titanium Rapid Library Preparation kit according to the manufacturer's instructions (Roche). The cDNA libraries were sequenced in a full Pico titer plate using the Genome Sequencer FLX Titanium platform (454-Roche).

2.5. Data filtering, trimming, and mapping

The output raw sequences were filtered according to length (> 100 bp), sequence complexity, and quality. Primer adaptors were trimmed using the SeqClean software (Chen et al., 2007). The filtered reads were mapped to the *Anopheles albimanus* transcriptome (Martínez-Barnetche et al., 2012) using GS Reference Mapper software v.2.5.3, with default parameters, and genome (Strain: STECLA, version Gene set: AalbS2.7), using Exonerate v.2.2 (Slater and Birney, 2005) with the EST2 genome mode, and a threshold score of 300, and a maximum intron length of 20,000 bp. Output bam files were used to verify mappings to the *An. albimanus* genome to identify putative transcribed genes that were not annotated. The count of reads by gene was done with the HTseq count v0.11.1 (Anders et al., 2015) software. The dataset used in this work is available at *Ny. albimanus* Brain Study Files at http://201.131.57.23:8080/nyssorhynchus_albimanus/ ("201.131.57.23:8080/Ny. albimanus Brain Study," 2018).

2.6. Transcriptome brain analysis

To characterize the *Ny. albimanus* brain transcripts that we obtained, we retrieved gene ontology (GO) annotations and performed comparisons between identified GOs using BLAST2GO v4.0.2. Additionally, we conducted a large-scale data mining with our *Ny. albimanus* brain transcripts as queries to retrieve a set of 1:1 orthologs in the *An. gambiae, Aedes aegypti* and *Drosophila melanogaster* genomes with the web interface BioMart in VectorBase (Giraldo-Calderón et al., 2015) and the *D. melanogaster* genome in Flybase (Thurmond et al., 2019). The *Ny. albimanus* ortholog set was used to identify genes with recognized expression in the brain of the aforementioned insects (Dwivedi et al., 2014; Matthews et al., 2016; Thurmond et al., 2019). A GO type enrichment analysis for biological processes, molecular functions and cellular components was performed using the R TopGO library (Alexa and Rahnenfuhrer, 2016). Transcripts were translated into six frames and analyzed using InterProScan against the InterPro database (Zdobnov and Apweiler, 2001). Transcripts containing neuropeptides and or hormone domains were selected and validated with tBLASTx and BLASTp using a cut-off e-value of 1.0e-5. BLAST outputs were retrieved, listed and compiled by descending sequence identity percentage and score, and ascending e-value. Each transcript was used to verify mappings to our *Ny. albimanus* brain transcriptome.

2.7. Neuropeptide identification

To identify putative neuropeptides in both *Ny. albimanus* groups (*P. berghei*-infected and control), we compiled a dataset (*reference-dataset*) of FASTA sequences from the Database for Insect Neuropeptide Research (DINeR) (Yeoh et al., 2017), as well as previously published neuropeptides and neuropeptide receptors of *An. gambiae* (Dwivedi et al., 2014) and *Ae. aegypti* (Matthews et al., 2016), and neuropeptide and brain expressed genes of *D. melanogaster* (Thurmond et al., 2019). The *reference-dataset* was used to perform multiple BLAST searches (BLASTn, tBLASTx and BLASTp) against the genome of *An. albimanus* using a cut-off e-value of 1.0e-5. BLAST outputs were retrieved, listed and compiled in the order of descending sequence identity percentage and score, and ascending *e*-value. The putative neuropeptides and neuropeptide receptors of *Ny. albimanus* were identified through BLAST (Altschul et al., 1990), pfam (El-Gebali et al., 2019), prosite (Sigrist et al., 2013), superfamily (Gough et al., 2001), smart (Letunic and Bork, 2017), panther, gene3D (Yeats et al., 2006), Conserved Domain Database (CDD)(Lu et al., 2020), prints (Attwood et al., 2012) and ProDom ("ncbi.nlm.nih.gov/ProDom," 2020) (Bru et al., 2005). Recognized domain signatures were visually inspected and compared against the genes of the *reference-dataset* to corroborate their architecture similarities. Also, we retrieved from VectorBase 1:1 ortholog of *An. albimanus, An. gambiae, Ae. aegypti* and *D. melanogaster* to investigate their neuropeptide orthologue relationships. The set of neuropeptides and neuropeptide receptors was annotated with BLAST2GO v4.0.2 (Conesa et al., 2005). The signal peptide prediction of putative neuropeptides was conducted with SignalP v5.0 (Almagro Armenteros et al., 2019). The presence of neuropeptide precursors were detected with the software NeuroPID (Ofer and Linial, 2014); the prediction of cleavage sites and neuropeptides were analyzed using the NeuroPred web application (Southey et al., 2006) and to predict transmembrane helices of neuropeptide receptors we used TMHMM Server v.2.0 (Krogh et al., 2001).

2.8. Expression and differential expression of neuropeptides in Ny. albimanus mosquitoes

Based on the available genomic data, we generated specific oligonucleotides for eighteen *Ny. albimanus* identified neuropeptides (Additional file 1: Table S1) using Oligo Analyzer v3.1 ("PrimerQuest – design qPCR assays | IDT, 2018," n.d.). We conducted real-time PCR assays and amplification of each potential neuropeptide region by triplicate using an Applied Biosystems (ABI) Step One Plus Real-Time PCR System. The qRT-PCR program was 95 °C for 10 min, 95 °C for 15 s and 64 °C for 1 min, repeated for 40 cycles; then 95 °C for 15 sec, 64 °C for 15 s, and 95 °C for 15 s, for one cycle. As an internal control, a fragment of actin was amplified (Herrera-Ortiz et al., 2011) (Additional file 1: Table S1), reactions without template were tested as negative controls. The specificity of the SYBR green PCR signal was confirmed by melting curve analysis and 1.5% agarose gel electrophoresis.

The expression of adipokinetic hormone/corazonin-related peptide, tachykinin related peptide, SIFamide, myosuppressin, hugin, corazonin, ILP-2, ILP-3, and IL-5 were investigated in the brain of *P. berghei*-infected and control mosquitoes through real-time PCR assays. For every neuropeptide gene, three biological replicates with 100 mosquito brains each were dissected as previously described and were conducted for both conditions. The real-time PCR program was conducted as described above. Generated qRT-PCR Ct values were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001) and tested with one-way ANOVA, followed by a Kruskal-Wallis post-test ($\alpha = 0.05$).

3. Results

3.1. Brain transcriptome analysis

Sequencing of *Ny. albimauns* brain yielded 101,520 raw reads from the control group and 109,383 from the *P. berghei*-infected group. The average read lengths in both groups were of 145 bp. Almost half of the total reads of the infected and control group mapped to the *An. albimanus* genome (45.1%, and 45.9%, respectively). The reference mapping identified 3811 transcripts, 31.29% of the total transcripts set currently registered in the transcriptome of *An. albimanus* (12,179 transcripts, AalbS2.7), 947 transcripts were located in control, 1220 transcripts in infected, and 1644 were shared by both groups (Fig. 1). Of these, 2131 have been previously annotated, and 1671 lack of available metadata (Additional file 2: Table S2). Most transcripts identified in *Ny. albimanus* brain were associated with a biological process that may include translation, oxidation-reduction, transmembrane transport, and proteolysis. A molecular function such as protein binding, ATP binding, structural constituent of ribosomes, nucleic acid binding and of cellular components like an integral component of membrane, intracellular, ribosome, nucleus, and cytoplasm. Molecular functions were the most representative sequences identified in the GO analysis. These included sequences associated to ATP synthesis coupled to proton transport (GO:0015986); SRP-dependent co-translational protein targeting to membranes (GO:0006614); retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum biological processes (GO:0006890); cytoplasm (GO:0005737); mediator complex (GO:0016592); integrator complex (GO:0032039), cellular component; NADH dehydrogenase (ubiquinone) activity (GO:0008137); transcription co-regulator activity

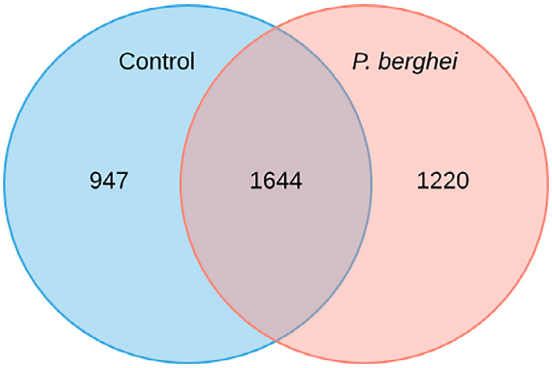


Fig. 1. Venn diagram depicting transcripts detected in uninfected (947) and *Plasmodium berghei*-infected brains (1220) of *Ny. albimanus*, 1644 transcripts were identified in both groups.

(GO:0003712) and ATP binding (GO:0005524). (Additional file 3: Table S3).

The InterPro analysis identified more than 300 domains; among these, Na+ channel auxiliary subunit TipE (IPR031578), choline/carnitine acyltransferase domain (IPR039551), Wnt (IPR005817), mitoguardin (IPR019392), ribosomal RNA small subunit methyltransferase H (IPR002903) and actin-related protein 2/3 complex subunit 4 (IPR008384). (Additional file 4: Table S4). Also, we identified transcripts that codified proteins related to the central nervous system (Additional file 4: Table S4).

3.2. Identification of neuropeptide transcripts

We identified 335 reads in *Ny. albimanus* brain in both conditions that mapped with 16 transcripts associated to neuropeptides in the *An. albimanus* genome (Fig. 2), SIFamide, pigment dispersant hormone, insulin-like peptide 3, diuretic hormone 31, and trissin being the most abundant and hugin, bursicon alpha and tachykinin related peptide with more reads in infected condition. In contrast, adipokinetic hormone and neuropeptide F transcripts were identified only in the infected condition, other neuropeptide transcripts identified were prothoracicotropic hormone, corazonin, allatostatin C, orcokinin, adipokinetic hormone/corazonin-related peptide and capa (Fig. 2).

The comparative *in silico* analysis using neuropeptide transcripts sequences of *An. gambiae, Ae. aegypti* and *D, melanogaster* yielded twelve neuropeptides in addition to the above mentioned: partner of bursicon, insulin-like peptide-2, insulin-like peptide-5, ecdysis triggering hormone, eclosion hormone, crustacean cardioactive peptide, allatotropin, allatostatin A, short neuropeptide F, glycoprotein GPA2, neuropeptide-like precursor 1 and diuretic hormone 44 (Table 1). In addition to these 28 neuropeptide transcripts, we identified a potential open reading frame within the sequence of the AALB009255 gene that codes for myosuppressin (details below). To confirm these identities, eighteen nucleotide sequences corresponding to these pre-pro-peptides were matched against *An. gambiae* genome in Vectorbase. Matching sequences were used to design oligonucleotides for amplification by RT-PCR of *P. berghei*-infected and uninfected mosquito brain samples

The identified transcripts encoded at least 60 putative neuropeptides. Their sequences are shown in Table 2. We also identified six neuropeptide receptor transcripts that belong to CAPA (AALB009517-RA), eclosion hormone (AALB000935-RA), and leucokinin, myosuppressin, short neuropeptide F and tachykinin orthologues receptors (AALB010632-RA, AALB004350-RA, AALB003789-RA, and AALB002458-RA) (Table 1).

For brevity, we described only neuropeptides with increased expression in the brains of the infected group compared to those of the control group. We also described a myosuppressin, identified within the deduced translated sequence of transcript AALB009255-RA. Details of the sequences of the other transcripts are presented in the Supplementary material (Additional file 5 Fig. S1).

3.3. Identification of myosuppressin gene

Gene AALB009255 annotated in *An. albimanus* database predicts a polypeptide of 1166 amino acid residues long containing C2 domains (calcium-dependent membrane-targeting module involved in signal transduction or membrane trafficking) and is coded by fourteen exons and thirteen introns (Fig. 3A and B). When we aligned the reads of *Ny. albimanus* transcriptome brain, many recognized and mapped the intergenic zone of intron 2 of transcript AALB009255-RA (Fig. 3C). Therefore gene region predicted to be an intron is an exon. A close sequence analysis, identified an open reading frame (ORF) between the first two exons and the second intron of AALB009255-RA (Fig. 3D). This ORF of 97 amino acid residues; was identified in the Locus_31294_Length_691 of the annotated *An. albimanus* transcriptome version 2 (Martínez-Barnetche et al., 2012) and the first 75 amino acid residues are identical to those of the peptide precursor AALB009255-PA (Fig. 3E and F, highlighted in red and yellow). This sequence encoded a fragment of a signal peptide of 20 amino acid residues (Fig. 3F, highlighted in red) and a predicted neuropeptide-coding sequence of 11 amino acid residues (TDVDHVFLRF) (Fig. 3F, highlighted in green) with a glycine residue (potential amino acid residue of amidation, Fig. 3F, highlighted in blue) and flanked by lysine-arginine (KR) residues (underlined in red). When we were aligning the ORF of myosuppressin precursor, we detected that neuropeptide sequence was identical to myosuppressins from several insects such as *An. gambiae, D. melanogaster, M. domestica* and *Ae. aegypti* (Fig. 3G). Another data that reinforces the myosuppressin precursor's identification is that in other insects this precursor has not presented C2 domains (Nichols et al., 2002; Predel et al., 2008). Therefore, according to the OFR identified this analysis is the correct characterization of the myosuppressin gene in *Ny. albimanus* as well as other Diptera such as *An. gambiae* or *D. melanogaster* that has myosuppressin. Probably, AALB009255 gene in *An. albimanus* database could be erroneously annotated.

3.4. Adipokinetic hormone/corazonin-related peptide (ACP)

One transcript-encoding a precursor of ACP was identified (AALB008450-RA). This transcript encodes for a 106-residue product with a predicted signal peptide of 31 amino acid residues in the N-terminus, followed by a predicted neuropeptide-coding sequence for 11 amino acid residues (QVTFSRDWNAG). It has a G residue and KR cleavage sites in the C-terminus (Fig. 4A). ACP is identical to the ACP of *An. darlingi* (ADAC008744-PA), *An. gambiae* (AGAP002430-PA) *An.stephensi* (ASTE011407-PA), *An. funestus* (AFUN002979-PA), *Ae. albopictus* (AALF004964-PA), *P. papatasi* (PPAI001823-PA), *C. quinquefasciatus* (CPIJ001379-PA), and *Ae. aegypti* (AAEL010950-PC) (Giraldo-Calderón et al., 2015; Hansen et al., 2010; Kaufmann et al., 2009; Kaufmann and Brown, 2006; Wahedi and Paluzzi, 2018) (Fig. 4B).

3.5. Hugin

One transcript encoding a precursor of hugin was identified (AALB008609-RA) with 199 amino acid residues. It has a predicted signal peptide of 22 amino acid residues in the N-terminus, and five predicted neuropeptide-coding sequences (Fig. 5A) (Giraldo-Calderón et al., 2015). One such sequence codes for a peptide of 11 amino acid residues (AAAMWFGPRLG) which corresponds to the neuropeptide PK-1 and is identical to the *An. darlingi* (ADAC000637-PA), *An. gambiae* (AGAP002292-PA) and *Ae. aegypti* (AAEL012060-PA) peptides (Choi et al., 2013; Giraldo-Calderón et al., 2015; Olsen et al., 2007). The second one codes for a peptide with 14 amino acid residues (PQPLFYHTAAPRLG), identical to that of *An. darlingi*; The third one codes for a peptide with 16 amino acid residues

Table 1
Neuropeptide and receptor transcripts identified in *An. albimanus* brain and annoted transcriptome.

Name	VectorBase ID (*An. gambiae, Ae. Aegypti*)[1]	SignalP[2]	TMH[3]	NPPs	VB AALB ID[4]	NPPs	InterPro shared domains	Reference *An.gambiae/Ae. Aegypti*
Adipokinetic Hormone	AGAP008834	Yes	–	1	AALB015568	1	Adipokinetic hormone/red pigment-concentrating hormone (IPR010475)	Neurostresspep database
Adipokinetic hormone/ corazonin-related peptide	AGAP002430	Yes	–	1	AALB008450	1	Adipokinetic hormone, conserved site (IPR002047)	Neurostresspep database
Allatostatin A**	AGAP003712	Yes	–	4	AALB015793	7	Allatostatin (IPR010276)	
Allatostatin C	AGAP010157	No	–	3	AALB007903	1	Allatostatin, insect (IPR020161)	Neurostresspep database
Allatotropin**	AGAP012130	Yes	–	1	AALB004051	1	EF-Hand 1, calcium-binding site (IPR018247)	
Bursicon alpha*	AGAP002537	No	–	1	AALB009577	1	Cystine-knot cytokine (IPR029034)	
Capa	AGAP000347	Yes	–	4	AALB006405	2	None predicted IPR	Neurostresspep database
Corazonin	AGAP003675	Yes	–	3	AALB010867	1	Procorazonin (IPR020190)	Neurostresspep database
Crustacean cardioactive peptide (CCAP)**	AGAP009729	Yes	–	1	AALB000113	1	Crustacean cardioactive peptide (IPR024276)	
Diuretic hormone 31	AAEL008070	No	–	2	AALB009260	1	Peptide hormone DH31-like (IPR034439)	
Diuretic hormone 44 (Dh44)**	AGAP003269	Yes	–	1	AALB009504	1	Corticotropin releasing factor (IPR000187)	
Ecdysis triggering hormone (ETH)**	AGAP007062	Yes	–	1	AALB004998	2	None predicted IPR	
Eclosion hormone (EH)*, **, "	AGAP010437	Yes	–	1	AALB007602	1	Eclosion hormone (IPR006825)	
Glycoprotein GPA2*	AGAP008301	Yes	–	2	AALB001547	1	DAN (IPR004133)	Neurostresspep database
Hugin	AGAP002292	Yes	–	5	AALB008609	5	Pyrokinin, conserved site (IPR001484)	Neurostresspep database
Insulin Like Peptide 3 (ILP- 3)*	AGAP010604, AGAP010603	No	–	2	AALB010411	7	Insulin like superfamily (IPR036438)	
Insulin Like Peptide 2 (ILP-2)**	AGAP010600	Yes	–	1	AALB010410	2	Insulin like superfamily (IPR036438)	Matthews et al. 2015)
Insulin Like Peptide 5 (ILP-5)**	AGAP003927	Yes	–	2	AALB008758	3	Insulin like superfamily (IPR036438)	
Myosuppressin*, %	AGAP001474	Yes	–	1	AALB009255	1	C2 domain superfamily (IPR035892)	
Neuropeptide F	AGAP004642	No	–	1	AALB002883	1	None predicted IPR	Neurostresspep database
Neuropeptide-like precursor 1 (NPLP1)*	AGAP010366	Yes	–	0	AALB001239	1	None predicted IPR	Neurostresspep database
Orcokinin	AGAP012220	No	–	6	AALB009966	5	None predicted IPR	Neurostresspep database
Partner of Bursicon (PBURS)**	AGAP004506	Yes	–	1	AALB003562	1	Bursicon subunit beta (IPR034441)	
Pigment-dispersing hormone	AGAP005776	Yes	–	1	AALB006094	1	Pigment-dispersing hormone (IPR009396)	Neurostresspep database
Prothoracicotropic hormone*	AGAP000859	Yes	–	0	AALB007627	1	Cystine-knot cytokine (IPR029034)	Neurostresspep database
short neuropeptide F (sNPF)*	AAEL012542	No	–	3	AALB007863	3	None predicted IPR	
SIFamide	AGAP007056	Yes	–	2	AALB005004	1	None predicted IPR	Neurostresspep database
Tachykinin-related peptide*	AGAP010014	No	–	2	AALB000888	4	None predicted IPR	Neurostresspep database
Trissin*	AGAP012496	Yes	–	2	AALB015467	1	None predicted IPR	Neurostresspep database
Capa receptor*	AAEL017335	No	Yes	–	AALB009517	–	Neuromedin U receptor (IPR005390)	Matthews et al. 2015)
Eclosion hormone receptor*	AAEL008387	Yes	Yes	–	AALB000935	–	Receptor, ligand binding region (IPR001828)	
Leucokinin receptor*	AAEL006636	No	Yes	–	AALB010632	–	G protein-coupled receptor, rhodopsin-like (IPR000276)	
Myosuppressin receptor*	AAEL006283	No	Yes	–	AALB004350	–	G protein-coupled receptor, rhodopsin-like (IPR000276)	
short neuropeptide F (sNPF) receptor*	AAEL007924	No	Yes	–	AALB003789	–	G protein-coupled receptor, rhodopsin-like (IPR000276)	
Tachykinin-like receptor (TkR99D)*	AAEL006947	No	Yes	–	AALB002458	–	G protein-coupled receptor, rhodopsin-like (IPR000276)	

* No metadata available for this ID transcript in AalbS2.7.

** No found in putative *An. albimanus* brain transcriptome but identified in AalbS2.7 data base."With Eclosion hormone domain (Pfam).All transcripts have start and stop codon, except allatostatin A and diuretic hormone 31 transcripts without start codon.% Identified at intronic sequense of AALB009255.

[1,4] https://www.vectorbase.org/.

[2] http://www.cbs.dtu.dk/services/SignalP/.

[3] http://www.cbs.dtu.dk/services/TMHMM/.

Table 2
Alphabetical list of peptides and their best BLAST hit on NCBI.

Neuropeptide	Transcript ID	Best BLAST hit (NCBI)	Identity	*E*-value	Accession number
ADIPOKINETIC HORMONE/CORAZONIN-RELATED PEPTIDE (ACP)	AALB008450-RA				
QVTFSRDWNAa KR		adipokinetic hormone/corazonin-related peptide [*Anopheles gambiae*]	100%	0.010	ABD60145.1
ALLATOSTATIN A	AALB015793-RA				
R SPKYNFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.007	ETN63487.1
KR RTYDFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.005	ETN63487.1
KR LPHYNFGLa KR		preproallatostatin [*Aedes aegypti*]	100%	3e-04	AAB08870.1
KR LPNRYNFGL		preproallatostatin [*Aedes aegypti*]	100%	0.020	AAB08870.1
KR ATSGNGAGGAYRYHFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	4e-12	ETN63487.1
KR YFEAEEFN KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.001	ETN63487.1
KR RYIIEDVPa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	93%	0.005	ETN63487.1
ALLATOSTATIN C	AALB007903-RA				
KR QIRYRQCYFNPISCF KK		allatostatin 2 [*Anopheles darlingi*]	100%	2e-12	ETN 61475.1
ALLATOTROPIN	AALB004051-RA				
R SIRAPFRNSEMMTARGFa KR		Allatotropin-like [*Anopheles albimanusi*]	100%	6e-13	XP_035793846.1
ADIPOKINETIC HORMONE (AKH)	AALB015568-RA				
QLTFTPAWa KR		AGAP008834-PA [*Anopheles gambiae* str. PEST]	100%	2.20e-02	XP_001238167.1
BURSICON	AALB003562-RA				
***Sequence too long to be given here.**		partner of bursicon [*Anopheles darlingi*]	100%	9e-96	ETN65201.1
CORAZONIN	AALB010867-RA				
QTFQYSRGWTNa KR		Pro-corazonin [*Eumeta japonica*]	100%	7e-06	GBP37784.1
CRUSTACEAN CARDIOACTIVE PEPTIDE (CCAP)	AALB000113-RA				
KR PFCNAFTGCa KK		cardioactive peptide-like isoform X1 [*Anopheles albimanus*]	100%	2.00e-5	XP_035783869.1
DIURETIC HORMONE 31 (Dh31)	AALB009260-RA				
KR TVDFGLSRGYSGAQEAKHRMAMAVANFAGGPa RK		diuretic hormone class 2 [*Anopheles stephensi*]	100%	5e-17	XP_035899591.1
DIURETIC HORMONE 44 (Dh44)	AALB009504-RA				
RR TKPSLSIVNPLDVLRQRIILEMARRQMREN-TRQVELNKALLREIa KR		AGAP003269-PA [*Anopheles gambiae* str. PEST]	98%	1.00e-22	XP_001230569.1
ECLOSION HORMONE	AALB007602-RA				
***Sequence too long to be given here.**		eclosion hormone [*Anopheles darlingi*]	94%	3e-23	ETN65246.1
ECDYSIS TRIGGERING HORMONE	AALB004998-RA				
TESPGFFIKLSKSVPRIa RR		ecdysis-triggering hormone [*Anopheles darlingi*]	100%	8.00e-11	ETN66573.1
RR GDLENFFLKQSKSVPRIa RR		ecdysis-triggering hormone [*Anopheles darlingi*]	100%	2.00e-13	ETN66573.1
INSULIN LIKE PEPTIDE (ILP- 3)	AALB010411-RA				
RR GHYCGRILSETLAKVCNSYNGMR KK		bombyxin-related peptide B-like isoform X2 [*Anopheles albimanus*]	100%	2.00e-19	XP_035793540.1
***Sequence too long to be given here.**		insulin-like peptide 7 precursor [*Anopheles gambiae*]	42%	1.9	AAQ89699.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles stephensi*]	53%	0.061	ETN61522.1
KR YCGAELVKVLSFLCDEFPDLHSTN KK		insulin-like peptide 3 precursor [*Anopheles darlingi*]	100%	3.00e-20	ETN61522.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles darlingi*]	100%	2e-43	ETN61522.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles darlingi*]	96%	1e-10	ETN61522.1
KK AGSFFGDAFDPTETGLSFPIPARF RR		uncharacterized protein LOC118466995 [*Anopheles albimanus*]	96%	2.00e-17	ETN61522.1
INSULIN LIKE PEPTIDE 2 (ILP-2)	AALB010410-RA				
TSTPKGDALISTLTRSRYCa RR		LIRP-like [*Anopheles albimanus*]	100%	9.00e-13	XP_035792810.1
***Sequence too long to be given here.**		insulin-like peptide 2 precursor [*Anopheles sinensis*]	97%	3e-31	KFB49854.1
INSULIN LIKE PEPTIDE 5 (ILP-5)	AALB008758-RA				
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles gambiae*]	90%	4e-29	AAQ89696.1
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles gambiae*]	70%	2e-04	AAQ89696.1
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles sinensis*]	97%	2e-17	KFB46716.1

(*continued on next page*)

Table 2 (*continued*)

Neuropeptide	Transcript ID	Best BLAST hit (NCBI)	Identity	*E*-value	Accession number
MYOSUPPRESSIN	AALB009255-RA				
KR TDVDHVFLRFa KR		Dms [*Drosophila busckii*]	100%	2.00e-06	ALC47282.1
NEUROPEPTIDE F	AALB002883-RA				
LTAARPQDGDAASVAAAIRYLQELETKHAQHARPRLa		neuropeptide F isoform X4 [*Anopheles albimanus*]	97%	2.00e-16	XP_035774874.1
ORCOKININ	AALB009966-RA				
KR NFDEIDRFARFNa KR		uncharacterized protein LOC118466927 [*Anopheles albimanus*]	100%	6.00e-09	XP_035792706.1
KR NFDEIDRFNAGFNYRLNGDEVAAIa KR		uncharacterized protein LOC118466927 [*Anopheles albimanus*]	100%	4.00e-21	XP_035792706.1
KR NFDEIDRFGRFSNFa KR		uncharacterized protein LOC118466927 [*Anopheles albimanus*]	100%	5.00e-07	XP_035792706.1
KR SLNNSDRRTLLYNYYSRGLAPMYE KR		uncharacterized protein LOC118466927 [*Anopheles albimanus*]	100%	9.00e-21	XP_035792706.1
KR NLDYEPSYVGTMDHGYSGRMS KR		uncharacterized protein LOC118466927 [*Anopheles albimanus*]	100%	1.00e-17	XP_035792706.1
PARTNER OF BURSICON (PBURS)	AALB003562-RA				
*__Sequence too long to be given here.__		partner of bur [*Anopheles darlingi*]	100%	3e-81	ETN65201.1
PIGMENT DISPERSING HORMONE (PDH)	AALB006094-RA				
KR NSELINSLLSLPKSMNDAa K		PREDICTED: pigment-dispersing hormone type 1 [*Musca domestica*]	100%	1.00e-12	XP_005181776.2
capavility/CAPA-PVK/PK	AALB006405_RA				
KR GPTVGLFAFPRVa R (PVK-1)		hypothetical protein RP20_CCG009807 [*Aedes albopictus*]	100%	1.00e-06	KXJ76345.1
KR QGLVPFPRVa R (PVK-2)		hypothetical protein RP20_CCG009807 [*Aedes albopictus*]	100%	7.00e-04	KXJ76345.1
KR ASGSGANGGMWFGPRLa KR (PK-1)		cardio acceleratory peptide 2b-like isoform X1 [*Anopheles albimanus*]	100%	9.00e-12	XP_035790045.1
HUGIN	AALB008609-RA				
KR AAAMWFGPRLa KR (PK-1)		PBAN-type neuropeptides-like [*Anopheles albimanus*]	100%	1.00e-06	XP_035796452.1
RK PQPLFYHTAAPRLa RR		PBAN-type neuropeptides-like [*Anopheles albimanus*]	100%	1.00e-09	XP_035796452.1
R NLPFSPRLa R (PK-2)		PBAN-type neuropeptides-like [*Anopheles albimanus*]	100%	5.90e-02	XP_035796452.1
RR DSVGENHQRPPFAPRLa R (HUGIN)		PBAN-type neuropeptides-like [*Anopheles albimanus*]	100%	1.00e-11	XP_035796452.1
RR EDDSGLEGNGVS KR		PBAN-type neuropeptides-like [*Anopheles albimanus*]	100%	2.00e-06	XP_035796452.1
SIFamide	AALB005004-RA				
RK PPFNGSIFa KR		IFa [*Drosophila busckii*]	100%	5.00e-04	ALC40751.1
TACHYKININ	AALB000888-RA				
RR VPSGFNGVRa KK		uncharacterized protein LOC118458604 [*Anopheles albimanus*]	100%	2.00e-04	XP_035777173.1
KR APSGFLGMRa KK		tachykinins isoform X1 [*Aedes aegypti*]	100%	1.00e-04	XP_001652135.2
KR APTGFTGMRa RR		tachykinins-like isoform X1 [*Anopheles stephensi*]	100%	5.00e-05	XP_035917287.1
KR VPNGFMGLRa KK		tachykinins-like isoform X1 [*Anopheles stephensi*]	100%	6.00e-04	XP_035917287.1
GLYCOPROTEIN GPA2	AALB001547-RA				
*Sequence too long to be given here.		glycoprotein hormone alpha 2 [*Anopheles darlingi*]	100%	2.00e-68	ETN58907.1
PROTHORACICOTROPIC HORMONE (PTH)	AALB007627-RA				
*Sequence too long to be given here.		prothoracicotropic hormone preproprotein [*Culex pipiens*]	79%	2.00e-59	ADO51754.1
NEUROPEPTIDE LIKE PRECURSOR 1 (NPLP1)	AALB001239-RA				
*Sequence too long to be given here.		PREDICTED: neuropeptide-like 1 [*Aedes albopictus*]	74%	1.00e-38	XP_019547010.1
TRISSIN	AALB015467-RA				
ALSCDSCGRECASACGTRHFRTCCFNYLR KR		AGAP012496-PA [*Anopheles gambiae* str. PEST]	100%	9.00e-14	XP_001230636.2
SHORT NEUROPEPTIDE F (sNPF)	AALB007863-RA				
RK AVRSPSLRLRFa RR		short neuropeptide F [*Culex quinquefasciatus*]	100%	1.00e-06	XP_038111065.1
R APQLRLRFa R		short neuropeptide F [*Culex quinquefasciatus*]	100%	0.058	XP_038111065.1
R APQLRLRFa R		short neuropeptide F [*Culex quinquefasciatus*]	100%	0.058	XP_038111065.1

*__Sequence too long to be given here. Supplemetary figures show each neuropeptide sequence__

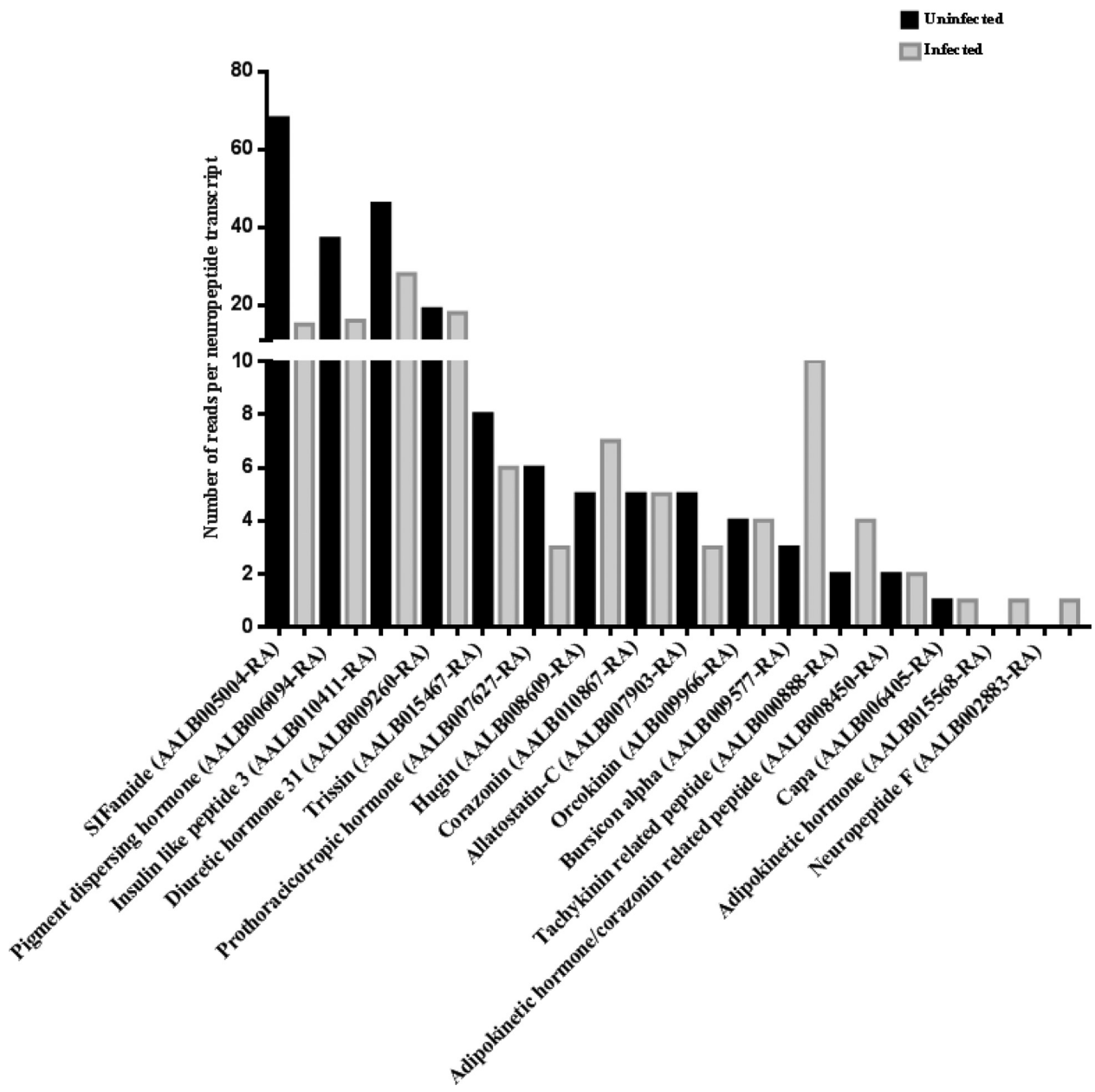


Fig. 2. Neuropeptides transcripts identified in *Ny. albimanus* brain transcriptome. SIFamide (AALB005004-RA), pigment dispersing hormone (AALB006094-RA) and insulin like peptide 3 (AALB010411-RA) transcripts with more represented.

(DSVGENHQRPPFAPRLG, neuropeptide PK-2), identical to peptides of *An. darlingi* and *An. gambiae*; and the fourth codes for a peptide with nine amino acid residues (NLPFSPRLG, neuropeptide PK-2-2), identical to peptides of *An. darlingi, An. gambiae* and *Ae. aegypti*. All these have a G residue and PRL-conserved motif in their C-terminus, and are flanked by KR, RK, RR and R cleavage sites (Fig. 5A and B), PRLamide conserved motif it's a feature of PK-2 related peptides. Even more, the first peptide also has the extended conserved motif of WFGPRLamide which is a feature of the PK-1 related peptides (Choi et al., 2013; Olsen et al., 2007). Also, we identified a fifth sequence codes for a peptide with 12 amino acid residues (EDDSGLEGNGVS), which is flanked by RR and KR cleavage sites, indicating that this peptide may be processed, but does not have the conserved motif FXPRL and G residue in its C-terminus (Fig. 5A, highlighted in gray).

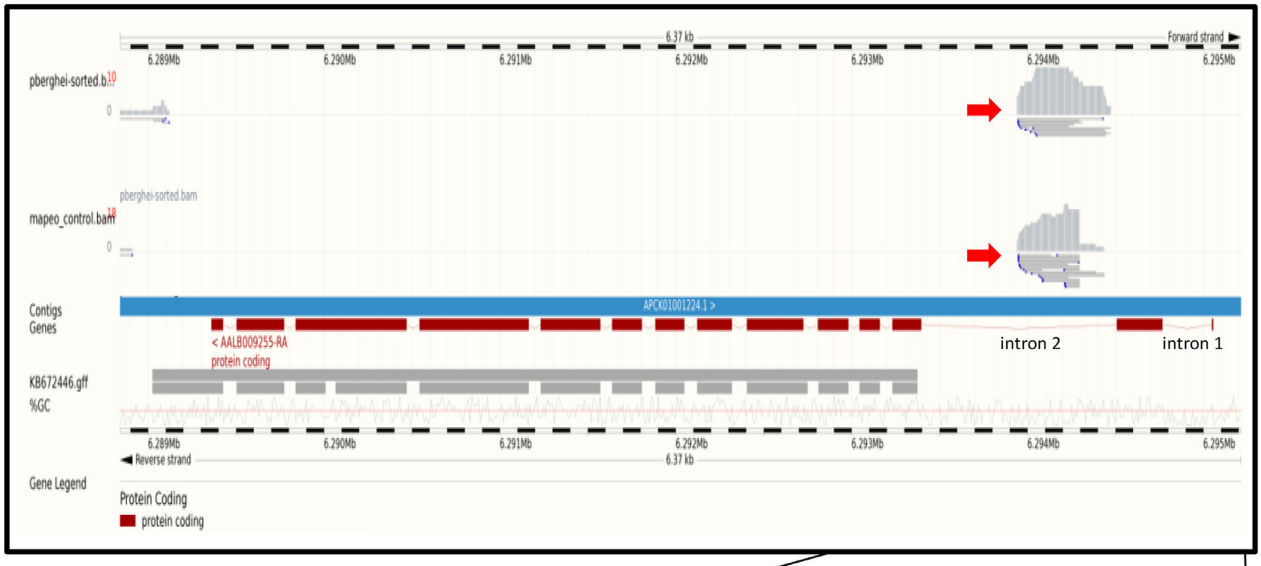
3.6. Corazonin

One transcript encoding a precursor of corazonin was identified (AALB010867-RA). It has 156 amino acid residues, with a predicted signal peptide of 20 residues in the N-terminus, followed by one predicted neuropeptide-coding sequence with 12 amino acid residues (QTFQYSRGWTNG). It has a G residue and KR cleavage sites in the C-terminus (Fig. 6A). This predicted corazonin peptide is identical to the corazonin of, *An. darlingi* (ADAC011092-PA), *An. dirus* (ADIR005352-PA), *Ae. aegypti* (AAEL005252-PA), *Ae. albopictus* (AALF024162-PA), *M. domestica* (MDOA008580-PA), *L. scapularis* (ISCI014429-PA), *O. furnacalis* (XP_028169596.1), *G. morsitans* (GMOY012060-PA) and *G. pallidipes* (GPAI024124-PA)(Fig. 6B) and others corazonin of several insect families (Giraldo-Calderón et al., 2015; Predel et al., 2007).

A)

```
-M--G--S--Q--H--Q--P--A--L--R--A--V--T--C--I--V--L--L--L--S-
-I--V--C--T--L--V--A--G--S--A--M--P--P--L--C--E--N--R--L--I-
-E--E--L--P--P--K--F--R--K--V--C--A--A--L--E--N--S--N--Q--F-
-A--E--A--L--N--A--Y--I--R--K--E--A--A--G--N--G--R--D--N--S-
-I--G--S--G--L--P--A--D--R--M--K--V--K--V--L--A--G--R--N--L-
```

B)



C)

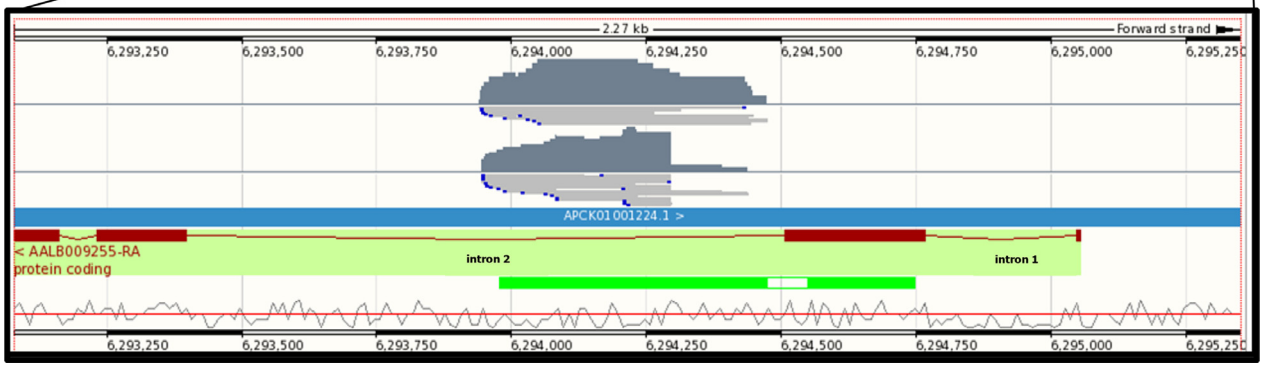


Fig. 3. Myosuppressin identification. **A)** Partial deduced amino acid sequence of AALB009255-PA (red, signal peptide and yellow, polypeptide). **B)** Structure of AALB009255 gene, exons (14 red boxes) and introns (13 red lines) are shown, besides reads from the transcriptome brain of *Ny albimanus* that aligned with the second intron of AALB009255 gene are indicate by red arrows. **C)** Magnification from the zone of introns 1 and 2. **D)** Partial genomic sequence of AALB009255, the first two exons (purple italic letters) and introns (black letters) and a sequence that should correspond to intron 2 (black boxes) are shown, in the second intron we identified the sequence for the neuropeptide myossuppresin (green), KR cleavage sites (red), and a stop codon (gray). **E)** Deduced nucleotide sequence of Locus_31294_Length_691 of the transcriptome of *An. albimanus* in red and yellow shows the sequence of the ORF identical to AALB009255-PA and the sequence encoding myossupressin neuropeptide (green), amidation site (blue), KR cleavage sites (red) and a stop codon (gray). **F)** Deduced amino acid sequence of myosuppressin of *An. albimanus*. **G)** Multiple sequence alignment of the *An. albimanus* myosuppressin prepropeptide with homologs from other insects. Note the conserved peptide **DVDHVFLRF**amide. *D. melanogaster, C. capitata, M. domestica, Ae. aegypti, An. gambiae, A. mellifera, P. clarkii* and *R. prolixus*.

D)

AALB009255

exon 1
ATGGGCAGGTACAGTGAACACTGCACAGCACGTTTATAGAAATTATGCTTGGAAAATCTC
TCAGATTAATGTTATATCGAAATAGTTGTACAACACTTAAAGTGTAATGCAATCGCTTCA
TTTTCTTTCAAACAAACAAATTCAAAATTGAATGTTGGATAACGCTTCCTTATTTTAGAA
intron 1
ACCTTAACACTTGAAATAAAAAACTAAAGCTAGCATATTACACCTACCTTTCCGTTACTT
CATCATGTGATGGCTAAAAGTCGATTGAAAATCTTATTGTGTCCCCCAGCCAACATCAGC
exon 2
CAGCATTGCGAGCAGTAACGTGTATCGTTCTGCTTCTCTCGATAGTCTGCACTTTGGTGG
CCGGCTCCGCTATGCCACCTCTTTGTGAGAACAGACTGATTGAGGAGCTCCCGCCAAAGT
TTCGAAAAGTGTGTGCAGCATTAGAAAACTCAAATCAGTTTGCCGAAGCCCTGAACGCGT
ACATACGAAAGGAGGCGGCTGGTAATGCGTAGAGATAACTCTATTGGTTCCGGGCTACCCG
CAGATCGAAGTAACTAGTGACACATTTCCGCGGCTTCTTTTCAGCGTTTCTCTATTTAGA
CGACGACATGTTGGTGCCGGGACAGAATGGCAAACGGACAGATGTAGACCACGTTTTCTT
intron 2
GCGATTCGGCAAACGACGTTAAGCTTACCGTCGCTGGTGGTTCACAGAGATGGACCATAA
TGCCATGGTCCAAGTCATGGACCACCACGGCGAGCAGCGGGAGGAATGGAATACACGTAT
GCAGACACGTTTCAAACGAACGAGCTAAATCTATTGAGAATTCATGGAAAACGTATAAGT
AGCTTTTTAAGCAGAACAGCATGGCGTCCCTTCCTTTAGCTTATAATTTTACATAATATG

E)

>Locus_31294_Length_691
GACGCAGTAACGTGTATCGTTCTGCTTCTCTCGATAGTCTGCACTTTGGTGGCCGG
CTCCGCTATGCCACCTCTTTGTGAGAACAGACTGATTGAGGAGCTCCCGCCAAAG
TTTCGAAAAGTGTGTGCAGCATTAGAAAACTCAAATCAGTTTGCCGAAGCCCTGA
ACGCGTACATACGAAAGGAGGCGGCTGGTAATGCGTTTCTCTATTTAGACGACGA
CATGTTGGTGCCGGGACAGAATGGCAAACGGACAGATGTAGACCACGTTTTCTTG
CGATTCGGCAAACGACGTTAAGCTTACCGTCGCTGGTGGTTCACAGAGATGGACC
ATAATGCCATGGTCCAAGTCATGGACCACCACGGCGAGCAGCGGGAGGAATGGAA
TACACGTATGCGGACACGTTTCAAACGAACGAGCTAAATCTATTGAGAATTCATG
GAAAACGTATAAGTAGCTTTTTAAGCAGAACAGCATGGCGTCCCTTCCTTTAGCT
TATAATTTTACATAATATGAACTGAACTCTCCATTCCCAGACAGCGGTGTGATTA
TAATGTAGCCACAGAATATATTGCAAAGCCAGCCAGTGAAGCATAAGACTGTGAG
ATGCAGATTCAGATATATCAAACATATGGCACATTTCATCGAACAATTTTTATTC
AGGGTGGTCAAAACAATGCGTTCGATGGGAG

F)

>Locus_31294_Length_691 5-3' Frame 3
-A--V--T--C--I--V--L--L--L--S--I--V--C--T--L--V--A--G--S--A
-M--P--P--L--C--E--N--R--L--I--E--E--L--P--P--K--F--R--K--V
-C--A--A--L--E--N--S--N--Q--F--A--E--A--L--N--A--Y--I--R--K
-E--A--A--G--N--A--F--L--Y--L--D--D--D--M--L--V--P--G--Q--N
-G--K--R--T--D--V--D--H--V--F--L--R--F--G--K--R--R--*

G)

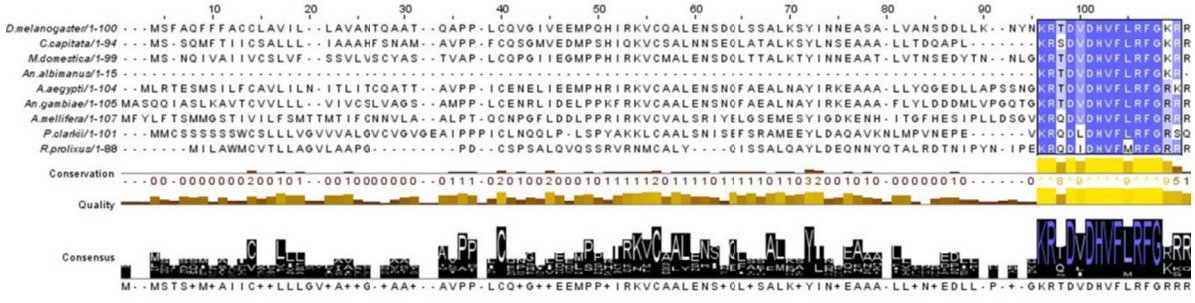


Fig. 3. Continued

3.7. *Neuropeptide brain expression in* P. berghei *experimental infection*

When we compared the expression of various precursors, we observed a significantly increased expression of ACP (4.94-fold), hugin (4.40-fold), and corazonin (1.75-fold) ($p < 0.001$) in the *P. berghei* infected group. By contrast, transcripts encoding tachykinin-related peptide, SIFamide, myosuppressin, ILP-5, ILP-2 and ILP-3, showed no differential expression following *P. berghei* infection (Fig. 7).

4. Discussion

In this work, we reported on a transcriptome in *Ny. albimanus* brain and deduced polypeptide precursors with a potential for coding neuropeptides. Besides, we analyzed the expression of several of them during infection with *P. berghei* parasite.

All neuropeptides transcripts precursors reported herein have orthologues in other insect transmitters of disease vectors

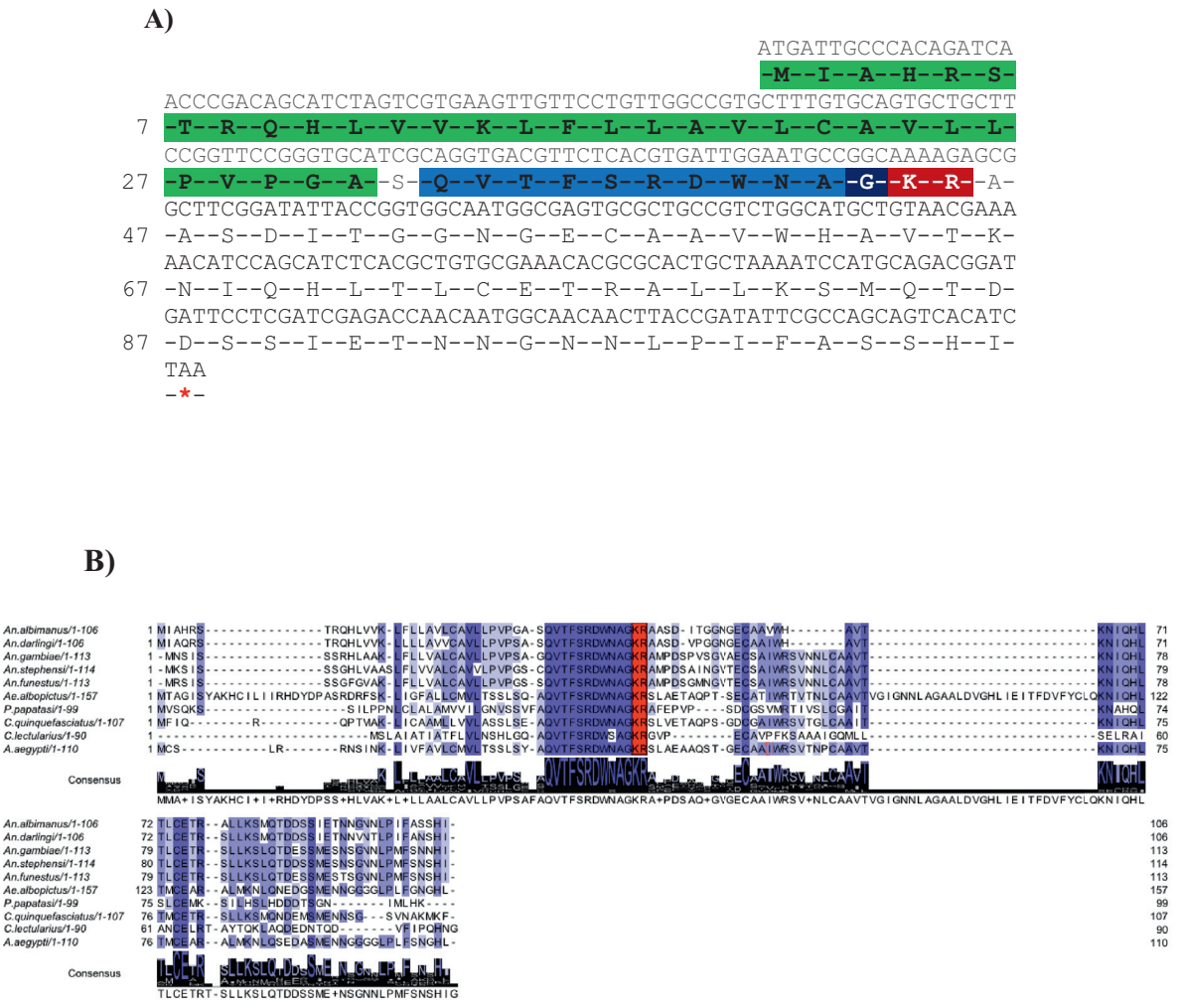


Fig. 4. Adipokinetic hormone/corazonin-related peptide (ACP). **(A)** Nucleotide sequence and deduced amino acid sequence. Signal peptide (green), ACP neuropeptide (blue), glycine residue for amidation (G), K-R cleavage site (red) and stop codon (*) are shown. **(B)** Multiple alignment of the *An. albimanus* ACP prepropeptide with homologs from other insects. Note the conserved peptide motif **QVTFSRDWNA** amide *An.darlingi, An. gambiae, An. stephensi, An. funestus, Ae. albopictus P. papatasi, C. quinquefasciatus C. lectularius and Ae. aegypti.*

(Ons et al., 2011; Predel et al., 2010) and agricultural insect pest (Binzer et al., 2014; Tanaka et al., 2014). Although most of the encoded peptides are identical or similar to orthologs, some of them exhibit amino acid sequence variations (Olsen et al., 2007; Predel et al., 2007; Wahedi and Paluzzi, 2018). Our results in *Ny. albimanus* indicate that the prepropeptide encoded by the allatostatin A transcript has the potential to yield seven neuropeptides; while allatostatin A precursor of *D. melanogaster* (Nässel and Zandawala, 2019) and *M. domestica* that can generate four and five neuropeptides (Liu et al., 2013), respectively. Whereas the tachykinin-related peptide transcript precursor of *Ny. albimanus* is identical to that of *An. darlingi* and has the potential for generating four neuropeptides, like *Ae. aegypti* (Siju et al., 2014), in *D. melanogaster* six neuropeptides are generated (Nässel and Zandawala, 2019). Other organisms, such as *Procambarus clarkii* (Decapoda: Cambaridae) generate seven tachykinin neuropeptides (Veenstra, 2015).

Although insulin-like peptide precursors ILP2 (AALB010410-RA) and ILP5 (AALB008758-RA) presented the most remarkable amino acid sequence variation (Mizoguchi and Okamoto, 2013; Wu and Brown, 2006) also presented the key characteristics to produce ILPs neuropeptides as a signal peptide, six cysteine residues and cleavage sites (RR, KR and RK) necessary to produce A, B and C peptides identified in other insects (Riehle et al., 2006; Sharma et al., 2019). Furthermore, the precursor of ILP3 (AALB010411-RA) has 495 amino acid residues and contains 14 cysteine residues, while ILP3 precursors of *An. gambiae, Ae. aegypti and D. melanogaster* have 160, 120 and 167 amino acid residues respectively with 6 cysteine residues (Giraldo-Calderón et al., 2015; Riehle et al., 2006) (Grönke et al., 2010). Interestingly, the analysis of AALB010411-RA proposes that probably this precursor forms a eukaryotic operon controlled by a single promoter as in *Ae. aegypti* (Riehle et al., 2006), the search for signal peptide identified 3 potential sequences, by separating each potential ORF and align against ILPs precursors of *Ae. aegypti* and *An. gambiae* resulted that ILP3 (AALB010411-RA) could contain also transcripts of ILP7 and ILP4 (Additional file 6 Fig. S2). However, this *in silico* result needs to be validated experimentally in order to elucidate this hypothesis.

Full reconstruction of the AALB009255-RA transcript identified a myosupressin precursor, not yet characterized in *Ny. albimanus* which is identical to that of other insects, including several anophelines orthologs (Giraldo-Calderón et al., 2015; Vilaplana et al., 2004).

Despite the importance of neuropeptides in adult insects, information about their function is limited for anophelines. Neuropeptides are associated with most of the physiological processes in insects, including the immune response activation (Eleftherianos et al., 2009; Ishii et al., 2013, 2010) and suppression (Pietri et al., 2015) after infection by different microorganisms. Interestingly, we detected an increase in the tran-

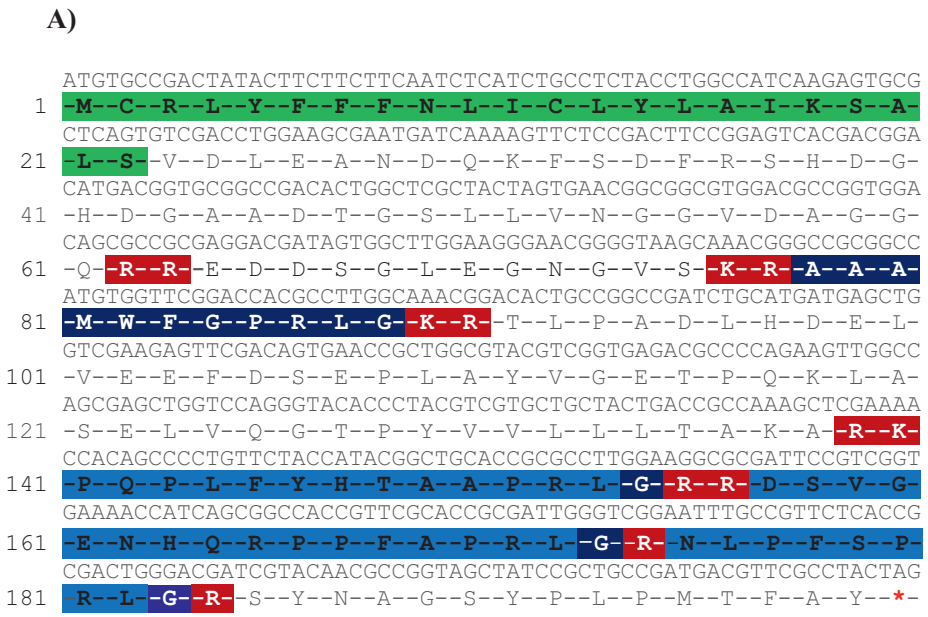


Fig. 5. Hugin. **(A)** Nucleotide sequence and deduced amino acid sequence. Signal peptide (green), neuropeptides **XXXXXPRLG** (blue), glycine residue for amidation (G), K-R, R-K, R-R and R cleavage site (red) and stop codon (*) are shown. **(B)** Multiple alignment of the *An. albimanus* Pk/PBAN prepropeptide with homologs from other insects. Note the conserved peptide motifs **XXXXXPRL**amide and **XXXWFGPRL**amide of *An. darlingi, An. gambiae, Ae. aegypti, T. castaneum, N.lugens* and *B. mori*.

scription of ACP, hugin, and corazonin precursors in *Ny. albimanus* infected with *P. berghei*.

The study of adipokinetic hormone/corazonin-related peptide (ACP) (Hansen et al., 2010), initially characterized as adipokinetic hormone II (AKH II) (Kaufmann et al., 2009; Kaufmann and Brown, 2006; Siegert, 1999), structurally intermediate between corazonin and adipokinetic hormone, has provided new evidence and questions on the role of neuropeptides in insects (Hansen et al., 2010), including anophelines (Kaufmann and Brown, 2006). ACP is primarily expressed in the nervous system and, to a lesser extent, in other insect organs and tissues (Li et al., 2008; Zandawala et al., 2015). ACP transcripts were detected in the head and thorax of larvae, pupae, and adult of *Ae. aegypti* (Wahedi and Paluzzi, 2018). Furthermore, ACP transcripts expression increases prominently in the brain and thoracic ganglia of *Ae. aegypti* after adult eclosion, suggest this neuropeptide may function in the regulation of post-ecdysis activities and development (Wahedi and Paluzzi, 2018; Zandawala et al., 2015), ACP transcripts in the brain of *Ny. albimanus* is consistent with previous results in *An. gambiae* where ACP (called AKHII) is expressed 72 h after feeding (blood or sugar) (Kaufmann and Brown, 2006). The differential expression of ACP between uninfected blood-fed and *P. berghei*-infected *Ny. albimanus* suggests that the midgut invasion by this parasite activates or modifies physiological pathways dependent on ACP. Thus, it could be possible that the increased level of this precursor indicates that an ACP neuropeptide production in *Plasmodium*-infected mosquitoes is generated and is part of the immune response activation against this parasite. Further studies are necessary to unravel the function of the ACP system; currently, no definitive functional studies for ACP in Anophelines has been determined.

Pyrokinins have been identified in various insects (Baggerman et al., 2002; Li et al., 2008; Predel et al., 2010), including mosquitoes (Hellmich et al., 2014; Olsen et al., 2007; Riehle et al., 2002). In *D. melanogaster* two genes, (*capa*) encode pyrokinins PVK-1 (GANMGLYAFPRV), PVK-2 (ASGLVAFPRV), PK (TGPSASSGLWFGPRL) and CPPB (GDAELRKWAHLLALQQVLD), and (*hugin*) encode *hug*-PK(SVPFKPRL) and *hug-γ* (LRQLQSNGEPAYRVRTPRL) (Nässel and Zan-

A)

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                ATGCTTCACACTCGTACGATCGCAGTGTTGGTTGTTGGATTAATAG
                -M--L--H--T--R--T--I--A--V--L--V--V--G--L--I--
   TGCTGGTCAATGCTCAAACCTTCCAATACTCTCGTGGATGGACAAACGGTAAACGCTCTT
16 V--L--V--N--A--Q--T--F--Q--Y--S--R--G--W--T--N--G--K--R--S--
   CTCCAACAGAACCGGGAGCTAATCCTGTGTTCTTGTCCCGATTGAACCAAGCCGGCCTAG
36 S--P--T--E--P--G--A--N--P--V--F--L--S--R--L--N--Q--A--G--L--
   ACTCTTTGAAACCAAACGAAAAGGCGTTATTGCGGCGTTTCTTGCACAACCCGTGTGACC
56 D--S--L--K--P--N--E--K--A--L--L--R--R--F--L--H--N--P--C--D--
   TCAGGATAGCTAGTTTACTAGGCAATCACCAGAGTAAAGATCTGCTGCCGGGTGGTGATC
76 L--R--I--A--S--L--L--G--N--H--Q--S--K--D--L--L--P--G--G--D--
   TATTCGACAGTGCGGAGACCAGTGGATCAACATTCGTTTTGCCGCAGTTTCTGATCGATT
96 L--F--D--S--A--E--T--S--G--S--T--F--V--L--P--Q--F--L--I--D--
   CCGAGGAGGGTAATGCCGGCTCCAGCAGTGCCAATTCAGTCAATGGACGATCTGTCGGGG
116 S--E--E--G--N--A--G--S--S--S--A--N--S--V--N--G--R--S--V--G--
   AAGCGCTTCGATTCAAGCGTGAGACGCCTGGAACGCTAGGCGAATCACGTTCTCAAATTG
136 E--A--L--R--F--K--R--E--T--P--G--T--L--G--E--S--R--S--Q--I--
   TTTAA
156 V--*-
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B)

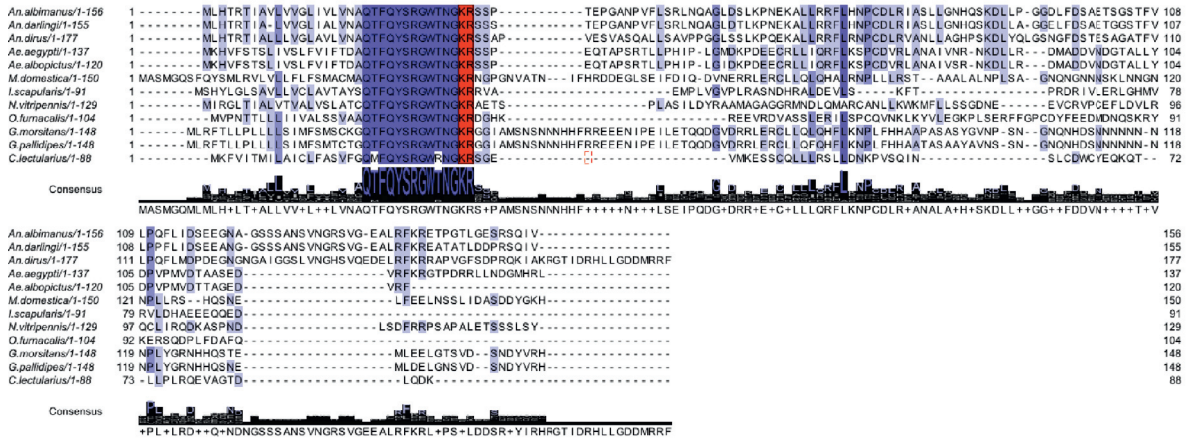

Fig. 6. Corazonin. **(A)** Nucleotide sequence and deduced amino acid sequence. Signal peptide (green), corazonin neuropeptide (blue), glycine residue for amidation (G), K-R cleavage site (red) and stop codon (*) are shown. **(B)** Multiple alignment of the *An. albimanus* corazonin prepropeptide with homologs from other insects. Note the conserved peptide motif **QTFQYSRGWTN** amide. *An. darlingi, An. dirus, Ae. aegypti, An. albopictus, M. domestica, I. scapularis, N. vitripennis O. furnacalis, G. morsitans, G. pallidipes, and C. lectularius.*

dawala, 2019). In *Ny. albimanus* we identified that *capa* produces at least two peptides with consensus sequence GLXXFPRV (PVK-1 and PVK-2) and one with consensus sequence XXWFGPRL (PK-1), while *hugin* produces one peptide with sequence XXWFGPRL (PK-1), two with sequence XXXFXPRL and one with sequence XXXPRL (PK-2) (Table 2). Several functions for these peptides were described in various insects, including stimulation of pheromone biosynthesis (Raina et al., 1989), induction of melanization (Matsumoto et al., 1990), induction of embryonic diapause (Suwan et al., 1994), stimulation of visceral muscle contraction (Predel and Nachman, 2001), inhibition of feeding, stimulates locomotion (Schlegel et al., 2016) and termination of pupal diapause development (Xu and Denlinger, 2003). But no evidence exists for the participation of pyrokinins in immune response mechanisms. The hugin transcript increased in *Ny. albimanus* brain infected with *Plasmodium*; suggesting that, at least one (PK-1 or PK-2) could be involved in signaling or activation mechanisms of the immune response of mosquitoes to these parasites.

Corazonin is widely conserved across insect genera (Predel et al., 2007) with various functions described including heart physiology, cardioacceleratory activity (Veenstra, 2009, 1989), silk spinning rate (Tanaka et al., 2002), ecdysis initiation (Kim et al., 2004), sperm transfer (Zer-Krispil et al., 2018), cuticular melanization (Tanaka et al., 2002; Tawfik et al., 1999) and regulation of stress responses (Veenstra, 2009). However, in Anophelines, until now, corazonin does not appear to modulate heart physiology (Hillyer et al., 2012). Therefore, the function of this neuropeptide in these mosquitoes remains unknown.

Although it has not yet been associated in immune response mechanisms, the increase in the expression of the corazonin precursor in brains could suggest a role in signaling mechanisms of the immune response in infected *Ny albimanus* mosquitoes modulating the expression of other neuropeptides.

It is important to mention that although most of the dissection was of the brain, for the identified precursors, the retrocerebral complex and the gnathal/subesophageal ganglion were dissected, which contributed

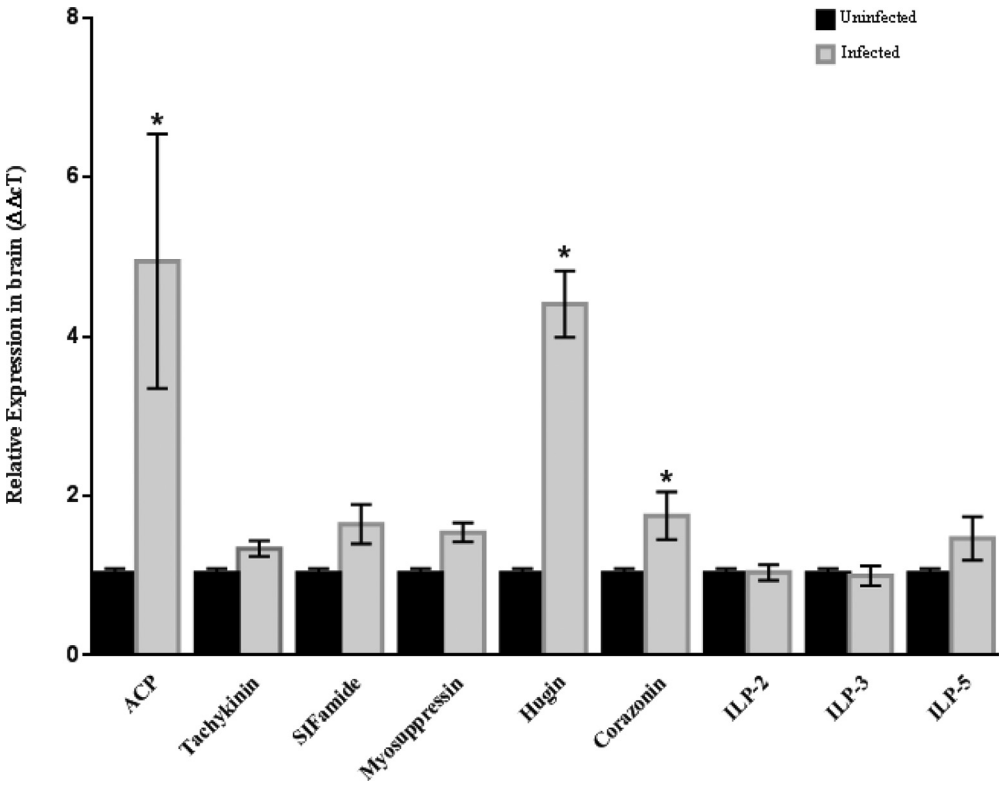

Fig. 7. Relative expression of neuropeptide transcripts in the brain of *Ny. albimanus* mosquitoes infected with *P. berghei* ookinetes. Expression of neuropeptide transcripts in the uninfected group was normalized to a fold change of 1, to which the other condition (infected) was compared. ACP (4.94*), tachykinin-related peptide (1.34), SIFamide (1.64), Myosuppressin (1.54), hugin (4.41*), corazonin (1.75*), ILP-2 (1.04), ILP-3 (1.00) and ILP-5 (1.46). For statistical analysis, data were analyzed by ANOVA followed by Kruskal-Wallis post-test. () = Fold change vs uninfected group, * = significative fold change Kruskal-Wallis post-test, neuropeptide infected group vs uninfected group (α= 0.05).

to identifying a greater number of neuropeptides precursors. The next step will be to analyze each of the neuropeptides that are generated from the transcripts identified in this work, and their specific participation in immune processes studies that to date are null.

In summary, we provide a brain neuropeptidome repertoire composed by 29 transcripts coding for at least 60 potential neuropeptides in *Ny. albimanus*. Mainly we report the increased expression of the adipokinetic hormone/corazonin-related peptide (ACP), hugin, and corazonin transcripts after *P. berghei* infection. At present, the functions of neuropeptides in insects during infection with parasites or viruses are partially understood. Further investigation is required as to whether significant changes in ACP, hugin and corazonin transcription in the brain reflect an increase in the production or release of the respective neuropeptides.

5. Conclusions

Most of the neuropeptides identified here showed high similarity with those previously reported in other insects and other mosquito vectors. Our results indicate that *P. berghei* promoted a modification of neuropeptide transcript expression in the mosquito brain at 24 h post-infection. The pattern of differential expression in adipokinetic hormone/corazonin-related peptide, hugin, and corazonin indicates that the invasion of midgut tissue by *Plasmodium* triggered a brain response. However, it is still necessary to explore the mechanisms activated by neuropeptides up-regulated in the *Ny. albimanus* brain by a *Plasmodium* infection. Whether this change is due to stress or immune response remains unclear since the function of these neuropeptides is practically unknown in anophelines. Nevertheless, these findings provide insights on the behavior and immune response of *Anopheles* during a *Plasmodium* invasion and contribute initial leads for the understanding of its neuroregulation.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Dedication

The authors dedicate this work to Rosa Elena Goméz Barreto†, rest in peace.

Declaration of Competing Interest

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Alejandro Alvarado-Delgado: Formal analysis, Writing – review & editing. **Jesús Martínez-Barnetche:** Formal analysis, Data curation, Writing – original draft. **Mario H. Rodríguez:** Writing – review & editing. **Everardo Gutiérrez-Millán:** Formal analysis. **Federico A. Zumaya-Estrada:** Formal analysis. **Humberto Lanz-Mendoza:** Writing – review & editing.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The datasets supporting the results are available at: http://201.131.57.23:8080/nyssorhynchus_albimanus/.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2021.100014.

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