THE ROLE OF PEPTIDE HORMONES IN INSECT PHYSIOLOGY, BIOCHEMISTRY, AND MOLECULAR BIOLOGY PROCESSES

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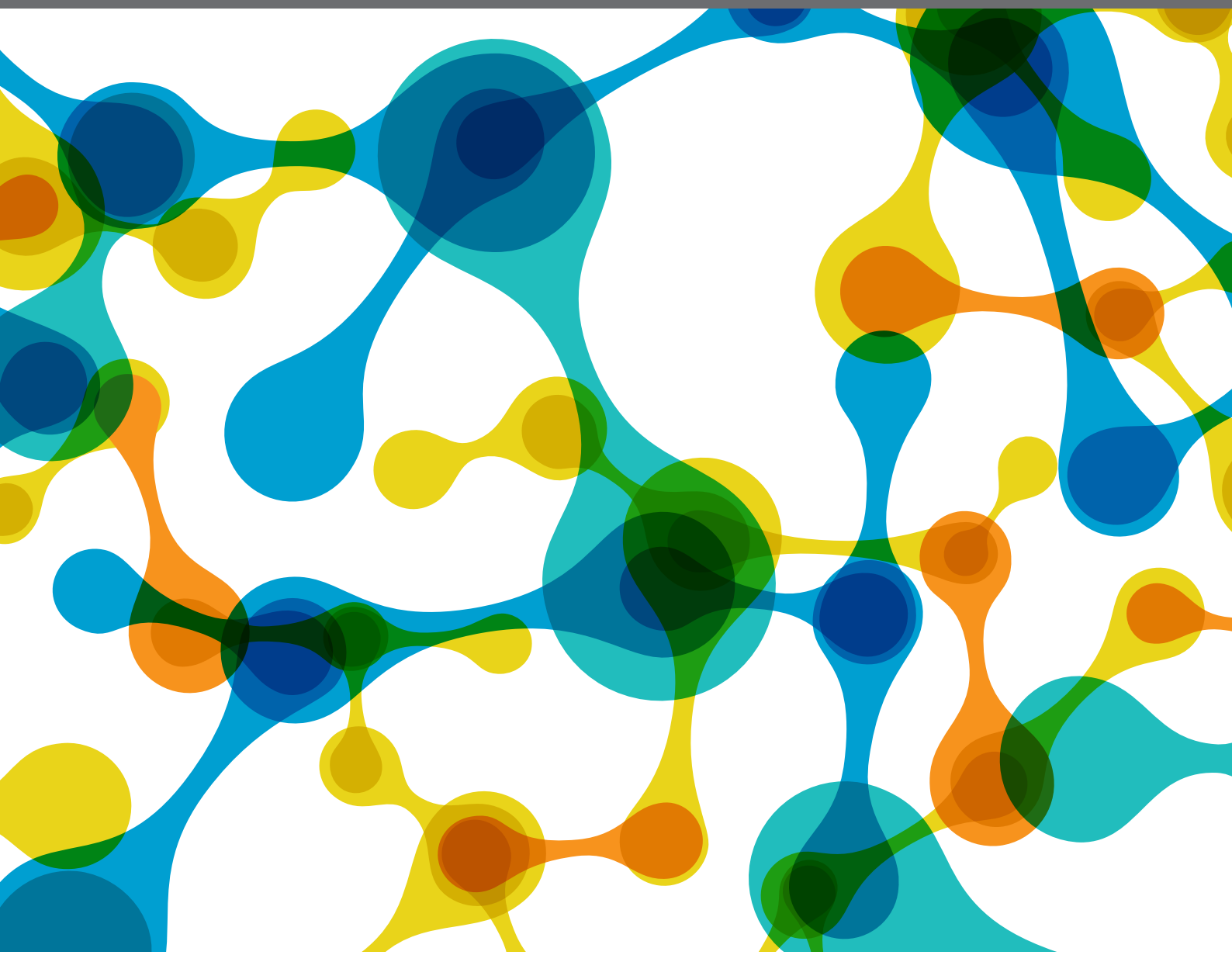

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THE ROLE OF PEPTIDE HORMONES IN INSECT PHYSIOLOGY, BIOCHEMISTRY, AND MOLECULAR BIOLOGY PROCESSES

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EDITORIAL
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Editorial: The Role of Peptide Hormones in Insect Physiology, Biochemistry, and Molecular Biology Processes

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Keywords: cellular immunity, venom proteins, insect midgut, cold stress, muscle contraction, pyrokinins, TMOF, hypertrehalosemic hormone

Editorial on the Research Topic

The Role of Peptide Hormones in Insect Physiology, Biochemistry, and Molecular Biology Processes

The study on the effect of the ectoparasitoid *Pachycrepoideus vindemmiae* on the cellular and humoral immunity in *Drosophila melanogaster* by Yang et al. was investigated at the pupal stage. The authors describe the cellular and humoral response to *P. vindemmiae* by pupal *D. melanogaster* using, *in vivo*, and *in vitro* studies showing that the Toll and Imd immune pathways are activated upon parasitization followed by the JAK/STAT pathway.

Venom proteins in the pupal ectoparasitoid *Pachycrepoideus vindemmiae* of *Drosophila* were analyzed by Yang et al. Expression studies show that 20 venom proteins are expressed 419-fold higher in tissues that produce venom. Relationships between venom proteins of other five species from three parasitoid families detected ancient orthologs in Pteromalidae. The authors assigned the venom proteins to orthologous groups showing that considerable interspecific variation of venom proteins is found in parasitoids using phylogenetic classification.

Neuropeptides and G-protein coupled receptors (GPCR) were studied in the red palm weevil by Zhang et al. They reported that the weevil causes damage to palm trees and induces highly tuned physiological and behavioral adaptability. The authors identified neuropeptide precursors and their cognate receptors in infected and uninfected tissues and identified 43 putative neuropeptides and 44 putative receptors. Using RT-qPCR analyses they showed that genes coding for several neuropeptides were expressed in various tissues but not in the nervous system suggesting that some of the peptides and their receptors may have other roles. Pathogens infections upregulated several of the neuropeptides and their receptors like tachykinin related peptide receptor.

Peptide hormones play an important role in insect midgut, they regulate growth, development, immunity, homeostasis and stress. Wu et al. reviewed the function and importance of these hormones in the insect midgut. The authors described that not only peptide hormones play an important role in the control of insect gut physiology, but several other hormones that are secreted from the corpus allatum, prothoracic gland and neural cells also have a role in inducing and secreting insects' digestive enzymes. These hormones also recognize pathogenic bacteria and may signal the gut cells to produce peptide hormones to control pathogens.

Neuropeptides act like important messenger molecules in neurotransmission. Marciniak et al. reported that FMRFamide-related peptides signal and regulate muscle contraction in tenebrionid beetles. These authors reported that neuropeptides that exhibit myotropic properties in insects also

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known as FMRFamide-like peptides (FaLPs) are regulating the contractile activities of the heart, ejaculatory duct, oviduct, and the hindgut in two beetle species. The authors identified *in silico* a putative receptor in both species. They showed that the putative receptors are expressed in various tissues including the visceral organs. Amino acid sequencing identified the receptor as G-protein coupled receptor. A synthetic receptor FaLP peptide was synthesized by the authors and was shown to have cardioactivity in one beetle and an inhibitory effect in a different beetle and myostimulatory effect on the visceral organs.

Pyrokinins are neuropeptides with myotropic, pheromonotropic, and melanotropic roles in several insects. In this study Lajevardi and Paluzze characterized pyrokinin1 and 2 receptors in the mosquito *Aedes aegypti* using an heterologous cell system. The authors measured the transcript level expression of these receptors in adults and found that PK1-R transcript is highest in the posterior hindgut (rectum), whereas PK2-R was highly expressed in the anterior hindgut (ileum) and in the reproductive organs. Immunoreactivity studies confirmed that PK1-R transcripts are found in the rectum and form the target of Pk1 regulating excretion.

Trypsin modulating oostatic factor (TMOF), a mosquito decapeptide that regulates blood digestion was cloned and expressed by Borovsky et al. in the methylotrophic yeast *Pichia pastoris*. Ten gene copies of TMOF were expressed by the engineered *Pichia* cells. The cells were analyzed by Southern, and Northern blotting followed by ELISA showing that the engineered cells (1.65×10^8 and 8.27×10^7 cells) synthesize 229 and 114 μM of TMOF, respectively, causing 100% mortality to larval mosquito. Therefore, engineered *P. pastoris* expressing TMOF could be used in the future to control mosquito larvae.

Insects are capable of surviving in arid to freezing climates. Lubawy et al. reviewed the role of insect neuroendocrine system during cold stress. These authors reported that neuropeptides and biogenic amines play a central role in regulating cold hardiness. Cold stress caused the release of chemical signals that control osmoregulation by various peptides such as capability peptides (CAPA), inotocin (ITC), ion transport peptide (ITP), diuretic hormones and calcitonin (CAL), tachykinin related peptides (TRPs) and peptides that are involved in mobilization of body reserves. The authors' review summarizes the current knowledge on the role of neuropeptides in cold stress response.

Two adipokinetic neuropeptide hormones (AKH) are synthesized by the corpora cardiaca of the Indian stick insect. Katali et al. reported that these neuropeptides increase the trehalose levels in the hemolymph if the insect is ligated between the head and thorax. They tested the potency of 19 AKH analogs on trehalose levels and change in the heartbeat rate with AKH analogs containing single amino acid substitution at different positions. Analogs that were modified at the termini did not show reduced activity as compared with control AKH. Shorter AKH peptides did not bind to the AKH receptor and substitutions of a single amino acid in most places of the native AKH abolished binding to the receptor. These results indicate that binding of AKH to its receptor is sequence specific.

Lipids are considered the primary storage molecules and essential energy source for insects during physiological events of reproduction, flight, starvation, and diapause. Lipid metabolism occurs is the fat body. The role of peptide hormones in insect metabolism was reviewed by Toprak describing the roles of peptide hormones in lipogenesis by adipokinetic hormone and brain insulin-like peptides (ILP2, 3, and 5). On the other hand, lipolysis is controlled by insulin-growth factor (ILP6), neuropeptide F, allatostatin-A, corazonin, leucokinin, tachykinins, and limostatin. Several neuropeptides that induce lipogenesis were also described. The functions of peptide hormones in lipid metabolism during reproduction, flight, diapause, starvation, infection and immunity were highlighted.

The roles of the *pyrokinin and capa* GPCRs genes in the stink bug *Halyomorpha halys* were reported by Ahn et al. These genes express multiple neuropeptides that were tested in cell-based assays and by RT-PCR. Ahn et al. showed that *H. halys* pyrokinin receptor-1 responds to pyrokinin 2 peptides. Whereas, *H. halys* pyrokinin receptor 2 responded to pyrokinin 1 type peptides that are similar to diapause hormone. The expression of the receptor genes in different tissues and life stages was determined by RT-PCR. The experiments described by Ahn et al. show that several of the peptides tested have specificities to 6 GPCRs and several are activated by pyrokinin 2 and pyrokinin 1 peptides.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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The Pupal Ectoparasitoid *Pachycrepoideus vindemmiae* Regulates Cellular and Humoral Immunity of Host *Drosophila melanogaster*

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The immunological interaction between *Drosophila melanogaster* and its larval parasitoids has been thoroughly investigated, however, little is known about the interaction between the host and its pupal parasitoids. *Pachycrepoideus vindemmiae*, a pupal ectoparasitoid of *D. melanogaster*, injects venom into its host while laying eggs on the puparium, which regulates host immunity and interrupts host development. To resist the invasion of parasitic wasps, various immune defense strategies have been developed in their hosts as a consequence of co-evolution. In this study, we mainly focused on the host immunomodulation by *P. vindemmiae* and thoroughly investigated cellular and humoral immune response, including cell adherence, cell viability, hemolymph melanization and the Toll, Imd, and JAK/STAT immune pathways. Our results indicated that venom had a significant inhibitory effect on lamellocyte adherence and induced plasmatocyte cell death. Venom injection and *in vitro* incubation strongly inhibited hemolymph melanization. More in-depth investigation revealed that the Toll and Imd immune pathways were immediately activated upon parasitization, followed by the JAK/STAT pathway, which was activated within the first 24 h post-parasitism. These regulatory effects were further validated by qPCR. Our present study manifested that *P. vindemmiae* regulated the cellular and humoral immune system of host *D. melanogaster* in many aspects. These findings lay the groundwork for studying the immunological interaction between *D. melanogaster* and its pupal parasitoid.

Keywords: *Drosophila melanogaster*, *Pachycrepoideus vindemmiae*, ectoparasitoid, venom, cellular immunity, humoral immunity

INTRODUCTION

Parasitoids are unique venomous organisms among hymenopteran insects, with an estimated number of species ranging from 150,000 to 600,000 (Mrinalini and Werren, 2017). There is great potential for developing parasitoids as a crucial means of biological control. They lay eggs into the hemocoel (endoparasitoids) or on the surface (ectoparasitoids) of hosts (Moreau and Asgari, 2015).

To ensure the survival and development of their offspring, parasitoids circumvent the host immune system with different adaptive strategies, namely, active immune suppression and passive immune evasion (Labrosse et al., 2003). Immune suppression is usually related to virulence factors, including venom (Asgari and Rivers, 2011), polydnaviruses (PDVs) (Gundersen-Rindal et al., 2013), virus-like particles (VLPs) (Grgacic and Anderson, 2006) and ovarian secretion (Mabiala-Moundoungou et al., 2010), and they work alone or cooperate with each other to regulate the cellular and humoral immune responses of hosts (Burke and Strand, 2014). In contrast, immune evasion occurs when wasp eggs are either covered with a fibrous layer or tightly adhered to host tissue, and the hosts fail to recognize it as non-self (Asgari et al., 1998; Eslin and Prévost, 2000; Hu et al., 2003). Both strategies are how parasitic wasps outwit their hosts.

Unlike mammals, insects lack acquired immune response, however, multiple innate defense responses have been highly developed to resist the invasion of parasitoids during the long-term arms race (Hoffmann, 1995). Insect innate immunity consists in cellular and humoral innate immunity (Lemaitre and Hoffmann, 2007). Cellular immunity response is mainly mediated by hemocytes. Much of our current understanding of hemocyte-mediated resistance to wasps lies in encapsulation. The *Drosophila* parasitoids are supposed to be valuable models for investigating their immunological interactions at the cellular immunity level (Carton et al., 2008). In general, *Drosophila melanogaster* recognizes parasitoids' eggs as non-self once parasitoids lay eggs (Russo et al., 1996), followed by recruiting and spreading of plasmatocytes. Ultimately, lamellocytes collaborate with plasmatocytes to surround the wasp eggs and melanin is then deposited to seal it off, which is indispensable for killing the invaders (Williams, 2007). As a prominent humoral immune response of *Drosophila*, melanization plays an important role in fighting against parasitization (Tang, 2014). Prophenoloxidase (PPO) secreted by crystal cells and lamellocytes mainly contributes to the melanization of the wasp eggs (Dudzic et al., 2015). In addition to the melanization, the Toll, immune deficiency (Imd) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) immune pathways associated with humoral immune responses are other exhaustively studied areas in confronting pathogenic infection and parasitization by parasitic wasps. In the Toll pathway, *Dif*/*Dorsal,* once activated, translocates into the nucleus and initiates the expression of antimicrobial peptides (AMPs) (Lehming et al., 1995; Nicolas et al., 1998; Wu and Anderson, 1998). In contrast, *caspase*-mediated N-terminal cleavage of *relish* activates the Imd pathway (Kim et al., 2006). Subsequently, N-terminal RHD-containing fragment (Rel-68) enters the nucleus, and a battery of AMPs are robustly produced (Stöven et al., 2000; Stoven et al., 2003). Unlike the complicacy of the Toll and Imd pathways, the JAK/STAT signaling pathway is more succinct, including three cytokine-like ligands named *unpaired* (upd) (Harrison et al., 1998), upd2 (Hombria et al., 2005), and upd3 (Agaisse et al., 2003; Wright et al., 2011), a transmembrane receptor, JAK and a Stat transcription factor (Rawlings et al., 2004; Myllymaki and Ramet, 2014). In *D. melanogaster*, an increasing number of studies have revealed that there is a connection between parasitization and the Toll, Imd and JAK/STAT pathways, while the regulatory effects vary greatly in different *Drosophila* parasitoid models (Wertheim et al., 2005; Martinson et al., 2014; Schmid, 2014; Yang et al., 2015; Louradour et al., 2017). Moreover, reactive oxygen species (ROS), a field of humoral innate immunity that has not been exploited fully, increased sharply in posterior signaling center (PSC) cells under parasitism, which conferred *Drosophila* resistance to wasp parasitism (Louradour et al., 2017). In short, they elaborately exploit cellular innate immunity and humoral innate immunity to cope with successful parasitism during the long-term antagonistic interaction between *Drosophila* and their parasitoid wasps.

Meanwhile, parasitoids have developed corresponding defense strategies to aid their progeny's survival by virtue of various virulence factors. Venom, the fundamental parasitic factor either in endoparasitoids or ectoparasitoids, is the topic of interest in their host immune regulation. The primary functions of venom include inducing hosts paralysis, interrupting host development, suppressing the immunity of their hosts, etc (Coudron et al., 1990; Edwards et al., 2006; Tian et al., 2010; Kryukova et al., 2011). In a larval solitary endoparasitoid *Leptopilina boulardi*, long gland products induced a drastic decrease and an alteration of actin cytoskeleton in lamellocyte cells to inhibit the encapsulation (Labrosse et al., 2005a,b). Additionally, venom from *L. boulardi* inhibited the PO cascade of the *D. yakuba* larval hemolymph (Colinet et al., 2009). As a previously uncharacterized *Drosophila* parasitoid, *Ganaspis* sp.1 venom suppressed plasmatocyte calcium burst, resulting in its failure to migrate toward parasitoid eggs (Mortimer et al., 2013). There is some evidence indicating that secretions from the venom gland and ovary collaborate to regulate host physiology in *Asobara japonica* (Mabiala-Moundoungou et al., 2010). Furthermore, PDV is another thoroughly studied virulence factor, which is equally as important as venom in host immune regulation. In Braconidae and Ichneumonidae, Bracoviruses and Ichnoviruses imitated inhibitor kB (IkB) proteins of *Drosophila* to regulate immune NF-kappa B signaling by virtue of *ankyrins* (Bitra et al., 2012; Gueguen et al., 2013). In addition to the venom and PDVs, VLPs from *L. heterotoma* and *L. victoriae* also triggered immune suppression responses and further weakened the encapsulation phenotype in the host *Drosophila* (Morales et al., 2005; Heavner et al., 2017). In short, the mechanism of immunological interactions between parasitoids and their hosts is extremely complex and finely modulated.

Pachycrepoideus vindemmiae (Hymenoptera: Pteromalidae) is a versatile and solitary pupal ectoparasitoid of many flies whose hosts range from Drosophilidae to Anthomyiidae, Calliphoridae, Muscidae, Sarcophagidae, Tachinidae, Tephritidae, and so on (Marchiori and Borges, 2017). Unlike the multiple virulence factors in many koinobiont parasitoids, venom is the only required parasitic factor for successful parasitism of *P. vindemmiae*. Although there are many lines of research addressing the physiological mechanisms of immune modulation in the larval parasitoids of *Drosophila*, little has been investigated about the pupal parasitoids, let alone the research of the *P. vindemmiae-D. melanogaster* model. The main objective of

the present study is to investigate the immune response of the host *D. melanogaster* once parasitized by *P. vindemmiae*. Our results demonstrated that great changes took place in cellular and humoral immunity of the host, including cell adherence, cell viability, hemolymph melanization and the Toll, Imd, and JAK/STAT immune pathways. Further studies illustrated the necessity of venom in the process of immune modulation. In brief, our present research opens a precedent for studying the pupal parasitoid-*Drosophila* system, which will contribute to a better understanding of the immunological interactions between the pupal parasitoids and their hosts.

MATERIALS AND METHODS

Fly Strains

Host *D. melanogaster* stocks were raised on standard medium at 25°C with 60 ± 5% relative humidity and 16 h:8 h (light: dark) photoperiod. The stock w^{1118} originating from an indoor reared population was used as wild-type control. The following stocks were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IL, United States): Dipt-lacZ, Drs-GFP (stock ID: 55707), 10*Stat92E-GFP (stock ID: 26198), and Hop^{Tum-1} (stock ID: 8492).

Parasitoid Collection and Rearing

The colony of *P. vindemmiae* was kindly provided by Prof. Yongyue Lu (South China Agricultural University, Guangzhou, China) in January 2016. Subsequently, *P. vindemmiae* was maintained with *D. melanogaster* pupae at 25°C with a photoperiod of 14 h: 10 h (light: dark) as previously described (Chen et al., 2015). Once closed, adults were held in glass containers and fed on 20% (v/v) honey solution.

Cell Adherence Ability Assay

We could not separate pure hemocytes from *D. melanogaster* pupae considering that the bled fluids contained many fat granules. As a result, third instar larvae were used to obtain the hemocytes. The cuticle of Hop^{Tum-1} third instar larvae was gently pricked by forceps. Hemocytes from three larvae were bled on a glass slide containing 30 μl 10 mM phosphate buffer (PBS), pH 7.4 or different venom reservoir equivalents (VRE) dissolved in 30 μl PBS and allowed to adhere for 1 h. Thus, 1 VRE represents protein equivalents isolated from one venom reservoir and 2 VRE represents that of two venom reservoirs. Once adhered, it was easy to distinguish plasmatocytes and lamellocytes, which constituted the majority of all blood cells in Hop^{Tum-1} *Drosophlia* (Hanratty and Dearolf, 1993). Namely, plasmatocytes are small spherical cells and lamellocytes are large discoid cells. The adhered cells were washed three times with PBS, fixed with 3.7% paraformaldehyde solution (Sangon Biotech, Shanghai, China) for 15 min, and then washed three times with PBS before being permeabilized for 15 min with 0.1% Triton X-100 (Sangon Biotech, Shanghai, China). Subsequently, the fixed samples were incubated with 1% bovine serum albumin (BSA) for 30–60 min once washed three times with PBS. The F-actin was visualized by staining the cells with 1:1000 phalloidin-iFluor 488 (Abcam, Cambridge, United Kingdom) diluted in 1% BSA for 1 h. After this, the cells were washed 3 times with PBS and mounted by the SlowFade™ Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA, United States). Photos were taken under white light and fluorescence view, respectively, by a Nikon eclipse TS-100 (Nikon, Japan). In the following analysis, the cell area of fluorescence staining larger than 400 μm^2 was considered as lamellocytes.

Hemocyte Viability Assay

Hemocytes were collected into the wells of a 96-well plate (Corning, New York, NY, United States) as mentioned above and then allowed to adhere for 1 h. Thirty microliter PBS, 1 VRE or Lysis Buffer (Promega, Madison, WI, United States) was added and incubated for 30 min. Cellular mortality was monitored by using the CellTox™ Green Dye (Promega, Madison, WI, United States) according to the manufacturer's instruction. Dead cells exhibited enhanced fluorescence as a result of the stable bond between dye and DNA, while no appreciable increase was observed in viable cells because of the integrity of the cell membrane. Photos were taken as mentioned above. The fluorescence value was measured with a setting of 485–510 nm as the excitation wavelength and 520–530 nm as the emission wavelength.

Melanization Assay

For the melanization analysis *in vivo*, w^{1118} pupae within 12 h pupation were collected and then injected with 23 nl 1 mg/ml BSA, saturated phenylthiourea (PTU) or 0.5 VRE (half dilution of 1 VRE), 1 VRE or 2 VRE, respectively. Three hours later, pupae were inspected under a Leica DFC425 Camera attached to a stereomicroscope Leica M205 A (Leica, Wetzler, Germany). The melanization analysis was performed according to the previous study with some modifications (Gregorio et al., 2002). Briefly, about 15 *D. melanogaster* pupae (20 mg in total) were ground in liquid nitrogen and immediately suspended in 100 μl PBS, PTU or VRE dissolved in Tris buffer (100 mM, pH 7.2) followed by 16,000 g for 20 min centrifugation, and the supernatant was transferred to a new tube. After 30 min incubation at room temperature, 10 μl aliquot was mixed with the PO assay mixture prepared as previously described and subjected to assay by measuring the optical density (OD) at 520 nm (Gregorio et al., 2002).

Fluorescence Microscopy and LacZ Activity Analysis

To determine the effects of parasitism on the Toll, Imd and JAK/STAT pathways, downstream transcription factors, AMPs or stress factors, including *drosomycin*, *diptericin, stat92E* and *thioester-containing protein 1(Tep1)*, were used as indicators. Hence, Dipt-lacZ; Drs-GFP stocks were used for both lacZ activity analysis and fluorescence microscopy. GFP detection was also conducted in 10*Stat92E-GFP stock. Briefly, pupae were collected and parasitized for 1 h within 12 h after pupation. It was assumed that successful parasitism occurred when the

envenomation lasted for more than 1 min, and the remaining that did not meet the criteria were removed. Pupae were reared as mentioned above. Then, pupae were photographed at 1, 6, 12, 24, 48, and 72 h after parasitization with GFP fluorescence channel using Nikon AZ100M (Nikon, Tokyo, Japan). Unparasitized *D. melanogaster* pupae were used as control.

LacZ activity was analyzed by quantifying the enzymatic activity of β-galactosidase as the previous study (Romeo and Lemaitre, 2008). In brief, five pupae were collected into a 2.0 ml Eppendorf tube. Then, 250 μl buffer A (60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 8.0) was added and homogenized for 30 s followed by supplementation of 250 μl buffer A and quickly vortexed. The mixture was centrifuged at 6000 × g for 5 min and the supernatant was transferred to a new 1.5 ml Eppendorf tube. Protein concentration was determined by a Modified Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. Finally, 30 μl aliquot was transferred to 96-well plates and 250 μl of 0.35 mg/ml O-nitrophenyl-β-D-galactoside dissolved in buffer A was added to each well followed by 37°C incubation. The β-galactosidase activity was determined at regular time intervals (10 min) by measuring the $OD_{420\ nm}$, and lacZ activity was calculated as Miller's description: $[(\Delta OD_{min})/\Delta T_{min}]$/protein concentration/0.0045 (Romeo and Lemaitre, 2008).

RNA Extraction and Quantitative Real-Time PCR

Both parasitized and unparasitized w^{1118} pupae (5 each) were homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, United States). The total RNA was extracted according to the manufacturer's protocol and cDNA was synthesized by using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Beijing, China). Quantitative RT-PCR (qPCR) was carried out using the TB Green™ *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Beijing, China) and run on a Bio-Rad CFX Connect (Bio-Rad, Hercules, CA, United States) instrument according to the manufacturer's instructions. Relative expression levels of *drosomycin*, *diptericin* and *Tep1* were quantified and further normalized to reference gene *RPL32* (also referred as *R49*) using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All the primers used were based on the previous study (Neyen et al., 2014).

Data Analysis

The phalloidin staining area of hemocytes and the fluorescence intensity of pupal microscopy were measured by using image processing software, Image J 1.8.0 (Image J, NIH, United States). The total corrected fluorescence (TCF) of pupal microscopy was calculated as follows: integrated fluorescence density – (area of photograph) × (mean fluorescence of background). Data for two groups or more than three groups were analyzed by unpaired two-tailed student's *t*-test and one-way analysis of variance (ANOVA) with Tukey's test, respectively. In addition, a Chi-square test was conducted to test the difference in data of **Supplementary Figure S1**. There was statistical significance if $P < 0.05$. All statistical analyses were carried out using the data processing system (DPS) package version 9.5 (Tang and Zhang, 2013). All figures were plotted using GraphPad Prism 7.0 (GraphPad, San Diego, CA, United States).

RESULTS

Effects of Venom on Cell Adherence

As shown in **Figure 1**, plasmatocytes and lamellocytes adhered to the plate within 1 h after PBS treatment. The fluorescence area of 985 cells was measured and the results indicated that total hemocyte population comprised approximately 22% lamellocytes (**Supplementary Figure S1**). However, compared to the PBS control (**Figure 1A**), the ratio of lamellocytes was significantly decreased to 16% when treated with 1 VRE (**Figure 1B**). With the increasing dose of VRE, fewer adherent lamellocytes were observed, and the total number of lamellocytes declined to about 4% following 2 VRE incubation (**Figure 1C**). In contrast, low-concentration venom (0.67 VRE, 0.33 VRE and 0.17 VRE) had limited effects on lamellocyte adherence (data not shown).

Effects of Venom on Cell Viability

To investigate whether the dysfunction in lamellocyte adherence was attributed to cell death, cell viability was detected using cellTox™ green dye staining (**Figure 2**). In contrast to the considerably high survival rate under PBS treatment and abrupt decline in viable hemocytes under lysis treatment (**Figures 2A,C**), the percentage of GFP-positive cells was about twice as high as that in the PBS control after 1 VRE incubation (**Figure 2B**). Similar results were obtained when fluorescence value was measured, that is, crude venom induced a significant increase (2.12-fold) in fluorescence value ($P < 0.05$)

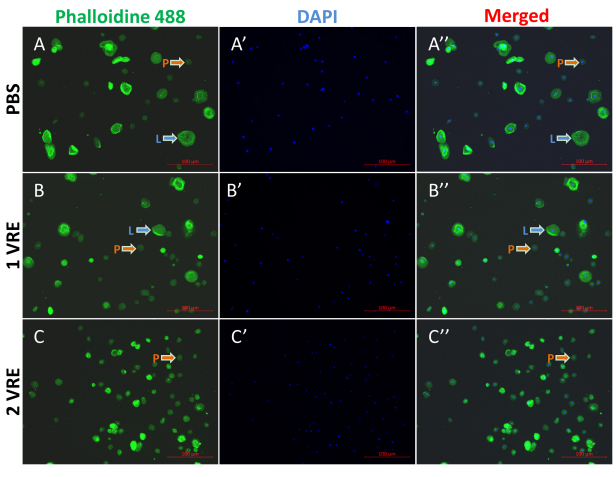

FIGURE 1 | Immunofluorescence detection of hemocytes after venom treatment. The stained hemocytes incubated with PBS **(A–A″)**, 1 VRE **(B–B″)**, or 2 VRE **(C–C″)** were photographed using fluorescence microscope. Subsequently, the staining area was counted. Plasmatocytes (P) and lamellocytes (L) are each indicated. Blue represents the nuclei stained with 4′, 6-diamidino-2-phenylindole (DAPI) and green represents the cytoskeleton marked with phalloidin 488.

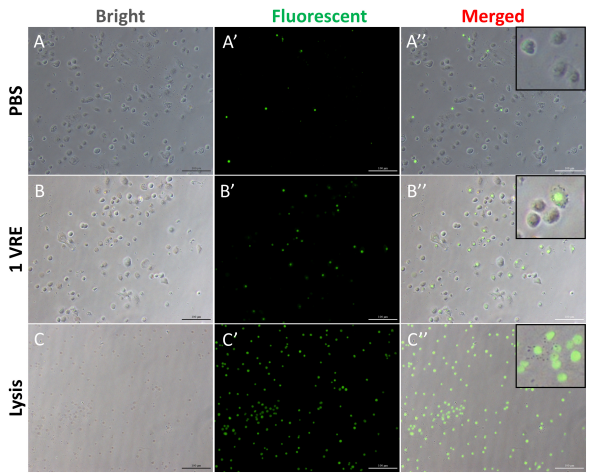

FIGURE 2 | Cell viability assay after venom treatment. The hemocytes were incubated with PBS **(A–A″)**, 1 VRE **(B–B″)**, and lysate **(C–C″)** followed by microscopic examination under white, fluorescent, and merged view, respectively. Fluorescently labeled cells represent dead cells.

(**Supplementary Figure S2**). Surprisingly, our findings indicated that GFP-positive cells were mainly occupied by plasmatocytes rather than lamellocytes in the presence of venom. Therefore, it was inferred that the functional disturbance of adherence in lamellocytes was unrelated to the cell death.

Effects of Venom on Melaninization of the Hemolymph

To determine whether crude venom inhibited the melaninization of hemolymph, PO activity of pupal hemolymph was determined both *in vivo* and *in vitro*. As shown in **Figure 3A**, there was a slight melanization at the wound after 1 VRE injection compared to the strong melanization induced by BSA injection. In addition, quantitative analysis of PO activity was also conducted. Our results showed that 0.5, 1 and 2 VRE inhibited the melanization of the host hemolymph to different degrees (**Figure 3B**). Thus, 2 VRE significantly blocked the blackening of the hemolymph even after 40 min incubation. By comparison, a weaker inhibitory effect was observed in 1 VRE treatment. It appeared that 0.5 VRE had a small but significant effect on melanization after 10, 20, and 30 min incubation; however, the effect disappeared after 40 min incubation. These findings demonstrated that the inhibition of hemolymph melanization by crude venom was dose-dependent. Furthermore, these results shed light on the inhibitory effect of venom components on the host PO cascade.

Effects of Parasitism on the Toll, Imd, and JAK/STAT Immune Pathways

To investigate the effect of parasitism on the Toll immune pathway, the expression level of *drosomycin*, a specific marker gene of the Toll immune pathways, was quantified. Results showed that the transcription levels of *drosomycin* increased sharply (46-fold) after 6 h of parasitism, and this effect lasted up to 72 h post-parasitism, although it was not shown at 48 h (**Figure 4A**). To further confirm the reliability of the results, protein expression level of *drosomycin* was quantified by measuring the fluorescence of drosomycin-GFP in *Drosophila* (**Figure 4B**). As **Figure 5** showed, stronger fluorescence was generated on the parasitized *Drosophila* compared to unparasitized ones. Additionally, the fluorescence intensity was compared between parasitized and unparasitized *Drosophila*. Results showed that a higher level of *drosomycin* was induced both at the transcription level and the protein expression level once parasitized, whereas contrary circumstances happened at 48h post-parasitism (**Figure 4**). This unusual result might be explained by the smaller difference in qPCR results between unparasitized (0.52) and parasitized (0.40). Overall, it can be concluded that parasitism by *P. vindemmiae* activates the Toll pathway-dependent immune response.

Similarly, the Imd immune pathway is equally as important as the Toll pathway in immune defense response in *D. melanogaster*. Once parasitized by *P. vindemmiae*, the expression level of the Imd pathway specific marker gene *diptericin* was upregulated. As shown in **Figure 6A**, the mRNA transcriptional level of *diptericin* increased more than 10-fold at 1 h post-parasitism, and the high fold change lasted to 72 h compared with the unparasitized group. At the same time, lacZ enzymatic activity was basically consistent with qPCR results. As **Figure 6B** shows, the enzymatic activity of parasitized *Drosophila* was significantly higher than unparasitized both in the early and late stage of parasitism. However, the higher enzymatic activity was observed in unparasitized *Drosophila* at 48 h. Regardless of this point, parasitism contributed to the activation of the Imd immune pathway both at the transcription and the protein expression levels.

Unlike the multifunctionality of the Toll and Imd immune pathways, the JAK/STAT immune pathway plays roles specifically in the process of immune responses against parasitoids and viruses. The Transcriptional level of *Tep1*, a marker gene of the JAK/STAT pathway, was detected. Similar with the previous results, the mRNA level of *Tep1* increased several times in *D. melanogaster* pupae once parasitized by *P. vindemmiae*, which lasted until the later stage of parasitization (**Figure 7A**). To clarify the facticity of the qPCR results, the expression levels of *Stat92E*, a transcription factor of *Tep1*, were further investigated by analyzing the fluorescence of 10*Stat92E-GFP *Drosophila* both in unparasitized and parasitized pupae at 1, 6, 12, 24, 48, and 72 h. Experimental results indicated that there was a stronger GFP fluorescence signal at 1, 6, and 12 h in parasitized *Drosophila* (**Figure 7B**). However, no significant difference was shown at 24 h between the parasitized and unparasitized (**Figure 7B**). Conversely, as shown in **Figure 8**, a significant inhibitory effect on the activation of *Stat92E* at 48h post-parasitism was revealed, that is, weaker fluorescence intensity was recorded. These results indicated that the JAK/STAT immune pathway was activated during the early stage of parasitization.

DISCUSSION

The innate immune system of host insects involves cellular and humoral aspects. They orchestrate together to resist

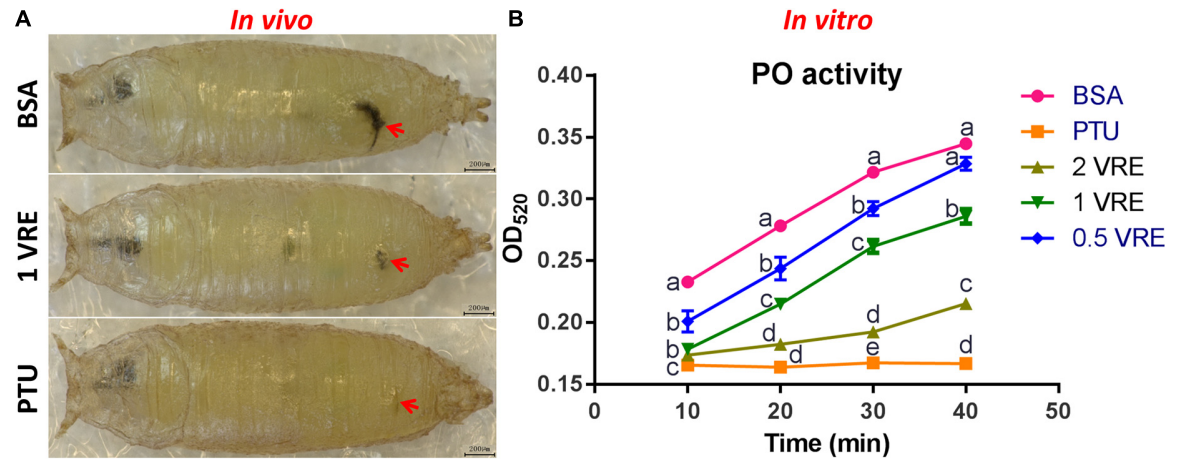

FIGURE 3 | Effects of venom on melaninization of the host hemolymph. **(A)** Microscope inspection of w^{1118} pupae was performed 3 h after injection with BSA, 1 VRE, or PTU. **(B)** PO activity was assayed every 10 min by measuring the OD_{520} after hemolymph was co-incubated with BSA, PTU, or VRE. The results are shown as the mean ± standard error (n = 3); different lowercase letters above bars for the same time point indicate significant difference ($P < 0.05$).

the successful parasitism of parasitic wasps. In *Drosophila*, hemocytes are the key players in cellular immunity response of *Drosophila*, which constitute the crucial adjective weapons in cellular immunity, especially lamellocytes, and are only produced by the immune system under parasitization or aberrant conditions (Rizki and Rizki, 1992). The precondition for encapsulation is that plasmatocytes are recruited and spread over the egg surface followed by adhesion of lamellocytes to the plasmatocyte-covered wasp eggs (Anderl, 2015). It has been reported that *A. japonica* venom significantly disturbed the spreading behavior of *D. melanogaster* hemocytes (Furihata et al., 2013). In a *L. Boulardi*/*D. melanogaster* model, virulent extracts of venom reservoir induced a drastic decrease in lamellocyte counts (Labrosse et al., 2005a). More research showed that more than 80% of plasmatocytes and granular cells from the host *Pseudoplusia includens* were unable to spread after oviposition by *Microplitis demolitor* within 2 h (Strand and Noda, 1991). In a closely related species, *Nasonia vitripennis*, the total number of plasmatocytes declined sharply after 60 min envenomation and further *in vitro* experiment proved that isolated crude venom indeed blocked adhesion and spreading of hemocytes (Rivers et al., 2002). In the present study, we primarily focused on alterations in lamellocyte adherence ability following crude venom treatment. Consistent with the above studies, *P. vindemmiae* crude venom exerted a similar inhibitory effect on adherence of lamellocytes as that of *N. vitripennis*, which disturbed normal lamellocyte function involved in encapsulation. In addition, the ratio of adhered lamellocytes gradually decreased with the increasing venom dosage. To investigate whether the disability of adherence in lamellocytes was caused by cell death, cell viability was evaluated. The results showed that cell death was indeed induced by venom but mainly on plasmatocytes. Hence, the underlying mechanism for inactivation in adhesion of lamellocytes is still unknown and further investigations are still needed.

Humoral immunity, equally important as cellular immunity, is common to all insects as well. As one of the prominent humoral immunity responses, melanization plays important roles in resisting microbial infection and parasitization. During melanization, phenols are oxidized to quinones followed by the formation of melanin (Tang, 2014). Successful parasitism is greatly determined by the dysfunction in hemolymph melanization. As a counter-immune strategy, venom proteins act in inhibiting the process of PO cascades in host insects. Several lines of research have identified venom proteins that are involved in the process, such as 50-kDa serine proteinase homolog (Vn50) (Asgari et al., 2003), Serpin (Colinet et al., 2009; Yan et al., 2017), Kazal-type serine protease inhibitors (Qian et al., 2015) and defensin-like peptide (Tian et al., 2010). Given that the immune response of *D. melanogaster* was successfully suppressed by venom, successful parasitism occurs and the wasp eggs hatch followed by larval feeding. This is a consequence of co-evolution in the arms race between parasitoids and their hosts. In this study, we thoroughly investigated the effects of the venom cocktail on the melanization of *Drosophila* pupae. First, *in vivo* injection indicated that venom significantly inhibited the blackening of the wound. Furthermore, in contrast to BSA treatment *in vitro*, hemolymph incubated with venom melanized to a lesser extent, and this inhibitory effect was gradually enhanced as the concentration increased from 0.5 VRE to 2 VRE, showing a dose-dependent effect. However, it is still a black box regarding how many venom proteins really function in this process, let alone their inhibitory mechanism.

As the core portion of humoral response, the Toll, Imd and JAK/STAT pathways play important roles against parasitism. For instance, the loss-of-function mutations of the JAK/STAT and Toll pathways in *Drosophila* larvae exhibited inadequate capacity to encapsulate the eggs of *L. boulardi* (Sorrentino et al., 2004). On the contrary, transgenic *Drosophila* of gain-of-function in the JAK/STAT or Toll pathway led to the plentiful formation of melanotic tumors (William and Jan, 1981;

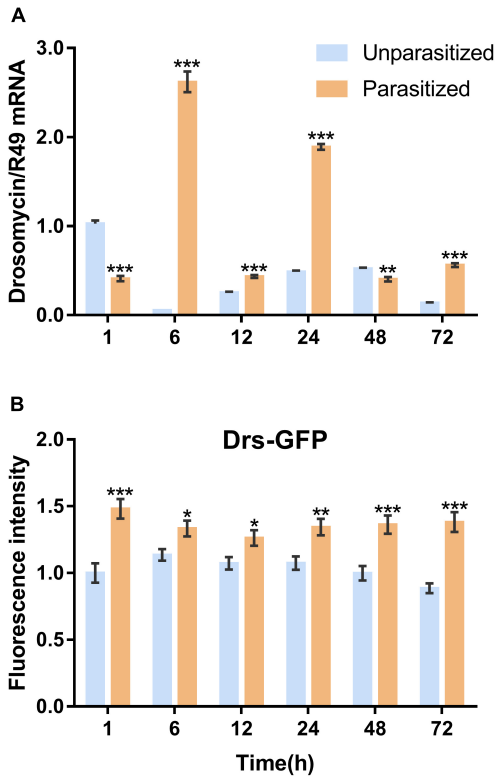

FIGURE 4 | Effects of parasitism on the Toll pathway. **(A)** mRNA expression levels of *drosomycin* at 1, 6, 12, 24, 48, and 72 h both in parasitized and unparasitized *Drosophila* ($n = 3$), and ribosomal protein 49 (*R49*) was used as internal reference. **(B)** Fluorescence intensity measurement of drosomycin-GFP in parasitized and unparasitized *Drosophila* based on the time points mentioned above ($n \geq 10$). The fluorescence intensity of 1 h unparasitized was normalized to 1.0. The results are shown as the mean ± standard error; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to unparasitized.

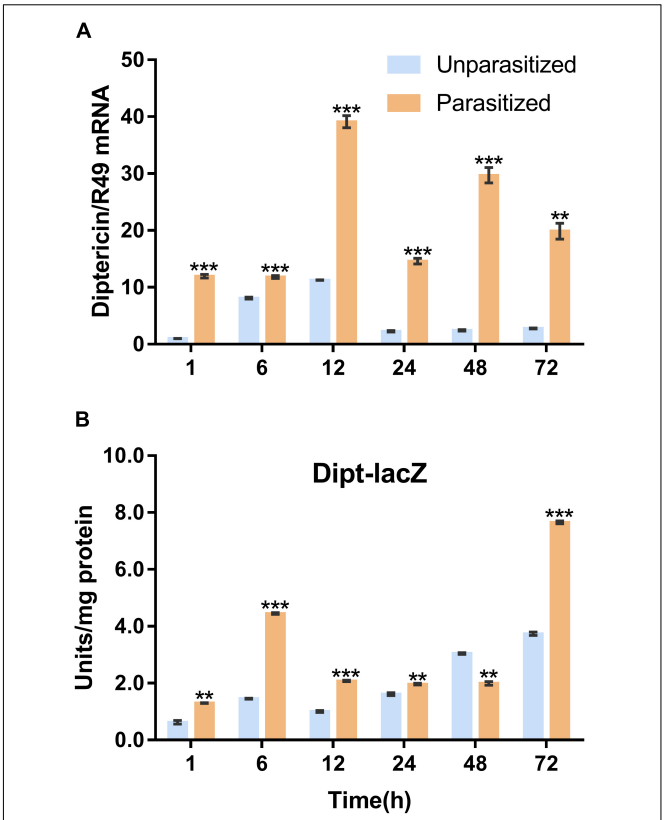

FIGURE 6 | Effects of parasitism on the Imd pathway. **(A)** mRNA expression levels of *diptericin* at 1, 6, 12, 24, 48, and 72 h in parasitized and unparasitized *Drosophila* ($n = 3$). *R49* was used as internal reference. **(B)** Enzymatic activity assay of diptericin-lacZ both in parasitized and unparasitized *Drosophila* based on the time points mentioned above ($n \geq 10$). The enzymatic activity of 1 h unparasitized was normalized to 1. The results are shown as the mean ± standard error; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to unparasitized.

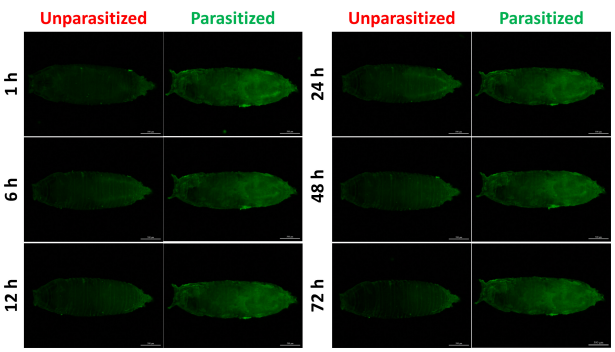

FIGURE 5 | Fluorescence microscopy of unparasitized and parasitized drosomycin-GFP in *Drosophila* at different times. The green fluorescence was visualized through the cuticle using a florescence microscope.

Gerttula et al., 1988; Harrison et al., 1995; Luo et al., 1995). In *L. boulardi*, the JAK/STAT signaling was significantly activated 27 h post-parasitization (Yang et al., 2015). Different from studies about the activation of the JAK/STAT and Toll pathways on parasitization, there have been limited studies about the crosslink between parasitization and the Imd immune pathway except for several lines of research based on high-throughput omics analysis. For instance, *L. heterotoma* venom specifically inhibited the Toll and Imd pathway signaling in fat body by microarray analysis (Schlenke et al., 2007). In contrast, genome-wide analysis indicated that transcription factor *Relish* and several AMPs downstream of the Imd pathway were strongly unregulated in response to *A. tabida* attack (Wertheim et al., 2005). In *N. vitripennis*, significant increases of several immune-related genes of the Toll and Imd pathways provided evidence that venom activated certain immune responses in envenomated hosts by whole gene expression profile analysis (Martinson et al., 2014). Thus, an 8.6-fold and 2.4-fold upregulation on *spätzle* and *relish* were shown, respectively. As a closely related species of *Nasonia*, it is inferred that parasitization may have similar effects on *Drosophila* immune signaling by *P. vindemmiae*. As expected, here we showed that the Toll and Imd immune pathways were activated by monitoring the transcription and expression

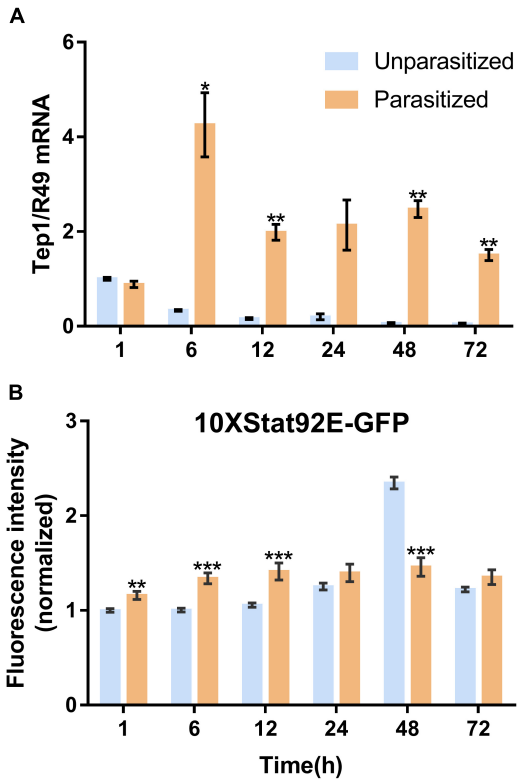

FIGURE 7 | Effects of parasitism on the JAK/STAT pathway. **(A)** mRNA expression levels of *Tep1* at 1, 6, 12, 24, 48, and 72 h both in parasitized and unparasitized *Drosophila* (n = 3), and *R49* was used as internal reference. **(B)** Fluorescence intensity analysis of 10*Stat92E-GFP *Drosophila* both in parasitized and unparasitized based on the time points mentioned above ($n \geq 10$), and the fluorescence intensity of 1 h unparasitized was normalized to 1. The results are shown as the mean ± standard error; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to unparasitized.

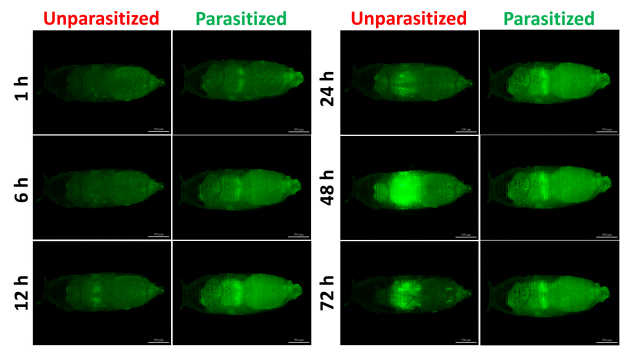

FIGURE 8 | Fluorescence microscopy of unparasitized and parasitized 10*Stat92E-GFP in *Drosophila* at different times. The green fluorescence was visualized through the cuticle using a florescence microscope.

of transcription factors and AMPs *in vivo* after parasitization. Similarly, our findings also indicated that the JAK/STAT pathway was activated during the early stage of parasitization, lagging behind the Toll and Imd pathways during this process. One unanticipated finding was that contrary circumstances occurred between the transcription level and the protein expression level at 48h post-parasitism. This observation could be due to the fact that pupae experienced a tissue regeneration process during this period. Separation of the pupal cuticle was initiated at 24 h after puparium formation and at around 48–50 h, the adult cuticle was formed (Carol et al., 1982). The transcript levels of a set of genes changed significantly during this process (Arbeitman et al., 2002). The latter point proposed by Wright et al. also found that a set of five L71 genes encoding polypeptides resembling AMPs were activated owing to the protection of pupal cuticle from bacterial infections in the late pupal stage (Wright et al., 1996). Therefore, their transcription or protein expression levels might not truly reflect the response. After 72 h in the unparasitized pupae, the eclosion of adults begins within the next few hours. Consequently, we did not monitor the transcription level and the protein expression level beyond 72 h. In fact, it is more complicated to evaluate the definite effect of parasitism on the Toll, Imd and JAK/STAT pathways. We postulate that venom plays major roles during this process.

Previous studies on the regulation of *Drosophila* immune response by venom were mainly performed on larval endoparasitoids. In particular, the genera *Leptopilina*, *Ganaspis* and *Asobara* have been well studied in the context of immunology. Our findings provided preliminary information on the immunological interplay between *D. melanogaster* and its pupal ectoparasitoid *P. vindemmiae* for the first time (**Figure 9**). Based on the reported evidence, the importance of host immune regulation by ectoparasitoids could be summarized as follows. First, many immune-related proteins have been identified in ectoparasitoid venoms such as serine proteases and serine protease inhibitors (De Graaf et al., 2010; Zhu, 2016). Our unpublished results showed that immune-related proteins occupy the major categories of proteins in *P. vindemmiae* venom, a result consistent with these reports. It is reasonable then to believe that venom is important for ectoparasitoids to regulate the host immune response. In ectoparasitoid *S. guani*, venom was essential for the survival of the larvae to avoid host cellular immune attack, considering that they would contact with the hosts' hemolymph released from the puncture wound (Li et al., 2018). Similarly, *P. vindemmiae* injected venom into the host hemocoel prior to laying eggs, and it is inferred that the persistently virulent effects on cellular defense of the host *Drosophila* also contributes to the successful development of their offspring, allowing the wasp offspring to feed more readily. More evidence revealed that envenomation by *N. vitripennis* induced significant increases in AMPs and their corresponding regulatory genes (Martinson et al., 2014), and our finding is consistent with the reported data. Based on this elaboration, we guess that the activation of the immune pathway of the host *Drosophila* is a preventative measure against bacterial or fungal infection and further enhances the nutritional quality of the host for larval feeding. In the host *Sarcophaga bullata*, melanization is an elaborative humoral immune response against foreign proteins such as parasitoid venom, and was inhibited by calreticulin from *N. vitripennis* venom (Siebert et al., 2015). Thus, it is speculated that the suppression of the

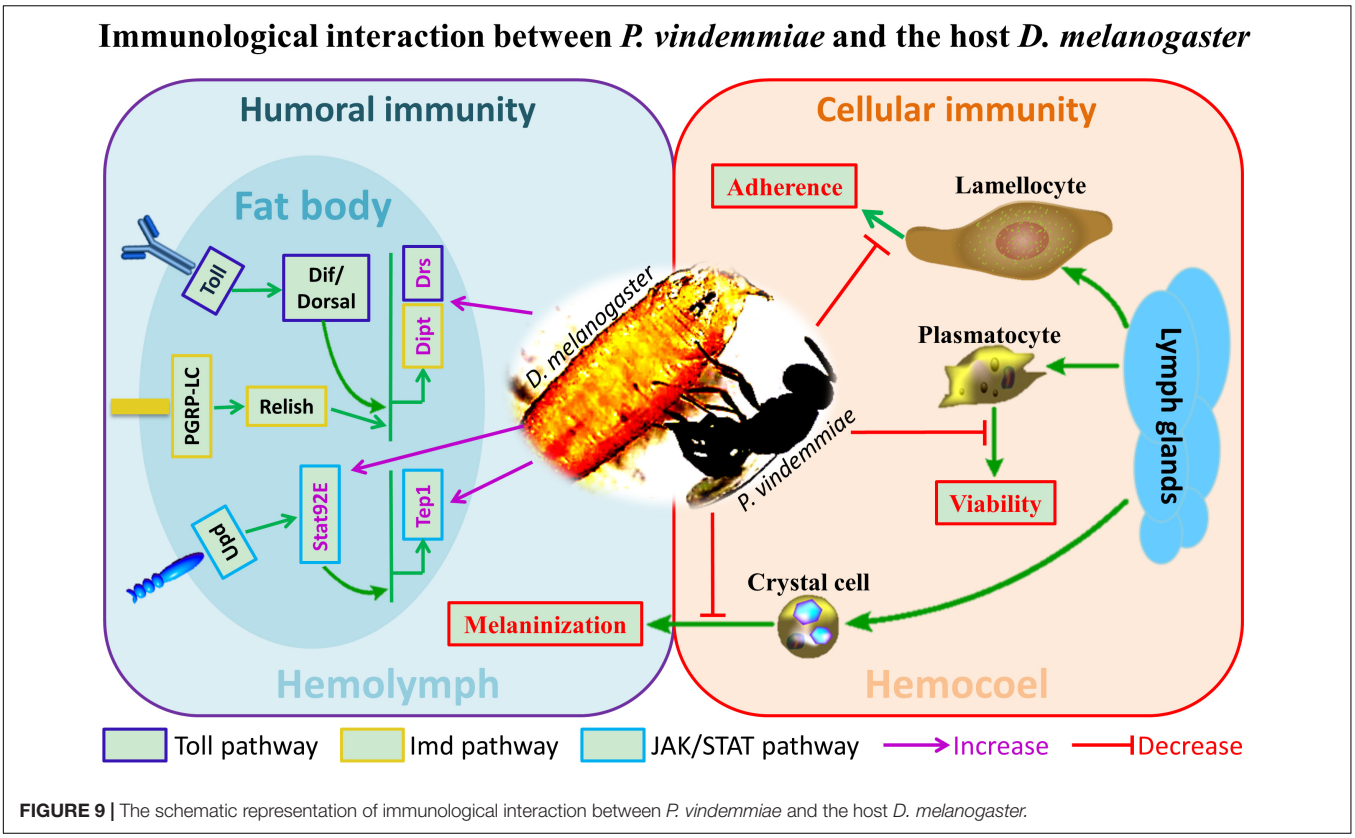

FIGURE 9 | The schematic representation of immunological interaction between *P. vindemmiae* and the host *D. melanogaster*.

host melanization is a coping strategy to avoid the dysfunction of venom for *P. vindemmiae*. Taken together, it is of vital importance for ectoparasitoid *P. vindemmiae* to regulate the host immune response. However, more research is still needed for understanding the underlying regulatory mechanisms.

As a groundbreaking research into the pupal ectoparasitoid of *D. melanogaster*, *P. vindemmiae* is attracting considerable interest in the field of biological control of fruit flies. Several attempts have been made on its potential for biological control in the last few years. One of the practical cases was that *P. vindemmiae* has prospects for control of olive fruit fly, *Bactrocera oleae* (Hoelmer et al., 2011). Additionally, a recent article reviewed that *Trichopria drosophilae*, a pupal parasitoid of *D. suzukii*, is desirable for high efficacy in biological control (Woltering et al., 2019). Similarly, as a well-known generalist pupal parasitoid of Diptera Cyclorrhapha, *P. vindemmiae* also possesses enormous potential on control of *D. suzukii* (Bonneau et al., 2019; Schlesener et al., 2019). To sum up, our explorative work on the crosstalk between ectoparasitoid *P. vindemmiae* and the host *Drosophila* will lay a foundation for providing new insights into biological control of fruit flies, and vastly propels the application of bio-control agent.

CONCLUSION

The ways that host immune systems are regulated vary greatly in different host/parasitoid systems. Here, we proposed that venom of *P. vindemmiae* functioned as the crucial regulator in cellular and humoral immune signaling of the host *D. melanogaster*. Our test results showed that the decreased cell adhesion and viability, weakened hemolymph melanization and dysfunctions of the Toll, Imd and JAK/STAT pathways were associated with the high potency of venom. However, where the actual targets of venom lied is still unknown. The data presented in this study clearly advance the knowledge of the immunological interaction between *Drosophila* and its pupal ectoparasitoid *P. vindemmiae*.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

We declare that appropriate ethical approval and licenses were obtained during our research.

AUTHOR CONTRIBUTIONS

LY, BW, and B-BW performed the experiments. LY, M-ML, and QF analyzed the data. LY, QF, Q-SS, and G-YY designed the experiments. LY, Q-SS, and G-YY

wrote the manuscript. All authors gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01282/full#supplementary-material

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Identification and Comparative Analysis of Venom Proteins in a Pupal Ectoparasitoid, *Pachycrepoideus vindemmiae*

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Parasitoid wasps inject venom containing complex bioactive compounds to regulate the immune response and development of host arthropods and sometime paralyze host arthropods. Although extensive studies have been conducted on the identification of venom proteins in larval parasitoids, relatively few studies have examined the pupal parasitoids. In our current study, a combination of transcriptomic and proteomic methods was used to identify 64 putative venom proteins from *Pachycrepoideus vindemmiae*, an ectoparasitoid of *Drosophila*. Expression analysis revealed that 20 tested venom proteins have 419-fold higher mean expression in the venom apparatus than in other wasp tissues, indicating their specialization to venom. Comparisons of venom proteins from *P. vindemmiae* and other five species spanning three parasitoid families detected a core set of "ancient" orthologs in Pteromalidae. Thirty-five venom proteins of *P. vindemmiae* were assigned to the orthologous groups by reciprocal best matches with venoms of other pteromalids, while the remaining 29 were not. Of the 35 categories, twenty-seven have orthologous relationships with *Nasonia vitripennis* venom proteins and 25 with venoms of *Pteromalus puparum*. More distant relationships detected that five and two venom proteins of *P. vindemmiae* are orthologous with venoms of two Figitidae parasitoids and a Braconidae representative, respectively. Moreover, twenty-two venoms unique to *P. vindemmiae* were also detected, indicating considerable interspecific variation of venom proteins in parasitoids. Phylogenetic reconstruction based on a set of single-copy genes clustered *P. vindemmiae* with *P. puparum*, *N. vitripennis*, and other members of the family Pteromalidae. These findings provide strong evidence that *P. vindemmiae* venom proteins are well positioned for future functional and evolutionary studies.

Keywords: parasitoid, venom, *Pachycrepoideus vindemmiae*, *Drosophila*, Pteromalidae

INTRODUCTION

Hymenopteran parasitoids dampen oscillations of insect populations, including those of agricultural and livestock pests. Parasitoids differ in egg-laying behavior (Pennacchio and Strand, 2006). Ectoparasitoids lay eggs on the surface of the host. In contrast, endoparasitoids oviposit into the host hemocoel (Asgari and Rivers, 2011). To assist successful development of offspring, female wasps inject multiple virulence factors into the host, including venom proteins (Asgari and Rivers, 2011; Moreau and Asgari, 2015; Wan et al., 2019), polydnaviruses (PDVs) (Ye et al., 2018), ovarian fluids (Teng et al., 2019), virus-like particles (VLPs) (Harvey et al., 2013). Venom proteins, an indispensable part of virulence factors, inhibit immunity, interrupt development, and regulate metabolism of the host (Asgari and Rivers, 2011; Moreau and Asgari, 2015).

Venom proteins comprise various bioactive molecules, such as enzymes, protease inhibitors, recognition and binding proteins, and other unknown compounds (Asgari and Rivers, 2011; Moreau and Asgari, 2015). So far, many venom components have been extensively investigated, such as RhoGAP (Labrosse et al., 2005a,b; Colinet et al., 2007), calreticulin (CRT) (Zhang et al., 2006; Wang et al., 2013; Siebert et al., 2015), sarco/endoplasmic reticulum calcium ATPase (SERCA) (Mortimer et al., 2013), GH1 β-glucosidase (Hubert et al., 2016), α-amylases (Wang et al., 2019), Crp32B (Teng et al., 2019). They inhibit host cell spreading, prevent host cellular encapsulation, suppress host hemolymph melanization or hydrolyze nutrients of the host to guarantee survival of wasp progeny. Besides, venom proteins also cooperate with other virulence factors to outwit the host. For instance, venom proteins of endoparasitoids, *Cotesia melanoscela*, *Pseudoplusia includens* and *Cotesia rubecula*, acted synergistically with PDVs in altering host physiological status (Stoltz et al., 1988; Michael and Noda, 1991; Zhang et al., 2004b). Additionally, previous studies have demonstrated the synergized effects of venom and calyx fluids on host's development and immune responses (Strand and Dover, 1991; Teng et al., 2016).

Identification of these venom proteins is the first step to study their functions. Recently, high-throughput sequencing and mass spectrometry have made it technically feasible to isolate and identify venom proteins in parasitoids (Danneels et al., 2010; Vincent et al., 2010; Goecks et al., 2013; Mortimer et al., 2013; Hubert et al., 2016; Laurino et al., 2016; Yan et al., 2016; Teng et al., 2017), as well as in snakes (Ching et al., 2006), scorpions (Ma et al., 2010), and spiders (Haney et al., 2014). Although there are as many as 600,000 parasitoid species (Heraty, 2009), few exhaustive studies have been carried out on their venom compositions and functions. To date, venom proteins of endoparasitoids are relatively well studied (Labrosse et al., 2005a,b; Colinet et al., 2009; Furihata et al., 2013; Goecks et al., 2013; Heavner et al., 2013; Mortimer et al., 2013). However, the venom protein repertoire of ectoparasitoids is less well known (Marchiori and Barbaresco, 2007; Marchiori et al., 2013; Chen et al., 2015; Marchiori and Borges, 2017).

Pachycrepoideus vindemmiae belongs to the family Pteromalidae (Hymenoptera). It is a versatile and solitary pupal ectoparasitoid that parasitizes various flies, including the genera of *Drosophila*, *Musca*, *Anastrepha*, *Calliphora*, and so on (Marchiori and Barbaresco, 2007; Marchiori et al., 2013; Marchiori and Borges, 2017). In this study, we examined the diversity of *P. vindemmiae* venom compositions based on both transcriptome-sequencing and proteome analysis, and compared it to venoms of three parasitoid families. Given the amazing toolkit available in its host drosophilids, we propose that future studies combining the power of *Drosophila* as a model system with its ectoparasitoid *P. vindemmiae* have great potentials for advancing our understanding of the functions and evolution of venom proteins, and assessing their pharmacological possibilities (Danneels et al., 2010, 2015; Moreau and Asgari, 2015; Huerta-Rey et al., 2017).

MATERIALS AND METHODS

Insect Rearing

The *Pachycrepoideus vindemmiae* colony was kindly provided by Prof. Yongyue Lu (South China Agricultural University, Guangzhou, China) in January 2016. Subsequently, *P. vindemmiae* was maintained with *D. melanogaster* pupae at 25°C, with a photoperiod of 14:10 hr (light:dark), as described (Chen et al., 2015). After eclosion, the adults were held in glass containers and fed with 10% (v/v) honey solution.

Venom Apparatus Collection and Isolation of Total RNA

Mated female wasps aged 2–5 days were anesthetized at 4°C for 10 min, rinsed in 75% ethanol (v/v) once, and then rinsed in sterile phosphate-buffered saline (PBS, pH 7.2) thrice. Subsequently, the females were dissected in PBS containing 1 unit/μL Murine RNase inhibitor (Vazyme, Nanjing, China) on an ice plate under a Leica MZ 16A stereomicroscope (Leica, Wetzlar, Germany), the venom apparatus (venom reservoirs and associated glands, henceforth, called the VG) and carcasses (the female body minus venom apparatus, henceforth, called the CA) were collected into 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, United States), respectively. Total RNA was extracted according to the manufacturer's protocol. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, United States). RNA concentration was measured using the Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, United States). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, United States).

Construction and Sequencing of the cDNA Library

A total amount of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, United States) following the

manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was performed using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptas. Second strand cDNA synthesis was subsequently performed. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, NEBNext adaptors with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments of preferentially 150∼200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, United States). Then, 3 μl USER Enzyme (NEB, United States) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primers. Eventually, PCR products were purified (AMPure XP system), library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

Transcriptomic Data Analysis

Clean data were obtained by processing raw data of fastq format through in-house perl scripts. In this step, reads containing adapters, reads containing ploy-N and low quality reads were removed. At the same time, Q20, Q30, GC-content, and sequence duplication levels of the clean data were calculated. Then, clean reads were assembled using Trinity v2012-10-05 without a reference genome (Grabherr et al., 2011). After assembling, the longest cluster sequences from each transcript were chosen as the reference sequences for subsequent analyses (henceforth, called unigenes). All unigenes were annotated on NCBI non-redundant protein sequences (Nr) database using blastx with *e*-value $< 1e^{-5}$. We estimated the expression levels of transcripts using the software RSEM (Li and Dewey, 2011). Differentially expressed unigenes were defined using DESeq software with strict screening thresholds of a corrected *p*-value < 0.05, $|\log_2$ (VG readcount/CA readcount)$| > 1$ and FPKM ≥ 10 (Anders and Huber, 2010). Gene Ontology (GO) enrichment analysis was implemented using the GOseq R packages based on Wallenius non-central hyper-geometric distribution (*e*-value $< 1e^{-6}$) (Young et al., 2010). Additionally, KOBAS software was used for testing the statistical enrichment of differential expression genes in Kyoto Encyclopedia of Genes and Genomes pathways (KEGG, *e*-value $< 1e^{-10}$) (Mao et al., 2005).

Venom Protein Collection

Mated female wasps aged 2–5 days were anesthetized at 4°C for 10 min as described above, and then dissected in sterile PBS containing 1 mM ProteinSafe^{TM} Protease Inhibitor Cocktail (Transgen, Beijing, China) on an ice plate under a stereoscope (Leica, Wetzlar, Germany). The venom reservoir was separated and washed thrice, and then transferred into 1.5 mL Eppendorf tubes. After centrifugation at 18,000 *g* for 10 min, the supernatant was transferred into a new 1.5 mL Eppendorf tube and stored at −80°C until use. The concentration of the venom proteins was determined by a Modified Bradford Protein Assay Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol.

SDS-PAGE and LC-MS/MS Analyses of Venom Proteins

Proteins from *P. vindemmiae* venom reservoirs were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250. After quality inspection, the solution containing venom proteins was digested into peptides with trypsin and analyzed on a liquid chromatography tandem mass spectrometry (LC-MS/MS) system (LTQ-VELOS; Thermo Finnigan, San Jose, CA, United States). Subsequently, samples were desalted on Zorbax 300 SB-C18 columns (Agilent Technologies, Wilmington, DE, United States), and then separated on a RP-C18 column (150 m i.d., 150 mm length) (Column technology Inc., Fremont, CA, United States). Buffer A was water with 0.1% formic acid; Buffer B was 84% acetonitrile with 0.1% formic acid. The Buffer B gradient was: 0–3 min, from 3% to 9%; 3–93 min, from 9% to 32%; 93–108 min, from 32% to 40%; 108–113 min, from 40% to 100%; and 113–120 min, 100%. The raw data from one proteome were generated and the identified peptide fragments were searched against the translated transcriptomic sequences of VG using the Sequest search algorithm (Eng et al., 1994). The parameters were set as follows: carbamidomethyl was set as a fixed modification, and oxidation was set as a variable modification, the cross-correlation scores (Charge = 1, XCorr $\geq$ 1.9; Charge = 2, XCorr $\geq$ 2.2; Charge = 3, XCorr $\geq$ 3.75, and delta CN $\geq$ 0.1) were used as the filter criteria. This part of experiment was conducted by Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China).

qPCR

Total RNA of the VG and CA were separately extracted. cDNA was synthesized from 1 μg RNA using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing, China). All specific primers for qPCR were designed by AlleleID 6 software (PREMIER Biosoft, Palo Alto, CA, United States). The qPCR was run in the CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States) using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) according to the manufacturer's protocol. The programs were set as follows: enzyme activation at 95°C for 30 s, followed by 40 cycles with denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s, and a melting curve analysis. mRNA expression levels were normalized to the reference (28S rRNA) (Ballinger and Perlman, 2017), and quantified based on the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The experiments were repeated 3 times.

Sequence Analysis and Phylogenetic Construction of Venom Proteins

The online software SignalP 4.1 was used for predicting signal peptides. Protein tertiary structure was modeled by the homology-modeling server SWISS-MODEL, as described (Biasini et al., 2014). Multiple sequence alignment was performed by Clustal Omega, and visualized using the ESPript 3.0 server (Robert and Gouet, 2014). We used a web resource, Simple Modular Architecture Research Tool, for identification and annotation of protein domains (Letunic and Bork, 2017). The motif-based sequence logo was generated using WebLogo (Crooks et al., 2004). For phylogenetic construction of "His_Phos_2 domain," low quality regions were removed by Gblocks Server. Subsequently, the phylogenetic tree was constructed by Mega 6 software using the maximum likelihood method with 1000 bootstrap values (Tamura et al., 2013), and visualized using the interactive tree of life (iTOL) v3 (Letunic and Bork, 2016).

Comparative Analysis of Parasitoid Venom Repertoires

Venom protein sequences of six parasitoids were obtained from previous literature (De Graaf et al., 2010; Goecks et al., 2013; Yan et al., 2016; Teng et al., 2017), including 55 entries in *Cotesia chilonis*, 169 in *Leptopilina boulardi*, 176 in *Leptopilina heterotoma*, 79 in *N. vitripennis*, 70 in *P. puparum* and 64 in *P. vindemmiae* (this study). Venom proteins were assigned to orthologous groups using OrthoMCL with a cutoff *p*-value of $1e^{-5}$ (Doerks et al., 2002). The categories best-reciprocal matches with other parasitoids' venom proteins were defined as orthologs, and vice versa, as species specific venom proteins. Then, the numbers of orthologs and species specific venom proteins were counted, respectively, and a clustered heatmap was constructed using TBtools v0.6669 based on the proportion of orthologs in total venom proteins (Chen et al., 2018).

Phylogeny of *Pachycrepoideus vindemmiae*

The phylogeny was reconstructed based on a data set of 107 proteins (Lindsey et al., 2018). Protein sequences from *P. vindemmiae* (this study), *P. puparum* (GECT00000000.1), *Aphelinus abdominalis* (GBTK00000000.1), and *Leptomastix dactylopii* (GBNE00000000.1) transcriptomes were assigned to these 107 protein groups by the best-reciprocal hits from searching between the translated transcriptome and the *N. vitripennis* genome (OGSv2.0[1]). Blastp was conducted with an *e*-value cut-off $< 1e^{-5}$. All 107 protein sequences were aligned by Mafft v7.123b, using the L-INS-i alignment algorithm (Katoh and Standley, 2013). The alignments were filtered by trimAI version 1.2rev59, with automated settings, and then concatenated using AMAS. The tree was conducted using RAxML v8.0.20 by setting the substitution model as "PROTGAMMAAUTO" with 1000 bootstraps (Stamatakis, 2014), and visualized with iTOL v3 (Letunic and Bork, 2016).

[1]http://arthropods.eugenes.org/genes2/nasonia/genes/

RESULTS AND DISCUSSION

Identification of Venom Proteins by Transcriptomic Method

Analyses of Venom Apparatus and Carcasses Transcriptome

Four cDNA libraries were separately generated and then sequenced, including three replicates from *P. vindemmiae* VG and one from CA. We obtained 50,757,700 bp, 50,391,012 bp, 42,563,398 bp, and 47,774,240 bp sequenced raw reads from VG1, VG2, VG3, and CA, respectively. Then, raw reads were filtered to eliminate low quality reads, and 49,355,482 bp clean reads for VG1, 48,780,604 bp for VG2, 41,347,730 bp for VG3, and 46,348,590 bp for CA were acquired. Assembly statistics showed that the transcripts result in N50, N90, and mean lengths of 2,186 bp, 267 bp, and 819 bp. For all transcripts, the longest from the same transcription locus were regarded as unigenes. Finally, 61,747 unigenes representing 161,819 transcripts were obtained. N50, N90, and the mean lengths were 3,566 bp, 622 bp, and 1,684 bp. Among 61,747 unigenes, 36,122 (58.5%) got matches in the Nr database using blastx with an *e*-value $< 1e^{-5}$.

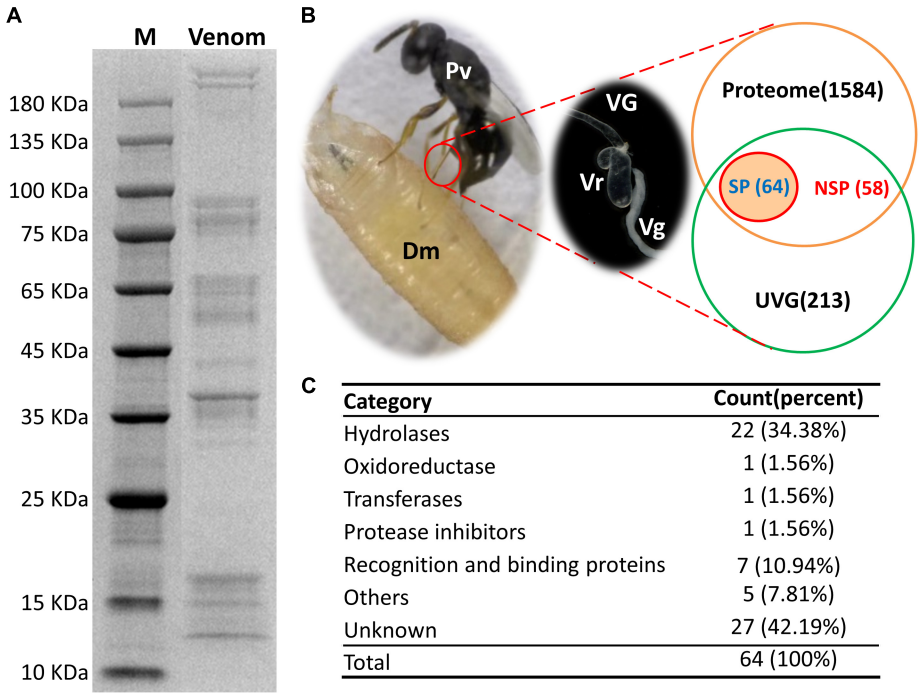
Functional Characterization of Upregulated Unigenes in Venom Apparatus

To define a robust set of venom proteins by transcriptomic method, we assumed that venom proteins were significantly higher expression in VG relative to CA. Hereby, differentially expressed genes were defined with the screening thresholds of a corrected *p*-value < 0.05, $|\log_2$ (VG readcount/CA readcount)$| > 1$ and FPKM ≥ 10. By these criteria, 398 differentially expressed unigenes were identified, including 335 upregulated unigenes in VG (UVG) compared to CA (**Supplementary Table S1**) and 63 downregulated items. We annotated the 335 UVG in the GO consortium database. Genes categorized as having "hydrolase activity" (GO: 0016787) were most abundant (97) (**Supplementary Figure S1**). It is inferred that the "hydrolase activity" proteins participate in immune and metabolic regulation of the host. In contrast, the 63 downregulated unigenes were enriched for GO categories myosin complex, actin cytoskeleton and motor activity.

To represent our knowledge of 335 UVG on molecular interaction, reaction and relation networks, their participation in KEGG pathways were assessed. On the basis of annotations, we sorted them into different categories. Among the top 20 annotated pathways, the most enriched categories pertained to RNA degradation (5 unigenes) and aminoacyl-tRNA biosynthesis pathways (5 unigenes) (**Supplementary Figure S2**). These unigenes may regulate host metabolism, degrade host nutrients and eventually provide additional energy for wasp larval development.

Identification of Venom Proteins by Proteomic Approach

Pachycrepoideus vindemmiae venom proteins (20 μg) were analyzed by 12 % SDS-PAGE. As shown in **Figure 1A**, venom proteins' molecular weights ranged from 12 kDa



C

Category	Count(percent)
Hydrolases	22 (34.38%)
Oxidoreductase	1 (1.56%)
Transferases	1 (1.56%)
Protease inhibitors	1 (1.56%)
Recognition and binding proteins	7 (10.94%)
Others	5 (7.81%)
Unknown	27 (42.19%)
Total	64 (100%)

FIGURE 1 | Identification of putative venom proteins in *Pachycrepoideus vindemmiae* combining transcriptomic and proteomic analyses. **(A)** 12% SDS-PAGE analysis of *P. vindemmiae* venom proteins followed by Coomassie Brilliant Blue staining. "M" denotes molecular weight marker. **(B)** Venn diagram of putative venom proteins combining transcriptomic and proteomic analyses. Pv, *P. vindemmiae*; Dm, *D. melanogaster*; VG, venom apparatus (including venom reservoirs and associated glands); Vr, venom reservoir; Vg, venom gland; UVG, upregulated unigenes in VG compared to CA, Proteome: identified unigenes from proteome; SP, unigenes with a secretory signal peptide in their amino acid sequences; NSP, unigenes without a secretory signal peptide in their amino acid sequences. **(C)** Categories of the 64 putative *P. vindemmiae* venom proteins based on annotations in the Nr database.

to more than 180 kDa and 38 kDa venom proteins were particularly abundant. After quality inspection, 100 μg original venom proteins were directly digested with trypsin and analyzed by LC-MS/MS. Raw reads from one proteome were generated, and the identified peptide fragments were searched against the translated transcriptomic sequences of VG. Finally, 1,706 unigenes from the venom reservoirs got matches in transcriptome of VG with a strict filtration standard (FDR < 0.01) (**Supplementary Table S2**).

Identification of Putative Venom Proteins Combining Transcriptomic and Proteomic Analyses

Venom proteins were identified under the assumption that they would show significantly higher expression in VG relative to CA (335) and were confirmed by proteome (1706) (**Figure 1B**). Based on these standards, 122 unigenes were identified. It is noticeable that a venom gene does not have to be higher expressed in VG, such as *PpS1V* in *P. puparum* (Yan et al., 2017). Further research should be undertaken to investigate low abundance venom components. However, we decided to focus further on this set of genes showing significantly higher expression in VG relative to other tissues and present in the venom reservoir proteome.

Venom proteins secreted by parasitoid wasp' venom gland cells are expected to be secretory (Yan et al., 2016). Therefore, unigenes with initiation codons of the 122 candidates were computationally translated into peptides followed by a signal peptide prediction. The remaining unigenes without initiation codons were searched against the Nr database and their best matched sequences were retrieved from NCBI as references for a signal peptide prediction. Ultimately, 64 proteins were identified as putative venom proteins based on enhanced expression in VG, proteomic analysis, and a predicted signal peptide (**Figure 1B**). Besides, the expression fold-changes of 64 putative venom proteins ranged from 6.66 to infinity in VG relative to CA (**Table 1**), consistent with their likely venom functions. These candidates were subjected to further study.

Verification of Putative Venom Proteins by qPCR

Venom proteins can be more broadly expressed in the wasp VG, and highly specialized for their functions (Siebert et al., 2015; Martinson et al., 2017). To reveal the expression levels of 64 putative venom proteins in VG and to contrast with CA, we randomly selected 20 venom genes for qPCR verification. The expression fold-changes of 20 tested genes ranged from 1.50 to 4909.41, with a mean value of 418.53 and 10 of those (50%) showed greater than 100-fold increase in VG relative to CA, with 16 (80%) greater than 10-fold (**Figure 2**), indicating their specialization for venom functions.

TABLE 1 | Venom proteins identified in *P. vindemmiae* by combined transcriptomic and proteomic analyses.

Gene ID	VG1	VG2	VG3	Carcass	Log_2 (VG readcount/CA readcount)	NR Description (Blastp)		
	FPKM	FPKM	FPKM	FPKM		Accession number	*E*-value	Putative function
Hydrolases								
Cluster-8535.6348	19557	19579	97.08	11	11.081	XP_008214647.1	1.10E−43	PREDICTED: serine proteinase stubble isoform X2 [*Nasonia vitripennis*]
Cluster-8535.6313	836.53	957.24	220.24	0.64	11.06	XP_001599566.1	2.50E−54	PREDICTED: ribonuclease Oy-like [*Nasonia vitripennis*]
Cluster-8535.6305	153.65	162.55	10.17	2.18	6.7107	XP_001600074.1	1.80E−186	PREDICTED: serine protease easter [*Nasonia vitripennis*]
Cluster-8535.6218	363.86	363.47	48	0	Inf	XP_008214285.1	1.70E−66	PREDICTED: chymotrypsin-1-like [*Nasonia vitripennis*]
Cluster-8535.6181	353.98	395.1	186.31	0	Inf	NP_001166082.1	2.70E−32	Serine protease 64 precursor [*Nasonia vitripennis*]
Cluster-8535.6139	48.33	62.27	13.84	0	Inf	XP_001600770.1	1.00E−104	PREDICTED: venom acid phosphatase Acph-1-like [*Nasonia vitripennis*]
Cluster-8535.6129	709.9	861.32	129.65	0.07	14.111	XP_001600770.1	8.40E−126	PREDICTED: venom acid phosphatase Acph-1-like [*Nasonia vitripennis*]
Cluster-8535.6096	787.64	840.59	55.25	0	Inf	XP_001599566.1	1.20E−50	PREDICTED: ribonuclease Oy-like [*Nasonia vitripennis*]
Cluster-8535.6041	722.84	819.52	290.18	0.13	13.254	NP_001155016.1	4.90E−86	Serine protease homolog 29 precursor [*Nasonia vitripennis*]
Cluster-8535.6027	392.38	479.24	22.41	0.04	13.94	NP_001166082.1	1.30E−47	Serine protease 64 precursor [*Nasonia vitripennis*]
Cluster-8535.5971	476.58	559.55	322.5	0	Inf	XP_001606746.2	2.60E−51	PREDICTED: venom metalloproteinase 2-like [*Nasonia vitripennis*]
Cluster-8535.5960	252	261.04	32.79	0.17	11.122	XP_008216710.1	3.70E−170	PREDICTED: lipase 3-like [*Nasonia vitripennis*]
Cluster-8535.5821	203.77	241.77	103.02	0	Inf	NP_001166092.1	8.60E−07	Serine protease 87 precursor [*Nasonia vitripennis*]
Cluster-8535.25150	356.01	388	123.85	0	Inf	XP_003424313.1	8.10E−113	PREDICTED: LOW QUALITY PROTEIN: xaa-Pro aminopeptidase 1 [*Nasonia vitripennis*]
Cluster-8535.25095	135.52	168.77	38.94	0	Inf	XP_003424313.1	1.80E−111	PREDICTED: LOW QUALITY PROTEIN: xaa-Pro aminopeptidase 1 [*Nasonia vitripennis*]
Cluster-8535.25089	270.84	86.54	74.05	0	Inf	NP_001164348.1	4.10E−70	Serine protease precursor [*Nasonia vitripennis*]
Cluster-8535.25073	164.9	179.82	73.26	0.04	12.665	XP_008214271.1	2.10E−106	PREDICTED: chymotrypsin-2-like [*Nasonia vitripennis*]
Cluster-8535.25068	300.9	349.25	22.27	0.04	13.668	XP_003424313.1	1.70E−111	PREDICTED: LOW QUALITY PROTEIN: xaa-Pro aminopeptidase 1 [*Nasonia vitripennis*]
Cluster-8535.25024	301.44	311.46	98.65	0.5	9.9185	XP_012262898.1	4.70E−120	PREDICTED: neprilysin-like [*Athalia rosae*]
Cluster-8535.25017	4271.4	4371.7	1724.6	0	Inf	NP_001155017.1	2.10E−78	Serine protease 33 precursor [*Nasonia vitripennis*]
Cluster-8535.24980	5233.6	5127.5	704.54	0	Inf	NP_001155087.1	3.50E−102	Endonuclease-like venom protein precursor [*Nasonia vitripennis*]
Cluster-8535.24978	536.25	533.46	19.91	0.58	10.384	ACA60733.1	3.10E−169	Venom acid phosphatase [*Pteromalus puparum*]
Oxidordeuctase								
Cluster-8535.6260	556.41	462.2	177.86	3.49	7.8363	XP_001600327.1	7.60E−225	PREDICTED: glucose-6-phosphate 1-dehydrogenase [*Nasonia vitripennis*]
Transferase								
Cluster-8535.10302	1306.7	1291.7	47.55	0.01	16.952	XP_001607488.2	1.70E−261	PREDICTED: gamma-glutamyltranspeptidase 1 [*Nasonia vitripennis*]

(Continued)

TABLE 1 | Continued

Gene ID	VG1	VG2	VG3	Carcass	Log_2 (VG readcount/CA readcount)	NR Description (Blastp)		
	FPKM	FPKM	FPKM	FPKM		Accession number	*E*-value	Putative function
Protease inhibitor								
Cluster-8535.6176	97.85	125.3	19.56	0.05	11.552	NP_001164350.1	2.30E−23	Kazal type serine protease inhibitor-like venom protein 2 precursor [*Nasonia vitripennis*]
Recognition/binding proteins								
Cluster-8535.6319	1048.3	1079.4	68.23	1.3	10.211	XP_001604854.1	5.50E−235	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Cluster-8535.6269	118.07	99.09	62.88	0.14	10.33	XP_003425456.1	5.30E−108	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Cluster-8535.6043	691.26	901.49	234.82	1.7	9.5069	NP_001164343.1	2.80E−25	Chitin binding protein-like venom protein precursor [*Nasonia vitripennis*]
Cluster-8535.25625	20.94	19.82	55.3	0	Inf	XP_003425456.1	5.60E−108	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Cluster-8535.25152	37.77	34.53	52.24	0	Inf	XP_003425456.1	5.40E−108	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Cluster-8535.25072	208.28	169.21	41.39	0	Inf	XP_003425456.1	3.50E−221	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Cluster-8535.24989	856.24	894.02	42.53	1.12	9.955	XP_001604854.1	1.20E−35	PREDICTED: low-density lipoprotein receptor-related protein 2-like [*Nasonia vitripennis*]
Others								
Cluster-8535.6175	494.74	415.13	870.57	0.05	14.205	NP_001155022.1	1.80E−18	Cysteine-rich/TIL venom protein 2 precursor [*Nasonia vitripennis*]
Cluster-8535.6002	206.01	173.37	110.46	0.75	8.7008	XP_001604583.1	4.40E−37	PREDICTED: probable salivary secreted peptide [*Nasonia vitripennis*]
Cluster-8535.5294	188.51	143.89	210.12	0.14	11.104	XP_003708569.1	9.20E−08	PREDICTED: venom allergen 3-like [*Megachile rotundata*]
Cluster-8535.13847	138.85	179.77	66.68	0	Inf	XP_003423804.1	1.20E−42	PREDICTED: probable salivary secreted peptide [*Nasonia vitripennis*]
Cluster-8535.12135	67.17	30.36	14.72	0.36	7.6995	NP_001154978.1	2.80E−149	Major royal jelly protein-like 9 precursor [*Nasonia vitripennis*]
Unknown proteins								
Cluster-8535.6347	286.78	376.42	39.01	0	Inf	XP_011502473.1	3.50E−07	PREDICTED: uncharacterized protein LOC105365896 [*Ceratosolen solmsi marchali*]
Cluster-8535.6343	763.62	906.56	151.8	0.01	17.273	XP_008206401.1	5.70E−87	PREDICTED: uncharacterized protein LOC103316135 [*Nasonia vitripennis*]
Cluster-8535.6342	8523.7	8690.2	3580.7	0	Inf	XP_003426464.2	1.50E−23	PREDICTED: uncharacterized protein LOC100679170 [*Nasonia vitripennis*]
Cluster-8535.6295	7027	6416.4	2015.4	0	Inf	XP_008206613.1	1.80E−25	PREDICTED: venom protein H isoform X1 [*Nasonia vitripennis*]
Cluster-8535.6272	636.42	745.66	221.15	0	Inf	XP_003398548.1	3.60E−10	PREDICTED: uncharacterized protein LOC100649303 [*Bombus terrestris*]
Cluster-8535.6259	2842.6	2169.4	500.37	0.05	16.055	XP_003427828.1	4.90E−13	PREDICTED: uncharacterized protein LOC100678638 [*Nasonia vitripennis*]
Cluster-8535.6248	1980.5	2043.6	2440.1	0	Inf	XP_003424286.1	1.10E−17	PREDICTED: uncharacterized protein LOC100678044 [*Nasonia vitripennis*]
Cluster-8535.6242	301.5	302.67	157.3	0.02	15.009	XP_008206401.1	7.90E−83	PREDICTED: uncharacterized protein LOC103316135 [*Nasonia vitripennis*]
Cluster-8535.6206	915.67	1327.3	451.62	0.16	13.398	XP_001606832.1	1.30E−42	PREDICTED: uncharacterized protein LOC100123223 [*Nasonia vitripennis*]

(Continued)

TABLE 1 | Continued

Gene ID	VG1	VG2	VG3	Carcass	Log_2 (VG readcount/CA readcount)	NR Description (Blastp)		
	FPKM	FPKM	FPKM	FPKM		Accession number	*E*-value	Putative function
Cluster-8535.6090	1490.1	1773.6	1235.4	0.38	12.858	NP_001155029.1	9.00E−19	Venom protein L precursor [*Nasonia vitripennis*]
Cluster-8535.6065	408.8	511.71	390.53	0	Inf	NP_001155028.1	1.30E−13	Venom protein K precursor [*Nasonia vitripennis*]
Cluster-8535.5978	194.01	237.28	19.94	0.07	12.229	XP_001601835.2	8.40E−206	PREDICTED: uncharacterized protein LOC100117668 [*Nasonia vitripennis*]
Cluster-8535.5949	251.98	157.94	283.57	0.16	11.384	NP_001164349.1	4.50E−69	Venom protein N precursor [*Nasonia vitripennis*]
Cluster-8535.5822	566.96	673.72	346.48	0	Inf	XP_008214317.1	1.20E−39	PREDICTED: uncharacterized protein LOC103317616 [*Nasonia vitripennis*]
Cluster-8535.25723	145.24	149.64	77.56	0.02	13.436	XP_001603409.1	5.60E−240	PREDICTED: uncharacterized protein LOC100119678 [*Nasonia vitripennis*]
Cluster-8535.25580	27.42	27.49	16.69	0.2	7.8349	NP_001155170.1	9.50E−44	Venom protein U precursor [*Nasonia vitripennis*]
Cluster-8535.25386	81.38	101.64	22.74	0.34	8.7077	XP_001601022.2	2.00E−250	PREDICTED: uncharacterized protein LOC100116563 [*Nasonia vitripennis*]
Cluster-8535.25243	38.36	37.63	10.68	0.06	9.947	XP_008217420.1	2.60E−14	PREDICTED: venom protein J isoform X1 [*Nasonia vitripennis*]
Cluster-8535.25157	41.59	38.61	10.18	0.36	7.4324	NP_001155170.1	1.90E−44	Venom protein U precursor [*Nasonia vitripennis*]
Cluster-8535.25146	216.01	280.26	82.17	0	Inf	XP_008210187.1	1.00E−50	PREDICTED: uncharacterized protein LOC103316723 [*Nasonia vitripennis*]
Cluster-8535.25086	695.81	768.91	450.62	0.03	15.253	NP_001155041.1	4.00E−14	Venom protein V precursor [*Nasonia vitripennis*]
Cluster-8535.25078	393.08	470.5	33.21	0.18	11.769	XP_008210187.1	3.80E−240	PREDICTED: uncharacterized protein LOC103316723 [*Nasonia vitripennis*]
Cluster-8535.24993	168.07	87.31	15.91	0.04	12.065	XP_001601177.1	1.50E−203	PREDICTED: uncharacterized protein LOC100116763 [*Nasonia vitripennis*]
Cluster-8535.17059	1212.9	1223.6	588.32	4.33	8.839	XP_003424551.1	1.80E−43	PREDICTED: uncharacterized protein LOC100680008 [*Nasonia vitripennis*]
Cluster-8535.16907	309.8	281.19	22.68	1.87	7.8547	XP_008210468.1	2.00E−303	PREDICTED: uncharacterized protein LOC100680448 isoform X3 [*Nasonia vitripennis*]
Cluster-8535.16090	270.75	290.36	182.01	0.38	10.333	XP_001604126.2	0.00E+00	PREDICTED: uncharacterized protein LOC100120484 [*Nasonia vitripennis*]
Cluster-8535.12555	124.88	120.27	64.48	0	Inf	XP_001604126.2	0.00E+00	PREDICTED: uncharacterized protein LOC100120484 [*Nasonia vitripennis*]

The remaining genes are more probably multifunctional (Martinson et al., 2017). This result also accorded with the data of high throughput RNA sequencing for quantifying the transcriptional levels of venom genes.

Classification of *Pachycrepoideus vindemmiae* Putative Venom Proteins

According to the annotations of 64 putative venom proteins in the Nr database, 37 proteins could be assigned to functional categories (hereafter called "knowns"), while 27 could not (hereafter called "unknowns") (**Table 1**). We noted that these functional categories were inferred by protein sequence similarities to annotated proteins from other organisms, or to recognized protein motifs. The functional categories of 37 "knowns" fell into hydrolases, oxidoreductases, transferases, protease inhibitors, recognition and binding proteins, and others (**Figure 1C**). Of the 37 categories, 22 hydrolases occupied the majority, and proteins commonly found in parasitoid venoms, such as serine proteases, metalloproteinase, acid phosphatases were included. This result is consistent with previous studies in pteromalid venom proteins, also demonstrated that hydrolases were the most abundant category (De Graaf et al., 2010; Yan et al., 2016). Furthermore, an oxidoreductase, a transferase, a protease inhibitor, five others, and seven venom proteins involved in the recognition and binding activities were also identified.

Hydrolases

In this study, 10 serine proteases (SPs) and serine protease homologs (SPHs) were identified in *P. vindemmiae* venom proteins, including the typical chymotrypsin. The functions of SPs have been thoroughly investigated in *D. melanogaster*, one of the predominant hosts of *P. vindemmiae*, indicating

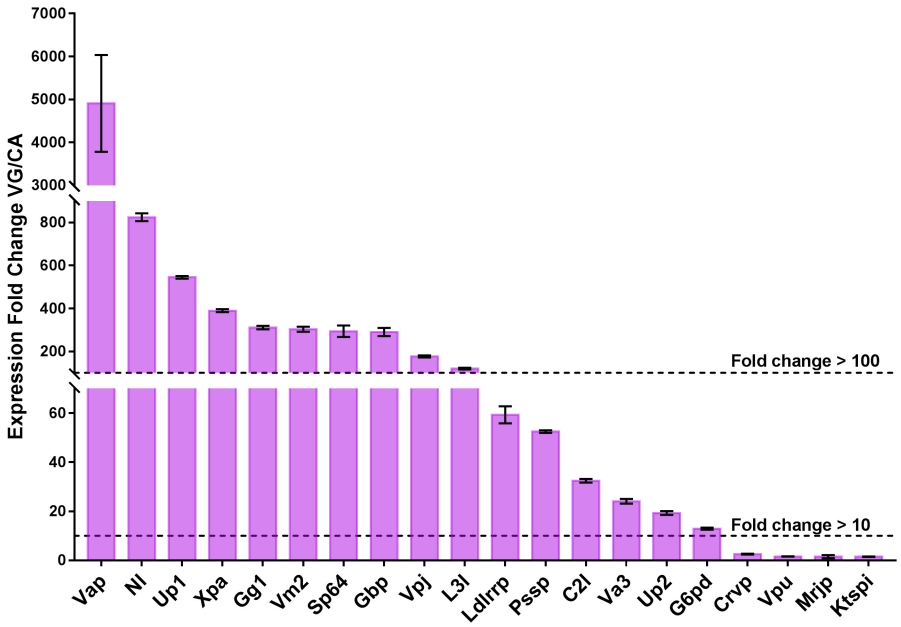


FIGURE 2 | qPCR verification of the 20 selected putative venom proteins. The expression levels of 20 venom proteins in VG are normalized to their mean expression levels in CA, and shown as the mean ± standard deviation. The primers are listed in **Supplementary Table S3**. Gene full names and sequence accession numbers are provided as follows. Xpa, Cluster-8535.25068, xaa-Pro aminopeptidase; Up1, Cluster-8535.6242, uncharacterized protein LOC103316135; Va3, Cluster-8535.5294, venom allergen 3-like; Up2, Cluster-8535.16090, uncharacterized protein LOC100120484; C2l, Cluster-8535.25073, chymotrypsin-2-like; Gbp, Cluster-8535.6043, chitin binding protein-like venom protein precursor; Vm2, Cluster-8535.5971, venom metalloproteinase 2-like; Mrjp, Cluster-8535.12135, major royal jelly protein-like 9 precursor; Vap, Cluster-8535.24978, venom acid phosphatase; Ldlrrp, Cluster-8535.6269, low-density lipoprotein receptor-related protein 2-like; Crvp:, Cluster-8535.6175, cysteine-rich/TIL venom protein 2 precursor; L3l, Cluster-8535.5960, lipase 3-like; Nl, Cluster-8535.25024, neprilysin-like; Gg1, Cluster-8535.10302, gamma-glutamyltranspeptidase 1; Vpj, Cluster-8535.25243, venom protein J isoform X1; Ktspi, Cluster-8535.6176, Kazal type serine protease inhibitor-like venom protein 2 precursor; Pssp, Cluster-8535.6002, probable salivary secreted peptide; G6pd, Cluster-8535.6260, glucose-6-phosphate 1-dehydrogenase; Sp64, Cluster-8535.6027, serine protease 64 precursor; Vpu, Cluster-8535.25580, venom protein U precursor. The experiments were repeated 3 times.

that these SPs were involved in the activation of Toll immune pathway and prophenoloxidase (PPO) cascade reaction (Jang et al., 2008). In contrast, researches on parasitoid venom SPHs have been paid more attention. For instance, a serine proteinase homolog Vn50 of *C. rubecula* interfered with the proteolytic cascade and inhibited the melanization of host hemolymph (Asgari et al., 2003; Zhang et al., 2004a). Recently, combined genomic and transcriptomic approaches identified six SPs and two SPHs in *P. puparum* venom proteins, and some SPs showed higher expression levels after immune stimulation, implying that they might participate in antimicrobial immunity processes (Yang et al., 2017). As demonstrated in previous studies, the "Tryp_SPc domain" is crucial for SPs and SPHs (Jang et al., 2008; Veillard et al., 2016). According to this point, we aligned the "Tryp_SPc domain" of SPH (Cluster-8535.6041) and SPs (Cluster-8535.25017, Cluster-8535.6181) from *P. vindemmiae* with those from *N. vitripennis* and *P. puparum*, and uncovered many conservative residues between *P. vindemmiae* and its relatives (**Figure 3**). Thus, we infer that venom SPs and SPHs of *P. vindemmiae* suppresses the host humoral immunity in similar manners to other pteromalids.

The ribonuclease Oy-like (Cluster-8535.6096) and endonuclease-like venom proteins (Cluster-8535.24980) of *P. vindemmiae* display higher identities with *N. vitripennis* (Blastp, 60% and 43%, respectively). A possible speculation for the presence of nucleases in *P. vindemmiae* venoms was that they cleave RNA of the host to confront its defensive responses (Trummal et al., 2016).

Three venom acid phosphatases (Cluster-8535.6129, Cluster-8535.6139, and Cluster-8535.24978) packed with a conserved "His_Phos_2 domain" were originally identified in *P. vindemmiae* venom proteins. As commonly known venom components in hymenopteran parasitoids, venom acid phosphatases also exist in *P. puparum* (Zhu et al., 2008), *L. heterotoma* (Heavner et al., 2013), *L. boulardi* (Colinet et al., 2013), *N. vitripennis* (Danneels et al., 2010), *Pimpla hypochondriaca* (Dani et al., 2005), and *Hyposoter didymator* (Doremus et al., 2013). Based on the phylogenetic analysis of "His_Phos_2 domain," we found that *P. vindemmiae* venom acid phosphatase (Cluster-8535.24978) and venom acid phosphatase Acph-1-like (Cluster-8535.6129) evolved into two branches (**Figure 4**), both clustering together with other pteromalids. Although studies noted the importance of venom acid phosphatases in parasitoids (Zhu et al., 2008), there were few exhaustive studies yet. We propose that the venom acid phosphatases of *P. vindemmiae* may play a special role in affecting the host's physiology and acquiring nutrients from the host hemolymph (Xia et al., 2000; Zhu et al., 2008).

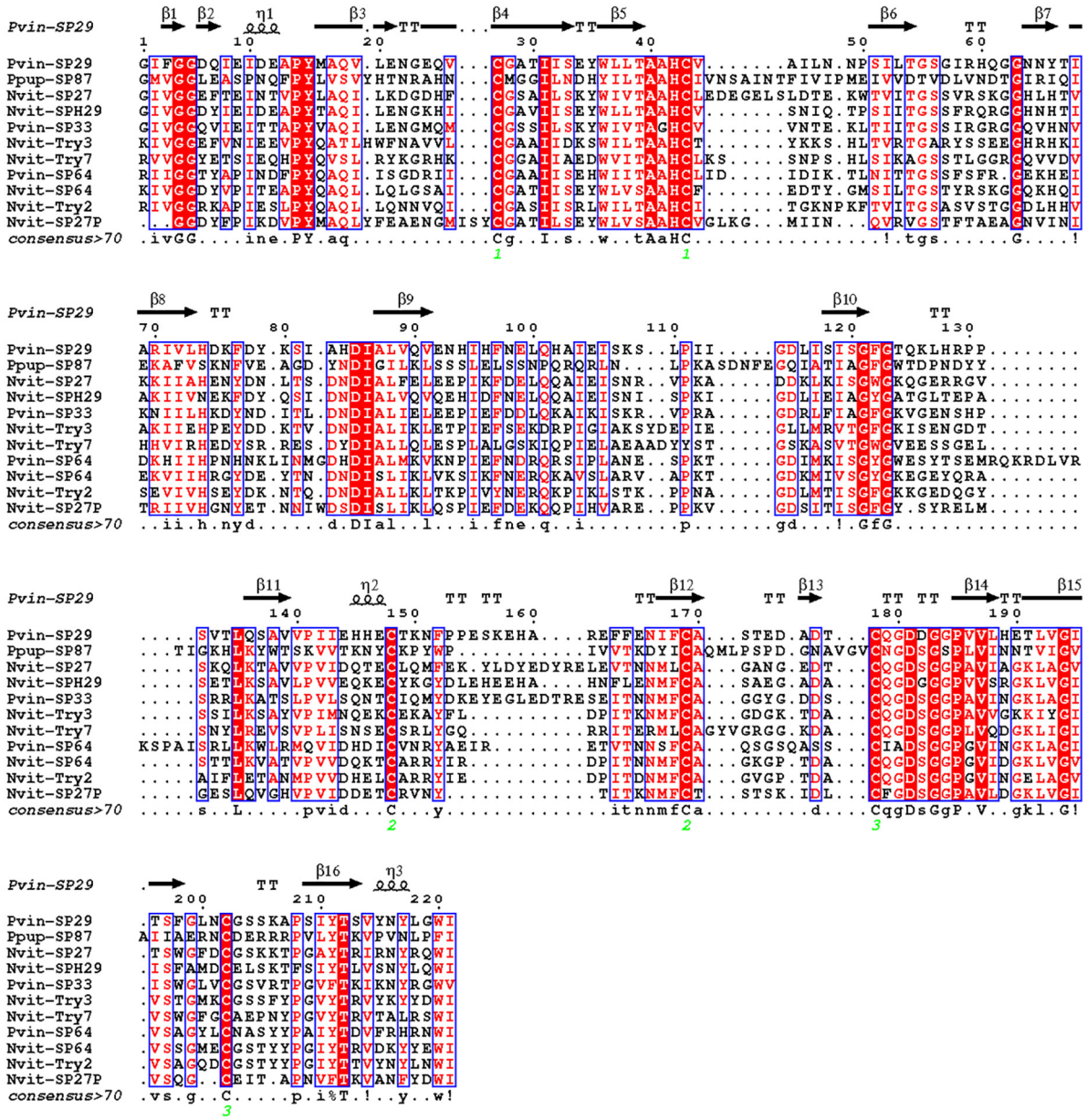


FIGURE 3 | Multiple sequence alignment of "Tryp_SPc domain" in SPs and SPHs. Multiple alignment was conducted using Clustal Omega and visualized by ESPript 3.0. Protein full names and sequence accession numbers are provided as follows. Pvin-SP29, *P. vindemmiae* serine protease homolog 29 precursor (Cluster-8535.6041); Pvin-SP33, *P. vindemmiae* serine protease 33 precursor (Cluster-8535.25017); Pvin-SP64, *P. vindemmiae* serine protease 64 precursor (Cluster-8535.6181); Ppup-SP87, *P. puparum* serine protease 87 precursor (comp43143_c1) (Yan et al., 2016); Nvit-SP64, *N. vitripennis* serine protease 64 precursor (NP_001166082.1); Nvit-Try2, *N. vitripennis* trypsin-2-like (XP_016843706.1); Nvit-SP27, *N. vitripennis* serine protease 33 precursor (NP_001155017.1); Nvit-Try3, *N. vitripennis* trypsin-3 (XP_001603705.2); Nvit-SP27P, *N. vitripennis* serine protease 27 precursor (NP_001166077.1); Nvit-SPH29, *N. vitripennis* serine protease homolog 29 precursor (NP_001155016.1); Nvit-Try7, *N. vitripennis* trypsin-7 (XP_016838732.1). Pvin-VAP, *P. vindemmiae* venom acid phosphatase (Cluster-8535.24978); Pvin-VAPA, *P. vindemmiae* venom acid phosphatase Acph-1-like (Cluster-8535.6129); Ppup-VAP, *P. puparum* venom acid phosphatase (ACA60733.1); Nvit-VAPP, *N. vitripennis* venom acid phosphatase-like precursor (NP_001155147.1); Nvit-VAPA1, *N. vitripennis* venom acid phosphatase Acph-1-like (XP_001605452.1); Nvit-VAPA1.1, *N. vitripennis* venom acid phosphatase Acph-1-like (XP_001600770.1); Lhet-VAPA1.1, *L. heterotoma* venom acid phosphatase Acph-1-like1 (comp1442_c0_seq1); Lhet-VAPA1.2, *L. heterotoma* venom acid phosphatase Acph-1-like2 (comp2636_c0_seq1); Lbou-VAPA1, *L. boulardi* venom acid phosphatase Acph-1-like (comp9544_c0_seq1); Tpre-VAPA1.2, *Trichogramma pretiosum* venom acid phosphatase Acph-1-like isoform X1 (XP_014234174.2); Fari-VAPA, *Fopius arisanus* venom acid phosphatase Acph-1-like (XP_011310108.1); Tsar-VAP, *T. sarcophagae* venom acid phosphatase (OXU23470.1); Mpha-VAPA, *Monomorium pharaonis* venom acid phosphatase Acph-1-like (XP_012537166.1); Ccin-VAPA, *Cephus cinctus* venom acid phosphatase Acph-1 (XP_015589422.1); Sinv-VAP, *Solenopsis invicta* venom acid phosphatase (XP_025987662.1); Veme-VAP1, *Vollenhovia emeryi* venom acid phosphatase Acph-1-like isoform X1 (XP_011864393.1); Veme-VAP2, *V. emeryi* venom acid phosphatase Acph-1-like (XP_011872642.1); Aros-VAP, *A. rosae* venom acid phosphatase Acph-1-like (XP_012251812.1).

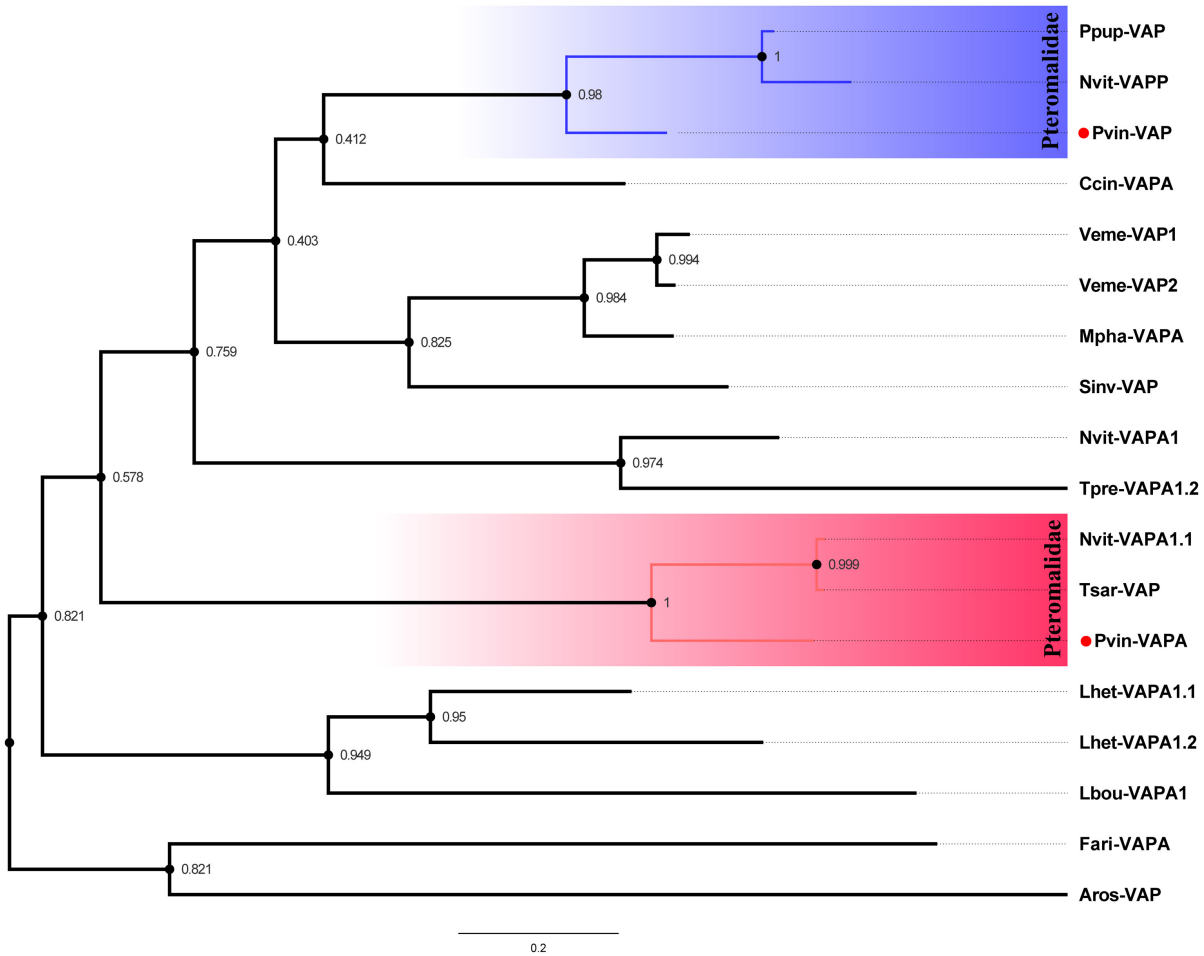


FIGURE 4 | Phylogenetic reconstruction based on "His_Phos_2 domain" of venom acid phosphatases. The phylogenetic tree was constructed by maximum likelihood method using the program Mega 6. 48 different amino acid substitution models were tested and the "LG+G+I" was considered to be the best model. The bootstrap values are presented on the nodes. Both venom proteins and non-venom orthologs were used for phylogenetic analysis. Full names of abbreviations are listed as follows. Pvin-VAP, *P. vindemmiae* venom acid phosphatase (Cluster-8535.24978); Pvin-VAPA, *P. vindemmiae* venom acid phosphatase Acph-1-like (Cluster-8535.6129); Ppup-VAP, *P. puparum* venom acid phosphatase (ACA60733.1); Nvit-VAPP, *N. vitripennis* venom acid phosphatase-like precursor (NP_001155147.1); Nvit-VAPA1.1, *N. vitripennis* venom acid phosphatase Acph-1-like (XP_001600770.1); Lhet-VAPA1.1, *L. heterotoma* venom acid phosphatase Acph-1-like1 (comp1442_c0_seq1); Lhet-VAPA1.2, *L. heterotoma* venom acid phosphatase Acph-1-like2 (comp2636_c0_seq1); Lbou-VAPA1, *L. boulardi* venom acid phosphatase Acph-1-like (comp9544_c0_seq1); Tpre-VAPA1.2, *Trichogramma pretiosum* venom acid phosphatase Acph-1-like isoform X1 (XP_014234174.2); Fari-VAPA, *Fopius arisanus* venom acid phosphatase Acph-1-like (XP_011310108.1); Tsar-VAP, *T. sarcophagae* venom acid phosphatase (OXU23470.1); Mpha-VAPA, *Monomorium pharaonis* venom acid phosphatase Acph-1-like (XP_012537166.1); Ccin-VAPA, *Cephus cinctus* venom acid phosphatase Acph-1 (XP_015589422.1); Sinv-VAP, *Solenopsis invicta* venom acid phosphatase (XP_025987662.1); Veme-VAP1, *Vollenhovia emeryi* venom acid phosphatase Acph-1-like isoform X1 (XP_011864393.1); Veme-VAP2, *V. emeryi* venom acid phosphatase Acph-1-like (XP_011872642.1); Aros-VAP, *A. rosae* venom acid phosphatase Acph-1-like (XP_012251812.1).

A venom protein of *P. vindemmiae* was annotated as metalloproteinase (Cluster-8535.5971). Metalloproteinases also present in *N. vitripennis* (De Graaf et al., 2010), *Chelonus inanitus* (Vincent et al., 2010), *L. boulardi* and *L. heterotoma* (Goecks et al., 2013), *H. didymator* (Doremus et al., 2013), *Microctonus hyperodae* (Crawford et al., 2008), *Microplitis demolitor* (Burke and Strand, 2014; Lin et al., 2018), and *Eulophus pennicornis* (Price et al., 2009). It was shown that recombinant venom protein metalloproteinase from *E. pennicornis* resulted in partial host mortality during molt and a delay in growth and development of the host (Price et al., 2009). Moreover, a compelling study suggested that the metalloproteinase from *M. mediator* venom reservoir interfered with host immune signaling cascades by binding to host nuclear factor kappa B (Lin et al., 2018). These studies will provide clues for functional characterization of metalloproteinase in *P. vindemmiae* venom proteins.

A lipase (Cluster-8535.5960) exists in *P. vindemmiae* venom reservoirs. Previous studies showed that *N. vitripennis* venom proteins induced alteration in lipid metabolism and arrested larval development of the host, implying that the lipase is an indispensable part of venom proteins (Rivers and Denlinger, 1995; Mrinalini et al., 2014). There is an abundant

room for determining the specific mechanism of lipase from *P. vindemmiae* venom proteins in regulating host lipid metabolism.

In this study, three aminopeptidases (Cluster-8535.25150, Cluster-8535.25095, and Cluster-8535.25068) were originally identified in *P. vindemmiae* venom proteins. In previous documented literatures, peptidases are crucial components in endoparasitoid venom proteins (Yan et al., 2016; Teng et al., 2017). It is inferred that aminopeptidases of *P. vindemmiae* are involved in the hydrolysis of host peptides, and further provide essential amino acids required for growth and development of offspring.

We identified a neprilysin in *P. vindemmiae* venom proteins (Cluster-8535.25024). Neprilysins have been characterized in many fly parasitoids, such as *Psyttalia lounsburyi*, *Psyttalia concolor* (Hubert et al., 2016), *L. boulardi* (Goecks et al., 2013), and *Ganaspis* sp. 1 (Mortimer et al., 2013). There is a great possibility that venom protein neprilysin modulates the host's immune responses. More investigations about their roles in parasitism are imperatively needed.

Oxidoreductase

An oxidoreductase of *P. vindemmiae* venom proteins was annotated as glucose-6-phosphate 1-dehydrogenase (Cluster-8535.6260). Glucose dehydrogenases also exist in venom reservoirs of *P. puparum* (Yan et al., 2016), *N. vitripennis* (De Graaf et al., 2010), *L. boulardi* and *L. heterotoma* (Goecks et al., 2013). We assume that it participates in the carbohydrate catabolism of the host and provides nutrition for the development of parasitoid offspring.

Transferase

Here we originally identified a gamma-glutamyltranspeptidase (Cluster-8535.10302) in *P. vindemmiae* venom proteins. Venom protein gamma-glutamyltranspeptidase was exhaustively described in *Aphidius ervi*, inducing cell apoptosis of the host ovariole by altering GSH metabolism and oxidative stress (Falabella et al., 2007). Whether the venom gamma-glutamyltranspeptidase of *P. vindemmiae* performs functions similar to that of *A. ervi* needs to be investigated.

Protease Inhibitor

A Kazal-type serine protease inhibitor (Cluster-8535.6176) was characterized in *P. vindemmiae* venom proteins. Similar with other parasitoids, the Kazal-type serine protease inhibitor of *P. vindemmiae* is packed by a conservative "Kazal domain." The motif prediction based on "Kazal domain" revealed several conservative amino acid residues between *P. vindemmiae*, *N. vitripennis* and *P. puparum* (**Figure 5**). Previous study showed that Kazal-type serine protease inhibitors from *N. vitripennis* venom proteins inhibited the PPO activation of host hemolymph (Qian et al., 2015), strongly supporting the speculation that the Kazal-type serine protease inhibitor from *P. vindemmiae* venom proteins may suppress the host humoral immunity, especially in melanization.

Recognition and Binding Proteins

Low density lipoprotein receptors were extensively identified in parasitoids, including *P. vindemmiae* (Cluster-8535.6319, Cluster-8535.6269, Cluster-8535.25625, Cluster-8535.25152, Cluster-8535.25072 and Cluster-8535.24989), *P. puparum*, and *N. vitripennis* (De Graaf et al., 2010; Yan et al., 2016). It was first reported that an insect homolog of low density lipoprotein receptor mediated the endocytosis of high density lipophorin in the circulatory compartment (Dantuma et al., 1999). We infer that the low density lipoprotein receptors of *P. vindemmiae* venom proteins participate in the internalization of high density lipophorin (Danneels et al., 2010).

Other than low density lipoprotein receptors, a venom protein annotated as chitin binding protein-like (Cluster-8535.6043) was also identified in *P. vindemmiae*. It was reported that chitin binding proteins present in the venom proteins of *N. vitripennis* and *P. puparum* (De Graaf et al., 2010; Zhu et al., 2015). Based on the previous investigation, we suspect that the chitin binding venom protein of *P. vindemmiae* selectively binds chitin, and likely facilitates wound healing of the host exoskeleton (Zhu et al., 2015).

Others

Venom allergens are common components in stinging insects, such as bees, fire ants and vespids. In *P. vindemmiae*, the venom allergen (Cluster-8535.5294) showing 25% identity (Blastp, *e*-value = $9e^{-08}$) with *M. demolitor* venom allergen 5 possibly leads to the anaphylaxis of the host.

In hymenopteran insects, major royal jelly proteins are necessary for social behavior. They were identified in *P. vindemmiae* (Cluster-8535.12135) and *P. puparum* (Yan et al., 2016) venom proteins. In honeybee, the royal jelly protein regulated the larval development, induced queen differentiation (Peiren et al., 2005, 2008), and embodied the novel nutritious function as major components of royal jelly (Klaudiny, 2007). Hereby, we infer that the major royal jelly venom protein of *P. vindemmiae* is related to the storage of nutrients.

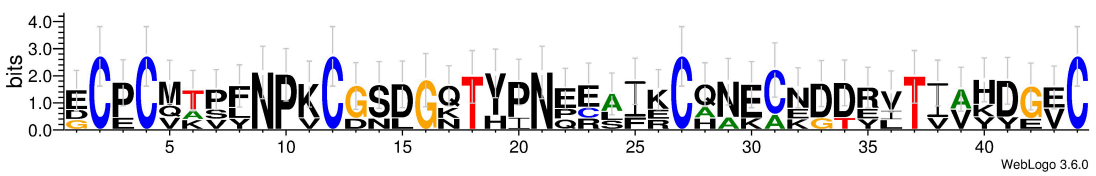


FIGURE 5 | Motif-based sequence analysis of "Kazal domain" in pteromalids. The sequence logo was generated using WebLogo based on "Kazal domain" of *P. vindemmiae* (Cluster-8535.6176), *N. vitripennis* (NP_001164350.1) and *P. puparum* (comp22195_c0) (Yan et al., 2016).

Alongside the above-mentioned proteins, a venom protein was annotated as cysteine-rich peptide (Cluster-8535.6175) in *P. vindemmiae*, and highly expressed in VG. In accordance with the previous study in *Nasonia* venom proteins (De Graaf et al., 2010), the cysteine-rich peptide of *P. vindemmiae* contains six conservative cysteine residues forming three disulfide bridges. Its function in parasitism is an important issue for future research. Besides, one unanticipated observation was the category of two salivary secreted peptides (Cluster-8535.6002, Cluster-8535.13847). It is speculated that they function in hydrolyzing the host nutrients.

Unknown Proteins

A total of 27 *P. vindemmiae* venom proteins were categorized into "unknowns" based upon the absence of recognized protein motifs. In contrast to our current study, 23 and 17 "unknowns" were originally identified in venom proteins of other pteromalids, *N. vitripennis* and *P. puparum*, respectively (De Graaf et al., 2010; Yan et al., 2016). Comparing the 27 "unknowns" venom expression of *P. vindemmiae* to that of the 37 "knowns" showed a similar expression pattern, ranging from 7.43 to 17.27-fold higher in VG than that in CA (mean value of 11.76) among "unknowns" compared to 6.71-16.95-fold higher (mean value of 11.18) among "knowns."

Comparative Analysis of Parasitoid Venom Proteins

Previous studies have found both considerable diversity of venom proteins between species and also evidence of functional redundancy within species (Goecks et al., 2013; Yan et al., 2016; Martinson et al., 2017). Hereby, a comparative analysis was conducted using OrthoMCL by all-against-all blastp on the basis of a set of venom protein sequences from *P. vindemmiae* and other pteromalids (*N. vitripennis* and *P. puparum*) (Doerks et al., 2002). Putative orthologs were identified by reciprocal best matches. Based on this criterion, thirty-five venom proteins of *P. vindemmiae* were assigned to orthologous groups, while the remaining 29 were not. **Figure 6** shows a Venn diagram of orthologous relationships between *P. vindemmiae* venom proteins and those of *N. vitripennis*, *P. puparum*. Among the 35 orthologs in *P. vindemmiae*, 27 have orthologous relationships with *N. vitripennis* venom proteins and 25 with venoms of *P. puparum* (**Figure 6**). Besides, 23 "knowns" and 12 "unknowns" were included in the 35 categorized orthologs. As can be seen, the proportion of "unknown" orthologs in total "unknown" venom proteins (12 of 27) was not different from that of "knowns" (23 of 37) (p = 0.52, Fisher Exact Test). Of the 23 "known" orthologs, 16 categories have orthologous relationships with *N. vitripennis* venom proteins and 18 with *P. puparum* venoms. These orthologs represent a set of conserved venom proteins in pteromalids, possibly contributing to their adaptation to parasitism. In contrast, of the 12 "unknown" orthologs, 11 have orthologous relationships with *N. vitripennis* venom proteins and 7 with *P. puparum* venoms, representing a set of unique venom proteins in pteromalids. Many of these venoms were highly expressed in VG. For instance, the expression level of venom protein J in VG was 176.73-fold higher than that in CA (**Figure 2**). The observations that these "unknowns" are orthologous with the related pteromalid species, do not have clear homologies to other parasitoid venom proteins, and have higher VG expression levels suggest their specialized venom functions.

Additionally, we compared the venom proteins of *P. vindemminae* with those from other parasitoids (*L. boulardi*,

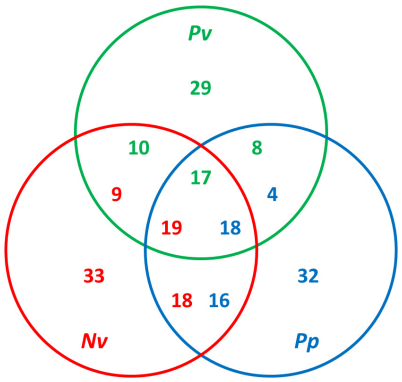


FIGURE 6 | Comparative analysis of venom proteins in pteromalids. The orthologs were identified using OrthoMCL by all-against-all blastp with a p-value cut-off of $1e^{-5}$. The green numbers indicate hits from *P. vindemmiae* venoms, the red numbers indicate hits from *N. vitripennis* venoms, and the blue numbers indicate hits from *P. puparum* venoms. The numbers of orthologs can be different in different venom sets. *Pv*, venom proteins of *P. vindemmiae*; *Nv*, venom proteins of *N. vitripennis*; *Pp*, venom proteins of *P. puparum*.

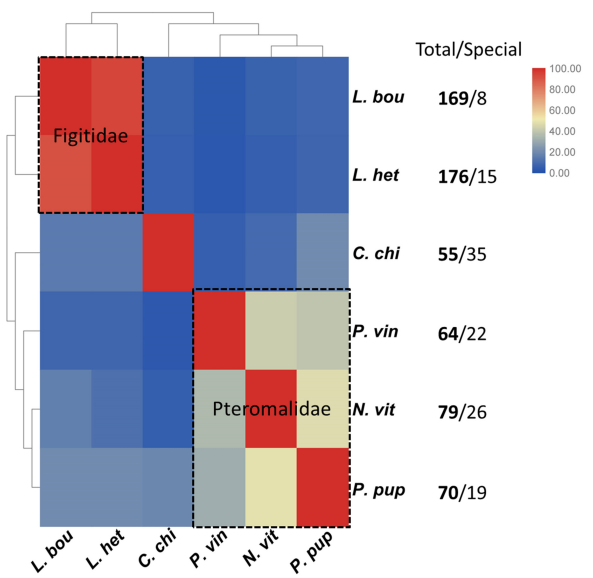


FIGURE 7 | Cluster analysis of the orthologs in six parasitoids. The heatmap was plotted using TBtools v0.6669 based on the proportion of orthologs in total venom proteins (Chen et al., 2018). Red indicates larger proportion of the orthologs and blue indicates the smaller. Each grid shows the proportion of orthologous venom proteins in total venom proteins of the horizontal axis labeled species by reciprocal best matches with the venom proteins of vertical axis labeled species. *C. chi*, *C. chilonis*; *L. bou*, *L. boulardi*; *L. het*, *L. heterotoma*; *N. vit*, *N. vitripennis*; *P. pup*, *P. puparum*; *P. vin*, *P. vindemmiae*.

L. heterotoma, and *C. chilonus*) encompassing two families, Figitidae and Braconidae. The sequences of venom proteins were obtained from previous literatures (Goecks et al., 2013; Teng et al., 2017), using species containing 50 or more venom proteins. Venom proteins of each species were assigned to orthologs according to the above classification criterion (Doerks et al., 2002). Results were displayed in clustered heatmap based on the proportion of orthologs in total venom proteins. As expected, closely related species shared a greater proportion of orthologs, even though they have more abundant venom proteins (e.g., *L. boulardi* and *L. heterotoma*). Two representative groups clustered together, one representing Pteromalidae and another

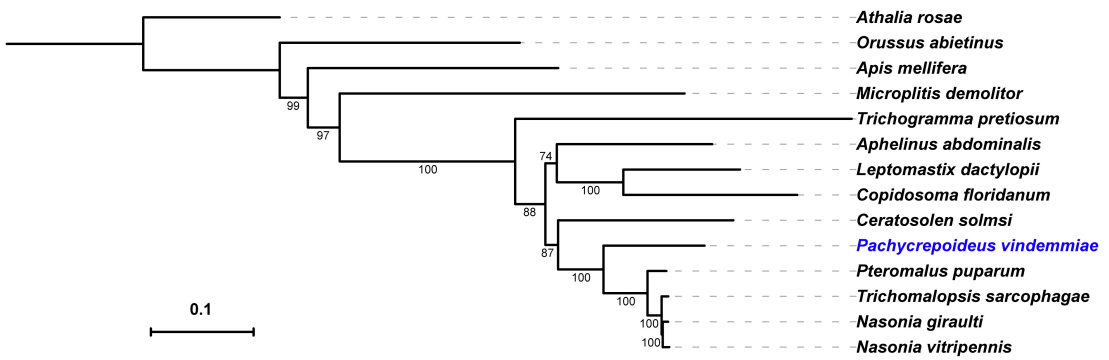


FIGURE 8 | Phylogeny of *P. vindemmiae*. A phylogeny was generated based on a set of 107 proteins, and visualized using iTOL v3 (Letunic and Bork, 2016). Indicated are members of the Pternomalidae and the chalcidoid superfamily. Proteins sequences from *P. vindemmiae* (this study), *P. puparum* (GECT00000000.1), *Aphelinus abdominalis* (GBTK00000000.1), and *Leptomastix dactylopii* (GBNE00000000.1) transcriptomes were assigned to these 107 proteins groups. Bootstrap values are shown at each node. *P. vindemmiae* is labeled in blue.

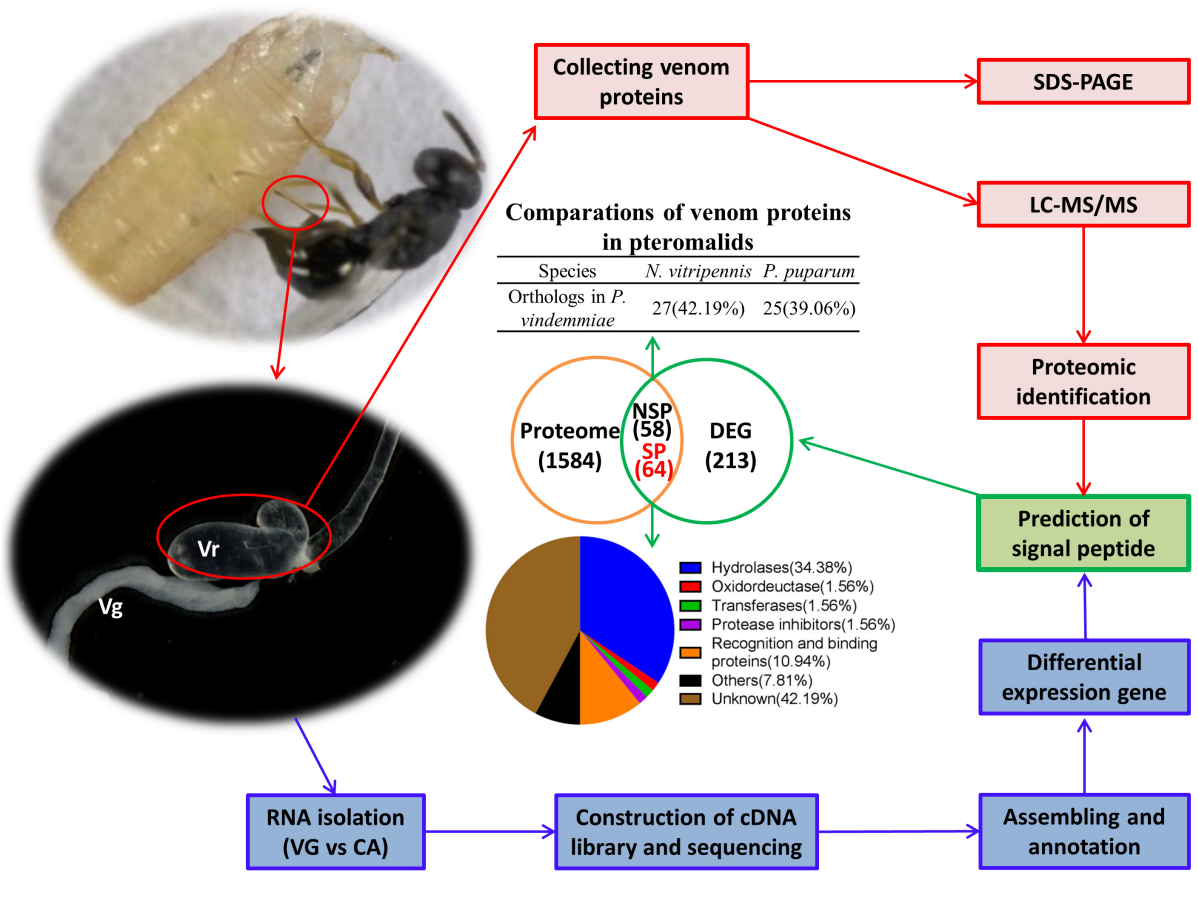


FIGURE 9 | Schematic representation of the identified venom proteins in *P. vindemmiae* by combined transcriptomic and proteomic analyses.

representing Figitidae (**Figure 7**). Five *P. vindemminae* venom proteins (venom allergen: Cluster-8535.5294, serine protease: Cluster-8535.6305, venom acid phosphatase: Cluster-8535.24978, lipase: Cluster-8535.5960, and neprilysin: Cluster-8535.25024) in this set have orthologous relationships with venoms of Figitidae representatives, and two orthologs were identified in *P. vindemminae* venoms ("unknowns": Cluster-8535.25078 and Cluster-8535.25723) by reciprocal best matches with venom proteins of the single representative of Braconidae. The large differences among this set of venoms may be due to their specific adaptations during the co-evolution between parasitic wasps and host species, and are consistent with the observed rapid turnover even in closely related species' venom proteins (Goecks et al., 2013; Martinson et al., 2017).

Phylogenetic Analysis of *Pachycrepoideus vindemmiae*

To better understand the evolutionary position of *P. vindemmiae*, a phylogenetic tree was constructed based on a data set of 107 proteins across a larger set of single-copy orthologs described in Lindsey et al. (best-reciprocal hits with *p*-value cut-off $< 1e^{-5}$) (Lindsey et al., 2018). What stood out in the **Figure 8** was that *P. vindemmiae* clustered together with other pteromalids, namely *P. puparum, N. vitripennis*, *Trichomalopsis sarcophagae*, and *Nasonia giraulti*, suggesting that they have evolved from the same ancestor. The majority of pteromalids are specialists, such as *P. puparum*, *N. vitripennis* and *N. giraulti*. Our observation that *P. vindemmiae* has a relatively close evolutionary relationship with these specialists provides a wonderful model for comparing the phylogeny between specialist and generalist, particularly in the variations of parasitic behavior. In the closely related species, the generalist *L. boulardi* and specialist *L. heterotoma*, comparison of their venom proteins profiles revealed several different venom compositions (Poirié et al., 2014), and may thus reflect long-term adaptations to their hosts (Goecks et al., 2013). Our examination on orthologous venom proteins also provides clues about the different parasitic behavior between *P. vindemmiae* and other specialists in pteromalids. This finding also provides corroborative evidence that the venom components of pteromalids are somewhat similar, and lays a foundation for functional research on venom proteins of *P. vindemmiae*.

As a generalist in dampening oscillations of Dipteran Cyclorrhapha, *P. vindemmiae* possesses vast potentials on host-killing capacity. Several practical cases have addressed its high efficacy at reducing flies' populations, one of which was that *P. vindemmiae* decreased the fitness of the host *Bactrocera oleae* (Hoelmer et al., 2011). A follow-up study also underlined that *P. vindemmiae* has been attracting considerable interest in control of *D. suzukii*, a destructive pest in American and European orchard (Bezerra Da Silva et al., 2019). More remarkably, the investigation into its ability to control *D. suzukii* populations showed an efficient reduce in host numbers by 32% (Bonneau et al., 2019). Our current study about the venom protein repertoire of ectoparasitoid *P. vindemmiae* will vastly propel its possible utilization as a biological agent to the control of flies. Taken as a whole, these explorative works expand our knowledge that *P. vindemmiae* has a tremendous biocontrol potential against flies.

CONCLUSION

In this study, we described the venom protein repertoire of *P. vindemmiae* (**Figure 9**), an ectoparasitoid that utilizes pupae of *Drosophila* species as a host. Both transcriptomic and proteomic analyses were used to identify 64 putative venom proteins in *P. vindemmiae*. Our striking observation revealed 35 core orthologous venom proteins from *P. vindemmiae* by best-reciprocal matches with other pteromalids. More distant relationships detected that five venom proteins of *P. vindemmiae* have orthologous relationships with venoms of Figitidae parasitoids and two with those of a Braconidae representative. In addition, twenty-two venoms unique to *P. vindemmiae* were detected, consistent with observations of rapid turnover and evolution of new parasitoid venom proteins. Our findings will deepen the understanding of the phylogeny as well as inner- and inter-taxon variations of venom proteins.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Venom apparatus and carcasses transcriptome of *Pachycrepoideus vindemmiae* (https://www.ncbi.nlm.nih.gov/sra/PRJNA573955), the mass spectrometry proteomic data of *Pachycrepoideus vindemmiae* were deposited on the ProteomeXchange Consortium with the dataset identifier PXD015627 (Ma et al., 2019).

ETHICS STATEMENT

We declare that appropriate ethical approval and licenses were obtained during our research.

AUTHOR CONTRIBUTIONS

LY, YY, and M-ML performed the experiments. LY, Z-CY, L-MQ, and FW analyzed the data. LY, QF, JW, and G-YY designed the experiments. LY, JW, and G-YY wrote the manuscript. All authors gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00009/full#supplementary-material

FIGURE S1 | GO functional categories of the 335 UVG. The 335 UVG were annotated on the GO consortium database using the GOseq R packages with e-value $< 1e^{-6}$.

FIGURE S2 | Top 20 KEGG functional categories of the 335 UVG. KEGG annotations were implemented by KOBAS software with e-value $< 1e^{-10}$.

TABLE S1 | 335 upregulated unigenes in VG compared to CA.

TABLE S2 | 1706 unigenes identified by proteomic approach.

TABLE S3 | Primers used for qPCR analysis of 20 venom genes.

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Neuropeptides and G-Protein Coupled Receptors (GPCRs) in the Red Palm Weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Dryophthoridae)

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The red palm weevil *Rhynchophorus ferrugineus* is a devastating, invasive pest that causes serious damages to palm trees, and its invasiveness depends on its strong ability of physiological and behavioral adaptability. Neuropeptides and their receptors regulate physiology and behavior of insects, but these protein partners have not been identified from many insects. Here, we systematically identified neuropeptide precursors and the corresponding receptors in the red palm weevil, and analyzed their tissue expression patterns under control conditions and after pathogen infection. A total of 43 putative neuropeptide precursors were identified, including an extra myosuppressin peptide was identified with amino acid substitutions at two conserved sites. Forty-four putative neuropeptide receptors belonging to three classes were also identified, in which neuropeptide F receptors and insulin receptors were expanded compared to those in other insects. Based on qRT-PCR analyses, genes coding for several neuropeptide precursors and receptors were highly expressed in tissues other than the nervous system, suggesting that these neuropeptides and receptors play other roles in addition to neuro-reception. Some of the neuropeptides and receptors, like the tachykinin-related peptide and receptor, were significantly induced by pathogen infection, especially sensitive to *Bacillus thuringiensis* and *Metarhizium anisopliae*. Systemic identification and initial characterization of neuropeptides and their receptors in the red palm weevil provide a framework for further studies to reveal the functions of these ligand- and receptor-couples in regulating physiology, behavior, and immunity in this important insect pest species.

Keywords: neuropeptides, GPCRs, immunoregulation, *Rhynchophorus ferrugineus*, expression profiling

INTRODUCTION

Neuropeptides are a class of signal molecules secreted by neuroendocrine cells for regulating the transmission of intercellular signals. Neuropeptides regulate behavioral activities of insects via their interactions with the corresponding receptors and subsequent signal transduction, and those behavioral activities can be further categorized into behaviors (involving feeding, reproduction, learning and memory, stress and addiction, circadian rhythms, sleep, wakefulness, social behavior) and physiological processes (including growth and development, digestion, energy homeostasis, water and ion balance, and metabolism) (Caers et al., 2012; Schoofs et al., 2017; Yeoh et al., 2017; Nässel and Zandawala, 2019). Neuropeptides are usually produced from the cleavage of larger precursors and are usually modified post-transcriptionally to form isopeptides, which are then transported to target cells to activate corresponding receptors (Veenstra, 2000; Pauls et al., 2014; Yeoh et al., 2017). A large number of neuropeptides and their receptors have been extensively characterized and functionally validated in various insects, such as *Drosophila melanogaster* (Nässel and Winther, 2010), *Tribolium castaneum* (Li et al., 2007; Hauser et al., 2008), *Locusta migratoria* (Veenstra, 2014; Hou et al., 2015), and *Bombyx mori* (Roller et al., 2008). These results suggest significant neuropeptide and receptor variation between different orders, even between different species from the same order (Veenstra, 2019). Insect neuropeptides and their GPCRs are promising targets for a novel generation of pesticides. Thus, identification and functional characterization of neuropeptides and receptors from insect pests may provide useful information for pest management and for enhancing our basic understanding of neuropeptide-related signal transduction.

The red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae) is a devastating pest, which has been spread to various regions with palm trees in southern China, causing serious damage to the palm industry and landscape (Hou et al., 2011; Wang et al., 2015, 2017; Muhammad et al., 2017; Ali et al., 2018). Red palm weevil larvae have a long life span, strong ability to drill collar, and strong adaptability to different environments (Shi et al., 2014; Peng et al., 2016; Dawadi et al., 2018; Habineza et al., 2019; Xiao et al., 2019). Due to damage to vascular tissues and consumption of large amounts of crown tissues, red palm weevil larvae can cause the death of palm trees (Butera et al., 2012; Muhammad et al., 2017). When symptom appears, it is usually too late to save the trees (Peng and Hou, 2017). Therefore, it is urgent to develop novel technologies for controlling this destructive pest at an early stage to reduce damage to palm trees (Pu and Hou, 2016; Pu et al., 2017). Neuropeptides and their receptors, the important behavioral and physiological regulators of insects, might be potential targets for developing novel methods for pest control (Audsley and Down, 2015). Very limited information is available on neuropeptides and their functions on the red palm weevil. Base on genomics, transcriptomics, and peptidomics, numerous neuropeptides and receptors have been identified from Coleopterans (Weaver and Audsley, 2008; Cunningham et al., 2017; Pandit et al., 2018; Veenstra, 2019). The types and numbers of neuropeptides and receptors in Coleopterans are quite different from those from other insect species. Significant variation has also been observed even among species within the order Coleoptera (Veenstra, 2019). For those identified genes, tissue and developmental expression profiles have been used in many insect species to mine for functional information (Hou et al., 2015; Xu et al., 2016; Wang et al., 2018). Identification and expression analyses of neuropeptides and corresponding receptors from the red palm weevil should provide useful information for comparative studies and exploration of practical application.

In addition to regulating various physiology and behavior of insects, neuropeptides may participate in immune responses of insects (Urbanski and Rosinski, 2018). One of the reasons for the red palm weevil succeeds in spreading globally is its strong immunity to pathogen's attack. It would be interesting to explore any role of neuropeptides in immunity of the red palm weevil. In this study, we have systematically identified neuropeptides and their receptors in the red palm weevil following a transcriptomic approach. We then analyzed sequence variation and phylogenetic relationship of the identified neuropeptides and receptors together with those identified from other insect species previously. Tissue expression profiles of neuropeptide precursors and corresponding receptors were examined via quantitative real time-PCR (qRT-PCR). Potential impact of pathogenic microbes on the expression of the newly identified neuropeptide precursors and corresponding receptors was also examined.

MATERIALS AND METHODS

Insect Rearing

The colony of the red palm weevil used in this study was originated from adults trapped from the campus of Fujian Agriculture and Forestry University in September 2017. The colony has been maintained in incubators since then. Adult males and females were fed with sugarcane stems in pairs at 27 ± 1°C, 75% relative humidity (RH), and a light: dark cycle of 12:12. Eggs were regularly collected on wet filter papers. The collected eggs were used to inoculate cuts of sugarcane stems right before larval hatch. Larvae were reared individually with regular diet changed until pupation and emergence.

Identification of the Neuropeptides and Their Putative G Protein-Coupled Receptors

The transcriptomic (RNA-seq) data derived from larvae and pupae of the red palm weevil were used for identification of the neuropeptides and receptors. Larvae data was downloaded from the published database (NCBI Sequence Read Archive: SRX096969), and pupae data was obtained from our laboratory (unpublished data). After assembly, unigenes encoding neuropeptides and receptors were identified by BLAST searches against a local database with amino acid sequences of the neuropeptide precursors and receptors of *D. melanogaster*, *T. castaneum*, *Hylobius abietis,* and other insects as queries. The cut-off Expectation Value (E) threshold

was 1.0 for putative neuropeptides, and 0.001 for receptors. Candidate genes identified from searches were further verified by additional BLAST searches against the NCBI non-redundant protein database (BLASTx) to remove false positives and repeat sequences.

Structural, Domain, and Sequence Analyses

Open reading frames (ORFs) of candidate genes were predicted using the NCBI ORF finder[1]. Secretion signal peptides were identified using SignalP 4.0[2] (Petersen et al., 2011). Sequence logos for neuropeptide motifs were analyzed using Weblogo[3] (Crooks et al., 2004). Sequences for multiple sequence alignments were downloaded from DINeR[4] (Yeoh et al., 2017). Multiple alignments of amino acid sequences were performed with MAFFT (Katoh and Standley, 2013), and visualized with Jalview 2.10.3 (Waterhouse et al., 2009).

Phylogenetic Analysis

Amino acid sequences used for phylogenetic tree construction were aligned with the MAFFT (Katoh and Standley, 2013). Phylogenetic trees were constructed with FastTree (version 2.1.7) using the maximum-likelihood method with 1000 bootstrap replicates (Price et al., 2010). Phylogenetic trees were edited and visualized with FigTree 1.4.4[5].

Tissue Expression Analysis of Neuropeptide Precursors and Receptors

To examine tissue expression profiles of neuropeptide precursors and their putative receptors, total RNA was extracted from various tissues, including the hemocytes (HC), fat body (FB), gut (including the foregut, midgut, hindgut, Malpighian tubes) and CNS (including the brain and ventral nerve cord) from eighth instar larvae using TRIzol reagent (Invitrogen, United States) following the manufacturer's instructions. The hemocytes were collected according to the method for studying *Chilo suppressalis* (Xu et al., 2016). cDNA was synthesized using a PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China). Primers specific to individual genes for qRT-PCR analyses were designed using the Primer 3 program[6] and are listed in **Supplementary Table S5**. The RT-qPCR experiments were performed according to the Minimum Information Required for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Johnson et al., 2014). qRT-PCR were performed on a Light Cycler 480 System (Roche Applied Science) using a 10 μl reaction containing 5 μl 2 × SYBR Green PCR Master Mix (Roche, Germany), 0.5 μl of each primer (10 μM), 1 μl of cDNA template, and 3 μl of sterile H_2O. PCR reactions were proceeded at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 32 s. Dissociation curves for PCR products were generated by heating to 95°C for 15 s, followed by cooling to 60°C for 1 min; heating to 95°C for 30 s, followed by cooling to 60°C for 15 s. Each sample had four biological replicates and each replicate had three technical duplicates. Relative transcript abundance was determined using the $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008), with the red palm weevil *gapdh* and *tubulin* genes as an internal reference. Comparative analyses of each target gene among different tissues were determined using a one-way nested analysis of variance (ANOVA) followed by a least significant difference test (LSD) for mean comparison, and data analyses were done in Prism7.0 (GraphPad Software, San Diego, CA, United States). Heatmaps of gene expression for different neuropeptides and their receptors genes among different tissues were generated by R version 3.4.1.

Impact of Pathogens on Expression of Neuropeptide and Receptor Genes

Three pathogens were selected to examine their impact on the expression of genes coding for neuropeptides and receptors. These pathogens were *Serratia marcescens* (Gram−), *Bacillus thuringiensis* (Gram+), and *Metarhizium anisopliae* (Fungus). PBS was used as control. The bacteria *S. marcescens* and *B. thuringiensis* were cultured overnight on Nutrient Broth (NB) at 28°C in a shaker at 200 rpm. Bacterial cells were harvested by centrifuging and washed three times with sterilized PBS by re-suspending in PBS. *M. anisopliae* spores were scraped from PDA medium, dissolved in PBS, and filtered through sterile gauze to obtain a fungal spore suspension. Cell density of the three pathogens were estimated using a hemocytometer and adjusted to required density with PBS. Early third-instar larvae of the red palm weevil with an average weight of 150 mg were chosen for infection. Larvae were surface-sterilized with 70% ethanol before pathogen injection. Larvae were randomly selected and individually injected with 5 μl PBS (control), or 5 μl PBS containing either 1×10^4 *S. marcescens*, 1×10^2 *B. thuringiensis* cells, or 1×10^2 *M. anisopliae* spores. Solution was injected into the hemocoel of each larva via the last left proleg. After injection, insects were collected for RNA extraction at different time points, including 0, 3, 6, 12, 48 h. Four larvae at each time point were combined for RNA extraction in each sample. Four biological replicates were included in each time point. RNA extraction, cDNA synthesis, qRT-PCR and quantification of transcript abundance were carried out as described in earlier sections. Comparative analyses of each target gene among different time points for the same treatment and the same time point among different treatments were conducted using a one-way nested analysis of variance (ANOVA) followed by a LSD for mean comparison. All data analyses were done in Prism7.0 (GraphPad Software, San Diego, CA, United States).

RESULTS

Overview of Transcriptomes

We generated two transcriptomes, one from larvae and the other from pupae. The raw reads of larval was downloaded

[1]http://www.ncbi.nlm.nih.gov/

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

[3]http://weblogo.berkeley.edu/logo.cgi

[4]http://www.neurostresspep.eu/diner/infosearch

[5]http://tree.bio.ed.ac.uk/software/figtree

[6]http://bioinfo.ut.ee/primer3-0.4.0/

from NCBI Sequence Read Archive (SRX096969) for reassembly and annotation. A total of 74.9 million raw reads were obtained from the pupal transcriptome. After removing low quality reads, adaptor sequences, and reads shorter than 20 bp, the remaining high-quality reads were 73.0 million for pupal transcriptome. High quality reads were then assembled into unigenes separately. A total of 16,875 unigenes were obtained for the larval transcriptome, with the average length 1138 bp and N50 1427. A total of 37,210 uigenes were obtained for the pupal transcriptome, with the average length 2025 bp and N50 3320. Unigenes from both transcriptomes were used to identify neuropeptide and receptor genes.

TABLE 1 | Neuropeptide precursors identified from the red palm weevil.

					Homology search with known protein		
Neuropeptide	**Accession no.**	**Abbreviation**	**ORF (aa)**	**SP (aa)**	**Species**	**Protein ID**	**E-Value**
Allatostatin B	MK751535	Ast B	199	37	*Asbolus verrucosus*	RZC34805.1	3e−44
Allatostatin C	MK751536	Ast C	114[a]	26	*Tribolium castaneum*	EFA09152.2	4e−24
Allatostatin CC	MK751537	AstCC	71[a]	∼	*Tribolium castaneum*	XP_001810067.1	7e−15
Allatotropin	MK751538	AT	84[a]	∼	*Hylobius abietis*	SRP133355	2e−29
Bursicon	MK751539	Burs	80[a]	∼	*Dendroctonus ponderosae*	XP_019755391.1	8e−49
Calcitonin	MK751540	Cal	283	20	*Hylobius abietis*	SRP133355	3e−66
Capability/CAP2b	MK751541	CAPA	195	23	*Hylobius abietis*	SRP133355	2e−41
CCHamide−1	MK751542	CCHa-1	116[a]	33	*Tribolium castaneum*	XP_008201341.1	4e−10
CCHamide−2	MK751543	CCHa-2	118	28	*Tribolium castaneum*	XP_008190391.1	5e−11
CNMamide	MK751544	CNMa	130	21	*Hylobius abietis*	SRP133355	3e−07
Diuretic Hormone 31	MK751545	DH31	121	32	*Anoplophora glabripennis*	XP_018565090.1	8e−34
Diuretic hormone 44	MK751546	DH44	197	∼	*Dendroctonus ponderosae*	XP_019767380.1	7e−69
Ecdysis triggering hormone	MK751547	ETH	130	17	*Leptinotarsa decemlineata*	QBH70331.1	8e−19
Eclosion hormone	MK751548	EH	49[a]	∼	*Leptinotarsa decemlineata*	XP_023028729.1	8e−20
Elevenin	MK751549	Ele	100[a]	∼	*Dendroctonus ponderosae*	XP_019770585.1	2e−04
FMRFamide	MK751550	FMRFa	167[a]	18	*Dendroctonus ponderosae*	XP_019753890.1	8e−19
	MK751551						
Glycoprotein hormone alpha 2	MK751552	GPA2	122	16	*Asbolus verrucosus*	RZC35091.1	6e−54
Glycoprotein hormone beta 5	MK751553	GPA5	154[a]	18	*Tribolium castaneum*	NP_001280517.1	1e−73
locust insulin-related peptide	MK751554	LIRP	115	29	*Anoplophora glabripennis*	XP_018573725.1	4e−08
Ion transport peptide	MK751555	ITPa	96	31	*Anoplophora glabripennis*	XP_018574385.1	1e−30
Ion transport peptide	MK751556	ITPb	135	31	*Asbolus verrucosus*	RZC33292.1	1e−57
ITG-like peptide	MK751557	ITG	213	19	*Dendroctonus ponderosae*	XP_019771238.1	2e−110
Myosuppressin	MK751558	MS-1	95	24	*Anoplophora glabripennis*	XP_018573133.1	1e−25
Myosuppressin	MK751559	MS-2	91	24	*Anoplophora glabripennis*	XP_018573133.1	3e−14
Natalisin	MK751560	NTL	155	34	*Tribolium castaneum*	XP_015833286.1	1e−21
Neuroparsin	MK751561	NP	102	21	*Anoplophora glabripennis*	XP_018568084.1	2e−31
Neuropeptide F (long transcript)	MK751562	NPFa	129	25	*Dendroctonus ponderosae*	XP_019762446.1	1e−53
Neuropeptide F (short transcript)	Unigene0020442	NPFb	92	25			
Neuropeptide-like precursor 1a	MK751563	NPLP1a	308	27	*Dendroctonus ponderosae*	XP_019771539.1	6e−106
Neuropeptide-like precursor 1b	MK751564	NPLP1b	282	27	*Dendroctonus ponderosae*	XP_019771539.1	5e−84
NVP-like	MK751565	NVP	327	17	*Tribolium castaneum*	XP_008196925.1	3e−78
Orcokinin B	MK751566	OK-B	357	19	*Hylobius abietis*	SRP133355	2e−21
Pigment dispersing factor	Unigene0014866	PDF	105	30	*Sitophilus oryzae*	XP_030754781.1	5e−43
Proctolin	MK751567	Proc	90	26	*Dendroctonus ponderosae*	XP_019763695.1	1e−09
Prothoracicotropic hormone	MK751568	PTTH	82[a]	∼	*Rhynchophorus ferrugineus*	ATU47262.1	3e−43
Pyrokinin 1	MK751569	PK1	147[a]	26	*Dendroctonus ponderosae*	XP_019770936.1	5e−17
Pyrokinin 2	MK751570	PK2	105[a]	∼	*Dendroctonus ponderosae*	XP_019770936.1	8e−12
RYamide	MK751571	RYa	117	25	*Asbolus verrucosus]*	RZC32873.1	2e−23
Short neuropeptide F	MK751572	sNPF	100	25	*Dendroctonus ponderosae*	XP_019767920.1	1e−37
Sulfakinin	MK751573	SK	112	32	*Tribolium castaneum*	EFA04708.1	7e−16
Tachykinin-related peptide	MK751574	TRP	264	25	*Dendroctonus ponderosae*	XP_019770548.1	1e−91
Trissin	MK751575	Tris	87[a]	∼	*Dendroctonus ponderosae*	XP_019766470.1	8e−39
Vasopressin	MK751576	VPL	142	26	*Tribolium castaneum*	NP_001078831.1	1e−35

ORF, open reading frame; SP, signal peptide; [a]not full length; ∼no signal peptide.

Identification of Various Neuropeptides

A total of 43 transcripts encoding putative neuropeptide precursors and putative neuropeptides from precursors were identified from the red palm weevil transcriptomes (**Table 1** and **Supplementary Tables S1, S3**). Among them, 29 transcripts encode full length proteins, and most of the predicted proteins contain a signal peptide. The identified neuropeptide precursors share sequence similarity with homologs from *T. castaneum*, *Dendroctonus ponderosae*, and *Hylobius abietis*.

Two transcripts encoding two different myosuppressin precursors, MS-1 and MS-2, were identified, with each encoding a different myosuppresin peptide. MS-1 and MS-2 differ significantly in amino acid sequence in both signal peptide- and mature peptide-coding regions (**Figure 1**). A sequence alignment of MS-1 and MS-2 together with the corresponding sequences from other insects revealed that MS-1 is identical to the myosuppresin from the beetles *T. castaneum*, *D. ponderosae*, and *H. abietis*. However, amino acid substitutions occurred at the 3rd and 8th positions of MS-2, with Val at the third position and Leu at the eighth position replaced with Met and Trp, respectively, in MS-2 (**Figure 1**).

Two neuropeptide F (NPF) transcripts of different lengths produced by alternative splicing were identified in red palm weevil (**Supplementary Figure S1**), consistent with NPF precursors found in other insects (Nässel and Wegener, 2011; Veenstra, 2014). The long splice variant of the *npf* gene has an optional exon compared to the short splice variant (**Supplementary Figure S1**). However, it seems that only mature neuropeptides predicted from the short splice variant of *npf* gene have been identified in neuropeptidomic of these insects (Nässel and Wegener, 2011; Pandit et al., 2018), whether long splice variant of *npf* gene encodes different mature neuropeptide remains unclear.

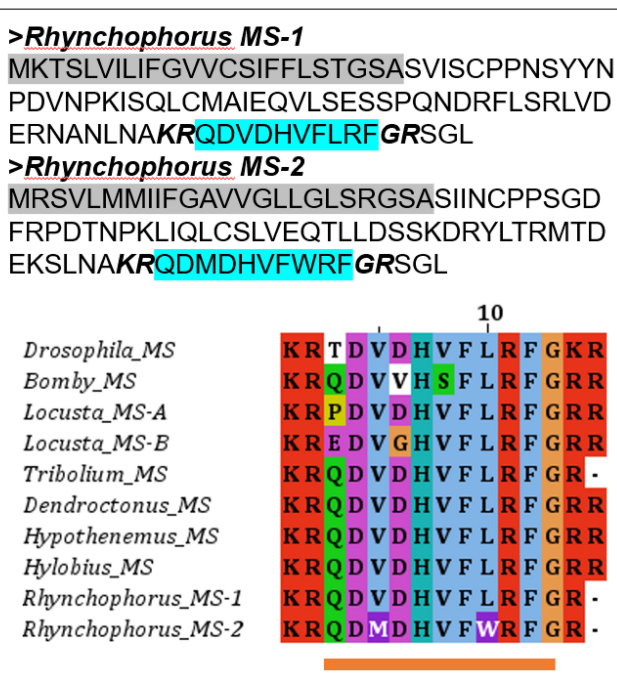

FIGURE 1 | Myosuppressin precursors of the red palm weevil and multiple sequence alignment of myosuppressin peptide of the red palm weevil with other insect species. Predicted signal peptides (highlighted in gray), cleavage signals (italics, bold) and supposed bioactive mature peptides are indicated. The sequence underlined in orange is the predicted mature peptide.

The Pigment dispersing factor (PDF) has undergone significant sequence changes in Coleoptera, especially in its C-terminal half (Veenstra, 2019). According to this, a transcript encoding the neuropeptide PDF was identified from the red palm weevil transcriptomes. An sequence alignment revealed that the predicted PDF from the red palm weevil showed high sequence similarity to those from Coleopterans (Veenstra, 2019), but lost the Arg- Lys cleavage sites at the C-terminal (**Supplementary Figure S2**).

Orcokinins were initially isolated from *Orconectes limosus* and have also been generally identified from insects (Stangier et al., 1992; Veenstra, 2014). In *T. castaneum*, two isoforms, named orcokinin-A (OK-A) and orcokinin-B (OK-B), have been identified, and are encoded by the same gene through alternative splicing (Jiang et al., 2015). Here we identified one orcokinin, which is similar to the B form. The orcokinin B precursor can be processed into several similar isopeptides, and the number of peptides varies from species to species. The number of iso-orcokinin-B is predicted 22 for the red palm beetle, more than 15 in *H. abietis* (Pandit et al., 2018), 10 in *T. castaneum* (Jiang et al., 2015), and only one in *D. melanogaster* (Veenstra and Ida, 2014). The consensus for Orcokinin B isopeptides is X(I, L, V)DXXGGG in N-terminal based on sequence alignments (**Supplementary Figure S3**).

Calcitonin-like diuretic hormone plays a role in regulating salt and water transport of insects and is considered to be the insect calcitonin ortholog (Zandawala, 2012; Veenstra, 2014). Calcitonin-like diuretic hormone is ubiquitous in insects, but calcitonin is only reported in several insects (Yeoh et al., 2017). Calcitonin can be separated into two distinct classes, calcitonin-A and calcitonin-B. In the red palm weevil, a transcript encoding calcitonin-B was identified. In both *T. castaneum* and *H. abietis*, two genes coding for calcitonin-B produce four and six isopeptides, respectively (Veenstra, 2014; Pandit et al., 2018). The red palm weevil transcript encodes seven mature isopeptides (**Supplementary Table S3**).

The number of insulin-like peptide (ILPs), also known as insulin-related peptide (IRP), varies widely in different insect species. For example, there are 50 in the silkworm (Aslam et al., 2011; Mizoguchi and Okamoto, 2013), but only one in the migratory locust (Veenstra, 2014). In *T. castaneum*, there are four insulin-like peptide-encoding genes assigned to three evolutionary groups based on their conserved motif (Li et al., 2007). Here in the red palm weevil, only one insulin gene was found, and the gene encodes a peptide similar to the *T. castaneum* ILP-B, with a CCxxxC motif. In Coleoptera, the number of insulin genes range from 2 to 10 and varies significantly in sequence (Veenstra, 2019). Our transcriptomes may not cover all of the insulin genes in the red palm weevil.

A pyrokinin and Capa precursor in hexapods can result in three types of neuropeptides: periviscerokinins (PVKs), pyrokinins (PKs), and tryptoPKs, and each one activates a specific

receptor (Terhzaz et al., 2012; Pandit et al., 2018). The number and combination of these three peptides differ in different species based on different ways of gene duplication and subsequent diversification (Derst et al., 2016). In the red palm weevil, transcripts encoding one complete capa and two incomplete pyrokinin precursors were identified, similar to that reported in several tenebrionid beetles (Neupert et al., 2018; Pandit et al., 2018). The CAPA precursor from the red palm weevil contains at least two potential PVKs and a single tryptoPK. Among the two PK genes, one coding for a PK precursor that can produce

TABLE 2 | Putative G protein-coupled receptors for neuropeptides identified from the red palm weevil.

				Homology search with known protein		
Neuropeptide receptor	**Accession no.**	**GPCR Class/Type**	**ORF (aa)**	**Species**	**Protein ID**	**E-Value**
Adipokinetic hormone	MK751489	Class A	377	*Dendroctonus ponderosae*	XP_019759039.1	0.0
Allatostatin C	MK751490	Class A	426	*Dendroctonus ponderosae*	XP_019756512.1	0.0
Capability	MK751491	Class A	266[a]	*Dendroctonus ponderosae*	XP_019756922.1	3e−132
CCHamide	MK751492	Class A	340[a]	*Dendroctonus ponderosae*	XP_019758999.1	0.0
Crustacean CardioActive Peptide	MK751493	Class A	152[a]	*Tribolium castaneum*	XP_015837746.1	1e−50
Ecdysis-triggering hormone	MK751494	Class A	465	*Dendroctonus ponderosae*	XP_019769415.1	0.0
FMRFamide receptor	MK751495	Class A	438	*Dendroctonus ponderosae*	XP_019768282.1	0.0
Long neuropeptide F	MK751496	Class A	449	*Leptinotarsa decemlineata*	XP_023018238.1	7e−174
Neuropeptide F 1	MK751497	Class A	414	*Dendroctonus ponderosae*	XP_019756679.1	0.0
Neuropeptide F 2	MK751498	Class A	429	*Dendroctonus ponderosae*	XP_019756688.1	0.0
Orphan1	MK751499	Class A	553	*Dendroctonus ponderosae*	XP_019754524.1	0.0
Orphan2	MK751500	Class A	447	*Sitophilus oryzae*	ALM55746.1	0.0
Orphan3	MK751501	Class A	342	*Anoplophora glabripennis*	XP_018566563.1	1e−174
Orphan4	MK751502	Class A	339	*Dendroctonus ponderosae*	XP_019754869.1	0.0
Proctolin	MK751503	Class A	552	*Dendroctonus ponderosae*	XP_019770777.1	0.0
Pyrokinin 1	MK751504	Class A	490	*Dendroctonus ponderosae*	XP_019763370.1	0.0
Pyrokinin 2	MK751505	Class A	526	*Dendroctonus ponderosae*	XP_019762501.1	0.0
RFLa peptide	MK751506	Class A	392	*Dendroctonus ponderosae*	XP_019764941.1	0.0
Sex peptide 1	MK751507	Class A	437	*Dendroctonus ponderosae*	XP_019758277.1	1e−166
Sex peptide 2	MK751508	Class A	380	*Dendroctonus ponderosae*	XP_019759112.1	0.0
Sex peptide 3	MK751509	Class A	317[a]	*Dendroctonus ponderosae*	XP_019769212.1	4e−101
	MK751510					
Sex peptide 4	MK751511	Class A	426[a]	*Dendroctonus ponderosae*	XP_019770807.1	3e−170
	MK751512					
Short neuropeptide F	MK751513	Class A	426	*Dendroctonus ponderosae*	XP_019761868.1	0.0
SIFamide	MK751514	Class A	446	*Dendroctonus ponderosae*	XP_019765870.1	2e−180
Sulfakinin	MK751515	Class A	424	*Dendroctonus ponderosae*	XP_019756917.1	0.0
Tachykinin-related peptide	MK751516	Class A	422	*Dendroctonus ponderosae*	XP_019768744.1	0.0
Trissin1	MK751517	Class A	410	*Dendroctonus ponderosae*	XP_019773394.1	0.0
Trissin2	MK751518	Class A	241[a]	*Dendroctonus ponderosae*	XP_019759003.1	5e−105
Inotocin (vasopressin-like)	MK751519	Class A	375	*Tribolium castaneum*	XP_015837046.1	0.0
Orphan5	MK751520	Class A	492	*Dendroctonus ponderosae*	XP_019761979.1	0.0
Calcitonin	MK751521	Class B	541	*Dendroctonus ponderosae*	XP_019756394.1	0.0
Diuretic hormone 31	MK751522	Class B	286[a]	*Dendroctonus ponderosae*	XP_019763869.1	3e−124
Diuretic hormone 44	MK751523	Class B	415	*Dendroctonus ponderosae*	XP_019758310.1	3e−90
Pigment-dispersing factor	MK751524	Class B	469	*Dendroctonus ponderosae*	XP_019773389.1	2e−162
Orphan6	MK751525	Class B	453	*Dendroctonus ponderosae*	XP_019757091.1	7e−144
Leucine-rich repeat-containing GPCR – FSH	MK751526	Type A	769	*Dendroctonus ponderosae*	XP_019769811.1	0.0
Leucine-rich repeat-containing GPCR – LGR4-like	MK751527	Type B	598	*Dendroctonus ponderosae*	XP_019753406.1	5e−153
Leucine-rich repeat-containing GPCR – Burs-like	MK751528	Type B	1169[a]	*Tribolium castaneum*	EFA02891.2	0.0
Leucine-rich repeat-containing GPCR – LGR5-like1	MK751529	Type B	672	*Dendroctonus ponderosae*	XP_019754543.1	0.0
Leucine-rich repeat-containing GPCR – LGR5-like2	MK751530	Type B	495	*Dendroctonus ponderosae*	XP_019760595.1	0.0
Leucine-rich repeat-containing GPCR – insulin-like1	MK751531	Type C	1391	*Dendroctonus ponderosae*	XP_019765260.1	0.0
Leucine-rich repeat-containing GPCR – insulin-like2	MK751532	Type C	1226	*Dendroctonus ponderosae*	XP_019755840.1	0.0
Leucine-rich repeat-containing GPCR – insulin-like3	MK751533	Type C	1319	*Dendroctonus ponderosae*	XP_019761347.1	0.0
Leucine-rich repeat-containing GPCR – relaxin 2-like	MK751534	Type C	424[a]	*Dendroctonus ponderosae*	XP_019760354.1	1e−160

three pyrokinins and one tryptoPK, and the other coding for a precursor that can produce two pyrokinins and one tryptoPK (**Supplementary Table S3**).

In addition, we identified two CCHamides, two ion transport peptides and two neuropeptide-like precursors from the red palm weevil transcriptomes. The two ion transport peptides and the two neuropeptide-like precursors are produced by alternative splicing and identical in sequence, but varied in sequence length. However, the two CCHamide precursors encode different types of peptides: CCHa-1 and CCHa-2. Multiple sequence alignments indicate that most insect mature CCHa-1 peptides have the consensus SCLSYGHSCWGAH, and CCHa-2 have the consensus GCSXFGHSCFG(G,A)H. However, the conserved Ser at position 8 of CCHa-1 is mutated to Ala in all Cucujiformia beetles, while the conserved Gly-His-amide at C-terminal of CCHa-2 is mutated to Gly-Met-amide in most Coleoptera beetles, the red palm weevil is even more strangely mutated to Ala-Leu-amide (**Supplementary Figures S4, S5**).

No transcripts encoding adipokinetic hormone, AKH/Corazonin-related peptide, Agatoxin-like, crustacean

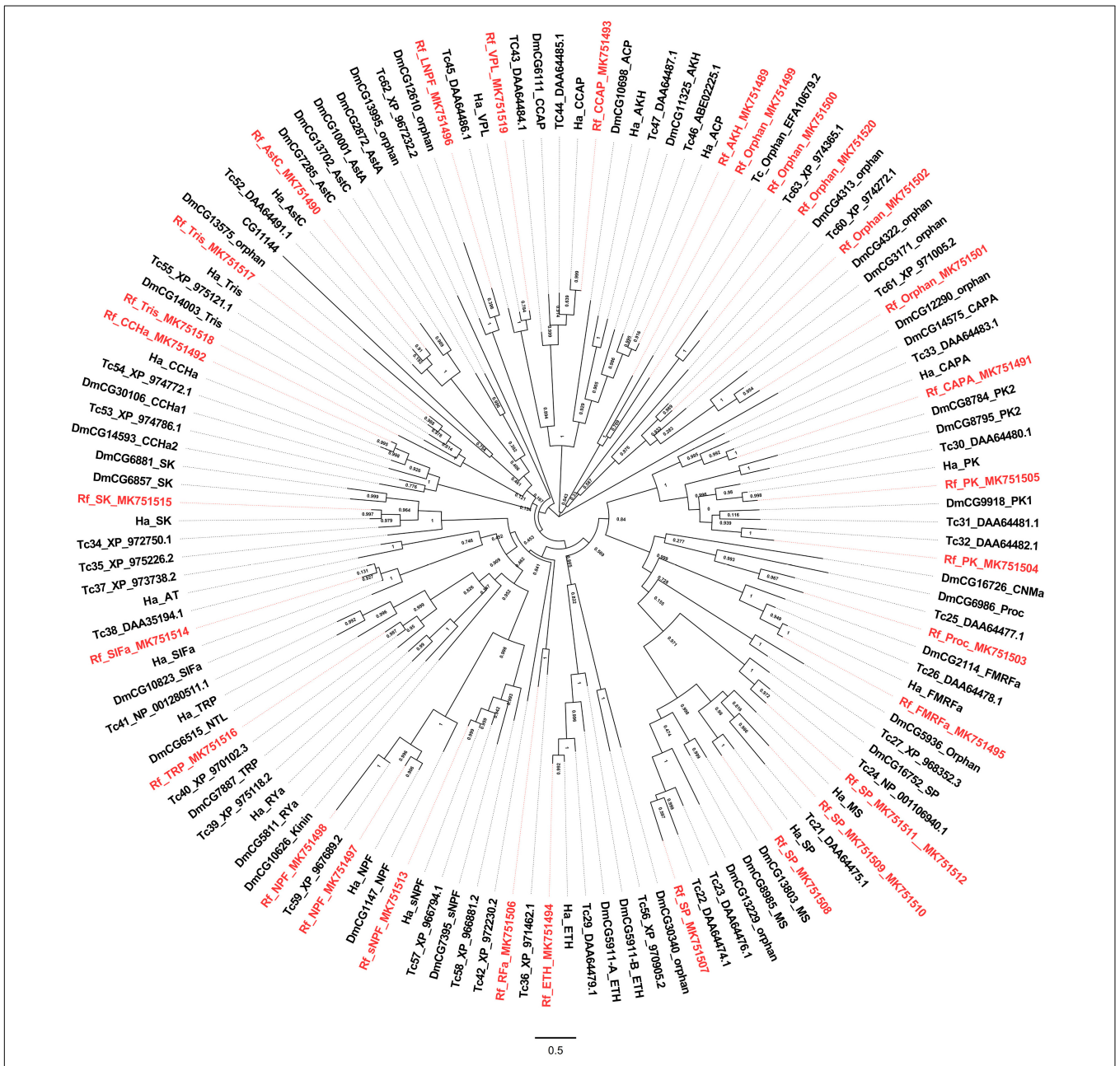

FIGURE 2 | Phylogenetic tree analysis of the Class A neuropeptide GPCRs from *Rhynchophorus ferrugineus* (Rf), *H. abietis* (Ha), *T. castaneum* (Tr), *Drosophilla melanogaster* (Dm).

cardio-active peptide, Hansolin, Relaxin, RFLamide, and SIFamide were found in the transcriptomes of the red palm weevil (**Supplementary Table S4**).

Identification of G Protein-Coupled Receptors (GPCRs) for Neuropeptides

A total of 44 putative neuropeptide GPCRs were identified from the red palm weevil transcriptomes (**Table 2** and **Supplementary Table S2**). These GPCRs were divided into three classes, 30 belonged to the Class A (Rhodopsin-like receptor family), five belonged to the Class B (Secretin-like receptor family), and the remaining nine belonged to LGRs (Leucine-rich repeat-containing GPCRs).

Class A contains the most receptors, and their potential neuropeptide ligands include Adipokinetic hormone, Allatostatin C, CCHamide 1, Ecdysis-triggering hormone, etc. A phylogenetic analysis showed that class A receptors from the red palm weevil are clustered with counterparts from other insect species including *T. castaneum*, *D. melanogaster*, and *H. abietis* (**Figure 2**) (Hauser et al., 2008; Pandit et al., 2018). GPCRs not found in the red palm weevil (but their corresponding neuropeptides were identified) included those receptors for allatotropin, CCHamide2, myosuppressin, and RYamide. GPCRs for adipokinetic hormone, the crustacean cardioactive peptide, RFa peptide, sex peptide, and SIFamide were identified from the red palm weevil. However, their corresponding neuropeptides were not found yet. The GPCRs for sex peptide, neuropeptide F, and trissin appeared to have expanded in the red palm weevil. Specifically, there were four sex peptide receptors, three receptors for neuropeptide F (including long neuropeptide F, neuropeptide F1, neuropeptide F2), and two trissin receptors identified in the red palm weevil, compared with only one sex peptide receptor, one neuropeptide F receptors, and one trissin receptor has been reported in *H. abietis*. In addition, five orphan GPCRs were identified from the red palm weevil, but their neuropeptide ligands remained unknown.

Class B receptors for calcitonin, diuretic hormones 31 and 44, and pigment-dispersing factor were identified along with an orphan GPCR from the red palm weevil. All the identified receptors were found to share evolutionary relationship (**Figure 3**).

Several leucine-rich repeats-containing GPCRs (LGRs) were also identified (**Figure 4**). The LGRs were divided into three types according to the numbers of leucine-rich repeat motifs, types A, B, and C. Type-A LGRs include follicle stimulating hormone receptor (FSH) and the choriogonadotropin receptor. However, no choriogonadotropin receptor was found in the red palm weevil. Type-B LGRs include Bursicon-like as well as LGR4 and 5-like receptors, all of which were identified in the red

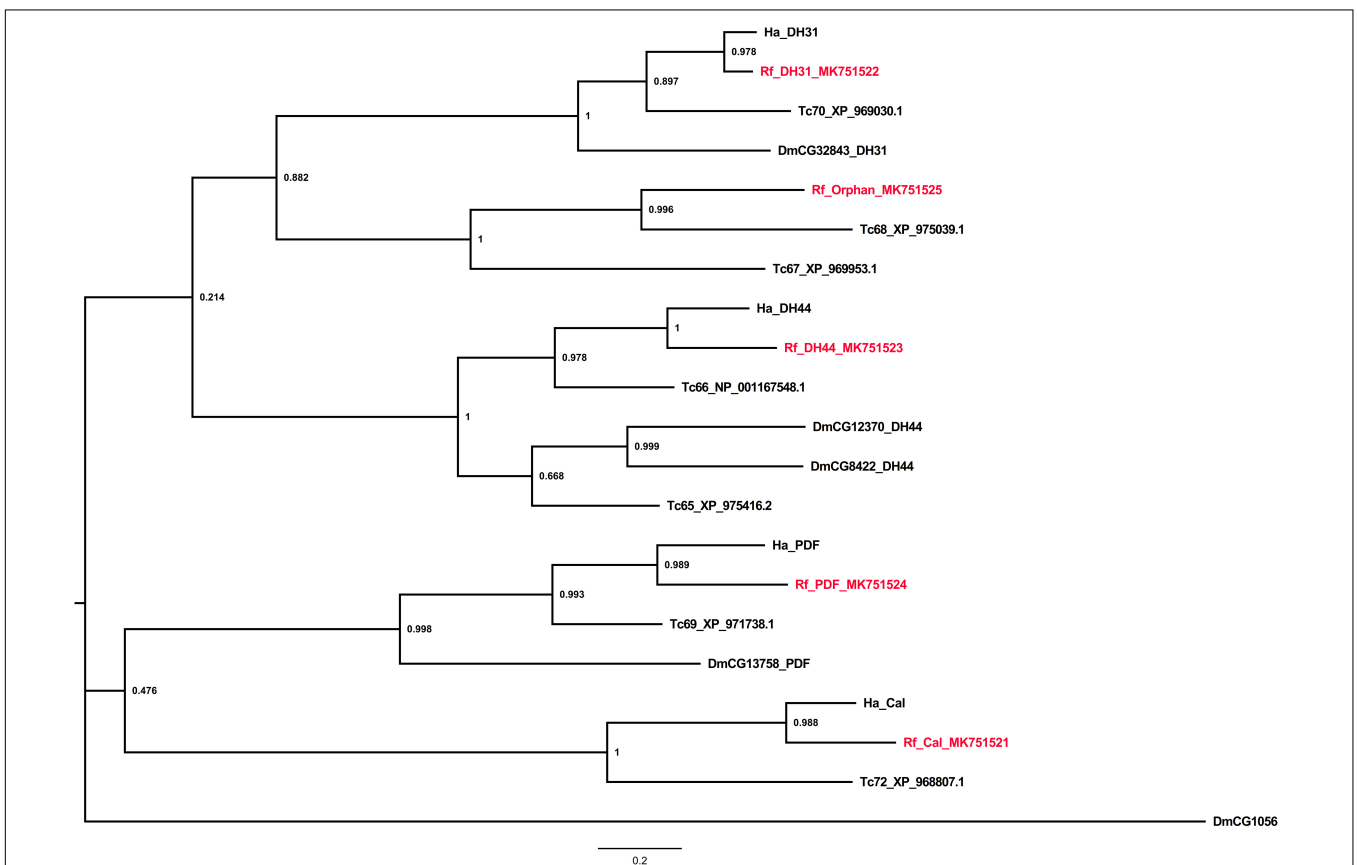

FIGURE 3 | Phylogenetic tree analysis of the Class B neuropeptide GPCRs from *R. ferrugineus*, *H. abietis*, *T. castaneum*, *D. melanogaster*.

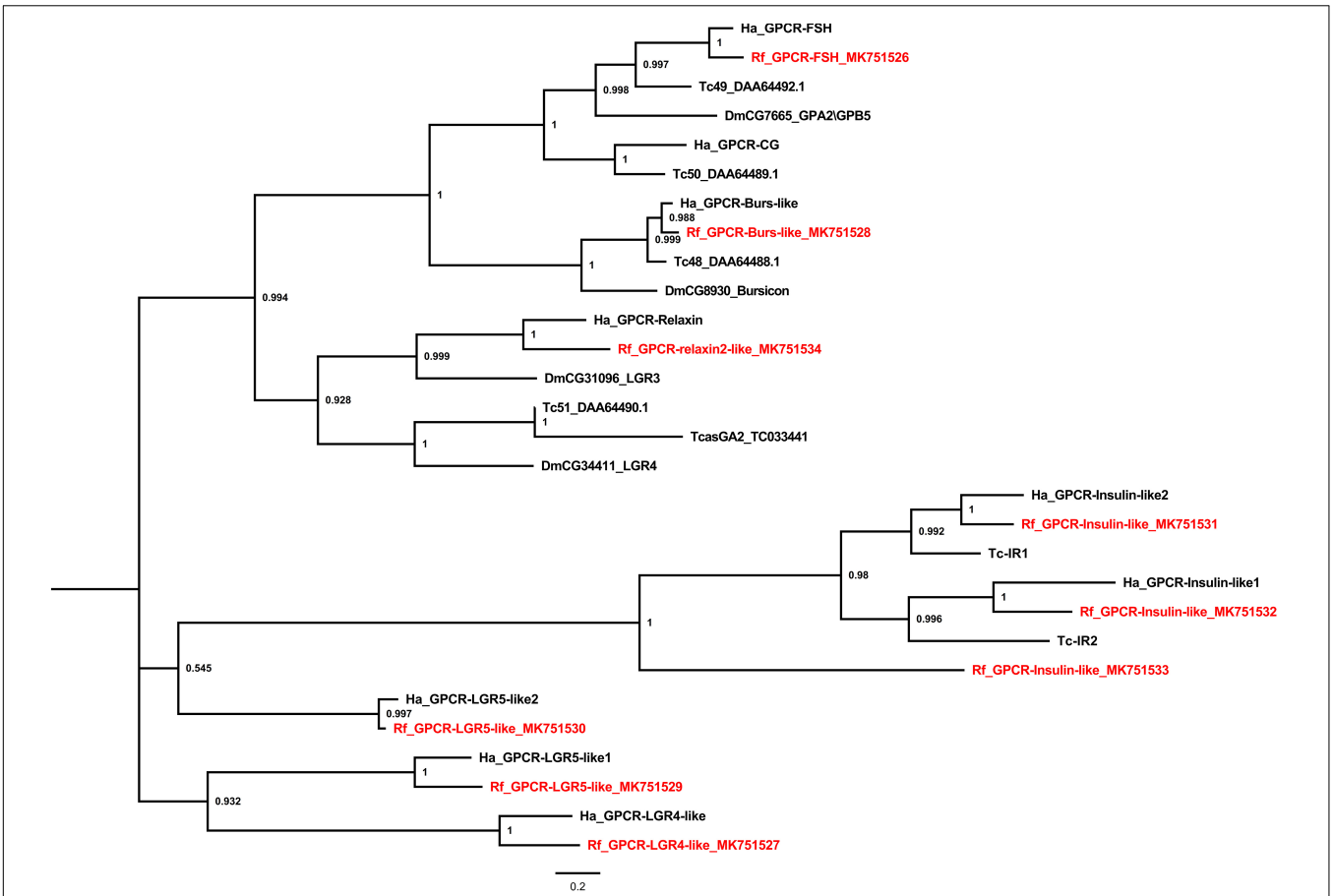

FIGURE 4 | Phylogenetic tree analysis of the Leucine-rich Repeat-containing GPCRs (LGR) from *R. ferrugineus*, *H. abietis*, *T. castaneum*, *D. melanogaster*.

palm weevil. Type-C LGRs identified from the red palm weevil include one relaxin 2-like and three insulin-like receptors, which is unusual since Coleopterans, Hymenopterans, and Hemipteran were thought to have only two insulin receptors (Sang et al., 2016), as is the case in *T. castaneum* and *H. abietis* (**Figure 4**).

Expression Profiles of the Neuropeptides and Neuropeptide Receptors

Gene expression profiles were analyzed in four types of tissues, including the central nervous system (CNS) (brain and ventral nerve cord), the gut (foregut, midgut, hindgut, and Malpighian tubes), hemocytes, and fat bodies. Most neuropeptide precursors were expressed at the highest levels in CNS. The genes coding for allatostatin B, allatostatin C, Calcitonin, diuretic hormone 44, Ion transport peptide a and b, orcokinin B were mainly expressed in gut plus Malpighian tubules. The genes coding for CNMamide, insulin-related peptide, neuroparsin were mainly expressed in fat bodies (**Figure 5** and **Supplementary Figure S6**).

For neuropeptide receptors, genes coding for receptors for ecdysis-triggering hormone and SIFamide along with four orphan GPCRs were predominately expressed in hemocytes, whereas receptors for adipokinetic hormone, pyrokinin 2, and diuretic hormone 44 were expressed at the highest levels in fat bodies. Receptors for CCHamide 1, FMRFamide, long neuropeptide F, orphan GPCR2, RFa peptide, Sex peptide 1, 3 and 4, short neuropeptide F, inotocin, calcitonin, diuretic hormone 31, pigment-dispersing factor, and insulin along with leucine-rich repeat-containing GPCR–FSH, LGR4, and LGR5-1 were mainly expressed in the gut plus Malpighian tubules. Receptors for neuropeptide F1, proctolin, and trissin-1 were predominately expressed in CNS. No differences in the remaining receptors were detected among the tissues analyzed (**Figure 6** and **Supplementary Figure S7**).

Impact of Pathogen Infection on the Expression of Neuropeptide and Receptor Genes

The expression of genes encoding neuropeptides and receptors in the red palm weevil was analyzed after the insect was infected with either *S. marcescens* (Gram−), *B. thuringiensis* (Gram+), or *M. anisopliae* (Fungus). The expression of the gene encoding the locust insulin-related peptide precursor decreased in insects infected with either *B. thuringiensis* or *M. anisopliae*. The expression of the genes encoding allatostatin CC, the GPCR for allatostatin C, tachykinin-related peptide, the receptor for tachykinin-related peptide, neuropeptide F,

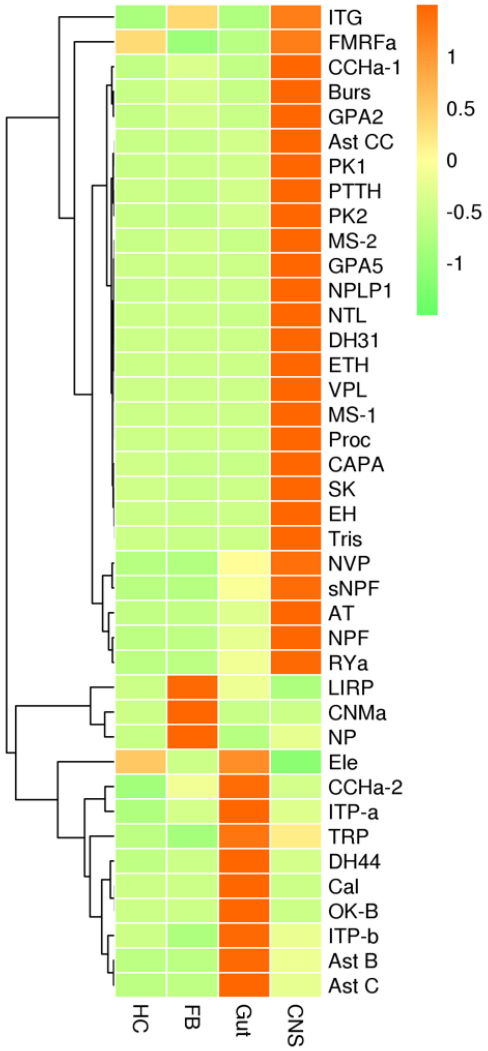

FIGURE 5 | qRT-PCR results showing the relative expression levels of the neuropeptide precursors in various tissues of the red palm weevil. HC, hemocytes; FB, fat bodies; Gut, including foregut, midgut, hindgut, and Malpighian tubes; CNS, central nervous system. The expression levels were estimated using the $2^{-\Delta CT}$ method. Red indicates overexpression, while green represents low expression.

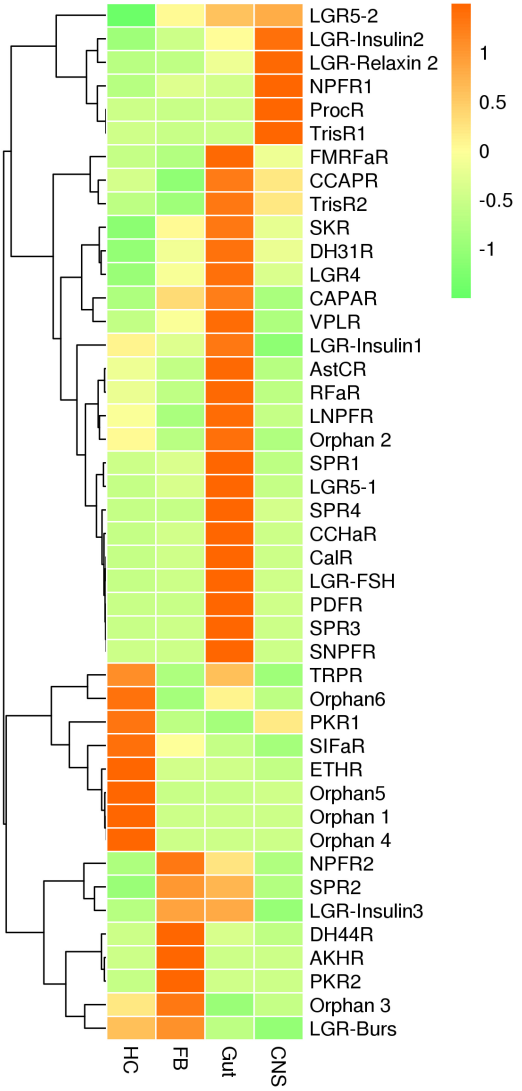

FIGURE 6 | qRT-PCR results showing the relative expression levels of the neuropeptide receptors in various tissues of the red palm weevil.

calcitonin, LGR-insulin 1, LGR-insulin 2, and LGR-insulin 3 increased after infection with either *B. thuringiensis* or *M. anisopliae*. The expression levels of genes encoding capability and the GPCR for short neuropeptide F increased significantly in 48 h after *S. marcescens* infection. The remaining genes were either not responsive to pathogen infection or irregularly expressed after pathogen infection (**Figure 7**).

DISCUSSION

In this study, we systematically identified neuropeptide precursors and GPCRs from the red palm weevil following a transcriptomic approach. The quality of the transcriptomes from both larvae and pupae appeared to be comparable with those from other insect species based on total numbers and average lengths of assembled unigenes, and N50 values. A total of 43neuropeptide precursors were identified from the red palm weevil, compared with 48 from *H. abietis* and 64 from *T. castaneum* (Pandit et al., 2018; Veenstra, 2019). A total of 44 neuropeptide GPCRs were identified from the red palm weevil, compared with 25 identified from *H. abietis* and 48 from *T. castaneum* (Hauser et al., 2008). The total numbers of genes coding for both neuropeptides and GPCRs were largely comparable to those from other insect species.

Despite similar numbers of neuropeptides and GPCRs identified from the red palm weevil, the repertoires of neuropeptides and GPCRs showed uniqueness in this insect species. First, two genes encoding two very different forms of

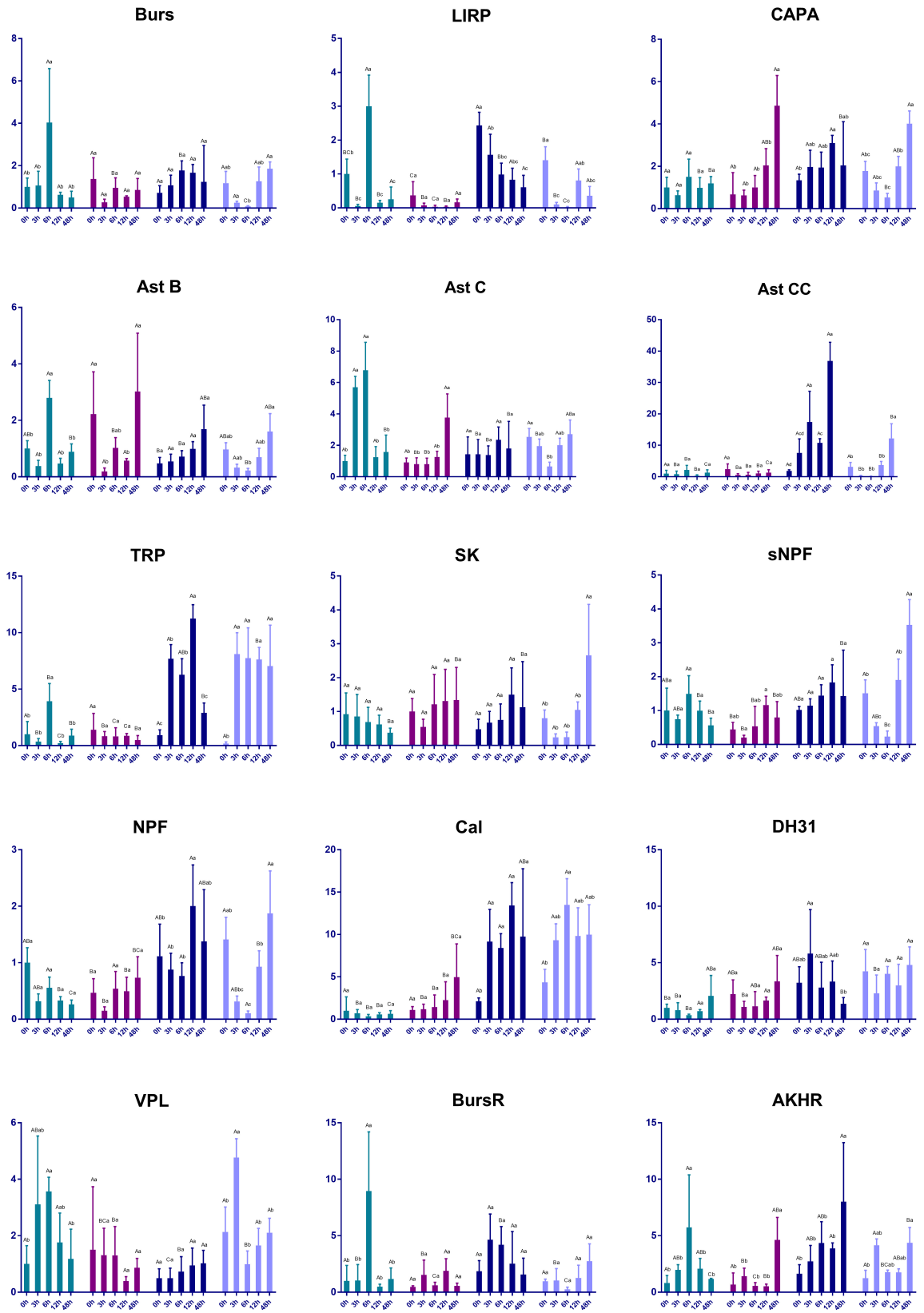

FIGURE 7 | Continued

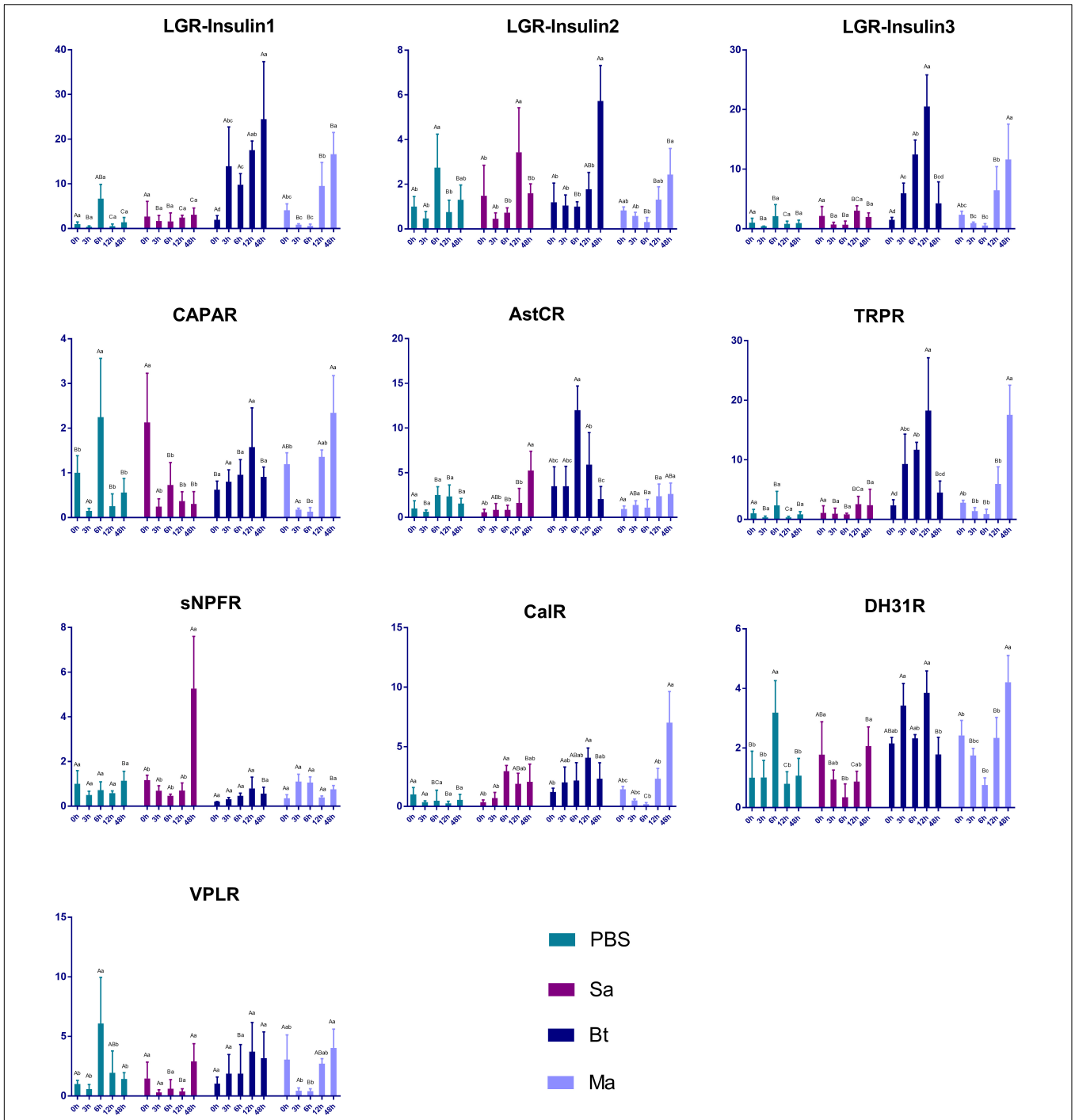

FIGURE 7 | Impact of pathogen infection on the expression of neuropeptide and receptor genes. Different small letters indicate statistically significant difference among different time points for the same treatment. Different capital letters indicate statistically significant difference among different treatments of the same time point.

myosuppressins were identified from the red palm weevil. This is striking because only one gene has been reported in most other insect species so far in the literature. Two gene encoding two myosuppresins are reported in the migratory locust, but these two neuropeptides are produced by alternative splicing and conserved at all consensus sites (Veenstra, 2014). Myosuppressins with the consensus XDVXHXFLRFamide generally play roles in regulating the gut and heart muscle contraction in insects (Nässel and Winther, 2010). Amino acid substitutions in myosuppresins can result in different developmental and

tissue-specific synergetic or antagonistic effects (Dickerson et al., 2012). The two myosuppresins from the red palm weevil are very diverged, with the residues at both the 3rd and 8th positions of Val and Leu replaced with Met and Trp (**Figure 1**). Because of the diverged sequences, the functions for these two myosuppresin isoforms would be quite different even though the exact function of the new myosuppresin remains to be delineated.

Second, several gene expansions were observed in the red palm weevil. The most obvious expansion is the GPCRs for neuropeptide F and insulin related peptides. Neuropeptide F plays a role in regulating feeding and sleep-wake behavior of insects (Chung et al., 2017). A long and short neuropeptide F usually exist in insect species. However, four CPCRs were found in the red palm weevil. Two CPCRs correspond to the long and short NPF, respectively, but the remaining two receptors remain unknown if they are involved in similar activation pathways. Three insulin receptors were found in the red palm weevil, but only two in other insect species. The two insulin receptors in *T. castaneum* were expressed in different developmental stages, and have functionally diverged with respect to the development and reproduction (Sang et al., 2016). The newly expanded insulin receptors in the red palm weevil may also have a unique regulatory effect on its growth and development.

It is quite interesting that several neuropeptide genes were apparently not found in our study, including adipokinetic hormone, AKH/Corazonin-related peptide, agatoxin-like, crustacean cardio active peptide, hansolin, relaxin, SIFamide andRFLamide, but some of their putative corresponding receptors were identified. Similarly, GPCRs, like allatostatin B, myosuppressin and RYamide, were not found in this study, but their corresponding hormones were identified. These neuropeptides and GPCRs are ubiquitous in other Coleoptera (Veenstra, 2019). Thus most of the absence may be due to insufficient sequencing depth or incomplete annotation. However, some neuropeptides have been reported got lost repeatedly in Coleoptera, like elevenin, AKH/Corazonin-related peptide, and relaxin (Veenstra, 2019), this suggests that a few neuropeptides may have been lost if not identified. The RFLamide and receptor was not found in either of the two Curculionids, *Hypothenemus* and *Dendroctonus* (Veenstra, 2019), but the receptor was identified in the red brown weevil. So it's still unclear whether this neuropeptide signaling is still present in the red palm weevil.

Some genes encoding neuropeptides and receptors were found to be highly expressed in tissues other than the CNS. For example, genes encoding several neuropeptides, including AstC, CCHa-2, CCAP, capa, calcitonin, FMRFamide, sNPF, myosuppressin, diuretic hormones, ion transport peptide, orcokinin and NVP-like peptide precursors, were all predominately expressed in gut plus Malpighian tubules, suggesting their important roles on feeding, digestion, diuresis, and energy homeostasis in insects. Similar observations were also found in other insect species (Audsley and Weaver, 2009; Schoofs et al., 2017). What is unique for the red palm weevil is the expression of the two genes encoding NPFs. Since NPF-genes are highly expressed in the gut of *C. suppressalis* and *L. migratoria*, it was thought that NPFs are associated with the regulation of feeding behavior (Hou et al., 2015; Xu et al., 2016). But both the long NPF and short NPF in the red palm weevil were highly expressed in the central nerve system, their corresponding receptor genes were highly expressed in gut. On the other hand, the gene encoding AstB was highly expressed in the gut of the red palm weevil larvae, but the same gene is primarily expressed in the central nerve system in *C. suppressalis* (Xu et al., 2016). These conflicting observations indicate that different insect species may regulate their signaling network differently, and there may be different recognition partners under different physiological conditions.

Neuropeptides are well-known to regulate various physiological processes and behaviors of insects, but little is known about their roles in regulating insect immunity. In this study, we found that some genes encoding neuropeptides and receptors responded to pathogen attack in the red palm weevil. The two pathogens that induced the most responses are *B. thuringiensis* and *M. anisopliae*. The induction of neuropeptide and receptor genes by pathogens indicate that these neuropeptide and receptor pairs play roles in immune response of the insect. For example, the gene encoding the tachykinin-related peptide was strongly induced in red palm weevils 3 h after *B. thuringiensis* and *M. anisopliae* infection and the effects lasted for a long time. Tachykinin-related peptide is a multifunctional neuropeptide, which may regulate insect immune system and metabolic homeostasis based on structure and functional homologs of vertebrate tachykinins (Urbanski and Rosinski, 2018). In *D. melanogaster*, intestinal microbiota and the microbial metabolite have also been found to activate innate immunity and promote the expression of the tachykinin-related peptide to promote host metabolic homeostasis (Kamareddine et al., 2018).

CONCLUSION

We have systematically analyzed genes encoding neuropeptides and their corresponding receptors in the destructive pest red palm weevil via establishing a larval and a pupal transcriptome, resulting in the identification of 43 putative neuropeptide precursors and 44 neuropeptide receptors. A novel form of myosuppressin was discovered, which carries distinct amino acid residues at two conserved sites. Genes encoding neuropeptide F receptors and insulin receptors have expanded. We also analyzed the expression of the identified genes in different tissues. Some genes encoding neuropeptide precursors and receptors were highly expressed in tissues other than the CNS and may play roles other than neural signaling. Four orphan receptors may play a role in regulating immune cell activity based on their high expression in hemocytes. Moreover, some neuropeptides and receptors, like the tachykinin-related peptide and receptor, were significantly induced by pathogen infection, especially sensitive to *B. thuringiensis* and *M. anisopliae*, which may regulate insect immune system. Our research laid the foundation for future functional studies on neuropeptides and

their receptors, which may lead to the development of novel pest control strategies.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI GenBank, Red Palm Weevil neuropeptide receptors accession numbers: MK751489–MK751534, Red Palm Weevil neuropeptide precursors accession numbers: MK751535–MK751576.

ETHICS STATEMENT

Rhynchophorus ferrugineus is exempted from above mentioned requirements.

AUTHOR CONTRIBUTIONS

YH and JL conceived and designed the research. HZ, JB, SH, and HL performed the experiments. HZ performed the analysis and wrote this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00159/full#supplementary-material

FIGURE S1 | Multiple sequence alignment of short NPF **(A)** and long NPF **(B)** of a number of insect species. The sequence in the box are the predicted mature peptides and the sequence underlined in red is absent from the short NPF precursors.

FIGURE S2 | Multiple sequence alignment of PDF mature peptides from a number of Coleoptera species. The sequence underlined in orange is the predicted mature peptide.

FIGURE S3 | Sequence logos for the putative Orcokinin B mature peptides that are repeated in the pre-propeptide of each insects.

FIGURE S4 | Multiple sequence alignment of the putative CCHa-1 mature peptides.

FIGURE S5 | Multiple sequence alignment of the putative CCHa-2 mature peptides.

FIGURE S6 | qRT-PCR histogram results showing the relative expression levels of the neuropeptide precursors in various tissues of the red palm weevil. The expression levels were estimated using the $2^{-\Delta\Delta CT}$ method. Bars represent standard error of four independent biological replicates with three technical duplicates for each replicate. Different small letters indicate statistically significant difference between tissues ($p < 0.05$, ANOVA, HSD).

FIGURE S7 | qRT-PCR histogram results showing the relative expression levels of the neuropeptide receptors in various tissues of the red palm weevil.

TABLE S1 | List of neuropeptide genes of red palm weevil.

TABLE S2 | List of neuropeptide receptor genes of red palm weevil.

TABLE S3 | Putative Neuropeptides/Neuropeptide-Like Peptides from precursors of red palm weevil.

TABLE S4 | Neuropeptide genes in the red palm weevil and other Coleopteran beetles. √, identified; ×, not identified.

TABLE S5 | Primers of candidate neuropeptide precursors and receptors in used for RT-qPCR.

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REVIEW
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Peptide Hormones in the Insect Midgut

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Insects produce many peptide hormones that play important roles in regulating growth, development, immunity, homeostasis, stress, and other processes to maintain normal life. As part of the digestive system, the insect midgut is also affected by hormones secreted from the prothoracic gland, corpus allatum, and various neuronal cells; these hormones regulate the secretion and activity of insects' digestive enzymes and change their feeding behaviors. In addition, the insect midgut produces certain hormones when it recognizes various components or pathogenic bacteria in ingested foods; concurrently, the hormones regulate other tissues and organs. In addition, intestinal symbiotic bacteria can produce hormones that influence insect signaling pathways to promote host growth and development; this interaction is the result of long-term evolution. In this review, the types, functions, and mechanisms of hormones working on the insect midgut, as well as hormones produced therein, are reviewed for future reference in biological pest control.

Keywords: insect, peptide hormones, midgut, symbionts, immunity, bio-control

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Insect hormones are secreted by the endocrine system within the body and participate in the regulation and control of processes such as growth, metamorphosis, and reproduction (Jindra et al., 2013; Slama, 2015; Roy et al., 2018; Santos et al., 2019). In contrast, pheromones, also known as chemical signals, that can allow insects to communicate within species (Yew and Chung, 2015). Insect hormones can be categorized into steroids, sesquiterpenes, polypeptides, and others. Ecdysone is a steroid hormone synthesized by the prothoracic gland (PG) (Ou et al., 2016). Juvenile hormone (JH) is a sesquiterpene compound produced and secreted by the corpus allatum (Roller et al., 1967; Jindra et al., 2013). These two hormones are very important for insect growth and development. In addition, numerous peptide hormones in insects also exhibit important regulatory effects on insect growth and development. Insect endocrine peptides can have three main functions: promotion of digestion, transmission of information to the central nervous system, and promotion of nutrient absorption and conversion (Wegener and Veenstra, 2015). Peptide hormones also affect the synthesis or secretion of 20-hydroxyecdysone (20E), JH, and other hormones. The many types of hormones in the insect body cooperate to ensure normal growth, development, reproduction, and other life processes in each insect.

Insect peptide hormones are a subset of neuropeptides, which are expressed and secreted by the central and peripheral nervous systems and then act on other organs, including the PG, corpus allatum, corpora cardiaca, and other glands. The insect gut is divided into three parts: the foregut,

midgut, and hindgut. The gut is responsible for digesting food and absorbing nutrition; it is also regulated by neuropeptide hormones (as in mammals) (Audsley and Weaver, 2009; Nassel and Winther, 2010; Mayer, 2011; Pool and Scott, 2014), which is manifested in the peristalsis of the gut, cellular secretion of digestive enzymes, and intake of nutrition. In addition, JH and ecdysone affect the gut. For instance, JH can stimulate proliferation of enterocytes in the mated *D. melanogaster* females (Reiff et al., 2015); ecdysone can promote the midgut programmed cell death (Nicolson et al., 2015). The insect gut can also produce hormones, including peptide hormones, that ensure a normal physiological state, especially after intake of different foods. Furthermore, peptide hormones produced by the gut can act on other tissues, including nerve tissues; therefore, some peptide hormones produced by the gut are regarded as brain-gut peptides. There have been many studies and reviews regarding insect hormones. Peptide hormone genes and their receptors in insects have been reviewed previously (Riehle et al., 2002; Reiher et al., 2011; Wegener and Veenstra, 2015; Strand et al., 2016). Although there have been several reviews on hormones produced by the intestinal endocrine cells (Wegener and Veenstra, 2015), the most recent literatures has not yet been summarized. This review discusses recent research regarding peptide hormones related to the insect midgut. Specifically, this review describes the functions of peptide hormones produced in the insect gut, which provides a reference for future works on the molecular mechanisms of insect gut peptide hormones and the application for the pest biological control.

PEPTIDE HORMONES PRODUCED IN THE GUT

The insect digestive tract secretes many enzymes to digest proteins, lipids, and carbohydrates, which are digested extracellularly (Weidlich et al., 2013, 2015; Holtof et al., 2019). In the insect midgut, epithelial cells can produce several digestive enzymes and direct distribution of nutrients and transport of ions and water (Caccia et al., 2019).

Generally speaking, insect midgut cells include intestinal endocrine cells, intestinal epithelial cells, columnar cells with intestinal villi, and intestinal stem cells (Billingsley and Lehane, 1996; Caccia et al., 2019). Some insects can regulate intestinal pH via goblet cells (e.g., *Lepidoptera*) or copper cells; endocrine cells can produce peptide hormones (Huang et al., 2015; Caccia et al., 2019). In addition to the relationship between endocrine cells and hormones, columnar cells can secrete the signaling protein Hedgehog to regulate nutrient availability and developmental timing (Rodenfels et al., 2014); columnar cells also produce and secrete digestive enzymes, lysozyme, and antimicrobial peptides, and are able to absorb nutrition (Caccia et al., 2019).

The critical mechanisms of peptide hormones secreted by the insect gut have been relatively well studied in *Drosophila*. The gut endocrine cells of *Drosophila melanogaster* can produce six peptides, including allatostatins A, B, and C, neuropeptide F, diuretic hormone 31 (Dh31), and tachykinin (Veenstra et al., 2008). However, more than 45 neuropeptide genes were recently found in the genome of *D. melanogaster*. Based on reverse transcription polymerase chain reaction and *in situ* hybridization analyses, there are at least 10 neuropeptides expressed in midgut endocrine cells (Chen et al., 2016). Endocrine cells in different parts of the intestine produce distinct peptide hormones. For instance, Dh31, CCHamide-1 (CCHa1), Allatostatin A (AST-A), and Myoinhibiting peptide (MIP = Allatostatin B) are expressed in the posterior midgut; MIP is also expressed in middle midgut, and the whole midgut can produce AST-C, CCHamide-2 (CCHa2), and tachykinin (Chen et al., 2016). CCHa1 and CCHa2, as brain-gut peptides, are expressed in both midgut and in brain nerves (Ren et al., 2015). Use of the CRISPR/Cas9 gene editing system to disrupt CCHa1 and CCHa2 led to significant reduction in the food intake of CCHa2 mutants. Moreover, CCHa2 mutations can delay the development of larva, which may be related to the 80% reduction in mRNA concentrations of insulin-like peptides (ILPs) 2 and 3 induced by *ccha2* mutation (Ren et al., 2015). Therefore, peptide hormones secreted by the insect gut are very important for development and physiological functions. **Table 1** provides a detailed introduction of several important peptide hormones produce by insect gut cells.

Insulin-Like Peptides

Insulin-like peptides (ILPs) are important peptide hormones in insects that play roles in metabolism, growth, reproduction, and aging (Garofalo, 2002; Defferrari et al., 2016; Nassel and Vanden Broeck, 2016; Zhang et al., 2017). ILP generation is regulated by insulin/insulin-like growth factor signaling (Nassel and Vanden Broeck, 2016). The insulin signaling pathway plays an important role in insect development and organ growth. Garofalo analyzed the genetics of insulin signaling in *Drosophila*, and compared the insulin signaling pathways among different species (Garofalo, 2002). Lin and Smagghe reviewed these pathways in detail (Lin and Smagghe, 2018).

ILPs can be expressed in the midgut and other tissues, where they can concurrently regulate the growth and development of insects. *D. melanogaster* has eight ILPs (DILP1–8) and two receptors, but mammals have many more receptors (Nassel and Vanden Broeck, 2016). The ILP1 gene of beet armyworm (*Spodoptera exigua*) can encode a 95-amino acid peptide, which is expressed in the fat body and epidermis, but not in the blood cells or intestine (Kim and Hong, 2015). When 5th-stage larvae of *S. exigua* were starved for 48 h, the transcription of SeILP1 was reduced, whereas the content of trehalose in hemolymph was increased more than twofold. Furthermore, RNAi of SeILP1 resulted in a significant increase in the content of trehalose in hemolymph. These findings showed that SeILP1 has an inhibitory effect on trehalose levels in hemolymph (Kim and Hong, 2015).

Factors released by the gut and adipocytes of *Drosophila* can regulate glucose-mediated DILP secretion. For example, DILP2 is produced by brain nerve cells, DILP3 is produced by gut muscles, DILP5 is produced by ovaries and Malpighian tubules, and DILP6 is mainly produced in the fat body (Nassel and Vanden Broeck, 2016). The expression levels of *dilp4* and *dilp5* in the gut of *Drosophila* larvae are high, whereas the expression level of *dilp6* in the gut is

TABLE 1 | Peptide hormones produced in midgut of insects.

Peptide	Location	Function	Insect species and references
Allatotropin	Digestive tract	Control JH biosynthesis	*Spodoptera frugiperda* (Abdel-Latief et al., 2004)
Allatostatin A, B(MIP), C	A: Endocrine cells in the posterior midgut	A: Regulate gut contraction, K^+ absorption.	*Diploptera punctate*, *Aedes aegypti*, *Anopheles albimanus*, *Drosophila melanogaster* (Reichwald et al., 1994; Hernandez-Martinez et al., 2005; Spit et al., 2012; Vanderveken and O'Donnell, 2014; Nouzova et al., 2015)
	B: Endocrine cells in the midgut	B: Inhibit fore- and hind-gut contractions and food intake.	
	C: Entire midgut	C: Inhibit the synthesis of JH III	
CCHamide 1, 2	1: Posterior midgut	1. Regulate gut muscle contractions	*Drosophila melanogaster* (Reiher et al., 2011; Veenstra and Ida, 2014; Chen et al., 2016)
	2: Entire midgut	2. Digestion, release to hemolymph	
Neuropeptide F	Endocrine cells of the midgut	Release to hemolymph, modulate the physiology of feeding and digestion	*Helicoverpa zea* (Huang et al., 2011)
Orcokinin	Enteroendocrine cells, anterior and middle midgut	Regulate ecdysis	*Bombyx mori*, *Drosophila melanogaster*, *Rhodnius prolixus* (Yamanaka et al., 2011; Chen et al., 2015; Chen et al., 2016; Wulff et al., 2018)
Ryamide	Enteroendocrine cells of the anterior midgut	Regulation of feeding and digestion	*Bombyx mori* (Roller et al., 2016)
Tachykinin-related peptides	Midgut	Adaptation to different nutritional conditions; development	*Drosophila*, *Bombyx mori* (Van Loy et al., 2010; Nagai-Okatani et al., 2016; Yamagishi et al., 2018)
Diuretic hormone 31	Midgut endocrine cells	Regulate midgut contraction frequency	*Drosophila melanogaster* (Reiher et al., 2011; Vanderveken and O'Donnell, 2014)
Kinin	Hindgut	Regulate diuretic, digestive and myotropic activities and hindgut contractions	*Rhodnius prolixus* (Bhatt et al., 2014)
Myosuppressin	Posterior midgut	Regulate contractions of the anterior midgut and hindgut	*Rhodnius prolixus* (Lee et al., 2012)
Insulin-like peptides 2, 4, 5	Midgut	Related to lifespan, body size, growth	*Drosophila melanogaster* (Brogiolo et al., 2001; Zhang et al., 2009; Gronke et al., 2010)
Prothoracicotropic factors (PTTH)	Proctodaea/gut	Promote ecdysone and 3-dehydroecdysone production in the PG	*Ostrinia nubilalis*, *Lymantria dispar*, *Sesamia nonagrioides* (Gelman et al., 1991; Perez-Hedo et al., 2010)
Head peptide	Midgut endocrine cells	Inhibit host-seeking behavior	*Aedes aegypti* (Stracker et al., 2002)

Most peptide hormones are also expressed in the nervous system, which is not marked in this table.

low; in the embryo stage, *dilp2* is highly expressed in the midgut (Brogiolo et al., 2001). Thus far, there have been multiple studies on DILPs produced by the brain and fat body, but few studies on DILPs produced by the intestine (Nassel and Vanden Broeck, 2016).

Although there are various mechanisms by which nutrients can regulate insect body size, the insulin/insulin-like growth factor signaling pathway and the target of rapamycin pathway both respond to food nutrition levels and regulate growth rate and duration; they also regulate the synthesis and concentrations of other important developmental hormones (Koyama and Mirth, 2018). The insulin receptor of *Drosophila* regulates body size and organ size by altering cell number and size (Brogiolo et al., 2001). In addition, ILP can regulate vitellogenin production and oviposition of the green lacewing, *Chrysopa septempunctata* (Zhang et al., 2017). In female *Drosophila*, DILP6 can regulate the metabolism of JH and dopamine (Rauschenbach et al., 2017). Mutation of *dilp6* reduced JH hydrolysis activity in mutant female *Drosophila*; it also increased dopamine synthesis enzymes activity. Under heat shock treatment, the reproductive capacity of females also increased. However, the fecundity and heat stress ability of the mutant flies did not differ from the characteristics of control flies when they were fed with precocene (JH inhibitor) for 10 h. Therefore, DILP6 can promote JH degradation and hinder its synthesis.

Other Peptide Hormones

Nerves are distributed throughout the insect gut, and the central and gastrointestinal nervous systems can affect the insect gut. Peptide hormones control food intake and digestion; therefore, destroying specific nerves can reduce eating behavior (Spit et al., 2012). In addition to regulating the nervous system via the intestinal tract, the peptides produced by various parts of the intestinal tract function to regulate the digestive system itself. These peptides are typically expressed in precursor form and are activated when needed (Reiher et al., 2011). For example, in female black

blowfly (*Phormia regina*) adults that have fed on beef liver, the midgut releases a peptide hormone with a relative molecular weight of 5,000–11,000 that acts on the brain and causes a neuroendocrine cascade reaction to promote egg formation (Yin et al., 1994). In the pre-molting and molting periods of the arthropod blue crab, *Carcinus maenas*, the level of hemolymph hyperglycemic hormone is more than 100 times higher than that in other developmental periods. Crustacean hyperglycemic hormone is released by endocrine cells in specific areas of the foregut and hindgut, which may be involved in molting (Chung et al., 1999). In the hindgut of European corn borer (*Ostrinia nubilalis*) and Gypsy moth (*Lymantria dispar*), a large number of prothoracicotropic factors exist; these prothoracicotropic factors can promote ecdysone and 3-dehydroecdysone production in the PG of the moth (Gelman et al., 1991). If the head of a gypsy moth is tied sufficiently to prevent brain-generated prothoracicotropic hormone from acting on the PG, the larva can also be induced to progress from molting to pupation when injected with intestinal extracts (Gelman et al., 1991). In summary, the gut can accept peptide hormones produced by other tissues to affect growth and development; similarly, the gut can produce peptide hormones that can regulate the physiological functions of insect development.

It was originally presumed that tachykinin-related peptide was produced by the midgut and released into hemolymph circulation to enable adaptation to different nutritional conditions (Winther and Nassel, 2001). The expression of tachykinin-related peptide in the midgut of silkworm has been demonstrated (Nagai-Okatani et al., 2016). When silkworm larvae midgut is incubated *in vitro* with nutritive substances such as glucose, amino acids, or the plant secondary metabolite chlorogenic acid, the midgut can discriminate and the endocrine cells secrete tachykinin-related peptide into the buffer of the cultured gut (Yamagishi et al., 2018). Prothoracicotropic hormone promotes the production of ecdysone by activating the target of rapamycin-receptor/ERK pathway to regulate metamorphosis (De Loof et al., 2015). The larvae and pupae of noctuid (*Sesamia nonagrioides*) can molt without the presence of the brain, although the PG is needed; this indicates that prothoracicotropic hormone can be produced in other tissues. Further analysis showed that prothoracicotropic hormone could be expressed in the intestine (Perez-Hedo et al., 2010).

With the sequencing of a growing number of insect genomes, 35 genes were found to encode regulatory peptides, among which five are ILPs (Riehle et al., 2002). The head peptide gene was identified in the genome of *Anopheles gambiae*; recombinant and purified head peptide was then injected into female mosquitoes that were not fed with blood, which prevented them from finding a host (Riehle et al., 2002). The head peptide gene of *Aedes aegypti* is expressed in the brain, ganglion end, and midgut (Stracker et al., 2002). This localization suggests that head peptide may be involved in more complex functional regulation; thus, additional meaningful peptide hormones may be identified by making full use of genomic information.

PEPTIDE HORMONES PRODUCED IN OTHER TISSUES AND AFFECTING THE GUT

During insect growth and development, some hormones can also affect intestinal activity. For example, ion transport peptide, a peptide hormone in *Drosophila*, is an antidiuretic peptide expressed in the brain, abdominal nerve cells, and other nervous areas. When *Drosophila* experiences thirst, ion transport peptide gene expression levels increase, regulating water balance by promoting water intake and inhibiting food intake and water excretion (Galikova et al., 2018). When ion transport peptide is overexpressed, water balance can be maintained by regulating defecation times. RNA interference to modify ion transport peptide levels can cause food to pass through the intestine quickly, similar to diarrhea (Galikova et al., 2018).

Interestingly, some social insects [e.g., ants (*Camponotus floridanus*)] can exchange certain hormones through oral fluid exchange (trophallaxis); the oral fluid contains various microRNAs and JH (LeBoeuf et al., 2016). The ingested RNA can be absorbed by the gut cells and then transferred to hemolymph (Maori et al., 2019). Compared to other social insects, many of the proteins in oral fluid are related to the regulation of growth, development, and maturation (LeBoeuf et al., 2016; He et al., 2018; Maori et al., 2019).

Adipokinetic Hormone

Adipokinetic hormone (AKH) is a type of neuropeptide produced by the corpora cardiaca. It can mobilize energy metabolism in the fat body in many insects; it can also act on other tissues, such as the PG and aorta of fly larva, and the brain and crop of *Drosophila* adults (Lee and Park, 2004). AKH influences the intestine, mainly by controlling the activity of digestive enzymes in the intestine (Kodrik et al., 2012; Bodlakova et al., 2017). When Pyrap-AKH is injected into the firebug (*Pyrrhocoris apterus*), the amount of protein and lipids in the midgut increase; the level of triacylglycerols are mainly increased in the fore-midgut, while diacylglycerols are in the middle-midgut. AKH has no effect on lipase activity, but increases peptidase and glucoamylase activity in the middle and hind midgut (Kodrik et al., 2012).

Under starvation conditions, flies without AKH neurons do not show hyperactivity to search for food like wild-type flies, and they are resistant to starvation-induced death (Lee and Park, 2004). AKH is very important for maintaining basic nutrition levels; therefore, mutating the amino acid sequence of AKH in *Drosophila*, or constructing AKH-deficient and overexpressing *Drosophila* mutants, can promote the study of AKH mechanisms (Sajwan et al., 2015; Mochanova et al., 2018). For example, in blood-sucking insect (*Rhodnius prolixus*), the expression of AKH receptor *RhoprAkhr* in the fat body increases with triacylglycerol mobilization in the starvation state; if the AKH receptor gene is knocked down, triacylglycerol will accumulate in the fat body in the starvation state. However, if synthetic Rhopr-AKH is simultaneously

injected into *R. prolixus*, the transcription levels of acyl-CoA-binding protein-1 and mitochondrial glycerol-3-phosphate acyltransferase, which are involved in fat metabolism, can be altered (Alves-Bezerra et al., 2016).

AKH can act on the intestine and influence digestive enzyme activity through AKH receptors present in the intestine. AKH receptor is a specific G protein-coupled receptor that exists on the target cell membrane. AKH receptors of the tsetse fly *Glossina morsitans morsitans* (i.e., *Glomo-akhr-a* and *Glomo-akhr-b*) are splicing variants of an open reading frame (Caers et al., 2016). AKH/corazonin-related peptide is the intermediate of AKH and corazonin, which is homologous to gonadotropin-releasing hormone of vertebrates. The AKH/corazonin-related peptide receptor gene of *A. aegypti* can form three variants through selective splicing of nine exons (Wahedi and Paluzzi, 2018). AKH/corazonin-related peptide and its receptor are mainly expressed in the central nervous system of *R. prolixus*; AKH/corazonin-related peptide is also expressed in the hindgut and posterior midgut (Zandawala et al., 2015). AKH can induce carbohydrate and fat metabolism only by binding to a rhodopsin-like G protein-coupled receptor (Marchal et al., 2018). In a study of AKH receptors of seven representative insect species, each receptor was able to be activated by endogenous AKH at very low doses. However, AKH receptors of mosquitoes could only be activated by endogenous ligands, while AKH receptors of locusts could be activated by AKHs of other insects (Marchal et al., 2018).

Hormones also play an important role in oxidative stress. AKH is the main stress hormone in the response pathway of oxidative stress (Kodrik et al., 2015); relevant work requires further study.

Allatostatin

Although allatostatins were first found in the cockroach (*Diploptera punctata*) brain where they inhibit the production of JH by the corpus allatum, allatostatins are also expressed in midgut endocrine cells and other tissues (Reichwald et al., 1994). There are three types of allatostatins: A, B, and C (Hernandez-Martinez et al., 2005). The C-terminus of allatostatin type A (AST-A) is a conserved FGL amine motif, which can be detected in the brain and midgut of insects; its receptor is a G protein-coupled receptor. AST-A peptide, which can inhibit the secretion of JH, can also regulate food intake through inhibition or reduction of food intake (Felix et al., 2015). After *Anopheles* mosquitoes sucked blood, the expression levels of AST-A receptor genes *GPRALS1 and 2* in the midgut were significantly higher than after the mosquitoes ingested glucose, however, these expression levels were reduced in the head and ovary (Felix et al., 2015). Therefore, AST-A may be involved in the digestion of blood by the midgut.

Other Hormones

In addition to the abovementioned hormones, several other hormones play important roles in regulating the physiological functions of insects. Both diuretic hormone 44 and leucokinin can induce the secretion of fluid from the Malpighian tubules; they can also regulate stress, feeding, rhythm, and other behaviors. Ganglion cells in the abdomen can produce both diuretic hormone 44 and leucokinin peptides simultaneously. Research has shown that knockdown of diuretic hormone 44 causes reduction of food intake, while knockdown of leucokinin does not; conversely, leucokinin knockdown can increase water retention (Zandawala et al., 2018). Thus, hormones produced by abdominal ganglion cells can affect the digestive system and homeostasis.

Ecdysone is synthesized by the PG; in addition, PG production of ecdysone is regulated by many neuropeptides. Neuropeptides mainly affect PG activity through hormones and neural pathways. For example, a group of neuropeptides identified in the silkworm, orcokinins, are neuronal prothoracicotropic factors that are mainly secreted by neuroendocrine cells in ventral ganglia; orcokinins can also be produced by small nerves of the central nervous system, as well as by endocrine cells of the midgut (Yamanaka et al., 2011). Orcokinins can regulate ecdysis in the hemimetabolous insect, *R. prolixus*, through the peptidergic signaling pathway (Wulff et al., 2017). Orcokinin neuropeptides are very important in the molting activity of *R. prolixus*. The *Orcokinin* gene can form two different neuropeptide precursors through selective splicing. RhoprOKA affects differential expression of molting-related neuropeptide precursors. RhoprOKB is related to feeding and midgut physiological function, since OKB is a brain-gut peptide in insects that can improve the frequency of spontaneous peristalsis and contraction of the midgut (Wulff et al., 2018). Endocrine cells with orcokinin B/C-like immune activity in the fore-midgut were identified by immunohistochemistry. The number of immune-active cells after 1 h of blood sucking was lower than that without feeding, which may be due to the release of OKB peptide from cells (Wulff et al., 2018).

HORMONES PRODUCED BY INTESTINAL SYMBIONTS

In the long evolutionary process, there have been many symbionts in the insect gut. Symbionts inhabiting the gut can help insects digest food, regulate growth and development, prolong life, detoxify, immunize, and communicate (Engel and Moran, 2013; Wu et al., 2016). Insects provide nutrition and habitats for symbiotic bacteria; these bacteria also affect their hosts. The gut symbionts of insects are regulated by molecules in midgut epithelial cells, the most important of which are reactive oxygen species and immune deficiency pathway-produced antimicrobial peptides (Huang et al., 2015). The existence of intestinal bacteria can affect the development of the midgut; for example, these bacteria can induce the proliferation of intestinal stem cells and the renewal of epidermal cells. In honey bee (*Apis mellifera*), a γ-proteobacterial species can encode pectin-degrading enzymes, which may be related to the degradation of pollen walls to help pollinate crops (Engel et al., 2012).

Rational use of honeybee symbiotic bacteria may help to improve the health conditions of honeybees with respect to various factors, including pathogens, parasites, pesticides, environmental changes, and habitat loss (Crotti et al., 2013). In addition, the intestinal microorganisms of the honeybee

can promote individual weight gain by mediating changes in vitellogenin production, insulin signaling, and the gustatory response of the host (Zheng et al., 2017). Compared with sterile bees, metabonomic analysis revealed that the short-chain fatty acids produced by the intestinal bacteria of normal bees affect the gut environment (e.g., by reducing intestinal pH and redox capacity). This environmental change alters the insulin/insulin-like growth factor signaling pathway. Honeybees have two ILP genes and two putative insulin receptors. In addition, vitellogenin production is closely related to the insulin/insulin-like growth factor signaling pathway. The expression levels of *ilp1* and *Vg* in the head and abdomen were shown to be several times higher in normal bees than in sterile bees; the expression levels of *ilp2* and *inR1* were also higher in normal bees (Zheng et al., 2017).

In addition to the above-described metabolite regulation, symbiotic bacteria can affect the growth and development of the host through the use of bacterial enzymes. In *Drosophila*, the symbiotic bacterium *Acetobacter pomorum* can regulate insulin/insulin-like growth factor signaling, body size, energy metabolism, and intestinal stem cell activity through PQQ-ADH enzyme activity (Shin et al., 2011).

Gut microbes can stimulate gut nerves to transmit signals to brain nerves (Forsythe and Kunze, 2013). In mammals, the vagus nerve mediates the connection between intestinal bacteria and the central nervous system. However, in insects, gut bacteria exhibit clear regulation of host behavior. Some pheromones produced by symbiotic bacteria in the gut can affect insect behavior. For example, *Pantoea agglomerans*, a bacterium isolated from the gut of locusts, produce a mixture of cohesion pheromone (guaiacol, which causes locusts to gather) and a small amount of phenol when added to sterile feces; however, the mixture cannot be produced by addition of bacteria pathogenic to locusts (Dillon et al., 2002). In *Drosophila*, through ingestion of different foods, biases in adult mating can be induced. For example, *Drosophila* fed sucrose medium tend to mate with *Drosophila* fed the same medium, while *Drosophila* fed starch medium tend to mate with others fed starch medium. However, addition of antibiotics to food can inhibit this phenomenon, which may be related to changes in sex pheromone levels from symbiotic bacteria, such as *Lactobacillus plantarum* (Sharon et al., 2011).

HORMONES AND IMMUNITY

Insects rely on innate immunity to resist the invasion of pathogens, but the immune level of an individual is closely related to development. For the silkworm, the immune level of the midgut changes dynamically in the stages of larva molting, mulberry eating, and wandering, in accordance with the expression of antimicrobial peptide (Xu et al., 2012; Yang et al., 2016). Injection of ecdysone into newly molted silkworm larvae can lead to induction of the larvae-to-larvae molting stage; gene expression differences in the midgut are also similar (Yang et al., 2016). In the prophase of molting, c-type lysozyme and pyrrhocoricin-like antimicrobial peptide are significantly increased in the midgut, which could reduce the number of *Burkholderia* intestinal symbionts (Kim et al., 2014). Because injection of 20-hydroxyecdysone can reduce the number of intestinal bacteria (Kim et al., 2014), changes in immune levels are presumably related to hormone changes. In addition to the gut, changes in hormone levels appear to enhance the cellular immunity of insects. When ecdysone is added to the *Drosophila* l(2)mbn cell line, the phagocytosis ability and antimicrobial peptide expression can be enhanced under immune stimulation (Dimarcq et al., 1997).

However, during the process of insect reproduction, hormone regulation reduces immune levels (Schwenke and Lazzaro, 2017). For example, during reproduction in female *Drosophila*, mating, semen protein, and sex peptide lead to reduced systemic immune activity against bacteria (Short et al., 2012; Schwenke et al., 2016). Sex peptide can stimulate the synthesis of JH in female flies after mating. However, JH also inhibits immunity because JH can reduce the humoral immunity and especially antagonize the 20E-dependent immune activities (Rolff and Siva-Jothy, 2002; Flatt et al., 2008). Therefore, after mating, female flies are more likely to be infected with pathogens and die (Fedorka et al., 2007; Miest and Bloch-Qazi, 2008; Schwenke and Lazzaro, 2017). If JH signaling is blocked, the survival rate of female flies that have engaged in mating will recover after pathogen infection (Schwenke and Lazzaro, 2017). The seminal fluid of male *Drosophila* contains sex peptide, which can be transferred to female flies during mating. Sex peptide can bind to specific sites of the central nervous system, peripheral nervous system, and reproductive tract of females. Receptors in the nervous system can recognize and activate the signal pathway after mating. In addition, receptor proteins in the genital tract can transfer sex peptide to hemolymph (Kubli, 2003).

PEPTIDE HORMONES AND PEST CONTROL

The harm from abuse of traditional chemical insecticides is becoming more and more serious, and new biological insecticides have been given increasing attention due to their green and friendly characteristics. Studies have increasingly shown that ecdysone, JH, and their analogs can be used for biological control of pests (Dhadialla et al., 1998; Retnakaran et al., 2003; Wilson, 2004). With respect to hormone regulation of insect reproduction, energy metabolism, water balance, feeding behavior, sexual attraction, and growth and development, Gade and Goldsworthy proposed in 2003 that insect peptide hormones could be used to design new, safe, and selective complexes for insect pest control (Gade and Goldsworthy, 2003). A normal level of allatostatin is critical for the growth and development of insects. Mannose-binding lectin (*Galanthus nivalis* agglutinin) can bind to the intestinal epithelial cells of insects and then enter hemolymph. Feeding the purified fusion protein of *G. nivalis* agglutinin and allatostatin to *Lacanobia oleracea* larvae can inhibit their feeding and growth, however, there is no such effect when *G. nivalis* agglutinin or allatostatin are fed alone, or when they are fed together (Fitches et al., 2002).

Kinin neuropeptide is a type of neuropeptide with a Phe^{1}-Xaa^{2}-Yaa^{3}-Trp^{4}-Gly^{5}-NH_2 structure at the C-terminus, which exists in many insects (Zhang et al., 2015a). It can promote the excretion of urine and stimulate contraction of the hindgut, as well as release of digestive enzymes. Zhang et al. designed and synthesized insect kinin analogs via a peptidomimetic strategy and obtained a biological insecticide with high aphid-killing activity: analog II-1. Its LC_{50} is 0.019 mmol/L, which is lower than that of commercial pymetrozine (LC_{50} = 0.034 mmol/L) (Zhang et al., 2015a).

When AKH and the pathogenic nematode *Steinernema carpocapsae* were applied to adults of *P. apterus*, the mortality rate of the adults increased 2.5-fold within 24 h; in addition, the metabolism of the insects was intensified and the production of carbon dioxide increased in the insect body. However, if AKH receptor expression is knocked down in *P. apterus*, the mortality rate is significantly reduced (Ibrahim et al., 2017).

CONCLUSION

Peptide hormones have been shown to cause no chemical pollution and to have specific action. Therefore, hormonal peptides can be used as new and safe biological insecticides; relevant research requires greater attention. The production and functions of peptide hormones are becoming increasingly clear, especially with respect to the mechanisms of action of steroids and several neuropeptides. In addition to the functions discussed above, insects have certain peptide-regulated behaviors; for example, insect SIFamide is a neuropeptide produced by four medial interneurons, which can affect sexual behavior, sleep, death, and pupa number (Lismont et al., 2018). Insect diapause hormone is a type of peptide that can help individuals survive in a changing environment, such as overwintering; some insects exhibit embryonic diapause, while others exhibit pupal diapause (Zhang et al., 2015b). The research regarding mechanisms underlying these behaviors in insects is very important for understanding human life activities, as well as for biological control.

Some neuropeptides and peptide hormones of insects can be used to treat mammalian diseases, as they exhibit antifungal, antitumor, and antiviral properties (Chowanski et al., 2016). Notably, in addition to peptide hormones, insects can produce antimicrobial peptides. Insects have no adaptive immunity to produce antibodies, however, they can produce antimicrobial peptides through the innate immune system, which eliminate pathogens from the body. Insect antimicrobial peptides also reportedly have bactericidal effects on many human pathogenic bacteria (Chowanski et al., 2016). Some antimicrobial peptides, such as CopA3, were found to increase the proliferation of colonic epithelial cells and enhance intestinal mucosal barrier function (Kim et al., 2016). CopA3 of Dung beetle (*Copris tripartitus*) is a 9-mer disulfide dimer peptide. *In vitro*-synthesized CopA3 can increase the proliferation of HT29 cells, as well as the levels of proliferation and apoptosis of colonic epithelial cells in mice treated with CopA3; these findings suggest that CopA3 can promote the regeneration of mouse colonocytes, given that CopA3 can downregulate the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$. Moreover, CopA3 could prevent enteritis in mice that were treated with *Clostridium difficile* toxin A (Kim et al., 2016).

The study of insect peptide hormones is also very useful in analysis of decapods. Neuropeptides exhibited significant similarity among seven shrimp and crab species; these included elongated pigment dispersing hormone, a newly identified peptide. Compared with insects, the only neuropeptides not found in decapods were allatotropins (Veenstra, 2016).

At present, research on insect hormones is mainly focused on the interactions of JH, ecdysone, and other hormones, while studies on peptide hormones in the midgut have been sparse and unsystematic. However, midgut hormones are important in nutrition regulation, water balance, development, and pest control. Therefore, research regarding midgut peptide hormones requires greater attention.

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REVIEW
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Role of the Insect Neuroendocrine System in the Response to Cold Stress

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Insects are the largest group of animals. They are capable of surviving in virtually all environments from arid deserts to the freezing permafrost of polar regions. This success is due to their great capacity to tolerate a range of environmental stresses, such as low temperature. Cold/freezing stress affects many physiological processes in insects, causing changes in main metabolic pathways, cellular dehydration, loss of neuromuscular function, and imbalance in water and ion homeostasis. The neuroendocrine system and its related signaling mediators, such as neuropeptides and biogenic amines, play central roles in the regulation of the various physiological and behavioral processes of insects and hence can also potentially impact thermal tolerance. In response to cold stress, various chemical signals are released either via direct intercellular contact or systemically. These are signals which regulate osmoregulation – capability peptides (CAPA), inotocin (ITC)-like peptides, ion transport peptide (ITP), diuretic hormones and calcitonin (CAL), substances related to the general response to various stress factors – tachykinin-related peptides (TRPs) or peptides responsible for the mobilization of body reserves. All these processes are potentially important in cold tolerance mechanisms. This review summarizes the current knowledge on the involvement of the neuroendocrine system in the cold stress response and the possible contributions of various signaling molecules in this process.

Keywords: cold stress, neurohormones, insects, biogenic amines, neuroendocrinology, neuropeptides

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INTRODUCTION

Insects are the largest group within the arthropod phylum. They are capable of surviving in virtually every environment from the deserts of Africa through the grasslands of temperate zones to the freezing permafrosts of Arctic regions (Chown and Nicolson, 2004). A major factor determining insect species distributions is their cold tolerance and water availability (Addo-Bediako et al., 2000). During their evolution, insects subjected to low temperature have developed distinct adaptations to overcome and thrive in suboptimal thermal conditions (Wharton, 2007; Lee, 2010). To survive in environments where the temperature drops below freezing, insects have evolved diverse mechanisms, which can be divided into two main strategies: (i) freeze-tolerance and (ii) freeze-avoidance (Lee, 1991; Sømme, 1999; Sinclair et al., 2003). In freeze-tolerant species, freezing is limited only to

extracellular matrix (ECM), as the formation of ice crystals inside of the cell inevitably leads to death of most animals (Storey and Storey, 1989; Block, 2003). The second strategy is much more widespread among arthropod phyla (Block, 1990; Lee and Costanzo, 1998), and freeze-avoiding insects utilize mechanisms which raise their ability to stay unfrozen by supercooling (Sformo et al., 2010). Cold and freezing stress affects a large number of physiological processes (Teets and Denlinger, 2013), causing mechanical damage to cells or their DNA (Lubawy et al., 2019), changes in main metabolic pathways (Chowanski et al., 2015, 2017b) or cellular dehydration, which results in increased acidity, toxic metabolic intermediate concentrations and osmotic stress (Storey and Storey, 2012; Pegg, 2015; Des Marteaux and Sinclair, 2016; Andersen et al., 2018). In both of these strategies, cryoprotectants are necessary for survival. Cryoprotectants like glycerol, trehalose or glucose, which lower the lowest lethal temperature are synthetized by freeze-avoiding species. The increase in their concentration results also in a drop of supercooling point (SCP) (Zachariassen, 1985). The freeze-tolerant insects in turn utilize these molecules to reduce cellular dehydration since ice formed in the ECM attracts water out of cells (Storey and Storey, 1988). Species that cannot tolerate freezing also remove any particles that can start the ice nucleation process, such as food, dust or bacteria from gut or ECM. This may be achieved for example by inhibiting feeding (Olsen and Duman, 1997). The latest findings indicate that water and ion balance is crucial for withstanding chilling injuries that lead to chill coma and death (Overgaard and MacMillan, 2017). Therefore, nerves and muscles are highly susceptible to cold stress (Garcia and Teets, 2019). However, little is known about the role of the nervous system in orchestrating these finely tuned processes.

The nervous and endocrine systems, through the process called neuroendocrine integration, interplay together to regulate a number of physiological functions and maintain system-wide homeostasis in regular as well as stressful situations (Hartenstein, 2006; Adamski et al., 2019). A number of physiological processes are mediated by two main classes of neurosecretory molecules i.e., neuropeptides and biogenic amines (Hartenstein, 2006; Chowanski et al., 2016, 2017a). They are produced mainly in the central nervous system (CNS) and take part in the regulation of metabolism, ion homeostasis and muscle contractions, including the heartbeat (Chowanski et al., 2017c). In different insect species, neuropeptides with homologous structures very often have similar functions (Bendena, 2010). As these compounds play central roles in physiological and behavioral processes, directly affecting the survival of adverse environmental conditions, it can be expected that in response to cold stress, molecules responsible for osmoregulation, such as capability peptides (CAPA), inotocin (ITC), ion transport peptide (ITP), diuretic hormones (DH_{31} and DH_{44}), kinins and calcitonin (CAL), will take part. Substances related to the general response of insect organism to stressors such as tachykinin-related peptides (TRPs) and/or peptides responsible for the mobilization of reserve substances (e.g., glycogen, trehalose, and glucose) such as adipokinetic hormones (AKHs), insulin-like peptides (ILPs), or neuropeptide F (NPF) may also take part in the cold stress response, as they regulate metabolic homeostasis, the circadian clock and feeding (Gäde, 2004, 2009; Fadda et al., 2019). However, not only neuropeptides can be key players. Current knowledge shows that biogenic amines such as octopamine (OA), dopamine (DA), and serotonin (5-HT) are involved in the stress response (Gruntenko et al., 2004, 2016). The levels of the above-mentioned biogenic amines have been found to change in various insect species under unfavorable conditions, including high- or low-temperature stress (Hirashima et al., 2000; Chentsova et al., 2002). Hence, this paper summarizes the existing knowledge on the role of the neuroendocrine system in response to cold stress and research perspectives in this area.

BIOGENIC AMINES

Biogenic amines play a crucial role in the regulation of basic life processes (Farooqui, 2012; Sinakevitch et al., 2018). They act not only as neurotransmitters and neuromodulators in nervous tissues but also, depending on the situation, they can be released into body fluids and act as neurohormones (Sinakevitch et al., 2018). Biogenic amines bind to G-Protein coupled receptors (GPCRs) and, depending on the receptor type and target tissue, stimulate different types of secondary messengers, mainly cAMP or Ca^{2+} (Farooqui, 2012).

The main biogenic amines identified in insects are octopamine (OA), serotonin (5-HT), dopamine (DA), histamine (HA), and tyramine (TA) (Blenau and Baumann, 2001). Current knowledge about the role of biogenic amines in insects suggests a wide spectrum of actions. They participate in the regulation of many behaviors, such as locomotion, feeding or social interactions (Blenau and Baumann, 2001; Armstrong and Robertson, 2006; Pflüger and Duch, 2011). Biogenic amines also evoke systemic responses to different environmental factors, including stressful conditions or pathogen infection (Gruntenko et al., 2004; Adamo, 2008). For instance, research has shown that OA and DA are released into insect hemolymph in the first minutes after exposure to stress, which evokes a cascade of reactions leading to the re-attainment of homeostasis (Hirashima and Eto, 1993; Chentsova et al., 2002; Gruntenko et al., 2004). Interestingly, the release of these biogenic amines during stress conditions appears non-specific to stressor type. For example, heat, vibration and starvation trigger the same response (Orchard et al., 1981; Hirashima et al., 2000; Gruntenko et al., 2004). Likewise, winter conditions such as low temperatures and a short-day photoperiod induce changes in the concentrations of biogenic amines, which allow insects to survive unfavorable conditions and/or prepare them for prolonged stress conditions (Isabel et al., 2001; Armstrong and Robertson, 2006). However, the changes in biogenic amine concentrations and their cause may be different in the case of the response of insects to rapid exposure to cold and during the acclimation process before winter.

One of the most important effects of biogenic amine release to the insect hemolymph is the mobilization of energy (Lorenz and Gäde, 2009). The mobilization of energy prepares insects to higher metabolic activity related to the stress response and is useful during the recovery period (Farooqui, 2012). The mobilization of energy under the control of biogenic amines

is the result of the stimulation of glycogen conversion into trehalose, glucose and trehalose oxidation and the release of lipids from fat body (Gruntenko et al., 2004). This action of biogenic amines may be very important through the prism of response to short-term and prolonged cold. Elevated levels of biogenic amines also intensify the process of energy mobilization by stimulating the release of other hormones, especially neuropeptides, which participate in the regulation of insect metabolism. The cooperation between biogenic amines and neuropeptides may evoke a reaction cascade that is crucial for the response of insects to environmental stressors, including cold. Pannabecker and Orchard (1986) showed that OA stimulates the release of AKHs. AKHs are neuropeptides that are considered the main insect stress hormones because, similar to OA, they enhance available energy by inducing lipolysis and suppressing life processes that have relatively low priority during stress conditions (Gäde, 2009; Ibrahim et al., 2018). The close interplay between these two hormones highlights the fact that receptors for AKHs were also found in dorsal unpaired median neurons (DUMs), which are among the main components of the insect octopaminergic system (Wicher et al., 2006; Wicher, 2007). Another example of close relations between biogenic amines and neuropeptides is the fact that the activity of neurosecretory cells producing ILPs is mediated by the serotonin receptor 5-HT_{1A} and octopamine receptor OAMB (Luo et al., 2012). The detailed relationship between biogenic amines and ILPs is described in the Neuropeptides section (subsection Metabolism).

Cold acclimation allows the maintenance of metabolic homeostasis and insect survival under prolonged stress (Lalouette et al., 2007; Colinet et al., 2012; Enriquez and Colinet, 2019). Generally, acclimation is associated with changes in insect metabolites, including sugars, polyols, free amino acids (FAAs), proteins and also biogenic amines (Isabel et al., 2001; Lalouette et al., 2007; Colinet et al., 2012). A study by Isabel et al. (2001) clearly showed that the concentration of DA in diapausing *Pieris brassicae* pupae was higher than that in non-diapausing individuals. Moreover, the DA level progressively increases during diapause. In the case of the 5-HT level, in the initial phase of the pupal stage, Isabel et al. (2001) did not observe any changes between diapausing and non-diapausing pupae. However, the 5-HT concentration in diapausing pupae was stable, while in non-diapausing individuals, it dropped during this developmental stage. High concentrations of DA likely lead to the arrest of insect development. High 5-HT levels could participate in the inhibition of pupal metabolism, which may be crucial for survival during insect overwintering at this developmental stage. Interestingly, the results of Isabel et al. (2001) suggest that in the case of *P. brassicae,* the accumulation of biogenic amines is the result of changes in the photoperiod but not exposure to lower temperature. On the other hand, research conducted on the beetle *Alphitobius diaperinus* indicates the influence of cold exposure on increasing concentrations of tyrosine (Tyr), a precursor of many hormones, including OA and DA, in insect hemolymph (Lalouette et al., 2007). A strict correlation between biogenic amines and Tyr was also shown in a study performed by Rauschenbach et al. (1995), who demonstrated that during an increase in DA concentration, a simultaneous decrease in Tyr was observed in *Drosophila*. Moreover, research conducted on *Drosophila virilis* showed that cold acclimation led to the upregulation of genes encoding serotonin receptor 7 and the serotonin transporter, which may also suggest that 5-HT is likely important in regulation of response to prolonged thermal stress (Vesala et al., 2012).

Current research addressing the role of biogenic amines in response to short-term and prolonged cold suggests that these hormones are not only important in the regulation of insect metabolism but also participate in neuroprotection. Generally, insects enter coma at critical high and low temperatures (Rodgers et al., 2010; Armstrong et al., 2012; Srithiphaphirom et al., 2019). This physiological state partly results from the progressive loss of ion homeostasis. The alteration of ion equilibrium provokes the depolarization of membranes, altering the action potentials of muscles and neuron cells, leading to a loss of neuromuscular functions and coma (Overgaard and MacMillan, 2017). The alteration of ion concentrations across membranes is associated with a decrease in Na^+/K^+-ATPase activity at low temperature (McMullen and Storey, 2008). Interestingly, after temperature acclimation in three cockroach species, *Periplaneta americana, Leucophaea maderae*, and *Blaberus craniifer,* their nervous tissues were excitable at temperatures lower than previously determined temperatures, which induced a chill coma in non-acclimated individuals (Anderson and Mutchmor, 1968). Similar results were observed in *Drosophila* species. The pre-exposure of flies to low temperatures decreases the value of the critical thermal minimum (CT_{min}), the temperature at which individuals lose responsiveness (Overgaard et al., 2011; Andersen et al., 2018). Interestingly, in *Locusta migratoria* after the application of OA decreasing of CT_{min} was observed (Srithiphaphirom et al., 2019). These data suggest that OA may play some role(s) in modulating the responsiveness of the nervous system under thermal stress. The OA mode of action is most likely associated with the indirect modulation of Na^+/K^+-ATPase activity and compensation for the negative effect of low temperatures on this pump (Srithiphaphirom et al., 2019). The activation of the OA receptor (i.e., OAR3) leads to the stimulation of cAMP production and activation of cAMP-dependent protein kinase A (PKA), which may regulate K^+ channels and Na^+/K^+-ATPase pumps (Feschenko et al., 2000; Armstrong and Robertson, 2006; Srithiphaphirom et al., 2019).

Many studies have been conducted on the neuroprotective role of biogenic amines in maintaining the muscle activity of different crustaceans under stress conditions, including low temperature (Stephens, 1985, 1990; Hamilton et al., 2007). Generally, an increase in the OA and 5-HT concentration in the hemolymph associated with exposure to cold causes an increase in excitatory postsynaptic potential (EPSP) amplitude in lobster and crayfish muscles. The effect of the application of biogenic amines is very often temperature dependent; for example, 5-HT induces changes in EPSP only at temperatures lower than optimal. This phenomenon may help neuromuscular junctions remain functional at low temperatures (Hamilton et al., 2007; Zhu and Cooper, 2018). Based on these results,

similar dependencies may also be observed in insects. This supposition is partially confirmed by the results of Zhu et al. (2016) in a study on the *D. melanogaster* heart. The authors demonstrated a strong excitatory effect of biogenic amines on the larval heart during cold exposure but only in the case of 5-HT. Interestingly, at room temperature, all of the tested biogenic amines (OA, DA, and 5-HT) evoke positive chronotropic effects on the *Drosophila* heart. Moreover, high concentrations (10 μM) of OA and DA at a low temperature led to a decrease in heart rate frequency or heart cessation. This negative chronotropic effect was not observed in the cases of OA and DA at low concentrations (1 μM). However, the strict physiological role of this OA and DA action is not fully understood. However, as suggested by Zhu et al. (2016), different modes of action of OA at different temperatures may be related to the activation of different subunits of the G protein-coupled receptor. The Gαq subunit, whose activation evokes a positive chronotropic effect, is most likely suppressed, but Gαi-coupled receptors are activated, which may lead to the opposite effect of OA on insect heart during cold stress (Zhu et al., 2016). Generally, biogenic amines may be needed to maintain heart functioning during chronic exposure to cold. This is essential for circulating nutrients/cryoprotectants and immune function, which undoubtedly influence insect survival during exposure to cold (Zhu et al., 2016).

All insects have preferred temperature (Tpref) that maximize their metabolic activities and fitness (Crickenberger et al., 2019). Hence, they actively choose to occur in certain microenvironments to remain close to these temperatures (Dillon et al., 2009). In addition, in many situations, insects can avoid stressful conditions by moving into protected buffer microhabitats (Dillon et al., 2006). Recent research has shown that biogenic amines, especially DA and HA, participate in the regulation of Tpref in insects (**Figure 1**) (Hong et al., 2006; Bang et al., 2011; Tomchik, 2013). Bang et al. (2011) demonstrated that dopaminergic neurons located in mushroom bodies participate in the regulation of Tpref in *D. melanogaster*. The targeted inactivation of these neurons caused a loss of cold avoidance by flies. Moreover, mutation in the DA receptor gene led to a decrease in Tpref in *Drosophila* flies (Bang et al., 2011). Similar results were observed in DA transporter-defective mutants. Interestingly, in these mutants, a higher metabolic ratio was observed, which may suggest that differences in Tpref may be associated with disturbance to the equilibrium of heat gain and heat loss (Ueno et al., 2012). Additionally, the mutation of genes involved in HA signaling gave similar effects as previously mentioned for mutations in the DA system. Since HA participates in visual reception, these results indicate a putative relationship between temperature perception and the circadian clock, which may be crucial for the acclimation process (Hong et al., 2006).

Despite the regulatory actions of biogenic amines during the direct response to cold, these compounds protect against other environmental stressors, such as starvation (Krashes et al., 2009; Yang et al., 2015; Damrau et al., 2017). Moreover, biogenic amines inhibit energetically costly processes, such as reproduction, by stimulating JH degradation (Chentsova et al., 2002; Gruntenko et al., 2016). Starvation and reproductive arrest are processes that are particularly relevant to the cold tolerance of insects.

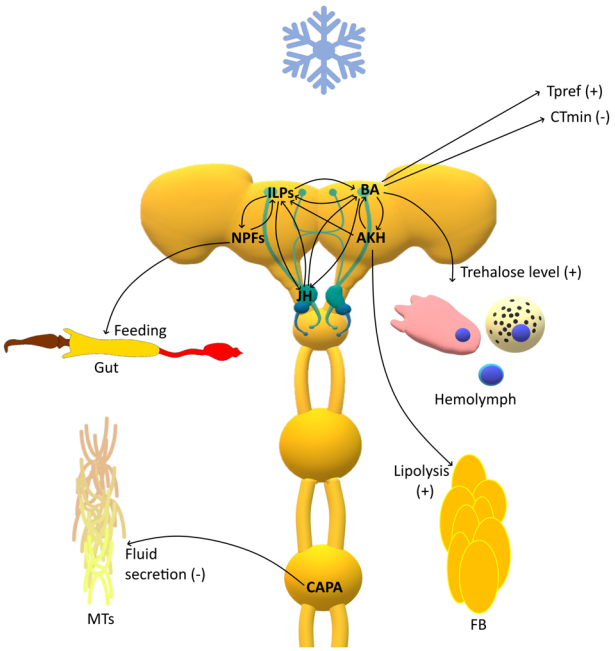

FIGURE 1 | Schematic representation of the response of insect neuroendocrine system to cold stress. The role of certain compound can be multiple and tightly regulated by a number of feedback loops which actions add up to the effect of regulating these finely tuned processes. ILPs, Insulin like peptides; BA, Biogenic amines; JH, Juvenile hormone; NPFs, Neuropeptides F; AKH, Adipokinetic hormone; CAPA, capability peptide; MTs, Malpighian tubules; FB, Fat body; CT_{min}, critical thermal minimum; T_{pref}, preferred temperature; (+), increase; (–), decrease.

NEUROPEPTIDES

Diuresis

Below a certain low temperature, insects generally enter into chill coma, a state associated with neuromuscular paralysis (Mellanby, 1939; MacMillan and Sinclair, 2011b; Findsen et al., 2014). During this state, insects lose ion and water homeostasis and regain it during a process called chill coma recovery (CCR) (MacMillan et al., 2012). In insects, Malpighian tubules (MTs) and the gut are mainly responsible for the regulation of ion homeostasis. This process may vary quite noticeably between different insects, depending, for example on diet (O'Donnell, 2008). Typically, the MTs are responsible for production of the primary urine, which is more or less isosmotic with the hemolymph. Ions K^+ and Cl^- (and Na^+ in blood feeding insects) flow from hemolymph to the lumen due to the coupling of the V-ATPase and H^+-cation exchangers. This allows to maintain a water gradient mediated by aquaporins, and movement of waste products by specific transporters into the lumen of the MTs (Ramsay, 1954; O'Donnell, 2009; Spring et al., 2009). The main neuropeptides contributing to the functioning of MTs are CAPAs,

which stimulate or inhibit secretion depending on the insect species and life stage (Davies et al., 2013; Halberg et al., 2015); kinins, which, in addition to stimulating secretion in MTs, also control the activity of gut muscles (Coast et al., 1990; Dow, 2009); and diuretic hormones (DH_{31} and DH_{44}) (Te Brugge et al., 2011; Cannell et al., 2016). In insects, hormones causing a reduction in diuresis have been identified, including ITP (Audsley et al., 1992; Gáliková et al., 2018), neuroparsins, glycoproteins GPA2/GPB5 which possibly act as Cl^- transport stimulating hormone (CTSH) (Paluzzi, 2012; Paluzzi et al., 2014; Rocco and Paluzzi, 2016), CCHamide which affect both MTs and midgut (Capriotti et al., 2019) and antidiuretic factors a and b (ADFa and ADFb) (Eigenheer et al., 2002, 2003; Massaro et al., 2004). As cold and desiccation both may result in a reduction in hemolymph volume and an increase in osmolarity and are closely linked at the molecular level (Sinclair et al., 2007, 2013; Rajpurohit et al., 2013), these two stressors should always be considered together. Terhzaz et al. (2015) showed that in drosophilids, the non-lethal exposure to low temperature significantly increases the mRNA levels of *capa*. The increase in *capa* expression was dependent on the duration of stress and came back to the levels before stress, after 4 h of recovery. During recovery, CAPA neuropeptides are released from neuroendocrine cells, improving (reducing) CCR (Terhzaz et al., 2015). On the other hand, recently published study by Li et al. (2020) showed significant decrease in *capa* expression level after 4 h of cold stress (4°C) and no changes after 1 h, in *Bemisia tabaci*. Together with the changes in neuropeptide precursor level, decrease in expression level of CAPA receptor was also observed (Li et al., 2020). The Terhzez's group also showed that during recovery from cold stress, the mRNA level of leucokinins increases in *Drosophila suzukii* (Terhzaz et al., 2018). Similar effect was also observed in *B. tabaci.* After prolonged cold stress (4°C for 4 h) a tendency to an increase in LK expression was noted (Li et al., 2020). These peptides also affect the function of MTs in *Aedes aegypti,* depolarizing them and increasing fluid secretion (Veenstra et al., 1997). This in turn shows that kinins may also take part in the response to cold stress (Terhzaz et al., 2018). Alford et al. (2019a) tested the effects of biostable analogs of kinin, CAPA, and PK in *D. suzukii* and *D. melanogaster*. They studied five CAPA/PK and three kinin analogs *ex vivo* to elucidate their roles in the modulation of fluid secretion through the MTs and *in vivo* to evaluate impacts of these neuropeptides on starvation, desiccation and cold stress tolerance. Out of all the tested peptides, the kinin analogs increased the fluid secretion in the MTs of both flies, whereas none of the other analogs affected this process. Although they did not affect the secretion of fluids, CAPA/PK analogs could be important regulators of stress response under desiccation conditions. Indeed, the injection of CAPA/PKs analogs increased survival under desiccation stress (Alford et al., 2019a). At low temperatures, injections of these analogs caused the protective effect, but only in *D. melanogaster* males and not in females of this species or in both sexes of *D. suzukii* (Alford et al., 2019a). In another study this group showed that 9 out of 10 tested analogs increased the mortality of cold stressed aphid *Myzus persicae* (Alford et al., 2019b). It has to be noted that in aphids MTs are not present due to evolutionary loss of these organs, and the osmoregulatory function of these organs was taken over by the gut (Jing et al., 2015). MacMillan et al. (2018) also showed that CAPA was connected to the cold tolerance of *D. melanogaster*, although the effects were dose-dependent (**Figure 1**). When administered at very low, femtomolar concentrations, CAPA was anti-diuretic and reduced tubule K^+ clearance rates and chill tolerance by significantly increasing the CCR time. However, at high doses, it facilitated K^+ clearance from the hemolymph and increased chill tolerance by reducing the CCR time and increasing survival (MacMillan et al., 2018).

As mentioned before, DH_{31} and DH_{44} represent another potential candidates for the investigation of the role of the neuroendocrine system in the cold stress response. However, the number of studies on this topic is limited. Although the results found by Terhzaz et al. (2018) showed no change in the mRNA level of DH_{31} and DH_{44} after cold stress, one should consider the possibility that results solely obtained from a single strain of *Drosophila* spp. may not be representative of the whole family/genus, as there is significant genetic variability in stress response among various populations of Drosophilidae (Schiffer et al., 2013). Especially since it has been shown that DH_{44} play a significant role in desiccation (Cannell et al., 2016) which regulatory pathways cross-talk with cold stress pathways (Sinclair et al., 2013). This indicates that the regulation of diuresis and ion homeostasis by the nervous system and its association with cold stress resistance is a complicated process. During cold stress, the regulation of ion and water homeostasis is extremely important, as the loss of balance in both causes neuromuscular disfunction and initiates chill coma (Macmillan and Sinclair, 2011a; MacMillan et al., 2012). Therefore, future research should focus on linking changes in the levels of individual diuretic and anti-diuretic peptides with changes in water and ion homeostasis and the administration of synthetic analogs to determine whether they affect the ability to survive cold stress.

Metabolism

At low temperature, the changes in the composition of body fluids controlled by diuresis (Neufeld and Leader, 1998; Williams and Lee, 2011) are accompanied by major metabolic changes – mainly related to carbohydrate metabolism, which is under neuroendocrine control. This is related to the production and storage of substances that are used to tolerate stress and survive low temperatures (Terhzaz et al., 2018). These substances are (a) ice nucleating agent (INA) proteins and lipoproteins in the hemolymph or cells, which induce freezing in a controlled way; (b) anti-freeze proteins (AFPs), which adsorb to the surface of small ice crystals, inhibiting its growth; and c) compatible solutes/cryoprotectants (CPAs) such as polyols and sugars (Fuller, 2004). Glycerol is the most widely occurring CPA in insects, although other polyhydric alcohols and some sugars such as trehalose have similar cryoprotective functions. These compounds, by adding to the pool of solute molecules, affect the osmotic pressure of the hemolymph and help to regulate cell volume during extracellular ice formation, and they also stabilize proteins. They are produced mainly from glycogen stored in the fat body and, in many insects, begin to accumulate at the beginning of the overwintering period (Doucet et al., 2009).

Among the different neurohormones, two highly conserved neural signaling systems have been found that are crucial for different aspects of insect metabolism and food response and are particularly involved in resistance to low temperatures (Lingo et al., 2007). These are ILPs and their receptor and NPFs, an analog of mammalian neuropeptide Y (NPY) (Lingo et al., 2007).

The tuning of insulin signaling during stress is one of the most important response of the neuroendocrine system to unfavorable conditions (Luo et al., 2012). In insects, different numbers of ILPs have been found in various species. One such peptide was found in the locusts *Locusta migratoria* and *Schistocerca gregaria*, whereas 38 in the silkmoth *Bombyx mori* (Nässel and Vanden Broeck, 2016). The classification of insect ILPs as insulin-like is mainly based on similarities in the amino acid sequences of the mature peptides to those of insulins in mammals. The number and positions of cysteine residues (Nässel and Vanden Broeck, 2016) and the arrangement of the precursor with the B, C, and A chains, which can be processed into dimeric peptides or with the maintenance of C peptides as in the insulin-like growth factor (IGF) are crucial in this assignment. Most of the research on the exact mechanisms of ILPs release and modes of action have been performed only on the model insect *Drosophila melanogaster*. It was shown that ILPs release from brain insulin producing cells (IPCs) in adult flies is triggered by a sugar meal through the direct activation of these cells via autonomous glucose-sensing capacity (Park et al., 2014). Thus, the mechanisms of glucose-induced ILPs release resemble those in pancreatic beta cells of mammals and include an ATP-sensitive potassium channel (K_{ATP}), a glucose transporter (GluT1) and voltage-sensitive calcium channels. However, different neurotransmitters, neuropeptides and peptide hormones have been implicated in acting on IPCs to modify the expression of ILPs. These are GABA, 5-HT, OA, sNPF, TRP, corazonin, allatostatin A, CCHamide, AKHs, adiponectin, and limnostatin (Nässel and Vanden Broeck, 2016). Thus, in insects, the synthesis and release of multiple ILPs is under complex control. The system is tightly regulated and probably, as shown below fragile to unfavorable conditions such as low temperature (Li et al., 2020). This whole precisely regulated system in insects is responsible for the regulation of a number of functions, including reproduction and development, growth, metabolic homeostasis, longevity and stress response (Rauschenbach et al., 2008).

In the endocrine stress response, ILPs have been shown to play a crucial role together with biogenic amines (5-HT, OA, and DA), 20-hydroxyecdysone and juvenile hormone (JH) – hormone in adult insects, levels of which act and change similarly under stress (Gruntenko and Rauschenbach, 2018). The insulin/IGF signaling system (IIS) has been shown to respond to various stress signals such as starvation and oxidative stress. Recently, it was also suggested to play a role in temperature stress. Its participation is crucial in the regulation of the JH, OA, and DA levels, and it controls catecholamine metabolism indirectly *via* JH. Possibly one of the pathway in which the IIS is involved in the control of stress resistance is mediated through JH/DA signaling (Gruntenko and Rauschenbach, 2018). Moreover, different studies suggest the existence of a feedback loop in the interplay of JH and the IIS (Yamamoto et al., 2013). JH serves as a positive regulator of the IIS, whereas IIS negatively regulate the JH level – feedback loop (Gruntenko and Rauschenbach, 2018). Recent studies have shown that JH and DA regulate carbohydrates at the circulating carbohydrate level, mainly trehalose (used as a cryoprotectant) (**Figure 1**). It was shown that increases in JH and DA decrease the levels of trehalose and glucose under normal conditions but after stress exposure bring them to values close to normal. Thus, the roles of DA and JH in the neuroendocrine stress reaction in *D. melanogaster* are related to normalizing it after stress (Karpova et al., 2019). As ILPs have been shown to regulate JH and DA levels, they indirectly regulate carbohydrate levels. Moreover, Luo et al. (2012) demonstrated that IPCs in the *Drosophila* brain may be inactivated by serotonergic signaling *via* serotonin receptor 5-HT_{1A}. On the other hand, OA stimulates the activity of IPCs by binding to OAMB receptors, which results in an increase in cAMP and the activation of cAMP-dependent PKA (Crocker et al., 2010). Despite the knowledge about the action of biogenic amines on IPCs, we still do not know the physiological role of the antagonistic action of OA and 5-HT. As Luo et al. (2012) suggested, this action of OA and 5-HT may be associated with the tuning of insulin signaling during stress conditions.

A second signaling system that is widely known for the regulation of metabolism in insects and is connected with stress response is NPF signaling. These neurohormones were first identified in invertebrates (the tapeworm *Moniezia expansa*) based on pancreatic polypeptide antiserum (Maule et al., 1991). The first analysis showed that they are similar to mammalian NPY. In insects, an additional group of short peptides (8–10 amino acids) with similar C-terminal sequences was also discovered and named short neuropeptides F (sNPF). Recent phylogenetic analysis revealed that they are evolutionarily distinct from one another and that only long (36 amino acid) neuropeptides F are related to NPY (Fadda et al., 2019).

In insects, NPF have been involved in the regulation of different biological processes, including growth and reproduction, nociception, the circadian clock, learning, feeding and metabolism, and they act mainly as neuromodulators or neurohormones (Fadda et al., 2019). The most extensive studies of NPF functions in insects have been conducted in *D. melanogaster*. First studies of NPF physiological function were performed on their role in feeding regulation. This was based on two evidences. Firstly, earlier studies showed that NPF signaling in model nematode *C. elegans* is involved in foraging behavior and secondly NPF and its receptor (NPFR1) are similar in structure to mammalian NPY and NPY receptor (NPYR) in whom they have been known to regulate feeding (Nässel and Wegener, 2011). In studies conducted on *D. melanogaster* it was shown that NPFR is connected with transient receptor potential channel (TRP) – *painless* (pain) when respond to noxius stimuli or various stress conditions (Rosenzweig et al., 2008; Xu et al., 2008). This TRP channels are crucial for the response of flies to temperature, mechanosensory stimuli or noxious chemicals (Tracey et al., 2003). The receptor is activated by fructose so it can trigger behavior which is related to food aversion. It was shown that it is inactivated by NPF during feeding when larva reside in environment very rich in sugar (Nässel and Wegener, 2011). Thus, NPF signaling is crucial for metabolism

and food acquisition. During exposure to low temperature (11°C for 120 min), the overexpression of the NPF receptor in *D. melanogaster* was sufficient to trigger cold-resistant feeding activity normally associated with fasted larvae (Lingo et al., 2007). This is evidence that during exposure to low temperature, NPF signaling may be responsible for food acquisition to store carbohydrates, which will work as cryoprotectants.

Among the 32 NPF families in insects, there are many neurohormones that might also be responsible for the regulation of metabolism during various unfavorable conditions, including cold. These neurohormones may include tachykinins and/or AKHs. However, the number of studies on the neuropeptidergic regulation of metabolic adaptations to low temperature is rather limited. AKHs have been shown to regulate, together with JH, the ice nucleator level (mainly lipoprotein) in the hemolymph of the stag beetle *Ceruchus piceus* (Xu et al., 1990) (**Figure 1**). Two hours after injection of AKH the level of ice nucleator increased and this increase appeared to be the result of the release of lipoproteins from the fat body (Xu et al., 1990). This is probable especially when we consider that the major function of AKH in insects is to regulate the lipids, carbohydrates and amino acid metabolism (Gäde, 2009). This might be especially important during cold. Xu et al. (1990) showed also that in lipoprotein release JH is also involved. This hormone decreased the ice nucleator activity but increased its level (Xu et al., 1990). However, the released lipoprotein pool was inactive in *Ceruchus piceus* beetle. This is in line with all the other mechanisms described above showing the crucial role of JH in the endocrine stress response.

Very recently the first detailed study about neuroendocrine stress response has been released. It describes changes in neuropeptide and neuropeptide receptors expression in *Bemisia tabaci* (Li et al., 2020). The authors showed that when insects were exposed to low temperature (4°C) for 1 and 4 h the expression level of several neuropeptides genes and neuropeptides receptor genes have been changed – for details see **Table 1**. These include neurohormones known for diuresis regulation, metabolic peptides and peptides which regulate reproduction and development. Remarkably peptides so far known only for myotropic properties such as proctolin or myosupressin were also changed.

TABLE 1 | Changes in mRNA level of insect neuropeptides after short (≤1 h) and prolonged (≥4 h) cold exposure.

Neuropeptide	Short cold stress	Prolonged cold stress
Adipokinetic hormone	n.e.	↑
Allatostatin A (FGL/AST)	↑	↓
Allatostatin CCC	↑	↑
Capability peptide	↑	↓/↑**
CCHamide	↓	n.e.
CNMamide	↓	↑
Corazonine	↓	n.e.
Eclosion hormone	n.e.	↓
Insulie-like peptide	n.e.	↑
Ion transport peptide	n.e./↑	↓
Kinin	n.e.	n.e./↑*
Myosuppressin	n.e.	↓
Orcokinin	n.e.	↑/↓
Proctolin	n.e.	↓
RYamide	↑	↓

*Prepared based on the Li et al. (2020), Terhzaz et al. (2015), and Terhzaz et al. (2018). *The effect was observable during recovery time after cold stress in D. melanogaster and no effect was observed in B. tabaci during cold stress. **Li et al. (2020) showed decrease in capa level in B. tabaci after 4 h of cold stress, whereas Terhzaz et al. (2015) a significant increase in D. melanogaster after 6 and 24 h of cold stress, the arrows represent an increase (↑) and a decrease (↓) in gene expression. n.e., no effect observed.*

CONCLUSION AND PERSPECTIVES

In this review, we summarize the current knowledge about the neuroendocrine stress response to low temperature. Remarkably, despite the recent advance in insect neuroendocrinology, very little is known about the neurohormonal regulation of this process. Of course, some universal mechanisms typical of physiological adaptations to various unfavorable conditions are known, and only a few studies focus on cold stress.

To date, it has been shown that three major groups of compounds are involved in the response to temperature stress: biogenic amines (5-HT, OA, DA), gonadotropins (JH, 20E) and neuropeptides (ILPs, CAPA, kinins). The adjustments during exposure to low temperature include changes in overall metabolism, mainly the production and storage of cryoprotectants and loss of ion and water homeostasis due to a switch in MT and/or gut physiology. These two processes seem to be independent. First, the central role might be played by IPCs in the brain, that release ILPs which regulate DA metabolism *via* JH. This model was proven at least for high-temperature stress (Gruntenko and Rauschenbach, 2018). It should also be evaluated for low-temperature stress. IPCs are also under strict neuroendocrine control from other neuropeptides, such as sNPF, TRPs, corazonin, and AKH. Taken together, the single reports on the influence of neuropeptides on different physiological processes under stress conditions, indicate that the involvement of these peptides in neuroendocrine cold stress response should be evaluated. The roles of certain peptides could be multiple, such as the regulation of ILPs release and the regulation of the functioning of other processes, for instance, in the fat body.

On the other hand, large scale analysis of neuropeptidome showed that neuroendocrine response to cold might be complex and involve several neuropeptides, at least on mRNA level. So far only *Drosophila* has been studied, so further analysis should be performed in non-model species including bigger insects such as cockroach *Gromphadorhina coquereliana* or beetles *Tenebrio molitor* and *Nicrophorus vespilloides*. These responses might be also species specific.

Second, the exact role of biogenic amines under cold stress should also be studied. In the response to heat stress, they were shown to be intermediary in the interplay between JH and 20E, and DA metabolism is regulated by ILPs indirectly by JH. However, the other properties of catecholamines indicate that they might play pleiotropic roles in the cold response, also regulating the level of neuropeptides. Finally, the exact

signaling mechanism of fluid secretion during exposure to low temperature and the involvement of all neurohormones in this process should be evaluated in detail. To date, CAPA neuropeptides and kinins have been shown to be involved. However, no neuroendocrine-controlling mechanism has been proposed thus far.

AUTHOR CONTRIBUTIONS

JL, AU, and PM were responsible for the general idea of the manuscript and text editing. JL coordinated the Introduction and Diuresis sections. AU coordinated the biogenic amine description. PM coordinated the Metabolism and Conclusion sections. JL, AU, PM, HC, and H-JP were responsible for the review and writing of the first draft of the manuscript. JL and AU acquired funding.

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Structure-Activity Studies on the Hypertrehalosemic Hormone II of the Stick Insect *Carausius morosus* (Phasmatodea): Carbohydrate-Mobilization and Cardio-Stimulatory Activities

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The corpora cardiaca of the Indian stick insect, *Carausius morosus*, synthesize two decapeptide neuropeptides of the adipokinetic hormone (AKH) family, both of which can increase the trehalose levels in the hemolymph when the stick insect is ligated between the head and the thorax. Here, we use two biological assays to assess the potencies of 19 AKH analogs in ligated *C. morosus*: the carbohydrate-mobilizing assay measures the change in the levels of circulating carbohydrates following injection of a substance, while the semi-exposed heart assay measures a change in heart beat rate after the peptide is applied onto the heart. With the endogenous AKH (Carmo-HrTH-II) as lead peptide, we report here on seven naturally-occurring AKH peptides (bioanalogs) selected for testing because of a single or double amino acid replacement, or for being octapeptides. Single amino acid substitutions by an alanine residue at all positions of Carmo-HrTH-II, as well as analogs modified at the termini were also investigated to give a comprehensive view of ligand-receptor interaction at the physiological level in a hemimetabolous insect that practices thanatosis (feigning death). Only small changes are elicited in the bioassays, but the results from the two tests are comparable bar one or two anomalies. Results show that analogs modified at the termini have no or reduced activity. Regarding structural requirements of a ligand, the *C. morosus* AKH receptor appears to be strict: octapeptides are not preferred and many of the decapeptide analogs failed to reach 50% activity relative to Carmo-HrTH-II. The data implies that the AKH receptor in *C. morosus* mostly does not tolerate shorter peptides and single amino acid replacements in most places of the native AKH peptide. This information is important if environmentally friendly insect-specific pesticides are made based on an insect AKH as lead peptide: stick insects that are normally not viewed as pest insects may not be easily targeted by cross-reactive AKH mimetics directed at harmful insects, due to the very specific amino acid requirements to activate the *C. morosus* AKH receptor.

Keywords: adipokinetic hormone, hypertrehalosemic hormone, fuel mobilization, heart beat rate, *Carausius morosus*, Carmo-HrTH-II, structure-activity studies, stick insect

INTRODUCTION

Insects are not only well-known for their diversity and abundance but also for their influence on the biosphere and human life. The major anthropomorphic division in insect groups is made between those that are beneficial to mankind and those that are health risks or agricultural pests (Burn et al., 1987; Capinera, 2010; Gullan and Cranston, 2014). Increasingly, there is a strong interest in developing environmentally friendly insecticides that are selective and affect only the target (pest) species instead of all insects. Specifically, the development of hormone-like compounds that can be used in specific drug design to act as targeted pesticides are being considered (Altstein et al., 2000; Gäde and Goldsworthy, 2003; Audsley and Down, 2015). The compounds in mind are the insect's neuropeptide hormones that control most of the key physiological processes such as development, reproduction, metabolism, behavior, muscle contraction including heart beat rate and diuresis.

A well-researched family of hormones are the adipokinetic hormones (AKHs) which are synthesized and released from the retrocerebral corpora cardiaca (CC); the AKHs are mainly tasked with mobilizing fuels (energy-rich metabolites) from fat body stores into the hemolymph and are identified as putative targets to develop new insecticides (see review by Marco and Gäde, 2020). The AKH peptide family is generally characterized by peptides having (a) a chain length of 8–10 amino acids; (b) post-translationally modified termini: a pGlu residue at the N-terminus and a carboxyamide at the C-terminus; (c) either a Leu, Ile, Val or Phe residue at position 2; (d) a Thr or Asn residue at position 3; (e) an aromatic Phe or Tyr residue at position 4; (f) the branched amino acids Thr or Ser at position 5; (g) the aromatic residue Trp at position 8; (h) the simple amino acid Gly at position 9, and (i) variable amino acids at positions 6, 7, and 10 (see Gäde, 2009).

AKHs exert a biological effect via a G protein-coupled receptor (GPCR) and this system, the ligand-receptor pair, is the target for peptide mimetics to be developed (see reviews by Audsley and Down, 2015; Verlinden et al., 2015) in a fashion similar to the well-known beta blockers treating hypertension in human medicine. Two standard methods have been used to investigate AKH ligand-receptor interactions in structure-activity relationship (SAR) studies; the oldest being an indirect *in vivo* biological assay in which ligands are tested in live animals, and the result of a signal transduction cascade is measured, e.g., the release of lipids/carbohydrates into the hemolymph, or the activation of glycogen phosphorylase. This has been done for AKH bioanalogs and synthetic analogs in locusts (see, for example, Stone et al., 1978; Gäde, 1990, 1993; Poulos et al., 1994; Goldsworthy et al., 1997), lepidopterans (Fox and Reynolds, 1991; Ziegler et al., 1991, 1998; Marco and Gäde, 2015, 2019) and cockroaches (Gäde, 1986, 1990, 1992; Ford et al., 1988; Hayes and Keeley, 1990; Gäde and Hayes, 1995). The second and more recent method of conducting SARs is via a direct *in vitro* receptor assay; the prerequisite is to have knowledge of the AKH receptor sequence: this is in general (with a few exceptions) only the case for those insects where the whole genome is known. To date, detailed receptor assay SAR studies with bioanalogs and specifically modified peptides have only been performed with dipteran species, *Drosophila melanogaster*, *Anopheles gambiae* and *Glossina morsitans* (Caers et al., 2012, 2016). In general, the results of these *in vitro* assays agree with those generated by *in vivo* biological assays, i.e., that the conserved aromatic amino acids at position 4 and 8 are important for receptor-peptide interactions, as well as (in the majority of cases) the blocked termini, whereas amino acids at positions 7 and 10 are not always that crucial. It also appears to emerge that the receptor of those species that have two or more endogenous AKHs, such as *Periplaneta americana*, *Locusta migratoria*, and *Hippotion eson*, seem to tolerate a wider variety of ligand modifications than an insect with a single endogenous AKH, such as *Blaberus discoidalis* and *Aedes aegypti* (see references above; Marchal et al., 2018; Wahedi et al., 2019).

AKHs are also known for their myotropic effects, especially to increase the rate of heart beat (Chowański et al., 2016); SAR studies have only been done in the cockroach *P. americana* to a certain extent (Baumann et al., 1990), and a few bioanalogs were tested on the heart of the stick insect *Baculum extradentatum* (Malik et al., 2012).

In the current study, a member of the order Phasmatodea is investigated with respect to metabolic (hypertrehalosemic) and myotropic (cardio-stimulatory) activity of AKH. Insects of this order are well known to be kept as pets, although there are also reports on the pest status of certain phasmid species, especially in private gardens on ornamental plants in the United States of America (Griffiths and Picker, 2011). The subject of the current study, the Indian stick insect *Carausius morosus*, is already well-known in laboratory research of neuropeptide hormones (see for example Miksys et al., 1997; Predel and Gäde, 1999; Lorenz et al., 2000; Liessem et al., 2018), and has also become a model organism for neurobiology, especially studying the control of locomotion (see for example, Bidaye et al., 2018).

With regards to AKH research on *C. morosus*, two near-identical decapeptide members of the AKH family were isolated from the corpora cardiaca, and these peptides had no effect on hemolymph metabolite levels in conspecific assays (Gäde, 1979). Three years later, a hypertrehalosemic effect of conspecific CC extract was demonstrated in adult *C. morosus* only when a ligature was applied to the insect (between the head and the first pair of legs) before injection of the CC extract (Gäde and Lohr, 1982). The exploratory study of 1982 further revealed that the increase in circulating trehalose was relatively small (about 4–7 mg ml^{-1}); the highest response was recorded in ligated 6th instar larvae shortly before the final molt; and there was evidence of ligand preference, i.e., the AKH receptor of the stick insect recognized the conspecific AKHs but not that of lepidopteran species, the migratory locust or of the decapod crustaceans (Gäde and Lohr, 1982). The two stick insect AKHs were sequenced by fast atom bombardment mass spectrometry (FABMS) (Gäde, 1985; Gäde and Rinehart, 1987): the later eluting 2nd peak contained most of the peptidic material and has the code-name Carmo-HrTH-II; the earlier eluting peak (code-name Carmo-HrTH-I) was shown to be identical in the primary sequence to Carmo-HRTH-II but a hexose is bound in an unorthodox manner to the Trp residue at position 8 (Gäde et al., 1992).

Through the heroic collection of 2000 CC and the use of a sensitive 800 MHz nuclear magnetic resonance spectrometer equipped with a cryoprobe, it was possible to show unequivocally that an α-mannopyranose is bound to Trp^8 of Carmo-HrTH-I in an unusual C-glycosylated fashion (Munte et al., 2008). Recently, a transcriptomic and neuropeptidomic analysis of the central nervous system of *C. morosus* revealed only one AKH precursor although both Carmo-HrTH-I and -II were confirmed in mass spectrometry of tissues (Liessem et al., 2018), thus, the one peptide precursor is modified post-translationally to form both versions of *C. morosus* AKHs.

Recently it was also shown that both AKH peptides of *C. morosus* are capable of increasing the rate of heart contraction in ligated stick insects: the application of synthetic Carmo-HrTH-II in doses above 6.67×10^{-8} M increased the heart beat rate significantly and maximally (i.e., higher peptide doses had no further increase on the heart beat rate; Marco et al., 2018). The frequency of heart contractions in *C. morosus* at rest was found to be much lower than that recorded in more active insect species and again, the endogenous AKH peptide could not affect heart contractions in non-ligated *C. morosus* while small increases in the heart beat rate were recorded in ligated stick insects (Marco et al., 2018). Prior to this study, an influence of AKH on stimulating the heart frequency of the Vietnamese stick insect, *B. extradentatum*, was also shown in decapitated specimens (Malik et al., 2012). The low heart rate and the small increases in metabolic reactions to endogenous AKHs are interpreted as evolutionary consequences of the cryptic defenses of stick insects where they shut down the metabolism to play dead (thanatosis) to avoid detection from predators, instead of entering into the fight or flight mode (Marco et al., 2018).

Based on the above-mentioned results, the present study was initiated with the following aim: to understand in detail the requirements of the AKH receptor with respect to structural features of the ligand. Although a number of detailed SAR studies have been performed on the AKH system in insects (references see above), there is only one case according to our information where an insect that has one decapeptide as AKH ligand has been tested; this is the cockroach *B. discoidalis* and its AKH Bladi-HrTH (Ford et al., 1988; Hayes and Keeley, 1990). That study and the companion research on *P. americana* (Gäde and Hayes, 1995), an insect with AKH octapeptides, show that the receptors have some different properties. Since the AKH receptor of *C. morosus* was not structurally known at the time of the current study, we performed *in vivo* bioassays and tested for a metabolic response (hypertrehalosemic activity) and a myotropic response (heart beat rate). In this way we also hoped to establish whether the putative receptor in the fat body tissue and in the heart muscle tissue are very likely identical. Bioanalogs and specifically altered peptides were chosen/designed to obtain the following information on structural requirements for interaction with the *C. morosus* AKH receptor: (a) acceptable chain length of the ligand, (b) the importance of the AKH termini, and (c) the side chain of each amino acid in the decapeptide.

MATERIALS AND METHODS

Insects

Indian stick insects (*Carausius morosus)* were reared under crowded conditions at approx. 25 ± 2°C, 65% RH and a 12 h light: 12 h dark regime. The stick insect nymphs were fed with fresh ivy (*Hedera helix)* leaves supplied twice a week, while the adults were provided with fresh twigs of mirror bush (*Coprosma repens*) about twice a week.

Peptides and Corpora Cardiaca Extract

For sequence information of the various synthetic peptides (see **Tables 1–3**).

The endogenous hypertrehalosemic hormone Carmo-HrTH-I was isolated from the CC of *C. morosus* as previously described by Gäde (1985). Synthetic Carmo-HrTH-II was purchased from

TABLE 1 | The biological effect (metabolic) of the termini of Carmo-HrTH-II in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (10 µl injected) and amino acid sequence[a] (20 pmol peptide injected)	Hemolymph carbohydrates (mg ml^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	0 min	90 min	Difference	P[#]	
Distilled water	13	8.1 ± 3.1	8.5 ± 2.5	0.4 ± 1.5	NS	NS
Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	9.4 ± 1.5	14.9 ± 1.9	5.4 ± 1.1	0.00001	100
Carmo-HrTH-I						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp* -Gly-Thr amide	12	8.0 ± 1.6	12.9 ± 1.9	4.9 ± 1.1	0.00001	91[b]
Carmo-HrTH-II: [N-Ac]Ala[1]						
[N-Ac]Ala -Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	9.0 ± 1.3	10.6 ± 2.0	1.5 ± 1.1	0.002	28
Carmo-HrTH-II: Nonapeptide, free N-terminus, Leu[1]						
Leu -Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	14	8.8 ± 1.7	9.5 ± 1.5	0.7 ± 1.3	NS	NS
Carmo-HrTH-II: free C-terminus						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly- Thr OH	21	8.8 ± 2.1	10.4 ± 2.6	1.6 ± 1.6	0.0002	30

*The data are presented as Mean ± S.D. [a]Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. *Tryptophan residue is mannosylated. [b]Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe's test. [#]Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

TABLE 2 | The biological effect (metabolic) of selected AKH bioanalogs in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (10 μl injected) and amino acid sequence[a] (20 pmol peptide injected)	Hemolymph carbohydrates (mg ml^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	0 min	90 min	Difference	P[#]	
Decapeptides						
Distilled water	13	8.1 ± 3.1	8.5 ± 2.5	0.4 ± 1.5	NS	NS
Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	9.4 ± 1.5	14.9 ± 1.9	5.4 ± 1.1	0.00001	100
Phyle-CC						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Ser amide	10	8.2 ± 1.9	14.1 ± 1.7	5.9 ± 1.7	0.00001	109[b]
Locmi-AKH-I						
pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	12	8.9 ± 1.3	9.9 ± 1.2	1.0 ± 1.2	0.002	19
Phymo-AKH						
pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Ser amide	10	9.6 ± 1.8	10.5 ± 1.4	0.9 ± 1.7	NS	NS
Rommi-CC						
pGlu-Val-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	8.0 ± 1.4	10.6 ± 1.6	2.6 ± 1.5	0.0004	48
Octapeptides						
Peram-CAH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp amide	18	9.3 ± 1.8	11.0 ± 1.1	1.7 ± 2.0	0.002	31
Aedae-AKH						
pGlu-Leu-Thr-Phe-Thr-Pro-Ser-Trp amide	10	8.1 ± 1.4	8.8 ± 1.2	0.7 ± 1.1	NS	NS
Pyrap-AKH						
pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp amide	10	8.7 ± 1.8	9.2 ± 1.8	0.5 ± 1.0	NS	NS

[a]*Amino acid substitutions in Carmo-HrTH-II analogs are highlighted.* [b]*Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe's test. The data are presented as Mean ± S.D.* [#]*Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

TABLE 3 | The biological effect (metabolic) of single amino acid substitutions in Carmo-HrTH-II in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (10 μl injected) and amino acid sequence[a] (20 pmol peptide injected)	Hemolymph carbohydrates (mg ml^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	0 min	90 min	Difference	P*	
Distilled water	13	8.1 ± 3.1	8.5 ± 2.5	0.4 ± 1.5	NS	NS
Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	9.4 ± 1.5	14.9 ± 1.9	5.4 ± 1.1	0.00001	100
Ala^{2}-Carmo-HrTH-II						
pGlu-Ala-Thr-Phe-Thr- Pro-Asn-Trp-Gly-Thr amide	10	11.4 ± 2.3	16.8 ± 1.7	5.4 ± 1.7	0.00003	100
Ala^{3}-Carmo-HrTH-II						
pGlu-Leu-Ala-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	10	10.3 ± 2.4	11.1 ± 1.8	0.8 ± 1.6	NS	NS
Ala^{4}-Carmo-HrTH-II						
pGlu-Leu-Thr-Ala-Thr-Pro-Asn-Trp-Gly-Thr amide	20	8.6 ± 2.2	9.1 ± 2.4	0.5 ± 2.3	NS	NS
Ala^{5}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Ala-Pro-Asn-Trp-Gly-Thr amide	10	10.2 ± 1.2	10.1 ± 1.3	−0.1 ± 0.1	NS	NS
Ala^{6}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Ala-Asn-Trp-Gly-Thr amide	10	9.5 ± 1.2	11.1 ± 0.9	1.6 ± 1.1	0.001	30
Ala^{7}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Ala-Trp-Gly-Thr amide	10	11.3 ± 2.0	12.0 ± 2.2	0.7 ± 0.6	0.003	13
Ala^{8}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Ala-Gly-Thr amide	10	8.5 ± 2.1	8.3 ± 1.9	−0.1 ± 1.3	NS	NS
Ala^{9}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Ala-Thr amide	10	9.5 ± 1.9	11.0 ± 1.5	1.5 ± 0.9	0.0004	28
Ala^{10}-Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Ala amide	10	9.9 ± 2.3	12.4 ± 1.6	2.5 ± 1.0	0.00002	46

[a]*Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. Data presented as Mean ± S.D. *Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

Peninsula Laboratories (Belmont, CA, United States) together with Locmi-AKH-I and Peram-CAH-I. Phyle-CC and Pyrap-AKH were synthesized by Dr. R. Kellner (Merck, Germany). Aedae-AKH was made by Genscript Corporation (United States). Carmo-HrTH-II as free acid and not as amidated form, as well as Carmo-HrTH-II minus pGlu (thus a nonapeptide), and Phymo-AKH were synthesized by Dr. S. Kyin (Biotechnology Centre, University of Illinois, Urbana-Champaign, United States). Analogs of Carmo-HrTH-II with single amino acids replaced by Ala were purchased from Pepmic Co., Ltd (Suzhou, China). Stock solutions were made by dissolving 1 mg of each peptide in 1 ml of 60% acetonitrile, including 0.10% trifluoroacetic acid (TFA), and diluted further to 5 pmol μl^{-1} using distilled water. The various peptide solutions were subsequently monitored for purity and quantified via reversed phase-high performance liquid chromatography (Gilson RP-HPLC system) with fluorescence detection (276 nm excitation, 350 nm emission; Gäde, 1985) and with a gradient of 43–53% solvent B in 20 min at a flow rate of 1 ml min^{-1} (Nucleosil C18 column; Solvent A: 0.11% TFA in water; solvent B: 60% acetonitrile with 0.10 TFA).

Corpora cardiaca (with the corpora allata attached) were dissected from the head of adult *C. morosus* into 80% methanol, the cell contents were extracted on ice via sonication (Branson sonifier cell disruptor), followed by centrifugation and the resulting supernatant was dried in a vacuum concentrator. The dried corpora cardiaca extracts were then reconstituted in distilled water for use in carbohydrate-mobilization assays.

Bioassays

Carbohydrate-Mobilization Assay

Sixth instar nymphs that were 1–2 days before molting were neck-ligated and used in the hypertrehalosemic *in vivo* assay as described in detail by Gäde and Lohr (1982). Stock peptide solutions (5 pmol μl^{-1}) were diluted with distilled water to the desired concentration in 10 µl, which was injected into the stick insect nymphs.

Semi-Exposed Heart Bioassay

The semi-exposed heart bioassay was carried out as described (Marco et al., 2018) with adult stick insects between 1 and 2 months old that were ligated at the neck. Briefly, a ventral incision of the abdomen exposed the ventral cavity with the internal organs; a few drops of stick insect saline (pH 6.6; 15 mM NaCl, 18 mM KCl, 7.5 mM $CaCl_2$, 2 mM HEPES, 50 mM $MgCl_2$, and 184 mM glucose according to Ejaz and Lange, 2008) were added to the exposed part. The semi-isolated heart preparation was viewed with a dissecting microscope (12-fold magnification), and the number of observed heart beats (contractions of the heart muscle) was counted during a fixed period using a manual tally counter and a timer. Once the heart had stabilized, saline was replaced with the test solution. For this the peptide stock solution of 5 pmol μl^{-1} was diluted with stick insect saline to the desired concentration and a final volume of 150 µl was used for each test.

Heart rates under normal saline served as controls. The change in heart rate after the application of the test solution was calculated as an average of the first four counts and was compared to the average heart rates counted in saline before the application of the test solution. This comparison of change in heart rates was done for all the peptides tested.

Statistical Analyses

Student's paired *t*-test was used to compare the concentrations of carbohydrates in the hemolymph, as well as the heart rates of *C. morosus* before and after the subjection to the test solution. One-way analysis of variance (ANOVA) followed by Scheffe's multiple comparison test was used to compare the hypertrehalosemic and cardio-stimulatory effects among *C. morosus* tested with different peptides. Differences were considered significant at $p < 0.05$ for all the tests.

RESULTS

Hypertrehalosemic Effects of the Endogenous Peptides

In the first series of experiments, various doses of synthetic Carmo-HrTH-II and corpora cardiaca (CC) extract of *C. morosus* were used to determine the maximal hypertrehalosemic response of *C. morosus.* Hemolymph samples were taken from 17 to 18 days old 6th instar nymphs 2 h after they were neck-ligated and then 90 min after either CC extract or synthetic Carmo-HrTH-II was injected. Ligated nymphs injected with distilled water served as controls for handling stress. As depicted in **Figures 1A,B**, various doses of both Carmo-HrTH-II and CC extract increased the level of carbohydrates in the hemolymph

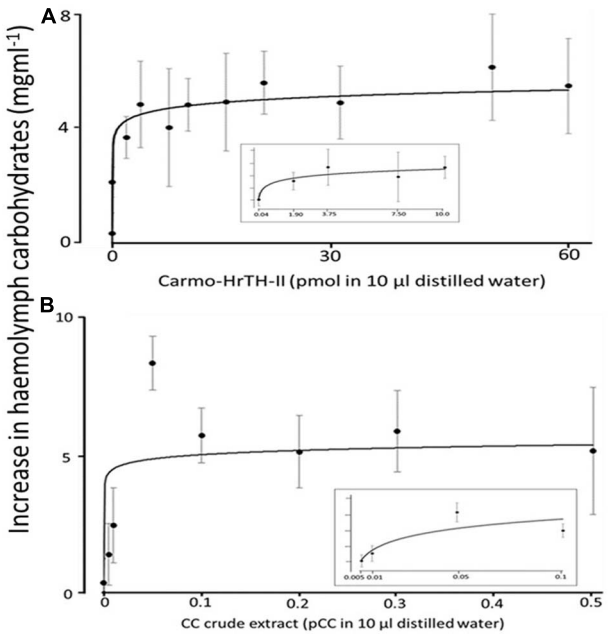

FIGURE 1 | *In vivo* hypertrehalosemic assays. The effects of the increasing doses of **(A)** synthetic Carmo-HrTH-II and **(B)** native CC extracts, on carbohydrate mobilization in ligated 6th instar nymphs of *C. morosus* (17–18 days old). Each data point represents the mean ± SD in mg ml^{-1}. Between 7 and 12 insects were used for each dose.

in a dose-dependent manner. A dose of 0.04 pmol of synthetic Carmo-HrTH-II and the equivalent of 0.005 pairs of corpora cardiaca (pCC) were sufficient to give significant increases in hemolymph carbohydrates ($p < 0.05$). With synthetic Carmo-HrTH-II, doses of 7.5–60 pmol gave an average increase of 5.2 ± 0.4 mg ml^{-1} and the *post hoc* Scheffe's test revealed that there was no significant difference between the effect of these doses ($p > 0.05$). Doses of 0.1–0.5 pCC gave an average increase of 5.6 ± 0.6 mg carbohydrates ml^{-1} hemolymph (**Figure 1B**); again, statistical analyses reveal no significant difference between the effect of these doses ($p > 0.05$). An anomalous result was obtained after injection with 0.05 pCC extract: a much higher increase in carbohydrate concentration (8.5 ± 1.0 mg ml^{-1}) was measured than from any other dose tested (**Figure 1B**), and this increase differed significantly ($p < 0.05$) from all the higher CC extract doses; moreover, such an increase was not achieved by any injection of synthetic Carmo-HrTH-II even at high doses (see **Figure 1A**).

Based on these pilot experiments, we selected a dose of 20 pmol for testing the analogs of Carmo-HrTH-II since such a dose of our lead compound, Carmo-HrTH-II had achieved maximal hypertrehalosemic activity.

Hypertrehalosemic Response of Synthetic Analogs of Carmo-HrTH-II

The Biological Effect of the Termini of Carmo-HrTH-II in *C. morosus*

AKH peptides are characterized by a pGlu in position 1 and an amidated C-terminus. This is believed to be an effective block against exopeptidases in the hemolymph of the insect, resulting in a longer half-life of the peptide (Oudejans et al., 1996). Carmo-HrTH-II analogs were designed to specifically explore how carbohydrate mobilization is affected by changes to the N terminal amino acid of the decapeptide. The results are shown in **Table 1**. When the termini of Carmo-HrTH were modified (i.e., N-terminal pGlu replaced with an acetylated Ala residue, or a free acid at the C-terminus instead of an amidation) the hypertrehalosemic activity was severely reduced from 100% to a mere 30%, while a nonapeptide lacking a pGlu and having, thus, a Leu residue in position 1 had no significant biological effect.

The Biological Effect of AKH Bioanalogs in *C. morosus*

Functional AKHs are either composed of eight, nine, or 10 amino acids; *C. morosus* synthesizes only decapeptide AKHs. The biological effect of shorter chain lengths (octapeptides) were therefore investigated, along with single or double amino acid substitutions in naturally occurring decapeptide AKHs to ascertain the flexibility of the *C. morosus* receptor that usually sees and responds only to two decapeptides of the same amino acid sequence.

Peram-CAH-II has the same 8 amino acids as in the endogenous decapeptide of the stick insect, Carmo-HrTH; this is, however, only sufficient to elicit a 30% hypertrehalosemic response (**Table 2**). The remaining octapeptides tested with single substitutions at position 3 and 7 had no significant activity.

Of the decapeptide bioanalogs, only Phyle-CC (Ser^{10} instead of Thr^{10}) increased the hemolymph carbohydrates as high as Carmo-HrTH-II ($p > 0.05$), while Rommi-CC (Val^2-Asn^3 instead of Leu^2-Thr^3), elicited a response of 48% of the maximal possible hypertrehalosemic effect. The remaining analogs were virtually unable to increase the hemolymph carbohydrates (**Table 2**).

The Biological Effect of Single Amino Acid Replacements in Carmo-HrTH-II

The relative importance of each amino acid of Carmo-HrTH-II was investigated via a series of analogs in which one amino acid was substituted with an alanine residue (**Table 3**). In this way we can make inferences about the relative importance of the side chains of the original residues in activating the stick insect AKH receptor. The substitution of an aromatic amino acid residue (i.e., Phe^4 or Trp^8) with Ala eliminated AKH activity (**Table 3**). An Ala replacement of Thr in position 3 and 5 also gave no hypertrehalosemic effect, while a very small increase in carbohydrates was observed after injection of an Ala^7 analog (thus, replacing Asn^7). Ala^6 and Ala^9 replacements of Pro and Gly, respectively (**Table 3**) resulted in a marked reduction of biological activity (around 30%), whereas Ala^{10}-Carmo-HrTH-II (in place of Thr^{10}) resulted in about half of the maximal possible hypertrehalosemic effect. In contrast, Ala^2-Carmo-HrTH-II increased the hemolymph carbohydrates to the same extent as did Carmo-HrTH-II (**Table 3**).

Effect of Endogenous Neuropeptides and Synthetic Analogs of Carmo-HrTH-II on the Heart Rate of *C. morosus*

Adult Indian stick insects without a neck ligature are unable to respond with an increased heart beat rate upon the application of various doses of Carmo-HrTH-II (see Marco et al., 2018). In neck-ligated stick insects, however, the application of 20 pmol of the endogenous AKH peptides (Carmo-HrTH-I and -II) on the heart preparation increases the heart rate significantly from 41 beats min^{-1} to 53 beats min^1 ($p < 0.05$; **Table 4**). The modified Carmo-HrTH-II with the acetylated Ala residue at the N-terminus did not significantly alter the rate of heart beat, whereas the heart rate increased (58% of the maximal response possible) after the application of the free acid-Carmo-HrTH-II and the Leu^1 nonapeptide (**Table 4**). The *post hoc* test revealed that there was no significant difference between the potencies of Carmo-HrTH-I and -II ($p > 0.05$) but there was a significant difference between the result of Carmo-HrTH-II and those of the terminal modified analogs (**Table 4**).

Of the bioanalogs tested, the increase in heart rate caused by the decapeptides Rommi-CC and Phyle-CC was highly significant and did not differ significantly from that caused by Carmo-HrTH-II ($p > 0.05$).

None of the systematically altered Ala analogs of Carmo-HrTH-II and none of the octapeptidic bioanalogs tested (**Tables 5**, **6**) increased the heart rate significantly, except seemingly Ala^3-Carmo-HrTH-II (**Table 6**). However, one-way ANOVA followed by the *post hoc* Scheffe's test revealed that the response to Ala^3-Carmo-HrTH-II is not significantly different from those of the rest of the Ala-analogs nor does it differ from the saline effect ($p > 0.05$). The data from all the systematically

TABLE 4 | The biological effect (myotropic) of the termini of Carmo-HrTH-II in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (150 μl applied) and amino acid sequence[a] (20 pmol peptide)	Heart rate (beats min^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	Pre-application	Post-application	Difference	P[#]	
Saline	12	40 ± 4	41 ± 1	1 ± 2	NS	NS
Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
Carmo-HrTH-I						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn- Trp* -Gly-Thr amide	8	41 ± 2	54 ± 2	13 ± 2	0.002	108[b]
Carmo-HrTH-II: [N-Ac]Ala[1]						
[N-Ac]Ala -Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	8	39 ± 6	39 ± 6	0 ± 0	NS	NS
Carmo-HrTH-II: Non-apeptide, free N-terminus, Leu[1]						
Leu -Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	9	39 ± 6	46 ± 5	7 ± 2	0.0001	58
Carmo-HrTH-II: free C-terminus						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly- Thr OH	7	40 ± 5	47 ± 3	7 ± 3	0.0008	58

[a]*Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. *Tryptophan residue is mannosylated.* [b]*Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe's test. The data are presented as Mean ± S.D.* [#]*Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

TABLE 5 | The biological effect (myotropic) of selected AKH bioanalogs in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (150 μl applied) and amino acid sequence[a] (20 pmol peptide)	Heart rate (beats min^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	Pre-application	Post-application	Difference	P[#]	
Saline	12	40 ± 4	41 ± 1	1 ± 2	NS	NS
Decapeptides						
Carmo-HrTH-II						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
Phyle-CC						
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly- Ser amide	5	38 ± 7	46 ± 6	8 ± 2	0.0005	67[b]
Locmi-AKH-I						
pGlu-Leu- Asn -Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	4	40 ± 4	43 ± 4	3 ± 1	0.002	25
Phymo-AKH						
pGlu-Leu- Asn -Phe-Thr-Pro-Asn-Trp-Gly- Ser amide	6	37 ± 3	38 ± 4	1 ± 1	0.04	8
Rommi-CC						
pGlu- Val - Asn -Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	6	39 ± 3	57 ± 4	18 ± 6	0.0007	150[b]
Octapeptides						
Aedae-AKH						
pGlu-Leu-Thr-Phe-Thr-Pro- Ser -Trp amide	5	42 ± 6	43 ± 6	1 ± 1	0.03	8
Pyrap-AKH						
pGlu-Leu- Asn -Phe-Thr-Pro-Asn-Trp amide	6	39 ± 5	41 ± 4	2 ± 2	0.02	16

[a]*Amino acid substitutions in Carmo-HrTH-II analogs are highlighted.* [b]*Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe's test. The data are presented as Mean ± S.D.* [#]*Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

altered analogs and saline differed significantly from that of Carmo-HrTH-II ($p < 0.0005$).

DISCUSSION

The peptides of the AKH/RPCH family are mainly known for their involvement in the regulation of energy metabolism, specifically the mobilization of stored fuel metabolites (lipid, carbohydrates or proline), although the AKHs are pleiotropic (Gäde, 1997; Marco and Gäde, 2020). In the stick insect, *Carausius morosus*, the endogenous members of the AKH/RPCH family act as hypertrehalosemic hormones (HrTHs) and have a stimulatory effect on heart contraction under certain conditions. The present study, thus, aimed to investigate the importance of structural features of the native HrTHs necessary to interact with the receptor on the fat body and on dorsal vessel cells of *C. morosus* to trigger a response that culminate in the release of carbohydrates into the hemolymph, and an increase in heart rate. Additionally, we were interested in whether the receptor features

TABLE 6 | The biological effect (myotropic) of single amino acid substitutions in Carmo-HrTH-II in ligated 6th instar Indian stick insects (*Carausius morosus*).

Treatment (150 μl applied) and amino acid sequence[a] (20 pmol peptide)	Heart rate (beats min^{-1})					% Activity relative to the effect of Carmo-HrTH-II
	n	Pre-application	Post-application	Difference	P*	
Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	8	41 ± 4	53 ± 5	12 ± 5	0.0002	100
Ala^2-Carmo-HrTH-II pGlu- Ala -Thr-Phe-Thr- Pro-Asn-Trp-Gly-Thr amide	5	42 ± 7	42 ± 5	0 ± 2	NS	NS
Ala^3-Carmo-HrTH-II pGlu-Leu- Ala -Phe-Thr-Pro-Asn-Trp-Gly-Thr amide	4	38 ± 6	40 ± 6	2 ± 1	0.035	16
Ala^4-Carmo-HrTH-II pGlu-Leu-Thr- Ala -Thr-Pro-Asn-Trp-Gly-Thr amide	5	40 ± 4	41 ± 4	1 ± 1	NS	NS
Ala^5-Carmo-HrTH-II pGlu-Leu-Thr-Phe- Ala -Pro-Asn-Trp-Gly-Thr amide	5	39 ± 7	40 ± 7	1 ± 2	NS	NS
Ala^6-Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr- Ala -Asn-Trp-Gly-Thr amide	5	43 ± 5	43 ± 5	0 ± 0	NS	NS
Ala^7-Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr-Pro- Ala -Trp-Gly-Thr amide	5	39 ± 4	39 ± 4	0 ± 1	NS	NS
Ala^8-Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr-Pro-Asn- Ala -Gly-Thr amide	5	42 ± 5	42 ± 4	0 ± 2	NS	NS
Ala^9-Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp- Ala -Thr amide	4	39 ± 2	38 ± 2	−1 ± 1	NS	NS
Ala^{10}-Carmo-HrTH-II pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly- Ala amide	4	41 ± 5	41 ± 5	0 ± 1	NS	NS

[a]*Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. Data presented as Mean ± S.D. *Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.*

of *C. morosus* would more or less mirror those of the cockroach *B. discoidalis* – the only insect with a sole decapeptide AKH that had been investigated via SAR studies to date.

Hypertrehalosemic Response in *C. morosus*

Pioneering metabolic studies with *C. morosus* (Gäde and Lohr, 1982) had revealed that the concentration of total hemolymph carbohydrates and the amount of fat body glycogen at the end of the 6th instar stage were usually higher than in adults, and that ligated stick insects show a clear and consistent hypertrehalosemic response when about to molt into adults (i.e., late-6th instar stage). The early study also demonstrated unequivocally that the ligature itself had no impact on the concentration of carbohydrates in the body over the measured period (Gäde and Lohr, 1982). The present study, thus, used carefully staged 6th instar *C. morosus* specimens to confirm that, indeed, a maximal hypertrehalosemic response was attained upon injection of a crude extract of conspecific corpora cardiaca, or the endogenous AKH members, Carmo-HrTH-I and -II. Once this was ascertained, we performed structure-activity response (SAR) studies to investigate how specific changes to the primary structure of Carmo-HrTH-II affected biological activity, and by inference, learn more about the ligand-receptor interactions in the stick insect using biological assays. The average carbohydrate concentration in the hemolymph of ligated *C. morosus* 6th instars before injection is calculated at 9.35 ± 0.96 mg/ml (n = 363) in the present study, which is in the same range as measured in Gäde and Lohr (1982).

Since *C. morosus* produces two functioning AKH peptides in its CC, and these decapeptides are identical in the primary amino acid sequence, we predict that the AKH receptor in this stick insect species may show a low tolerance for accommodating differences in peptide chain length (e.g., octapeptides and nonapeptides), as well as for particular amino acid substitutions in the peptide chain. The opposite was shown to be the case in the moth species, *Hippotion eson* that produces and reacts positively to five endogenous AKHs of varying chain length and sequence (Marco and Gäde, 2015, 2019).

One of the characteristic features of AKH peptides are their blocked termini: a pyroglutamic acid (pGlu) in position 1, and an amidated C-terminus, which renders protection to the ligand from exopeptidases in the hemolymph of the insect, and therefore results in a longer half-life of the peptide to achieve its hormonal effect. The current study confirms the importance of the N-terminal pGlu and C-terminal amide of Carmo-HrTH-II, the lead peptide: the terminally modified analogs showed only a slight hypertrehalosemic activity and none at all, when the N-terminal pGlu residue was eliminated and, hence, an unprotected nonapeptide was tested. Previous *in vivo* assays had also reported reduced or no activity when one of the blocking termini residues was removed or the pGlu was substituted by other blocked amino acids (Gäde, 1990; Ziegler et al., 1991, 1998; Gäde and Hayes, 1995; Lee et al., 1997; Marco and Gäde, 2015). In addition, *in vitro* receptor binding assays on the flies

D. melanogaster and *G. morsitans* reported a decline in activation of the expressed AKH receptor when N-terminal acetylated-Ala analogs of the native AKHs were tested, whereas 40–70% receptor activation was reported with non-amidated analogs of the native fly AKH peptides (Caers et al., 2012, 2016). Hence, it is most likely that the loss of biological activity in the current study is because the deaminated analog and the analog missing pGlu were not protected from amino- or carboxypeptidases in the hemolymph of *C. morosus* which may have resulted in the peptides being partially digested before reaching the receptor. The lack of relevant activity with the N-terminal acetylated-Ala analogs *in vivo* (current study) and *in vitro* (Caers et al., 2012, 2016) suggests that the conformation of the resulting peptide differs considerably from the native conformation with pGlu, preventing proper binding to the AKH receptor. The interaction of pGlu with the AKH receptor has been shown to occur in the model for AKH ligand-receptor binding in the desert locust, *Schistocerca gregaria* (Jackson et al., 2019). Moreover, the model for the RPCH receptor of the water flea, *Daphnia pulex*, also suggests that both termini of this octapeptide are involved in binding, i.e., to the extracellular part (Jackson et al., 2018).

In addition to the termini, the chain length of the ligand appears to be critical for the *C. morosus* AKH receptor: Peram-CAH-II, one of the octapeptide bioanalogs tested in the present study, is identical to the first 8 amino acids of the lead peptide, Carmo-HrTH-II, and yet it is not able to achieve a potent hypertrehalosemic response; two other octapeptides with one amino acid substitution relative to Peram-CAH-II failed completely to achieve hypertrehalosemia (see **Table 2**). The response of *C. morosus* to Peram-CAH-II is in agreement with a previous study (Gäde and Lohr, 1982) that tested the CC extract of *P. americana* (thus, containing Peram-CAH-II and also the octapeptide Peram-CAH-I) in the same stick insect bioassay. The poor or no biological activity suggests that the AKH receptor of *C. morosus* has a weak affinity for octapeptides and that the two amino acids (Thr and Gly) at the C-terminal of the native Carmo-HrTH peptides are needed for receptor interaction. This is reminiscent of the situation in *B. discoidalis* where octapeptides that differ from the endogenous Bladi-HrTH only by one amino acid (besides the amino acids Gly-Thr at positions 9 and 10, of course) are more than 30-fold less active than Bladi-HrTH (Hayes and Keeley, 1990). The relevance of AKH peptide chain length does not seem to be important in an insect like *S. gregaria*, where two octapeptides and a decapeptide (Locmi-AKH-I) are endogenous AKHs. The solution structure of these three *S. gregaria* AKH peptides and models of their binding to the endogenous locust receptor were recently reported (Jackson et al., 2019), wherein it was demonstrated that all three AKHs have the same binding site on the *S. gregaria* AKH receptor, interact with similar residues of the receptor and have comparable binding constants. Now that the *C. morosus* AKH receptor is reportedly known from RNA sequencing and a *de novo* transcriptome assembly (Duan Şahbaz and Birgül Iyison, 2019), it opens the way for future *in vitro* receptor assays and molecular modeling of ligand-receptor binding to understand why (if, indeed, at all) decapeptides seem to be favored by the *C. morosus* AKH receptor. As for the decapeptide AKH bioanalogs tested in the current study, Phyle-CC with Ser^{10} instead of Thr^{10} showed the same potency as Carmo-HrTH-II (**Table 2**) suggesting that Thr and Ser are interchangeable because both are polar amino acids with hydroxylated side chains. The other decapeptide bioanalog with a single amino acid replacement that was tested in the current study is Locmi-AKH-I with Asn^3 instead of Thr^3; unlike Phyle-CC, Locmi-AKH-I could only slightly activate the *C. morosus* AKH receptor. Taking this result together with the fact that Phymo-AKH, with the double amino acid substitution of Asn^3 and Ser^{10} instead of the endogenous Thr^3 and Thr^{10} residues, had no significant hypertrehalosemic effect (**Table 2**), it is concluded that the Asn residue in position 3 is detrimental for ligand-receptor interaction. Even though both amino acids are hydrophilic, the difference is the hydroxylated side chain (Thr), whereas the second carboxy group of Asn is "neutralized" by an amide formation, resulting in a carboxyamide side chain (see Marco and Gäde, 2015). The results imply that even the removal of a simple side chain, such as a hydroxyl group, can be quite crucial for ligand-receptor interaction. However, when Asn^3 appeared in combination with Val^2 as in Rommi-CC (instead of the endogenous Thr^3 and Leu^2), hypertrehalosemic action was restored to nearly 50% of maximal activity in the current study. In Rommi-CC, the hydrophilic-hydrophobic alternating pattern is maintained by this double substitution, and having established already that the single replacement of Thr^3 with Asn^3 results in little receptor activation (Locmi-AKH-I in the present study), these results could mean that the long side chain of Val^2 (as compared to Leu^2) may be the reason for restored activity. Gäde (1992) reported that Leu and Val at position 2 of Peram-CAH-II can be interchanged without affecting the affinity of the peptide for the AKH receptor in *P. americana*. This seems to be the case with the *C. morosus* AKH receptor too, although we have not tested an AKH analog with a single replacement of Val^2 for Leu^2 in the current study. The lack of biological response with Locmi-AKH-I in the current study is in agreement with those of Gäde and Lohr (1982), and in another case study with the stick insect *B. extradentatum* (that also has Carmo-HrTH-II as an endogenous peptide), where Locmi-AKH-I too effected a much reduced hypertrehalosemic response in comparison to Carmo-HrTH-II (Malik et al., 2012). The replacement of Thr^3 with Asn^3 in a peptide tested on the moth, *H. eson*, similarly resulted in no biological activity – it should be added that Thr^3 is conserved in all five endogenous AKH peptides of this moth (Marco and Gäde, 2015). Thus, clearly the AKH receptors of different species behave differently and it appears that co-evolution between endogenous peptide(s) and receptor has occurred for the "best fit."

It is clear from the bioassay results with various AKH bioanalogs that side chains and charge of amino acid residues are important for an effective ligand and a biological response. Hence, in order to gain greater clarity, we designed an Ala-replacement series of analogs in the current study starting with Ala^2 all the way to Ala^{10} to see how important side chain and charge is for the AKH activity of Carmo-HrTH-II in *C. morosus*. Ala was selected as substitution because this is a non-polar amino acid and it lacks a hydroxylated side chain. Given that Thr has a hydroxylated side chain, it is perhaps not a surprise that in this study, the single replacement of Thr at position 3,

5, or 10 of Carmo-HrTH-II, resulted in a complete loss of or a decline in biological activity (**Table 3**). A contributing factor for the loss of activity is that the substitution of Thr with Ala at position 3 and 5 disrupts the alternating hydrophilic-hydrophobic amino acid pattern of Carmo-HrTH-II (Thr^3, Thr^5, Asn^7), which in turn might interrupt peptide conformation and thus affect the binding efficacy of the peptide. Previous studies that used *in vitro* receptor binding assays with expressed AKH receptors of dipteran insects and Ala-replacement analogs of conspecific AKHs that are similar to Carmo-HrTH-II at position 1–4 (Drome-AKH and Glomo-AKH), also showed a lack of receptor activation by analogs where Thr is substituted by an Ala residue (Caers et al., 2012, 2016). Carmo-HrTH-II has two hydrophobic amino acid residues at position 8 and 9 (Trp^8-Gly^9) followed by a hydrophilic Thr in position 10; thus, replacing Thr^{10} with Ala^{10} in the current study increases the C-terminal hydrophobicity of Carmo-HrTH-II, but apparently this is not so crucial as breaking up the alternating hydrophilic-hydrophobic amino acid pattern preceding this hydrophobic tail, for about 50% activity was recorded with the Ala^{10} analog of Carmo-HrTH-II (**Table 3**). This might also mean that the hydroxyl group present on the terminal end (Thr^{10}) is less important for peptide-receptor interaction in *C. morosus* compared with that present on non-terminal residues (Thr^3, Thr^5). In a study with modifications of the Thr residues in Locmi-AKH-I, Poulos et al. (1994) demonstrated that in *L. migratoria*, the hydroxyl group of the Thr^5 of Locmi-AKH-I is important for biological activity, while that of Thr^{10} is not. Similarly, in *B. discoidalis* a single amino acid change at position 10 was well tolerated without much loss in bioactivity (Ford et al., 1988).

The current study revealed that the substitution of Leu^2 in Carmo-HrTH-II with Ala has no impact on the AKH receptor on the fat body of *C. morosus*. Both Leu and Ala residues are non-polar. The only difference between these residues is that Leu has a bulkier alkyl side chain than Ala [$-CH_2CH(CH_3)_2$ vs. $-CH_3$]. This might mean that only one methyl group of Leu is involved in the receptor interaction or the whole side chain may, indeed, not be so important. Similarly, in *B. discoidalis* Ala in position 2, instead of Val in the endogenous Bladi-HrTH, can also be tolerated well (Ford et al., 1988). Finally, two aromatic amino acids are conserved in AKHs and form part of the hallmark features of the AKH peptide family; these are Phe^4 and Trp^8. Not surprisingly, therefore, replacement of the aromatic side chains with Ala in the present study resulted in the complete loss of metabolic activity in *C. morosus*, as has been shown for other insect species too in *in vivo* and *in vitro* receptor assays (Ford et al., 1988; Gäde and Hayes, 1995; Ziegler et al., 1998; Caers et al., 2012, 2016; Marco and Gäde, 2015), thus signifying that these structural features are crucial for receptor-binding in insects. When these aromatic amino acids were swapped to construct a Locmi-AKH-I with Trp^4 and Phe^8, it was not very active in *L. migratoria* (Velentza et al., 2000). Moreover, single substitutions of Phe^4 with Trp of Locmi-AKH-I is tolerated (a 10-fold loss of potency), while the replacement of Trp^8 with Phe is not (>300 times decrease in potency), leading to the conclusion that position 4 requires a phenyl ring in the side chain, and position 8 an indole ring (Velentza et al., 2000). Ligand interaction diagrams for the two AKH receptor models (*S. gregaria*; *D. pulex*) show also clearly that the two aromatic residues in the molecule are essential for binding (Jackson et al., 2018, 2019).

AKHs are predicted to have a β-turn at position 5–8 (Hayes and Keeley, 1990; Zubrzycki and Gäde, 1994; Cusinato et al., 1998) and, although amino acid sequences and chain length of AKHs can vary, nuclear magnetic resonance experiments assigned turns for each of the examined AKHs (see, for example, Jackson et al., 2019). If this is true for Carmo-HrTH-II as well, then the single replacement of these amino acids with Ala, which results in the removal of the side chain and/or the interruption of alternating hydrophilic pattern, might disrupt the peptide β-conformation. Interrupting the stability of the conformation may hinder the interaction of the peptide with its receptor through the backbone hydrogen bonding (Gäde and Hayes, 1995). In the current study, single replacements of amino acids at position 5–9 of Carmo-HrTH-II with Ala resulted in complete loss of potency. This is quite unique. In most studies position 7 could be replaced without major loss of activity (see, for example, Ford et al., 1988; Gäde and Hayes, 1995). In *B. discoidalis* positions 2, 7, and 10 were the ones least affected by single substitution. Unexpectedly the *C. morosus* AKH receptor did accept the change at position 2 very well (**Table 3**).

Cardio-Stimulatory Activities in *C. morosus*

The effect of neuropeptides on the contraction of the dorsal heart was studied before in stick insects, including *C. morosus* (Ejaz and Lange, 2008; da Silva et al., 2011; Malik et al., 2012; Marco et al., 2018). Although members of the AKH/RPCH family are reported to stimulate the heart beat rate in insects, including stick insects that were either decapitated or ligated behind the head (see Chowański et al., 2016; Marco et al., 2018), it is not known whether these peptides act directly or indirectly on the heart. It is, nevertheless, thought that the stimulation of the heart by the AKH peptides is a mechanism for assisting the faster distribution of fuel metabolites (Gäde and Marco, 2013). In the current study we applied the same bioanalogs and analogs of Carmo-HrTH-II that were tested in *in vivo* metabolic assays also in a semi-isolated heart assay with *C. morosus* to see whether the respective biological output could lead to a conclusion about the receptor identity or receptor needs in the two physiological systems.

The application of the N- or C- terminal modified analogs on the *C. morosus* heart preparations resulted in 58% increase in the frequency of heart contractions compared to that of Carmo-HrTH-II, while the analog that had a pGlu replaced with N-acetyl-Ala did not increase the heart rate. These results indicate that the native peptide may lose some of its binding affinity when the pGlu is removed, or with a free acid at the C-terminus; presumably, the breakdown of the peptides are not as rapid in this assay where the peptides are directly applied to the heart, as opposed to the case of the metabolic assay where the peptides are in the hemolymph and exposed to exopeptidases for 90 min. Further, these results suggest that the peptide analogs with the free termini retain a conformation that can bind to the *C. morosus* AKH receptor, whereas the peptide conformation brought about

through the N-acetyl-Ala in position one is not conducive for ligand-receptor binding. The metabolic and the heart assay results in the current study largely indicate the same outcome. In *P. americana,* the affinity is completely lost, with no stimulation of the heart rate when the pGlu or amide is removed in both *in vivo* and semi-isolated heart assays (Baumann et al., 1990).

Single replacements of amino acids with Ala at all positions of Carmo-HrTH-II resulted in the peptide losing its efficacy completely. The data indicate that the side chains of all the amino acids of Carmo-HrTH-II are crucial for eliciting cardio-stimulatory action in *C. morosus*. This is mostly comparable with the data from the metabolic assays *in vivo* where small metabolic changes are measured, while the heart beat is totally unresponsive to the analogs. There is, however, one anomaly that is not easily explained: the Ala^2 analog was as active biologically as the lead peptide Carmo-HrTH-II in raising the carbohydrate concentration in the ligated stick insects (**Table 3**); this same analog did not have a significant effect on the heart contractions in the semi-exposed heart assay (**Table 6**). We are not able to explain this phenomenon at present.

The myotropic effect measured with the selection of bioanalogs yielded largely comparable results to those obtained in the *C. morosus* metabolic assay, with Rommi-CC and Phyle-CC standing out as active peptides, and only a very small effect measured with the other decapeptide analogs and the octapeptides.

Since the data trends between the two assays and the peptide analogs tested are so comparable, we conclude that the same receptor is at play in both physiological systems: the rate of heart beat and the mobilization of carbohydrates from the fat body stores in ligated stick insects.

Further studies could be carried out to investigate the mode of action of AKH/RPCH peptides in non-ligated *C. morosus* and other stick insect species since they respond with a biological effect only when the circulation is disrupted between the head and the rest of the body. Wicher et al. (2006) demonstrated that the AKHs of the cockroach *P. americana*, in addition to the metabolic function, act directly on the central nervous system through the release of octopamine from the thoracic dorsal unpaired median (DUM) neurons, and this release of octopamine stimulates locomotor activity. Octopamine is a well-known "fight-or-flight" stress hormone in insects (Verlinden et al., 2010), and its action as heart stimulant has been studied in many insect species (see Chowański et al., 2016). Indeed, direct octopaminergic innervation of the insect heart is observed in several insect species, arising from the DUM neurons, and is responsible for cardioacceleratory responses in *D. melanogaster*, *P. americana*, and *M. sexta* (see Johnson et al., 1997; Zornik et al., 1999; Papaefthimiou and Theophilidis, 2011). In *C. morosus*, however, octopamine unequivocally inhibits the contraction of the heart of *C. morosus* in a dose-dependent manner, and this is interpreted as an appropriate response in an insect species that relies on cryptic biology to escape predators (Marco et al., 2018). *C. morosus* masquerades as a stick or twig in its habitat, engages in slow movements to keep up the pretense of being part of the food plant, and escapes the interest of predators through thanatosis (i.e., playing dead when detected; Gullan and Cranston, 2014). The Indian stick insect, thus, relies on a strategy of concealment (low energetic costs) in which fight, flight and energy mobilization play no role. By extension, it is expected that the role of stress hormones, such as octopamine and AKHs, would have an opposite physiological effect in such a species.

Lessons for Green Insecticide Design

Phasmids are generally not regarded as serious pest insects since they do not pose a direct threat to food security, nor are they known to be vectors of diseases, nevertheless quite damaging outbreaks of stick insect population numbers have been recorded over the years in Australia, North America, China and other geographical areas, where they defoliate economically important timber crops (Baker, 2015). Such defoliated timber trees respond in subsequent years with a smaller stem diameter which is deleterious to the pulp industry. Although many stick insect species are apterous and can therefore not spread as rapidly and widely as winged insects, they can have a serious local impact in the event of an outbreak (Baker, 2015). Pest status notwithstanding, are there any lessons to learn from our work here on the physiological action of AKH ligands in the Indian stick insect *C. morosus* that may be useful for the design of a peptide mimetic that could act specifically to target known pest insects without interfering with other insects?

To date, only a small number of stick insect species have been studied with respect to their AKH neuropeptides. Besides *C. morosus*, primary structures are known from *Sipyloidea sipylus*, *Extatosoma tiaratum,* and *Baculum extradentatus* which all synthesize the decapeptide Carmo-HrTH-II (pELTFTPNWGTa) in their CC as the major AKH family neuropeptide, as well as a less abundant neuropeptide that is also biologically active (Gäde, 1989; Gäde and Rinehart, 1990; Malik et al., 2012). In the case of *C. morosus* and *B. extradentatus*, the less abundant peptide was characterized as a post-translational variant of Carmo-HrTH-II, viz. at position 8 the Trp is C-mannosylated (Carmo-HrTH-I, Munte et al., 2008) or modified to kynurenine (Malik et al., 2012). While the structural identity of the additional neuropeptide in *S. sipylus* and *E. tiaratum* was not pursued (due to a lack of sufficient material at the time), we speculate that it may also be Carmo-HrTH-II with a post-translationally modified Trp, and this may be a trait in other stick insects with Carmo-HrTH-II. Recent genomic data sets (Veenstra, 2019) revealed that Carmo-HrTH-II is encoded in the New Zealand stick insect *Clitarchus hookeri* whereas in the evolutionary basal stick insect *Timema christinae* an octapeptide (pEVNFSPSWa) is encoded; this octapeptide is well-known as Anaim-AKH and found in certain dragonflies (Gäde and Marco, 2005) and other basal orders of insects such as Archaeognatha (Marco et al., 2014) and Ephemeroptera (Gäde and Marco, 2012). Carmo-HrTH-II and Anaim-AKH are structurally vastly different peptides – not only in sequence length but also in the actual amino acid sequence. To base a putative lead peptide for AKH insecticide use on the octapeptide would certainly also affect dragonflies which are mostly endangered species, whereas it is envisaged that *C. morosus* will not be targeted by most AKH mimetics as

pesticides because of the very specific needs required for ligand-receptor binding in this species, as deduced here from biological assays: the AKH receptor of *C. morosus* accepts decapeptides and only the amino acids in positions 2 and 10 may deviate from the Carmo-HrTH-II primary sequence.

This seem to be good criteria for specificity but what do we know about the effect of Carmo-HrTH-II on other insects? SAR data only exist from *in vivo* assays and they revealed the following:

(1) Carmo-HrTH-II was more than 300-fold less active in the cockroach *B. discoidalis* than the endogenous Bladi-HrTH (Hayes and Keeley, 1990).
(2) Carmo-HrTH-II was as, or slightly more active than the endogenous nonapeptide Manse-AKH in the lepidopteran *Manduca sexta* (Fox and Reynolds, 1991; Ziegler et al., 1991).
(3) Carmo-HrTH-II was more or less as active in *P. americana* as the endogenous octapeptides Peram-CAH-I and -II (Gäde, 1990).
(4) Carmo-HrTH-II was only slightly less active in *L. migratoria* than the endogenous decapeptide Locmi-AKH-I (Gäde, 1990).

Hence, the effect of an insecticidal peptide based on Carmo-HrTH-II would very likely also be effective against some serious pest insects such as a number of lepidopteran larvae, migratory locusts and blattid cockroaches which is not undesirable.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

HM and GG conceptualized the research project, financed the work, and supervised OK. OK and HM performed experiments, reared the animal cultures, and analyzed the data. GG contributed chemicals and peptides, helped with interpretation of the data, and writing the draft manuscript. OK performed most biological assays, was involved in data interpretation, and drafting of the manuscript. HM helped with interpretation and analyses of data, writing, and refining the draft manuscript. All the authors agreed to be accountable for the content of the work presented here.

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REVIEW
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The Role of Peptide Hormones in Insect Lipid Metabolism

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Lipids are the primary storage molecules and an essential source of energy in insects during reproduction, prolonged periods of flight, starvation, and diapause. The coordination center for insect lipid metabolism is the fat body, which is analogous to the vertebrate adipose tissue and liver. The fat body is primarily composed of adipocytes, which accumulate triacylglycerols in intracellular lipid droplets. Genomics and proteomics, together with functional analyses, such as RNA interference and CRISPR/Cas9-targeted genome editing, identified various genes involved in lipid metabolism and elucidated their functions. However, the endocrine control of insect lipid metabolism, in particular the roles of peptide hormones in lipogenesis and lipolysis are relatively less-known topics. In the current review, the neuropeptides that directly or indirectly affect insect lipid metabolism are introduced. The primary lipolytic and lipogenic peptide hormones are adipokinetic hormone and the brain insulin-like peptides (ILP2, ILP3, ILP5). Other neuropeptides, such as insulin-growth factor ILP6, neuropeptide F, allatostatin-A, corazonin, leucokinin, tachykinins and limostatin, might stimulate lipolysis, while diapause hormone-pheromone biosynthesis activating neuropeptide, short neuropeptide F, CCHamide-2, and the cytokines Unpaired 1 and Unpaired 2 might induce lipogenesis. Most of these peptides interact with one another, but mostly with insulin signaling, and therefore affect lipid metabolism indirectly. Peptide hormones are also involved in lipid metabolism during reproduction, flight, diapause, starvation, infections and immunity; these are also highlighted. The review concludes with a discussion of the potential of lipid metabolism-related peptide hormones in pest management.

Keywords: peptide hormones, neuropeptides, lipid metabolism, lipolysis, lipogenesis, adipokinetic hormone, insulin

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INTRODUCTION

In all living being, carbohydrate, protein and lipids are the three main energy sources for vital activities of insects. Among these sources, lipids are the primary storage molecules and an essential source of energy for growth and development, reproduction, periods of prolonged flight, starvation, and diapause. Many insect sex pheromones, cuticular wax, as well as various defensive secretions, such as phenols, quinones and carboxylic acids, contain or are synthesized from lipids (Downer and Matthews, 1976; Klowden, 2007; Yew and Chung, 2015).

The center for insect lipid metabolism is the fat body, which is analogous to vertebrate adipose tissue and the liver. The fat body is primarily composed of adipocytes, which accumulate

triacylglycerols (TAGs) in intracellular lipid droplets (LDs). Genomics and proteomics, together with functional analyses, such as RNA interference (RNAi) and CRISPR/Cas9-targeted genome editing, have revealed that storage of lipids and their metabolism are conserved, sophisticated and complicated processes. These studies identified various genes expressed by adipocytes that are involved in lipid metabolism and elucidated their functions. Briefly, lipid metabolism starts with the hydrolysis of the dietary lipids in midgut via lipases, lipid transport into target sites, primarily the fat body, and muscles and ovaries by lipophorins, cellular uptake and transport by fatty acid transport and fatty acid binding proteins, synthesis, accumulation and hydrolysis of lipids in the fat body by Fatty Acid Synthase (FAS) and perilipins. These processes occur at the mRNA level by transcription factors and post-transcriptional modifications of proteins. Most of these events are under the control of the insect endocrine system.

The insect endocrine system consists primarily of neurosecretory cells and endocrine glands (e.g., corpora cardiaca, corpora allata, and prothoracic glands). Organs such as the midgut, fat body, ovaries and testes are also considered endocrine glands as they synthesize various hormones. Insect hormones could be classified as amine-type (e.g., octopamine, serotonin, and tyramine), steroids (ecdysteroids), sesquiterpenes [juvenile hormone (JH)], peptide-type [e.g., prothoracicotropic hormone, adipokinetic hormone (AKH)] and lipid-type (e.g., prostaglandin). Among these, ecdysteroids and JHs are the most-studied and indirectly affect lipid metabolism due to their general effect on growth and development. However, the essential and key hormones affecting insect lipid metabolism are peptide-hormones.

Peptide hormones are central to many aspects of insect life, such as molting (Nässel et al., 2015), development (Oudejans et al., 1993), reproduction (Hou et al., 2017), digestion (Borovsky et al., 1990), behavior (Wu et al., 2003; Gospocic et al., 2017), and pheromone production (Sato et al., 1993), in addition to lipid metabolism. Peptide hormones are produced by neurosecretory cells and endocrine glands. Most of these hormones are produced by the central nervous system and specifically referred to as "neuropeptides." Regardless of their origin, many peptide hormones perform their tasks by binding into their cognate G protein-coupled receptors (GPCRs) (Park and Adams, 2010; Duan-Şahbaz and Ýyison, 2018). Studies on the genome of the common fruit fly, *Drosophila melanogaster* (stated as *Drosophila* since here throughout the article), have revealed more than 35 neuropeptide and 45 GPCR genes (Duan-Şahbaz and Ýyison, 2018); similar numbers have been reported from other species (Wang et al., 2018).

Various peptide-hormones have been shown to affect insect lipid metabolism and the current review focuses on these peptide hormones and their role(s) in insect lipid metabolism. The role of these peptide hormones in lipid metabolism-related biological events, such as reproduction, flight, diapause, starvation, infection and parasitism including the potential of peptide hormones in pest management is discussed.

Abbreviations: ACBP1, Acyl-CoA-Binding Protein 1; ACC, Acetyl-CoA Carboxylase; ACP, AKH/Corazonin-related Peptide; Adipo, Adiponectin; AdipoR, Adiponectin Receptor; AGPAT3, 1-Acylglycerol-3-Phosphate Acyltransferase 3; AKH, Adipokinetic Hormone; AKHR, Adipokinetic Hormone Receptor; AKT, Serine-Threonine Protein Kinase; AMPK, AMP-activated Protein Kinase; APRP, AKH Precursor-Related Peptide; AstA, Allatostatin-A; ATGL, Adipose Triglyceride Lipase; Bmm, Brummer; CaM, Calmodulin; CaMKII, Calcium/Calmodulin-dependent Protein Kinase-II; cAMP, cyclic Adenosine monophosphate; CaN, Calcineurin; CCHa2, CCHamide-2; CCHa2R, CCHamide-2 Receptor; CREB, cAMP Response Element-Binding Protein; CRTC, cAMP-Regulated Transcriptional Co-activator; Crz, Corazonin; CrzR, Corazonin Receptor; DAG, Diacylglycerol; DAR2, Drosophila Allatostatin Receptor 2; DGAT1, Diacylglycerol O-Acyltransferase 1; DH, Diapause Hormone; DHR, Diapause Hormone Receptor; DILP, Drosophila Insulin-like Peptide; DOR, Diabetes and Obesity Regulated; DYRK1A, Dual−specificity Tyrosine Phosphorylation−regulated Kinase 1A; EcR, Ecdysone Receptor; ERK1, Extracellular signal-Regulated Kinase 1; ERK2, Extracellular signal-Regulated Kinase 2; E75, Ecdysone-induced Protein 75; 4EBP, 4E Binding Protein; 20E, 20-Hydroxyecdysone; FAS, Fatty Acid Synthase; FoxO, Forkhead Box Class O; FTZ-F1, Fushi Tarazu Factor 1; GABA, Gamma-aminobutyric acid; GPAT1, Glycerol-3-Phosphate-O-Acyltransferase 1; GPAT4, Glycerol-3-Phosphate-O-Acyltransferase 4; GPCR, G Protein-Coupled Receptor; Gαq, G Protein α q Subunit; Gγ1, G Protein γ 1 Subunit; HDAC4, Histone Deacetylase 4; HR3, Hormone Receptor 3; HSL, Hormone Sensitive Lipase; IGF, Insulin-like growth-factor; IGFBP, Insulin Growth- Factor Binding Protein; ImpL2, Imaginal Morphogenesis Protein-Late 2; ILP, Insulin-like Peptide; InR, Insulin Receptor; IPBP, Insulin-related Peptide Binding Protein; IPC, Insulin producing cell; IP_3, Inositol-1,4,5-trisphosphate; IP_3R, IP_3 Receptor; JH, Juvenile hormone; JHAMT, Juvenile Hormone Acid Methyltransferase; JNK, Jun N-Terminal Kinase; Kr-h1, Kruppel Homolog 1; LD, Lipid droplet; Lk, Leucokinin; LKB1, Liver Kinase B1; LkR, Leucokinin Receptor; LSD1, Lipid Storage Droplet-1; Lst, Limostatin; Mdy, Midway; miRNA, MicroRNA; Mnb, Minibrain; NPF, Neuropeptide F; Orai1, Plasma Membrane Calcium Channel Protein 1; PAP, Phosphatidic Acid Phosphatase; PBAN, Pheromone Biosynthesis Activating Neuropeptide; PEPCK, Phosphoenolpyruvate Carboxykinase; PI_3K, Phosphoinositide 3−Kinase; PIP_2, Phosphatidylinositol bisphosphate; PIP_3, Phosphatidylinositol trisphosphate; PKA, Protein Kinase A; PKB, Protein Kinase B; PKC, Protein Kinase C; PLC, Phospholipase C; PLIN 1, Perilipin 1; PTEN, Phosphatidylinositol-3,4,5-Trisphosphate 3-Phosphatase; RNAi, RNA interference; SERCA, Sarco/Endoplasmic Reticulum Calcium-ATPase; SIK, Salt-inducible kinase; SIK2, Salt-Inducible Kinase 2; SIK3, Salt-Inducible Kinase 3; sNPF, Short Neuropeptide F; sNPFR, Short Neuropeptide F Receptor; SOCE, Store-operated calcium entry; SREBP, Sterol Regulatory Element-Binding Protein; STIM, Stromal Interaction Molecule; TAG, Triacylglycerol; TGL, Triglyceride Lipase; Tk, Tachykinin; TkR, Tachykinin Receptor; TOR, Target of Rapamycin; TORC1, Target of Rapamycin Complex 1; UPD1, Unpaired 1; UPD2, Unpaired 2.

PEPTIDE HORMONES INVOLVED IN INSECT LIPID METABOLISM

The major peptide hormones directly or indirectly involved in insect lipid metabolism are Adipokinetic Hormone (AKH), Insulin-like Peptides (ILPs), Diapause Hormone-Pheromone Biosynthesis Activating Neuropeptide (DH-PBAN), Short Neuropeptide F (sNPF), Neuropeptide F (NPF), Allatostatin-A (AstA), Corazonin (Crz), Leucokinin (Lk), CCHamide-2 (CCHa2), Tachykinins (Tk), Cytokines (Adipokines), and Limostatin (Lst) (**Table 1**).

Adipokinetic Hormone (AKH)

Adipokinetic Hormones (AKHs) are glucagon-like peptides and are produced by the neurosecretory cells of the corpora cardiaca (Beenakkers et al., 1985; Goldsworthy and Mordue, 1989; Goldsworthy et al., 1997; **Table 1**). These cells might be present both in larval and adult stages, and release AKH in response to developmental stage or conditions (Lee and Park, 2004). AKH is synthesized as a preprohormone consists of a hydrophobic

TABLE 1 | Peptide hormones involved in insect lipid metabolism and their features.

Name/Abbreviation	Major synthesis site	Other synthesis sites	Length (amino acid)	Roles	Lipid-specific role
Adipokinetic hormone (AKH)	Corpora cardiac	Ganglia located in ovaries, midgut, fat body, accessory glands and muscle	79- D. melanogaster	• Stimulation of heart beat • Increase of muscle tonus • Stimulation of general locomotion • Enhancement of immune response • Protection against oxidative stress • Mobilization of lipid stores for reproductive activities, flight, diapause preparation, and starvation	Lipolysis
INSULIN-LIKE PEPTIDES					
Brain insulin-like peptide 2 (ILP2)	Insulin producing cells in adult brain	Embryonic and larval midgut, salivary glands and mesoderm	137- D. melanogaster 136- L. decemlineata	• Regulation of carbohydrate metabolism • Inhibition of foxO activity • Activation of Sterol regulatory element-binding protein (SREBP) for *de novo* lipogenesis • Reproductive activities, fecundity • Lipid accumulation for diapause preparation	Lipogenesis
Brain insulin-like peptide 3 (ILP3)	Insulin producing cells in adult brain	Intestinal muscle	120- D. melanogaster	• Regulation of carbohydrate metabolism • Inhibition of foxO activity • Activation of Sterol regulatory element-binding protein (SREBP) for *de novo* lipogenesis • Lipid accumulation for diapause preparation	Lipogenesis
Brain insulin-like peptide 5 (ILP5)	Insulin producing cells in adult brain	Ovaries and Malpighian tubules	108- D. melanogaster	• Regulation of carbohydrate metabolism • Inhibition of foxO activity • Activation of Sterol regulatory element-binding protein (SREBP) for *de novo* lipogenesis • Lipid accumulation for diapause preparation	Lipogenesis
Insulin-like growth factor insulin-like peptide 6 (ILP6)	Larval and adult fat body	Salivary glands, heart and glial cells in the ventral nerve cord	107- D. melanogaster	• Suppression of brain ILPs • Induction of lipid uptake, activation of lipid turn over in oenocytes in fasting • Induction of starvation tolerance	Lipolysis
Diapause hormone-pheromone biosynthesis activating neuropeptide (DH-PBAN)	Neurosecretory cells in the subesophageal ganglion		192-B. mori 194-H. armigera (Active peptide: 24)	• Induction of diapause • Activation of extracellular signal-regulated kinase phosphorylation	Lipogenesis
Short neuropeptide F (sNPF)	Brain lateral neurosecretory cells	Midgut, hindgut, antennae, Malpighian tubules and ovary	281- D. melanogaster (Active peptide: 6–19)	• Regulation of feeding behavior • Locomotor activity Circadian rhythm • Appetitive olfactory behavior • Sleep homeostasis • Hormone release • Gut epithelial integrity • Stimulation of ovarian development	Lipogenesis
Neuropeptide F (NPF)	Brain	Subesophageal ganglion and midgut	102- D. melanogaster (Active peptide $\geq$ 28)	• Regulation of feeding behavior and food choice • Adult longevity Wakefulness • Modulation of odor-aroused appetitive behavior • Reproduction • Suppression of the inhibitory influence of AstA activity	Lipolysis
Allatostatin-A (AstA)	Brain	Gut	151- D. melanogaster	• Inhibition of starvation-induced feeding behavior • Regulation of AKH and ILP signaling	Lipolysis

(Continued)

TABLE 1 | Continued

Name/Abbreviation	Major synthesis site	Other synthesis sites	Length (amino acid)	Roles	Lipid-specific role
Corazonin (Crz)	Brain lateral neurosecretory cells		154- D. melanogaster (Active peptide: 11)	• Cardioactivity • Regulation of the ecdysis initiation • Melanization Stress responses Sperm transfer and copulation • Regulation of ethanol sedation • Induction of food uptake	Lipolysis (starvation-induced)
Leucokinin (Lk)	Brain, insulin producing cells and ventral ganglia		160- D. melanogaster (Active peptide: 6–15)	• Myotropic activity • Regulation of water and ion homeostasis in Malpighian tubules and hindgut • Meal size regulation • Regulation of feeding, metabolic rate, post-feeding physiology and behavior • Regulation of locomotor activity • Regulation of starvation-induced sleep suppression	Lipolysis (starvation-induced)
CCHamide-2 (CCHa2)	Fat body and midgut		136- D. melanogaster	• Stimulation of feeding • Stimulation of insulin signaling	Lipogenesis
Tachykinin (Tk)	Gut	Central nervous system	297- D. melanogaster	• Locomotor activity • Olfactory responses • Midgut immunity • Reduction of insulin signaling and lipid storage	Lipolysis
Cytokines					
Unpaired 1 (UPD1)	Brain		413- D. melanogaster	• Sensing of the fed-state • Activation of insulin signaling	
Unpaired (UPD2)	Fat body		406- D. melanogaster	• Sensing of the fed-state • Activation of insulin signaling	Lipogenesis in adipose tissue, Lipolysis in oenocytes
Limostatin	AKH-producing neurons in corpora cardiaca	Fat body	139- D. melanogaster	• Suppression of insulin production	Lipolysis

signal peptide, a bioactive neuropeptide and an AKH-associated peptide, which is also known as AKH Precursor-Related Peptide (APRP) (Van der Horst et al., 2001; Van der Horst, 2003). The signal peptide is removed co-translationally and the remaining prohormone is stored in the CC. The bioactive peptide is cleaved from the prohormone prior to its release into the hemolymph (O'Shea and Rayne, 1992; Oudejans et al., 1999). The APRPs can be further processed to form smaller peptides; however, their exact role is not known (Baggerman et al., 2002; Huybrechts et al., 2002). AKH bioactive peptides have 8–10 amino acids, an amino terminus blocked by pyroglutamate, a carboxy terminus blocked by amidation, aromatic residues at positions 4 (Phe or Tyr) and 8 (Trp), and a Gly residue at position 9 (Gäde et al., 1997; Gäde, 2004; Gäde and Marco, 2013; **Table 1**). It is noteworthy that expression of AKH genes is not restricted to the corpora cardiaca as different mRNA variants are produced by the ganglia located in the ovaries, midgut, fat body, accessory glands and muscle tissues (Abdel-latief and Hoffmann, 2007; Kaufmann et al., 2009).

The first report on the presence of an AKH goes back 50 years describing the involvement of this peptide in the mobilization of lipids during flight in the migratory locust *Schistocerca gregaria* (Beenakkers, 1969; Mayer and Candy, 1969). However, the AKH signaling system has been studied mainly in the tobacco hornworm, *Manduca sexta*, and its AKH was first sequenced in 1985 (Ziegler et al., 1985). Not surprisingly, *Drosophila* is another model that adds significantly into our knowledge on AKHs (Grönke et al., 2007; Baumbach et al., 2014b; Gáliková et al., 2015). The AKH signaling system is also present in coleopterans, hemipterans, orthopterans, blattodeans and hymenopterans, and more than 60 different kinds of AKHs have been identified (Kaufmann and Brown, 2006; Konuma et al., 2012; Gäde and Marco, 2013; Marchal et al., 2018). The number and sequences of insect AKHs are diverse, for example, three different AKHs (AKH-I to III) with different bioactivities are present in the migratory locust, *Locusta migratoria* (Oudejans et al., 1993; Vroemen et al., 1998), whereas two AKHs are present in *S. gregaria*

(Oudejans et al., 1991) and a single AKH is present in *Drosophila* (Grönke et al., 2007).

AKH has been shown to be involved in various events, such as the stimulation of heart beat (Scarborough et al., 1984), general locomotion (Socha et al., 1999), neuronal signaling (Milde et al., 1995), increase of muscle tonus (O'Shea et al., 1984), immunity (Goldsworthy et al., 2002), and protection of insects against oxidative stress (Bednářová et al., 2013). However, its primary role is to initiate the lipid/carbohydrate mobilization from the fat body (Van der Horst et al., 2001). The lipid mobilization occurs through the action of AKH on the Triglyceride Lipase (TGL) (Arrese et al., 2006; Arrese and Soulages, 2010). Additionally, there is a second system initiating lipolysis, the Brummer (bmm) lipase (homolog of mammalian Adipose Triglyceride Lipase, ATGL) (Grönke et al., 2005). *Bmm*-mutant flies are lipid mobilization-impaired and obese (Grönke et al., 2005). Notably, the AKH system functions in response to rapid changes in lipid demands, while bmm functions to maintain the lipid levels for a metabolic baseline (Grönke et al., 2007), therefore, bmm is also necessary during the periods of energy demand.

AKH exerts its effects on lipid mobilization via signal transduction (Canavoso and Wells, 2001; **Figure 1**). AKH binds to its GPCR, the Adipokinetic Hormone Receptor (AKHR) related to the mammalian gonadotropin-releasing hormone receptor (Lindemans et al., 2009) and was first identified from *Drosophila* and the silkworm *Bombyx mori* (Park et al., 2002; Staubli et al., 2002). AKHR is produced primarily in the fat body (Arrese and Soulages, 2010), but also in other tissues, such as the midgut, muscles, brain and reproductive organs (Kaufmann and Brown, 2006; Ziegler et al., 2011; Zandawala et al., 2015; Alves-Bezerra et al., 2016; Hou et al., 2017). Binding of AKH to its cognate AKHR results in the activation of two different second-messenger systems involved in lipid mobilization (Park et al., 2002; Staubli et al., 2002; **Figure 1**). In one pathway, binding of AKH to AKHR leads to stimulation of Phospholipase C (PLC), which cleaves membrane lipid phosphatidylinositol 4,5-diphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Finally, release of IP_3 activates IP_3 Receptor (IP_3R) in the endoplasmic reticulum, leading to mobilization of the second messenger calcium from the endoplasmic reticulum to cytosol (Gäde and Auerswald, 2003; **Figure 1**). The increase in cytosolic concentrations of calcium transmits the AKH signal, however, the exact mechanism is not known (Arrese et al., 1999; Van der Horst et al., 1999; Baumbach et al., 2014a,b). In brief, calcium stored within the endoplasmic reticulum represents an important signal for lipid mobilization in the first pathway. In the second pathway, binding of AKH to its receptor on the fat body cell surface activates adenylate cyclase and mediates a rapid increase of the second messenger cyclic Adenosine monophosphate (cAMP) leading to activation of cAMP-dependent Protein Kinase (PKA), which promotes the phosphorylation of downstream elements, such as the LDs, TGL and Perilipin 1/Lipid Storage Droplet-1 Protein (PLIN1/LSD1) (Arrese and Wells, 1994; Arrese et al., 1999; Arrese et al., 2008; **Figure 1**). PLIN1 phosphorylation has been shown to increase the accessibility of LDs for TGL, thereby allowing lipid mobilization. In accordance with this, *Drosophila PLIN1*-mutants are obese (Beller et al., 2010). On the other hand, PKA inhibits the activity of a member of the AMP-activated Protein Kinase (AMPK) family, the Salt-Inducible Kinase 3 (SIK3) by phosphorylating a conserved serine residue (Ser^{563} in *Drosophila* SIK3) (Wang et al., 2011; **Figure 1**). This leads to translocation of a class IIa histone deacetylase, the Histone Deacetylase 4 (HDAC4), from cytosol into the nucleus, where it deacetylates and activates the transcription factor Forkhead Box Class O (foxO) (Wang et al., 2011; Choi et al., 2015; **Figure 1**). This results in the activation of foxO targets, such as bmm, leading to lipolysis. Overexpression of *HDAC4* leads to up-regulation of the *bmm*, indicating that HDAC4 regulates *bmm* expression in the fat body (Choi et al., 2015). In line with this, *SIK3*-null mutants exhibit a lipodystrophic (lean) phenotype and display up-regulated *bmm* expression and increased lipase activity as expected. By contrast, constitutive over-expression of active *SIK3* completely blocks the *bmm* expression (Choi et al., 2015). Interestingly, deletion of *SIK3* reversed both the lipid accumulation and the reduced *bmm* expression phenotypes of *AKHR*-mutant flies (Choi et al., 2015). It is noteworthy that a serine/threonine kinase known as Liver Kinase B1 (LKB1) also plays an important role in governing lipid metabolism by activating SIK3 in a kinase activity-dependent manner (Choi et al., 2015). Thus, *Drosophila LKB1*-mutants display decreased lipid storage and increased expression of *bmm*, suggesting that the AKH pathway inhibits the kinase activity of LKB1 (Choi et al., 2015). In line with this, foxO is dephosphorylated, therefore activated, and localized to the nucleus during lipolysis. In brief, the LKB1-SIK3 pathway is upstream of HDAC4, whereas LKB1, SIK3, HDAC4, and foxO are downstream elements of AKH signaling. Additionally, AKH signaling works in a manner opposite to LKB1-SIK3 signaling.

Null mutations in *AKH* or *AKHR* result in obese *Drosophila* adults, whereas their over-expression leads to a dramatic reduction of lipid stores (Grönke et al., 2007; Bharucha et al., 2008; Baumbach et al., 2014b; Gáliková et al., 2015). In accordance with this, LDs accumulated in adipocytes of *AKHR* deletion mutants (obese) and in flies subjected to RNAi-mediated knockdown of *AKHR* in the fat body. In contrast, LDs are largely depleted from adipocytes of the flies subjected to *AKH* or *AKHR* overexpression (lean). In accordance with this, silencing of *AKHR* reduces DAG levels leading to TAG accumulation in the fat body in the kissing bug *Rhodnius prolixus* (Alves-Bezerra et al., 2016), the two-spotted cricket *Gryllus bimaculatus* (Konuma et al., 2012) and the oriental fruit fly *Bactrocera dorsalis* (Hou et al., 2017). Additionally, double-mutant flies, which lack the lipolytic PLIN1 and AKHR, were found to be more obese than the single *PLIN1*- or *AKHR*-mutants and remained lipolysis-competent (Grönke et al., 2007; Beller et al., 2010). Dual knockout of the *AKHR* and *bmm* genes in *Drosophila* yields flies also that are obese and not starvation-tolerant (Grönke et al., 2007). Notably, overexpression of *AKH* in *bmm-Drosophila* mutants was still found to reduce the excessive TAG storage; however, *bmm* expression was found to be higher in *AKHR*-mutants, suggesting AKH/AKHR signaling is not a prerequisite for bmm activity (Grönke et al., 2007). Females of tsetse fly of which *bmm* or AKH/AKHR systems were silenced individually or together were found to have prolonged lifespan under starvation and elevated lipid levels at the time of

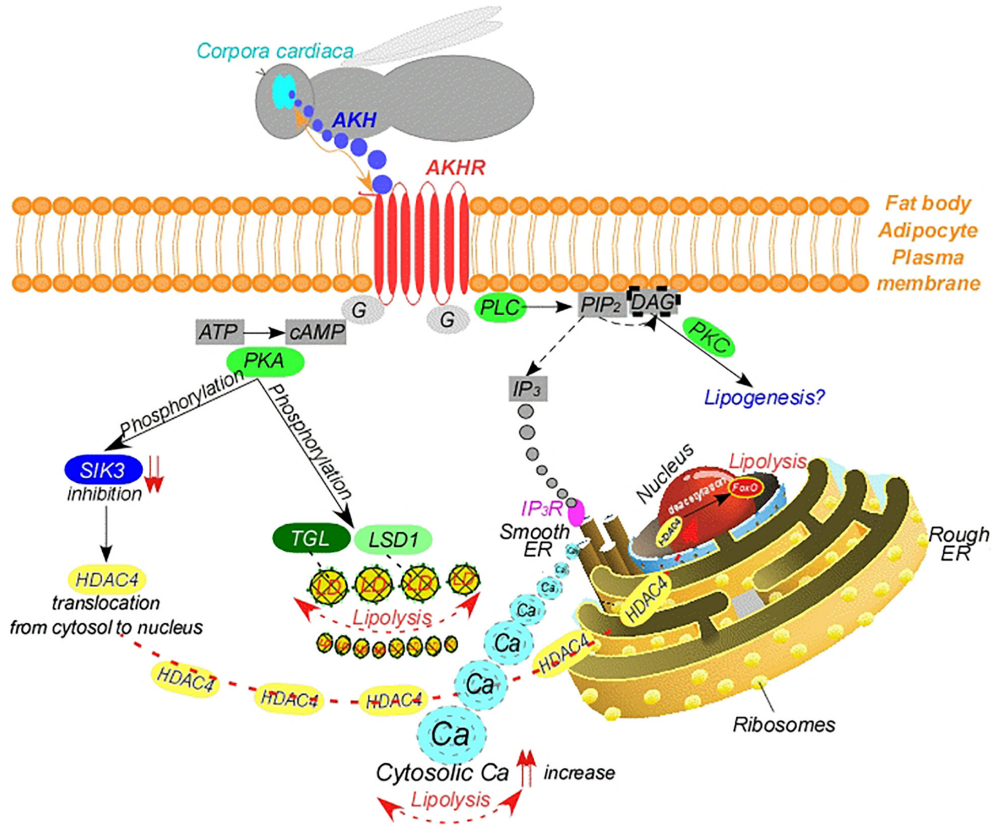

FIGURE 1 | Mode of action of adipokinetic hormone. AKH, adipokinetic hormone; AKHR, adipokinetic hormone receptor; Ca, calcium; DAG, diacylglycerol; ER, endoplasmic reticulum; G, g-protein couple; HDAC4, histone deacetylase 4; IP_3, inositol-1,4,5-trisphosphate; IP_3R, inositol-1,4,5-trisphosphate receptor; LD, lipid droplet; LSD1, lipid storage droplet 1 protein; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-diphosphate; PKA, cAMP-dependent protein kinase; PKC, calcium-dependent protein kinase C; SIK3, salt induced kinase 3; TGL, triglyceride lipase.

death, suggesting extended survival is likely due to the reduced rate of lipolysis during starvation and the inability to utilize lipid reserves (Attardo et al., 2012). On the other hand, *bmm* expression shows an antagonistic response to disturbed AKH and fat body calcium homeostasis, as opposed to the lipogenic gene *midway* (*mdy*) encoding the Diacylglycerol O-Acyltransferase 1 (DGAT1) (Buszczak et al., 2002), which is down-regulated in response to increased cytosolic calcium levels in fat body cells (Bi et al., 2014). Conversely, depletion of cytosolic calcium levels in the fat body of adult flies up-regulates *mdy*, and down-regulates *bmm* expression (Baumbach et al., 2014a), suggesting AKH signaling via calcium promotes *bmm* expression (Baumbach et al., 2014a; Choi et al., 2015). In addition, AKH has been shown to be involved in the lipid mobilization only in the adult stage, but not in larval stages in *Drosophila* (Gáliková et al., 2015). Lee and Park (2004) also reported that fat body TAG content did not change in *AKH*-deficient mutants, suggesting that lipid metabolism might occur normally also in the absence of AKH.

The extracellular or intracellular calcium is important in the lipolytic response by AKH as mentioned above and has been demonstrated in the adults of the orthopterans *S. gregaria* (Ogoyi et al., 1998), *L. migratoria* (Auerswald and Gäde, 2006), *G. bimaculatus* (Anand and Lorenz, 2008), lepidopteran *M. sexta* (Arrese et al., 1999), the coleopterans *Pachnoda sinuata* (Auerswald et al., 2005) and *Zophobas atratus* (Slocinska et al., 2013) and *Drosophila* (Grönke et al., 2007; Baumbach et al., 2014a; Gáliková et al., 2015). For example, incubation of fat body from *P. sinuata* in calcium-free medium reduces the elevation of cAMP in comparison to that in calcium-containing medium, further indicating the importance of calcium in the AKH-induced lipolytic response (Auerswald and Gäde, 2001). However, no effect of calcium signaling on TAG levels has been detected in *Drosophila* larvae, which is in accordance with the fact that AKH mobilizes lipids only in the adult stage (Baumbach et al., 2014a; Gáliková et al., 2015).

Calcium homeostasis is primarily coordinated by a process called "Store-Operated Calcium Entry (SOCE)," which has two major components; the SERCA (Sarco/Endoplasmic Reticulum Calcium-ATPase) that pumps calcium from the cytosol into the endoplasmic reticulum lumen and the IP_3R that releases calcium from endoplasmic reticulum into cytosol. Binding of AKH to AKHR triggers an IP_3 second messenger response via GPCR signal transducer G-proteins, such as G Protein α q Subunit (Gαq) and G Protein γ 1 Subunit (Gγ1), and phospholipase C (PLC) (Baumbach et al., 2014b). Binding of IP_3 to IP_3R in the endoplasmic reticulum membrane causes calcium

efflux, which is sensed by the "Stromal Interaction Molecule (STIM)." STIM interacts with the Plasma Membrane Calcium Channel Protein 1, Orai1, to elevate cytosolic calcium levels (Cahalan, 2009). At resting stage, STIM is bound to calcium and spread evenly throughout the endoplasmic reticulum membrane. Upon activation, STIM translocates to junctions between endoplasmic reticulum and plasma membrane, where it couples with Orai1. This coupling results in the import of calcium from the extracellular compartment to the cytosol, providing spatial calcium replenishment into the endoplasmic reticulum lumen through SERCA.

It is not surprising that genes involved in calcium homeostasis affect lipid metabolism when the calcium/AKH interaction is taken into consideration. For example, impaired SERCA activity leads to reduced fat storage in adipose tissue in *Drosophila* (Baumbach et al., 2014a). This appears to be opposite to the effects of impaired endoplasmic reticulum calcium homeostasis on fat storage in mammalian hepatocytes (Bi et al., 2014). On the other hand, loss of IP_3R leads to obesity in *Drosophila* adults (Subramanian et al., 2013a,b). Chronic silencing of *STIM* leads to obesity and dysfunction of lipid mobilization due to reduced AKH signaling in adult fly fat body, whereas *AKHR* is up-regulated in the fat body of flies continuously expressing *STIM*, suggesting an impairment of AKH upon *STIM* disruption (Xu et al., 2019). In line with this, *bmm* was down-regulated and *mdy* was up-regulated upon *STIM* knock down, however, the *mdy* up-regulation was found only at day 1 of silencing (Xu et al., 2019). Functional impairment of the PLC, and Gαq, Gγ1, STIM, and AKHR, lowers the intracellular calcium concentration and increases the fly body TAG content (Baumbach et al., 2014b). At the onset of PLC-dependent adiposity, *mdy* was found to be up-regulated and *bmm* down-regulated (Baumbach et al., 2014b). Similarly, a Gγ1-dependent body fat increase correlated with an almost doubled expression of the lipogenic *mdy* gene and reduction of *bmm* expression. Over-expression of *Gαq* or *STIM* leads to lean flies which down-regulated *mdy*, and up-regulated *bmm* (Baumbach et al., 2014b). Notably, silencing calmodulin (*CaM*) encoding a calcium-binding messenger protein in the adult fat body also leads to an increase in fly body TAG content as CaM is also regulated by intracellular calcium (Baumbach et al., 2014b). In addition, silencing *CaM* leads to a similar transcriptional response of the *mdy* (upregulation) and *bmm* (downregulation) genes (Baumbach et al., 2014b). In brief, changes in calcium homeostasis directly impact fat deposition and AKH signaling employs the Gαq/Gγ1/PLC/STIM module of GPCR-dependent calcium signaling to regulate lipid mobilization (Baumbach et al., 2014b). *Gαq*, *Gγ1*, *PLC*, *STIM*, IP_3R, and *CaM* act as anti-obesity genes, whereas *SERCA* acts as an obesity gene.

GPCRs can also activate calcium-dependent Protein Kinase C (PKC) (Ojani et al., 2016) and Calcium/Calmodulin-dependent Protein Kinase II (CaMKII) (Van Marrewijk et al., 1991; Liu P. et al., 2015). The membrane-bound DAG produced by signal-induced activation of PLC could activate PKC, which might phosphorylate other molecules, leading to lipogenesis. However, the CaMKII is likely to lead to a lipolytic response. AKH signaling via CaMKII has been demonstrated to inhibit secretion of the adipokine Unpaired 2 (UPD2), which triggers systemic insulin signaling from the central brain (Rajan and Perrimon, 2012) and impairs TAG mobilization (Rajan and Perrimon, 2012; Rajan et al., 2017). Additionally, AKH induces the transcriptional factor cAMP Response Element-Binding Protein (CREB) via increased cAMP through PKA signaling (Iijima et al., 2009; **Figure 2**). This occurs through the activity of the CREB co-activator, "cAMP-Regulated Transcriptional Co-activator (CRTC)," which works in cooperation with the foxO during fasting in mammalians (Koo et al., 2005; Dentin et al., 2007; Matsumoto et al., 2007). The CRTC-related lipolytic response requires its dephosphorylation (at Ser^{157}), which is accomplished by the calcium-dependent calcineurin (CaN), a calcium/calmodulin-dependent serine/threonine phosphatase that binds directly to CRTC (Wang et al., 2008; Yang et al., 2013; **Figure 2**). Thus, increases in intracellular calcium stimulate CREB target gene expression (Screaton et al., 2004; Koo et al., 2005) and induces CRTC dephosphorylation, therefore activation (**Figure 2**). As will be discussed under "Insulin-like Peptides," ILPs inhibit CRTC activity in a phosphorylation-dependent manner, which occurs primarily in the feeding stages.

Genetic activation of AKH signaling suppresses the expression of lipogenic *midway* encoding the DGAT1. On the other hand, knockdown of *AKHR* leads to upregulation of the lipogenic gene encoding Acyl-CoA-Binding Protein-1 (ACBP1), which was

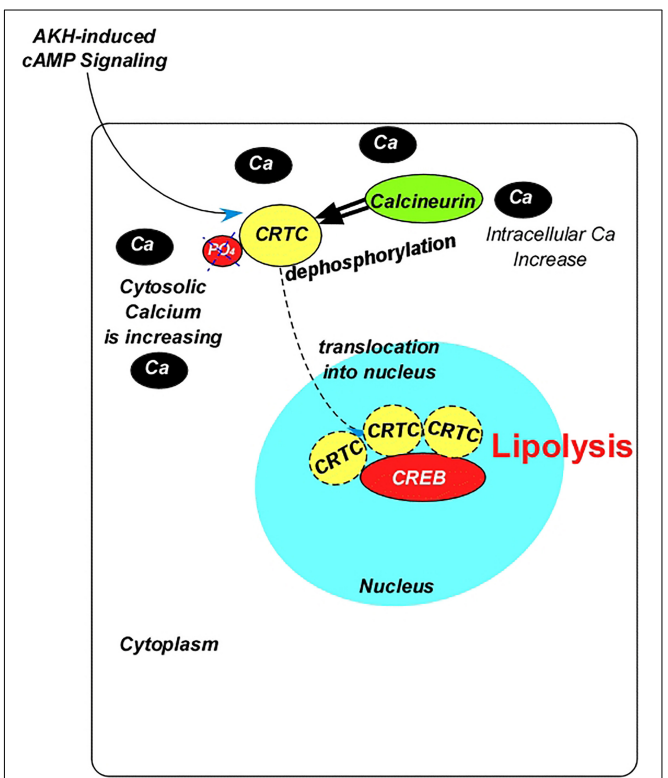

FIGURE 2 | Diagram summarizing the interaction between the adipokinetic hormone and the transcription factor cAMP response element-binding protein. AKH, adipokinetic hormone; Ca, calcium; CREB, cAMP response element-binding protein; PO_4, phosphate; CRTC, cAMP-regulated transcriptional co-activator.

shown in *R. prolixus*. ACBP1 is required for binding of the acyl-CoA produced from fatty acids that are released during TAG hydrolysis, as well as delivery to the acyltransferases involved in TAG synthesis (Alves-Bezerra and Gondim, 2012; Alves-Bezerra et al., 2016). Downregulation of the CREB target genes, *ACBP1* in this case, is in accordance with CRTC dephosphorylation. *AKHR* silencing leads to also downregulation of the gene encoding mitochondrial-like *GPAT1* (Glycerol-3-Phosphate-O-Acyltransferase 1) required for the first and committed step in the synthesis of TAG. This may be related to avoiding excessive TAG synthesis exceeding the cellular capacity of storage (Alves-Bezerra et al., 2010, 2016). *AKHR* knockdown leads to TAG accumulation in fat body and flight muscles, and reduced hemolymph lipid levels after starvation in *R. prolixus*, also indicating the requirement of AKHR in TAG mobilization (Zandawala et al., 2015; Alves-Bezerra et al., 2016). Notably, injection of the ligand, AKH, induces expression of both *ACBP1* and *GPAT1* when the AKHR is also highest in the fat body (Alves-Bezerra et al., 2016). Similarly, knockdown of *AKHR* in *B. dorsalis* resulted in TAG accumulation both in feeding and starving flies (Hou et al., 2017). In brief, cAMP and calcium signals stimulate CRTC dephosphorylation cooperatively through their effects on Salt-Inducible Kinases (SIKs) and phosphatases. Notably, other kinases, such as the Extracellular signal-Regulated Kinase 1 and 2 (ERK1 and ERK2) could be activated by GPCRs (Lim et al., 2015).

Obesity formed upon the impairment of AKH/AKHR has also been examined in terms of feeding behavior. Adipose tissue dysfunction promotes hyperphagia, which may be related to increased secretion of AKH (Xu et al., 2019). Thus, silencing *AKHR* causes hyperphagia in *G. bimaculatus*, while reducing hemolymph lipid levels (Konuma et al., 2012). In *Drosophila*, *AKHR* knockdown reduces fat body intracellular calcium leading to obesity as mentioned before. The obesity formed by functional impairment of STIM also depends on hyperphagia (Xu et al., 2019). The STIM-dependent hyperphagia is related to the remote up-regulation of the orexigenic *sNPF* gene that is expressed in the central nervous system (Baumbach et al., 2014a,b). Thus, over-expression of *sNPF* increases both food consumption and overall body size, whereas loss of *sNPF* decreases food intake (Lee et al., 2004). Notably, neurons use both extracellular and intracellular sources of calcium (Berridge et al., 2000; Bednářová et al., 2013). In this manner, various peptide hormones trigger IP_3-induced release of calcium from non-mitochondrial intracellular storage compartments (Berridge and Irvine, 1989). On the other hand, there are controversial results in regard to the effect of AKH on feeding. Gáliková et al. (2017) reported a decrease in food intake in adult *Drosophila* upon *AKH/AKHR* mutation. Expression of *sNPF* has been reported to be unaffected by *AKH* mutation or by the *AKH* overexpression. However, another orexigenic peptide, Neuropeptide F (NPF), which encodes the fly counterpart of the mammalian orexigenic Neuropeptide Y (Nässel and Wegener, 2011), is up-regulated in *AKH*-mutants (Gáliková et al., 2017). Therefore, other mechanisms unrelated to AKH secretion could affect food uptake.

AKH interferes with the expression of other neuropeptide genes, such as Tachykinin (*Tk)*, Corazonin (*Crz)*, and Limostatin (*Lst)* (Gáliková et al., 2017). *Tk*, which encodes a hormone that positively regulates expression of *ILP2* and *ILP5* (Birse et al., 2011), is up-regulated in the fly gut upon food deprivation (Song et al., 2014). *AKH*-mutants have upregulated *Tk* mRNA levels. Nevertheless, overexpression of *AKH* is not sufficient to downregulate *Tk* (Gáliková et al., 2017). Tk is a negative regulator of fat storage (Song et al., 2014) and the increased expression of this gene in the *AKH*-mutants indicates that the de-repression of *Tk* might contribute to AKH deficiency-triggered obesity (Gáliková et al., 2017). On the other hand, genes encoding the cardioacceleratory peptides *Crz* and *Lst* are down-regulated in *AKH*-mutants, suggesting that other interactions likely to affect lipid metabolism (Xu et al., 2019). Thus, partial loss of *STIM* has been found to reduce Crz signaling leading to impaired larval development which might affect lipid metabolism (Megha Wegener and Hasan, 2019).

Insulin-Like Peptides (ILPs)

Similar to mammalian insulin, insect Insulin-like Peptides (ILPs) are able to regulate circulating levels of carbohydrates in the hemolymph (Wu and Brown, 2006), thus, their temporal production is increased by hemolymph carbohydrate levels and decreased by starvation. ILPs are key elements of insect growth, reproduction, regulation of stress responses and life span. ILPs are primarily produced by the medial or lateral neurosecretory cells, known also as the insulin producing cells (IPCs) of the brain and the corpora cardiaca (Cao and Brown, 2001; Ikeya et al., 2002). Thus, the insulin signaling pathway in insects links metabolism and growth with the availability of nutrients. The fat body could also remotely control the secretion of ILPs from the IPCs through the Target of Rapamycin (TOR) pathway (Colombani et al., 2003; Géminard et al., 2009).

The classification of insect ILPs is based on similarities in the amino acid sequence of mature peptides to those of mammalian insulins, especially the number and locations of cysteine residues (Brogiolo et al., 2001; Grönke et al., 2010). Another conserved feature is the arrangement of the precursor (pre-proinsulin) protein with B-C-A domains that can be processed into dimeric peptides with an A and a B-chain linked by disulfide bridges (Nässel and Vanden Broeck, 2016). An exception to this structure has been detected for the insulin-like growth-factors (IGFs), where a short C-peptide is retained and the extended peptide is a single chain with internal cysteine bridges (Nässel and Vanden Broeck, 2016).

The first ILP to be identified in insects was bombyxin, or small prothoracicotropic hormone (Yoshida et al., 1998), and many ILPs from a variety of insects have been reported since. In *Drosophila*, eight ILPs [*Drosophila* Insulin-like Peptide 1-8 (DILP1-8)] (Kannan and Fridell, 2013), but only two receptors, a tyrosine kinase (Brogiolo et al., 2001) and the relaxin receptor-like leucine-rich repeats (Colombani et al., 2015) are found. DILP2, DILP3 and DILP5 resemble mammalian insulins and are primarily produced by IPCs in the adult brain and are therefore, denoted as "brain ILPs" (Brogiolo et al., 2001; Rulifson et al., 2002; **Table 1**). *DILP2* is also expressed in the embryonic and larval midgut, salivary glands and mesoderm (Brogiolo et al., 2001; **Table 1**). *DILP3* and *DILP5* transcripts are not detectable until larval stages (Brogiolo et al., 2001). *DILP3* is also expressed

by the intestinal muscle (Veenstra et al., 2008), and *DILP5* is expressed in ovaries and Malpighian tubules (Ikeya et al., 2002; **Table 1**). DILP6 resembles IGFs structurally and functionally, and is produced in the larval and adult fat body, as well as in the salivary glands, heart and glial cells in the ventral nerve cord (Okamoto et al., 2009; Slaidina et al., 2009; **Table 1**). DILP7 and DILP8 have been proposed to be relaxin-like peptides (Yang et al., 2008; Colombani et al., 2012; Garelli et al., 2012). *DILP7* is expressed in the embryonic midgut during development and abdominal ganglia in third instar larvae and adults (Rulifson et al., 2002; Veenstra et al., 2008; Yang et al., 2008). *DILP8* is primarily expressed in the imaginal discs of the larva and ovaries of adults (Colombani et al., 2012; Garelli et al., 2012; Nässel et al., 2015) and shown to coordinate *Drosophila* tissue growth by delaying the onset of metamorphosis (Colombani et al., 2012; Garelli et al., 2012). *DILP1* is primarily expressed in IPCs mainly during the pupal stage, as well as in the adult stage (Slaidina et al., 2009; Liu W. et al., 2016). *DILP4* is expressed in the embryonic midgut and mesoderm during late-stage embryogenesis (Brogiolo et al., 2001). Together, this suggests that different ILPs are produced in different cell types and tissues at different developmental stages and may have multiple roles in other pathways (Nässel and Vanden Broeck, 2016).

The insulin signaling pathway appears to be highly conserved in insects (**Figure 3**). It is expected that the ILPs act similarly to insulin. When nutrients are abundant, the pathway is activated as ILPs released from the brain bind to an "Insulin Receptor (InR)" at the cell membrane (Fernandez et al., 1995; Chen et al., 1996). This leads to the recruitment of the InR substrate, Chico (Böhni et al., 1999), and subsequent activation of class I Phosphoinositide-3-Kinase (PI_3K), which catalyzes the addition of a phosphate group to PIP_2 forming phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Oldham et al., 2002; Brown and Auger, 2011; **Figure 3**). The elevated PIP_3 recruits Protein Kinase B (PKB), also known as Serine-Threonine Protein Kinase (AKT) to the membrane (Verdu et al., 1999; Britton et al., 2002). AKT can directly inhibit foxO activity by phosphorylation (Puig et al., 2003; Wang et al., 2008; **Figure 3**). An indirect route through AKT occurs through the activation of SIK3. In this route, AKT phosphorylates LKB1, and LKB1 phosphorylates and activates AMPKs, including SIK3 (Thr^{196} in *Drosophila* SIK3) (DiAngelo and Birnbaum, 2009; Funakoshi et al., 2011; Choi et al., 2015; **Figure 3**). In line with this, depletion of AKT enhances the activity of the CREB Co-activator, CRTC; therefore, insulin signaling pathway inhibits CRTC activity (Wang et al., 2008). Furthermore, over-expression of *LKB1* increases the level of phosphorylated AMPK (Funakoshi et al., 2011). This results in the phosphorylation and inhibition of HDAC4 by LKB1-activated SIK3 in the fat body, leading to dissociation of the HDAC4 from nucleus to the cytosol and inhibition of the lipolytic foxO. Thus, loss of *SIK3* leads to elevated expression of *bmm* and decreased lipid stores (Wang et al., 2011; Choi et al., 2015). Notably, *Drosophila* ILPs induce AKT-mediated SIK3 phosphorylation independently of increasing LKB1 kinase activity (Dentin et al., 2007; Wang et al., 2011; Choi et al., 2015). Thus, overexpression of *LKB1* induces lipid levels and downregulates *bmm*, suggesting LKB1 plays a critical role in lipid storage (Choi et al., 2015). On the other hand, AKT indirectly regulates TOR, a central regulator of cellular metabolism. In this manner, activation of AMPK leads to down-regulation of TOR signaling (Shaw et al., 2004) and phosphorylation of Raptor, a component of the TOR complex (Gwinn et al., 2008). Therefore, LKB1 suppresses TOR activity (Dentin et al., 2007). Briefly, in either route resulting in foxO inhibition, directly by AKT or indirectly via LKB1/SIK3, the inhibition of foxO leads to decrease in bmm activity, which leads to accumulation of lipids during feeding (Puig et al., 2003; Wang et al., 2011; Choi et al., 2015). This is in accordance with the increase in insulin in feeding stages. These findings suggest that foxO plays a central role in connecting insulin signaling to TAG metabolism (Heier and Kühnlein, 2018; **Figure 3**). By contrast, *bmm* is up-regulated when insulin signaling is low (Wang et al., 2011; Lee and Dong, 2017). Thus, reduction of insulin signaling, for example in starvation, stimulates dephosphorylation and nuclear translocation of foxO (Jünger et al., 2003; Puig et al., 2003), which in turn up-regulates genes encoding lipases involved in TAG hydrolysis (Vihervaara and Puig, 2008; Wang et al., 2011), supplying energy to the insect (**Figure 1**). In brief, the insulin-induced kinase activity of SIK3 controlled by LKB1 is critical for lipid storage in the fat body (Choi et al., 2015; **Figure 3**).

As mentioned before, the transcription factor, CREB induces lipolytic responses via the action of AKH. In accordance with this, down-regulation of *CREB* in the fat body leads to obesity in flies (Iijima et al., 2009). CREB also serves as a transcriptional factor target of ILPs, which occurs via the CREB co-activator CRTC (**Figure 4**). In parallel to the increase in insulin signaling, CRTC activity is inhibited during feeding through the phosphorylation of Ser^{157} by the Salt-Inducible Kinase 2 (SIK2) (Wang et al., 2008) leading to lipid accumulation. -Mutation of the inhibitory PKA phosphorylation site at Ser^{1032} to Ala in SIK2 further increased the amount of phosphorylated CRTC (Wang et al., 2008). Notably, CRTC is dephosphorylated by CaN, and SIK2 is inhibited during starvation (**Figure 4**). Additionally, deletion of *CRTC* induced the lethality of *LKB1*- and *SIK3*-null mutants (Choi et al., 2015).

Brain ILPs, and the ILP6 that suppresses the production of brain ILPs, are the most commonly studied ILPs (**Table 1**). Knock down of ILPs leads to various defects, such as loss of weight, reduced fecundity and body size, impaired development and metamorphosis or even lethality (Fernandez et al., 1995; Chen et al., 1996; Okamoto et al., 2009; Slaidina et al., 2009; Grönke et al., 2010; Fu et al., 2016; Deng et al., 2018), indicating their critical role in insect survival. Transgenic ablation of ILP-producing neurons in *Drosophila* leads to elevation of total blood sugar (Rulifson et al., 2002). Not surprisingly, ILPs are also key regulators of lipid metabolism (Broughton et al., 2005; DiAngelo and Birnbaum, 2009) and insulin signaling promotes TAG accumulation (DiAngelo and Birnbaum, 2009; Lehmann, 2018). This is indeed a complex interaction influenced by multiple factors, such as transcription factors, neuropeptides, neurotransmitters, lipases, and the internal interaction among ILPs and other actors originating from fat body and midgut. Basically, insulin (brain ILPs) inhibits the activity of foxO and activates the Sterol Regulatory Element-Binding Protein (SREBP), a transcription factor that regulates a variety of genes

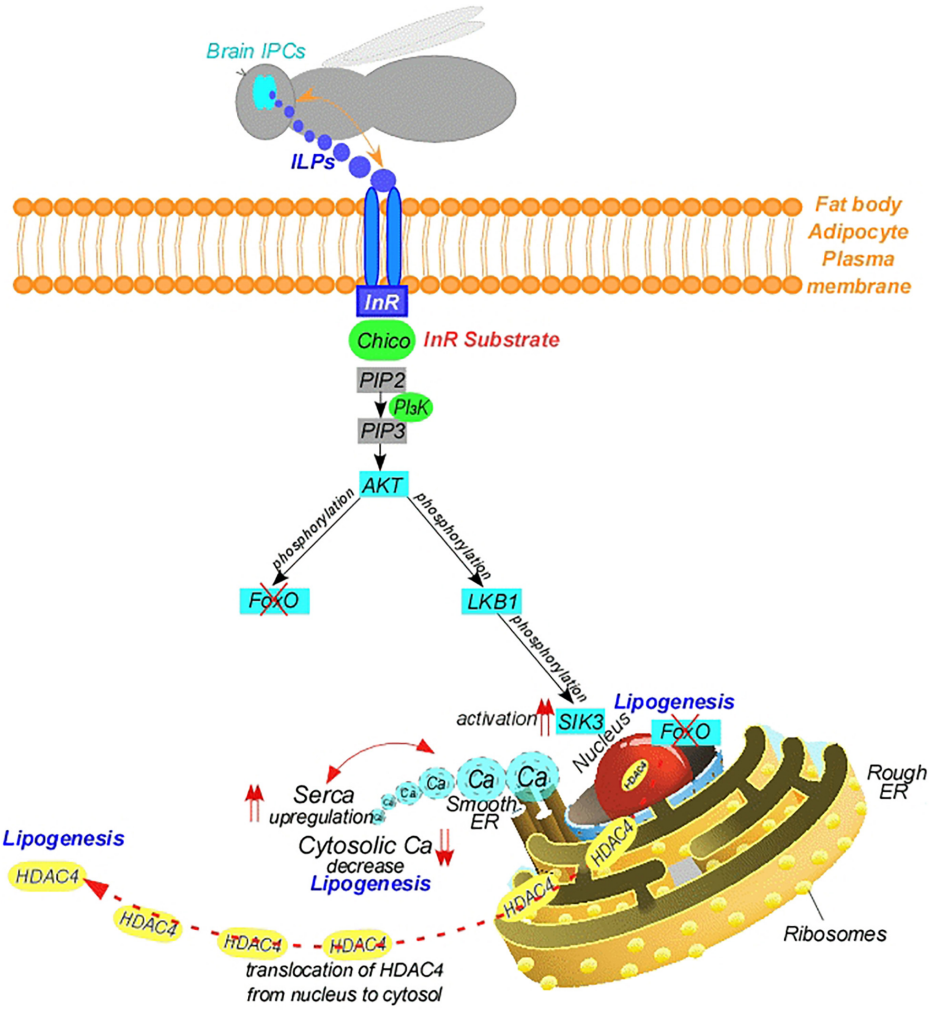

FIGURE 3 | Mode of action of brain insulin-like peptides. AKT, serine-threonine protein kinase; Ca, calcium; ER, endoplasmic reticulum; G, g-protein couple; HDAC4, histone deacetylase 4; ILP, insulin-like peptide; InR, insulin-receptor; IPC, insulin producing cells; LKB1, liver kinase B1; PI_3K, phosphoinositide-3-kinase; PIP_2, phosphatidylinositol 4,5-diphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; SERCA, sarco/endoplasmic reticulum calcium-ATPase; SIK3, salt induced kinase 3.

involved in *de novo* lipogenesis, leading to the accumulation of reserves. Not surprisingly, genes encoding several lipases and acyl-CoA synthetase or acyl-CoA dehydrogenases are also down-regulated by dietary sugars, therefore, repressed by the action of ILPs via foxO (Zinke et al., 2002; Wang et al., 2011; Mattila et al., 2015). Feeding on a high carbohydrate diet also induces the expression of genes encoding other lipogenic enzymes, such as Acetyl-CoA Carboxylase (ACC) and FAS (Zinke et al., 2002; Musselman et al., 2013; Mattila et al., 2015), which is coordinated by Mondo/Bigmax. This transcription factor likely binds to the promoter of *FAS* and/or *ACC* as *Bigmax*-mutant *Drosophila* larvae have decreased *ACC* and *FAS* gene expression, and store less TAG (Mattila et al., 2015). In addition, Mondo/Bigmax controls expression of genes encoding other transcription factors, such as *Sugarbabe*, which is highly induced by sugar and positively regulates lipogenic gene expression (Zinke et al., 2002; Mattila et al., 2015). Notably, reducing Mondo/Bigmax also reduces Seven-up, a positive regulator of insulin signaling (King-Jones and Thummel, 2005). Seven-up promotes glucose clearance and lipid turnover by inhibiting ecdysone signaling in the larval fat body (Musselman et al., 2018b). On the other hand, ablation of the IPCs, which leads to elimination of DILP2, DILP3, and DILP5 synthesis results in elevated levels of circulating carbohydrates levels in the hemolymph (hyperglycemia), elevated lipid storage in the fat body, increased starvation resistance, and extended life span in *Drosophila* adults (Brogiolo et al., 2001; Rulifson et al., 2002; Broughton et al., 2005). Likewise, silencing genes encoding ILPs increases the levels of lipid and carbohydrate in the hemolymph in *R. prolixus* (Defferrari et al., 2016) and *Spodoptera exigua* (Kim and Hong, 2015). In another study, elevated levels of TAG and obesity in *IP_3R-Drosophila* mutants were also rescued after insulin expression, further suggesting the involvement of ILPs in lipid metabolism (Subramanian et al., 2013b). On the other hand, *DILP2*-knockdown leads to an

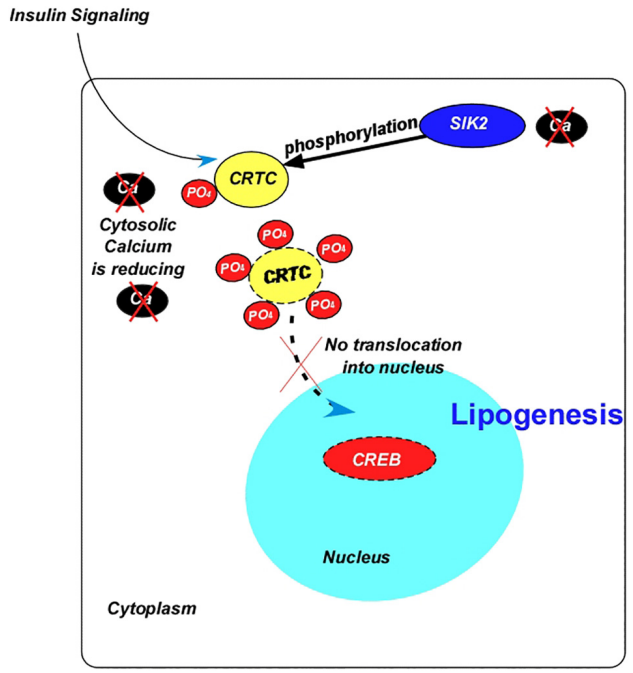

FIGURE 4 | Diagram summarizing the interaction between the insulin signaling and the transcription factor cAMP response element-binding protein. Abbreviations: Ca, calcium; CREB, cAMP response element-binding protein; PO_4, phosphate; SIK2, salt induced kinase 2; CRTC, cAMP-regulated transcriptional co-activator.

up-regulation in *DILP3* and *DILP5* levels (Brogiolo et al., 2001), suggesting a compensatory balance mechanism amongst brain ILPs. Furthermore, the IGF-like DILP6 represses brain ILPs, thus, its over-production leads to a decrease in the expression of *DILP2* and *DILP5* in the brain, and DILP2 level in hemolymph in adult flies (Bai et al., 2012). In accordance with this, deletion of brain ILPs leads to up-regulation of *DILP6* in the fat body; however, *DILP6* deletion was not found to change the expression of brain DILP genes (Zhang and Xi, 2015). *DILP6* is also under the control of foxO and strongly induced upon starvation in a foxO-dependent manner in the larval fat body (Slaidina et al., 2009). During late larval and pupal stages when insects do not feed, *DILP6* expression is strongly induced (Okamoto et al., 2009; Slaidina et al., 2009). Thus, *DILP6*-mutants were shown to have elevated lipid levels (Grönke et al., 2010). As expected, loss of *DILP6* does not affect lipid stores in feeding larvae (Slaidina et al., 2009). Furthermore, DILP6 induces lipid uptake in oenocytes in fasting adult flies, indicating it is required for lipid turnover when adult flies are starved (Chatterjee et al., 2014). *DILP3* and *DILP5* expression is down-regulated, while the TAG levels increased in *miR-14*-mutant flies; however, the hyperlipidemic defect in *miR-14*-mutants was rescued by over-expressing *DILP3* (Varghese et al., 2010). This suggests an indirect role for DILP3 in lipid metabolism. In brief, high-sugar feeding promotes lipid biosynthesis and inhibits lipid catabolism to channel excess carbon derived from sugars into TAGs (Mattila and Hietakangas, 2017).

Insulin Receptor (InR) is essential to insulin activity, but also affects lipid storage as up-regulation of *Drosophila InR* specifically increases TAG stores in the adult fat body (DiAngelo and Birnbaum, 2009). Larvae over-expressing *InR* accumulate more fat in the fat body (Britton et al., 2002). Additionally, *InR* knockdown increases lipid levels in the hemolymph, while reducing lipid content in the fat body in *R. prolixus* (Defferrari et al., 2018). Interestingly, the levels of carbohydrates in the hemolymph and the fat body were found to be unchanged. The activation of AKT and phosphorylation of foxO were also reduced in knockdown insects (Defferrari et al., 2018). *InR* expression was found to be up-regulated in response to the reduction of ILPs in 5th instar *M. sexta* larvae (Walsh and Smith, 2011). In *L. decemlineata*, knockdown of *ILP2* led to up-regulation of *InR* and the insulin signaling target *Thor* gene encoding the translational regulator 4E Binding Protein (4EBP) (Fu et al., 2016). *4EBP* was also induced in *Drosophila ILP6*-mutants (Grönke et al., 2010). Furthermore, *4EBP* was significantly up-regulated in these mutants when combined with knockout of brain ILPs (Grönke et al., 2010). On the other hand, the InR substrate chico, an important component of the insulin signaling pathway, also affects lipid storage. For example, *Drosophila chico*-mutant males had an almost twofold increase in lipid levels despite their size reduction compared to normal flies (Böhni et al., 1999). The Chico binding protein SH2B, a SH2 domain-containing adaptor protein directly promotes insulin signaling, therefore, its disruption decreases insulin signaling and increases hemolymph carbohydrate levels, whole-body lipid levels, suggesting SH2B in fat body plays a key role in regulating lipid metabolism and energy homeostasis (Böhni et al., 1999; Song et al., 2010). In brief, InR is involved in fat body lipid storage in both non-feeding stages and post-feeding stages. These effects are likely to be regulated by the activation of AKT in a manner similar to mammalian insulin signaling pathway.

The cell growth factor, myc, in the fat body was also shown to facilitate DILP2 release from the brain, induce accumulation of TAGs and confer resistance to starvation (Gallant, 2013; Parisi et al., 2013). DILP2 accumulates in the IPCs of *Drosophila* larvae after starvation; however, reduced myc levels in the fat body also lead to accumulation of DILP2 in the IPCs during feeding, whereas increased myc levels decreases the amount of trehalose in the hemolymph (Parisi et al., 2013). Furthermore, down-regulation of genes encoding brain ILPs blocks the effect of myc on systemic growth, suggesting that myc activity in the fat body requires ILPs to induce systemic growth. As another point, expression of the gene encoding the fat desaturase Desat1, an enzyme that is necessary for monosaturation and production of fatty acids, was found to be necessary for myc-induced TAG storage (Parisi et al., 2013).

AKH signaling can also affect insulin signaling and, therefore, affect lipid metabolism (Buch et al., 2008; Hentze et al., 2015; Gáliková et al., 2017). AKH activity is antagonistic to insulin activity (Rulifson et al., 2002), thus, AKH elevates hemolymph trehalose titers (Park and Keeley, 1995). Additionally, *AKH*-mutants have increased expression of genes encoding brain ILPs, whereas *AKH* over-expression decreases their transcription. AKH signaling has been also reported to be required in the

IPCs for sugar-dependent ILP3 release in *Drosophila* (Kim and Neufeld, 2015). However in another study, overexpression of *AKH* did not alter trehalose levels in adult *Drosophila* (Lee and Park, 2004). Nevertheless, silencing of *AKH* or ablation of the corpora cardiaca producing AKH inhibits fat body TOR activation in response to trehalose (Buch et al., 2008; Kim and Neufeld, 2015). This indicates that trehalose stimulates the corpora cardiaca to release AKH and AKH then acts directly on the IPCs to induce secretion of ILP3 leading to stimulation of TOR signaling (Kim and Neufeld, 2015). Therefore, the TOR pathway is directly activated by insulin. In line with this, *TOR*-mutant flies possess reduced fat body TAG levels, with a concomitant down-regulation in the lipogenic *Drosophila FAS* and an up-regulation in the lipolytic *bmm* (Luong et al., 2006). Furthermore, these mutants have also decreased hemolymph sugar levels, suggesting a sugar-regulatory role for TOR in addition to its involvement in the control of lipid metabolism (Luong et al., 2006). In accordance with these findings, adult flies lacking AKH are more resistant to starvation and do not exhibit starvation-induced hyperactivity (Lee and Park, 2004). Not surprisingly, ILP6 was found to be affected by the AKH deficiency in the opposite manner, thus, *AKH*-mutants have decreased *ILP6* mRNA (Gáliková et al., 2017) and, therefore, the obese phenotype in *AKH*-mutants could be also related to ILP6 reduction (Grönke et al., 2010; Gáliková et al., 2017). Briefly, ILP release by IPCs is stimulated by trehalose-activated AKH signaling leading to TOR activation (Kim and Neufeld, 2015).

One other factor that affects insulin signaling is the Insulin-related Peptide Binding Protein (IPBP), a homolog of the mammalian Insulin Growth-Factor Binding Protein (IGFBP) (Honegger et al., 2008). An IPBP, the Imaginal Morphogenesis Protein-Late 2 (ImpL2), which is a neural/ectodermal development factor in *Drosophila*, has been identified from cell culture of imaginal discs (Zapf et al., 1985; Honegger et al., 2008). In *Drosophila*, ImpL2 has been shown to bind to ILP2 and ILP5 and acts as a secreted antagonist of insulin signaling, as well as being essential for tolerance to starvation stress (Honegger et al., 2008). However, ImpL2 promotes insulin signaling in a subset of neurons in the larval brain (Bader et al., 2013). Sloth Andersen et al. (2000) identified a lepidopteran IPBP, which was also shown to be capable of inhibiting human insulin action at its receptor. Interestingly, ImpL2 is up-regulated in obese *AKH-Drosophila* mutants, suggesting that the peripheral insulin signaling decreases in response to AKH deficiency (Gáliková et al., 2017). As the expression pattern of the peripheral insulin targets does not reflect the increased expression of brain ILPs, up-regulation of ILPs could be a compensatory mechanism reflecting insulin resistance of *AKH*-mutants (Gáliková et al., 2017). Thus, AKH may act as an ILP antagonistic hormone by releasing foxO leading to the activation of genes involved in lipolysis and fatty acid oxidation.

Insulin signaling pathway also interferes with ecdysone and JH signaling (Nässel et al., 2015). FoxO plays a key role in these interactions, for example, higher concentrations of 20-hydroxyecdysone (20E) repress insulin-induced gene expression in the cotton bollworm, *Helicoverpa armigera* (Liu C.Y. et al., 2015). 20E antagonizes insulin signaling by up-regulating Phosphatidylinositol-3,4,5-Trisphosphate 3-Phosphatase (PTEN) expression, which represses AKT phosphorylation, thereby repressing foxO phosphorylation, leading to foxO nuclear localization and lipolysis (Rusten et al., 2004; Colombani et al., 2005; **Figure 3**). On the other hand, ILPs stimulates growth of prothoracic gland and/or ecdysone biosynthesis and release in *Drosophila* (Colombani et al., 2005), *B. mori* (Gu et al., 2009), *R. prolixus* (Vafopoulou and Steel, 1997), and *M. sexta* (Kemirembe et al., 2012). Moreover, ILPs also activate ecdysteroidgenesis in ovaries in the yellow fever mosquito, *Aedes aegypti* and *Drosophila* (Brown et al., 2008; Wen et al., 2010). Similarly, knocking down ILP2 was found to decrease 20E titer and repressed the expression of two 20E-response genes, those encoding the nuclear receptors HR3 (Hormone Receptor 3) and FTZ-F1 (Fushi Tarazu Factor 1) in *L. decemlineata* (Fu et al., 2016). Conversely, insulin signaling inhibits foxO activity by phosphorylation and 20E by controlling the expression of the gene encoding the transcriptional co-activator, "Diabetes and Obesity Regulated (DOR)" during feeding (Francis et al., 2010). Additionally, the relaxin-like ILP8, which is produced and secreted from abnormally growing imaginal discs, has been shown to delay metamorphosis by suppressing ecdysone biosynthesis in developing larvae (Garelli et al., 2012). On the other hand, the link between JH and insulin signaling was first demonstrated in *Drosophila* as *InR*-mutants were found to possess reduced JH biosynthesis (Tatar et al., 2001). Similarly, knockdown of *ILP2* in *L. decemlineata* resulted in a decrease in JH titers, as well as impaired pupation and adult emergence (Fu et al., 2016). In accordance with this, the levels of an allatostatin (Ast-C), which inhibits JH synthesis, were significantly increased upon silencing of *ILP2* in *L. decemlineata* (Meng et al., 2015; Fu et al., 2016). Knockdown of *ILP2* in the 3rd instar larvae also significantly reduced the transcript levels of the early JH target gene Krüppel-homolog 1, a zinc finger transcription factor, and a JH biosynthesis gene encoding the Juvenile Hormone Acid Methyltransferase (JHAMT) (Fu et al., 2016). Thus, knockdown of *ILP2* delayed the onset of the wandering in *L. decemlineata* larvae (Meng et al., 2019). Additionally, genes encoding the InR substrate *chico* and PI_3K, which meditate insulin signaling, were also down-regulated upon *ILP2* silencing (Deng et al., 2018; **Figure 3**). In line with this, knockdown of *Chico* or PI_3K reduced expression of several 20E- [*EcR* (Ecdysone Receptor), *HR3* and *E75* (Ecdysone-induced Protein 75)] and JH- [JHAMT, *Kr-h1* (Kruppel Homolog 1) and *Hairy*] signaling genes, leading to retardation of larval development and inhibition of larval growth (Deng et al., 2018). In another study, insulin was found to stimulate JH production in the German cockroach, *Blattella germanica* (Suren-Castillo et al., 2012). Notably, insulin signaling might modulate JH synthesis by affecting the allatotropins that stimulate JH production (Klowden, 2007). Therefore, JH and insulin signaling appear to interact through a positive feedback loop (Fu et al., 2016). It is noteworthy that foxO is also a critical factor in the regulation of lipid metabolism by JH, which was shown in tsetse flies (Baumann et al., 2013) and diapausing mosquitoes (Sim and Denlinger, 2013). These data all together suggest that the brain

ILPs triggers JH signaling pathway during larval feeding and activates 20E signaling pathway at the late stage onset molting. The interaction between insulin, ecdysone and JH pathways affect the regulation of lipid metabolism.

Phosphatidic Acid Phosphatase (PAP), also known as lipin, converts phosphatidic acid into DAG, and therefore is also an important factor for insulin signaling (Finck et al., 2006; Schmitt et al., 2015). Thus, insulin signaling positively affects the role of lipins in LD formation (Schmitt et al., 2015). In *Drosophila*, lipin is localized to cytosol or nucleus (Valente et al., 2010), however, it translocates into the cell nucleus when nutrient availability and TOR signaling are low (Schmitt et al., 2015). Notably, down-regulation of the insulin pathway does not lead to nuclear translocation of lipin (Schmitt et al., 2015). Instead, reduced InR activity strongly promotes the small LD phenotype observed after reduction of lipin (Lehmann, 2018). In addition, reduced expression of *lipin* or knockdown of the *GPAT4* (Glycerol-3-Phosphate-O-Acyltransferase 4) and *AGPAT3* (1-Acylglycerol-3-Phosphate Acyltransferase 3, also known as Lysophosphatidic Acid O-Acyltransferase 3), the genes encoding enzymes preceding the dephosphorylation of phosphatidic acid by lipin, decreases PIP_3 levels in the fat body. In accordance with this, impaired signaling through the InR-controlled PI_3K-AKT pathway leads to increased hemolymph sugar levels in *Drosophila* larvae (Schmitt et al., 2015). In another study, a *GPAT4*-mutant was found to exhibit elevated levels of *DILP2* and *DILP3* mRNA, and decreased insulin responsiveness (Yan et al., 2015). In brief, PAP activity and an intact glycerol-3 phosphate pathway are required for regular insulin signaling (Schmitt et al., 2015).

As mentioned before, the gene encoding the leptin-like cytokine, UPD2, is induced in the adult fat body in response to either a high-sugar or a high-fat diet, and promotes systemic insulin secretion from IPCs (Rajan and Perrimon, 2012; Zhang and Xi, 2015). This occurs through the activation of the JAK/STAT (Janus Kinase/Signal Transducers and Activators of Transcription) signaling cascade in GABAergic neurons (Géminard et al., 2009; Rajan and Perrimon, 2012). Thus, knockdown of fat body *UPD2* reduces adult body size by inhibiting the release of DILP2 from IPCs (Géminard et al., 2009; Wright et al., 2011; Rajan and Perrimon, 2012). On the other hand, sNPF-dependent increase in food consumption and body size is related to the effect of sNPF on insulin secretion as sNPF regulates the release of DILPs from IPCs (Lee K.S. et al., 2008).

Several microRNAs (miRNAs) have been also reported as critical regulators of ILP gene expression. For example, *miR-14*-mutants have a reduced lifespan with increased levels of TAG and DAG and an enlarged LDs, as well as decreased *DILP3* and *DILP5* expression, suggesting that miR-14 serves as a critical regulatory factor of lipid metabolism by down-regulating TAG and DAG synthesis (Xu et al., 2003). Additionally, specific down-regulation of *miR-14* in IPCs of the adult *Drosophila* brain increased lipid storage, whereas down-regulation in the fat body had no effect on fat stores (Varghese et al., 2010). miR-14 was found to regulate insulin metabolism through its direct target, *sugarbabe,* which encodes a predicted zinc finger protein that negatively regulates expression of several ILP genes, including *ILP3* and *ILP5* (Varghese et al., 2010). Thus, miR-14 exerts its effect on lipid storage indirectly through inhibition of an inhibitor of ILP expression. By contrast, *miR-278*-mutants were found to possess significantly reduced TAG levels, indicating that they induce lipogenesis (Teleman et al., 2006). Indeed, brain ILPs and trehalose levels increases in *miR-278*-mutants, suggesting that miR-278 interferes with the insulin pathway, and the reduction of lipid stores in *miR-278*-mutants is an outcome of a direct action of miR-278 on brain ILPs (Teleman et al., 2006). Another miRNA, miR-277, was also found to target other ILP genes (*ILP7* and *ILP8*) in the regulation of lipid deposition and mobilization in the mosquito *A. aegypti* (Ling et al., 2017). Another miRNA, miR-33, which is derived from an intron in *SREBP*, regulates genes involved in fatty acid metabolism and insulin signaling (Gerin et al., 2010; Dávalos et al., 2011). A genetic screen aiming to identify the miRNAs leading to inhibition of body growth in *Drosophila* revealed that miR-9a also acts on insulin signaling and body growth by controlling the expression of *sNPF* (Suh et al., 2015). Thus, IPC-specific over-expression of *miR-9a* reduces the insulin signaling and body size, and loss of *miR-9a* enhances the level of sNPF (Suh et al., 2015).

Another interesting topic on ILPs is their interaction with the Store-Operated Calcium Entry system, the SOCE, which also leads to changes in the lipid metabolism. For example, chronic knockdown of *STIM* leads to hyperglycemia, impairment of insulin signaling in fat body tissue, and formation of larger LDs accompanied by up-regulation of *4EBP* and a decrease in phosphorylated AKT levels (Xu et al., 2019). In addition, loss of function of the three brain ILPs was not found to prevent the extra fat accumulation in these knockdown insects (Xu et al., 2019). In accordance with these data, the insulin-promoting gene, *CCHa2,* was upregulated, whereas insulin-inhibiting genes, *ImpL2* and *Lst*, were down-regulated upon *STIM* down-regulation (Xu et al., 2019). Notably, *CCHa2*, which is expressed in the larval fat body and gut, is induced in response to dietary glucose (Sano et al., ı2015). Thus, mutants that lack *CCHa2* or the *CCHa2R* (CCHa2 Receptor) exhibit reduced DILP2 secretion and *DILP5* expression (Sano et al., ı2015). Lst suppresses DILP2 secretion, and Lst deficiency leads to hyperinsulinemia, hypoglycemia, and excess adiposity (Alfa et al., 2015). These results suggest that obesity is an outcome of the *STIM*-knock down related insulin signaling impairment which interferes with other neuropeptides, such as CCHa2 and Lst. Notably, other yet unknown neuropeptides could be also involved in this interaction.

Insulin signaling could lead to different outcomes in larval and adult stages (Kannan and Fridell, 2013; Owusu-Ansah and Perrimon, 2014); this is likely to be related to the differences in the physiology and feeding behavior. Thus, genetic ablation of the IPCs in larval stages of *Drosophila* leads to retardation in development and an increase in carbohydrate levels in the hemolymph (Rulifson et al., 2002). However, IPC ablation in the adult *Drosophila* reduces fecundity, increases stored TAG and sugars, and lifespan (Broughton et al., 2005). The overall evidence obtained to date suggest that brain ILPs are primarily controlled

by sNPF, ecdysone, and foxO in larval stages, whereas miRNAs, foxO, and UPD2 are the major regulatory molecules involved in the transcriptional control of ILP genes in the adult stage, at least in *Drosophila* (Kannan and Fridell, 2013).

Diapause Hormone-Pheromone Biosynthesis Activating Neuropeptide (DH-PBAN)

Diapause is a developmental arrest to overcome seasonal challenges, such as winter and the absence of food, and can occur in any developmental stage depending on the species (Denlinger et al., 2005). Various insects, such as the silkworm *B. mori* and the cotton bollworm *H. armigera*, have been shown to possess a specific peptide called the Diapause Hormone (DH) to regulate the process of diapause (Hasegawa, 1957; Xu et al., 1995; Zhang et al., 2004b).

DH is produced by neurosecretory cells in the subesophageal ganglion and possesses three regions; the N-terminal region that facilitates binding of the hormone to the Diapause Hormone Receptor (DHR), the middle region with its duplicated amino acid structure for full potency, and the carboxy-terminal essential core structure for biological activity (Saito et al., 1994). The Arg^{23} and Leu^{24} in the carboxy-terminal core structure are essential for binding to the DHR, whereas Trp^{19} and Phe^{20} contribute to functional activity (Shen et al., 2018). Interestingly, the carboxy-terminal active peptide (24 amino acids) is homologous to the carboxy-terminus of Pheromone Biosynthesis Activating Neuropeptide (PBAN), which is involved in female sex pheromone biosynthesis; therefore, DH is encoded by a PBAN gene and has been named DH-PBAN (Sato et al., 1993; **Table 1**).

B. mori overwinters in embryonic diapause, whereas *H. armigera* overwinters in pupal diapause (Zhang et al., 2004a,b). In the silk moth, DH-PBAN is secreted from the mother's subesophageal ganglion and induces diapause in the eggs (Yamashita, 1996). Interestingly, while DH-PBAN induces diapause in *B. mori* (Xu et al., 1995), it reactivates metabolism in pupae and terminates pupal diapause in *H. armigera* (Xu and Denlinger, 2003; Zhang et al., 2004a,b). Therefore, DH-PBAN activation may lead to different outcomes for diapause initiation or termination.

DH-PBAN has been shown to affect lipid metabolism. For example, lipid content of eggs is slightly elevated by DH-PBAN (Hasegawa and Yamashita, 1965), however, this effect is likely to be a secondary consequence of the hormone's effect on carbohydrate metabolism. DH-PBAN has been also reported to regulate the expression of genes involved in lipid metabolism in *H. armigera* (Majerowicz and Gondim, 2013).

Recent studies have revealed new insights into the DH-PBAN pathway. Binding of DH-PBAN to DHR induces activation of ERK phosphorylation through the signal transducer G-protein-linked PLC, PKC, and PI_3K pathways (Jiang et al., 2016). More specifically, DHR is coupled with PLC via Gαq protein, leading to the production of DAG and the second messenger IP_3 (Jiang et al., 2016). DAG directly activates PKC, and IP_3–mediated release of calcium from endoplasmic reticulum to cytosol leads to an indirect activation of PKC (Litosch, 2015). It would be interesting to further examine the SOCE components in DH-PBAN-controlled diapausing events.

Short Neuropeptide F (sNPF) and Neuropeptide F (NPF)

Peptides of the Short Neuropeptide F (sNPF) and Neuropeptide F (NPF) (NPF) family are widely distributed throughout the Arthropoda phylum. NPF is functional homolog of mammalian Orexigenic Neuropeptide Y; the sNPF system is conserved across protostomes, but is not present in vertebrates (Fadda et al., 2019). Both systems are involved in the coordination of feeding behavior and metabolism (Lee et al., 2004; Nässel and Wegener, 2011; Mirabeau and Joly, 2013). Both systems also share common structural features; however, they are evolutionary distinct. The sNPF is characterized by an "M/T/L/FRF(W)" amide, and the NPF by an "RXRF(Y)" amide carboxy-terminal motif (Fadda et al., 2019). Most of the studies on these peptides in relation to lipid metabolism are on the sNPF.

The first insect sNPF was reported from *L. decemlineata* (Spittaels et al., 1996), followed by the discovery of sNPFs from the locust *S. gregaria* (Schoofs et al., 2001) and the fruit fly (Lee et al., 2004). The precursor is around 281 amino acid in length and the amidated peptide consists of 6–19 amino acids and possesses a carboxy-terminal "RLRF" sequence (Wegener and Gorbashov, 2008; Fadda et al., 2019; **Table 1**). sNPFs bind to the receptors (sNPFR), which are also from the rhodopsin-like GPCRs superfamily, like other neuropeptide receptors.

sNPFs are typically expressed by brain lateral neurosecretory cells, as well as in the midgut (Reiher et al., 2011), hindgut (Caers et al., 2016), antennae, Malpighian tubules, and ovaries (Jiang et al., 2017; **Table 1**). In most species, multiple sNPF isoforms exist and are derived from a single peptide precursor. For example, the sNPF precursor generates four sNPF isoforms in *Drosophila* (Vanden Broeck, 2001; Baggerman et al., 2002), the tsetse fly, *Glossina morsitans morsitans* (Caers et al., 2016) and *B. dorsalis* (Jiang et al., 2017), three sNPF isoforms in *B. mori* (Yamanaka et al., 2008) and *A. aegypti* (Veenstra, 1999), two peptides in *L. decemlineata* (Spittaels et al., 1996) and a single peptide in *R. prolixus* (Ons et al., 2011).

The main function of sNPFs is to regulate feeding behavior (Lee et al., 2004; Dillen et al., 2013); however, they may also be involved in locomotor activity (Kahsai et al., 2010), circadian rhythm (Johard et al., 2009; Geo et al., 2019), appetitive olfactory behavior (Root et al., 2011; Jiang et al., 2017), sleep homeostasis (Chen et al., 2013) and release of other hormones such as ILPs and AKH (Nässel et al., 2008). Regarding their primary role, sNPFs promote food intake and feeding in *Drosophila*, therefore, they could be considered as hunger hormones (Lee et al., 2004; Root et al., 2011). Over-expression of *sNPF* produces larger flies (Lee et al., 2004). sNPF also induces feeding in *B. mori* (Nagata et al., 2011). Presence of sNPF during feeding and its absence in the diapausing stage in *L. decemlineata* also suggests a positive correlation between feeding and sNPF activity (Huybrechts et al., 2004). On the other hand, inhibitory effects of sNPFs on feeding have been demonstrated in *A. aegypti*

(Nässel et al., 2008), *S. gregaria* (Dillen et al., 2013), and *Culex quinquefasciatus* (Christ et al., 2018). sNPF has been shown to inhibit the serotonin-induced peristaltic contractions and ion transport in the anterior stomach of *A. aegypti* larvae (Onken et al., 2004). sNPF also inhibits the release of digestive enzymes in the cockroach *Periplaneta americana*, indicating an inhibitory effect of sNPF on digestion (Mikani et al., 2012). The inhibitory effect on the digestive process might be one of the reasons for sNPF-reduced feeding behavior. Notably, the sNPF level in the antennal lobes drops following a blood meal, indicating an inhibition of odor-mediated host seeking behavior (Onken et al., 2004). In *S. gregaria*, RNAi-mediated silencing of the sNPF precursor leads to an increase in food intake (Dillen et al., 2013, 2014). These findings suggest that there is not an obvious correlation between sNPF and feeding in the class of insecta and that sNPF can act as a stimulatory or inhibitory factor on feeding.

sNPF also affects lipid metabolism, for example, down-regulation of *sNPF* reduces food intake (Lee et al., 2004), increases starvation sensitivity (Kahsai et al., 2010), and causes lean flies in *Drosophila* (Baumbach et al., 2014a). A recent study also revealed that knockdown of *sNPF* in circadian clock neurons reduced TAG level (Geo et al., 2019). In accordance with this, over-expression of *sNPF* causes hyperphagia and body fat accumulation in *Drosophila* adults (Baumbach et al., 2014a); this effect could be via the effect on ILPs. Thus, *sNPF* expression is up-regulated in starved flies, where insulin levels are low, leading to initiation of food search behavior (Root et al., 2011). The Mnb/Dyrk1a kinase (Minibrain/Dual-specificity Tyrosine Phosphorylation-regulated Kinase 1A), which is localized to *sNPF*-expressing neurons and activates foxO, was found to be the most pronounced and up-regulated gene after sNPF administration, further showing a connection between sNPF and lipolysis (Hong et al., 2012). In support of this, activation of Mnb/Dyrk1a kinase occurs through PKA and CREB which also induce lipolysis (Hong et al., 2012). *CREB* is up-regulated by its binding partner cAMP-regulated transcription co-activator (CRTC) to induce the expression of *sNPF*, resulting in an increased starvation resistance (Shen et al., 2016). Therefore, sNPF is a direct target of CREB and CRTC. On the other hand, sNPF has been demonstrated to stimulate the production of ILPs in larval and adult IPCs in *Drosophila* (Lee K.S. et al., 2008; Kapan et al., 2012). In this manner, sNPF activates extracellular activated receptor kinases in IPCs, which leads to production of insulin (Lee K.S. et al., 2008; Kapan et al., 2012). More specifically, the fat body of *sNPF*-mutant *Drosophila* had down-regulated *AKT* expression and nuclear-localized foxO, up-regulated translational inhibitor *4E-BP* and reduced cell size with elevated glucose levels (Lee K.S. et al., 2008). On the other hand, knockdown of *sNPF* in the dorsal lateral peptidergic neurons results in diminished *DILP2* and *DILP5* expression leading to increased starvation resistance and increased levels of carbohydrates and lipid (Lee K.S. et al., 2008). These findings are indeed contradictory to the proposed role of sNPF as a hunger signal in *Drosophila*, however, up-regulation of DILP genes by sNPF might relate more to regulating metabolism and growth, rather than feeding (Lin et al., 2019).

The first insect NPF identified from *Drosophila* consisted of 36 amino acids with a characteristic "RVRF" carboxy-terminal sequence (Brown et al., 1999; **Table 1**). The carboxy-terminal tyrosine residue in vertebrate NPYs is replaced with a phenylalanine residue; therefore, these peptides were designated as "NPF" (Maule et al., 1991). The NPF precursor is around 102 amino acid in length and the active amidated peptides typically consist of at least 28 amino acids and share the common "RXRF/Y" carboxy-terminal motif (Fadda et al., 2019; **Table 1**). NPF is localized in the midgut and brain in *Drosophila* (Brown et al., 1999; Lee et al., 2006). Similarly, NPF was detected in the midgut and the subesophageal ganglion in *A. aegypti* (Onken et al., 2004; **Table 1**).

The *Drosophila* NPF has been shown to be important for adult longevity, wakefulness and feeding behavior, modulation of odor-aroused appetitive behavior, and reproduction (Gendron et al., 2014; Chung et al., 2017; Harvanek et al., 2017). However, most of the studies focused on the activation of NPFs by sugars, in particular in *Drosophila* larvae. Interestingly, the causative agent for the NPF activation is not sugar ingestion itself, instead taste perception induces the *NPF* expression (Shen and Cai, 2001). Additionally, *NPF* expression was found to be high in young larvae, whereas it was low in older larvae that avoid food (Wu et al., 2003). The interaction between the NPF and sugar feeding may indicate that insulin might also have an effect on *NPF* expression. In a recent study, NPF has been shown to function downstream of insulin signaling to regulate feeding in *Drosophila* larvae (Fadda et al., 2019). NPF not only affects feeding, but also regulates food choice as opposed to sNPF (Wu et al., 2005a,b). In support of this, NPF induces feeding on lower quality or noxious foods in starved fruit flies, whereas NPF-induced feeding response toward noxious food is inhibited in satiated fruit flies (Wu et al., 2005b). Furthermore, the inhibitory effect was found to be insulin-related. In *S. gregaria*, injection of NPF increases food intake and weight, while silencing *NPF* decreases food intake and weight, suggesting a stimulatory role of NPF in feeding (Van Wielendaele et al., 2013). Similarly, knockdown of *NPF* resulted in a reduction of food intake and growth in *B. mori*, also suggesting a role for NPF as a positive regulator of feeding (Deng et al., 2014). A recent study indicated that the enzymatic cofactor tetrahydrobiopterin (also known as BH4) inhibits NPF release, and might be one of the signals that transmit the message of energy status from the fat body to the brain (Kim et al., 2017).

Evidence on the involvement of NPF in lipid metabolism is limited. Activation of the *NPF*-expressing neurons has been shown to decrease TAG levels in adult *Drosophila* (Chung et al., 2017). Another study revealed that adult male fruit flies exposed to female sex pheromone displayed reduced TAG levels in parallel to an increase in the levels of NPF in the brain, however, inhibition of *NPF*-expressing neuron activity and down-regulation of *NPF* reverses these effects (Gendron et al., 2014). Based on the limited evidence in these studies and, one could say that NPF activation leads to reduced TAG levels.

Allatostatin-A (AstA)

Allatostatin-A (AstA) is mainly expressed in the brain and gut (Veenstra et al., 2008; Hentze et al., 2015) and was originally

reported to be involved in the inhibition of JH synthesis in the cockroach *Diploptera punctata* (Yoon and Stay, 1995). However, subsequent studies revealed that AstA does not regulate JH in all insects and is involved in the inhibition of the starvation-induced feeding behavior in *Drosophila* (Hergarden et al., 2012; Hentze et al., 2015; Chen et al., 2016). Furthermore, activation of *NPF*-expressing neurons suppresses the inhibitory influence of AstA neuron activation on feeding, leading to increased feeding (Hergarden et al., 2012).

AstA regulates AKH and ILPs through its galanin-like receptor "DAR2 (*Drosophila* Allatostatin Receptor 2" that is expressed in AKH- and ILP-producing cells (Hentze et al., 2015; Nässel and Vanden Broeck, 2015; **Table 1**). Thus, both insulin and AKH signaling are stimulated by AstA via DAR2; silencing *DAR2* reduced both ILPs and AKH and increased starvation resistance (Buch et al., 2008; Nässel and Vanden Broeck, 2015; Hentze et al., 2015). Furthermore, Drosophila lacking *AstA* accumulate high lipid levels, indicating that reduced AKH and ILP signaling also promotes lipid accumulation in the fat body (Hentze et al., 2015). Notably, *AstA* and *DAR2* expression differ according to the diet and gender (Hentze et al., 2015). These data suggest that AstA regulates the balance between AKH and ILPs and is important for the maintenance of nutrient homeostasis in Drosophila.

Corazonin (Crz)

Corazonin (Crz) is a neuropeptide present as a 154 amino acid precursor and 11 amino acid of mature peptide (Choi et al., 2005; **Table 1**). It is produced by the brain lateral neurosecretory cells (Duan-Şahbaz and Ýyison, 2018). Crz was originally isolated as a cardioactive factor in *P. americana* (Veenstra, 1989). Subsequent studies revealed that it is also involved in the regulation of the ecdysis initiation (Kim et al., 2004), melanization (Žitòan and Daubnerová, 2016), stress responses (Kubrak et al., 2016), sperm transfer and copulation (Tayler et al., 2012), social behavior and caste identity (Gospocic et al., 2017), and ethanol sedation (Sha et al., 2014).

Crz is evolutionarily related to AKH (Veenstra, 1994) and expression of *Crz* is reduced by 50% in *AKH*-mutants (Choi et al., 2008). Additionally, ablation of *Crz* decreases trehalose levels (Choi et al., 2008; Lee K.S. et al., 2008). In this manner, Crz might modulate AKH-cell functions through neuronal pathways or trehalose levels by acting as a hormone on adipocytes and AKH-producing cells (Choi et al., 2008). On the other hand, activation of Crz-producing neurons leads to increased food uptake in adult flies (Lee G. et al., 2008; Zhao et al., 2010). The *Drosophila* Crz Receptor (CrzR) is also related to the family of AKH receptors; however, CrzR is highly selective for Crz (Park et al., 2002; Kim et al., 2004). Notably, intermediates similar to both AKHR and CrzR have been reported for a neuropeptide named as ACP (AKH/Corazonin-related Peptide) that is also structurally intermediate between AKH and Crz (Hansen et al., 2010). *Crz* is expressed primarily by dorsolateral peptidergic neurons, as well as abdominal ganglia, whereas *CrzR* is expressed in adult salivary glands and fat body (Sha et al., 2014; Kubrak et al., 2016).

Crz has been shown to affect lipid and carbohydrate metabolism (Kapan et al., 2012; Kubrak et al., 2016; Gáliková et al., 2017). Knockdown of *CrzR* in the fat body leads to a decrease in TAG levels and food intake, and an upregulation of *bmm* only after starvation (Kubrak et al., 2016). However, ablation or inactivation of the *Crz*-expressing dorsolateral peptidergic neurons in the brain resulted in increased TAG levels, suggesting that Crz decreases energy reserves (Zhao et al., 2010). Similarly, knockdown of *Crz* in dorsolateral peptidergic neurons in the *Drosophila* brain increases TAG levels and circulating glucose (Kapan et al., 2012). Knockdown of *CrzR* in the fat body or in the periphery also increases glucose levels in the hemolymph, but only in response to starvation as fed-flies did not show any altered levels of circulating glucose upon *CrzR* silencing (Kubrak et al., 2016). On the other hand, production of brain ILPs varies in response to diminished CrzR. For example, *ILP5* expression increased only in fed-flies, whereas *ILP3* expression decreased both in fed and starved flies, but *ILP2* was not found to change (Kubrak et al., 2016). Notably, expression of all three ILP genes decreased upon a longer period of starvation (36 h), but there was no significant change at the peptide level upon *CrzR* knockdown (Kubrak et al., 2016). These findings suggest that the effect on carbohydrate metabolism is mediated by Crz signaling to the periphery and this effect is stronger during stress conditions, such as starvation. Additionally, fat-body-derived humoral signals are affected by Crz activation of adipocytes. As a final point, the effect of Crz on lipid metabolism is likely to be indirect and occurs through insulin or another signaling system.

Leucokinin (Lk)

Leucokinin (Lk) is a myotropic neuropeptide and initially identified as a neurohormone that plays an important role in water and ion homeostasis by regulating fluid secretion in the Malpighian tubules and hindgut motility (Holman and Cook, 1983; Schoofs et al., 1992; **Table 1**). Lk is also involved in meal size regulation, feeding, metabolic rate, post-feeding physiology and behavior, regulation of stress, water homeostasis, locomotor activity and sleep (Al-Anzi et al., 2010; Zandawala et al., 2018; Yurgel et al., 2019). The first Lk was isolated from the cockroach *Leucophaea maderae* (Holman et al., 1986), followed by identification of other Lks from *L. migratoria* (Schoofs et al., 1992), *A. aegypti* (Veenstra, 1994) and *Culex salinarius* (Hayes et al., 1989). The Lks consist of about 160 amino acids and the active peptides vary from 6 to 15 amino acids in length and are characterized by a carboxy-terminal pentapeptide motif "FXXWG" amide, which is essential for biological activity (Radford et al., 2002; **Table 1**).

Drosophila has a single Lk gene encoding the longest known leucokinin, "Drosokinin" and a Leucokinin Receptor (LkR) has been also identified (Radford et al., 2002). There is no known mammalian counterpart for Lk, but LkR is homologous to the vertebrate "Tachykinin Receptor, TkR" (Radford et al., 2002). *Lk* and *LkR* are expressed in small subsets of neurons in the brain, IPCs and ventral ganglia, and *LkR* is also expressed in the foregut, hindgut, Malpighian tubules and genital tracts (Radford et al., 2002; Al-Anzi et al., 2010; Zandawala et al., 2018).

During feeding, *Lk*- and *LkR*-mutant adult flies consume larger meals, but exhibit reduced long-term food intake (Al-Anzi et al., 2010; Liu Y. et al., 2015; Zandawala et al., 2018). The activity

of Lk neurons is modulated by feeding with reduced activity in response to glucose and increased activity under starvation conditions (Yurgel et al., 2019). Thus, the effect of Lks on lipid metabolism is indirect and likely occurs through insulin signaling. In support of this, *Lk*- and *LkR*-mutants or flies with targeted knockdown of *LkR* in IPCs displayed altered expression of ILP genes, increased *DILP2* and *DILP3* in IPCs, and increased starvation resistance, suggesting a role for Lk in regulation of insulin signaling (Zandawala et al., 2018). Based on this data, Lk might act as a starvation-induced lipolytic agent; however, no study has examined this to date.

CCHamide-2 (CCHa2)

CCHamide-2 (CCHa2) is also a typical orexigenic brain-gut peptide without a known counterpart in mammals (Lin et al., 2019). *CCHa2* is expressed in the brain; as well as fat body and the midgut (Ren et al., 2015; Sano et al., 2015; **Table 1**). *CCHa2* expression decreases in response to starvation and levels can be rescued by feeding on glucose or yeast (Sano et al., 2015). In accordance with this, *Drosophila* larvae and adult flies lacking CCHa2 show reduced feeding activity, indicating the stimulatory effect of CCHa2 on feeding (Ren et al., 2015).

CCHa2 binds to its receptor, CCHamide-2 Receptor (CCHa2R), in IPCs to promote insulin signaling (Ren et al., 2015; Sano et al., 2015). Thus, disruption of CCHa2R was found to reduce ILP concentrations and larval growth, which is consistent with late pupariation observed in the *CCHa2*-mutants (Sano et al., 2015). Gáliková et al. (2017) suggested that the repression of the central ILPs by AKH might be at least partially mediated by the CCHa2. Overall, the effect of CCHa2 on lipid metabolism might be lipogenic; however, this has not been specifically reported. Nevertheless, the effect occurs indirectly through interaction with insulin signaling.

Tachykinins (Tk)

Gut peptide hormones play crucial roles in systemic lipid homeostasis (Song et al., 2014). The most abundant gut hormone is tachykinin (Tk), which produces six mature peptides (Tk1-Tk6) in Drosophila (Veenstra et al., 2008; Reiher et al., 2011; **Table 1**). Many other invertebrates, and even humans have also Tks. Notably, Drosophila Tks are also produced in the central nervous system, and brain Tks are involved in locomotor activity and olfactory responses (Winther et al., 2006; Birse et al., 2011; Reiher et al., 2011; **Table 1**). However, only gut Tks have been specifically shown to repress intestinal lipogenesis, which occurs via the G-protein-coupled Tachykinin Receptor (*TkR*) that is also expressed in gut (Song et al., 2014). TAG levels were dramatically increased in the midgut, fat body and hemolymph in the absence of gut Tks (Song et al., 2014). In accordance with this observation, genes encoding the intestinal lipase Magro, and the two key enzymes of lipogenesis, FAS and ACC, were all found to be up-regulated when Tk production was reduced, also confirming that Tk deficiency promotes midgut lipogenesis (Song et al., 2014). Notably, the suppressive role of Tks on lipogenesis occurs through repression of SREBP, a transcription factor that triggers lipogenesis. On the other hand, expression of the foxO target genes, *4EBP* and *InR*, in the midgut was not affected by removal of Tks (Song et al., 2014). However, knockdown of *TkR* in *Drosophila* induces expression of *ILP2* and *ILP3* in fed flies, and *ILP2* in starved flies, whereas expression of *ILP3* was reduced in starved flies (Birse et al., 2011) suggesting that gut Tks may affect insulin signaling in the midgut.

Cytokines (Adipokines)

Fat body adipocytes secrete protein hormones termed cytokines (also known as adipokines). One hormone in this group is the leptin-like cytokine, the UPD2 (Rajan and Perrimon, 2012; **Table 1**). UPD2 indeed acts on brain IPCs, which release ILPs under the control of the brain gamma-aminobutyric acid (GABA) (Nässel and Vanden Broeck, 2016). IPCs also possess metabotropic GABA receptors (Enell et al., 2010) and are hyperpolarized by GABA (Rajan and Perrimon, 2012). This GABAergic inhibition can be disengaged by UPD2 (Rajan and Perrimon, 2012). Notably, specific perturbation of UPD2 function in the fat body alters energy metabolism and inhibits development (Rajan and Perrimon, 2012). UPD2 release from the fat body is triggered by elevated levels of lipid or carbohydrate in the hemolymph, thus, UPD2 senses the fed state and regulates secretion of brain ILPs (Rajan and Perrimon, 2012). Therefore, in the fed state, circulating UPD2 binds to its transmembrane receptor, "domeless," which activates the JAK/STAT signaling in the GABAergic neurons, blocking GABA release and diminishing IPC hyperpolarization resulting in secretion of ILPs (Brown et al., 2001; Rajan and Perrimon, 2012; Lin et al., 2019). Thus, flies with *UPD2* knockdown in fat body exhibited increased ILP accumulation in the brain under a fed state (Rajan and Perrimon, 2012). By contrast, IPCs are inhibited by GABAergic neurons that hyperpolarize IPCs in the starved state, thus, *UPD2* is down-regulated in starving adults. In accordance with this, fat body-specific knockdown of *UPD2* resulted in hyperglycemic, lean flies and larvae with considerably reduced TAG and increased circulating sugar levels. It is noteworthy to mention that *UPD2*-mutant larvae had dramatic accumulation of LDs in oenocytes, suggesting an opposite function for oenocytes and adipocytes in lipid metabolism (Rajan and Perrimon, 2012; Lin et al., 2019). Thus, UPD2 suppresses stored fat breakdown in oenocytes during starvation (Rajan and Perrimon, 2012; Lin et al., 2019). *UPD2* was found to be downregulated in the fat body after *CrzR*-knockdown, suggesting UPD2 also serves as a messenger between the fat body and the brain by acting on brain ILPs. On the other hand, Unpaired 1 (UPD1), another fly leptin-like peptide, fulfills the roles of UPD2 upon *UPD2* knockdown in adults (Beshel et al., 2017). Unlike UPD2, which is secreted from fat body, UPD1 is produced by a small cluster of neurons in the brain (**Table 1**).

Another peptide belonging to this group is the Adiponectin (Adipo), which regulates glucose levels and fatty acid breakdown in mammals. No obvious Adipo homolog has been identified in *Drosophila*; however, an Adiponectin Receptor (AdipoR) with high homology to the human Adiponectin Receptor 1 has been discovered (Kwak et al., 2013; Laws et al., 2015). The *Drosophila AdipoR* is expressed in the IPCs of larval and adult brains (Kwak et al., 2013). Inhibition of AdipoR leads to elevated sugar levels in the hemolymph, TAG levels in whole body, and ILP2 accumulation in IPCs (Kwak et al., 2013). In contrast, the level

of circulating ILP2 and insulin signaling were reduced in the fat body (Kwak et al., 2013). A subsequent study revealed the requirement of AdipoR in germline stem cell maintenance in the *Drosophila* ovary (Laws et al., 2015). In brief, AdipoR modulates insulin secretion and lipid metabolism. Additionally, Adipo signaling is intrinsically required for stem cell maintenance independently of insulin signaling (Laws et al., 2015).

Limostatin (Lst)

Limostatin (Lst) is a known suppressor of insulin production and expressed by AKH-producing neurons in the corpora cardiaca and fat body, in particular during starvation (Alfa et al., 2015; **Table 1**). Thus, *Lst*-mutant flies were found to be hypoglycemic with increased levels of *DILP2*, *DILP3*, and *DILP5* mRNA (Alfa et al., 2015). Additionally, Lst levels decreased in an AKH-deficient background; however, *AKH* over-expression did not significantly increase *Lst* expression. In brief, Lst leads to lipolysis; however, this effect is an outcome of insulin suppression.

ROLE OF PEPTIDE HORMONES IN LIPID METABOLISM-RELATED BIOLOGICAL EVENTS

In this section, the peptide hormones involved in lipid metabolism-related biological events together with their role(s) in these events are examined.

Reproduction

Lipids plays a critical role in reproductive physiology and are mobilized as the major metabolic source during reproduction (Hansen et al., 2013), therefore, peptide hormones involved in lipid metabolism have also essential roles in reproduction. AKH, ILPs, and sNPF are the major peptide hormones involved in lipid metabolism in relation to reproduction.

Role of AKH signaling in insect reproduction has been studied in several insects. For example, AKHR knockdown led to obese females incapable of utilizing their lipid reserves during pregnancy for milk production *G. m. morsitans* (Attardo et al., 2012). Such silencing also resulted in delayed oocyte development with a reduction of 20% in fecundity (Attardo et al., 2012). Additionally, knockdown of the *AKH*/*AKHR*-mediated lipolytic system affected larvigenesis as suppression of *AKHR* expression lowered production (offspring per female). AKH also inhibits egg-laying indirectly in *G. bimaculatus* due to the reduction in fat body lipid stores by AKH during vitellogenesis (Lorenz, 2003). In *B. dorsalis*, *AKHR* silencing was found to lower lipolytic activity, delay oocyte maturation, and reduce fecundity (Hou et al., 2017). The inability of fat body to accumulate adequate nutrient reserves after AKH exposure has been also shown in the locust, *S. gregaria* (Gokuldas et al., 1988), and the mosquito, *A. aegypti* (Ziegler, 1997). The majority of the stored lipid in the oocytes is TAG and any failure to TAG accumulation and mobilization would affect fecundity and oocyte development, therefore, the increased rate of lipolysis might negatively affect reproduction (Lu et al., 2018b). Notably, AKH also affects sexual courtship activity, as was shown in *B. dorsalis* (Hou et al., 2017). There are also reports indicating no noticeable effects of AKH on reproduction as genetic manipulation of *AKH* in adult *Drosophila* flies did not cause any negative outcome in the reproductive capabilities and courtship behavior of flies (Lee and Park, 2004).

ILPs have been also shown to affect reproduction. CRISPR/Cas9-mediated depletion of *ILP7* and *ILP8* leads to reproductive defects related to lipid homeostasis and ovarian development (Ling et al., 2017), and ILP7 is involved in egg-laying behavior (Yang et al., 2008). Fecundity was found to be reduced in *Drosophila* mutants lacking *ILP2* (Grönke et al., 2010). In a recent study, knockdown of insulin signaling genes *Chico*, *TOR* and *Slimfast*, a membrane transporter of amino acids that is involved in Target of Rapamycin Complex 1 (TORC1) signaling, was found to reduce the number of ootheca in *B. germanica* (Li et al., 2019). In another recent study, the c-Jun N-Terminal Kinase (JNK)-initiated insulin-myc signaling loop was shown to promote mitochondrial respiration and biogenesis in *Drosophila* ovary, suggesting the insulin-myc signaling is important for mitochondrial biogenesis in the ovary (Wang et al., 2019). A miRNA acting on insulin signaling, miR-277, has also been shown to be important in the reproduction of the mosquito *A. aegypti* as CRISPR/Cas9 deletion of *miR-277* led to failures in ovary development (Ling et al., 2017).

NPF and sNPF also exhibit several effects on insect reproduction. In locusts (Cerstiaens et al., 1999; Schoofs et al., 2001) and the fruit fly (Mertens et al., 2002), sNPF stimulates ovarian development (De Loof et al., 2001). On the other hand, the level of NPF was been found to be elevated in male fruit flies exposed to the sex pheromone of females, while TAG levels decreased (Gendron et al., 2014; Harvanek et al., 2017). However, these effects may be non-specific and do not have to be due to direct interactions with reproductive physiology.

Flight

Many insects, in particular long distance flying insects, use lipids as the primary fuel for flight (Weis-Fogh, 1952). In this regard, AKH is a main determinant of successful energy demand (Ziegler and Schulz, 1986). During the first few minutes of flight, octopamine is released, inducing the first release of DAG from the fat body (Orchard et al., 1993), however, the subsequent, more prolonged phase of TAG mobilization occurs through the action of AKHs (Arrese and Soulages, 2010). As a result, the concentration of DAG in the hemolymph increases and constitutes the principal fuel for flight.

The effect of AKH on insect flight has been mostly studied in two locusts such as, *L. migratoria* and *S. gregaria*, and a moth, *M. sexta* (Van der Horst and Ryan, 2012). AKH peptides originate from pre-prohormones that are translated from different mRNAs and eventually enzymatically processed. Binding of the AKHs to their plasma membrane GPCRs on the fat body cells is the primary step in the induction of signal transduction events that lead to mobilization of lipids to be used by muscles as a fuel for flight (Vroemen et al., 1998). Such events require involvement of various other molecules, such as cAMP, PKA and IP_3 (Van Marrewijk et al., 1996; Vroemen et al., 1997), lipases (Arrese and Wells, 1994; Ogoyi et al., 1998), lipophorins (Izumi et al., 1987; Van der Horst and Rodenburg, 2010), fatty acid binding

proteins (Haunerland and Chisholm, 1990), and calcium ions (Van Marrewijk et al., 1991).

Diapause

Lipid reserves are the most important resources for insects to meet energy demand during the dormancy state known as diapause (Hahn and Denlinger, 2011). Insects accumulate lipid reserves prior to diapause and a failure to accumulate adequate amounts of lipids leads to incomplete diapause and possibly death (Toprak et al., 2014b).

AKHs do not contribute to diapause-associated alterations in metabolism (Hahn and Denlinger, 2011). AKH is produced in response to AMPK, which leads to the release of DAG into hemolymph from TAG stores in the fat body via a cyclic cAMP and calcium signaling cascade during diapause maintenance (Sinclair and Marshall, 2018). For example, AKH has been shown to release approximately twice as much lipid into the hemolymph in diapausing adult females of the firebug *Pyrrhocoris apterus* versus the non-diapausing counterparts, suggesting diapausing adults have greater sensitivity to lipid-mobilization by AKH (Socha and Kodrik, 1999).

Insulin signaling also plays an important role in the regulation of diapause (Sim and Denlinger, 2008). This is not surprising as insulin is central to energy storage and suppresses the lipolytic action and nuclear translocation of foxO (Baker and Thummel, 2007). The lipid accumulating effect of insulin associated with adult diapause has been shown in *Drosophila* (Tatar and Yin, 2001), and *C. pipiens* (Sim and Denlinger, 2008). Additionally, silencing *InR* in non-diapausing females inhibits ovary development, which simulates the diapause state (Sim and Denlinger, 2008). As expected, the insulin effect occurs mainly during feeding, therefore, before the initiation of the diapause. For example, adult females of *C. pipiens* increase feeding with sugar instead of blood in the prediapause period and accumulate much greater lipid reserves compared to non-diapausing counterparts (Robich and Denlinger, 2005). In this manner, specific ILPs, such as ILP1, contribute to the diapause regulation-related lipid accumulation in *C. pipiens* (Sim and Denlinger, 2009). Notably, JH and ecdysone interfere with insulin signaling in terminating diapause (Denlinger et al., 2005).

NPF and sNPF might also affect lipid metabolism-related diapause regulation as they are involved in feeding behavior, nutritional homeostasis, and insulin signaling (Brown et al., 1999; Wu et al., 2003; Huybrechts et al., 2004). For example, overexpression of *NPF* leads to prolonged feeding in *Drosophila* larvae (Wu et al., 2003; Chung et al., 2017), therefore, *NPF*-mutant larvae feed less. This induces insulin signaling and affects lipid sources permitting preparation for diapause (Fadda et al., 2019). As mentioned before, sNPF is also involved in feeding behavior. In Colorado potato beetles, feeding adults were found to possess sNPF; however, diapausing beetles lack sNPF (Huybrechts et al., 2004). The authors speculated that sNPF could play a role in the adult diapause process and possibly contribute to prediapause shifts in feeding behavior associated with lipid accumulation (Huybrechts et al., 2004).

DH/PBAN is another peptide hormone regulating diapause, in particular during embryonic diapause in several insects (Yamashita, 1996). The best known example for the involvement of DH/PBAN in diapause is the one that occurs in *B. mori* (Sato et al., 1993; Xu et al., 1995). The DH is produced by female adults during summer and induces diapause in developing eggs that would otherwise hatch and begin developing during the unfavorable autumn and winter months (Klowden, 2007). The induction of diapause by DH occurs through the stimulation of trehalase activity in the developing embryos, which leads to generation of glycogen and eventually glycerol and sorbitol which are necessary for diapause in these eggs (Su et al., 1994). The decline in sorbitol by the end of diapause leads to development of the embryo (Horie et al., 2000). Other molecules, such as dopamine and ecdysteroids, might also affect the embryonic diapause (Noguchi and Hayakawa, 2001; Denlinger, 2002).

Starvation

Lipids are the primary fuel consumed during starvation stress (Marron et al., 2003). The most important peptide to mobilize lipid during starvation is AKH as starvation induces AKH release into the hemolymph to signal hunger (Kim and Rulifson, 2004). Under fasting conditions, AKH induces utilization of stored energy by stimulating lipolysis, glycogenolysis and trehalose release through activation of cAMP signaling in the fat body (Kim and Rulifson, 2004; Bharucha et al., 2008). For example, injection of AKH into adult insects, such *L. migratoria* and *M. sexta,* stimulates the formation of DAG (Gäde and Beenakkers, 1977; Shapiro and Law, 1983). In *B. dorsalis*, *AKHR* was found to be up-regulated significantly upon starvation (Hou et al., 2017). In addition, knockdown of *AKHR* resulted in high levels of whole body lipids (obesity) at death, indicating an inability to mobilize lipid reserves during starvation (Attardo et al., 2012; Choi et al., 2015). This is likely due to the inability of flies to utilize lipid stores under starvation conditions (Grönke et al., 2007). In line with this, starvation was found to significantly induce the expression of *AKH* and *AKHR* also in the brown planthopper, *Nilaparvata lugens* (Lu et al., 2018a). Additionally, *AKHR* silencing decreased DAG levels in the hemolymph and increased TAG levels in the fat body, whereas AKH injection led to a critical accumulation of DAG in the hemolymph and a severe reduction of TAG content in the fat body. In addition, knockdown of *AKHR* resulted in prolonged lifespan and high levels of whole-body TAG, indicating an inability to mobilize TAG reserves during starvation. This is also similar to that reported for tsetse fly (Attardo et al., 2012). It is noteworthy that increased DAG levels in hemolymph during starvation could be independent of AKH activity as other lipolytic factors, such as bmm or carbohydrate metabolism (trehalose levels), could affect the rate of lipolysis (Heier and Kühnlein, 2018; Zhou et al., 2018).

Drosophila has been proposed as a good model to study the biochemical background of starvation (Gibbs and Reynolds, 2012). Starvation resistance is linked to lipid content in several *Drosophila* species (van Herrewege and David, 1997; Bharathi et al., 2003). Lipids stored in the fat body of *Drosophila* are consumed rapidly upon starvation (Zinke et al., 1999; Lee and Park, 2004). Various proteins, such as LSD1 and bmm, are activated by AKH-dependent phosphorylation to initiate

lipolysis to overcome the starvation stress (Canavoso and Wells, 2001; Grönke et al., 2007). Other lipolytic agents, such as the Hormone-Sensitive Lipase (HSL), is also likely to be involved in starvation-induced lipolysis as the HSL has been shown to be translocated to LDs during starvation in *Drosophila* (Kühnlein, 2012). The role of AKH in HSL secretion is a gray area and requires further studies. On the other hand, AKH has been shown to act as a metabolic stimulator causing hyperlipemia, an abnormally high concentration of lipids in the hemolymph (Lee and Park, 2004). Furthermore, starved flies devoid of AKH neurons lack starvation-induced hyperactivity and displayed strong resistance to starvation-induced death with a longer life span (Gáliková et al., 2015; Sajwan et al., 2015; Zemanová et al., 2016). These mutants were also not able to mobilize lipids efficiently and, therefore, do not utilize these reserves rapidly. In another study, Mochanová et al. (2018) reported that *AKH*-mutant females were more resistant to starvation with a longer life span compared to males. On the other hand, absence of AKH has been also shown to increase survival rate during starvation (Gáliková et al., 2015; Sajwan et al., 2015; Zemanová et al., 2016). However, knock down of the SOCE molecule, *STIM* that is involved in calcium transport leads into reduced AKH levels and life span, and abnormal lipid mobilization profile under starvation (Xu et al., 2019).

The interaction between the AKH and LKB1-SIK3 signaling and HDAC4 localization has been examined in terms of the starvation response in *Drosophila* (Choi et al., 2015). As already mentioned, PKA inhibits SIK3 via phosphorylation, which leads to the translocation of HDAC4 from the cytosol into the nucleus to activate foxO, resulting in lipolysis (**Figure 1**). In this manner, fasting inhibits or reduces the kinase activity of LKB1 on SIK3, and induces HDAC4 nuclear localization, which leads to foxO activation and up-regulation of *bmm* (Choi et al., 2015). In accordance with these findings, SIK3 Thr^{196} phosphorylation by LKB1 has been found to be reduced during fasting and when *AKH* was over-expressed compared to that in feeding conditions. Furthermore, HDAC4 was found to be localized to both cytoplasm and nuclei in *AKHR*-mutant larvae fasting for short periods (4 h); however, HDAC4 accumulated only in the nuclei of the fat body cells in mutants fasting for prolonged periods (∼10 hr) (Choi et al., 2015). This finding indicates the presence of mechanisms for HDAC4 localization during prolonged fasting, which are independent of AKH signaling. Interestingly, continuous production of active SIK3 blocked the prolonged fasting-induced nuclear localization of HDAC4 (Choi et al., 2015). Nevertheless, AKH plays a critical role in the localization of HDAC4 in fasting, in particular for shorter periods. Notably, these events work in parallel to reduce insulin signaling.

Insulin is another factor affecting starvation. Disruption of the insulin signaling promotes lipid accumulation and increases resistance to starvation (Clancy et al., 2001; Broughton et al., 2005). Insulin secretion is elevated in response to feeding and typically decreases during starvation (Britton et al., 2002; Ikeya et al., 2002; Géminard et al., 2009). A recent study in *Drosophila* described insulin as an orexigenic hormone during short periods of starvation (Sudhakar et al., 2020). InR activity is also reduced following starvation (Britton et al., 2002). Similarly, *DILP3* and *DILP5* are down-regulated during starvation (Ikeya et al., 2002). However, Chatterjee et al. (2014) demonstrated that insulin activation is specifically required in oenocytes during starvation to maintain starvation resistance. Thus, the fat body-derived ILP6 induces lipid uptake in oenocytes, promotes lipid turnover during fasting and increases starvation tolerance in fasting adult flies (Chatterjee et al., 2014). Notably, ILP6 resembles IGFs and suppresses brain ILPs. Furthermore, silencing of *ILP6* or inhibition of the insulin activity in oenocytes reduces starvation-induced accumulation of LDs, therefore, new lipid synthesis in oenocytes, and induced-sensitivity to starvation, indicating insulin signaling in oenocytes is crucial to maintain starvation resistance (Chatterjee et al., 2014). Interestingly, overexpression of *DILP6* in the fat body and gut did not induce starvation tolerance, but rather increased starvation sensitivity (Chatterjee et al., 2014). As such, starvation tolerance significantly decreased when *DILP6* expression was reduced in the fat body (Chatterjee et al., 2014). These findings are in accordance with the proposed analogy between oenocytes and mammalian hepatocytes (Gutierrez et al., 2007; Martins and Ramalho-Ortigao, 2012) and support the notion that oenocytes play a central role in the metabolic adaptation to starvation (Chatterjee et al., 2014). On the other hand, starvation induces a significant increase in the number and size of LDs in adult oenocytes as starvation also induces TAG levels in the mammalian liver (Ohama et al., 1994). Similarly, knockdown of the insulin target "*PEPCK* (Phosphoenolpyruvate Carboxykinase)" impaired starvation-induced lipid uptake in oenocytes. Overall, the study by Chatterjee et al. (2014) suggests the presence of an oenocyte-specific insulin activity, which is critical for the mobilization of stored lipid under fasting conditions in oenocytes. In brief, the role of insulin signaling on lipid metabolism is different between adipocyte and oenocytes.

In terms of the biochemical background of insulin signaling on lipid metabolism, inhibition of the foxO transcription by insulin is a central phenomenon (Giannakou and Partridge, 2007), however, foxO was not detected in adult oenocytes (Chatterjee et al., 2014), suggesting the effect of insulin signaling in oenocytes might be foxO-independent. Nevertheless, starvation leads to a decline in PIP_3 levels and dephosphorylation of AKT and translocation foxO to the nucleus in the fat body adipocytes. The decrease in ILPs during starvation leads to up-regulation of the insulin signaling target, 4EBP, encoded by *Thor*, and dephosphorylation of existing Thor protein (Gibbs and Reynolds, 2012). In parallel, decreases in insulin signaling stimulate the dephosphorylation and nuclear translocation of foxO (Jünger et al., 2003; Puig et al., 2003), therefore, phosphorylation of foxO decreases *Thor* expression, which occurs by feeding and increase in insulin signaling. On the other hand, starvation also triggers the activation of the CREB co-activator, CRTC, in *Drosophila* (Wang et al., 2008). *CRTC*- mutant flies have reduced glycogen and lipid stores and are sensitive to starvation (Wang et al., 2008). In line with this, the increase in insulin signaling inhibits CRTC activity during feeding through SIK2-mediated phosphorylation, leading to degradation of CRTC (**Figure 4**). CRTC was not

phosphorylated during refeeding in flies with defective of insulin signaling (Wang et al., 2008). Furthermore, depletion of neuronal SIK2 increases CRTC activity and resistance to starvation. In line with these findings, foxO activity was found to be elevated in *CRTC*-mutant flies in parallel to the depletion of lipid and glycogen (Wang et al., 2008). CRTC indeed acts in parallel with foxO as CRTC is dephosphorylated and activated during starvation (**Figure 2**). In support of this, silencing *AKT* increases CRTC activity (Wang et al., 2008). Overall, CRTC enhances survival during starvation (Wang et al., 2008).

Leucokinin is also involved in the lipid metabolism-related starvation response. *Lk*-and *LkR*-mutant flies eat excessively after starvation, but do not eat more than normal flies when continuously supplied with food, suggesting that the mutants consume abnormally large meals, but at a reduced frequency (Al-Anzi et al., 2010). The effect of Lk on starvation response might occur through its interaction with insulin signaling. *Lk*- and *LkR*-mutants or flies with targeted knockdown of *LkR* in IPCs displayed increased *DILP2* and *DILP3* expression in IPCs and increased starvation resistance (Zandawala et al., 2018). Notably, Lk has been also reported to be involved in the modulation of starvation-dependent changes in sleep (Zandawala et al., 2018; Yurgel et al., 2019). Lk neurons in the lateral horn of the fly brain are required for starvation-induced sleep suppression and activity of these neurons increases under starvation conditions (Yurgel et al., 2019). In line with this, knockdown of *Lk* in Lk-expressing neurons was found to induce sleep during starvation (Yurgel et al., 2019). Additionally, LkR function in the IPCs is required for starvation-induced sleep suppression as silencing *LkR* in DILP2 neurons prevented starvation-induced sleep loss. These finding suggest that LkR is required in DILP2 neurons for starvation-induced sleep suppression (Yurgel et al., 2019).

NPF and sNPF may also affect lipid metabolism in relation to starvation. In various insects, production of these peptides is induced following starvation and decreases with feeding (Chung et al., 2017; Lin et al., 2019). Thus, sNPFR genes are up-regulated by starvation in fruit flies (Lee K.S. et al., 2008; Jiang et al., 2017), cockroaches (Mikani et al., 2012), and foraging honeybees (Ament et al., 2011). A recent study in *Drosophila* also revealed that knockdown of *sNPF* in circadian clock neurons reduced TAG level, starvation resistance and increased the starvation-mediated hyperactivity response after 24 h of starvation (Geo et al., 2019). Additionally, knock down of *sNPFR* expressed in IPCs was found to increase starvation resistance, but reduced the starvation-induced hyperactivity response after 24 h of starvation. On the other hand, *NPF*-mutant flies have been shown not to suppress sleep following prolonged starvation conditions, suggesting that NPF also acts as a hunger signal to keep the animal awake (Chung et al., 2017). Furthermore, activation of *NPF*-expressing neurons was found to decrease whole body TAG levels and increase food consumption and sensitivity to starvation conditions (Chung et al., 2017). The decrease in TAG levels by an increase in food consumption could be related to the activity of the leptin, UPD1. The UPD1 receptor domeless is expressed in *NPF*-expressing neurons, *Drosophila* domeless can be activated by human leptin, and feeding behavior is perturbed in flies lacking UPD1 (Rajan and Perrimon, 2012; Beshel and Zhong, 2013). Additionally, UPD1/domeless signaling suppresses NPF activity; and absence of this signaling leads to food overconsumption (Beshel et al., 2017). On the other hand, disruption of UPD2 in adipose tissue contributes to a reduction in body size (Rajan and Perrimon, 2012), indicating differences between the actions of the two domeless ligands (UPD1 and UPD2) (Beshel et al., 2017). It is noteworthy that there are contradictory findings on the reaction of NPF and sNPF toward starvation. *sNPFR* is down-regulated upon starvation in *B. mori* (Nagata et al., 2012), *Solenopsis invicta* (Chen and Pietrantonio, 2006) or *S. gregaria* (Dillen et al., 2013), however, *sNPF*/*sNPFR* expression increases transiently after feeding in these insects. Overall, sNPF positively regulates feeding in most species.

AstA is also involved in the starvation response as it inhibits starvation-induced feeding behavior, which leads to lipolysis (Hergarden et al., 2012; Hentze et al., 2015; Chen et al., 2016). Hergarden et al. (2012) indicated that AstA activation is likely to be an outcome, not a cause, of metabolic changes that induce the state of satiety. Other hormones, such as octopamine, could also affect lipid-metabolism-related starvation responses, in particular by affecting the release of peptide hormones, such as insulin (Li et al., 2016). However, such interactions are not included in the current review as octopamine is an amine hormone.

Infections and Immunity

Infections or mutualistic interactions could alter lipid metabolism, therefore, immunity is also another factor affecting lipid homeostasis. Infections may lower whole body TAG levels according to the studies in Drosophila. For example, infection of *Drosophila* with *Listeria monocytogenes* leads to a decrease in both stored fats and glycogen (Chambers et al., 2012) and infections initiates host responses that lead to inhibition of TOR activity, which results in lipolysis in adipocytes (Lee et al., 2018). In accordance with this, microbe-free *Drosophila* flies possess elevated TAG levels compared to conventionally-reared counterparts (Wong et al., 2014). On the other hand, mutualistic bacteria, such as *Lactobacillus brevis* and *Acetobacter fabarum,* lower TAG levels in *Drosophila* (Sommer and Newell, 2019).

Several peptide hormones involved in insect lipid metabolism interfere with insect immunity. One example of this is AKH, which has been shown to activate the prophenoloxidase cascade (Goldsworthy et al., 2003; Mullen and Goldsworthy, 2006). In *L. migratoria,* phenoloxidase activity is induced more in response to laminarin when applied with AKH, compared to laminarin alone (Goldsworthy et al., 2002). In another study, the injection of a lipopolysaccharide from *Escherichia coli* with AKH resulted in the formation of a higher number of nodules compared to injection of the lipopolysaccharide only (Goldsworthy et al., 2003). The venom of the parasitoid *Habrobracon hebetor* up-regulated *AKH* expression in paralyzed adult females of *P. apterus* (Shaik et al., 2017). Furthermore, co-application of venom with AKH reduced paralysis compared to the application of venom only. Infection of *P. apterus* adults by the entomopathogenic nematode *Steinernema carpocapsae* up-regulated *AKH* (Ibrahim et al., 2017). These studies suggest that

AKH is elevated upon infection and this increase can induce immune responses.

Insulin is another peptide hormone that interferes with infections and insect immunity (Galenza and Foley, 2019). For example, infection by *Mycobacterium marinum* leads to a decrease in lipid and glycogen stores in fruit flies by impairing insulin signaling through reduced AKT activation (Dionne et al., 2006). A previous study showed that the InR substrate *chico*-mutant homozygous and heterozygous flies have increased resistance against two pathogenic bacteria, the Gram-negative *Pseudomonas aeruginosa* and the Gram-positive *Enterococcus faecalis*; however, the mutants displayed a nearly threefold increase in survival, but no alteration in the expression of antimicrobial peptide genes upon infection (Libert et al., 2008). Interestingly, *Thor* was up-regulated twofold in *chico*-mutant homozygous flies upon infection. In line with this, Thor has been previously implicated in pathogen resistance of *Drosophila* (Bernal and Kimbrell, 2000). Libert et al. (2008) suggested that decreased insulin signaling in *chico*-mutant flies causes higher foxO activity, which leads to up-regulation of its target *Thor*, and improved survival of *Chico* mutants (Libert et al., 2008). *Chico*-mutant *Drosophila* flies (hypomorphic, but not null alleles) exhibit increased phenoloxidase activity and melanization response, and reduced phagocytosis in response to the insect pathogen *Photorhabdus luminescens* and non-pathogenic *Escherichia coli* (McCormack et al., 2016). Furthermore, the mutants contained lower pathogen titers in response to *P. luminescens* infection compared to controls, suggesting *chico*-mutants have increased resistance to infection. On the other hand, infected flies showed reduced transcript levels of antimicrobial peptide genes in the *chico*-mutants; however, *chico* mutation does not affect the survival upon bacterial infection which is in contrast to the findings by Libert et al. (2008). Notably, foxO could also induce expression of antimicrobial peptide genes in the fat body (Becker et al., 2010). *Chico* silencing in the fat body promotes the expression of the gene encoding the peptidoglycan receptor protein PGRP-SC2, but suppresses the expression of *PGRP-SB2*; both are important for development (Musselman et al., 2018a). These findings indicate a complex role for insulin activity in the host response that is highly context-dependent and varies for individual pathogens (Galenza and Foley, 2019). Nevertheless, chico plays an important role in the regulation of the antibacterial immune function. This could be related to decreased insulin signaling, which increases longevity, a common phenomenon in immunometabolism.

The gut hormone Tk could also interfere with insect midgut immunity, for example, microbial-derived acetate has been shown to induce *Tk* expression (Kamareddine et al., 2018). As Tk reduces insulin signaling and lipid storage (Birse et al., 2011; Song et al., 2014), it may be one of the reasons for the infection-related TAG decrease.

sNPF could be also involved in insect immunity as loss of sNPF signaling disrupts gut epithelial integrity and up-regulates anti-microbial peptide genes (Shen et al., 2016). By contrast, over-expression of *sNPF* has been found to dampen the gut immune response (Shen et al., 2016).

POTENTIAL OF LIPID METABOLISM-RELATED PEPTIDE HORMONES IN PEST MANAGEMENT

Pest control strategies targeting insect lipid metabolism has great potential due to the essential roles of lipids in insect biology and physiology. Various molecules targeting insect lipid metabolism have been developed and used already in the field as registered insecticides against various pests. In this manner, the most-important group is the lipid synthesis inhibitors, such as spiromesifen, spirodiclofen and spirotetramat (Nauen and Konanz, 2005; Nauen et al., 2008). Other groups of insecticides including JH analogs such as pyriproxifen (Fotouhi et al., 2015), chitin synthesis inhibitors such as hexaflumuron (Mirhaghparast et al., 2015), or synthetic pyrethroids (Balabanidou et al., 2016), organophosphates (Li et al., 2016) and neonicotinoids (Clements et al., 2020) have been also shown to impair directly or indirectly insect lipid metabolism. As this review focuses on the peptide hormones involved in insect lipid metabolism, this section is also restricted to the developments and potential of approaches targeting these hormones.

Strategies targeting peptides hormones involved in lipid metabolism in pest control is a developing area; therefore the progress at this stage is limited; however, there are promising findings. In this manner, efforts have focuses on the development of peptide hormone agonists/antagonists that have the potential of being replacements to chemical insecticides, or at least being used within integrated pest management programs (Fónagy, 2006). One promising candidate is AKH. The co-application

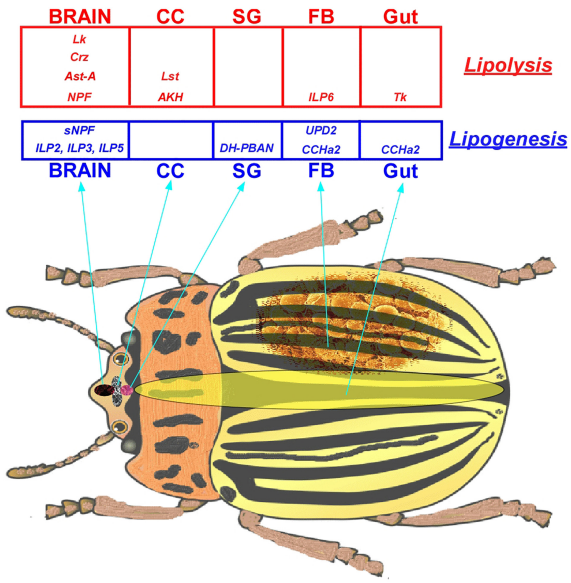

FIGURE 5 | Lipoliytic and lipogenic peptide hormones and their synthesis site in an insect. Abbreviations: AKH, adipokinetic hormone; ILPs, insulin-like peptides, DH-PBAN, diapause hormone-pheromone biosynthesis activating neuropeptide; sNPF, short neuropeptide F; NPF, neuropeptide F; AstA, allatostatin-A; Crz, corazonin, Lk, leucokinin; CCHA2, CCHamide-2; Tk, tachykinins; Upd2, unpaired 2; Lst, limostatin.

of insecticides with AKH has been shown to increase their efficacy (Kodrík et al., 2015; Plavšin et al., 2015). In another study, co-application *S. carpocapsae* with AKH was found to enhance the mortality of the host *P. apterus* adults about 2.5 times within 24 h (Ibrahim et al., 2017). By contrast, firebugs with reduced expression of *AKHR* displayed significantly lower mortality (Ibrahim et al., 2017). The stimulatory effect of AKH on the efficacy of entomopathogenic fungi or bacteria has been also reported (Goldsworthy et al., 2005; Mullen and Goldsworthy, 2006). These studies all suggest that AKH has a potential for use with other pest control tools.

Another promising peptide hormone to be targeted is DH. Synthetic DH agonists/antagonists were examined in terms of their effect on the pupal diapause of the corn earworm, *Heliothis zea*. These studies revealed that DH agonists leads to an inhibition in the entrance into pupal diapause or a premature termination of diapause, while DH antagonists block the termination of diapause, suggesting DH analogs and antagonists are promising candidates for pest management by disrupting diapause (Zhang et al., 2011; Zhang Q. et al., 2015).

RNAi also provide unique opportunities in pest control (Toprak et al., 2013, 2014a). This strategy could include application (sprays) of single dsRNA or a combination of dsRNA with synergistics, chemicals, microbials or other molecules with insecticidal action. Many genes involved in lipid metabolism, such as *FAS*, *AKT*, *ACC,* and *CaM* have been targeted by RNAi for functional analysis, which resulted in impairment of lipid, carbohydrate and calcium metabolisms; as well as, inhibition of development, growth, reproduction, and even death (Cheon et al., 2006; Roy et al., 2007; Sim and Denlinger, 2009; Zhang Y. X. et al., 2015; Tan et al., 2017; Wang W. et al., 2018). Targeting peptide-hormones or their receptors, such as AKHR (Konuma et al., 2012; Alves-Bezerra et al., 2016; Hou et al., 2017), ILPs (Kim and Hong, 2015; Meng et al., 2015; Defferrari et al., 2016; Fu et al., 2016), sNPF (Dillen et al., 2013, 2014), NPF (Van Wielendaele et al., 2013), CrzR (Kubrak et al., 2016), and LkR (Yurgel et al., 2019) by RNAi also led to similar outcomes. Although the laboratory results are quite promising, transfer of this technology into field, in particular in relation to security for non-target organisms and financial cost, requires selection of specific genes and efficient dsRNA-synthesis technologies therefore further studies.

One alternative use of peptide hormones in pest management could be within the development of pest monitoring and forecasting strategies that are currently based on ecological parameters, primarily temperature. For example, AKH might be used as a marker for the prediction of emergence time of pests from hibernation, which would be also important to estimate migration times of pests from overwintering sites into the field in spring. Thus, AKH levels are elevated toward the end of hibernation in order to mobilize lipids prior to migration. Another one could be the DH which is also elevated by end of pupal diapause in *Heliothis* (Zhang et al., 2011). In sum, use of peptide hormones as biochemical markers of insect emergence and possibly epidemics is promising and worthed to focus; however, the concept is in the initial phase and requires further studies.

Overall, peptide-hormones have the potential for use in pest management, in particular with biological or chemical insecticides; however, the difficulty and the cost of peptide synthesis, the need for extensive (field-scale) amounts for application, as well as the necessity of techniques for efficient delivery are still drawbacks at this stage.

CONCLUDING REMARKS

Lipid metabolism is an ancient pathway with various common genetic actors and/or functional homologs from microorganisms to mammals. Insects have great potential to study lipid metabolism in related human disorders as they also share many common pathways. The fat body adipocytes and the hepatocyte-analogous oenocytes harbor events related to lipid metabolism, which are controlled through differential gene expression by transcription factors, post-transcriptional modifications, secondary messengers and hormones. Various peptide hormones, including neuropeptides, have many different effects on lipid metabolism through various pathways. One could say that the two most important groups of peptide hormones affecting lipid metabolism are AKH and the brain ILPs (ILP2, ILP3, and ILP5), the former induce lipolysis and the latter induce lipogenesis. Notably, the IGF-like ILP6 suppresses the production of brain ILPs and, therefore, might contribute into lipolysis. AstA, Crz, Lk, CCHa2, Tks, Lst, UPD1/2 and AdipoR affect lipid metabolism via their modulation on insulin secretion. NPF and sNPF are primarily involved in feeding behavior, therefore, affect lipid metabolism. Overall, AKH, ILP6, NPF, AstA, Crz, Lk Tk, and Lst stimulate lipolysis, while ILP2, ILP3, ILP5, DH-PBAN, sNPF, CCHa2, UPD1, and UPD2 induce lipogenesis (**Figure 5**). Although peptide hormones have diverse roles, they are also involved in other events, such reproduction, flight, diapause, starvation, and immunity, which are related to lipid metabolism. Finally, peptide hormones have promising potential to be used in pest control, in particular with biological or chemical insecticides; however, further studies are required in order to carry the approach into field.

AUTHOR CONTRIBUTIONS

UT conceptualized the study and wrote the manuscript.

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ORIGINAL RESEARCH
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FMRFamide-Related Peptides Signaling Is Involved in the Regulation of Muscle Contractions in Two Tenebrionid Beetles

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Peptidergic signaling regulates various physiological processes in insects. Neuropeptides are important messenger molecules that act as neurotransmitters, neuromodulators or hormones. Neuropeptides with myotropic properties in insects are known as FMRFamide-like peptides (FaLPs). Here, we describe the myotropic effects of the endogenous FaLPs in the regulation of contractile activity of the heart, ejaculatory duct, oviduct and the hindgut in two beetle species, *Tenebrio molitor* and *Zophobas atratus*. A putative receptor was identified *in silico* in both species. Using RT-PCR these putative FaLPs receptors were found in the various tissues of both beetles, including visceral organs. Analysis of the amino acid sequence of the receptor indicated that it is similar to other insect FaLPs receptors and belongs to G-protein coupled receptors. A synthetic FaLP (NSNFLRFa) found as the bioanalogue of both species demonstrated concentration-dependent and organ-specific myoactive properties. The peptide had species–specific cardioactivity, in that it stimulated *Z. atratus* heart contractions, while slightly inhibiting that of *T. molitor* and had mainly myostimulatory effect on the examined visceral organs of both beetle species, with the lowest activity in the ejaculatory duct of these beetles. The peptide was the most active in the hindgut of both species, but only at high concentration of 10^{-5} M. The results suggest that FaLPs are potent modulators of endogenous contractile activity of the visceral muscles in beetles and may indirectly affect various physiological processes.

Keywords: G-protein coupled receptor, visceral organs, FMRFamide-like peptides, neuropeptides, beetles (Coleoptera), heart

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INTRODUCTION

FMRFamide is the four amino acid sequence (Phe-Met-Arg-Phe-NH_2) first found as an molluscan cardioacceleratory agent (Price and Greenberg, 1977). FMRFamide itself is not present in insects, but the name has been used to describe peptides with a C-terminal RFamide motif, which were grouped together in a family of FMRFamide-like peptides (FaLPs) or FMRFamide-related peptides (FaRPs). The FaLPs "family" originally included N-terminally extended FMRFamides, myosuppressins (MS), neuropeptides F (NPF), and short neuropeptide F (sNPF). These peptides

now are assigned to separate families, because they are encoded on different genes and have their own receptors. The insect FaLPs therefore comprises only the N-terminally extended FMRFamides (Coast and Schooley, 2011). A variable number of N-terminally extended FMRFamides are present in various insects along with extended IRF/Lamides (Coast and Schooley, 2011).

The physiological activity of insect FaLPs have been studied in different species and were shown to be mainly myoactive, however, with different effectivity. They regulate the heart and other visceral muscles contractility including male and female reproductive tracts (Merte and Nichols, 2002; Sedra and Lange, 2014; Suggs et al., 2016). Other known functions of FaLPs include involvement in regulation of circadian rhythm (Pyza and Meinertzhagen, 2003) and synaptic activity (Marques et al., 2003). Most of the research has been performed on different insect species, such as flies (Merte and Nichols, 2002), locusts (Robb and Evans, 1994), stick insects (Lange et al., 2009), or mosquitoes (Hillyer et al., 2014). However, scarce information is available regarding FaLPs in the largest insect order – the beetles. Thus far, only tetrapeptide FMRFa was shown to stimulate contractions of beetle reproductive tracts (Marciniak and Rosinski, 2010). Thus, the exact physiological role of FaLPs in beetles, as well as in other insects is still unclear.

Recently, due to the involvement of omics technologies, FaLPs precursors have been identified in several beetle species (Pandit et al., 2019). In all studied beetle species, the identified precursors contain several bioanalogues which the C-terminus contains either – FLRFa or – FIRFa (Veenstra, 2019). In tenebrionid beetles, FaLPs precursors' sequences are available for three species: *Tenebrio molitor* and *Zophobas atratus* (this study) and the first beetle with complete neuropeptidome *Tribolium castaneum* (Li et al., 2008). In all of these species, the precursor contains six bioanalogues (**Figure 1**). To evaluate the organ-specific myotropic properties of FaLPs in beetles, here, we report the activity of the last bioanalogue from the precursor FMRF6 (NSNFLRFa) which was identical in all three species (**Figure 1**).

Most of the insect neuropeptides act *via* G protein coupled receptors (Audsley and Down, 2015). GPCRs have seven α-helical transmembrane domains and are, therefore, also called seven transmembrane (7TM) receptors and are one of the most common and important molecules in living organisms (Hauser et al., 2008). In beetles, a complete set of GPCRs for neuropeptides was first described in *T. castaneum* (Hauser et al., 2008). In this study, we predicted also the FaLPs receptor (FMRFR) in *T. molitor* and *Z. atratus* in order to evaluate whether the observed effects of peptide applications are direct due to ligand receptor interactions.

MATERIALS AND METHODS

Insects

Tenebrio molitor adult males (4 weeks post-eclosion) were reared according to a previously described procedure (Rosinski et al., 1978). *Zophobas atratus* adults (4 weeks post-eclosion) were obtained from a colony maintained at the Department of Animal Physiology and Development, Adam Mickiewicz University, Poznań according to the Quennedy procedure (Quennedey et al., 1995).

Peptides

Peptide FMRF6 (NSNFLRFa) was synthesized according to the Fmoc procedure, as described previously (Marciniak et al., 2008; Lubawy et al., 2018). Proctolin (RYLPT), used as a control peptide, was obtained from Sigma Aldrich (Germany). The peptides were dissolved in physiological saline appropriate for beetles (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose, 5 mM, HEPES, pH 7.0) to yield a stock solution of 10^{-3} M, and it was stored at −20°C. Working dilutions were prepared from a stock solution in a physiological saline.

Transcriptome Sequencing, Database Search, and Sequence Comparison

Transcriptomic data from *T. molitor* and *Z. atratus* were obtained after Illumina Hiseq sequencing of total RNA extracted from the brains (n = 10) and retrocerebral complexes (n = 10) of adult beetles and sequenced at the Beijing Genomics Institute (Shenzhen, China). After initial filtering of low quality reads and adaptor removal, clean reads were *de novo* assembled using Trinity. The transcriptomic data has been submitted to NCBI Sequence Read Archive database (SRR11184806 and SRR11358229, BioProject PRJNA608239 for *T. molitor* and SRR11178058 and SRR11178059, BioProject PRJNA608269 for

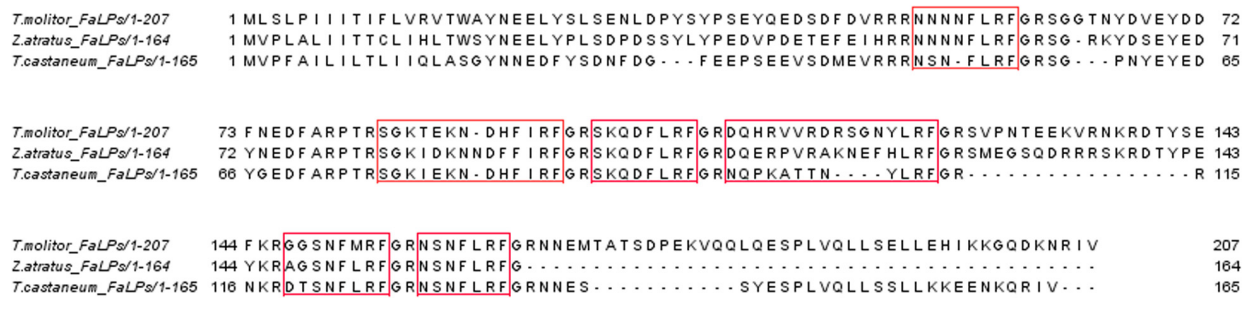

FIGURE 1 | Sequence alignment of FaLPs precursors from *T. molitor* (*T. molitor*_FaLPs – this study), *Z. atratus* (*Z. atratus*_FaLPs – this study) and *T. castaneum* (*T. castaneum*_FaLPs, EFA02863), all from Tenebrionidae. Peptides present in the precursor are marked in red boxes.

Z. atratus) and were used for local tblastn withFaLPs precursor sequence from *T. castaneum* (EFA02863) and FaLPs receptor sequence from *T. castaneum* (NP_001280540.1) to find *T. molitor* and *Z. atratus* FaLPs precursor and receptor sequence.

Receptor Transcript Distribution

Transcript profiles of Tenmo-FMRFR and Zopat-FMRFR were determined by reverse transcriptase PCR (RT-PCR) in various tissues of adults. RT-PCR was performed according to a modification of the method described by Marone et al. (2001). Suitable tissues/organs (nervous system – brain, retrocerebral complex CC/CA and ventral nerve cord, and heart, hindgut, ejaculatory duct and oviduct) after dissection were transferred to 150 μL of RNA lysis buffer (Zymo Research, United States) and homogenized for 3 min using a pellet homogenizer. The homogenized tissues/organs were immediately frozen in liquid nitrogen and then stored at –80°C. A Quick-RNA® Mini Prep kit (Zymo Research, United States) was used for RNA isolation. The RNA concentration was determined with a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, United States). Reverse transcription of the same amount of isolated RNA to cDNA was accomplished using the RevertAid^{TM} Reverse Transcriptase kit (Thermo-Fisher Scientific, United States) according to the manufacturer's protocol. PCR analyses were conducted using a T100^{TM} Thermal Cycler (Bio-Rad, United States). The primers were designed based on sequences of Tenmo-FMRFR and Zopat-FMRFR using Primer3 software (Untergasser et al., 2012). The primer pair for Tenmo-FMRFR was created to amplify fragments of 120 bp with the following sequences Fw 5′-AACATAATAGACACCTACTG-3′ and Rev 5′-CTTCTCACCGAATATCAC-3′, whereas the primers for Zopat-FMRFR amplify fragment of 143 bp and where Fw 5′-TACCTCCAGCTCTACCGCTT-3′ and Rev 5′-AGGCCGATGAGGAGGTAGTT-3′. The primers were synthetized by the Institute of Biochemistry and Biophysics of the Polish Academy of Science (Warsaw, Poland). PCR was performed in a 10 μL reaction volume containing 3.95 μL of DNase/RNase-free water, 1 μL of DreamTaq Green Buffer (Thermo-Fisher Scientific, United States), 1 μL of 2 mM dNTP, 1 μL of 10 μM forward primers, 1 μL of 10 μM reverse primers, 0.05 μL of DreamTaq DNA polymerase (Thermo-Fisher Scientific, United States) and 2 μL of cDNA. The obtained products were analyzed by electrophoresis using a 2% TAE agarose gel stained with ethidium bromide. The GeneRuler 100 bp DNA Ladder (Fermentas, United States) was run on each gel. Photos of the agarose gels were taken using ChemiDoc Touch (Bio-Rad, United States). PCR was with a minimum of five biological and three technical replicates. To confirm our results, the bands were sequenced with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, Foster City, CA, United States) according to manufacturer's protocols by the Molecular Biology Techniques Laboratory (Faculty of Biology, Adam Mickiewicz University, Poznań) and compared with transcriptomic data. "No template control" and "no RT control" reactions were included in the analysis to ensure that there was no foreign DNA or genomic DNA contamination.

In vitro Visceral Organs Contraction Bioassays

The heart bioassay was performed by microdensitometric whereas the oviduct, ejaculatory duct and hindgut bioassay by a video microscopy techniques as described previously (Marciniak and Rosinski, 2010; Marciniak et al., 2011). In all bioassays, eight peptide concentrations were tested ranging from 10^{-12} to 10^{-5} M. Proctolin as a positive control in concentration 10^{-7} M was used (**Supplementary Table S1**). In brief, in the heart bioassay a semi-isolated heart preparations in superfusion chamber were mounted under the microdensitometer MD-100 (Carl Zeiss, Germany), whereas in video microscopy technique isolated visceral organs on Sylgard filled chamber were placed under the Olympus SZX12 stereomicroscope equipped with a SD30 camera. In all bioassays, an open perfusion system was used, with an injection port (for peptides) 70 mm above the superfusion chamber. The organ was subjected to a constant perfusion with fresh saline at the rate of about 140 μL/min. All tested samples were applied at the injection port with a Hamilton syringe. Many pulse applications of samples could be sequentially assayed in a single preparation. After the initial 15 min stabilization, the activity of the isolated organ was recorded for 2 min. Next the peptide was applied and the heart activity was recorded for a further 2 min. In the heart bioassay, the apparatus equipped with photocell counts optically every contraction of the heart, and being connected with microdensitometer register system, computer generated the cardiogram. In the video microscopy technique, first the video recordings of superfused organs were performed and then analyzed with the edge tracking software (AnTracker) to create a trace of the movement of the side edge of the organ.

Statistics

All statistical comparisons non-parametric *t*-tests (Mann–Whitney test) were performed with usage of Graph Pad Prism 6 software (AMU license). Results were considered statistically significant with $p < 0.05$. Prior to the analysis Shapiro-Wilk normality test was done for all of the groups.

RESULTS

Analysis of *T. molitor* and *Z. atratus* FaLPs Precursor and FMRFR Sequences

A blast search in the transcriptomes assemblies of *T. molitor* and *Z. atratus* yielded the FaLPs precursors of *T. molitor* and *Z. atratus*. Precursors were similar in structure to the homologous precursor from *T. castaneum* and encode six peptides of which two are identical in all of the beetles (**Figure 1**).

The BLAST search with local databases of the transcriptomic assemblies of *T. molitor* and *Z. atratus* brains and retrocerebral complexes yielded one open reading frame of a putative FaLPs receptor in each assembly. The receptor in *T. molitor* is 1,278 bp and in *Z. atratus* is 1,266 bp (**Figure 2**). Both display the seven transmembrane domains typical for GPCRs (Bass et al., 2014) with an N-terminal ligand binding region

FIGURE 2 | Alignment of the FMRFRs sequences from *T. molitor* (Tenmo-FMRFR), *Z. atratus* (Zopat-FMRFR), and *T. castaneum* (Trica-FMRFR), all from the Tenebrionidae family. Predicted seven transmembrane domains are highlighted in black.

and a C-terminal intracellular region. The comparison of the putative sequences showed that there is a very high degree of similarity (90%) between Tenmo-FMRFR and Zopat-FMRF. Less but also very high similarity was observed between Tenmo-FMRFR and Trica-FMRFR –84% and between Zopat-FMRFR and Trica-FMRFR –86%.

Distribution of FMRFR Transcripts in Different Tissues of *T. molitor* and *Z. atratus*

In order to check whether the peptide is able to influence the visceral organs directly, we examined the FMRFR spatial distribution by RT-PCR. As a positive control, we used nervous tissues, as FMRFR was previously shown to be present in this tissue in other insects, such as *D. melanogaster* (Meeusen et al., 2002). Analysis of FMRFR transcripts distribution in both species revealed that it is present in all tissues used for RNA isolation. It proves that FMRFR is present in the nervous system as well as various visceral organs of tenebrionid beetles (**Figure 3**). Despite the fact that quantitative analysis was not performed, the band intensity indicates that the level of the transcript may vary between tissues tested and, as expected, is the highest in the nervous system of both beetles and much lower in the digestive tract and the reproductive tract (**Figure 3**).

Effect of FMRF6 on the *T. molitor* Visceral Muscles Contractility

Application of a synthetic tenebrionid FMRF6 peptide during superfusion with physiological saline caused differentiated effects in frequency of contractions of the heart, hindgut, ejaculatory duct and oviduct of *T. molitor* beetle (**Figure 4**). The observed effects were organ-specific.

The *T. molitor* adult heart rhythm *in vitro* remained regular during superfusion with saline and showed on average 124 beats/min. Application of FMRF6 caused immediate, dose-dependent and reversible slight decrease of the heart contractile activity. The significant cardioinhibition was caused only by the highest tested concentration – 10^{-5} M (**Figure 4A**). All other concentrations from 10^{-12} to 10^{-6} M caused no effects.

The hindgut contractions of adult *T. molitor* remained irregular during superfusion with saline (5 contractions/min on average). Contrary to the heart, application of the FMRF6 peptide to the hindgut caused an increase in contraction frequency (**Figures 4B**, **5B**) in almost all of tested concentrations apart from

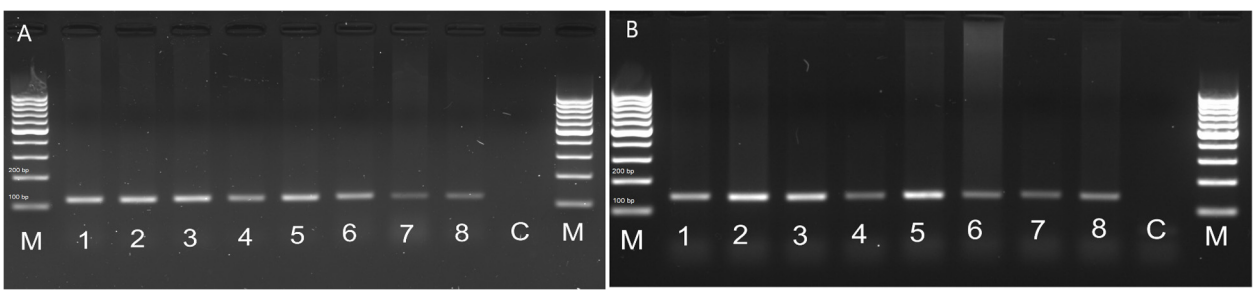

FIGURE 3 | Distribution of FMRFR transcript in different tissues of *T. molitor* **(A)** and *Z. atratus* **(B)**. 1, whole body; 2, brain; 3, ventral nerve cord; 4, retrocerebral complex; 5, heart; 6, hindgut; 7, ejaculatory duct; 8, oviduct; C, control H_20; M, marker.

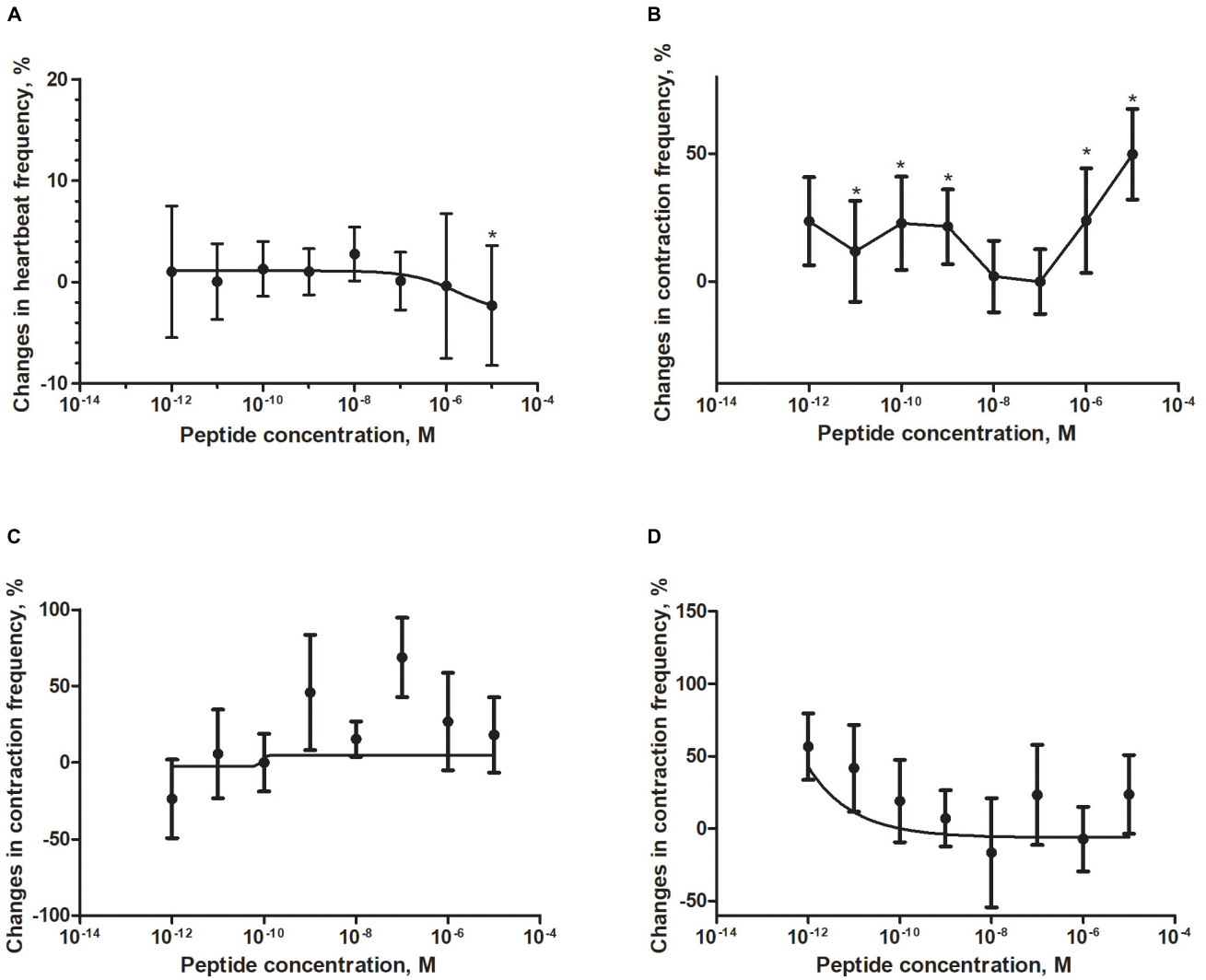

FIGURE 4 | Changes compared to control in the contractions frequency of heart **(A)**, hindgut **(B)**, ejaculatory duct **(C)**, and oviduct **(D)** of adult *T. molitor* beetle after application of FMRFa. Mean ± SEM are given from at least eight determinations. Statistically significant differences ($p \leq 0.05$) in the contractions frequency from control (saline application) are indicated by asterisks (*t*-Student test) $n = 10–15$.

10^{-8} to 10^{-7} M. The highest myotropic effect (51% stimulation) was observed after application of the highest concentration 10^{-5} M. Remarkably in lower concentrations (10^{-12}–10^{-11} M) less potent myostimulation also was observed with an effect half that observed after 10^{-5} M application.

The *T. molitor* ejaculatory duct contractions were very irregular during superfusion with physiological saline with the level of 2 contraction/min on average (**Figure 4C**). Application of FMRF6 peptide caused no statistically significant effects in the contractions frequency, however in single repetitions stimulation of 70% was sometimes observed.

Contrary to the ejaculatory duct, the oviduct of *T. molitor* females contracted more regular with an average of 7 contractions/min (**Figure 4D**). FMRF6 caused dose-dependent miostimulatory effects on the oviduct. Similarly to the hindgut bioassay, the highest increase in contraction frequency (50%) was observed in lower concentrations (10^{-12}–10^{-11} M). In higher concentration ranges, the stimulatory effect was not so evident.

Effect of FMRF6 on the *Z. atratus* Visceral Muscles Contractility

In *in vitro* bioassays with isolated *Z. atratus* visceral organs, peptide FMRF6 caused differentiated myotropic effects. Similar to other insect evaluated, the effect of FMRF6 in *Z. atratus* was organ-specific (**Figure 6**).

Semi-isolated heart of adult *Z. atraus* during superfusion with physiological saline showed regular rhythm with an average 35 beats/min, which was much slower than that of *T. molitor* (**Figure 5A**). In contrast to *T. molitor*, addition of the peptide resulted in a concentration-dependent positive chronotropic effects (increase in heartbeat frequency). The

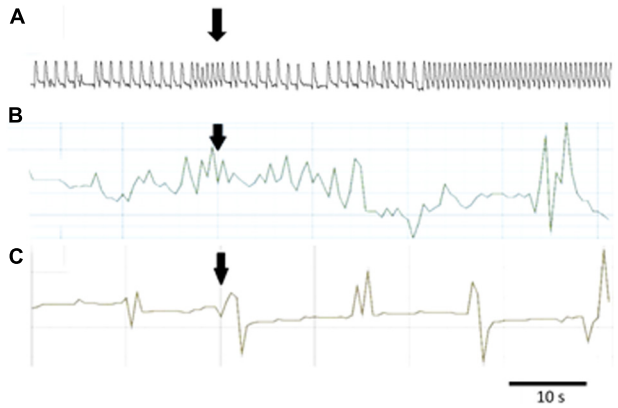

FIGURE 5 | Typical responses in the contraction frequency of *Z. atratus* heart after application of FMRF6 at the concentration 10^{-5} M **(A)**; *T. molitor* hindgut at the concentration 10^{-6} M **(B)**; *Z. atratus* oviduct at the concentration 10^{-6} M **(C)**. Peptide application is indicated by an arrow.

maximal effect was observed after application of one of the highest concentrations 10^{-6} M, as was shown for the previously described species, where also only the highest concentrations were cardioactive (**Figure 6A**).

The highest tested concentration (10^{-5} M) also was myostimulatory when applied on the isolated hindgut of *Z. atratus*. Average contraction frequency of the hindgut during superfusion was around 10 and increased to 193% after its application. Remarkably all other concentrations tested (10^{-12}–10^{-6} M) showed also an increase in contraction frequency of on average 37% (**Figure 6B**).

The irregular control ejaculatory duct contraction during superfusion with saline was on average 3 contractions/min. Similar to *T. molitor*, application of FMRF6 resulted in an increase in contraction frequency but the effect was much stronger than in *T. molitor*. The highest increase in contraction frequency (48%) was measured after application of 10^{-7} M. All tested concentrations between 10^{-9} and 10^{-6} M were myostimulatory with different efficiency (**Figure 6C**). Only the

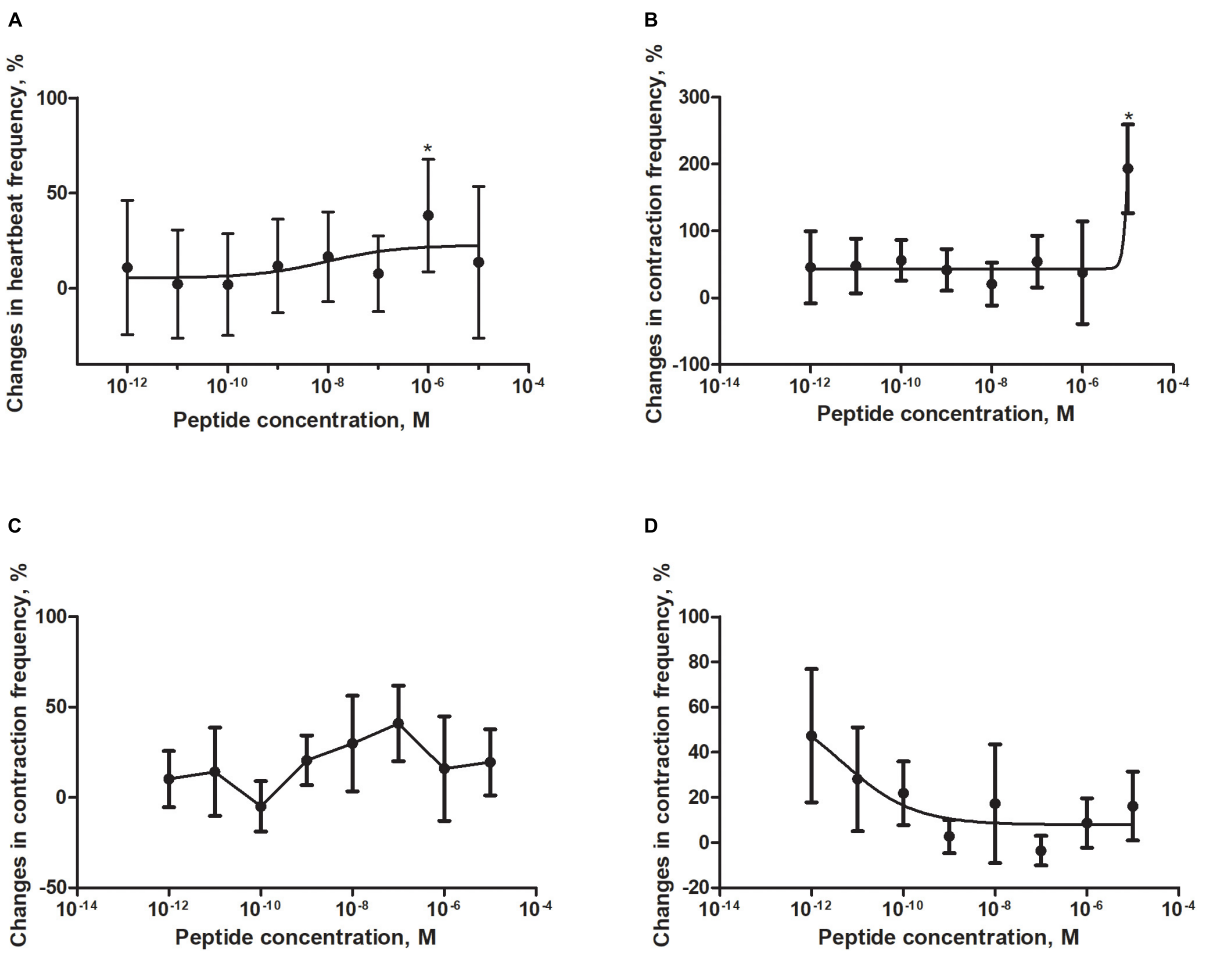

FIGURE 6 | Changes compared to control in the contractions frequency of heart **(A)**, hindgut **(B)**, ejaculatory duct **(C)**, and oviduct **(D)** of adult *Z. atratus* after application of FMRF6. Mean ± SEM are given from at least eight determinations. Statistically significant differences ($p \leq 0.05$) in the contractions frequency from control (saline application) are indicated by asterisks (*t*-Student test).

concentration of 10^{-10} M was inactive when applied on the oviduct (**Figure 6C**).

The last tested visceral organ – the oviduct from *Z. atratus*, had a control contraction rhythm at the level of 13 contractions/min on average. Similar to *T. molitor*, application of FMRF6 caused an increase in the contraction frequency but only in the lower tested concentration ranges (**Figure 6D**). The strongest effect was obtained after application of the lowest 10^{-12} M concentration and the increase in contractions frequency was about 47%. The highest concentrations from 10^{-9} to 10^{-5} M caused no effect (**Figure 5C**).

DISCUSSION

The present study expands the knowledge about the physiological role of FaLPs in the largest group of insects – Coleoptera. Here, we have examined the role of a N-terminally extended bioanalogue of FMRFa in the regulation of visceral organs contractions in *T. molitor* and *Z. atratus* beetle. We used a synthetic peptide FMRF6 (NSNFLRFa), which was identical in the FaLPs precursors of both tested beetles. We showed in *in vitro* bioassays, that the peptide was mainly myostimulatory and, with the exception of the *T. molitor* heart, it increased the frequency of contractions of beetles muscles organs with different efficiency. The effects appear to be organ-specific. An oviduct of both beetle species turned out to be the most sensitive to the peptide. The peptide acts *via* the putative GPCR characterized in both species.

FaLPs signaling has been studied in variety of insect species, including mosquitoes (Hillyer et al., 2014), stick insects (Lange et al., 2009), kissing bugs (Sedra and Lange, 2014), or flies (Merte and Nichols, 2002). However, the receptor for FaLPs was first discovered (Cazzamali and Grimmelikhuijzen, 2002) and very well characterized only in *D. melanogaster* (Maynard et al., 2013; Milakovic et al., 2014). As for most of the receptors activated by neuropeptides, FMRFRs belong to G-protein coupled receptors with seven transmembrane helixes, N-terminal extracellular segment and the intracellular C-terminus responsible for interactions with G proteins (Hauser et al., 2008). Moreover, a detailed analysis of insect FMRFR indicated that all belong to the subfamily A – rhodopsin like GPCRs thus far identified in flies, mosquitoes, moths and kissing bugs (Rasmussen et al., 2015). In beetles, the first FMRFR was identified in the model species for this order – *T. castaneum* (Hauser et al., 2008). Also, the receptor predicted in this study resembles this type of receptors containing some of typical rhodopsin-like amino acid patterns in its seven transmembrane domains: GN in helix 1, NLX3-DX8P in helix 2, SX3LX2IX2DRY in helix 3, WX8P in helix 4, FX2PX7Y in helix 5, FX3WXP in helix 6 and NPX2YX6F in helix 7 (**Figure 2**; Bass et al., 2014). Bioinformatical analysis demonstrated that the sequence similarity within tenebrionid beetles as expected is high.

The spatial tissue distribution of FMRFR was first studied in *D. melanogaster* (Meeusen et al., 2002). These authors showed that Drome-FMRFR is present in the nervous system (brain), guts and ovaries of larva and adult *D. melanogaster,* as well as trachea and fat body (Meeusen et al., 2002), suggesting that FaLPs might be involved in regulation of various physiological processes and in agreement with our study. We showed that the FaLPs receptor predicted here is present in the nervous system (brain, ventral nerve cord and retrocerebral complex CC/CA) as well as in all tested visceral organs suggesting the role of FaLPs in the regulation of functions of these organs. The data also is partially in agreement with studies performed on mosquitoes (Hillyer et al., 2014). The highest expression of FMRFR in *Anopheles gambiae* occurred in the head and thorax of this insects (Hillyer et al., 2014). It is surprising that in *A. gambiae* the expression level of FMRFR was so low in the abdomen. As in numerous insects, FaLPs immunoreactivity was observed in abdominal ganglia, midguts, and other visceral organs as well as in abdominal neural processes in *D. melanogaster*, *R. prolixus*, *Schistocerca gregaria*, *Phormia regina* (blow fly) and other insects (Myers and Evans, 1985; Nichols et al., 1995; Sedra and Lange, 2014), and therefore it was expected that the FaLPs receptors will be highly expressed in the abdomen. Probably this is the case in *D. melanogaster* and in the beetles studied here but not in the mosquito. This differences might be connected with role of FaLPs in visceral organs physiology. These beetles and mosquitos differ significantly in how they ingest and process food.

FaLPs are neuropeptides which are known to be mainly myoactive (Merte and Nichols, 2002). The best established activity of these peptides is their involvement in the regulation of the heart rhythm (Chowanski et al., 2016). The cardioactive properties of different FaLPs were shown in variety of insect species including *S. gregaria, B. extradentatum*, *D. melanogaster* and *A. gambiae* (Cuthbert and Evans, 1989; Nichols, 2006; Lange et al., 2009; Hillyer et al., 2014). These studies have not been performed on beetles before now. Most insect species exhibit dose-dependant cardioacceleratory properties and our study is partially in agreement. FMRF6 was cardio acceleratory on the heart of *Z. atratus*. Surprisingly the same peptide was cardio inhibitory on the *T. molitor* heart in the high concentration ranges. In the very high concentrations (10^{-3}–10^{-2} M), one of the N-terminally extended FaLPs was also cardioinhibitory in mosquitoes (Hillyer et al., 2014). However, in *A. gambiae* a FaLP peptide exerted bimodal effect. It was cardio acceleratory in low concentrations whereas in our study it exerted no cardiotropic effects.

Apart from the heart, FaLPs were shown to be myoactive on different visceral organs such as the gut and reproductive tract (ejaculatory duct and oviduct) of tenebrionids. As in the heart, the peptide was shown to be mostly myostimulatory. The results obtained in this study are in agreement with results obtained in other species, especially the extensively studied female reproductive tract. It was shown for example that various parts of the *R. prolixus* reproductive tract are stimulated by N-terminally extended FaLPs and the effect is similar to the FMRFa peptide (Sedra and Lange, 2014, 2016). In beetles, no previous studies were available for FaLPs in terms of the regulation of contractility of visceral organs. Previous studies examined only the tetrapeptide FMRFa on the motility of *T. molitor* and *Z. atratus* ejaculatory duct and oviduct (Marciniak and Rosinski, 2010). We showed that contractions of ejaculatory duct was inhibited whereas oviduct contractions were mainly stimulated after application of FMRFa in these tenebrionids.

In both cases, the effects were bimodal and dose-dependent (Marciniak and Rosinski, 2010). The different responses of the ejaculatory duct and oviduct to F**M**RFa and FMRF6 (NSNF**L**RFa) is probably due to difference in amino acid – M changed to L, which affects receptor binding.

In summary, we demonstrated the myostimulatory activity of N-terminally extended FaLP in two tenebrionid beetles. The tested peptide NSNFLRFa is present in both insects in the sequence of the FaLPs precursor and presumably act via G-protein coupled receptors, which were also predicted and identified in both species. We showed that in both beetles this peptide is myostimulatory to the gut, ejaculatory duct and oviduct and the effect is dose-dependent in almost all cases. The data suggest that FaLPs regulate functioning of this organs thus are involved in different physiological processes. The role of FaLPs in cardiac physiology is more complex. The effect of peptide is dose-dependent and species specific.

DATA AVAILABILITY STATEMENT

The transcriptomic data has been submitted to NCBI Sequence Read Archive database (SRR11184806 and SRR11358229, BioProject PRJNA608239 for *T. molitor* and SRR11178058 and SRR11178059, BioProject PRJNA608269 for *Z. atratus*).

AUTHOR CONTRIBUTIONS

PM and GR contributed conception of the study. PM designed the study, analyzed the data, and wrote the manuscript. PM, WW, and MS performed the physiological experiments. JP-B and SC performed the molecular experiments. MK performed the peptide synthesis. All authors contributed to manuscript, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00456/full#supplementary-material

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Receptor Characterization and Functional Activity of Pyrokinins on the Hindgut in the Adult Mosquito, *Aedes aegypti*

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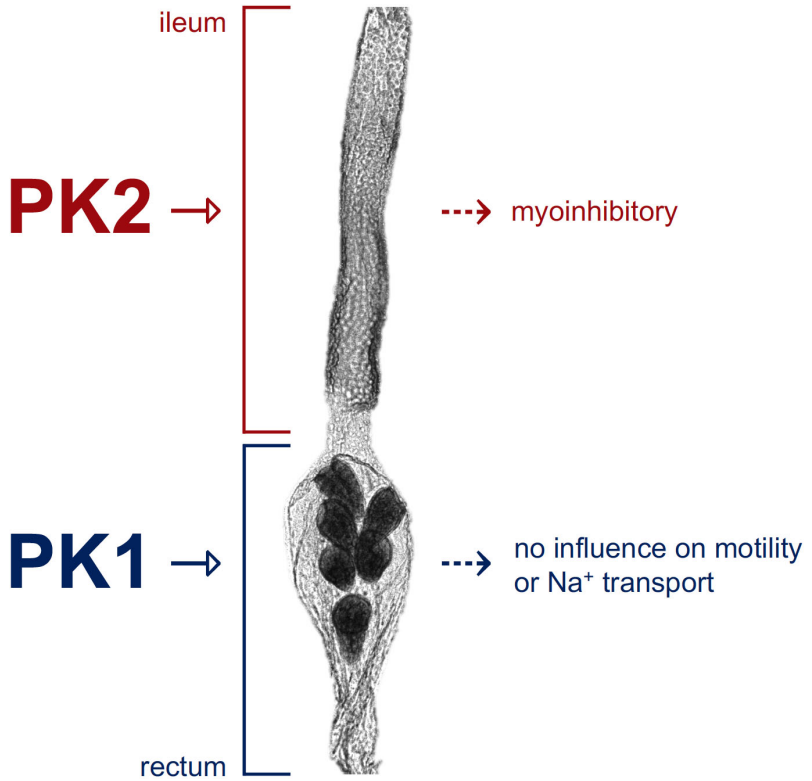
Pyrokinins are structurally related insect neuropeptides, characterized by their myotropic, pheromonotropic and melanotropic roles in some insects, but their function is unclear in blood-feeding arthropods. In the present study, we functionally characterized the pyrokinin-1 and pyrokinin-2 receptors (PK1-R and PK2-R, respectively), in the yellow fever mosquito, *Aedes aegypti*, using a heterologous cell system to characterize their selective and dose-responsive activation by members of two distinct pyrokinin subfamilies. We also assessed transcript-level expression of these receptors in adult organs and found the highest level of PK1-R transcript in the posterior hindgut (rectum) while PK2-R expression was enriched in the anterior hindgut (ileum) as well as in reproductive organs, suggesting these to be prominent target sites for their peptidergic ligands. In support of this, PRXa-like immunoreactivity (where X = V or L) was localized to innervation along the hindgut. Indeed, we identified a myoinhibitory role for a PK2 on the ileum where PK2-R transcript was enriched. However, although we found that PK1 did not influence myoactivity or Na^+ transport in isolated recta, the PRXa-like immunolocalization terminating in close association to the rectal pads and the significant enrichment of PK1-R transcript in the rectum suggests this organ could be a target of PK1 signaling and may regulate the excretory system in this important disease vector species.

Keywords: pyrokinin, G protein-coupled receptor, heterologous receptor functional assay, hindgut, motility, scanning ion-selective electrode technique, insect, disease vector



GRAPHICAL ABSTRACT | Schematic depicting pyrokinin target sites along the hindgut of *Aedes aegypti*. The *A. aegypti* PK2-R is expressed in the anterior hindgut region (ileum) and a pyrokinin-2 (PK2) peptide was shown to inhibit ileum motility, whereas the *A. aegypti* PK1-R is expressed in the posterior hindgut (rectum) indicating a pyrokinin-1 (PK1) peptide, such as *Aedae*CAPA-PK1, acts on the rectum, although its exact role remains unknown.

INTRODUCTION

Neuropeptides regulate an array of physiological processes in insects, including feeding, metamorphosis, diapause, and reproduction (Nässel and Winther, 2010). One such group of neuropeptides is the pyrokinin (PK) family. The first member to be identified, leucopyrokinin, was isolated from the cockroach, *Leucophaea maderae*, based on its stimulation of hindgut motility (Holman et al., 1986). Subsequently, a neuropeptide isolated based on its induction of sex pheromone production in female corn earworm moths (*Helicoverpa zea*; Raina et al., 1989), so named pheromone biosynthesis activating neuropeptide (PBAN), was found to have the same carboxyl (C) terminus, FXPRL-NH_2, and identification of the *pban* gene revealed additional encoded peptides with this conserved motif (Raina and Kempe, 1992; Sato et al., 1993). Related PKs have also been identified in other insects and shown to regulate cuticle melanization, pupariation, and feeding behavior (Matsumoto et al., 1990; Zdarek et al., 1997; Verleyen et al., 2004; Bader et al., 2007).

The existence of another PK subfamily with the highly conserved WFGPRL-NH_2 C terminus was revealed with the characterization of the diapause hormone (DH) that regulates the onset of embryonic diapause in the silkworm *Bombyx mori* (Yamashita, 1996). The role of DH in diapause has been identified in some lepidopteran species (Xu and Denlinger, 2003), and more recently in *Locusta migratoria* (Hao et al., 2019). Other peptides with this conserved sequence have been identified in Diptera (Predel et al., 2004; Predel et al., 2010), but their physiological roles remain unclear. These neuropeptides, which are commonly referred to as tryptopyrokinins (Veenstra, 2014), are classified as the pyrokinin-1 (PK1) type, while PBAN-related neuropeptides, characterized by their pentapeptide core motif (FXPRL-NH_2), are considered to be the pyrokinin-2 (PK2) type (Jurenka, 2015; Ahn and Choi, 2018). Members of the PK1 subfamily are encoded by two genes in most insects. The first was characterized for *B. mori* and called *pban* (Kawano et al., 1992; Sato et al., 1993), which also encodes PK2 peptides. A homologous gene has been characterized in *Drosophila melanogaster*, termed *hugin*, expressed in a subgroup of neurosecretory cells within the subesophageal ganglion (Meng et al., 2002; Bader et al., 2007), however, this gene in *D. melanogaster* only gives rise to PK2 peptides (Nässel and Winther, 2010). The second gene in insects encoding PK1 is the *capability* (*capa*) gene, which was first identified in *D. melanogaster* (Kean et al., 2002; Baggerman et al., 2002), which encodes not only a PK1 but also two additional neuropeptides known as CAPA or periviscerokinins that influence the activity of insect Malpighian tubules (Kean et al., 2002; Pollock et al., 2004; Terhzaz et al., 2012; Sajadi et al., 2018). Homologous *capa* genes were subsequently identified

across insect groups and found to be expressed predominantly in a pair of neurosecretory cells in the abdominal ganglia and a subset of neurons of the subesophageal ganglion (Predel and Wegener, 2006; Hellmich et al., 2014). PK-producing neurons localized in these ganglia have axons extending to perisympathetic organs, where peptides either act on the nervous system or are released into the hemolymph to exert their actions at peripheral targets (Choi et al., 2001; Hellmich et al., 2014).

Efforts to define PK signaling in target cells have identified G protein-coupled receptors that selectively bind PK1 or PK2 forms found in *D. melanogaster*, the African malaria mosquito (*Anopheles gambiae*), and the kissing bug (*Rhodnius prolixus*) (Cazzamali et al., 2005; Olsen et al., 2007; Paluzzi and O'Donnell, 2012). These studies were conducted with cell systems expressing putative PK receptors cloned from the insects of interest, as was accomplished for the yellow fever mosquito, *Aedes aegypti* (Choi et al., 2013). However, in this latter study, the PK1 and PK2 receptors (PK-Rs) were only tested using pyrokinins encoded by the *AAEL012060* (*hugin*) gene, whereas activity of PK1 encoded by the *AAEL005444* (*capa*) gene was not determined. Although no functions have yet been assigned for PK neuropeptides in mosquitoes or any other hematophagous arthropods, expression profiles of PK receptors in *R. prolixus* and the deer tick, *Ixodes scapularis*, show enrichment in the nervous system and reproductive tissues, and to a lesser extent, in the prothoracic glands and hindgut of *R. prolixus* (Paluzzi and O'Donnell, 2012; Gondalia et al., 2016).

Female *A. aegypti* are chief vectors of the chikungunya, dengue and yellow fever, and Zika viruses that are the causative agents of acute and chronic illnesses in humans globally (Kotsakiozi et al., 2017). Improving our understanding of mosquito biology and the regulation of underlying physiological processes by neuropeptides is imperative in order to develop new methods for vector control. Studying neuropeptide receptors in particular helps to unravel the neurocrine control of these uncharacterized regulatory mechanisms. The current study set out to examine the potential physiological roles of PK signaling in a vector mosquito by first examining the expression profiles of two PK receptors in different organs of adult *A. aegypti*. We then investigated whether the *A. aegypti* PK1-R and PK2-R identified previously (Choi et al., 2013) are activated by the *AAEL005444* (*capa*) gene-derived PK1 (*Aedae*CAPA-PK1), since this particular pyrokinin was not previously examined. Our current results along with the ability of PKs to stimulate hindgut motility in other insects (Holman et al., 1986) prompted us to further investigate the potential that these neuropeptides may influence myotropic and ionomodulatory activity in the hindgut, as these critical processes contribute toward maintenance of hydromineral balance in nectar- and blood-fed female mosquitoes.

MATERIALS AND METHODS

Animal Rearing

Aedes aegypti eggs (Liverpool strain) oviposited onto Whatman filter papers (GE Bioscience) were collected and hatched in plastic containers with distilled water, as previously described (Rocco et al., 2017). Larvae and pupae were reared in a 26°C incubator under a 12:12 h light:dark cycle. Larvae were fed daily with 2% brewers yeast:beef liver (1:1) powder solution (NOW foods, Bloomingdale, Illinois). All adult mosquitoes were fed 10% sucrose (w/v) *ad libitum*, and females in the colony cages were regularly fed with sheep blood in Alsever's solution (Cedarlane Laboratories, Burlington, ON, Canada) for egg production to maintain the colony. All experiments were performed on 4-day old female and male mosquitoes that were isolated during the pupal stage and transferred into glass mesh-covered jars.

Receptor Expression Profiles and Pyrokinin Immunolocalization

Tissue Dissections, RNA Isolation, cDNA Synthesis and RT-qPCR

Female (n = 20) mosquitoes were immobilized with brief exposure to CO_2, and submerged in nuclease-free Dulbecco's phosphate-buffered saline (DPBS; Wisent Inc., St. Bruno, QC, Canada). The midgut, Malpighian tubules, pyloric valve (midgut-hindgut junction), ileum, rectum, and reproductive organs (ovaries with accessory reproductive organs, including the common and lateral oviducts, and spermathecae, pooled together) were dissected and transferred into RNA lysis buffer containing 1% 2-mercaptoethanol. Whole-body total RNA samples were obtained from 7-8 females submerged in RNA lysis buffer and homogenized with a plastic microcentrifuge tube pestle and then frozen at −20°C overnight. Total RNA was subsequently extracted using the EZ-10 RNA Miniprep Kit (Bio Basic Inc., Markham, ON, Canada) following the manufacturer's protocol. The purified RNA was loaded onto a Take3 micro-volume plate and quantified using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, United States). cDNA was synthesized with 25 ng total RNA as template from each sample using iSCRIPT Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada) following the manufacturer's instructions and diluted 10-fold for subsequent qPCR analysis.

The synthesized cDNA from mosquito organs was used to assess PK1-R and PK2-R transcript expression by amplifying a 249-bp and 203-bp fragment, respectively, with the forward (5′-TGTACGCTCTGATTGGCCTGAA-3′; PK1-R) or (5′-TATTGTACTTTCTGTCGACGTGC-3′; PK2-R) and reverse (5′- GCACTAATGGATCGTTCGGCTG-3′; PK1-R) or (5′-ATTTGCACCCGTTTTGAAGGAG-3′; PK2-R) primer sets based on a previously identified sequence (Choi et al., 2013; GenBank accession: EAT35008.1, PK1-R; KC155994.1, PK2-R) using PowerUP SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, United States). A 214-bp fragment of the *rp49* (GenBank accession: AY539746) gene was also amplified as a reference control using primers described previously (Paluzzi et al., 2014) with all cycling conditions as follows: (1) 50°C for 2 min, (2) 95°C for 20 s, and (3) 40 cycles of (i) 95°C for 3 s and (ii) 62°C for 30 s. Relative expression levels were determined using the $\Delta\Delta C_T$ method and normalized to the *rp49* reference gene. Expression profiles were determined using three biological replicates, each of which included four technical replicates.

Enzyme-Linked Immunosorbent Assay (ELISA) and Whole Mount Immunohistochemistry

A custom synthesized rabbit polyclonal antibody (*Rhopr*CAPA-2 antigen sequence: EGGFISFPRV-NH_2, generously provided by Prof. Ian Orchard, University of Toronto Mississauga, ON, Canada) and recently used to localize CAPA (PRV-NH_2) immunoreactivity in *A. aegypti* (Sajadi et al., 2020), was used herein to visualize PRXa-like immunolocalization (X = V or L) along mosquito tissues as this antibody was believed to cross-react with structurally related peptides. To confirm that the antibody recognizes PKs, a competitive ELISA was performed. In brief, 96-well plates were coated with anti-*Rhopr*CAPA-2 primary antiserum diluted to 1:1000 in carbonate buffer (15 mM Na2CO3-H2O and 35 mM NaHCO3 in water; pH 9.4) and incubated overnight at 4°C. Plate contents were discarded, blotted, and rinsed three times with wash buffer (350 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 5.15 mM Na2HPO4-H2O, and 0.05% Tween-20 (v/v) in water). Wells were then incubated for 1.5 h on a rocker with block buffer [0.5% skim milk powder (w/v) and 0.5% BSA (w/v) in PBS]. The block solution was discarded, and standard solutions were added (100 μL/well). Standards consisted of *Aedae*CAPA-1 and *Aedae*CAPA-2 diluted in block buffer to achieve concentrations ranging from 250 pM to 250 nM, or *Aedae*CAPA-PK1 and *Rhopr*PK2b from 4.8 nM to 75 uM. After a 1.5 h incubation on a rocker, 1 nM biotinylated-*Drome*CAPA diluted in block buffer (MacMillan et al., 2018) was added (100 μL/well) to compete with the unlabeled peptide standards for antibody binding. Following overnight incubation at 4°C, the wells were washed four times with wash buffer, and incubated at 4°C for 1.5 h with Avidin-HRP (1:2000; Bio-Rad, Mississauga, ON, Canada). The wells were washed three times, and incubated with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (100 μL/well; Sigma-Aldrich, Oakville, ON, Canada) for 15 min at RT for color development. Reactions were stopped with 100 μL/well 2N HCl and absorbance at 450 nm was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, United States).

In light of the PK receptor transcript expression profile and antibody validation confirming pyrokinin cross-reactivity, the pyloric valve region separating the midgut and hindgut organs, as well as the hindgut, including both the ileum and rectum of 4-day old adult female mosquitoes were used to examine PRXa-like immunoreactivity, following a procedure described previously (Rocco et al., 2017). Tissues were incubated in primary antibody solution (diluted 1:1000) made up in 0.4% Triton X-100, 2% normal sheep serum (NSS) (v/v) and 2% BSA (w/v) in PBS. Negative controls involved primary antibody solution pre-incubated with 5 μM *Aedae*CAPA-PK1. Both experimental and control antibody solutions were prepared and left at 4°C overnight prior to incubating with tissues. Following a 48-h primary antibody incubation at 4°C with gentle agitation, tissues underwent three 10-min washes with PBS, and were then incubated with Alexa Fluor 568-conjugated AffiniPure goat anti-rabbit secondary antibody (1:200 dilution; Life Technologies) and 0.165 μM Alexa Fluor 488-conjugated phalloidin (Life Technologies) in 10% NSS (v/v) made up in PBS overnight at 4°C with gentle agitation. Tissues were then rinsed three times with PBS and mounted on slides with mounting media containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) to visualize cell nuclei in tissue preparations. Images were analyzed using a Lumen Dynamics X-Cite^{TM} 120Q Nikon fluorescence microscope (Nikon Instruments Inc., Melville, NY, United States) and a Zeiss Cell Observer Spinning Disk Confocal Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Heterologous Functional Receptor Assay

Preparation of Mammalian Expression Constructs With *A. aegypti* PK1-R and PK2-R

The complete open reading frame was amplified based on the partial (Nene et al., 2007) and complete sequences (Choi et al., 2013) reported earlier for the *A. aegypti* PK1-R (Genbank accession: EAT35008.1) and PK2-R (Genbank accession: KC155994.1). Forward 5′-ATGTTCAGTACAAACCTAAC-3′ (PK1-R) or 5′-ATGATGGAGCTGCAGCAGGTGTCA-3′ (PK2-R) and reverse 5′-TTAATGACGTACCTTGAAAGCTTG-3′ (PK1-R) or 5′-TCAGCGAATCTCATTGTTGATTTCGGCC-3′ (PK2-R) primers were designed over the start and stop codons (underlined), respectively, and used to amplify the complete coding sequence using Q5 High-Fidelity DNA polymerase following manufacturer recommendations (New England Biolabs, Whitby, ON). The 1125 bp (PK1-R) and 1917 bp (PK2-R) PCR products were purified using a Monarch PCR purification kit (New England Biolabs, Whitby, ON, Canada) and reamplified using the identical reverse primers but forward primers possessing the consensus Kozak translation initiation sequence (Kozak, 1984; Kozak, 1986), 5′-GCCACCATGTTCAGTACAAACCTAAC-3′ (PK1-R) or 5′-GCCACCATGATGGAGCTGCAGCAGGTGTCA -3′ (PK2-R). The resulting products were cloned into pGEM-T Easy sequencing vector, and miniprep samples were sequenced to verify base accuracy. The receptor constructs were excised using standard restriction enzyme digestion and subcloned into the mammalian expression vector, pcDNA 3.1^+ (Life Technologies, Burlington, ON, Canada). Transfection quality plasmid DNA was purified from an overnight bacterial culture using the PureLink MidiPrep Kit (Invitrogen, Burlington, ON, Canada) following manufacturer guidelines.

Cell Culture, Transfections, and Bioluminescence Assay

Chinese hamster ovary cells (CHO-K1) described previously (Paluzzi et al., 2012; Gondalia et al., 2016; Wahedi and Paluzzi, 2018) were grown in Dulbecco's modified eagles medium: nutrient F12 (DMEM:F12) media containing 10% heat-inactivated fetal bovine serum (FBS; Wisent, St. Bruno, QC, Canada), 200 μg/mL geneticin, and antimycotic-antibiotic mixture as described previously (Wahedi and Paluzzi, 2018). Cells were grown to approximately 80% confluency and were co-transfected with mammalian codon-optimized aequorin using Lipofectamine 3000 transfection reagent following recommended guidelines (Invitrogen, Burlington, ON, Canada) to transiently express either the *A. aegypti* PK1-R or PK2-R. Cells were then prepared for the functional assay that

was performed 48 h post-transfection following a protocol described previously (Wahedi and Paluzzi, 2018), at which point mCherry-expressing cells showed a transfection efficiency of about 90%. Various concentrations of synthesized PK peptides and other peptides (purity > 90%; Genscript, Piscataway, NJ, United States) were prepared in BSA media and loaded in quadruplicate into white 96-well luminescence plates (Greiner Bio-One, Germany).

Luminescence responses to *A. aegypti* CAPA-1 (GPTVGLFAFPRV-NH_2), CAPA-2 (pQGLVPFPRV-NH_2) and CAPA-PK1 (AGNSGANSGMWFGPRL-NH_2) peptides, along with the *R. prolixus* PK2 orthologs, PK2a (NTVNFSPRL-NH_2), and PK2b (SPPFAPRL-NH_2) were examined. A list of peptides used in this study and sequence comparison to native *A. aegypti* pyrokinin peptides is found in **Supplementary Table S1**. Cells prepared for the functional assay were loaded into each well of the plate using an automated injector unit and luminescent response was measured with a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, United States). Negative controls were carried out using BSA media alone whereas 50 μM ATP, which activates endogenously expressed purinoceptors (Iredale and Hill, 1993; Michel et al., 1998), was used as a positive control. Luminescence responses were normalized to ATP responses and analyzed in GraphPad Prism 7.02 (GraphPad Software, San Diego, CA, United States). EC_{50} values were determined using dose-response curves from multiple biological replicates.

Hindgut Contraction Assays

Preparation of Hindgut Tissues

Hindgut contraction assays were conducted on isolated ilea and recta of female *A. aegypti*. Mosquitoes were anesthetized with CO_2 and dissected under saline, containing 150 mM NaCl, 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfuronic acid (HEPES), 3.4 mM KCl, 7.5 mM NaOH, 1.8 mM $NaHCO_3$, 1 mM $MgSO_4$, 1.7 mM $CaCl_2$-$2H_2O$ and 5 mM glucose, titrated to pH 7.1. The isolated tissue was secured with minutien pins within a Sylgard-lined petri-dish.

Peptide and Neurotransmitter Dosages

Given its high sequence similarity to endogenous *A. aegypti* PK2 peptides (**Supplementary Table S1**), we used the *R. prolixus* PK2b peptide (Paluzzi and O'Donnell, 2012), henceforth referred to as *Rhopr*PK2b, for motility bioassays. Commercially synthesized peptides (Genscript, Piscataway, NJ, United States), *Aedae*CAPA-PK1 (AGNSGANSGMWFGPRL-NH_2) and *Rhopr*PK2b (SPPFAPRL-NH_2), were used to examine myomodulatory activity. Serotonin (5-hydroxytryptamine, 5-HT; Sigma-Aldrich, Oakville, ON, Canada), previously shown to stimulate *A. aegypti* hindgut contractions (Messer and Brown, 1995), was used as a stimulatory control. Given the inhibitory properties of myoinhibitory peptides (having the consensus $W(X_6)W$-NH_2 carboxyl terminus) on insect hindgut motility (Lange et al., 2012), *Rhopr*MIP-7 (AWNSLHGGW-NH_2; Genscript, Piscataway, NJ, United States; Paluzzi et al., 2015) was used as an inhibitory control. All hormones were diluted in saline to achieve a final concentration of 1 μM.

Electrophysiological Measurements of Recta

To assess whether PK1 peptides plays a role in regulating rectal motility, contractions were monitored in saline to obtain baseline contraction rates and subsequently following *Aedae*CAPA-PK1 or 5-HT treatments, using probes connected to an impedance converter (UFI model 2991, Morro Bay, CA, United States) connected to a Powerlab 4/30 and laptop computer running LabChart Pro 6.0 software (AD Instruments, Colorado Springs, CO, United States). The isolated rectum was contained in a small circular ridge of a Sylgard-coated dish which was bathed in saline. Probes were positioned on either side of the rectum by observation with a dissecting microscope (Olympus SZ61), and contractile responses were recorded on LabChart Pro 6.0 software (AD Instruments, Colorado Springs, CO, United States). Contractions were monitored for two minutes prior to, and after the addition of either *Aedae*CAPA-PK1 or 5-HT.

The number of contractions were recorded over a 2-min interval to obtain the contraction frequency (number of contractions min^{-1}). To account for variability in myoactivity between individual preparations, rectal myoactivity is expressed as a ratio in contraction frequency upon *Aedae*CAPA-PK1 or 5-HT application relative to the baseline contractile activity of the same tissue preparation in saline alone.

Video Measurements of Ilea

Unlike the rectum, the mosquito ileum produces weaker contractions that we were unable to measure using an impedance converter as described above. As a result, to examine the effects of a PK2 peptide on anterior hindgut motility, video recordings of dissected ilea were obtained using an Olympus SZ microscope connected to Luminera's INFINITY1-2CB video camera. The dissected gut was pinned in the midgut and rectum to allow the ileum to freely contract in saline and following treatments. Contractions were recorded for 2 min in saline that was used for baseline activity measurements, followed by three subsequent 2-min recordings, including: (i) additional saline, (ii) 1 μM *Rhopr*PK2b, and (iii) either 1 μM 5-HT with *Rhopr*PK2b or 1 μM *Rhopr*MIP-7 with *Rhopr*PK2b. The *Rhopr*PK2b was added along with the stimulatory or inhibitory compound (5-HT or *Rhopr*MIP-7, respectively), to maintain its 1 μM concentration in the bath.

Using the video recordings, the contraction rate (number of contractions min^{-1}), average duration of each contraction (sec), and average length of time between each contraction (sec) over the 2-min interval were recorded individually. To account for any potential changes in tissue contractile activity upon adding solutions to the bath, each of these variables were measured relative to baseline saline recordings of the same preparation as a ratio of change, where a value above or below one indicates an increase or a decrease, (respectively), in the rate, duration or length between each contraction in response to the treatment.

Ion Transport Along the Rectal Pad Epithelia

Preparation of Hindgut Tissues

Female mosquitoes were anesthetized on ice for 3 min and dissected in Ca^{2+}-free *A. aegypti* saline, as described previously

(Paluzzi et al., 2014), to limit spontaneous hindgut contractions during ion flux measurements. The isolated rectum was then transferred to a Petri dish with saline, pre-coated with poly-L-lysine (Paluzzi et al., 2014) to allow the tissue to adhere to the bottom of the dish.

Peptide Dosages and Saline Application

*Aedae*CAPA-PK1 or *D. melanogaster Droso*-leucokinin (NSVVLGKKQRFHSWG-NH_2, Genscript, Piscataway, NJ, United States; provided by Prof. Dick Nässel, Stockholm University, Sweden) were solubilized in double distilled water as a 1 mM stock and then diluted in the above-mentioned Ca^{2+}-free *A. aegypti* saline to achieve a 1 μM final concentration. Control measurements were obtained by applying an equal volume of saline only, referred to as saline control treatments.

Scanning Ion-Selective Electrode Technique (SIET)

To measure Na^+ flux across rectal pad epithelia, ion-selective microelectrodes and reference electrodes were used, as described previously (Paluzzi et al., 2014), with the following changes: the microelectrode was backfilled with 100 mM NaCl, front loaded with Na^+-selective ionophore (sodium ionophore II cocktail A; Fluka, Buchs, Switzerland), and calibrated before every preparation with 200 mM NaCl, and 20 mM NaCl containing 180 mM LiCl to equalize osmolarity of the standard solutions.

SIET measurements were obtained through the Automated Scanning Electrode Technique (ASET) software (Science Wares, East Falmouth, MA, United States). To obtain background recordings for every preparation, the microelectrode tip was positioned at a site located 3 mm away from the tissue. Voltage gradients were measured as the microelectrode moved perpendicularly to the tissue surface between two points separated by a distance of 100 μm. The sampling protocol used a wait time of 4 s after microelectrode movement and a recording time of 1 s after the wait period. Following background voltage readings, the ion-selective microelectrode tip was positioned at a distance of 2 μm from the rectal pad epithelia. A similar sampling protocol was used at the tissue surface. For each sample, several initial measurements were obtained at various sites across the length of one rectal pad and the site demonstrating maximal ion flow was used for all subsequent measurements. Specifically, the sampling protocol was repeated six times at this target site in saline solution, and the voltage difference between the two sites was used to calculate a voltage gradient by the ASET software. A treatment (either saline or peptide) was then directly applied to the dish to determine if this induced a change in ion flux by the rectal pad epithelia. The sampling protocol was then repeated 12 times. Following measurements at the rectal pad sites, background voltage readings were again recorded at a distance of 3 mm away from the tissue.

Calculation of ion flux used in this study has been described previously (Paluzzi et al., 2014). Approximately 70% of tissues initially exhibited Na^+ absorption in saline, whereas the remaining preparations (approximately 30%) were initially secreting Na^+ into the rectal lumen. In light of this potential source of variation, further experimental treatment was only carried out on preparations exhibiting hemolymph-directed ion transport (i.e. reabsorbing Na^+). The change in flux upon treatment application was calculated by subtracting flux values obtained during saline measurements, in which a positive change indicates increased ion absorption, and a negative change represents a decrease in absorption.

Graphical Representation

Data were transferred into GraphPad Prism 7.0 to create all figures and conduct statistical analyses, which are described as appropriate in the figure captions.

RESULTS

Receptor Expression Profile and Localization of PRXa-like Immunoreactivity

As a first step toward discovering physiological roles for pyrokinins in *A. aegypti*, prospective targets were examined. RT-qPCR was used to measure PK1 and PK2 receptor transcript levels in adult organs. Expression of PK1-R was only significantly enriched in the rectum compared to expression in the whole body (**Figure 1**). Comparatively, PK2-R was abundant in the anterior ileum and significantly enriched in reproductive organs relative to the whole body (**Figure 1**), and demonstrated significantly higher expression compared to PK1-R levels in these organs, which was consistent across all biological replicates.

Given this highly defined expression within the two segments of the hindgut, we next used immunohistochemistry to visualize peptide distribution along this region. Using a custom-synthesized antibody against a CAPA neuropeptide, which was

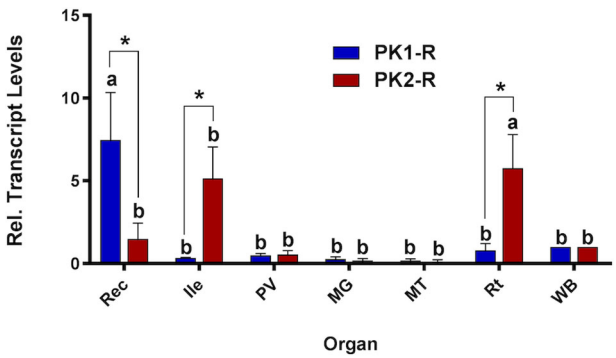


FIGURE 1 | Spatial expression patterns of PK1-R and PK2-R transcript in female organs relative to the whole body (WB), normalized to the reference gene *rp49*. Expression was analyzed in the rectum (Rec), ileum (Ile), pyloric valve (PV) region, midgut (MG), Malpighian tubules (MT) and reproductive organs (Rt). Levels significantly different from the WB are denoted with different letters, and significant differences in transcript abundance between the two receptors in an individual organ are denoted by an asterisk, as determined by a two-way ANOVA and Sidak post test ($p < 0.05$). Data represent the mean ± SEM ($n = 3$).

shown to cross-react with and bind to PKs (**Supplementary Figure S1**), PRXa-like immunostaining was observed in an axon net encircling the pyloric valve (**Figure 2A**), which separates the midgut and hindgut. Immunolocalization was detected in axonal projections over the ileum (**Figure 2B**), and innervating the rectal pads with immunoreactive projections terminating in close association and encircling 4-5 cells within the lumen of all six rectal pads (**Figure 2C**; **Supplementary Figure S2**). Staining was abolished in control preparations treated with antibody pre-incubated with *Aedae*CAPA-PK1 (**Supplementary Figure S3**).

PK1-R and PK2-R Functional Activation Assay

Heterologous expression and functional analysis of the *A. aegypti* PK1-R revealed a robust activation by *Aedae*CAPA-PK1 (EC_{50} = 37.6 nM), as demonstrated by the dose-dependent luminescent response (**Figure 3A**) in CHO-K1 cells transiently expressing *A. aegypti* PK1-R. Additionally, the *A. aegypti* PK1-R was responsive to the *R. prolixus* PK2 orthologs, *Rhopr*PK2a and *Rhopr*PK2b (EC_{50} = 1.57 and 0.4465 μM, respectively), encoded by the *R. prolixus ADA83379.1* gene (Jurenka and Nusawardani, 2011), albeit 12- to 42-fold reduced activity compared to *Aedae*CAPA-PK1. Structurally related peptides (*Aedae*CAPA-1 and -2) encoded by *A. aegypti AAEL005444* gene were also effective in activating PK1-R at high concentrations. The efficacy of these other peptides, however, was orders of magnitude lower than *Aedae*CAPA-PK1, which elicited a luminescent response significantly greater than that achieved with all other tested peptides. Notably, at 10 nM, *Aedae*CAPA-PK1 was the only peptide which elicited a significant luminescent response different from controls treated with BSA assay media alone (**Figure 3B**).

Cells transiently expressing the *A. aegypti* PK2-R (**Figure 3C**) were significantly more responsive to the PK2 peptides, *Rhopr*PK2a and *Rhopr*PK2b (EC_{50} = 17.47 and 8.54 nM, respectively), particularly at lower doses of 10 nM (**Figure 3D**) since no other tested peptide at this concentration had a significant effect on *A. aegypti* PK2-R response. Although *Aedae*CAPA-PK1 was still able to activate PK2-R at high concentrations (EC_{50} = 158 nM), it elicited an over four-fold greater potency on PK1-R expressing cells (**Figures 3A,C**). Similar to results observed in PK1-R functional expression, the structurally related CAPA peptides (*Aedae*CAPA-1 and -2) were only active on PK2-R at very high concentrations and did not achieve over 25% activation relative to the highly potent pyrokinins (**Figures 3C,D**).

Pyrokinins on Mosquito Hindgut Motility

Having verified specific sites of *A. aegypti* PK1-R and PK2-R transcript enrichment and functional activation of these receptors with greatest sensitivity to PK1 and PK2 peptides, we sought to determine the potential myotropic activity of these neuropeptides on the rectum and ileum, respectively. Serotonin (5-HT), a known myostimulator of *A. aegypti* hindgut (Messer and Brown, 1995), was used as a positive control to validate functionality of the bioassay setup. Application of 5-HT typically stimulated greater contractile activity compared to unstimulated baseline activity (**Figure 4A**) reflecting a 1.97-fold increase in contraction rate for female recta (**Figure 5A**) and a 2.48-fold

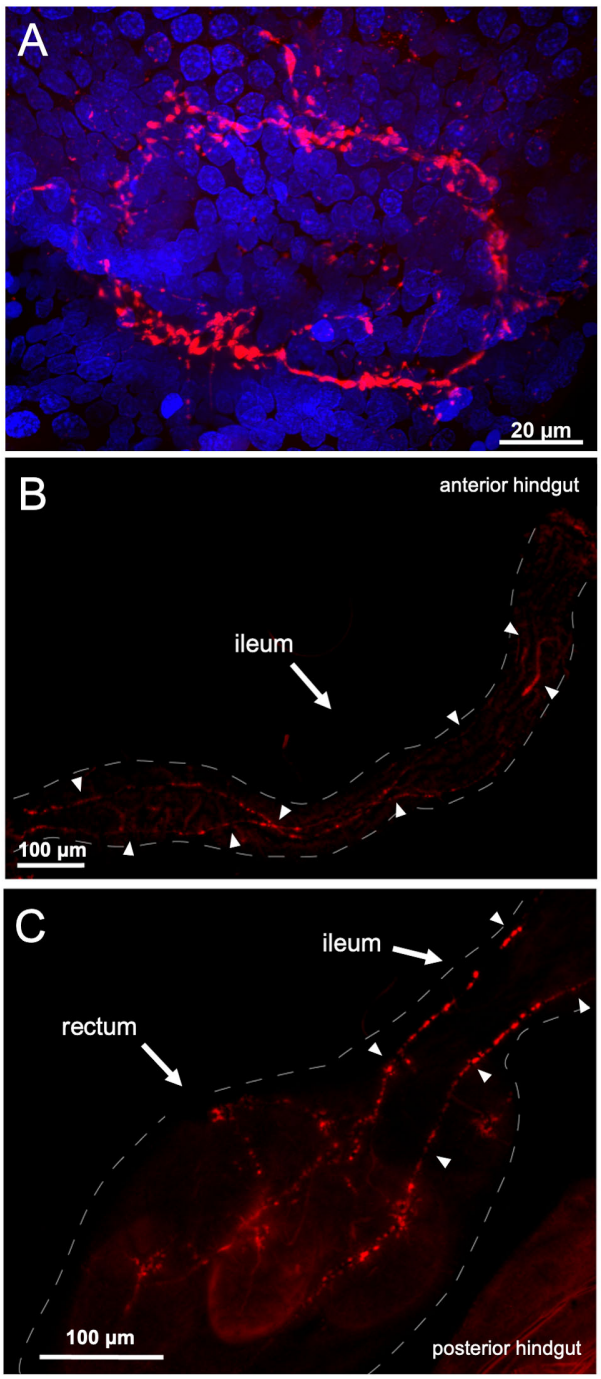


FIGURE 2 | PRXa-like immunolocalization associated with the female gut. Immunoreactive staining (red) was observed within an axon net encircling the pyloric valve at the junction between the midgut and hindgut with nuclei (blue) stained with DAPI **(A)**. PRXa-like immunoreactive axonal projections continue over the anterior hindgut, denoted by arrowheads **(B)** and terminate within the lumen of the six rectal pads **(C)**.

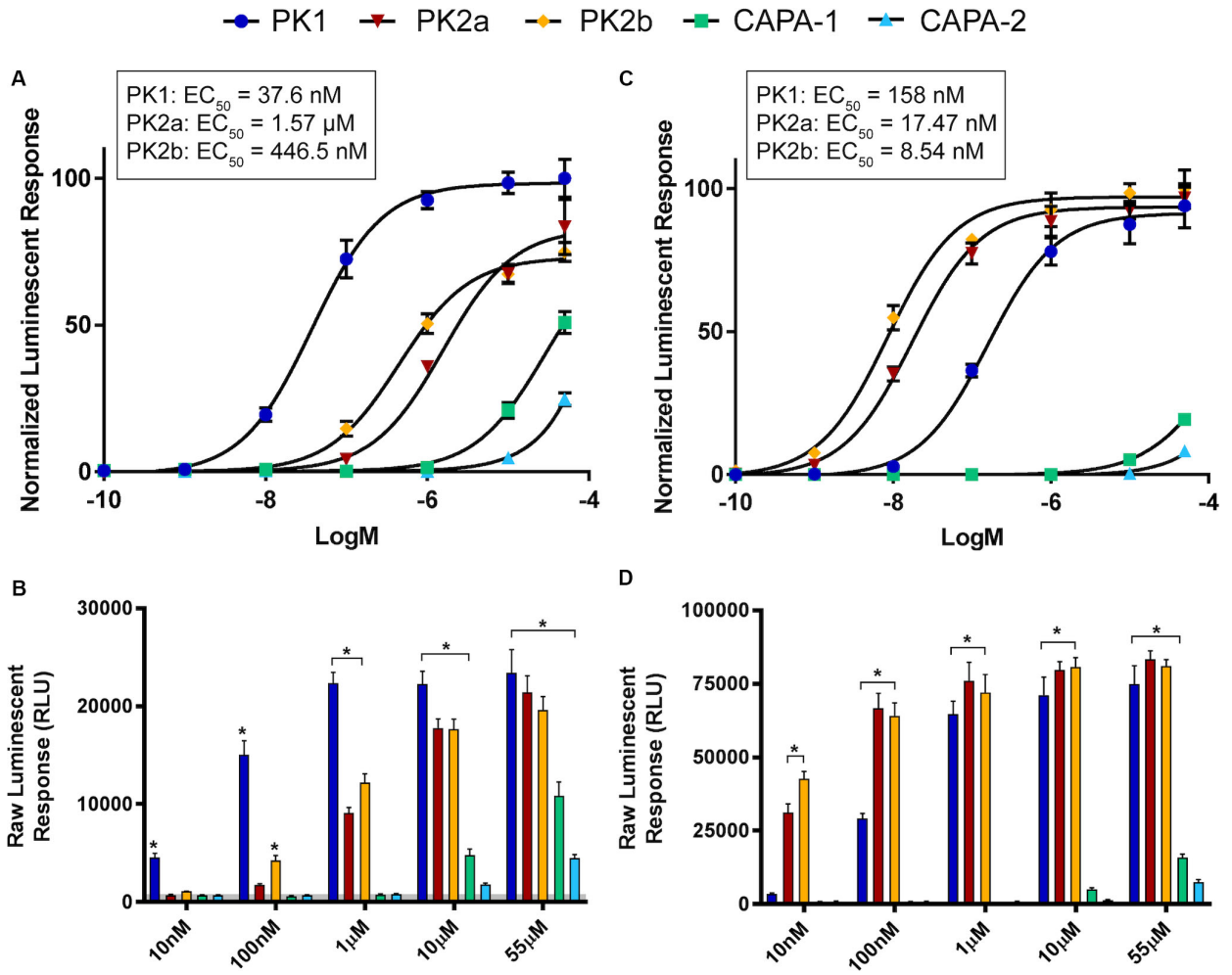


FIGURE 3 | Luminescent response of CHO-K1 cells expressing the *A. aegypti* PK1 **(A,B)** and PK2 **(C,D)** receptors. Transient expression of *A. aegypti* PK1-R in CHO-K1 cells was used to examine receptor functional activation by *Aedae*CAPA-PK1 (denoted as PK1) demonstrating a dose-dependent luminescent response following peptide application **(A,C)**. Structurally related peptides, including two exogenous PK2 analogs (denoted as PK2a and PK2b) along with endogenous *AAEL005444* gene-derived CAPA anti-diuretic peptides (denoted CAPA-1 and CAPA-2) demonstrated significantly lower activity on the heterologously expressed *A. aegypti* PK1 receptor **(B)**. Although *Aedae*CAPA-PK1 was able to activate both receptors, PK2-R displayed more selective activation by the PK2 analogs, PK2a and PK2b, derived from the *R. prolixus ADA83379.1* gene **(C,D)**. Luminescent responses were monitored after peptide treatment, with data representing average luminescence (mean ± standard error) over the first 10 s immediately following peptide application. Raw luminescent responses significantly different from background responses (BSA media alone shown in the gray shaded region) are denoted by an asterisk, as determined by a one-way ANOVA and Dunnett's multiple comparison *post hoc* test ($p < 0.05$). Data obtained from two individual biological replicates, each consisting of 4-8 technical replicates. Sequences of the tested peptides along with other related endogenous peptides are listed in **Supplementary Table S1** highlighting the conserved core bioactive sequence within each peptide family.

increase for male recta (**Figure 5B**). *Aedae*CAPA-PK1 had no apparent effect on contractile activity (**Figure 4B**) with no change in the contraction rate (0.99-fold) for female recta (**Figure 5A**) and an increase (1.28-fold) for male recta (**Figure 5B**).

To assess the role of PK2 along the anterior hindgut, we used both a stimulatory as well as an inhibitory control following *Rhopr*PK2b treatment. Given the potent bioactivity of *Rhopr*PK2b on the mosquito PK2-R (**Figures 3C,D**), this naturally occurring analog can be used as a proxy to determine the action of endogenous PK2 peptides in the mosquito. Relative to baseline levels, contraction frequency significantly decreased from 0.72-fold to 0.55-fold in response to *Rhopr*PK2b (**Figure 6A**; **Supplementary Video S1**). This inhibitory effect was reversed upon 5-HT treatment. Although the duration of each contraction did not significantly differ upon *Rhopr*PK2b application, the length of time between each contraction event increased. 5-HT effectively reduced both of these metrics, resulting in a 2.88-fold increase in ileal contraction rate (**Figures 6B,C**). Due to its inhibitory nature, we further assessed the effects of *Rhopr*PK2b with the addition of a known insect myoinhibitor, *Rhopr*MIP-7 (Lange et al., 2012). Although *Rhopr*PK2b significantly reduced ileal contraction frequency, *Rhopr*MIP-7 resulted in further inhibition by 0.38-fold (**Figure 7A**; **Supplementary Video S2**). The duration of each contraction increased in response to both

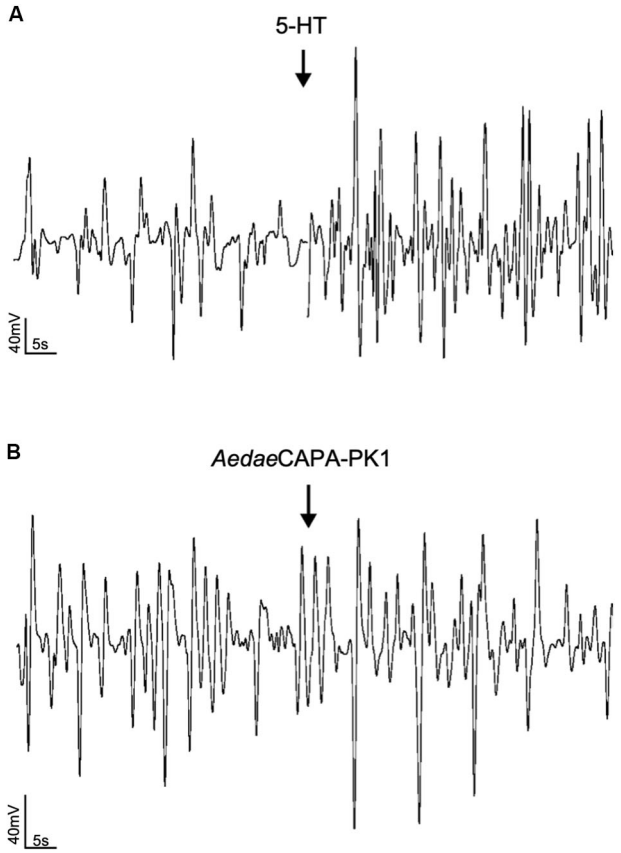


FIGURE 4 | Sample traces of 5-HT **(A)** and *Aedae*CAPA-PK1 **(B)** on spontaneous rectal contractions. Arrows indicate time of hormone application following baseline saline measurement. Contraction rate increased in response to 5-HT **(A)**, whereas no change in activity was observed following *Aedae*CAPA-PK1 application **(B)**.

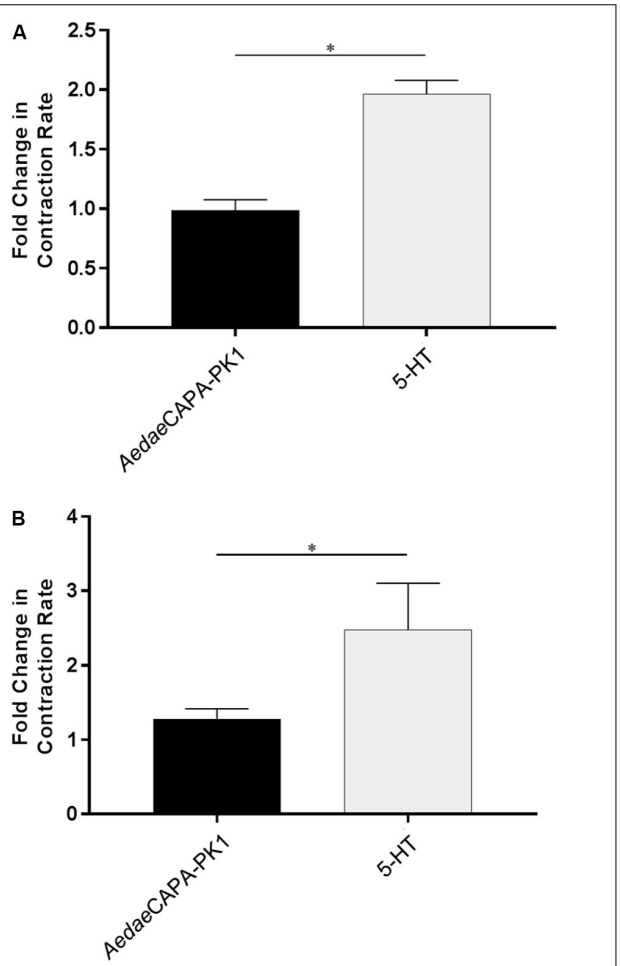


FIGURE 5 | Fold change in contraction frequency of recta isolated from female **(A)** and male **(B)** mosquitoes in response to 5-HT and *Aedae*CAPA-PK1 relative to baseline activity. Mean ± SEM are obtained from 6–18 preparations, with asterisks representing significant differences, as determined using an unpaired *t*-test ($p < 0.001$).

peptides by about 1.9-fold (**Figure 7B**), and *Rhopr*MIP-7 further enhanced relaxation duration by 3.9-fold (**Figure 7C**).

*Aedae*CAPA-PK1 on Hindgut Ion Transport

As determined by the SIET, most recta (∼70%) exhibited hemolymph-directed Na^+ transport (i.e. absorption) in saline prior to treatment, while the remaining recta exhibited lumen-directed transport (i.e. secretion); however, only absorptive rectal preparations were used to examine the potential for *Aedae*CAPA-PK1 in eliciting an ionoregulatory role. Furthermore, measurements of the preparations displayed variability in baseline transport activity in saline, therefore the difference in ion flux, following application of saline alone or containing peptide, relative to initial transport activity in saline was calculated. The resultant data showed that Na^+ absorption decreased by 52.8 ± 31.4 pmol cm^{-2} s^{-1} after saline application (**Figure 8A**); however, the net Na^+ transport remained absorptive. An earlier study showed that the receptor for *A. aegypti* kinins (*Ae*KR) was localized to the hemolymph-facing outer rectal pad membrane and *Ae*KR knockdown by RNA interference decreased excretion (Kersch and Pietrantonio, 2011). This result suggested that an available kinin analog (i.e. *Droso*-leucokinin) may decrease reabsorption over the rectal pads, given that mosquito kinins stimulate diuresis by the Malpighian tubules (Veenstra et al., 1997). Indeed, we found that the ionomodulatory effect of *Droso*-leucokinin along the rectum was significant leading to a four-fold decrease in Na^+ absorption (201.6 ± 39.0 pmol cm^{-2} s^{-1}) compared to saline control (**Figures 8A–C**). In response to *Aedae*CAPA-PK1, Na^+ transport decreased two-fold (104.4 ± 43.2 pmol cm^{-2} s^{-1}), although this was not significantly different from changes in ion transport following treatment with saline alone (**Figures 8A,D,E**).

DISCUSSION

In this study, we have characterized the functional activation of *A. aegypti* PK1 and PK2 receptors in response to various PKs and

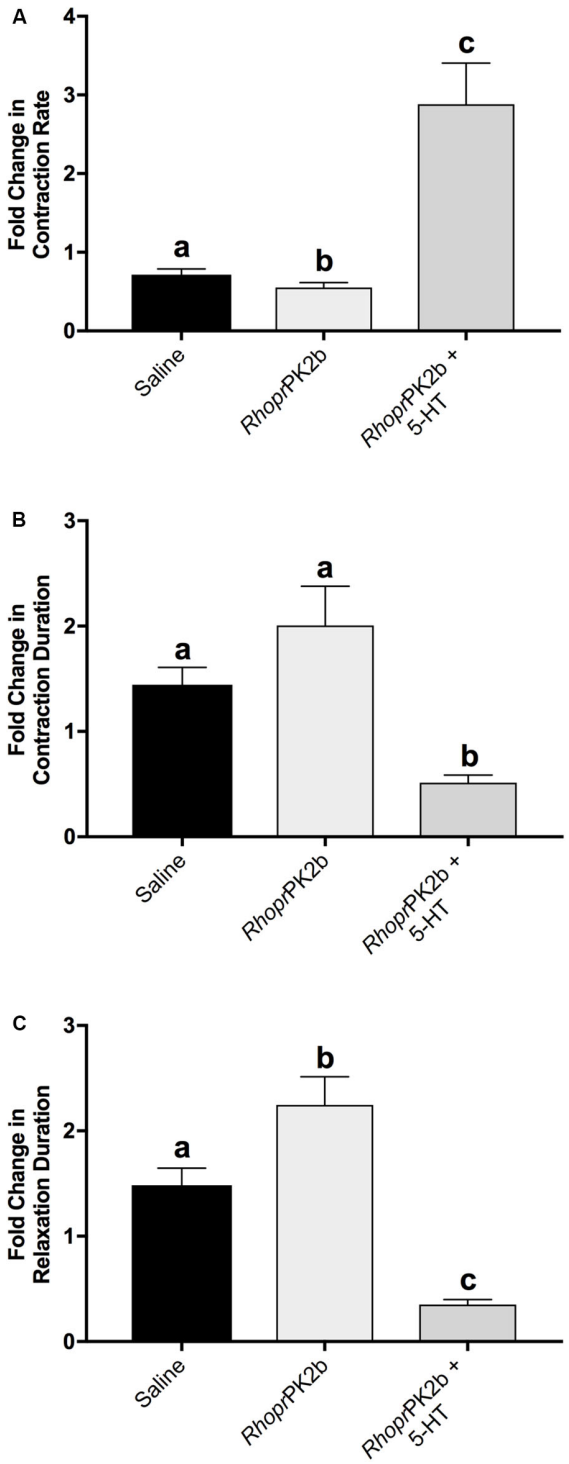


FIGURE 6 | Motility of ilea isolated from female mosquitoes in response to added saline (vehicle control), *Rhopr*PK2b and 5-HT (stimulatory control). The change in contraction frequency **(A)**, duration **(B)** and length of time between contractions **(C)** was measured relative to baseline recordings. Values are presented as mean ± SEM from 15 preparations. Significant differences between the treatments are denoted by different letters, as determined by a one-way repeated measures ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

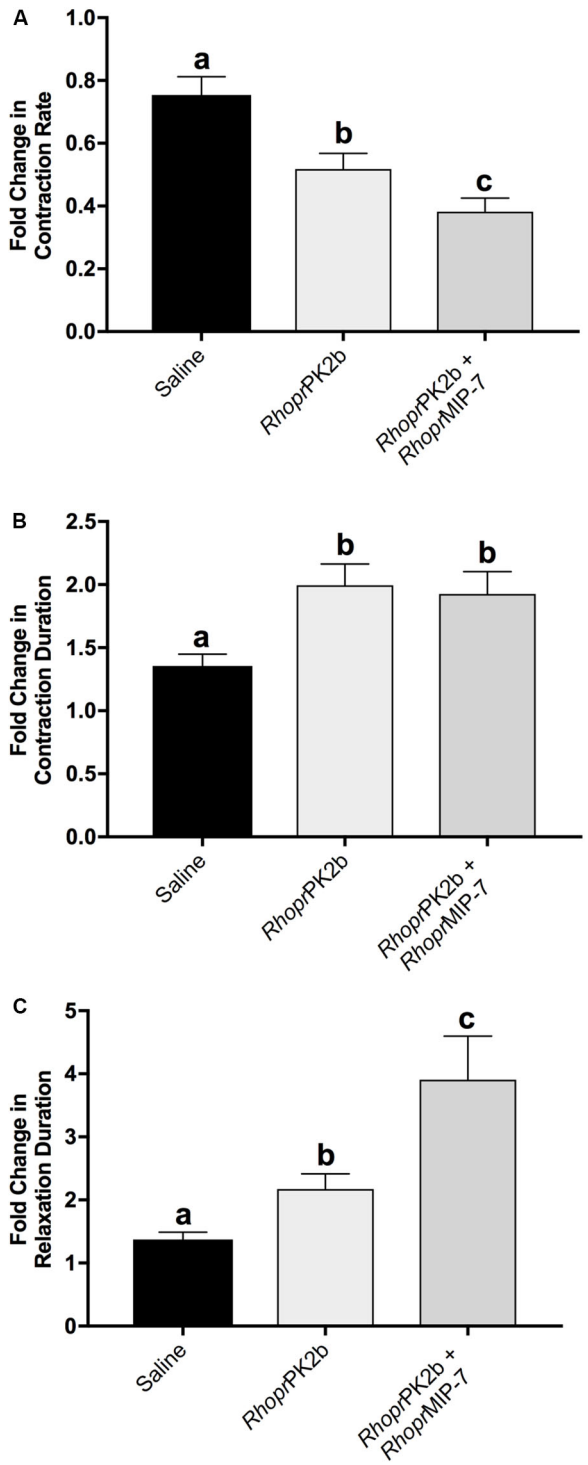


FIGURE 7 | Motility of ilea isolated from female mosquitoes in response to added saline (vehicle control), *Rhopr*PK2b and *Rhopr*MIP-7 (inhibitory control). The change in contraction frequency **(A)**, duration **(B)** and length of time between contractions **(C)** was measured relative to baseline recordings. Values are presented as mean ± SEM from 17 preparations. Significant differences between the treatments are denoted by different letters, as determined by a one-way repeated measures ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

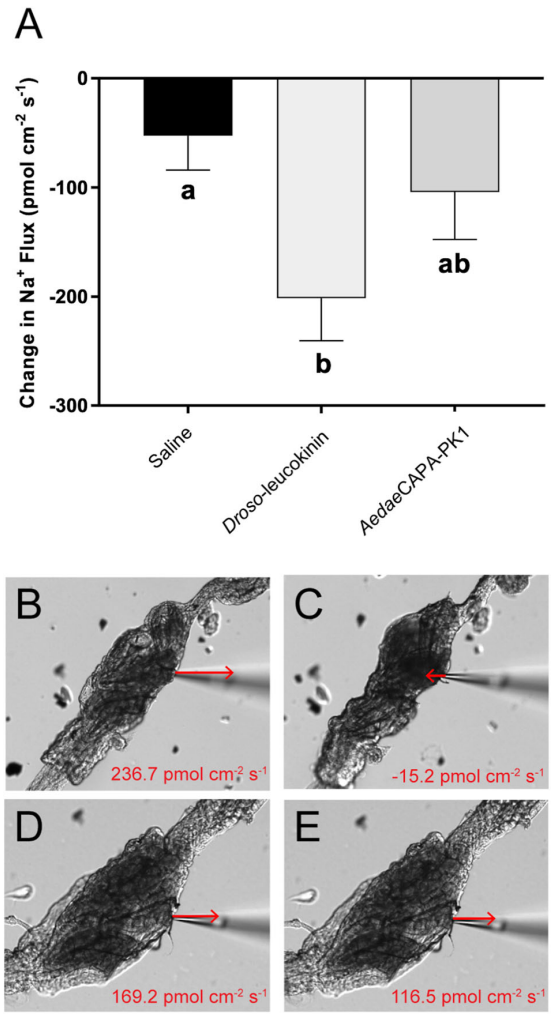


FIGURE 8 | Changes in Na^+ transport across female rectal pad epithelia in response to saline (vehicle control), *Droso*-leucokinin and *Aedae*CAPA-PK1. Experiments were run only using hindgut preparations that were initially exhibiting hemolymph-directed ion flux (absorption) when unstimulated. Mean ± SEM obtained from 10 to 14 tissue preparations. There was a significant reduction in Na^+ absorption in response to *Droso*-leucokinin ($p < 0.05$); however, ion transport did not significantly change following *Aedae*CAPA-PK1 treatment in comparison to basal transport activity **(A)**, determined by a one-way ANOVA and Tukey's *post hoc* test. Still images of the dissected rectum are demonstrated with ion flux recordings obtained from a sample measurement during baseline activity in saline **(B,D)** followed by either *Droso*-leucokinin **(C)** or *Aedae*CAPA-PK1 **(E)** application. Arrows indicate the direction and approximate the magnitude of Na^+ transport, but are not drawn to scale. Scale bars, 100 μm.

related neuropeptides. Our findings indicate that PK1-R is most sensitive to PK1 peptides possessing a WFGPRL-NH_2 carboxyl terminus, also referred to as tryptopyrokinins (Veenstra, 2014), whereas PK2-R is most sensitive to PK2 peptides characterized by their FXPRL-NH_2 motif. This selective activation was similarly observed in *A. aegypti*, *A. gambiae*, *R. prolixus* and the European corn borer, *Ostrinia nubilalis* (Olsen et al., 2007; Paluzzi and O'Donnell, 2012; Nusawardani et al., 2013; Choi et al., 2013), further confirming the binding specificity and selectivity of PK receptors to their subfamily specific ligands.

Pyrokinins were first discovered in insects based on their effects on hindgut physiology (Holman et al., 1986). The hindgut collects undigested foodstuff passed from the midgut through the pyloric valve, as well as fluid secreted from the Malpighian tubules, and guides these contents along the alimentary canal for waste excretion (Hine et al., 2014). Here we found that relative to other regions examined within the adult mosquito alimentary canal, PK2-R transcript was strongly enriched in the ileum, and PK1-R in the rectum. Our results partially agree with a previous RT-PCR analysis in *A. aegypti* that examined a subset of the tissues/organs we studied herein. Specifically, while enrichment of PK1-R in the midgut and ovaries was not evident as reported earlier (Hellmich et al., 2014), we found this receptor transcript to be significantly enriched in the rectum, similar to findings in other hematophagous arthropods, whereby the tick rectal sac, a structure analogous to the rectum, as well as the ovaries and nervous system were found to express the PK receptor (Yang et al., 2015). For PK2-R, this receptor was previously reported to be expressed within the ovaries in adult female mosquitoes (Hellmich et al., 2014), which is in agreement with our observations showing significant enrichment in female reproductive organs along with the enrichment we observed in the ileum. In contrast, mosquito CAPA receptor expression is highly enriched in the Malpighian tubules, but has no detectable expression in the hindgut (Sajadi et al., 2020). Further, this differential receptor expression supports that PRXa-immunolocalization in this study represents PK peptides, which was much weaker than CAPA-like immunoreactivity localized to neurosecretory cells in abdominal ganglia and associated neurohemal organs (Sajadi et al., 2020), yet the cross-reactivity may also reveal other structurally related peptides.

Due to the extensive network of musculature in the mosquito hindgut (Rocco et al., 2017), as well as the ion transporters dispersed along the epithelia (Patrick et al., 2006), this structure requires the coordination of ionomodulatory and myotropic activity for controlled waste elimination (Kwon and Pietrantonio, 2013). These processes are regulated by neuropeptides along with other neurochemicals and, considering the distinct expression profile of PK receptors in the hindgut that was herein identified along with PRXa-like immunohistochemical staining in association with the hindgut, we examined the potential involvement of pyrokinins in these processes. Specifically, immunostaining was observed in the axon net encircling the pyloric valve that was contiguous with axonal projections over the ileum and extending toward the rectum, revealing these sites as prospective targets for PKs. Although hindgut muscle contractions are myogenic, they can also be modified by neurochemical input from innervation extending from the ventral nerve cord (Audsley and Weaver, 2009). Previously, it was observed that immunostaining for the ovary ecdysteroidogenic hormone (OEH) isolated from *A. aegypti* was associated with the pyloric valve nerve net as well as axonal projections that continued toward the rectum; however, a direct source of this immunoreactivity was not observed although assumed to originate in the ventral nerve cord (Brown and

Cao, 2001). Comparatively, cells of the terminal abdominal ganglion innervate the locust hindgut to influence its motility (Donini et al., 2002). Some neurochemical factors have been identified as myotropins acting on *A. aegypti* hindgut, including serotonin and diuretic hormone 31 (Messer and Brown, 1995; Kwon and Pietrantonio, 2013), which also promote fluid secretion across the Malpighian tubules (Sajadi et al., 2018). The concerted action of these and other factors suggest coordination between the diuretic response and hindgut motility to regulate urine and blood bolus expulsion in female mosquitoes.

CAPA-like immunoreactivity was previously identified in pairs of neurosecretory cells of the abdominal ganglia, including the terminal ganglion from which projections that extend onto the hindgut may originate (Sajadi et al., 2020). Given the absence of CAPA receptor transcript in the hindgut (Sajadi et al., 2020), in this study we examined the role of PK1 and PK2 peptides on hindgut motility. The ability of *Rhopr*PK2b to significantly reduce ileal motility suggests endogenous PK2 peptides may have a role in regulating digestive and excretory processes. Visceral muscle contractions along the hindgut aid in the movement of undigested foodstuff following post-prandial diuresis to eliminate waste (Te Brugge et al., 2008). Inhibition of hindgut motility by a PK2 therefore warrants further study to determine its specific function in these processes and during different feeding states. This is the first study to establish a myoinhibitory role for a PK2 peptide on the insect hindgut, since members of this neuropeptide family sharing the conserved FXPRL-NH_2 carboxyl terminus have previously been characterized as having myostimulatory actions on the hindgut of *L. maderae*, *Periplaneta americana*, *Zophobas atratus*, and *Tenebrio molitor* (Holman et al., 1986; Predel and Nachman, 2001; Marciniak et al., 2012), demonstrating their diverse effects across various insect species.

*Aedae*CAPA-PK1 did not significantly influence myotropic activity in the mosquito rectum, despite receptor transcript enrichment in this organ, which prompted us to examine other potential functions. The rectum serves as the final site for reabsorbing ions, water and essential metabolites back into the hemolymph, ultimately determining the composition of excreted matter (Coast, 2009; Beyenbach and Piermarini, 2011). PRXa-like immunoreactivity revealed axonal projections terminating in close association with cells located within the lumen of the rectal pads (**Supplementary Figure S2**), structures that protrude from the rectal epithelium. This close association is indicative of a potential neurotransmitter/neuromodulatory role, in which local release at these synaptic terminals may activate receptors on these uncharacterized cells. To date, morphological studies of the mosquito rectum have been limited. Hopkins (1967) was one of the very few to study the ultrastructure of the mosquito rectal pads, revealing a single layer of epithelia surrounding a central canal. The central canal of the rectal pads carries a tracheal trunk, which extends into several tracheolar branches throughout the epithelia. Hopkins (1967) proposed the presence of tracheal and glial cells associated with this tracheal trunk. In some insects, cells along this region have been termed medullary cells, situated in close proximity to neurosecretory terminals (Gupta and Berridge, 1966). In *Blattella* and *Blaberus* rectal pads, axonal projections terminate at sites adjacent to the basal surface of secondary cells, which may help regulate fluid reabsorption (Wall and Oschman, 1973). By examining structural changes in the rectal pads following a blood meal in mosquitoes, these sites have been proposed to play crucial roles in maintaining iono- and osmoregulation to help restore hemolymph homeostasis (Hopkins, 1967). The presence of ion transporters within these structures was later revealed, whereby basolateral P-type Na^+/K^+-ATPase and apical V-type H^+-ATPase staining along the rectal pad epithelia supported that they serve as sites for ion transport, enhancing the overall absorption of ions and water back into the hemolymph prior to waste excretion (Patrick et al., 2006).

Although the exact mechanisms of ion and water transport within the rectal pads have not yet been characterized, the *Ae*KR was previously localized along the hemolymph-facing membrane surface of these structures (Kersch and Pietrantonio, 2011). Our results confirm that a structurally related kinin from *Drosophila*, *Droso*-leucokinin, inhibits Na^+ absorption at these sites, showing that ion transport mechanisms along the rectal pads may be regulated by neuropeptides. PK1-R transcript detection along with PRXa-like immunoreactivity within the rectum was initially suggestive of an ionoregulatory role at these sites. However, since *Aedae*CAPA-PK1 did not significantly influence Na^+ transport along the rectal pad epithelia, the function of this peptide on the rectum remains unclear. To better understand its role in mosquito hindgut physiology, it is critical in future studies to identify and characterize the cells closely associated with immunoreactivity within the rectal pad central canal (**Supplementary Figure S2**), which are distinct from the epithelial cells where *Ae*KR was immunolocalized over the outer rectal pad membrane (Kersch and Pietrantonio, 2011).

The *AAEL005444* gene (Strand et al., 2016) encodes *Aedae*CAPA-PK1 along with two anti-diuretic hormones (*Aedae*CAPA-1 and -2), with the latter regulating the inhibition of fluid secretion across the Malpighian tubules of larval and adult *A. aegypti* (Ionescu and Donini, 2012; Sajadi et al., 2018). In the adult *A. aegypti*, PK-like immunoreactivity has been previously localized in the central nervous system, including numerous cells in the brain, within three groups of neurons of the subesophageal ganglion, and the abdominal ganglia of the ventral nerve cord (Hellmich et al., 2014). In larval stage *A. aegypti*, genes encoding pyrokinins were molecularly characterized revealing *hugin* gene expression, which encodes both PK1 and PK2 neuropeptides, primarily within the subesophageal ganglion, whereas *capa* gene expression was detected mainly within the abdominal ganglia (Hellmich et al., 2014). In support of this observation, neuropeptidomic analyses have shown the presence of pyrokinins originating from both the *hugin* and *capa* gene in the subesophageal ganglion, whereas only *capa* gene-derived *Aedae*CAPA-PK1 was found within the abdominal ganglia, which supply the neurohemal perivisceral organs via the unpaired median nerve (Predel et al., 2010). Given that PK1 and anti-diuretic hormones are derived from a common precursor peptide, along with the proposed roles that rectal pads may play in osmoregulatory processes, there could be some functional relatedness between these peptides, such as binding to distinct receptors expressed along different target organs of the

alimentary canal to exert similar overall actions. Localization of PK-like immunoreactivity in close association with cells within the lumen of the rectal pads could suggest a role for *Aedae*CAPA-PK1 in the regulation of ion and water absorption owing to its specific receptor being enriched within this organ. Following nectar or blood feeding, the excess water and ions taken up from the meal pose a challenge to the hydromineral balance of the organism (Coast, 2009). Although *Aedae*CAPA-PK1 did not elicit changes to Na^+ transport across the rectal pad epithelia of unfed adults, examining other critical processes at these sites, such as anion or water transport, may uncover the role of this neuropeptide in the mosquito rectum. Since the rectal pads have been suggested to play a role in helping to alleviate this insult to hemolymph homeostasis (Hopkins, 1967), *Aedae*CAPA-PK1 may require the initiation of other signaling pathways involved during postprandial diuresis to exert its physiological actions and aid in the regulation of ion and water balance. However, the control of these processes along the hindgut is not yet well understood and, as a result, updating the current model of the *A. aegypti* rectal pad ultrastructure through modern electron microscopy approaches is necessary. This may in turn help unravel the function of *Aedae*CAPA-PK1 that activates its receptor (*Aedae*PK1-R) expressed in this organ, along with other neuropeptides that target these structures.

The presence of *A. aegypti* PK2-R transcript associated with both the ileum and reproductive organs indicate that its PK2 ligands may exhibit pleiotropic actions. Similar to our findings, it was earlier shown by RT-PCR that PK2-R is present in reproductive tissues in *A. aegypti*, where specifically the ovaries were examined (Hellmich et al., 2014), which is consistent with observations on PK receptors in other blood-feeding arthropods, including *R. prolixus, I. scapularis* and the cattle tick, *Rhipicephalus microplus* (Paluzzi and O'Donnell, 2012; Yang et al., 2015; Gondalia et al., 2016). Although their physiological role has not yet been characterized in these hematophagous arthropods, receptor transcript in reproductive organs indicates that PK2 peptides may target these regions to regulate processes critical to mosquito reproduction or development. In other insects, for instance, related PKs were shown to stimulate *L. migratoria, P. americana*, *Z atratus* and *T. molitor* oviduct contractions (Schoofs et al., 1993; Predel and Nachman, 2001; Marciniak et al., 2012), which promote the passage of eggs toward the common oviduct for fertilization (Wigglesworth, 1942). PKs also trigger embryonic diapause by binding to receptors in developing *B. mori* ovaries, upregulating trehalase expression, which promotes glycogen accumulation in oocytes required for the initiation of diapause (Su et al., 1994; Homma et al., 2006; Kamei et al., 2011). Similar roles have been reported in other lepidopteran species, such as the tussock moth, *Orgyia thyellina*, where a PK1 peptide was found to induce embryonic diapause and also promote ovarian development (Uehara et al., 2011).

In *A. aegypti* mosquitoes, females must feed on blood to initiate egg production within the ovaries (Clements, 2000). Upon feeding, neuropeptides such as insulin-like peptides and OEH are secreted from brain neurosecretory cells to stimulate nutrient uptake into developing oocytes by promoting ecdysone synthesis (Brown et al., 1998, 2008; Helbling and Graf, 1998; Riehle and Brown, 1999). PKs have been previously shown to regulate ecdysteroidogenesis upon receptor activation in *B. mori* prothoracic gland (Watanabe et al., 2007). To assess whether PK2 may be involved in similar processes in mosquitoes, future studies should investigate PK2-R expression upon blood feeding to further delineate the involvement of the PK signaling system in regulating these previtellogenic processes. Although no studies to our knowledge have examined the action of PKs on reproductive success in insects, PK2-R transcript expression associated with this organ warrants further investigation to reveal its putative role in mosquito reproductive biology. Given the importance of these PK receptor-enriched organs in a range of physiological activities, these insights may be useful in developing novel strategies to target processes critical to mosquito survival and reproduction, and could ultimately reduce the burden of these disease vectors.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AL performed all the experiments and wrote the initial manuscript. Both authors analyzed the data and contributed towards editing of the final manuscript submitted for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00490/full#supplementary-material

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Biochemical and Molecular Characterization of *Pichia pastoris* Cells Expressing Multiple TMOF Genes (*tmf*A) for Mosquito Larval Control

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Trypsin modulating oostatic factor (TMOF), a decapeptide hormone synthesized by female mosquito ovaries, ganglia and the central nervous system of *Aedes aegypti*, terminates trypsin biosynthesis in larvae, and blood-fed female mosquitoes. Earlier, TMOF was cloned and expressed as a single copy in *Chlorella dessicata* and in *Saccharomyces cerevisiae* cells as a potential larvicide. Here we report the use of a methylotrophic yeast cells, *Pichia pastoris*, that efficiently express multi copies of heterologous proteins, that are readily ingested by mosquito larvae. *P. pastoris* was engineered using pPICZB (Invitrogen, CA, United States), and 2 genes: *gfp-tmf*A and *tmf*A inserted between *Kpn*I and *Xba*I in the multiple cloning site. The plasmid carries a strong AOXI promoter and *P. pastoris* KM71 and KM71H cells were transformed by homologous recombination. The synthesis of GFP-TMOF was followed using UV and clones were analyzed using southern and Northern blot analyses. Cloning *tmf*A into KM71H and selection on high Zeocin concentration (2.0 mg/mL) identified a clone that carried 10 copies of *tmfA.* A comparison between a single and high copy (10 genes) insertions using Northern blot analyses showed that a *tmfA* transcript was highly expressed even after 120 h. SDS-PAGE analysis of KM71 cells transformed with *gfp-tmf*A identified a protein band that ran at the expected M_r of 31 kDa. Enzyme Linked Immunoadsorbant Assay (ELISA) analysis of the recombinant cells showed that 1.65×10^8 and 8.27×10^7 cells produce 229 and 114 μM of TMOF, respectively, and caused 100% larval mortality when fed to groups of 5 larvae in 25 mL water. These results indicate that the recombinant *P. pastoris* cells could be used in the future in the marsh to control mosquito populations.

Keywords: genetic engineering, *Pichia pastoris*, low and high volume fermentations, TMOF, larval control

INTRODUCTION

Environmental, resistance and human health concerns for using chemical pesticides have been a major reason in searching for new biorational insecticides to battle pests such as mosquitoes, transmitters of several detrimental human diseases including malaria, dengue, yellow fever, encephalitis (Spielman and D'Antonio, 2001), and Zika virus. These diseases cause health problem worldwide and Malaria alone causing death to more than one million people in Africa including 300–500 million clinical cases annually (World Health Organization [WHO], 1998). Traditional controls using chemical insecticides to control mosquitoes and agricultural pests often cause environmental and human health problems, as well as, the development of resistance. Thus, new approaches are urgently needed to overcome these challenges to health.

One approach is to utilize insect-specific peptide hormones to specifically control insects. These peptide hormones control multiple functions in insects; digestion, reproduction, water balance, feeding, metamorphosis, and sex attraction (Gäde and Goldworthy, 2003). Thus, disruption of these processes causes irreversible damage culminating in death. Unlike insecticides that are organic in nature and thus, xenobiotic peptide hormones do not cause harm to the environment, and are biodegradable. Thus, peptides that disrupt egg development and digestion in mosquitoes are good candidates for biodegradable mosquito control agents.

Factors that inhibit egg development (oostatic hormones and antigonadotropins) have been reported in cockroach, decapod crustaceans, and the house fly (Iwanov and Mescherskaya, 1935; Carlisle and Knowles, 1959; Adams et al., 1968; Kelly et al., 1984). In mosquitoes, the ovary secretes a humoral factor that inhibits yolk-deposition in less developed follicles (Else and Judson, 1972; Meola and Lea, 1972). These reports indicated that an ovarian factor synthesized by the ovary controls oogenesis in diverse insects. Borovsky (1985) demonstrated that the ovary contains a hormone that initially was named "oostatic hormone". Injection of the hormone into decapitated and ovariectomized female mosquitoes directly stopped trypsin biosynthesis and blood digestion in the females gut and, therefore, the hormone affects the activity of female mosquito midgut cells that synthesize trypsin and not the ovary or endocrine tissues (Borovsky, 1988). The hormone was then named Trypsin Modulating Oostatic Factor (TMOF) and was purified sequenced and characterized by mass spectroscopy as an unblocked decapeptide having two close sequences (YDPAPPPPPP and DYPAPPPPPP; Borovsky et al., 1990). NMR analyses (Curto et al., 1993) suggested that the polyproline portion of TMOF in solution is a left-handed alpha helix. Oral applications and injections of TMOF was shown to inhibit serine proteases biosynthesis by disabling mosquitoes, cat flea, stable fly, house fly, and midge from digesting their blood meals (Borovsky et al., 1990, 1993). These results indicate that TMOF can traverse the gut epithelial cells into the hemolymph, bind TMOF gut receptor(s) with high affinity, and modulate trypsin biosynthesis (Borovsky et al., 1994). Based on these observations, Borovsky and Mahmood (1995), and Borovsky et al. (1989) suggested that TMOF could be used to control adult and larval mosquitoes.

To control mosquito larvae in the field we selected several potential microorganisms that are readily consumed by mosquito larvae and can produce TMOF in large quantities for field applications. In order to not pose a danger to the environment, these cells were heat inactivated before the release without inactivating TMOF.

Recent reports show that TMOF can be successfully expressed by *Chlorella desiccate* and in combination with *Bti cry* toxins in *Pichia pastoris* and without *Bti cry* toxins by *Saccharomyces cerevisiae* (Borovsky et al., 2010, 2011, 1994, 2018). These reports show that mosquito larvae readily consume these engineered cells and die. One advantage of using *P. pastoris* to express foreign genes is the presence of the alcohol oxidase promoters (P_{AOX1} and P_{AOX2}). These promoters strongly depend on methanol but are also expressed in media containing other carbon sources like glycerol, glucose, and sorbitol (Thorpe et al., 1999). A second advantage for using these cells is the ease by which expression of foreign genes can be scaled up from shake-flask to high density fermenter cultures without the loss of yield. As a matter of fact, expression levels in shake flasks are usually lower than fermenter cultures make these cells ideal for industrial production of *tmf* A engineered cells.

This study expands the initial reports of engineered *P. pastoris* cells (Borovsky et al., 2010, 2011) and a book chapter (Borovsky, 2015) by describing a detailed biochemical and molecular biology analyses of the *tmf* A expressed in *P. pastoris*, detailed cloning strategies including Southern and Northern blot analyses, SDS PAGE of the recombinant *gfp-tmf* A expressed gene and high volume industrial production of *tmf* A by *P. pastoris* cells that have been approved by the EPA for use in the environment (Borovsky, 2007).

MATERIALS AND METHODS

Genes Construction

All primers used in this study to construct *gfp-tmf* A, *tmf* A, and *gfp*-IEGR were synthesized by Gemini Biotech (FL, United States) and previously described for *S. cerevisiae* (Borovsky et al., 2018; **Table 1**). Two genes *gfp-tmf* A and *gfp*-IEGR were amplified by PCR using cycle 3 mutant *gfp* from the jellyfish *Aequorea Victoria* (accession number 1B9C_C), whereas a synthetic *tmf* A was prepared by annealing synthetic primers (Borovsky et al., 2018).

Expression Vectors

All restriction enzymes used followed supplier recommendations (Gibco BRL, GA, United States). The constructed genes were digested with the appropriate restriction enzymes; *Kpn*I and *Xba*I for *gfp-tmf* A and plasmid pPICZB (Borovsky, 2015) was opened with the corresponding enzymes. For cloning *tmf* A, the vector was opened with *Xho*I and *Xba*I. Complete vector digestion was verified by agarose gel electrophoresis. Ligations were performed using T4 DNA ligase using overnight incubation at 14°C. Cloning into competent *Escherichia coli* cells was carried out by using heat shock at 42°C for 30 s.

TABLE 1 | Primers used for the genetic engineering of *P. pastoris* cells.

Primers	Primer sequence (5′-3′)	t_m(°C)
*tmf*A		
DB 192 (forward)	TCGAG**ATG**TATGATCCAGCACCT CCTCCTCCTCCTCCTTGAT	70
DB 193 (reverse)	CTAGA**TCA**AGGAGGAGGAGGAGGA GGAGGAGGTGCTGGATC ATACATC	68
*gfp-tmf*A		
DB 207 (forward)	AAGGTACC**ATG**GCTAGCAAAGGAGAAGAA	62
DB 209 (reverse)	TTTCTAGA**TCA**AGGAGGAGGAGGAGGA GGTGCTGGATCATA TCTACCTTCGATTTTGTAGAGCTCATCCAT	68
gfp		
DB 207 (forward)	Sequence as above	
DB 230 (reverse)	TTTCTAGATTCATTTGTAGAGCTCATCCAT	57
*AOX*1		
DB 533 (forward)	AGATCTAACATCCAAAGACGA	51
DB534 (reverse)	CTCGTTTCGAATAATTAGTTG	48
RNA probe		
Nt 855–875 (forward)	GACTGGTTCCAATTGACAAGC	55
Nt 1160–1180 (reverse)	GCAAATGGCATTCTGACATCC	55

Underlined sequences indicate restriction enzymes cleavage sites for the primers. DB 192 XhoI, DB 193 XbaI, DB 207 KpnI, DB 209, and DB 230 XbaI. For RNA probe the position of the forward and reverse primers on the AOX1 is given in parenthesis. Nt = nucleotides. Bold letters denote start and stop signal.

A control was included when an empty parental vector (without a gene inserted into its multiple cloning site) was cloned into competent *E. coli* cells. Transformants of *E. coli* InvαF' were selected on Low Salt Luria-Bertani plates (1% Tryptone, 0.5% Yeast Extract, 0.5%NaCl, pH7.5, and 1.5% agar) containing 25 μg/ml Zeocin™ (Invitrogen, CA, United States). Zeocin™-resistant transformants were grown on Low Salt LB medium (1% Tryptone, 0.5% Yeast Extract, 0.5%NaCl, and pH7.5) with 25 μg/ml Zeocin™ overnight at 37°C. Plasmids were extracted and purified using QIAprep Spin Miniprep kit (Qiagen, CA, United States). Screening of recombinants was done by restriction enzyme and PCR analyses. Plasmids that contained inserts were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977) with [α^{35}S]dATP and the enzyme T7Sequenase (version 2.0; US Biochemicals, OH; Tabor and Richardson, 1987) or with ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, MA, United States). Removal of excess BigDye™ terminators from completed DNA sequencing reactions was done using DyeEx kit (Qiagen, CA, United States) and DNA was analyzed using Applied Biosystems Model 377 DNA sequencer (Perkin Elmer, CA, United States).

Cloning Into *Pichia pastoris* and Screening for Multi-Copy Recombinants

Competent *P. pastoris* KM71 or KM71H cells were prepared using the *Pichia*.EasyComp™ transformation kit (Invitrogen, CA, United States) and aliquots (50 μl) were stored at −80°C. Transformation was performed following the LiAc/SS-DNA/PEG procedure (Gietz et al., 1995) using *Pichia*.Easycomp transformation kit (Invitrogen, CA, United States). Prior to transformation, the pPICZB vectors (Borovsky, 2015) were linearized with *BstXI* to facilitate homologous recombination at the *AOX*1 promotor locus. Transformants were selected on YPDS plates (1%Yeast Extract, 2% Peptone, 2% Dextrose, 1 M Sorbitol, and 2% Agar) with 100 μg/ml Zeocin™ at 30°C. Transformants selected on 100 μg/ml Zeocin plates were further tested on YPDS plates with increased concentrations of Zeocin™ (200 to 3000 μg/ml). The plates were incubated for several days at 30°C. Colonies that grew on the highest concentrations of Zeocin were selected.

Gene Expression by *Pichia pastoris* Cells (Shake Flask Fermentation)

Single colonies of the multi-copy transformants that were selected with Zeocin (100 and 3000 μg/ml) for single and multiple copies of *tmf*A on YPDS plates (above) were removed and grown at 30°C in 10 ml of MGYH (minimal glycerol medium + histidine; 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 1% glycerol, 4×10^{-5} biotin, and 0.004% histidine) overnight in a shaking incubator at 300 rpm or without histidine when KM71H cells were used. In the morning, MGYH (200 ml) was inoculated with the overnight culture and grown at 30°C until the culture reaches an (Optical Density) OD_{600} of 4. The cells were harvested by centrifugation and the pellet resuspended in 40 ml of MMH (minimal methanol medium +histidine) 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 0.004% histidine, and 0.5% methanol). The cultures were then stimulated for up to 144 h and every 24 h an aliquot (10 ml) was taken and frozen at −20°C until further analysis. To maintain induction, at 24 h intervals, 100% methanol was added to a final concentration of 0.5%.

Large Scale Fermentation

Large scale fermentation (150 L) of *P. pastoris* cells KM71 and KM71H engineered with *tmf*A and *gfp-tmf*A were done by BRI at NRC (Canada). Transformed cells were grown at 30°C with agitation rate of 400–1000 rpm, airflow of 7–14 L/min, pressure of 0.05 bar, and pH control at 6.0. During growth on glycerol ammonium hydroxide was used and during the induction phase phosphoric acid was used. Dissolved oxygen was at 20% saturation. At induction time 15 L of 5X BMGY (complex medium) medium without glycerol but with methanol (0.7% v/v) and 50% of the required phosphate buffer was added to prevent precipitation and to minimize potential nutrients starvation during induction. Additional nutrients were added at 16 and 40 h after induction. Fluorescence of cells engineered with *gfp-tmf*A was monitored during the fermentation and fluorescence reached a maximum at 144 h. Methanol concentration was maintained between 0.5 and 0.7% and monitored by GC. Samples were removed at different intervals during the fermentation (0, 21, 45, 69, 78, 93, 117, and 142 h) heated at 75°C for 3 h or subjected to large scale drying, using an oven blower in which the samples entered at 200°C and left at 85°C. The heat treatment of the cells weakened the cell wall inactivated the cells and allowed larvae to digest the yeast cells more efficiently thus, obtaining more TMOF.

ELISA (Enzyme Linked Immunoadsorbant Assay)

At intervals during the fermentation 1.5×10^8 cells/ml were collected and centrifuged down, supernatants discarded and to each pellet of Y-PER (400 μl) Yeast Protein Extraction Reagent (Pierce, IL) was added and incubated for 20 min at room temperature. Cells were then broken using Fast Prep Instrument FP120 (BIO 101, CA) at speed 6 for 20 s with glass beads. Broken cells were spun down for 5 min at 14,000 RPM at room temperature and the supernatant kept. The glass beads were then washed with 400 μl PBS buffer, pH 7.2, and added to the supernatant and stored at −20°C.

Enzyme Linked Immunoadsorbant Assay was determined using Reacti-Bind™ Maleic Anhydride Activated Polystyrene Plates from Pierce (Borovsky et al., 1992, 2010). Briefly, Samples with TMOF were diluted 1:400, 1:800,1:1000, and 1:1600-fold were bound to the plate wells in PBS (100 μl), pH7.2 (0.1 M Phosphate,0.15 M NaCl) overnight by gentle shaking. The wells were decanted and to each well blocking solution (300 μl) pH 7.5 containing 50 mM Tris–HCl, 0.15 M NaCl, 0.05%Tween® 20 and 0.3% BSA was added and incubated for 1 h at room temperature. The solutions were decanted from the wells and to each well 100 μl of the anti-peptide antibody (anti-TMOF) diluted in dilution buffer (PBS, 0.1% BSA, and Tween®20 0.05%) was added. The plates were then incubated for 1 h with gentle shaking. The wells were then washed three times with washing buffer (50 mM Tris–HCl, 0.15 M NaCl, 0.05%Tween®20, 2% polyvinylpyrolidone, 0.1% BSA, and pH7.4). After the third wash 100 μl of goat anti rabbit antibody linked to alkaline phosphatase that was diluted in dilution buffer was added. The wells were incubated for 1 h with gentle shaking. After three washes, 200 μl of alkaline phosphatase liquid substrate from Sigma was added for detection. After 30 min of incubation the plates were read at 405 nm in a Microplate reader from Bio-Tex. A calibration curve was constructed with known concentrations of the peptide TMOF.

Southern Blot Analysis

Pichia pastoris genomic DNA was isolated using a fast DNA kit (BIO 101, CA, United States) or a DNeasy tissue kit (Quiagen, CA, United States). For the fast DNA kit, yeast cells from each clone (1.5×10^8 to 3×10^8 cells) were broken in 2 ml tubes containing 0.25 inch Sphere and Garnet Matrix and 1 ml of CLS-Y (cell lysis/DNA solubilization solution) using a FastPrep instrument (FP120, BIO 101, CA, United States). Broken cells were centrifuged, and DNA was bound to DNA binding matrix solution. The bound DNA-matrix was then centrifuged, the pellet washed with salt ethanol wash solution and the DNA eluted from the matrix with DNA elution solution (BIO 101, CA, United States), the solution centrifuged, and the supernatant collected and stored at −20°C. For the Qiagen DNeasy Tissue kit yeast cells (3×10^7 cells) were centrifuged, and the pellet resuspended in PBS (200 μl), and AL buffer (200 μl; Qiagen, CA, United States). The suspended cells were broken with glass beads for 20 s using FastPrep (FP125, BIO101 Savant, CA, United States). To the broken cells Proteinase K was added and the homogenate incubated at 70°C for 10 min. After incubation, the broken cells homogenate was centrifuged for 5 min at 14,000 rpm and the supernatant transferred to a fresh tube and ethanol (200 μl) was added and the mixture adsorbed onto DNeasy spin columns and genomic DNA was eluted after several washes following manufacturer's guideline and stored at −20°C.

A 942 bp *AOX*1 probe was amplified by PCR using pPICZB and the following primers: DB 533 (forward), 5′-AGATCTAACATCCAAAGACGA-3′ and DB534 (reverse), 5′-CTCGTTTCGAATAATTAGTTG-3′. A 700 bp *gfp* probe was amplified by PCR using pYDB2*gfp* and primers, DB207, and DB230 (Borovsky et al., 2018). The probes were labeled with [α^{32}P] dCTP using Rediprime™II labeling system (Amersham Pharmacia Biotech, United Kingdom) and the labeled probes purified using Qiaquick PCR column (Qiagen, CA, United States). The 46 bp TMOF oligonucleotide (DB192; Borovsky et al., 2018) was labeled with [γ^{32}P] dATP using RTS T4 Kinase (GibcoBRL, MD). Ambion SouthernMax™ kit was used for Southern analysis (Ambion, TX, United States). *P. pastoris* genomic DNA, prepared using a FastDNA® Kit (BIO101), was digested with 5 U of *Eco*RI (Gibco-BRL, MD). DNA digests (10 μg per lane) were separated on a 0.8% agarose gel and transferred to BrightStar-Plus positively charged nylon membrane (Ambion, TX, United States) using a Turboblotter (Schleister & Schuell, Germany). The membrane was blocked and hybridized with a purified denatured radioactive labeled DNA probe or a radioactive labeled oligonucleotide. Prehybridization and hybridization was performed at 42°C in rotating hybridization bottles in a hybridization oven (Enprotech, OH, United States). After washing, the membrane was wrapped in plastic foil and exposed to an X-ray film. Between hybridizing with each probe, the membrane was washed with a boiling 0.1% SDS in sterile water solution for 15 min to strip the label.

Northern Blot Analysis

RNA from transformed *P. pastoris* cells (1 mL) were isolated using TRIzol (Gibco BRL, CA, United States). The cells were broken for 20 s using speed of 4 in a Fast Prep Instrument (Savant, CA, United States) using glass beads following manufacturer instructions. The RNA was precipitated with isopropanol (0.5 mL) and the pellet washed with 75% ethanol and dried at room temperature for 10 min. The dried RNA was redissolved in 100 mL of DEPC (diethyl pyrocarbonate) treated sterile water at 57°C for 10 min and the concentration was determined at 260 nm using GeneQuant spectrophotometer (Amersham Pharmacia, United Kingdom). To detect the RNA transcript a probe (360 bp) was prepared by PCR (Borovsky et al., 2018) using *AOX*1 as a template and forward and reverse primer pair (5′-GACTGGTTCCAATTGACAAGC-3′ and 5′-GCAAATGGCATTCTGACATCC-3′), respectively.

RNA transcript levels in engineered *P. pastoris* cells were determined according to Sambrook et al. (1989) using NorthernMax kit (Ambion, TX, United States). RNA aliquots (3 μg) and standards were separated by gel electrophoresis (80 V for 1.5 h) on 1% denatured agarose gels according to manufacturer's instructions (Ambion, TX, United States). Samples were mixed with formaldehyde loading dye and

incubated for 15 min at 65°C prior to electrophoresis. Following electrophoresis, the RNA was transferred for 3 h from the gels unto BrightStar-Plus positively charged nylon membranes using transfer buffer (Ambion, TX, United States), and a Turboblotter (Schleister & Schull, Keene, NH, United States). The RNA was cross linked to membranes at 120°C for 15 min and the membranes were stored at −20°C. Standards were visualized by methylene blue (Sambrook et al., 1989). The membranes were prehybridized for 4 h in ULTRAhyb (20 ml; Ambion, TX, United States) and hybridized overnight with purified denatured [^{32}P]DNA probe. Prehybridization, hybridization and washing steps were done at 42°C in rotating hybridization bottles in an oven (Enprotech, MA, United States). After low and high stringency washes (Ambion, TX, United States) the membranes were wrapped in plastic film to prevent drying and exposed to X-ray film for 24 to 72 h at −80°C.

To allow re-probing of the membranes with an actin probe (300 bp) based on *P. pastoris act*1 transcript (accession number AF216956) was amplified by PCR using primer pair (forward) 5′- TTAGTTATCGACAATGGTT-3′ (t_m 55°C) and (reverse) 5′-CCTCAGTCAAAAGAACTG-3′ (t_m 57°C), the membrane was incubated with SDS (0.1%) solution that was preheated to 100°C. The incubation mixture was cooled down to room temperature, the membrane removed, and scanned for radioactivity with a hand-held Geiger counter. The procedure was repeated until no radioactivity was detected. The hybridization procedure and the washings were repeated as mentioned above and the X-ray film was exposed for 24 to 72 h at −80°C (Borovsky et al., 2016).

The Northern blots were scanned using Sapphire Biomolecular Imager (azure biosystems) using 3 wavelengths (488, 520, and 658 nm) and the scanned *tmf*A transcript bands were divided by the scanned *act* transcript bands that were used as a reference gene. The ratios were then plotted against different fermentation times and were used to compare the *tmf*A transcripts of low copy and high copy engineered *P. pastoris-tmf*A cells.

3D Model

A three-dimensional ribbon drawing model of GFP-TMOF recombinant protein was built (**Figure 1B**) using SYBYL molecular modeling software (v. 6.3) and converted into a ribbon representation by the program Molecular Molscript (Kraulis, 1991; Borovsky et al., 1996; Yan et al., 1999) and is based on X-ray diffraction conformation for GFP (PDB 1EMA) and NMR presentation of TMOF (Curto et al., 1993).

Monitoring GFP-TMOF Expression

Expression of the GFP-TMOF in *P. pastoris* engineered cells was monitored using short wave UV-254 nm Lamp (model UVS-11, Ultra-Violet products, CA, United States) in a dark room. Fluorescent engineered cells were photographed and compared with the same cells that were radiated with normal light.

SDS PAGE Analysis

Engineered *gfp-tmf*A *P. pastoris* (1.5×10^8 cells) were pelleted at 14,000 rpm for 5 min and lysed by vortexing in 50 mM Tris–HCl, pH 7.9, 2% SDS, 5% β-mercaptoethanol using glass beads for 30 min at 4°C. The mixture was heated for 5 min at 90°C, centrifuged at 14,000 rpm for 5 min at room temperature and supernatants collected. Aliquots (80 μl) were removed and mixed with 20 μl of sample buffer [0.5 M Tris–HCl, pH 6.8, glycerol 10% (v/v), 25% β-mercaptoethanol, and 0.05% Bromophenol blue]. The extracted proteins were separated by SDS-PAGE on a slab poly acrylamide gel (Laemmli, 1970) and stained with Coomassie Blue R-250 and distained in MeOH acetic acid for 18 h. Distained gels were then dried using a gel drier at 70°C (BioRad, CA, United States).

Larvicidal Activity of the Engineered *P. pastoris* Cells

Aedes aegypti eggs were placed in deionized water supplemented with live yeast extract to induce egg hatching. Single newly hatched larvae were added to separated wells of 48 microtiter plates at room temperature. Each well contained sterile water (1 ml) and 2×10^7 engineered yeast cells. The yeast cells were washed three times by centrifugation before feeding them to larvae to get rid of the culture medium that the cells were growing in. Cells were then heat treated at 80°C for 3 h and after shake flask fermentation, or for large scale fermentation dried in an oven at 200°C when cell entered the oven and at 85° upon exit. Larval growth and mortalities were monitored daily up to 12 days. For large scale testing, larvae (100 per group) were tested in 200 ml water with different concentrations of recombinant *P. pastoris* KM71H-*tmf*A heat treated dried yeast cells in an oven as mentioned above.

Data Analysis

Data are expressed as means of 3 determinations ± SEM. Graphs were plotted using GraphPad Prism 5.0 (GraphPad, CA, United States).

RESULTS

Cloning and Expressing Genes in *P. pastoris*

Pichia pastoris cells were transformed using a multi copy integrating plasmid pPICZB (Invitrogen, CA, United States; **Figure 1A**). The plasmid has a multiple cloning site and a strong alcohol oxidase promoter (P_{AOX1}) allowing selection using Zeocin and ColE1 origin allowing replication and selection in *E. coli.* Two genes *tmf*A and *gfp-tmf*A were amplified by PCR using primer pairs DB 192 and DB 193 for *tmf*A and DB 207 and DB 209 for *gfp-tmf*A (**Table 1**). The *tmf*A and *gfp-tmf*A amplicons were digested with *Xho*I and *Xba*I and *Kpn*I and *Xba*I, respectively, to insert the two genes into the multiple cloning site of pPICZB (**Figure 1A**). The *gfp-tmf*A codes a Green Fluorescent protein TMOF fusion protein with an IEGR trypsin cleavage site between GFP and TMOF at R243 (**Figure 1B**). The TMOF nucleotides sequence is based on the amino acid sequence of the decapeptide (**Figure 2A**). After ligation of *tmf*A and *gfp-tmf*A into pPICZB the inserts were sequenced, the plasmids were linearized with *Bst*XI and *P. pastoris* strains KM71

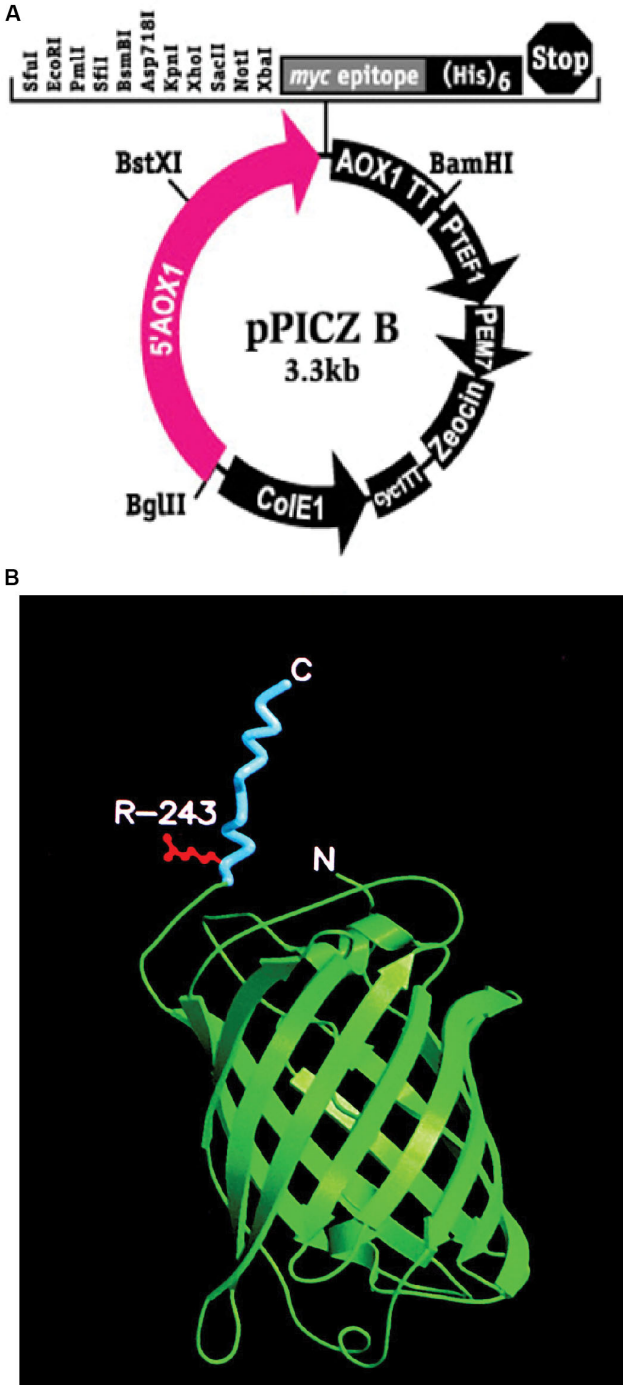

FIGURE 1 | (A) pPICZB plasmid that was used to transform KM71 and KM71H cells. The plasmid multiple cloning site is shown including the positions of the restriction enzyme pairs *Xba*I and *Kpn*I and *Xba*I and *Xho*I that were used to clone *gfp-tmf*A and *tmf*A, respectively. Shown also is the P_{AOX1} and the restriction enzyme position for *Bst*XI used to open the plasmid for homologous recombination. The arrows indicate the direction of replication. **(B)** A 3D model of the recombinant fusion protein GFP-TMOF including R243 where mosquito larva cleaves the fusion protein in the gut releasing TMOF. The carboxyl and amino ends are denoted as C and N, respectively. TMOF (blue) assumes a left handed conformation and GFP green a barrel conformation made of β-pleated sheets.

(*arg4aox1*Δ::*ARG4,his4*), and KM71H (*arg4aox1*Δ::*ARG4*) were transformed by homologous recombination at the P_{AOX1} locus (**Figure 1A**) and screened for multiple insertions using Zeocin (**Figure 2B**). KM71 and KM71H strain carry Mut^s with a phenotype that utilizes methanol slowly. They also have a mutation at the *aox*1 locus in which the *AOX*I gene is largely deleted and replaced with *S. cerevisiae ARG4 gene* and wild type *AOX2* gene that metabolizes methanol slower and therefore the cells grow slow in medium containing methanol. After transformation the transformants were screened for multiple insertions by selecting the colonies on increasing concentrations of Zeocin because there is a strong correlation between drug resistance and copy number. Colonies were selected on 2000 μg/ml from the KM71-*tmf*A (**Figure 2B**) and KM71-*gfp-tmf*A transformed cells. Several colonies were also selected from KM71H-*tmf*A transformed cells on 3000 μg/ml Zeocin and a colony was selected for comparison on 100 μg/ml Zeocin. The cells that were selected on Zeocin were fermented in methanol using shake flask and large scale (150 L) fermentations for up to 144 h at 30°C and aliquots (10 ml) were removed for ELISA at intervals and were also fed to mosquito larvae. *P. pastoris* cells KM71-*gfp-tmf*A that were fermented in shake flask (0–144) were monitored with UV light. In the absence of UV light, the cells did not fluoresce and increase in fluorescence was observed with increase time of the fermentation reaching a peak between 72 to144 h (**Figure 2C**, upper and lower panels).

Copy Number of *gfp* and *gfp-tmf*A

To find out how many *tmf*A and *gfp-tmf*A genes were incorporated into the genome of *P. pastoris* after homologous recombination, three genetic maps of *P. pastoris* were constructed. Wild type with mutated *AOX*1 gene is about 6000 bp if it is cut with *Eco*RI (**Figure 3**; Ellis et al., 1985). A single insertion of *tmf*A or *gfp-tmf*A would generate an additional *Eco*RI restriction site of 2500 bp and 6700 bp or 2500 bp and 7400 bp bands, respectively (**Figure 3**, single insertion), whereas double insertion of *tmf*A or *gfp-tmf*A will generate 3300 bp and 6700 bp or 4000 bp and 7400 bp bands, respectively (**Figure 3**, double insertions). Southern blotting analysis of genomic DNA of KM71 cells that were transformed with *gfp-tmf*A and *tmf*A and were digested with *Eco*RI identified two DNA bands (7400 and 2500 bp) for *gfp-tmf*A and one for *tmf*A (9200 bp) when probed with *AOX*1. The larger DNA band of 9200 bp indicate that the *Eco*RI restriction site was lost during the homologous recombination (**Figures 3**, **4** left blot; **Supplementary Material 1**). Two expected bands of 2500 bp and 6700 bp were detected in *P. pastoris* cells that were transformed with an empty pPICZB plasmid (**Figure 4** left blot, control; **Supplementary Material 2**). Probing the blot with *gfp* probe after the *AOX*1 probe was stripped from the blot identified a single band of 7400 bp when recombinant cells with *gfp-tmf*A were probed with *gfp* probe, and no DNA band was detected when recombinant cells carrying *tmf*A or transformed with empty plasmid were probed with the *gfp* probe (**Figure 4**, right blot). These results indicate that the KM71 cells that were transformed by homologous recombination have a single insertion of *gfp-tmf*A and *tmf*A. Transforming KM71H,

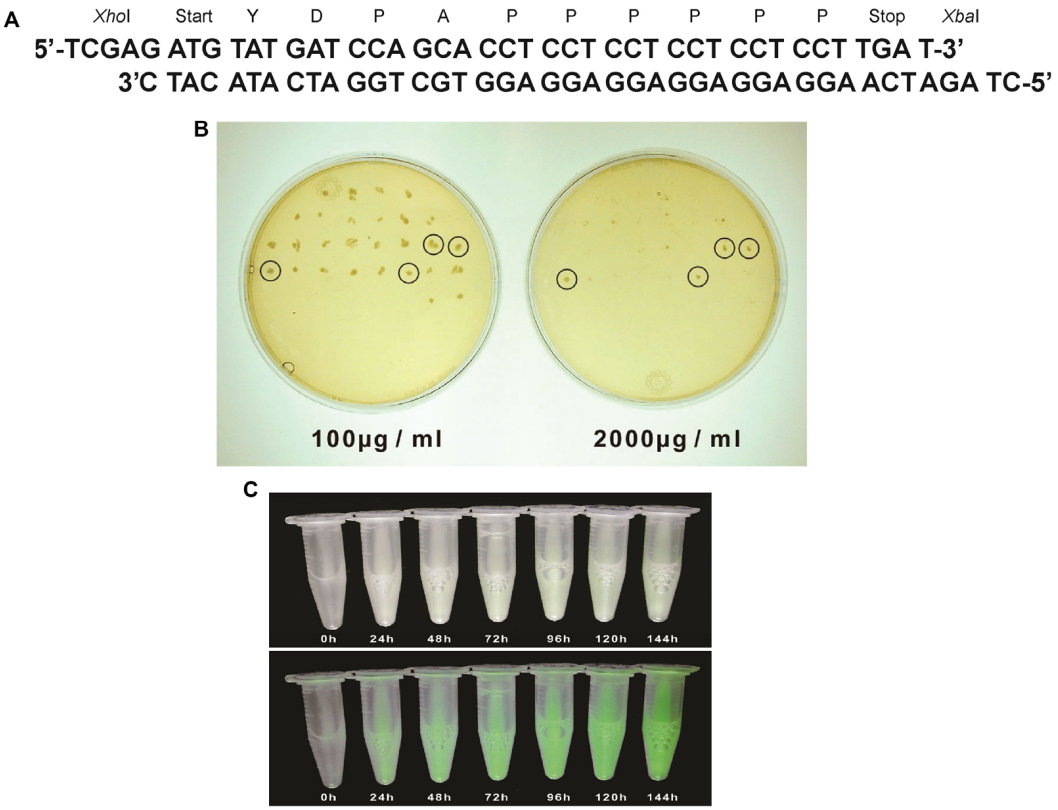

FIGURE 2 | (A) A synthetic *tmf*A made up of 2 oligonucleotides that were annealed and used for cloning *tmf*A into pPICZB. The two restriction site positions *Xho*I and *Xba*I and start and stop signals and the amino acids sequence of TMOF are also shown. **(B)** YPDS agar plates to select transformed KM71H-*tmf*A for high copy number and low copy number on Zeocin (100 μg/ml and 1000 μg/ml, respectively). Only 4 colonies survived in the presence of high Zeocin concentration, whereas many colonies survived at low Zeocin concentration. **(C)** KM71-*gfp-tmf*A engineered cells that were fermented by shake flask for (0–144 h). Cells that were exposed to regular light (upper panel) did not fluoresce, whereas cells that were exposed to UV light (lower panel) increased in fluorescence as the fermentation progressed.

however, with *tmf*A and analyzing two of the transformed colonies (#22 and #5) using Southern blot analysis and *AOX*1 probe show that in both cases the 3 expected bands of 2500, 3300, and 6700 bp are found (**Figure 5**), however, the ratio of the 3300 bp band to the 6700 bp band in colony #22 is 10 times higher as judged by the intensity of the bands (determined with KODAK EDAS 290 image analyzer) indicating that the transformed cells have at least 10 copies of *tmf*A (**Figure 5**). On the other hand, the intensities of the 3300 bp and the 6700 bp bands of colony #5 are equal (determined as above) indicating that this colony incorporated a single *tmf*A (**Figure 5**). The Southern blot analysis were repeated twice with similar results.

Transcript Analysis of KM71H-*tmf*A Cells

Two clones (#5 and #22) that integrated one and 10 copies of *tmf*A, respectively (**Figure 5**) were analyzed by Northern blot analysis by removing samples at different intervals during shake flask methanol fermentation of the cells (0, 24, 48, 72, 96, and 120 h; **Figure 6A** and **Supplementary Material 3**). A *tmf*A transcript of 450 bp was detected at 24 h and no message was detected before the methanol induction time (0 h) in cells with low (L) and high copy number (1 and 10 copies, respectively; **Figure 6A**). At different times during the fermentations the *tmf*A/*act* ratios of cells carrying 10 *tmf*A copies were much higher than cells carrying one copy (**Figure 6B**). When the *tmf*A/*act* ratios of cells carrying 10 copies were compared with the ratios of cells that carried one copy a 7-fold difference was found at 24 h, 16-fold difference at 40 h, 11-fold difference at 72 h, 10-fold higher at 96 h, and 15-fold higher at 120 h (**Figure 6B**). As the fermentation proceeded the intensity of the blots dropped, however, the ratios of *tmfA/act* transcripts in cells carrying 10 copies as compared with cell carrying a single copy were close. These results show that engineered cells with 10 copies of *tmf*A expressed more transcript throughout the fermentation than cells with one *tmf*A copy. The *act* a house keeping gene was used as a reference gene for the analysis of the Northern blot (**Figure 6B**) and to show a uniform transfer of the *tmf*A transcript in all the lanes (**Figure 6A**). The experiment was repeated twice with similar results.

Detection of GFP-TMOF by SDS PAGE

To detect the expressed GFP-TMOF fusion protein, *P. pastoris* cells that were transformed with KM71-*gfp-tmf*A were fermented for 93 h or immediately used (0 h). The cells were broken with glass beads in the presence of SDS and proteins were separated by SDS-PAGE and the gel stained with Coomassie Blue. Two heavily stained bands of 31 kDa and a second band that has a similar mobility with the GFP standard were detected (**Figure 7**,

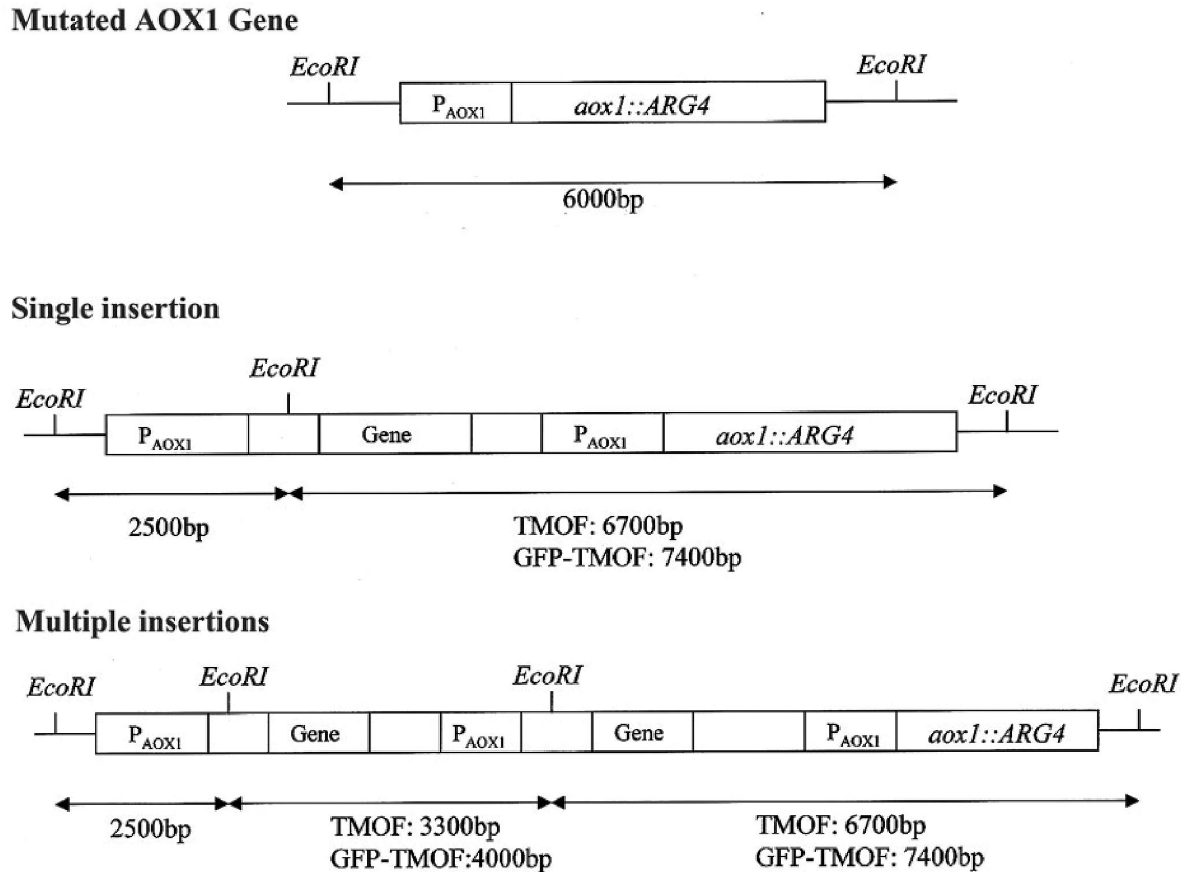

FIGURE 3 | Genetic map of KM71 and KM71H cells with mutation at the *aox*1 and its promoter showing the DNA size and *Eco*RI restriction sites. A genetic map after a single insertion of *tmf*A and *gfp-tmf*A including *Eco*RI restriction sites and expected restriction fragments after *Eco*RI digestion. A genetic map of multiple insertion of *tmf*A and *gfp-tmf*A including additional *Eco*RI sites and restriction fragments.

lanes 3, and 1). The protein band that ran at 31 kDa corresponds to the GFP-TMOF fusion protein, whereas the protein band that ran below the 31 kDa band with similar mobility of the GFP standard may be protease digested GFT-TMOF at the IEGR site (**Figure 1B**) during the glass beads extraction of the recombinant protein.

Detection of TMOF by ELISA

Although our Northern blot analysis (see above) clearly indicated that the amount of *tmf*A transcript is at least 2-fold higher if the intensities of the bands are observed and at 7 to 16-fold higher if the transcript bands are normalized with *act* as a reference gene (**Figures 6A,B**) it does not necessarily mean that the amount of TMOF expressed by the high copy transformed cells is different than cell expressing one copy. To find out if high expression of *tmf*A transcript results in higher amount of TMOF, the engineered *P. pastoris* KM71 cells, proteins were extracted from cells at 96 h when KM71-*gfp-tmf*A and KM71-*tmf*A cells were fermented by shake flask fermentation and analyzed by ELISA (Borovsky et al., 1992). The amount of GFP-TMOF after 96 h fermentation of 3×10^8 cells was 10.2 ± 1 ($\mu g \pm$ SEM). KM71-*tmf*A 3×10^8 engineered cells, produced 12 ± 1.4 ($\mu g \pm$ SEM). The same number of KM71H-*tmf*A cells (10 insertions, clone #22, and **Figure 5**) that were fermented by industrial fermenters produced about 4 to 10-fold more TMOF after 120 h using 5 to 150 L fermenters (**Table 2**). We also purified the extracted proteins by HPLC from 3×10^8 cells (Borovsky et al., 1990, 1993). Even though, high losses occurred during the lengthy purification steps we showed that after 120 h of shake flask fermentation of engineered KM71H-*tmf*A cells 136 ± 13 (ng $\pm$ SEM; $n = 3$) could be detected proving that the engineered cells synthesize TMOF.

Biological Activity of *P. pastoris* Engineered Cells

Shake Flask Fermentations

Engineered *P. pastoris* cells KM71-*gfp-tmf*A were fermented in shake flask and heated to 80°C for 3 h. After the heat treatment the cells were washed with distilled water (3 times) and the washed cells were fed to first instar *Ae. aegypti* larvae in 48 well plate (1 larva per well) containing engineered *P. pastoris* (2×10^7 cells) in 1 ml of distilled water. Three groups of 8 larvae were fed the recombinant cells for 12 days and mortality was daily monitored. 88% of the larvae died within 3 days, whereas larvae that that were fed *P. pastoris* KM71-*gfp* without *tmf*A all survived, controls (**Figure 8A**). In a second experiment *P. pastoris*

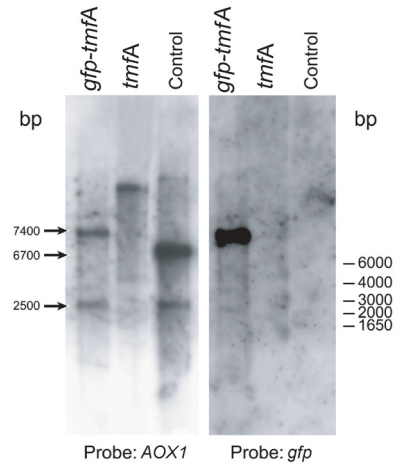

FIGURE 4 | Southern blot analyses of genomic DNA of transformed KM71-*tmf*A and KM71-*gfp-tmf*A cells. The blots were probed with *AOX*1 (left blot) and *gfp* (right blot) generated by PCR (**Table 1**). The size of DNA markers is given on the right and the sizes of the digested DNA bands is indicated with arrows on the left. The Southern blot was repeated a second time showing the same results.

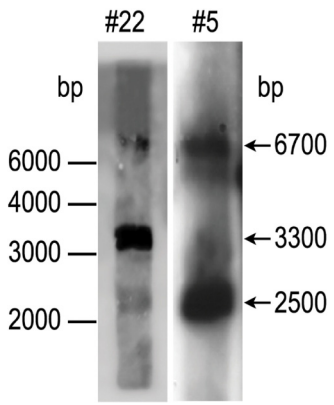

FIGURE 5 | Southern blot analysis of *P. pastoris* KM71-*tmf*A colonies #22 and #5. DNA was extracted and digested with *Eco*RI and analyzed by Southern blotting using *aox*1 probe. The size of the DNA markers is on the left and the sizes of the digested DNA bands is indicated with arrows on the right. The Southern blot was repeated a second time showing the same results.

KM71-*tmf*A (2×10^7 cells/ml) were heated at 55°C for 3 h and compared with control cells that were not heated. Six groups of 100 *Ae. aegypti* first instar larvae were each fed in pans containing 200 ml distilled water. The larvae that were fed heated cells all died within 8 days whereas the larvae that were fed nonheated cells all died at 13 days (**Figure 8B**). These results indicate that heating the recombinant yeast cells makes TMOF more available to the larvae perhaps by weakening the yeast cell wall and indicate that the recombinant cells are effective in larger volumes of water (100 ml) as well as in small volume of water (1 ml). Controls that were fed non recombinant *P. pastoris* or Brewers yeast did not die (results not shown).

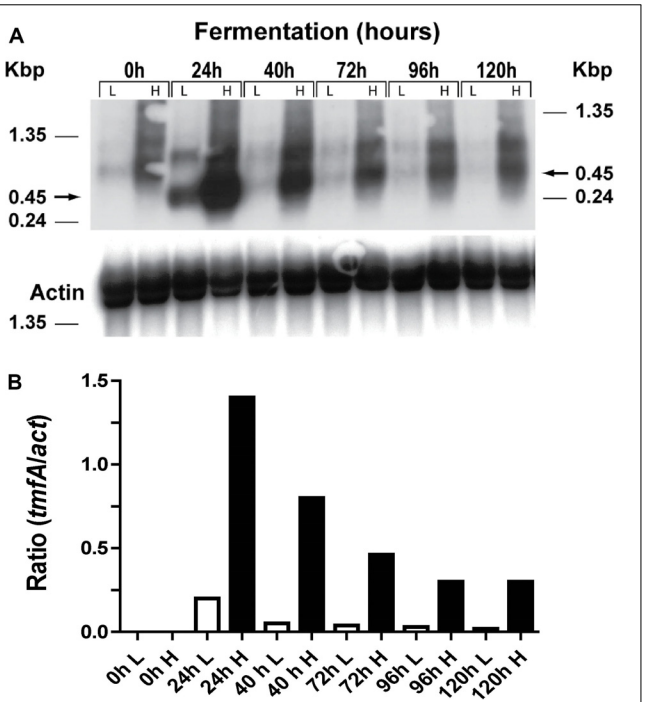

FIGURE 6 | Northern blot analysis of KM71H-*gfp-tmf*A cells (#5 and #22, low and high copy, respectively) that were fermented by shake flask for 0–120 h. **(A)** Total RNA (3 μg/lane) was analyzed using a specific probe amplified by PCR (**Table 1**). The size of the RNA markers is given on the left and the *tmf*A transcript (450 bp) is shown by an arrow on the right. **(B)** Scanned *tmf*A transcript blots (see a above) after different hours of the fermentation (0–120 h) of high and low copy colonies engineered with *tmf*A were compared with *act* transcript as a reference gene and the transcript ratios of *tmfA/act* were compared for cell that were engineered with 10 and 1 *tmf*A genes, respectively. White bars represent cells expressing a single *tmf*A and black bars cells expressing 10 *tmf*A genes. L = low copy colony #5 and H = high copy colony #22. The Northern blot analyses were repeated two times showing the same results.

High Volume Fermentations (Industrial)

After high volume fermentation of *P. pastoris* KM71H-*tmf*A cell for 120 h and drying the cells in an oven (210°C entry of wet yeast cells and 85°C exit of dried cells). The cells were rehydrated in water, broken with glass beads and analyzed by ELISA for TMOF content. The recombinant cells, different number of cells (3×10^7 to 1.65×10^8) were fed for 10 days to 21 groups of first instar *Ae. aegypti* larvae (1 larva/ml, 20 per group) in 25 ml sterile distilled water. Mortality of the fed larvae increased as the concentration of the TMOF (nM) in the fed engineered cells increased killing all the larvae tested at concentrations larger than 100 nM, whereas cells that did not express *tmf*A (0 nM TMOF) did not kill the larvae, control (**Figure 9**). Similarly, feeding 16 groups (5 larvae/group) in 25 ml at TMOF concentrations of 0–229 nM (4.97×10^7–1.65×10^8 cells/ml) killed all the larvae at concentrations of 114 and 229 nM within 8 to 10 days, respectively (**Table 3**). Lower TMOF concentrations (57 nM) killed only 25% of the larvae and *P. pastoris* cells that were not engineered (control) did not kill the larvae. *Culex quinquefasciatus* larvae that were fed *P. pastoris* KM71H-*tmf*A

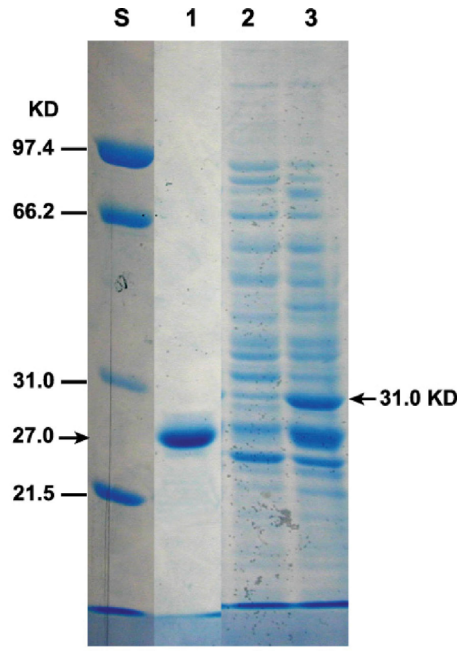

FIGURE 7 | SDS-PAGE of KM71-*gfp-tmf*A transformed cells. Each lane contains equal amount of proteins extracted from equal number of cells (1.6×10^7) and separated by SDS-PAGE. Protein were stained with Coomassie Blue. Marker protein of known size were run on the left lane (S). Lane 1 standard GFP (27.6 kDa), lane 2 protein extracted from non-stimulated cells, Lane 3 cells stimulated with 0.5% methanol for 93 h. GFP-TMOF expected protein band (31 kDa) is indicated with an arrow on the right.

TABLE 2 | Synthesis of GFP-TMOF and TMOF using shake flask and large volume fermentations of *P. pastoris* KM71-*gfp-tmf*A and KM71-*tmf*A cells.

Fermentation (h)	N	μg/3 × 10^8 cells	Size (L)
a. Shake flask			
KM71-gfp-tmfA			
96	3	10.2 ± 1	0.4
KM71-tmfA			
144	6	12 ± 1.4	0.4
b. Industrial fermentation			
KM71H-tmfA (colony #22)			
120	3	44 ± 6	5
120	3	101 ± 14	5
120	3	19.2 ± 5	20
120	3	32.4 ± 10	150

P. pastoris cells transformed with KM71-gfp-tmfA, KM71-tmfA (one copy), and KM71H-tmfA (10-copies) were fermented in a shake flask or in large volumes using industrial fermenters. Aliquots (3 × 10^8 cells) were removed at different times and assayed for TMOF using ELISA (Borovsky et al., 1992). Industrial fermented KM71H-tmfA (clone #22) cells were dried in an oven entry temperature 210°C and exit temperature 85°C. Results are average of 3 to 6 determinations ± SEM.

cells for 4 days did not grow like *Ae. aegypti* larvae, and large differences of size was observed when compared with larvae that were fed control *P. pastoris* cells that were not engineered (control; **Figures 10A,B**). These results indicate that *P. pastoris* engineered cells expressing TMOF can be used to control larval *Ae. aegypti* and *Cx. quinquefasciatus*.

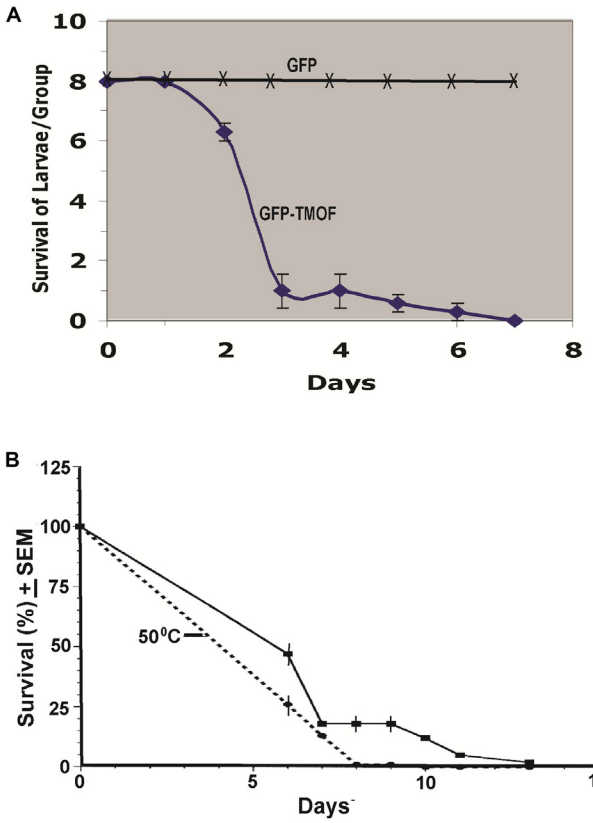

FIGURE 8 | (A) Feeding first instar *Ae. aegypti* larvae KM71-*gfp-tmf*A and KM71H-*gfp* (control) cells after shake flask fermentation and heat treatment (80°C for 3 h). Three groups (8 larvae per group) were fed the engineered cells (2×10^7 cells) in 48 well plate containing one larva in 1 ml water per well for 7 days. **(B)** Comparison between KM71-*tmf*A cells that were heated at 55°C for 3 h (broken line) and cells that were not heated (solid line) and were fed to 6 groups of 100 larvae per 200 ml for 13 days. Mortalities were followed daily and the results are expressed as means ± SEM.

DISCUSSION

In this report, we used *P. pastoris* cells to express TMOF a decapeptide hormone that was shown to be effective against mosquito larvae when expressed in *Chlorella desiccate*, and *S. cerevisiae* (Borovsky et al., 2016, 2018). The advantage of using a methylotrophic yeast rather than *C. desiccate* and *S. cerevisiae* is the presence of a strong alcohol oxidase promoter (P_{aox1}) and the ease in scaling up the production of heterologous genes by the cells in large fermenters. *P. pastoris* cells have shown to express high variety of different genes, from prokaryotes to higher eukaryotes (Cregg et al., 1987; Clare et al., 1991a; Brankamp et al., 1995; Fairlie et al., 2000; Fu et al., 2001). Since not all the heterologous genes are expressed at equally high level by the P_{aox1} due e.g., for rapid degradation of the foreign genes' transcripts, early transcriptional terminator

sequences or proteolytic degradation of the expressed protein. The biological activity of foreign proteins expressed by the cell depends on the cell wall thickness that prevents the protein from easy release from the cell, aggregation and precipitation of the foreign protein in the cell (Cassland and Jonsson, 1999). To increase the amount of heterologous protein production in *P. pastoris*, genes are inserted at the *AOX*1 locus by homologous recombination resulting in spontaneous multiple genes insertion at a low but detectable frequency (1–10%; Clare et al., 1991a,b). We transformed several *P. pastoris* cells (KM71 and KM71H) by homologous recombination using pPICZB (**Figure 1A**) carrying *tmf* A and *gfp-tmf* A. The *gfp-tmf* A carries an IEGR cleavage site (**Figure 1B**) allowing mosquito larvae to digest and release TMOF from the recombinant protein (GFP-TMOF) when it is fed to mosquito larvae, a similar strategy was also used with engineered *S. cerevisiae* cells (Borovsky et al., 2018). The engineered *P. pastoris* cells were grown on Zeocin (100–2000 μg/ml) to select colonies with up to ten insertions of *tmf* A (**Figure 2B**). Southern blot analyses of *P. pastoris* KM71-*gfp-tmf* A and KM71-*tmf* A and KM71H-*tmf* A confirmed that KM71 cells have a single insertion of *gfp-tmf* A or *gfp* (**Figure 4**), whereas KM71H-*tmf* A cells exhibited a single and 10 insertions (clones #5 and #22; **Figure 5**). These results confirm the genetic map of *P. pastoris* (**Figure 3**; Ellis et al., 1985). The Northern blot analysis of *P. pastoris* KMH71-*tmf* A cells harboring low and high copy number inserts (clones #5 and #22) show that a *tmf* A transcript (450 nt) was expressed only after the methanolic stimulation (**Figure 6A**). In low and high copy number cells the transcript reached a peak at 24 h, and then rapidly declined. To quantitate the abundance of the *tmf* A transcript expressed by the genetically engineered cells with a single (L) and ten insertions (H) after the Northern blot analysis (**Figure 6A**) the transcript ratios of *tmf* A transcript to the *act* transcript that was used as a reference gene for the Northern blot at 24 h was 7-fold higher than found in cells that were engineered with one copy of *tmf* A (**Figure 6B**) as was predicted by the Southern blot analysis (**Figure 5**). Even though the transcript levels of both low and high copy number cells declined during the fermentation the transcript ratios of cells with 10 copies to one copy cells stayed similar during the 120 h fermentation period (**Figure 6B**). The *tmf* A transcript level of high copy *tmf* A transcript even though it fell about 4-fold is still detected at 120 h. The transcript in low copy number cells, on the other hand, fell about 10-fold, and it almost completely disappeared and this was confirmed by ELISA determinations showing that the amount of TMOF produced in cells with high copy number is 3 to 8-fold higher than in cells expressing single *tmf* A copy (**Table 2** and **Figure 6A**). Our observations confirm a report by Sreekrishna et al. (1988) showing a 200-fold increase in the synthesis of tumor necrosis factor in *P. pastoris* cells harboring 20 copies of the gene as compared with copy. To monitor *P. pastoris* during the methanolic fermentation and confirm during the lengthy fermentation period that TMOF is continuously synthesized, we cloned and expressed GFP-TMOF in *P. pastoris* KM71-*gfp-tmf* A cells using shake flask fermentation and at intervals (0–144 h) determined fluorescence using UV light. Cells that where illuminated with normal light did not fluoresce, whereas cells illuminated by UV light highly fluoresced (**Figure 2C**). To confirm that the cells synthesize GFP-TMOF, cells before induction (0 h), and cells at 93 h after induction were extracted and their proteins separated by SDS-PAGE (**Figure 7**) and compared with Native *Aequorea Victoria* GFP (M_r 27.6 kDa, **Figure 7** lane 1). GFP (cycle 3) variant has a M_r of 30 kDA (Crameri et al., 1996) and TMOF has a M_r of 1.047 kDa, therefore the fusion protein GFP-TMOF has a M_r of 31 kDA. A strong protein band of 31 kDA was detected after 93 h fermentation (**Figure 7** lane 3). In cells that were not stimulated the 31 kDa band is missing (**Figure 7** lane 2). A second strong band running about M_r 27.6 kDa was also observed in the protein extract of the stimulated cells. This band is probably a proteolytic degradation of the GFP-TMOF at the IEGR site or other sites e.g., K5 and K26 on the GFP cycle 3 protein (Crameri et al., 1996) making the GFP-TMOF shorter by 15 or 36 amino acids the later will probably run at the observed M_r of about 28 kDa similar to the heavily stained band underneath the GFP-TMOF band (**Figure 7** lane 3). However, only MS/MS analysis, that was not done, will confirm whether this band is indeed a proteolytic moiety of the GFP-TMOF.

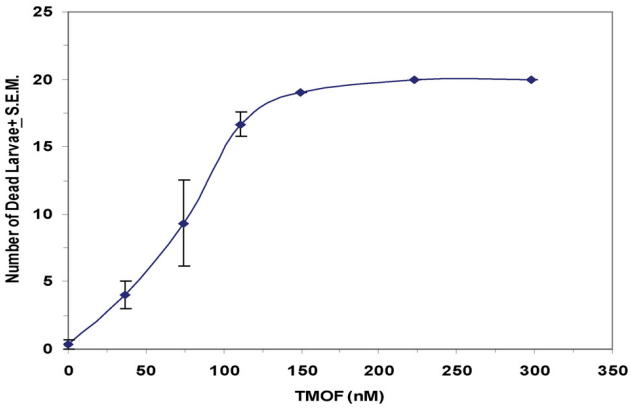

FIGURE 9 | Feeding KM71H-*tmf*A cells with increasing concentrations of TMOF (0–229 nM) to *Ae. aegypti* first instar larvae after high volume (150 L) fermentation and heat treatment in an oven (entry at 210°C and exit at 85°C). Heat treated cells were fed for 12 days and mortality was daily determined. Groups of 5 larvae were tested in 25 ml sterile tissue culture wells. The amount of TMOF in heat treated cells was determined by ELISA. Results are expressed as means ± SEM.

TABLE 3 | Feeding of industrial fermented *P. pastoris* KM71H-*tmf*A to *Ae. aegypti* Larvae.

Number of cells/ml	TMOF (nM)	Feeding days	Mortality (%) ± SEM
1.65×10^8	229	10	100
8.27×10^7	114	8	100
4.97×10^7	57	10	25 ± 3
1.6×10^8 (control)	0	10	0

Ae. aegypti larvae 24 h after emergence, 16 groups (5 larvae per group) were fed in tissue culture wells containing 25 ml of sterile distilled water different amounts of recombinant P. pastoris KM71H-tmfA cells for 10 days. The yeast cells were fermented in 150 L fermenter for 120 h and were heat treated in oven. Larval mortality was daily followed up to 10 days. P. pastoris cells that were not genetically engineered were used as control.

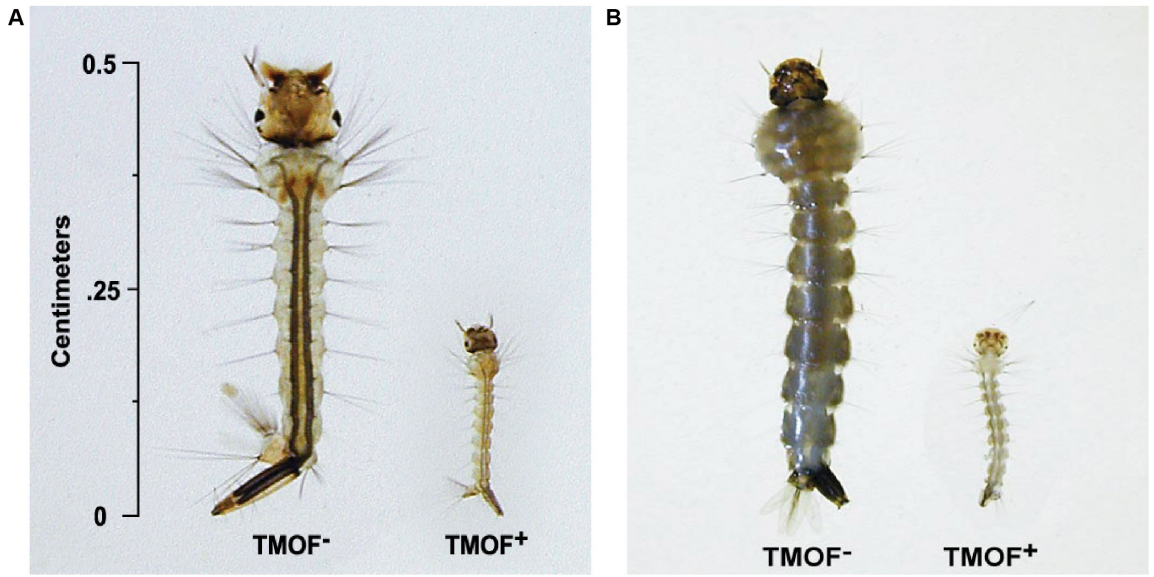

FIGURE 10 | *Cx. quinquefasciatus* **(A)** and *Ae. aegypti* **(B)** larvae were fed *P. pastoris* cells KM71H TMOF^{-} (control) and KM71H-*tmf*A cells TMOF^{+} for 4 days. Larvae that fed the recombinant cells expressing TMOF did not grow because they cannot digest their food.

The biological activity of the KM71-*gfp-tmf*A recombinant cells was followed by feeding cells to first instar larvae. A high mortality (88%) was observed at 3 days and all the larvae died at day 7. This was not due to the GFP because feeding engineered KM71-*gfp* cells (control) to the larvae did not cause mortality (**Figure 8A**) and GFP expressed in *S. cerevisiae* recombinant cells did not affect mosquito larvae that were fed on these cells (Borovsky et al., 2018).

Earlier biological testing of the recombinant cells was done in small volumes (1 ml) in 48 well plates. In future formulation for field applications it is important to test the recombinant cells biological activity in larger volumes that will be needed for field work. To find out the potency of the recombinant KM71-*tmf*A cells, 100 larvae were fed non treated and heat treated cells after shake flask fermentation in 200 ml water. Cells that were heat treated (55°C for 3 h) killed the larvae in 8 days, whereas cells that were tested without heat treatment killed all the larvae in 13 days (**Figure 8B**). These results indicate that heat treated recombinant cells are more potent. Large volume fermentation (150 L) of our recombinant KM71H-*tmf*A cells for 120 h and heat treatment of the wet cells after the fermentation in an oven in such a way that the cells entered the oven at 210°C and dry cells left at 85°C did not affect the potency of the recombinant TMOF (**Tables 2**, **3**, and **Figure 9**). All the tested larvae died when fed the recombinant yeast cells that expressed TMOF (114 to 125 nM). Heat treatment of recombinant *S. cerevisiae* cells expressing TMOF also enhanced the larvicidal potency of these cells (Borovsky et al., 2018). Heating yeast cells for prolonged time releases carbohydrases and proteases that can partially hydrolyze the cell wall (Jones, 1991; Nguyen et al., 1998) making TMOF more accessible in the gut after ingestion of the recombinant yeast cells by mosquito larvae. Heat treatment did not affect TMOF or GFP-TMOF when they were tested by ELISA (**Table 2**) and the heat treated recombinant cells inhibited the growth of *Ae. aegypti* as well as *Cx. quinquefasciatus* larvae by starving the larvae (**Figure 10**).

The recombinant proteins, GFP-TMOF, and TMOF, that are produced inside the heat treated *P. pastoris* cells are protected by the yeast's cell wall from sunlight and bacterial degradation as was reported for photosynthetic cyanobacterial species that were used for toxin delivery to control mosquito larvae (Manasherob et al., 2002; Khasdan et al., 2003). The engineered heat treated yeast cells are thus more palatable to mosquito larvae that are filter feeders and selectively eat particles found in the marsh (Clements, 1992) and could be used in the future to control mosquito larvae in the field. TMOF was found by the EPA to be safe to be used in the environment (Thompson et al., 2004; Borovsky, 2007).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DB and SN performed the experiments. DB and RS analyzed the data. DB designed the experiments and wrote the manuscript. All authors approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00527/full#supplementary-material

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ORIGINAL RESEARCH
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Identification and Characterization of GPCRs for Pyrokinin and CAPA Peptides in the Brown Marmorated Stink Bug, *Halyomorpha halys* (Hemiptera: Pentatomidae)

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The brown marmorated stink bug, *Halyomorpha halys*, is an invasive hemipteran that causes significant economic losses to various agricultural products around the world. Recently, the *pyrokinin* and *capa* genes that express multiple neuropeptides were described in this species. Here we report six *pyrokinin* and *capa* GPCRs including two splice variants, and evaluate their (a) ability to respond to neuropeptides in cell-based assays, and (b) expression levels by RT-PCR. Functional studies revealed that the *H. halys* pyrokinin receptor-1 (HalhaPK-R1a & b) responded to the pyrokinin 2 (PK2) type peptide. RT-PCR results revealed that these receptors had little or no expression in the tissues tested, including the whole body, central nervous system, midgut, Malpighian tubules, and reproductive organs of males and females. HalhaPK-R2 showed the strongest response to PK2 peptides and a moderate response to pyrokinin 1 (PK1) type peptides (= DH, diapause hormone), and was expressed in all tissues tested. HalhaPK-R3a & b responded to both PK1 and PK2 peptides. Their gene expression was restricted mostly to the central nervous system and Malpighian tubules. All PK receptors were dominantly expressed in the fifth nymph. HalhaCAPA-R responded specifically to CAPA-PVK peptides (PVK1 and PVK2), and was highly expressed in the Malpighian tubules with low to moderate expression in other tissues, and life stages. Of the six GPCRs, HalhaPK-R3b showed the strongest response to PK1. Our experiments associated the following peptide ligands to the six GPCRs: HalhaPK-R1a & b and HalhaPK-R2 are activated by PK2 peptides, HalhaPK-R3a & b are activated by PK1 (= DH) peptides, and HalhaCAPA-R is activated by PVK peptides. These results pave the way for investigations into the biological functions of *H. halys* PK and CAPA peptides, and possible species-specific management of *H. halys*.

Keywords: neuropeptide, GPCR, *pyrokinin*, CAPA, *Halyomorpha halys*

INTRODUCTION

Neuropeptides are the largest group of insect hormones. They regulate or modulate a variety of physiological actions, such as fat body homeostasis, feeding, digestion, excretion, circulation, reproduction, metamorphosis, and behavior throughout all life stages (Nässel and Winther, 2010; Nässel and Zandawala, 2019). The PRXamide peptide family (X, a variable amino acid) is a well-characterized neuropeptide component having a common amino acid sequence, PRX-NH_2, at the C−terminal end, which is conserved across Insecta (Jurenka, 2015). The peptides are classified into three subfamilies, each having diverse biological roles in insects: (1) pyrokinin (PK) includes the pheromone biosynthesis activating neuropeptide (PBAN) and the diapause hormone (DH), (2) the capability (CAPA) peptides (peptides produced from the *capa* gene), and (3) the ecdysis−triggering hormone (ETH).

The neuropeptides activate specific G protein-coupled receptors (GPCRs) that are involved in essential biological processes. Data mining and computational analyses of the *Drosophila* genome identified a variety of GPCRs for PRXamide peptides that are orthologous to the vertebrate neuromedin U receptor (NmU-R) (Hewes and Taghert, 2001; Vanden Broeck, 2001). The hypothesis of ligand-receptor coevolution was supported when GPCRs of *Drosophila* PRXamide peptides were first identified and found to be related to the vertebrate peptide neuromedin U (NmU) (Howard et al., 2000; Park et al., 2002). Since then, GPCRs for PRXamide peptides have been studied in other insect groups, and the first PBAN receptor was identified from *Helicoverpa zea* (Choi et al., 2003), followed by *Bombyx mori* (Hull et al., 2004). To date, many GPCRs for PBAN/PK2, DH/PK1, CAPA, and ETH peptides have been identified, characterized, and/or functionally expressed from various animal groups (reviewed by Jurenka, 2015).

The first CAPA peptides in hemipteran species were reported from the southern green stink bug, *Nezara viridula* (Predel et al., 2006). Later, various CAPA and PK peptides were identified from *Rhodnius prolixus*, a blood−feeding disease vector (Paluzzi et al., 2008; Predel et al., 2008; Paluzzi and Orchard, 2010; Ons, 2017), and the bed bug, *Cimex lectularius*, a global human ectoparasite (Predel et al., 2018). The GPCRs for CAPA and PK1 peptide variants were found to regulate the antidiuretic process in *R. prolixus* (Paluzzi et al., 2010; Paluzzi and O'donnell, 2012).

Halyomorpha halys is an invasive insect native to East Asia which has subsequently spread to Europe, North America and South America over the last couple of decades. This insect has become a serious polyphagous agricultural pest, using its proboscis to pierce and suck fluids from the stems of various crops, which in turn affects the plants' growth rates, fruit production and esthetics (Leskey and Nielsen, 2018). Current control measures are based on integrated pest management tactics, using the recently identified aggregation pheromone (Khrimian et al., 2014) for mass trapping, and native and exotic parasitoids for biological control (Herlihy et al., 2016). More recent studies have taken molecular approaches, using RNA interference technologies to control these sap sucking insects (Ghosh et al., 2018).

We recently identified two *H. halys* PRXamide genes: a *pyrokinin* gene encoding three PK2 peptides (PK2-1, PK2-2, and PK2-3), but no PK1 (= DH) peptide; and the *capa* gene encoding two periviscerokinin (PVK) peptides (CAPA-PVK1 and CAPA-PVK2) and a CAPA-DH (= PK1-like) peptide (Ahn and Choi, 2018). In the present study, we report the identification of six GPCRs including two variant receptors associated with these peptides. Receptor gene structures determined, their expressions in different tissues and developmental stages were evaluated, and their responses to *H. halys* PK and CAPA peptides were characterized *in vitro*.

MATERIALS AND METHODS

BLAST Search for PK and CAPA Receptor Sequences

Putative *H. halys* PK and CAPA receptors were identified via BLAST analysis of *H. halys* genomic and transcriptomic databases available through the i5k Workspace Project of the United States Department of Agriculture National Agriculture Library[1] using the sequences of PRXamide receptors from other insects as queries.

Cloning of PK and CAPA Receptor Genes

Total RNA was isolated from various tissues of male and female *H. halys*, and cDNA was synthesized using the Superscript IV First-Strand Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States) using random hexamers and oligo-dT primers as described previously (Ahn and Choi, 2018). Primers (**Supplementary Table S1**) were designed using the Primer 3 application within Geneious v8.1.5 software (Biomatters, Ltd., Auckland, New Zealand). The primers were used to amplify full-length HalhaPK/CAPA-Rs from cDNA using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). PCR conditions were as follows: an initial incubation at 98°C for 2 m, followed by 35 cycles of 98°C for 10 s, 60°C for 15 s, and 72°C for 90 s, followed by a final 10 m extension at 72°C. PCR products were electrophoresed on a 0.7% agarose gel, and fragments of the expected size for each target were excised from the gel and purified using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific). Following the manufacturer's instructions, purified PCR products were inserted into the pIB/V5-His TOPO expression vector (Thermo Fisher Scientific) which allows for *OpIE2*-driven constitutive expression of the gene of interest in various insect cell lines. Bacterial colonies were tested for the presence of modified pIB plasmids by PCR using DreamTaq polymerase (Thermo Fisher Scientific) and gene-specific primers to amplify full-length sequences using the following PCR conditions: an initial 8 min incubation at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. Three positive colonies were each inoculated into LB media and grown overnight. Plasmids were purified from bacterial cultures using the GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific) and Sanger sequenced to confirm

[1] https://i5k.nal.usda.gov

the correct sequence and orientation of each gene within the pIB vectors. One plasmid containing each HalhaPK/CAPA-R gene with the correct sequence and orientation was chosen for transfection of insect cell lines.

Expressing Receptors in Sf9 Cells

Five micrograms of each purified pIB/HalhaPK-R (or CAPA-R) plasmid and 15 μL of Cellfectin II reagent (Thermo Fisher Scientific) were diluted into 500 μL of serum free Insectagro Sf9 medium (Corning, Corning, NY, United States) and allowed to incubate for 10 min at room temperature, after which they were mixed together and allowed to incubate for an additional 15–20 min. Each pIB/HalhaPK-R (or CAPA-R) Cellfectin mixture was then added to T-25 cell culture flasks containing Sf9 cells at 50% confluency and incubated overnight at 28°C. The following morning the media was removed from each flask and replaced with media containing 20 μg/mL blasticidin (Corning). Each transfected cell line was cultured in the presence of 20 μg/mL blasticidin for ∼3 weeks until a blasticidin-resistant cell line was established, after which the blasticidin concentration was reduced to 10 μg/mL and the cell lines were frozen prior to testing in functional assays.

Preparation of PK and CAPA Peptides

Peptides encoded by *H. halys pyrokinin* and *capa* genes used in this study have been recently identified (Ahn and Choi, 2018). Four peptides (>95% purity): PK2 (FYAPFSPRLa), CAPA-PVK1 (DAGLFPFPRVa), CAPA-PVK2 (EQLIPFPRVa), and CAPA-DH (= PK1-like, NGASGNGGLWFGPRLa) were synthesized to contain an amide group on their C-termini (Peptide 2.0 Inc., Chantilly, VA, United States). Peptides were solubilized in water, aliquoted into 2–20 nM working stocks, dried using a DNA120 SpeedVac Concentrator (Thermo Scientific), and frozen at –20°C until use in binding assays.

Functional Assays of *H. halys* GPCRs

For each cell line expressing a specific *H. halys* PRXamide receptor, cells were thawed and cultured for at least three passages in a suspension culture containing blasticidin prior to functional assays. The transfected cell lines were grown as 20 mL suspension cultures in 50 mL-Erlenmeyer flasks with a screw cap, and shaken at 145 rpm at 28°C. For functional assays, cells were taken from a suspension culture in the exponential growth phase, and ∼50,000 cells were plated into each well of a tissue culture-treated, black-walled, 96-well plate (Corning). The plated cells were then incubated at 28°C for 48 h, after which the media was removed from the plates and the wells were rinsed with serum-free media, then loaded with 95 μL of 1X FLIPR Calcium 6 assay reagent (Molecular Devices, Sunnyvale, CA, United States). Plates were incubated at room temperature for 60 min then transferred to a FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) to measure cell fluorescence intensity from intracellular calcium influx after peptide-induced receptor activation. Wells of cells were then treated with 5 μL of water or 20X concentrations of each peptide using the automated injection system of the FlexStation plate reader. In all experiments, fluorescence was measured every 5 s for 30 s prior to treatment and for 2 min after exposure. In all cases, the maximum response obtained occurred ∼20 s after exposure to ligand. The mean response of four wells receiving the same treatment was calculated and expressed as a change in fluorescence from baseline (prior to treatment) to maximum response. For initial screening, each cell line was exposed to a single concentration of 500 nM of each peptide or control. Compounds that elicited responses were tested further in concentration-response experiments. Concentration-response curves and EC_{50} values were generated using the variable slope, four parameter non-linear regression function of GraphPad Prism data analysis (GraphPad Software, Inc., San Diego, CA, United States). Each cell line was tested for its response to peptides in at least three independent experiments.

Reverse Transcriptase (RT) and Quantitative (q) PCRs

The following tissues: central nervous system, midgut, Malpighian tubules, testis and ovary, were dissected from 5 to 10 days-old adults and the total RNA was extracted as described previously (Ahn and Choi, 2018). cDNA was synthesized using the Superscript IV First-Strand Synthesis Kit (Thermo Fisher Scientific) using random hexamers and oligo-dT primers from 1,000 ng of the previously isolated RNA. Primers (**Supplementary Table S1**) were designed with the Primer 3 application within Geneious v8.1.5 software (Biomatters) and were used to amplify (a) full-length glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control, and (b) HalhaPK/CAPA-Rs from cDNA using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). PCR conditions were as follows: initial incubation at 98°C for 2 m, followed by 35 cycles at 98°C for 10 s, 60°C for 15 s and 72°C for 90 s, then a final 10 m extension at 72°C.

Samples were collected from the following developmental stages: eggs, 1st to 5th nymphs, adult males and adult females (three biological replicates, each). Total RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) were performed as described previously (Ahn and Choi, 2018). Briefly, cDNA was synthesized from 1,000 ng of total RNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. A qRT-PCR reaction mixture was prepared in 20 μL with 1,000 ng of cDNA, PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 0.25 μM of each primer, and RNase-free water. The reaction was run at 95°C for 15 min, then 40 cycles at 95°C for 15 s, and 60°C for 1 min using StepOnePlus™ Real-Time PCR System (Applied Biosystems). Amplification specificity was followed by melting curve analysis over a 60–95°C range. Gene expression levels for the developmental stages were compared using *rpn2* as a reference gene.

RESULTS

H. halys Receptor Identification, Cloning, and Sequence Comparison

Six GPCRs for PRX family peptides were identified in *H. halys* (**Figure 1A**). Five of these were classified as *Halyomorpha halys*

PK receptors (HalhaPK-Rs). These were designated as HalhaPK-R1a, HalhaPK-R1b, HalhaPK-R2, HalhaPK-R3a, HalhaPK-R3b, and one, HalhaCAPA-R, as a CAPA receptor. Sequences of the six receptors were aligned and their transmembrane domains determined (**Figure 1B**).

HalhaPK-R1 was present in the i5k genomic database on contigs XM_014437522.1 and HHAL010714-RA, and was comprised of 7 exons spread across a 56,938 bp region of scaffold2666. HalhaPK-R1 had two transcript variants with one comprised of all 7 exons (HalhaPK-R1a) and one missing exon 6 (HalhaPK-R1b). In addition, there were 6 non-synonymous mutations between the two versions of the proteins, which were validated by independent PCR methods and their presence in the genome. In the genome, some of the exons code for the HalhaPK-R1a transcripts and some code for HalhaPK-R1b transcripts, indicating that the genome sequence is an amalgamation of different HalhaPK-R1 alleles that were present in the pooled DNA used to generate the genome. HalhaPK-R2 was comprised of 5 exons spread across a 99,734 bp region of scaffold629, and was found as a complete transcript in contigs XM_014427306.1 and XM_014427307.1 in the i5k database. HalhaPK-R2 was amplified via PCR and the sequence of the resulting product exactly matched that of the transcriptome and genome. HalhaPK-R3a and HalhaPK-R3b were both present in the i5k genome and were comprised of 5 exons spread across a 56,025 bp region of scaffold1013, with two alternatives of exon 5. Both HalhaPK-R3a and HalhaPK-R3b were present in the i5k transcriptome on contigs XM_014431884.1 and XM_014431883.1, respectively. HalhaCAPA-R was present in the i5k genomic database, comprised of 4 exons spread across scaffold1099 (exons 1, 2 and 4) and scaffold3190 (exon 3), and in the transcriptome spread across two un-assembled contigs (XM_014432674.1 and XM_014437682.1). The HalhaCAPA-R transcriptomic and genomic sequences were identical.

Functional Testing of GPCRs

In initial screening experiments, empty Sf9 cells and Sf9 cells expressing each of the six GPCRs were tested for ligand-induced receptor activation using each of the *H. halys* peptides or a negative vehicle control (**Supplementary Figure S1**). Empty Sf9 cells did not respond to any of the compounds tested. HalhaPK-R1a, -R1b, -R2, -R3a, and -R3b receptors all responded to PK2 and CAPA-DH peptides to varying degrees. HalhaPK-R2 responded strongly to PK2 peptides. HalhaPK-R3a and -R3b responded to PK1 (= CAPA-DH) and PK2 peptides, with the highest sensitivity to PK1 (= CAPA-DH), especially for HalhaPK-R3b (**Table 1** and **Figures 2**, **3**). HalhaCAPA-R responded specifically to PVK1 and PVK2 peptides (**Table 1** and **Figures 2**, **3**).

Peptides that activated HalhaPK-Rs in initial screening experiments were subsequently tested in concentration-response experiments. GPCRs that responded to PK2 peptides in screening experiments showed positive concentration-dependent activity, with varying sensitivities and degrees of absolute magnitude. Similarly, receptors HalhaPK-R2, -R3a, and -R3b showed concentration-dependent responses to PK1 (= CAPA-DH), with varying sensitivities and degrees of absolute magnitude (**Table 1** and **Figure 2**). HalhaCAPA-R had a concentration-dependent response to PVK1 and PVK2 (**Figure 2**), with similar sensitivity and degree of absolute magnitude.

Tissue-Specific Expression of *H. halys* Receptors

RT-PCR results revealed that *H. halys* GPCRs were differentially expressed in male and female adult tissues (**Figure 4**). Interestingly, HalhaPK-R1a and -R2b were both cloned from initial preps of cDNA made from RNA isolated from male whole bodies, however, they were not detected in any of the samples tested in RT-PCR experiments, despite repeated attempts using cDNA preps from multiple RNA isolations. HalhaPK-R2 was detected in all tissues tested, with the highest expression being in the male and female Malpighian tubules. HalhaPK-R3a and b were primarily detected in the Malpighian tubules and central nervous system. Varying expression levels of HalhaCAPA-R were detected in all tissues tested, with the highest expression observed in the Malpighian tubules.

Age-Specific Expression of *H. halys* Receptors

Among different life stages, all three PK receptor genes (HalhaPK-R1, -R2, and –R3) were expressed the most in the last nymphal stage (N5), whereas the CAPA receptor gene (HalhaCAPA-R) showed broad-range expression throughout the life cycle, except in the egg stage (**Figure 5**). Among different receptor genes, HalhaCAPA-R was most highly expressed (50–250), followed by HalhaPK-R3s (5–20), HalhaPK-R2 (1–6), and HalhaPK-R1s (0–0.5). The three PK receptor genes did not have sex-biased expression, but the HalhaCAPA-R was more abundant in adult females than in adult males (**Figure 5**).

DISCUSSION

G protein-coupled receptors (GPCRs) are readily identified from insect genome sequences based on the presence of a well conserved, seven-transmembrane-domain topography (Hewes and Taghert, 2001). We identified six GPCRs through sequence homology-based searches of *H. halys* genomic and transcriptomic databases. The GPCRs were functionally characterized using PK and CAPA peptides *in vitro*. Upon amplification of these receptors via PCR it was discovered that two PK receptors, HalhaPK-R1 and HalhaPK-R3, were transcribed as two alternatively-spliced variants, which is not unusual among PBAN/PK receptors in moths (Lee et al., 2012; Nusawardani et al., 2013). The "a" and "b" versions of the receptors were alternatively spliced at the C-terminal ends and physically resided in the last intracellular domain of the proteins (**Figure 1**). We did not find any variants of the CAPA receptor in this study, although an atypical and inactive variant of the CAPA receptor has been found in *R. prolixus* (Paluzzi et al., 2010).

H. halys PK and CAPA receptors are expected to respond to PK peptides encoded from the *PBAN/pyrokinin* gene, and CAPA-PVK peptides encoded from the *capa* gene, respectively. In this study, HalhaCAPA-R was clearly distinguished from

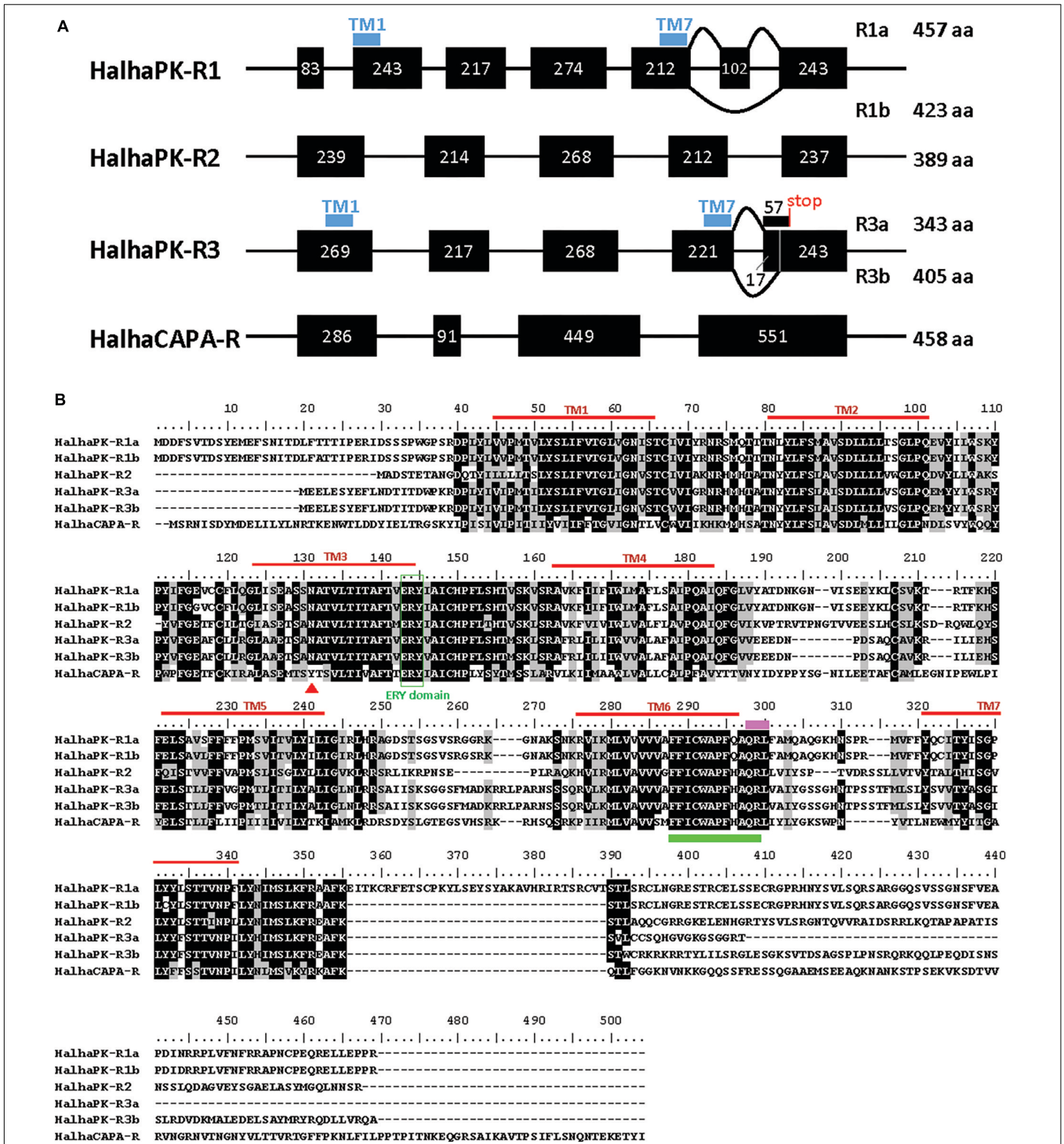

FIGURE 1 | PRXamide receptor sequences from *Halyomorpha halys*. **(A)** Schematic genome structure of the four PRXamide receptors. Black boxes represent exons in coding sequences with the length of nucleotides inside the box. HalhaPK-R1 and HalhaPK-R3 produce two transcript variants (a,b), presumably by alternative splicing. Positions of the first and last transmembrane (TM) domains (TM1 and TM7) are indicated by blue bars only in HalhaPK-R1 and HalhaPK-R3, showing that the splicing occurs after TM7. **(B)** Alignment of the deduced amino acid sequences from the six receptors. Sequences were aligned using MUSCLE algorithm in MEGA 7.0. Consensus sequences were shaded with 70% cut-off using BioEdit. Seven transmembrane (TM) domains (red lines) were predicted by TOPCONS (Tsirigos et al., 2015). ERY domain (green box) and QRL motif (pink bar) are conserved in PRXamide receptors, and the rotamer toggle switch (green bar) is conserved in GPCRs in general. The red triangle in TM3 indicates a receptor-specific amino acid position in the PK receptors with N, and CAPA receptor with Y.

TABLE 1 | Half-maximum effective concentrations (EC_{50}) of the peptides tested on heterologously-expressed PK/CAPA receptors of *Halyomorpha halys*.

Peptide	Sequence	HhalPK-R1a	HalhaPK-R1b	HalhaPK-R2	HalhaPK-R3a	HalhaPK-R3b	HalhaCAPA-R
PBAN/PK2	FYAPFSPRLa	>1 μM	420 nM	35 nM	199 nM	142 nM	>1 μM
CAPA-DH/PK1	NGASGNGGLWFGPRLa	>1 μM	>1 μM	800 nM	660 nM	91 nM	>1 μM
CAPA-PVK1	DAGLFPFPRVa	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM	80 nM
CAPA-PVK2	EQLIPFPRVa	>1 μM	>1 μM	>1 μM	>1 μM	>1 μM	57 nM

"a" to indicate amide.

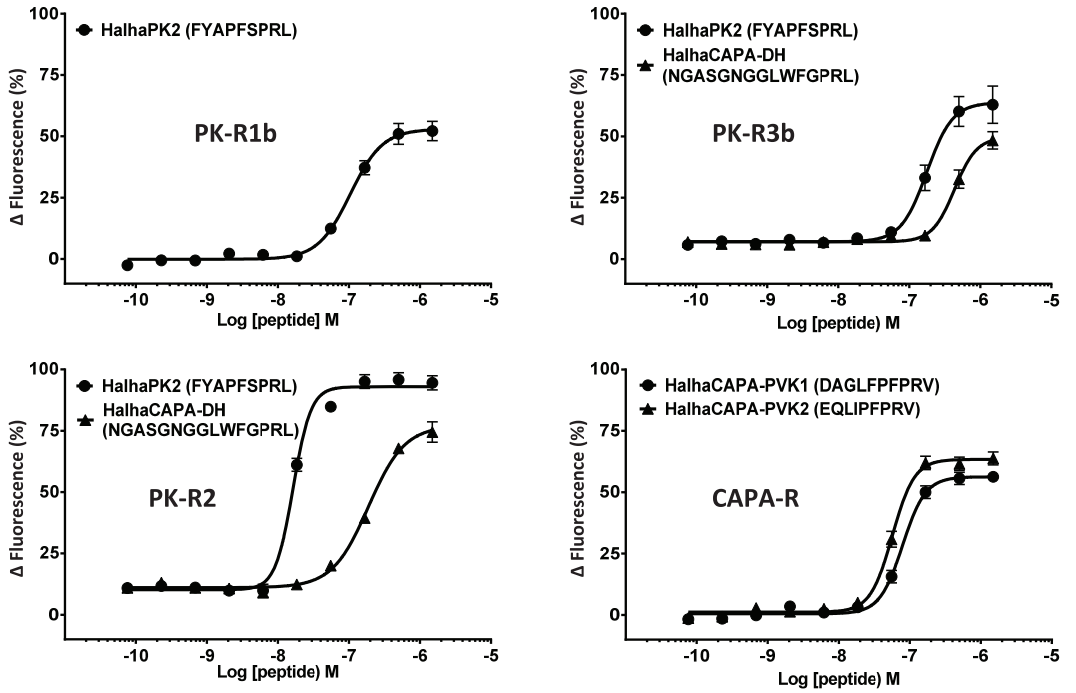

FIGURE 2 | Concentration-response profiles of *Halyomorpha halys* receptors to pyrokinin and CAPA peptides that elicited responses in screening experiments. Data represent the mean ± SEM response of cells from three independent experiments. EC_{50} values for all six GPCRs are in **Table 1**.

the other five receptors. It responded strongly only to CAPA-PVK peptides containing the PRVamide conserved motifs in the C-termini, but had no response to PK type peptides containing the PRLamide conserved motifs in the C-termini. Also, CAPA-PVK peptides did not activate any of the other PK receptors tested in a concentration-dependent manner. The CAPA receptor was expressed in all tissues and life stages we tested in this study. In addition, the CAPA receptor expression levels were much higher than the other *H. halys* receptors. In fact, *capa* genes are ubiquitously found across invertebrate phyla including Arthropoda (Predel and Wegener, 2006) and CAPA peptides and their receptors are more evolutionarily conserved (Choi et al., 2017). As expected, such a high expression level of CAPA receptor observed in Malpighian tubules suggests CAPA peptides might be involved in antidiuretic processes in the brown marmorated stink bug as in other hemipteran insects (Paluzzi et al., 2010; Paluzzi, 2012).

In our experiments, we found five GPCRs, HalhaPK-R1a/b, HalhaPK-R2 and HalhaPK-R3a/b, that belong to the PK receptor group based on their sequence homologies and binding responses to PK and CAPA peptides, however, functional studies did not clearly differentiate them as PK1 (= DH) or PK2 (= PBAN) receptors. Although HalhaPK-Rs 1a/b, 2, 3a/b all responded to PK2 peptides, HalhaPK-R1a/b responded weakly to PK2 peptides and had very low expression in the body, suggesting they might be losing their biological relevance in *H. halys*. Meanwhile, HalhaPK-R2 was much more sensitive to PK2 than the PK1 peptide, suggesting it might play a role as the primary receptor for PK2 peptides.

From the first report of a PBAN receptor in *Helicoverpa zea* (Choi et al., 2003) to the recent report of *Ostrinia furnacalis* (Luo et al., 2019), many GPCRs for PK2 (= PBAN) peptides have been identified and functionally characterized in a variety of insect groups. However, PK1 receptors (PK1-R or DH-R) have been identified and characterized in only 9 species, including the tick, since the first *Drosophila* PK1-R was reported (Cazzamali et al., 2005). Interestingly, HalhaPK-R3a/b responded to both types of PK2 and CAPA-DH (= PK1) peptides, but notably, HalhaPK-R3b was the most sensitive to CAPA-DH among the six GPCRs. Therefore, the result suggests HalhaPK-R3 is the

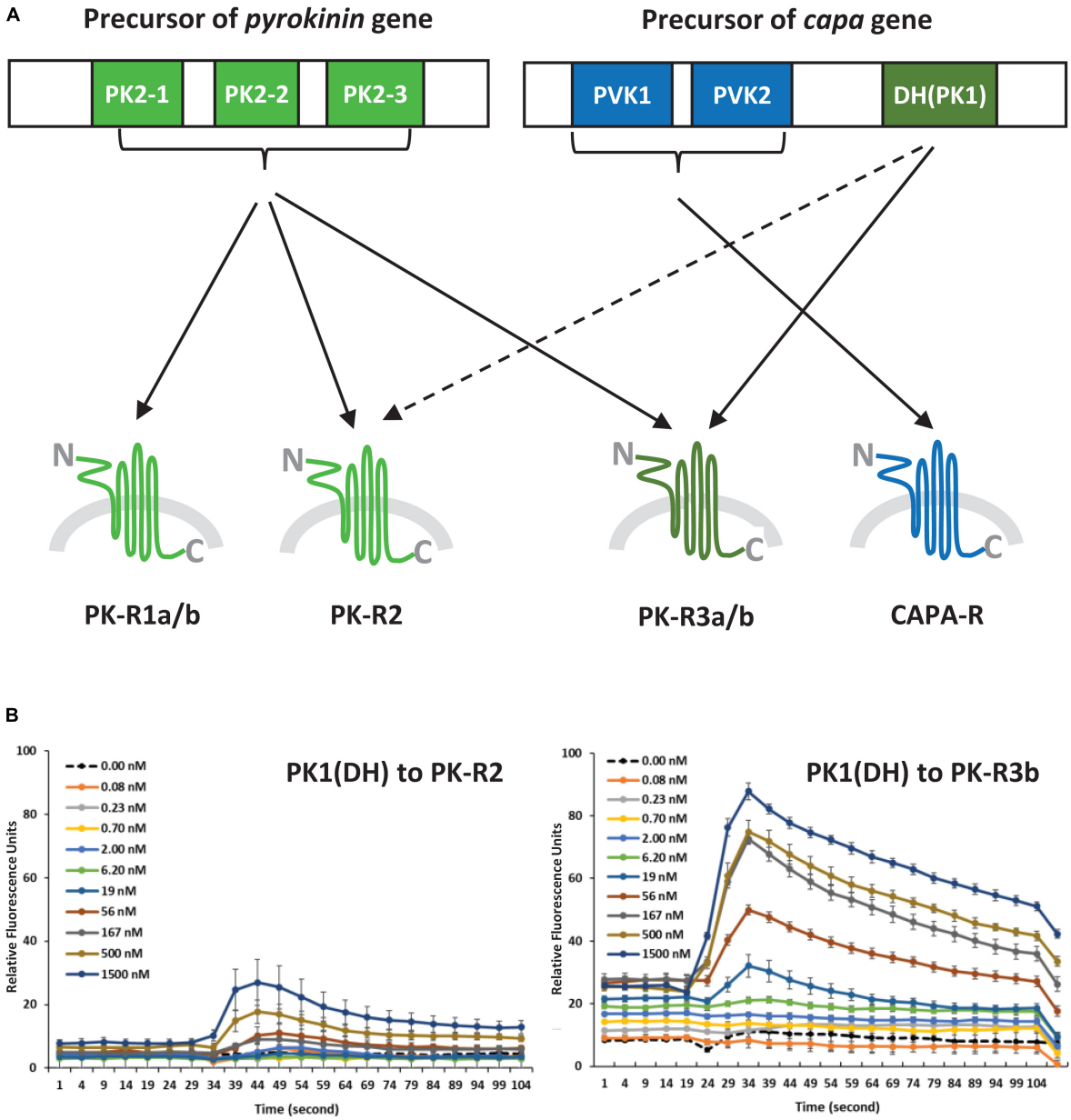

FIGURE 3 | (A) Schematic diagram of ligand-receptor pairings determined by the concentration-response profiles of pyrokinin and CAPA peptides on *Halyomorpha halys* receptors. The colors indicate three groups of the ligand and receptor types, and the broken line indicates a weak response. **(B)** Responses of *H. halys* receptor 2 (PK-R2) and receptor 3b (PK-R3b) to various doses of CAPA-PK1(DH) over time. Data are representative, showing the mean ± SEM response of four wells of cells in a single experiment. Experiments were repeated on three occasions using naïve cells and fresh reagents.

primary receptor for CAPA-DH (= PK1) in *H. halys*. Based on the responses of the *H. halys* GPCRs to peptide ligands, HalhaPK-R3 and HalhaCAPA-R can be activated by all PKs (PK1 and PK2s) or only CAPA peptides, respectively. Actually, these two receptors are the most dominant in the *H. halys* organs tested in this study. Although HalhaPK-R2 is expected to be the primary receptor for PK2 peptides, HalhaPK-R3b strongly responded to both PK1 and PK2 peptides, thus it may be an alternative receptor in *H. halys*. Previously, it was found that PK2 receptors are more promiscuous in other insects, responding to both PK2 and DH ligands, but insect DH receptors tend to respond specifically to DH type peptides (Olsen et al., 2007; Nusawardani et al., 2013). Other studies have found the opposite, that DH receptors cross reacted to the PBAN/PK2 and DH/PK1 (Jiang et al., 2014a,b). It is difficult to generalize; therefore, it is possible that the receptor sensitivities and specificities shown here for *H. halys* have evolved differently than in other previously studied insect groups.

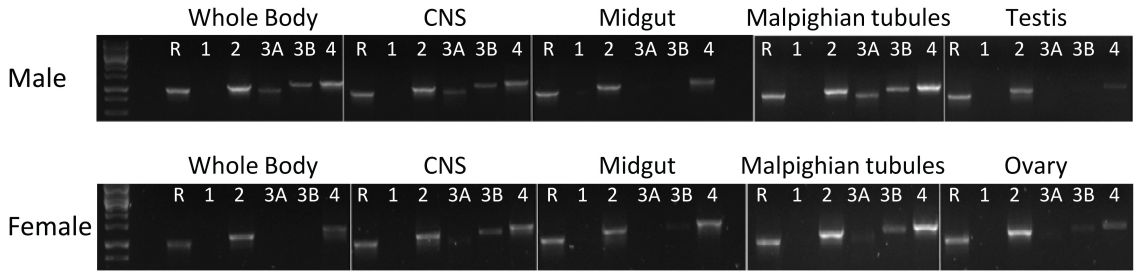

FIGURE 4 | Expression of the PK/CAPA receptors in whole body and various organs of male and female in *Halyomorpha halys* as determined by RT-PCR. R, reference gene (GAPDH); 1, HalhaPK-R1a/b; 2, HalhaPK-R2; 3A, HalhaPK-R3a; 3B, HalhaPK-R3b; and 4, HalhaCAPA-R.

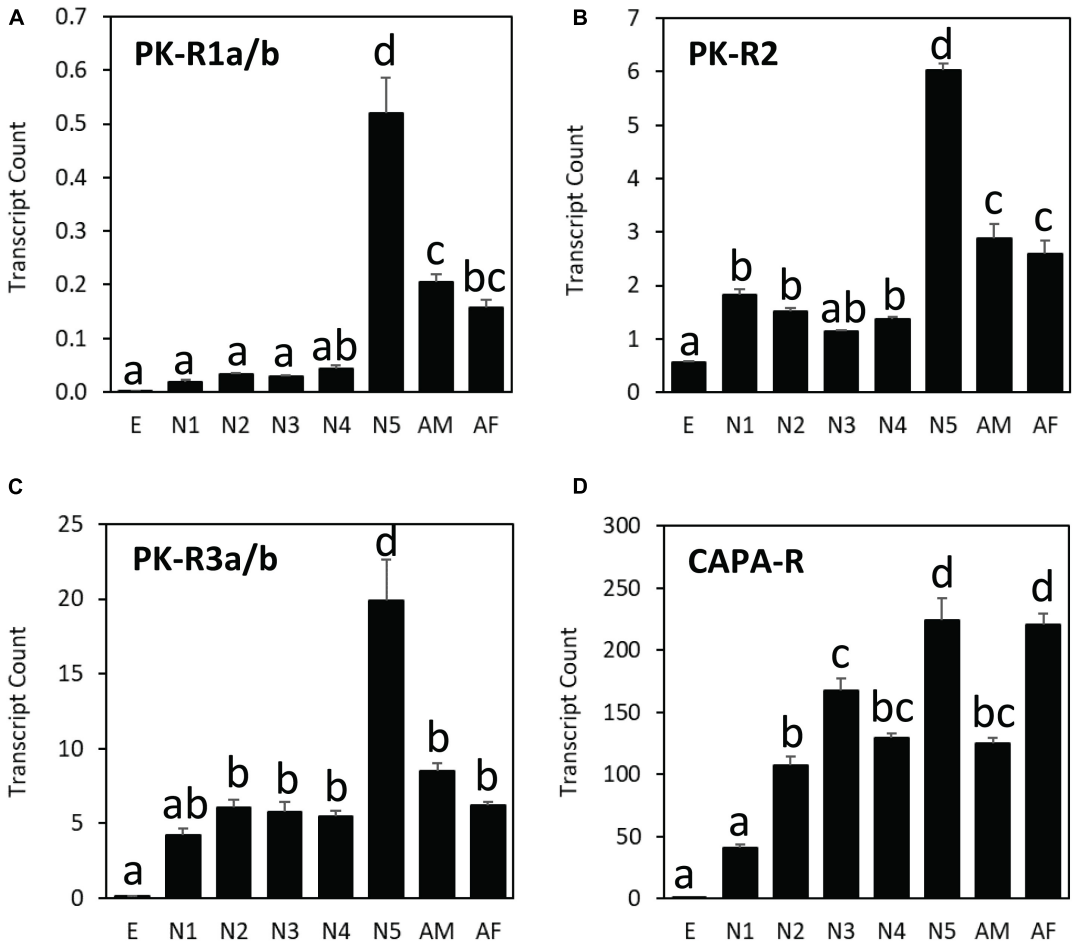

FIGURE 5 | Transcript levels of **(A)** HalhaPK-R1a/b, **(B)** HalhaPK-R2, **(C)** HalhaPK-R3a/b, and **(D)** HalhaCAPA-R receptor genes in different developmental stages of *Halyomorpha halys* as measured by quantitative PCR, expressed in terms of transcript copies per 1,000 Rpn2 transcripts. E, egg; N1–N5, 1st–5th nymphs; AM, adult male; AF, adult female (mean ± SEM). Different letters denote significant differences ($P < 0.05$) determined by One-way ANOVA multiple comparisons statistical analysis.

We then examined the expression patterns of the mRNAs of four *H. halys* PK GPCRs in selected tissues of *H. halys* and compared them between male and female. RT-PCR results revealed that the expression patterns of *H. halys* receptors were similar between male and female, with one exception being that HalhaPK-R2 was relatively more abundant in the Malpighian tubules and central nervous system of males than in female adults (**Figure 4**). Like the CAPA receptor, HalhaPK-R2 is ubiquitously expressed in *H. halys* tissues. Interestingly, strong transcription of HalhaPK-R2 was detected in testis and ovary, indicating a potential function involving muscle contraction or nutritional

metabolism in these reproductive organs. PK2 peptides were previously isolated from cockroaches, and demonstrated to stimulate the contraction of hindgut muscles (Holman et al., 1986). Our RT-PCR and qPCR results for certain receptors did not always correlate. It is possible that HalhaPK-R3a and -R3b genes separated or combined for two different PCR measurements. For example, in qPCR experiments HalhaPK-R1a & -R1b and HalhaPK-R3a & -R3b were all detected in both male and female whole body samples, however, in RT-PCR experiments HalhaPK-R1a & -R1b were not detected in male or female samples and HalhaPK-R3a & -R3b were not detected in female whole body samples. One possible explanation for the difference between RT-PCR and qPCR experiments is that the sizes of the amplicons differed significantly: in RT-PCR experiments the full-length open reading frames were amplified, whereas in qPCR experiments only ∼100 bp fragments were amplified. These results might suggest that the mRNAs are being selectively degraded or are present only as fragments in certain RNA preparations. Furthermore, expression levels of HalhaPK-R1a and -R1b variants were low or not detected at all in the tissues we tested despite being cloned from RNAs isolated from *H. halys*.

In this study, we identified six GPCRs that show varying response profiles to peptides produced from *pyrokinin* and *capa* genes in the brown marmorated stink bug. These receptors might have pivotal roles in the regulation of critical physiological processes that govern the development and survival of this insect. Now that these receptors have been identified and characterized, they can serve as biological targets in the development of *H. halys* specific pest control methods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

S-JA, JC, and MC performed all the experiments and analyzed the data. RV and the other authors wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00559/full#supplementary-material

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