Pyrokinin receptor silencing in females of the southern cattle tick Rhipicephalus (Boophilus) microplus is associated with a reproductive fitness cost

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Abstract

Background: Rhipicephalus microplus is the vector of deadly cattle pathogens, especially Babesia spp., for which a recombinant vaccine is not available. Therefore, disease control depends on tick vector control. However, R. microplus populations worldwide have developed resistance to available acaricides, prompting the search for novel acaricide targets. G protein-coupled receptors (GPCRs) are involved in the regulation of many physiological processes and have been suggested as druggable targets for the control of arthropod vectors. Arthropod-specific signaling systems of small neuropeptides are being investigated for this purpose. The pyrokinin receptor (PKR) is a GPCR previously characterized in ticks. Myotropic activity of pyrokinins in feeding-related tissues of Rhipicephalus sanguineus and Ixodes scapularis was recently reported.

Methods: The R. microplus pyrokinin receptor (Rhimi-PKR) was silenced through RNA interference (RNAi) in female ticks. To optimize RNAi, a dual-luciferase assay was applied to determine the silencing efficiency of two Rhimi-PKR double-stranded RNAs (dsRNA) prior to injecting dsRNA in ticks to be placed on cattle. Phenotypic variables of female ticks obtained at the endpoint of the RNAi experiment were compared to those of control female ticks (non-injected and beta-lactamase dsRNA-injected). Rhimi-PKR silencing was verified by quantitative reverse-transcriptase PCR in whole females and dissected tissues.

Results: The Rhimi-PKR transcript was expressed in all developmental stages. Rhimi-PKR silencing was confirmed in whole ticks 4 days after injection, and in the tick carcass, ovary and synganglion 6 days after injection. Rhimi-PKR silencing was associated with an increased mortality and decreased weight of both surviving females and egg masses (P < 0.05). Delays in repletion, pre-oviposition and incubation periods were observed (P < 0.05).

Conclusions: Rhimi-PKR silencing negatively affected female reproductive fitness. The PKR appears to be directly or indirectly associated with the regulation of female feeding and/or reproductive output in R. microplus. Antagonists of the pyrokinin signaling system could be explored for tick control.
Keywords: Pyrokinin/pheromone biosynthesis-activating neuropeptide/diapause hormone (PK/PBAN/DH) neuropeptide family, Tick CAPA gene, GPCR, RNA interference, Tick survival, Tick fitness, Tick reproduction, Tick feeding

Background

In arthropods, neuropeptides and their receptors play a critical integrative regulatory role in many physiological processes, such as feeding, excretion, mating, molting and diapause [1, 2]. Although neuropeptides are likely as important in ticks (Acari: Ixodidae), little is known about their physiological functions in comparison to other groups such as insects and crustaceans [1–3]. The recognized significance of emerging tick-transmitted pathogens to humans, cattle and wildlife led to the genome sequencing of the Lyme disease vector, *Ixodes scapularis* Say (Acari: Ixodidae) [4] and the cattle tick *Rhipicephalus microplus* Canestrini (Acari: Ixodidae) [5, 6]. In addition, *R. microplus* ticks have developed resistance to many acaricide classes and, thus, endocrinological research is crucial, specifically the study of neuropeptides and their G protein-coupled receptors (GPCRs), for the discovery of novel selective targets against ticks [1].

The Capability/Pyrokinin (CAPA/PK) endocrine signaling system is an ancient pathway in invertebrates, present in Nematoda and Arthropoda, that is putatively homologous of the Neuromedin U system in Vertebrata [7]. The ancestral neuropeptide CAPA/PK gene was duplicated and differentiated in insects into two genes, CAPA, which encodes periviscerokinin, and pyrokinin/pheromone biosynthesis-activating neuropeptide (PK/PBAN) [8].

The PK/PBAN gene encodes the pyrokinin/pheromone biosynthesis-activating neuropeptide/diapause hormone (PK/PBAN/DH) neuropeptide family. This family is defined by a conserved 5-amino acid C-terminal sequence (FXPRL-amide, X=G, T, I, V, K, A, P, S or D), which is the minimal sequence required for physiological activity [9]. This motif is conserved throughout several arthropod taxa, such as Arachnida [10–12], Protura [13], Crustacea [3, 14, 15] and Insecta (reviewed in [9]). Peptides of the PK/PBAN/DH family are myotropic and promote pheromone biosynthesis in different insect orders [9]. In addition, they regulate other physiological processes in Lepidoptera, such as melanization [16], induction of embryonic diapause and termination of pupal diapause [17, 18], puparium formation [19] and ecdysteroidogenesis in prothoracic glands [20].

PK/PBAN/DH orthologous sequences from insects, and their receptors, have been identified in ticks [4, 10, 21–23]. To date, only two functional studies with tick PK recombinant receptors of *R. microplus* [23] and *I. scapularis* [24] have been conducted, and only recently we reported the first in vitro analysis of tick pyrokinin 2 myotropic activity on *I. scapularis* and *Rhipicephalus sanguineus* tissues [25].

The pyrokinin receptor (PKR) is a GPCR, characterized by seven transmembrane helical regions, belonging to GPCR family A. The corresponding locus has been annotated in *I. scapularis* as pyrokinin-1 receptor LOC8040758. Three receptor isoforms corresponding to three transcripts (X1, XP_042145382.1; X2, XP_002401180.3; and X3, XP_029839423.1, respectively) have been predicted for this locus [4]. These three isoforms share a 94% identity in the overlapping amino acid sequences of the coding regions, and the X1 and X2 variants are longer than the X3 variant (by 83 and 80 amino acid residues, respectively) at the C-terminal region [4]. The pyrokinin-like receptor protein predicted in *Dermacentor variabilis* (Say) (Acari: Ixodidae) (ACC99623.1, unpublished) corresponds to the X1 variant predicted in *I. scapularis*, and the pyrokinin-like receptor protein predicted in *Dermacentor silvarum* Oleven (Acari: Ixodidae) (XP_03756591.1, unpublished) corresponds to the X3 variant of *I. scapularis*. Experimental confirmation only exists in *R. microplus* [23] and *I. scapularis* [24] by cloning of complementary DNAs (cDNAs) that correspond to the *I. scapularis* X3 variant. This is the only form identified in *R. microplus* by both analysis of the genomic sequence and by cDNA cloning, and is the focus of the present work. The genomic analyses of *R. microplus* and other tick species predicted the PKR as similar to the *I. scapularis* X3 variant [5, 6]. The transcript relative expression pattern in both tick species showed that the highest expression for PKR was in the synganglion, and secondly in the female reproductive system [23, 24]. However, a lower expression was observed in hindgut, and a very low expression was observed in other tissues, such as midgut, Malpighian tubules and salivary glands [23, 24]. This expression pattern suggests a functional role in feeding and reproduction, possibly associated with the regulation of myotropic activity, as we recently reported [25].

Beyond a few neuropeptides and cognate GPCRs characterized in ticks [10, 23, 26–29], little is known about the physiological role of GPCRs in this taxa, but some have shown a potential for interventions after RNA interference (RNAi) experiments by decreasing tick fitness [28]. Subsequently, high-throughput screens were conducted with the aim to discover new chemistries of antagonists of the tick kinin receptor [30]. The significance of these approaches is underscored by the lack of recombinant
vaccines to prevent deadly babesiosis of cattle caused by Babesia spp. which is transmitted by R. microplus, limiting this disease control to tick control [31, 32]. Further, R. microplus populations worldwide have developed resistance to amidines (amitraz), organophosphates, pyrethroids, fluazuron and ivermectins (reviewed in [33–37]). Therefore, there is a critical need to validate novel and selective pesticide targets in ticks to ensure the global security of cattle herds. To this end and to help elucidate the function of the tick pyrokinin system, we investigated the PKR loss of function through gene silencing in females of R. microplus.

**Methods**

**Tick rearing and animal care**

Rhipicephalus microplus ticks were reared at the Cattle Fever Tick Research Laboratory (USDA-ARS; Mission, TX, USA) under a cooperative agreement with Texas A&M AgriLife Research. Cattle breeds used for tick production or gene silencing experiments were R. microplus-naive Hereford, Charolais or Angus, with each individual weighing between 136 and 182 kg. All cattle were vaccinated, dewormed and acclimated for 2 weeks at the USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory prior to shipment to the USDA ARS Cattle Fever Tick Research Laboratory (CFTRL), a biosecure research facility near Edinburg, Texas, USA. Cattle used for routine tick rearing or RNAi experiments at the CFTRL were maintained under approved Animal Use Protocols (AUP). All procedures for handling and treating animals were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC), USA, TAMU AUP 2019-0197 EX, referring to IACUC USDA-ARS approved AUP 2021-12. Ticks used in this study were obtained from colonies of the R. microplus acaricide-susceptible Deutsch strain established from ticks collected in 2001 from an outbreak in Webb County, Texas, USA [38]. For the RNAi experiments, filial generation F65 was used for RNAi tests performed on December 2019; F66 for the March 2020 RNAi replicate; and F80 for the July 2021 RNAi replication.

**RNA isolation, cDNA synthesis and quantitative reverse-transcriptase PCR analysis**

Whole ticks and dissected tissues (synganglion, ovary and tick carcasses) were used for molecular analyses. For analyses of expression in different stages we used adults of both sexes, nymphs, larvae, egg masses and carcasses, at 5.65 Hz (m/s). Total RNA was extracted from whole ticks and individual tick tissues using the Zymo Quick-RNA™ MicroPrep kit (Zymo Research, Irvine, CA, USA), according to the manufacturer’s specifications. Two DNase 1 (deoxyribonuclease 1) steps were conducted: (i) during RNA extraction, by adding 30 U of DNase 1 to the sample and incubating for 15 min at room temperature (RT); and (ii) after solubilization of the sample in nuclelease-free water (NF-water), by adding 5 U of DNase 1 and incubating for 15 min at RT. After DNase 1 treatment, the RNA Clean & Concentrator™-5 Kit (Zymo Research) was used to clean the sample (following the manufacturer’s specifications), and the sample was recovered in 13 µl of NF-water. Clean total RNA (2 µl) was quantified spectrophotometrically using a Tecan Infinite M200 Pro plate reader (Tecan, Research Triangle Park, NC, USA).

Total RNA from whole ticks or tissues was used for cDNA synthesis in a final reaction volume of 22 µl containing 150–200 ng of total RNA as template (the same for all tissues), 1 µl oligo-dT20 (50 µM) and 1 µl random hexamers (50 ng/µl), using the SuperScript™ III First-Strand Synthesis System (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s specifications. The synthesized cDNA concentration (2 µl of a dilution 1:10) was checked using a Tecan Infinite M200 Pro plate reader (Tecan), and the undiluted cDNA was stored at −20 °C until further use.

Quantitative reverse-transcriptase PCR (qRT-PCR) analysis was performed in a reaction volume of 10 µl consisting of 5 µl PowerUp SYBR™ Green PCR Master Mix (Applied Biosystems™, Thermo Fisher Scientific), 1 µl of a primer mix (300 nM final concentration of each primer), 2 µl of cDNA (40 ng/µl) and 2 µl of NF-water. All reactions were performed in duplicate. Real-time relative quantification was performed using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The conditions for qRT-PCR cycling were: an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 95 °C 15 s and 60 °C 60 s. All oligonucleotide primers (Table 1) used for qRT-PCR analysis were synthesized by IDT Integrated DNA Technologies (Coralville, IA, USA).

To select reference genes for qRT-PCR analyses, we tested their stability across different treatments by selecting samples randomly. Four genes which had been used previously in published studies on R. microplus [23, 28, 39] were tested for this purpose, namely alpha and beta tubulin (Rhimi-A-Tub, XM_03743254.1 and Rhimi-B-Tub, XM_037427816.1 respectively), elongation factor 1 alpha (Rhimi-EF1A, EW679365.1) and ribosomal protein S4 (Rhimi-RPS4; CV436347). The stability and
suitability of these reference genes were evaluated with BestKeeper, Normfinder, Genorm and the comparative Delta-Ct method software tools [40]. *Rhimi-EF1A* and *Rhimi-RPS4* relative expression was the most stable and thus these two genes were selected as internal reference genes (Additional file 6: Table S1). The normalized relative quantity (NRQ) with respect to these reference genes was calculated for *Rhimi-PKR* and *R. microplus* beta-actin (*Rhimi-ACTB*) following the formulas in [41].

**Rhimi-PKR** relative expression throughout different stages of development

To determine *Rhimi-PKR* relative expression by qRT-PCR across different stages of development, unfed neolarvae (early first larval instar [42]), nymphs and adults were flash frozen within 24 h of emergence and kept in 500 µl of RNAlater™ (Invitrogen) at −80 °C until used for qRT-PCR analysis. All tick stages were maintained within two patches on a Hereford calf, with each patch infested with approximately 250 mg of tick neolarvae. To obtain newly molted nymphs, engorged larvae were removed from the host animal 6–7 days after neonate infestation. The larvae were allowed to molt in an environmental chamber kept at 25 ± 2 °C and a relative humidity (RH) of 95% [43]. Engorged nymphs were removed from the animal after 13 to 14 days post-infestation (dpi). These were placed in an environmental chamber maintained at the same temperature and RH as stated above, until ecdysis. Eight biological replicates (*n* = 8) for each of the developmental stages were used, as follows: for egg masses,
each replicate consisted of approximately 100 mg of eggs that were a pool from egg masses from different females (approx. 11.6 ± 0.9 mg per egg mass obtained 3–6 days after oviposition), neolarvae (10 whole bodies pooled per replicate), nymphs (5 whole bodies pooled per replicate), males (unmated, 2 whole bodies pooled per replicate) and females (unmated, 1 whole body per replicate). All ticks (generation F81) were kept under the conditions described under section: “Tick rearing and animal care”.

Total RNA extraction, cDNA synthesis, qRT-PCR conditions, references genes and target gene (Rhimi-PKR) were the same used for and described in section RNA isolation, cDNA synthesis and quantitative reverse-transcriptase PCR analysis.

Rhimi-PKR gene silencing by RNAi

In vitro RNAi evaluation of dsRNAs using the dual luciferase Rhimi-PKR reporter plasmid

It is critical to reduce the number of ineffective silencing dsRNAs tested on ticks placed on cattle due to the high cost, intensive labor and restrictions for the use of large animals in tick research. For these reasons, the silencing efficiencies of Rhimi-PKR dsRNA sequences were first tested in vitro in a dual luciferase system. The dual luciferase reporter was constructed for R. microplus as previously described [44] and subsequently adapted for in vitro assessment of RNAi efficacy targeting transcripts of Rhimi-PKR. The cDNAs cloned for dsRNA synthesis were amplified from messenger RNA isolated from whole unfed females of the Deutsch strain by RT-PCR using specific primers (Table 1). Primers were designed based on partial sequences derived from the Rhimi-PKR-cloned cDNA (KP126932 [23]), and from an additional 5′-untranslated region (UTR) sequence from the predicted transcript XM_037432703.1 (LOC119181450 pyrokinin-1 receptor-like R. microplus, southern cattle tick). RT-PCR was run with the AmpliTaq Gold™ Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer’s specifications, using approximately 25 ng of template (cDNA) and at the following temperature-cycling parameters: 1 cycle of 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final cycle of 72 °C for 5 min. The PCR product fragments were then cloned into the XmnI/SbfI sites in two dual luciferase reporter plasmids using an InFusionTM cloning kit (Clontech Laboratories, Mountain View CA, USA). The first of these clones was RmPyr_DualLuc-5′: PK-584–580.2/pCR 2.1-TOPO, with a Rhimi-PKR cDNA insert of 895 bp that encompassed 197 bp of the 5′-UTR end of the KP126932 (in bold in Additional file 1: Figure S1), having an extended 5′-UTR sequence of 629 bp upstream of this section, and 69 pb of the open reading frame (ORF) of Rhimi-PKR, which was used to test the in vitro silencing activity of the ds762-913 (green highlight in Additional file 1: Figure S1). The second of these clones was DualLuc RmPyr#3′: PK-618.2/pCR 2.1-TOPO, with a Rhimi-PKR cDNA insert of 1638 bp that encompassed the majority of KP126932 as follows: 133 bp of the 5′-UTR end, the ORF, and 184 nucleotides (nt) at the 3′-UTR end, which was used to test the in vitro silencing activity of the ds1485-1627 (gray highlight in Additional file 1: Figure S1).

In vitro transfection of the R. microplus embryonic cell line BmE26 [45] was accomplished by using a mix containing Effectene transfection reagent (Qiagen, Hilden, Germany), 150 ng of the dual luc/pyrokinin construct (either RmPyr_DualLuc-5′ or RmPyr_DualLuc-3′) and 50 ng of the corresponding dsRNA to be tested for silencing activity: ds762-913 for construct RmPyr_DualLuc-5′ and ds1485-1627 for cells transfected with RmPyr_DualLuc-3′ [44].

Wells with no dsRNA and those with a dsRNA whose sequence does not overlap with the Rhimi-PKR sequence, designated dsfsg, which corresponds to R. microplus cholinesterase-like transcript (XM_037420972.1; see Additional file 1: Figure S1) were included in each assay as negative controls. At 5 days post-transfection, the cells and supernatant were harvested. The supernatant luciferase activity was measured using the Nano-Glo Luciferase System (Promega, Madison, WI, USA), and the cells were lysed and tested using the Steady-Glo Luciferase System (Promega). As the transfection with the cell line (BmE26) used in this assay is highly variable, the ratio of activity of the two luciferases was used as an efficiency parameter. The ratio of cell activity/supernatant activity was recorded for each well (Table 2). The dual luciferase reporter system uses two independently controlled luciferases, which allows one promoter to function as an internal reference standard. By calculating the ratio of expression of the two reporters, differences in relative transfection efficiency and dosage between wells are normalized with respect to the internal reference luciferase, allowing comparison of the normalized second luciferase activity [44]. Since the release of the R. microplus genome [6], further analysis of the specificity of Rhimi-PKR dsRNAs has become possible. To this end, for each Rhimi-PKR dsRNA sequence, the algorithm BLASTn was used in searches (https://www.ncbi.nlm.nih.gov/) to identify similar sequences in the genome (R. microplus, assembly ASM1333972v1) that could lead to off-target RNAi effects (Additional file 2: Figure S2).

Synthesis of dsRNAs for RNAi

Target sequences for RNAi of 150–250 nt were selected from the Rhimi-PKR cDNA (KP126932.1) (Additional file 1: Figure S1), primarily from the 5′- and 3′-UTR
regions. dsRNAs for the in vitro and in vivo gene silencing experiments were synthesized following the manufacturer’s instructions using the T7 RiboMAX™ Express RNAi System (Promega) and the clones and plasmids mentioned above. Oligonucleotide primer sequences (Table 1) were selected using the software Oligo v6.71 (Molecular Biology Insight Inc., Cascade, CO, USA) and adapted by the addition of the T7 polymerase recognition sequence, as specified by the manufacturer for dsRNA synthesis (Table 1). The concentration was determined using a Nanodrop 1000 spectrophotometer using optical density, $A_{260}/A_{280}$ ratios (Thermo Fisher Scientific). The dsRNA for the negative (beta-lactamase) and positive ($Rhimi$-ACTB gene) dsRNA controls were synthesized using the same conditions mentioned above, with the primers listed in Table 1. The $BLA$ gene (beta-lactamase) encodes a bacterial enzyme that is not present in ticks. Beta-actin (used as a dsRNA positive control) is a gene that encodes for a ubiquitous protein in insects, involved in cell motility, structure, and integrity [46]. dsRNAs for both beta-lactamase (dsβ-lac) and beta-actin (dsβ-act) were previously used as a negative and positive controls, respectively, for silencing experiments in $R. microplus$ [28, 47].

### Microinjection of ticks

Ticks used in the RNAi experiments were unfed adult females, between 1 and 5 days after eclosion from collected nymphs, held in cotton-stoppered glass vials under constant temperature (25 ± 2 °C) and 95% RH conditions [43]. Four independent RNAi experiments were performed. Each replicate consisted of dsRNA-injected ticks and non-injected ticks (as negative control) held in round, cotton sleeves that were glued to the calves’ shaved backs [28, 48]. Each calf could accommodate up to eight screens, four on each side of the animal, and each animal was considered to be a randomized block. Each treatment corresponded to a sleeve, and treatments were randomly adjudicated to sleeves that were then labeled. The sleeves contained 35–40 females and 15–25 males. In total, five calves were used for these RNAi experiments.

Female ticks were microinjected with dsRNA specific for each treatment: dsRNAs for $Rhimi$-PKR (dsPKR) as the experimental group; dsβ-act as the positive controls; and dsβ-lac as the negative controls. These dsRNAs were diluted in 0.2 µl of NF-water at concentrations ranging from 5.21 to 7.5 µg/µl, with the exception of the dsβ-act, which was used at a dilution of 5.6–9.3 µg/µl (Additional file 7: Table S2), using methods previously described [49]. Injected ticks were moved to glass vials and kept at the same temperature and humidity conditions as with adult emergence (mentioned above) for 24 h post-injection, following which live and motile injected and non-injected females were transferred to the sleeves on the bovine host. The four independent biological replicates for each treatment were performed in 3 consecutive years: December 2019, March 2020 and July 2021. The delay between the second and third replicate was due to the SARS-COVID-19 pandemic causing the closure of the USDA-ARS facilities for animal experiments.

### Tissue collection, RNA extraction, cDNA synthesis and qRT-PCR assays for evaluation of gene silencing

For evaluating silencing, we collected three partially fed females per treatment and biological replicate on the third and fifth day on the animal. The ticks that fed for 3 days were pierced and individually kept in 250 µl of RNAlater™ (Invitrogen) at − 80 °C until use. Ticks that were 5 days on the animal were dissected in cold physiological saline, and tissues were individually kept at − 80 °C until RNA isolation [28, 49]. RNAlater was used to store each carcass (250 µl) and ovary (100 µl). The synanglion was kept in 50 µl Trizol reagent (Invitrogen) to ensure RNA stability and because the synanglion is a very small tissue that becomes fully transparent in RNAlater and cannot be visualized for recovery. Ovary samples

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**Table 2** Luciferase activity and silencing efficiency obtained from $Rhipicephalus microplus$ BmE26 cells at 5 days post-transfection with dsRNAs targeting the $Rhimi$-PKR sequence

| Treatment set | Treatment applied to $R. microplus$ PKR dual LUC-expressing cells | Size of dsRNA (bp)$^a$ | Luciferase ratio$^b$ | Silencing compared to no added dsRNA (%)$^c$ |
|---------------|-------------------------------------------------|------------------|-----------------|-------------------------------------|
| Control (−)   | No dsRNA                                         | −                | 0.699827        | 0.0                                 |
| Control (−)   | dsRNA fsg                                        | 357              | 0.376921        | 22.9                                |
| 1             | dsRNA 762–913$^d$                                | 207              | 0.011161        | 98.3                                |
| 2             | dsRNA 1485–1627$^d$                              | 198              | -0.039550       | 103.7                               |

$^a$ Length of the dsRNAs in bp including the amplified sequences and the 5'- and 3'-ends T7 tags

$^b$ Luciferase ratio Steady-Glo/Nano-Glo

$^c$ Silencing = 1 − (luciferase ratio for dsRNA treatments/luciferase ratio control value [no dsRNA])

$^d$ dsRNAs used for the in vivo study

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additionally included the vagina and seminal receptacle and likely muscles closely associated with these tissues. Samples were selected to analyze the silencing efficiency at the organism level (whole tick), and tissues were those in which a relative high expression for Rhimi-PKR transcript had been previously reported in *R.* microplus [23].

For the analyses of relative gene expression to evaluate the effect of the dsRNAs, total RNA extraction, cDNA synthesis, qRT-PCR variables, reference and target genes (*Rhimi-PKR* and *Rhimi-ACTB*) and preparation of reactions were the same as those described in section RNA isolation, cDNA synthesis and quantitative reverse-transcriptase PCR analysis.

To compare the relative gene expression among treatments, for the qRT-PCR assays all cDNA samples of non-injected, *Rhimi-PKR* (both dsRNAs 762–913 and 1485–1627) and dsβ-lac-injected ticks were loaded in the same 96 cell-well plate, and this was replicated in a total of three plates for each of the following: (i) ticks 3 days on the animal; (ii) carcasses; (iii) ovaries; and (iv) the synganglion of ticks that fed for 5 days. For each tissue (of replicates 1–4), relative expression was analyzed on two plates using primers for each reference gene (*Rhimi-ACTB* and *Rhimi-EF1A*) respectively, and on the third plate using primers for the gene of interest (*Rhimi-PKR*) and for *Rhimi-ACTB*. Therefore, a total of 48 plates were utilized (4 replicates x 4 tissues x 3 plates per tissue). The NRQ was calculated for *Rhimi-PKR* and *Rhimi-ACTB* following the protocol mentioned in section RNA isolation, cDNA synthesis and quantitative reverse-transcriptase PCR analysis.

The relative transcript abundance of *Rhimi-PKR* for each tissue was presented as a fold-change (FC) of dsβ-lac-injected ticks (FC = 1).

### Phenotypic data collection

To register potential phenotypic changes associated with PKR silencing, each day after ticks were placed on the animals, the sleeves were opened, and ticks were photographed (Additional file 3: Fig. S3). Any detached engorged ticks found were removed, weighed, transferred to a cotton-stoppered glass vial and held under the laboratory maintenance conditions previously mentioned. The date of “drop” (self-detachment from the bovine) as well as the “repletion period” (duration of the female feeding period) were recorded. Engorged females in vials were monitored daily to record the date of the first oviposition to calculate the pre-oviposition period, which extends from detachment to oviposition. Because *R.* microplus females die after oviposition [42], the dead female tick was removed from the vial and the egg masses were weighed. Egg masses were monitored to determine the incubation period, which corresponds to the time lapsed from egg laying to first hatch. The percentage of emergence was used to calculate the egg hatch per female, which corresponds to the eggs that hatched per egg mass. This assay was performed by visual estimation by the same experienced technician for the duration of each experiment, as described in [50]. The overall time period from “attachment to animal” until hatching of the first egg for each female tick was also recorded as the cumulative “observation period.” The reproductive efficiency index (REI) = [(egg mass/replete female weight) × 100] was calculated for each female [51]. In addition, more phenotypes were recorded for each female, as defined: (i) female mortality was recorded over the course of the experiments; (ii) females with no eggs, calculated from the number of females that survived (these ticks were placed in vials, and did not lay eggs at all); (iii) females with no hatch, which reflects the percentage of produced egg masses that do not hatch at all.

Results for both PKR RNAi treatments ds762–913 and ds1485–1627 were compiled for the analysis of all phenotypic variables: mortality, repletion period, female weight, pre-oviposition period, egg mass weight, REI, females with no eggs, incubation period, egg hatching per female, females with no hatch and observation period. As per approved AUP that assures maintenance of the animal health and taking into consideration that the expected detachment time from the host for untreated ticks is 6–9 days, an endpoint for the experiment of 2 weeks after tick attachment to the calf was chosen. Ticks that did not detach before this pre-determined date were considered dead and not included in subsequent analyses. Further, the data obtained after the endpoint are highly variable, and tick death is often associated with factors such as fungus on the tick females, confounding results.

### Statistical analysis

Results of phenotypic variables and qRT-PCR assays were analyzed with and graphs were produced using GraphPad Prism v6 software (Graphpad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) followed by a Tukey’s HSD test was performed to compare PKR relative expression throughout developmental stages. The qRT-PCR relative expression data were similarly analyzed to verify PKR silencing in whole ticks and in different female tissues. Results are presented as the mean ± standard error of the mean (SEM). Kruskal–Wallis one-way ANOVA followed by a corrected Dunn’s multiple comparisons test was used to analyze phenotypic data, and the results are presented as the mean ± SEM.
Results

Rhimi-PKR expression throughout different stages of development

The qRT-PCR analyses showed that the Rhimi-PKR transcript was expressed throughout all stages of development of *R. microplus* (Fig. 1). The lowest Rhimi-PKR relative expression was observed for egg and male (Fig. 1), and the relative abundance for egg was used as the reference ratio (calibrator) for the FC calculation. Rhimi-PKR relative abundance was similar for larva, nymph and female, surpassing the expression in eggs by a factor of 4.3–4.7 (*female*, surpassing the expression in eggs by a factor of 4.3–4.7 (*female*)). The qRT-PCR analyses showed that injections with ds762-913 caused a mRNA Rhimi-PKR knockdown of 76% (average for the 4 replicates) in whole ticks that were 3 days on the animal (Fig. 2b). Females injected with the same dsRNA that were dissected after 5 days on the animal showed a decrease in Rhimi-PKR transcript in the ovary, carcass, and synganglion of 85, 68, and 92%, respectively (*n*=12 (4 replicates x 3 ticks or tissues), *P*<0.05; Figs. 2c–e). For females injected with ds1485-1627, the mRNA of Rhimi-PKR showed a decrease of 77% (average for the 4 replicates) in whole ticks that were 3 days on the animal (Fig. 2b). For those ticks dissected after 5 days on the animal there were reductions in the ovary, carcass, and synganglion of 80, 76, and 92%, respectively (average for the 4 replicates) (Figs. 2c–e).

RNAi silencing of Rhimi-PKR

For the RNAi experiment, two dsRNAs, designated ds762-913 and ds1485-1627, respectively, were tested in vivo (Fig. 2a). The dsRNAs were considered suitable for in vivo experiments on the basis that they efficiently silenced the Rhimi-PKR transcript in the dual luciferase reporter assay (see section In vitro RNAi evaluation of dsRNAs using the dual luciferase Rhimi-PKR236 reporter plasmid) (Table 2). To test for possible off-target effects, we conducted BLASTn searches, but only sequences ≤17 nt were found with similarity to *Rhimi-PKR* dsRNA sequences (Additional file 2: Figure S2). For in vivo experiments, silencing efficiencies of dsRNAs are reported with respect to the dsβ-lac-injected females. qRT-PCR analyses showed that injections with ds762-913 caused a mRNA *Rhimi-PKR* knockdown of 76% (average for the 4 replicates) in whole ticks that were 3 days on the animal (Fig. 2b).

Phenotypes associated to the RNAi silencing of Rhimi-PKR

More than 95% of the ticks that were placed on the calves from all treatments dropped off and were collected within the 2 weeks of the experiment. After specific *Rhimi-PKR* silencing was verified by qRT-PCR (Fig. 2), the phenotypic traits of ticks under each treatment were evaluated; the results at the endpoint of the assay are summarized in Fig. 3. Tick pictures representative of the treatment effects after 6, 8, and 10 days on the calves are shown in Fig. 4. A complementary detailed record of the progression of tick sizes for all treatments in days 6 to 10 is shown in Additional file 3: Figure S3.

dsPKR-injected ticks showed an increased net mortality of 15% (*n*=110–198; *P*<0.05) relative to the dsβ-lac-injected females (negative controls) (Fig. 3a). All dsβ-act-injected females (positive control) died, as expected, and were not included in the remaining
Fig. 2 RNAi silencing of *Rhimi-PKR*. 

(a) Location of the PKR dsRNAs and qRT-PCR amplicon on *Rhimi-PKR* full-length cDNA sequence [23]. The full-length cDNA sequence is 1713 bp with a 1326-bp ORF (KP126932.1), which is depicted limited by vertical bars. Locations of PKR dsRNAs are highlighted as a darker box on the left for ds762-913, and as a light gray, black-dotted box for ds1485-1627, on the right. The PKR qRT-PCR amplicon is shown as a solid-gray box, below. An additional 5'-UTR sequence beyond the original cDNA cloned sequence was obtained based on the genomic sequence (not shown). The 5'-dsRNA762-913 identification numbers refer to the nucleotide positions that encompassed on that additional 5'-UTR sequence (Additional file 1: Figure S1, alignment of all PKR sequences), thus the numeric discrepancy between the cDNA clone positions and the ds762-913 shown on the left of the schematic. The ds762-913 nucleotide position in the cDNA KP126932.1 is from −65 to +66 nt. For more details see section In vitro RNAi evaluation of dsRNAs using the dual luciferase *Rhimi-PKR* 236 reporter plasmid and Table 1.

(b-e) qRT-PCR analyses of partially fed female ticks from all treatments to confirm *Rhimi-PKR* silencing: (b) whole tick, 3 days on calves; (c) ovary; (d) carcass; (e) synganglion, from females 5 days on calves. Primers used for the analysis are given in Table 1. A one-way ANOVA followed by a Tukey’s multiple comparisons test was used for the statistically analysis. Different lowercase letters at top of histogram bars indicate significant difference (*P* < 0.05).

Abbreviations: cDNA, Complementary DNA; ds, double-stranded; ORF, open reading frame; UTR, untranslated region.
Fig. 3  Phenotypic evaluation of Rhimi-PKR silencing in Rhipicephalus microplus females. Results for both treatments, PKR ds762-913 and ds1485-1627 were compiled for the analysis of all the phenotypic variables: a percentage of female mortality, b duration of repletion period, c female weight, d duration of pre-oviposition period, e egg mass weight, f REI, which represents the conversion of the blood intake to egg mass production, g females with no eggs, i.e. females that showed not oviposition at all, h duration of egg incubation period, i percentage of egg hatching per female, j females with no hatch, i.e. percentage of females whose egg mass had no eggs hatching at all, k total observation period, which represents the time period from “tick attachment to animal” until hatching of the first egg for each female. Positive control: Rhimi-ACTB dsRNA-injected ticks (dsβ-act). Treatments sharing the same lowercase letter (top of each graph) were not significantly different from each other (P < 0.05) as determined by the Kruskal–Wallis test followed by the corrected Dunn’s multiple comparisons test. Abbreviations: NI, Negative controls (non-injected ticks); dsβ-lac, beta-lactamase dsRNA-injected ticks; dsPKR, Rhimi-PKR dsRNA-injected ticks; REI, reproductive efficiency index.
analyses (Fig. 3a). The lowest mortality was for non-injected ticks (18.3%), followed by dsβ-lac-injected ticks with 32.7% mortality (Fig. 3a).

The repletion and pre-oviposition periods were 0.8 \((n = 103–125; P < 0.05)\) and 1.3 \((n = 96–118; P < 0.05)\) days longer, respectively, for dsPKR-injected female ticks in comparison to dsβ-lac-injected females (Fig. 3b, d). There were no significant differences between the negative control treatments for both traits. In addition, dsPKR-injected females had a net decreased weight \((n = 103–123; P < 0.05)\) and net decreased weight of the egg masses \((n = 96–118; P < 0.05)\) of 13% for both phenotypic traits relative to dsβ-lac-injected females (Fig. 3c; e, respectively). Similarly, there were no differences between the negative control treatments for both traits. However, the REI, which reflects the percentage of conversion of female weight to egg mass \((n = 96–119; P > 0.05)\), showed no difference between dsPKR-injected ticks and negative controls (Fig. 3f).

With respect to the number of females with no eggs (live females that did not oviposit), no significant statistical differences were observed \((n = 103–123; P > 0.05)\) among the three treatments (Fig. 3g). On the other hand, the incubation and observation periods were significantly longer by 1.1 \((n = 82–107; P < 0.05)\) days, respectively, for dsPKR-injected female ticks (Fig. 3h, k). The non-injected ticks did not differ from the dsβ-lac controls for both traits (Fig. 3h, k).

Finally, for the percentage of egg hatching per female \((n = 84–107; P > 0.05)\) and the number of egg masses that showed no hatch at all (females with no hatch) \((n = 82–107; P > 0.05)\), there was no statistical difference between dsPKR-injected ticks and negative controls (Fig. 3i, j).

Representative phenotypic image records of ticks for all treatments in patches for days 6, 8 and 10 after being placed on the animals are shown in Fig. 4. These phenotypic images accurately reflect the results of the phenotypic traits quantitatively evaluated in Fig. 3, but further provide visual evidence of the differential feeding progression for all treatments. Many of the non-injected ticks (NI) fed to repletion between days 6 and 8, and by day 10 most of them had dropped from the animal. For the dsβ-lac-negative controls, while the records appear to indicate a delay in their repletion with respect to the NI (compare day 8 NI, with β-Lac; Fig. 4), there was no significant difference between the two treatments in terms of repletion period by the endpoint (Fig. 3b); it should be noted that most of them had similarly dropped off the animal by day 10. In contrast, the PKR-silenced
ticks that survived appear to be repleted by day 10, significantly delayed in their repletion period (Fig. 4 PKR; Fig. 3b) and reached a significantly lower final weight (Fig. 4 PKR; Fig. 3c). The dsβ-act-injected ticks showed a rounded shaped body at day 6 (compare NI with β-Act), and although some appeared to continue to feed by day 10 while others appear to have reduced their size by the same day, all of them died by the endpoint of the assay (Fig. 3a), and none fed to repletion (compare day 6 NI, day 8 β-Lac and day 10 PKR, with β-Act).

Discussion

Pyrokinins are pleiotropic neuropeptides in insects, and one of their functions is to promote muscle contractions [8]. While we recently reported that pyrokinins and a pyrokinin peptide analog elicit the contraction of feeding-related tissues of R. sanguineus in vitro [25], the pleiotropic functions of the pyrokinin signaling system in ticks are still unexplored. Therefore, we performed loss-of-function experiments by RNAi of the pyrokinin receptor in live females of R. microplus to further investigate the physiological significance of the pyrokinin signaling system in ticks and to assess if this GPCR could be a good candidate for control interventions (e.g. acaricides or vaccines).

RNAi has proven effective in inducing specific gene silencing in R. microplus [28, 48, 52–54]. Previously, we established that the receptor transcript was expressed in female tissues [23]. In the present work, we determined that the PKR transcript was also expressed in male ticks and was present throughout all stages of development of R. microplus (Fig. 1), which would be crucial for a candidate control target. Several research studies have looked at the possibility of designing dsRNAs in the 5’-UTR or 3’-UTR [55]. The advantage of choosing these regions is that they are less conserved than the coding sequence, leading to much higher specificity [55]. Selected regions in the 5’- and 3’-UTRs of the PKR cDNA sequence were chosen as targets for RNAi, and both were found to be equally effective. These results are similar to those on the pea aphid, Acyrthosiphon pisum, in which no differences in efficiency were found between dsRNA targeting the 5’- and 3’-UTR ends of the hunchback gene [56]. However, to discard possible off-target effects, we first ran BLASTn searches against the R. microplus genome. The results of these searches indicated that Rhimi-PKR dsRNAs off-target effects are highly unlikely, since 17 bp in length was the maximum contiguous identical sequence for the alignment between Rhimi-PKR dsRNA sequences and non-target sequences (Additional file 2: Figure S2). These alignments are too short to trigger meaningful off-target effects according to the authors of previous studies who concluded that a sequence of 19–21 nt of contiguous identical sequence is required to produce significant biological activity in RNAi-sensitive insect species [55, 57].

In the present study, we used a successful RNAi protocol to characterize the PKR, a GPCR of R. microplus. This protocol was previously successful for RNAi of the kinin receptor, another neuropeptide tick GPCR [28]. The protocol included first the use of a dual-luciferase reporter system to validate the silencing efficiency of dsRNA in vitro, prior to performing in vivo experiments with ticks on cattle that was developed by [44]. These in vitro assays were performed to increase the probability of success and specificity of the RNAi, being cognizant that the molecular components of the RNAi pathway(s) are still being characterized in the Acari [58]. RNAi experiments in R. microplus are particularly expensive and difficult to conduct beyond the normal complexity of performing RNAi in ticks [59, 60] because this is a one-host tick and a quarantined species in the USA, and because APHIS-approved cattle facilities are required [28]. Both dsRNAs were highly effective in silencing the PKR transcript in the dual-luciferase assay (Table 2). We then performed PKR RNAi in female ticks using these validated dsRNAs. qRT-PCR analyses demonstrated that both dsRNAs were effective in silencing the receptor in vivo (Fig. 2b), and silencing was detected in whole ticks 4 days after injection (Fig. 2b), and in the ovary, carcass and synganglion of females after interference that had been 5 days on the calves (Fig. 2c-e). The significant silencing of the PKR transcript in vivo verified by qRT-PCR underscores the importance of verification by the in vitro system before studies are undertaken in ticks using cattle.

The results point to the direct or indirect involvement of the PKR signaling system in the regulation of female feeding and reproductive output in R. microplus. Through the Rhimi-PKR silencing by RNAi, two physiological processes related to feeding were affected: the repletion period was delayed, and female weight decreased (Fig. 3b, c). With respect to reproductive output, the pre-oviposition and incubation periods were longer, suggesting an overall delayed egg/embryo maturation (Fig. 3d, h). Moreover, the egg mass weight was lower in comparison to the controls (Fig. 3e). A R. microplus female produces a compact egg mass of about 3000 eggs [42]. Since the percentage of eggs hatching was not affected by Rhimi-PKR silencing (Fig. 3i), we hypothesize that the decrease in the weight of egg masses was due to the reduction in the number of eggs (quantity) rather than the quality of the eggs; however, eggs were not counted. Overall, Rhimi-PKR silencing increased female mortality and reduced the fitness of surviving females, which is reflected in the
delayed observation period that encompasses the female feeding and the day of first egg hatching (Fig. 3a, k).

Based on the results of this study, our recent work [25] and previous evidence in insect tissues reviewed in [9], the PK tick signaling system appears to activate muscles involved in feeding. Unlike blood-feeding insects that feed through blood vessels, ticks are pool feeders, with a complex and active feeding mechanism [61]. The feeding process involves host skin penetration by the hypostome, retraction of the cheliceral shafts and lateral movement of cheliceral digits [61]. The blood is sucked into the food canal and pharynx by contraction of the dilator muscles of the pharynx. Relaxation of these muscles allows the blood to pass through the esophagus into the midgut [62]. Consequently, a reduced activity of one or more of these muscles will result in a decreased feeding performance, in agreement with our results. Rhipicephalus microplus ticks cement themselves to the host, and once attached, the female takes a prolonged meal that is characterized by the interference of the pyrokinin signaling system: a delayed feeding progression (Fig. 4), a longer repletion period (Fig. 3b) and a decreased female weight (Fig. 3c). The photographs documenting feeding progression suggest that the slow phase is affected (compare day 8 β-Lac vs. PKR) (Fig. 4), but further studies are needed to determine if this or both phases were compromised. The hypothesis is further supported by our recent study that demonstrated the myotropic activity of PK in feeding-related tissues of two tick species, R. sanguineus and I. scapularis [25], being the first report to explore the functional activity of PK and a PK analog in ticks. Additional evidence supports that the pyrokinin signaling system is associated with the feeding-related tissues in arthropods. Drosophila melanogaster pyrokinins are encoded by the capability (CAPA) and hugin genes, respectively [64, 65]. In Drosophila larvae hugin-expressing neurons send projections to the pharyngeal muscles [66, 67], and Schlegel et al. [68] observed that hugin neuropeptides are associated with feeding regulation. In Lepidoptera, PK/PBAN is expressed in the subesophageal ganglion (SOG) [69], and several studies support the SOG as a taste and food intake control center in insects (reviewed in [70]). Consequently, we propose that the decreased reproductive output and the overall female fitness reduction observed after Rhimi-PKR silencing are consistent with defects in feeding.

There is some evidence that PKR transcript expression in tick reproductive tissues may be positively correlated with feeding. In unfed females of both I. scapularis [24] and R. sanguineus [25], the relative expression of the PKR is significantly higher in the synganglion, with reproductive tissues having lower relative expression. In contrast, in R. microplus partially fed females, the highest relative transcript expression is in reproductive tissues, followed by the synganglion [23]. These findings not only support a possible direct functional role for PK on the female reproductive tissue in ticks, but also, possibly, in a feeding-dependent manner. The quantity and quality of food intake are related to ovary development and egg maturation in insects [71, 72]. In the present work, the pre-oviposition and incubation periods were delayed, and the egg mass weight decreased after Rhimi-PKR silencing. These results could be associated to a decreased blood intake (Fig. 3b, c), or a possible reduction of PK activity in the reproductive tissues themselves, due to a decreased PKR expression and signaling in the latter.

In lepidopterans, diapause hormone (DH; a PK/PBAN family peptide) initiates embryonic diapause in the silkworm Bombyx mori Linnaeus [73, 74] however breaks pupal diapause in Heliothis and Helicoverpa spp. [18, 75–77]. Further, pupal diapause duration is modulated using DH analogs, both agonists and antagonists in Helicoverpa zea [78, 79]. The sequence WFGPRLα is the conserved C-terminal motif for the DH in insects [8]. None of the R. microplus predicted pyrokinins contain this exact sequence [25]. However, Suwan et al. [74] observed diapause induction in eggs after injecting B. mori pharate adults with DH-like peptides of varied length featuring the C-terminal motif FXPRLα (i.e. WFGPRLα and CFGPRLα), characteristic of the PK/PBAN/DH neuropeptide family [8]. The Rhimi-CAPA-PK2 is the only predicted PK from the cloned cDNA of R. microplus featuring the conserved motif in its sequence GTFVPRLα, ending in RLa while the other PKs end in RNA or RIα [25]. The Rhimi-CAPA-PK2 activates the recombinant R. microplus PK receptor with an EC50 of 188nM [30], therefore, it is expected to similarly activate the receptor in vivo. The identical sequence is present in R. sanguineus (Rhsa-CAPA-PK2), while this peptide in I. scapularis is GSFVPRLa, Ixosc-CAPA-PK2 [25]. After PK receptor loss of function in R. microplus females, we observed a delay in egg incubation period.

Several types of diapause have been observed in Prostratia and Metastratia ticks [80–83], including a delay of oogenesis in engorged females and a delay in the onset of embryogenesis in eggs [81]. Several factors are associated with the induction of developmental and behavioral diapause in ticks, such as daylight, temperature (high or low), starvation and humidity [80, 81]. In Ixodes ricinus
Linnaeus (Acari: Ixodidae). Belozerov observed that diapause induction in the unfed female was manifested as delayed embryogenesis within the eggs, rather than as delayed oogenesis [81]. In addition, a study conducted in D. silvarum, showed that egg masses laid by post-diapause females were significantly smaller than those laid by females that did not experience diapause [84], suggesting a correlation between female diapause and the reproductive output. PKR presents a similar expression pattern and a conserved myotropic functional activity in insects and ticks. In ticks, the PKR transcript is expressed in the female reproductive tissue [23, 24], and we observed a decrease in the weight of the female and egg masses, as well as delayed pre-oviposition and incubation periods after Rhimi-PKR silencing. Regarding hormonal control of diapause in ticks, the breaking of diapause in larvae of R. sanguineus and Dermacentor albipictus Packard (Acari: Ixodidae) was observed after ecdysteroid treatment [85, 86]. In addition, Khalil also suggested that the induction of female diapause in Argas arboresus Kaiser (Acari: Argasidae) is associated with a decrease in a gonadotropin hormone, which is regulated by an unknown hormone secreted by the synganglion [87]. Based in our results, a role for PK in the regulation of oviposition and/or embryonic development warrants exploration in R. microplus. CAPA-PK2 peptides ending in FXPRLa should be investigated for induction or breaking of tick diapause.

Rhimi-PKR silencing increased female mortality by 15% versus dsβ-lac-injected ticks (Fig. 3a). Previously, increased mortality in D. melanogaster [88] and the moths H. zeas and Heliotis virescens [89, 90] was reported following the application of PK/PBAN analogs and antagonists. The results on D. melanogaster [88] were obtained under stress conditions, and the authors suggested that the mortality could be associated with a deregulation of the water/ionic balance. PKR transcript expression has been detected in both the midgut-hindgut and Malpighian tubules in R. microplus and I. scapularis [23, 24]. Thus, PK could additionally have a role in fluid homeostasis regulation in ticks.

Conclusions

The RNAi results of the present study suggest that the PK signaling system is an attractive target for the use of PK antagonists for tick control because the loss of receptor transcript expression was detrimental to females. We had previously characterized the PKR from the cattle fever tick R. microplus [23]. Recently, we demonstrated the myotropic activity of PKs in feeding-related tissues of two tick species, R. sanguineus and I. scapularis [25], and with the present work, we took a further step in the characterization of the PKR transcript in ticks. These results support a role for PK neuropeptides throughout all stages of tick development and in the regulation of tick female feeding and reproduction. It appears that PKs may have pleiotropic functions in ticks, similar to what they have in insects.

Abbreviations

AUP: Animal use protocols; cDNA: Complementary DNA; CFTRL: Cattle fever tick research laboratory; DNase 1: Deoxyribonuclease 1; FC: Fold-change; GPCR: G-protein-coupled receptor; NF-water: Nuclease-free water; nt: Nucleotides; OD: Optical density; PK: Pyrokinin; PK/PBAN/DH: Pyrokinin/pheromone biosynthesis-activating neuropeptide/diapause hormone; PKR: Pyrokinin receptor; qRT-PCR: Quantitative reverse-transcriptase PCR; REI: Reproductive efficiency index; RNAi: RNA interference; RT: Room temperature; SD: Standard deviation; SEM: Standard error of the mean; SGIC: Subesophageal ganglion; USDA-ARS: United States Department of Agriculture-Agricultural Research Service; UTR: Untranslated region.

Supplementary Information

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Author contributions
PVP and KBT conceptualized the experiments. JPW, KBT, KGS, JPT, CX and PVP performed the experiments and JPW performed the statistical analyses and produced the figures. JPW and PVP wrote the manuscript, and JPW, KBT, and PVP reviewed and edited the manuscript. KTL edited the final version of the manuscript and provided administrative oversight of experiments at the CFTRL in Edinburg, TX. All authors read and approved the final manuscript.

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Availability of data and materials
All relevant data are provided in the manuscript.

Declarations

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Competing interests
The authors declare that there are no competing financial interests.

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