Periviscerokinin (Cap$_{2b}$; CAPA) receptor silencing in females of Rhipicephalus microplus reduces survival, weight and reproductive output

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Abstract

Background: The cattle fever tick, Rhipicephalus (Boophilus) microplus, is a vector of pathogens causative of babesiosis and anaplasmosis, both highly lethal bovine diseases that affect cattle worldwide. In Ecdysozoa, neuropeptides and their G-protein-coupled receptors play a critical integrative role in the regulation of all physiological processes. However, the physiological activity of many neuropeptides is still unknown in ticks. Periviscerokinins (CAP$_{2b}$/PVKs) are neuropeptides associated with myotropic and diuretic activities in insects. These peptides have been identified only in a few tick species, such as Ixodes ricinus, Ixodes scapularis and R. microplus, and their cognate receptor only characterized for the last two.

Methods: Expression of the periviscerokinin receptor (Rhimi-CAP$_{2b}$R) was investigated throughout the developmental stages of R. microplus and silenced by RNA interference (RNAi) in the females. In a first experiment, three double-stranded (ds) RNAs, named ds680-805, ds956-1109 and ds1102-1200, respectively, were tested in vivo. All three caused phenotypic effects, but only the last one was chosen for subsequent experiments. Resulting RNAi phenotypic variables were compared to those of negative controls, both non-injected and dsRNA beta-lactamase-injected ticks, and to positive controls injected with beta-actin dsRNA. Rhimi-CAP$_{2b}$R silencing was verified by quantitative reverse-transcriptase PCR in whole females and dissected tissues.

Results: Rhimi-CAP$_{2b}$R transcript expression was detected throughout all developmental stages. Rhimi-CAP$_{2b}$R silencing was associated with increased female mortality, decreased weight of surviving females and of egg masses, a delayed egg incubation period and decreased egg hatching ($P < 0.05$).

Conclusions: CAP$_{2b}$/PVKs appear to be associated with the regulation of female feeding, reproduction and survival. Since the Rhimi-CAP$_{2b}$R loss of function was detrimental to females, the discovery of antagonistic molecules of the CAP$_{2b}$/PVK signaling system should cause similar effects. Our results point to this signaling system as a promising target for tick control.

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Background

Ticks (Acari: Ixodidae) are vectors of pathogens causing diseases in humans and livestock worldwide [1]. Tick infestation also causes monetary losses by direct stress effects on domestic and farm animal health [2, 3]. Further, the prevalence of tick-borne diseases has increased recently in humans and livestock, and tick distribution is continually expanding due to direct and indirect human actions, such as livestock transportation and climate change [3, 4].

The cattle fever tick (CFT) or Asian blue tick, *Rhipicephalus (Boophilus) microplus*, known as the southern cattle tick in the USA, is the vector of pathogens causative of babesiosis and anaplasmosis, two highly lethal bovine diseases that affect cattle globally [5–7]. CFT control mainly focuses on pesticides that target the nervous system or that inhibit chitin deposition in the cuticle [8, 9]. However, the CFT populations worldwide have developed resistance to several pesticides, such as amidines (amitraz), organophosphates, pyrethroids, fluazuron and imidacloprid [10–13]. The lack of recombinant vaccines prevent deadly cattle babesiosis [14] reinforces the critical need to validate novel and selective pesticide targets to control this and other tick species.

In the Ecdysozoa, neuropeptides and their receptors play a critical integrative role regulating all physiological processes, such as feeding, excretion, molting, diapause and mating [15, 16]. Neuropeptides have a ubiquitous role in insects as well, and many of them display a pleiotropic function [17]. Therefore, these and their receptors (G protein-coupled receptors (GPCRs)), have been suggested as potential targets for pest control [18, 19]. Among other arthropods, neuropeptides and their receptors may similarly have integrative functions in ticks (Acari: Ixodidae) [20, 21]. However, little is known about their physiological functions in comparison to other groups, such as insects and crustaceans [17, 22], beyond a few neuropeptides and cognate GPCRs characterized in ticks, such as those described in [23–29].

The study of neuropeptides and their GPCRs in ticks has also been suggested as a strategy for discovering novel selective targets for tick control [20]. A few GPCRs for small neuropeptides have been investigated in *R. microplus* through RNA interference (RNAi), and the results suggested a role in feeding and reproduction [29, 30]. High-throughput screens have been used to discover small molecule antagonists of the tick kinin receptor with the goal of reproducing in vivo the effects observed after kinin receptor silencing by RNAi [29, 31]. The myotropic activity of pyrokinins in feeding-related tissues of two tick species, *Rhipicephalus sanguineus* and *Ixodes scapularis*, was recently demonstrated [32].

In the present study we focus on another receptor for a small neuropeptide, the periviscerokinin (CAPA/PVK) receptor of the southern cattle tick [25], for which the cognate ligand in insects is coded by the Capability gene (CAPA). The Capability/Pyrokinin (CAPA/PK) is an ancient endocrine signaling system in Ecdysozoa, and putatively homologous of the Vertebrata Neuromedin U system [33]. In insects, the ancestral CAPA/PK gene was duplicated and differentiated into two genes [34]; however, active peptides encoded under each gene can differ among species. In *Drosophila melanogaster*, the CAPA gene codes for two CAPA/PVKs, namely CAPA-PVK-1 and -2, both with a PRVamide ending [35], and for a single trypto-pyrokinin, CAPA-PK-3, with a C-terminal WFGPRL-amide ending [35], later designated CAPA pyrokinin Diapause Hormone 1 (DH-1) [34, 36]. The *Drosophila* hugin gene encodes only pyrokinins (PKs) [37]. A similar arrangement exists in *Tribolium castaneum*, where the CAPA gene codes for PKVs and the trypto-PK [38]. Regarding the insect CAPA-related peptide receptors, these have been characterized in several insect species (see, for example, [39–41]). The insect CAPA/PVK GPCR belongs to PRX-amide peptide receptor family [33].

Regarding CAPA/PVK function, in several insect species these peptides stimulate fluid secretion from Malpighian tubules [42–44] and act as antiuretic hormones [44, 45]. In *Aedes aegypti*, CAPA/PVKs display either diuretic or antiuretic activity in isolated Malpighian tubules, depending on their concentration [46]. However, antiuretic activity appears to be the main role of CAPA/PVKs in *Ae. aegypti*, having a potent inhibitory activity against diuretic hormones [47]. The myotropic activity of CAPA/PVKs was first discovered in *Manduca sexta* and has also been observed in several tissues of Blaberoidea and Blattidae cockroaches [48]. In roaches, CAPA/PVKs were initially detected from the abdominal neurohemal organs, designated perisympathetic organs (PSOs) or perivisceral organs, from which the name periviscerokinins derives. CAPA/PVKs are neurohormones released from PSOs into the hemolymph, and their hormonal
actions on different target tissues may differ between insect species; they may also be neuromodulators [49].

CAP_{2b}/PVKs have been identified in only a few tick species, such as *Ixodes rici

us*, *I. scapularis* and *R. microplus* [50–52]. In *R. microplus*, two CAP_{2b}/PVKs were identified in a cloned cDNA of the CAP_{2b}/PVK precursor, namely Rhimi-CAPA-PVK1 [52] and Rhimi-

CAPA-PVK2, with sequences pQGLIFPRVa and pQLVPVRNa, respectively [32]. CAPA-PVK2, which features the unusual PRN-amide C-terminal ending, was also observed in *R. sanguineus* and *I. scapularis* [32, 52]. In insects, the PRN-amide is an uncommon ending and has only been identified in Blattodea (e.g.: genera Deropeltis and Periplaneta), as reported in the DIneR neuropeptide insect database that analyzed 201 species and their 539 PVK corresponding sequences [53]. The tick CAP_{2b}/PVK receptor has been annotated in *I. scapularis* [51] and *R. microplus* [25], but the complementary DNA (cDNA) was cloned and functionally analyzed only for the second species [25]. In *R. microplus*, the CAP_{2b}/PVK receptor transcript was detected in synganglion, salivary gland, Malpighian tubules and the ovary of partially fed female ticks [25]. Regarding the physiological role of CAP_{2b}/PVKs in ticks, a myotropic activity was observed in the hindgut of *I. scapularis* [54]. The expression of the CAP_{2b}/PVK transcript receptor in Malpighian tubules and ovaries [25] may suggest a role in diuresis and reproduction, but these have not been studied.

We previously performed CAP_{2b}/PVK immunolocalization in the synganglion of *I. ricinus* with an anti-PVK2 from *Periplaneta americana* [50, 55] and have also demonstrated the functionality of the *R. microplus* CAP_{2b}/PVK receptor [25] and identified the cDNA of *R. microplus* CAP_{2b}/PVK precursor [52]. From this precursor we determined that the Rhimi-CAPA-PVK1 sequence pQGLIFPRVa, which activates the receptor at the nanomolar level (half maximal effective concentration \( EC_{50} = 64 \text{ nM} \)) [25], differs from the sequence previously identified by MALDI-TOF, PALIFPRVa, in their two N-terminal residues (highlighted in bold) [50] but identical to the *I. scapularis* PVK [25, 52]. In the present work, we used RNAi to investigate the role of the CAP_{2b}/PVK receptor in females of the cattle tick *R. microplus* (Rhimi-CAP_{2b}R).

**Methods**

**Tick rearing and animal care**

Ticks were reared at the Cattle Fever Tick (CFT) Research Laboratory (US Department of Agriculture-Agricultural Research Service [USDA-ARS]; Mission, TX, USA) under a cooperative agreement with Texas A&M AgriLife Research (College Station, TX, USA). Cattle used for tick production or gene silencing experiments were CFT-naive Hereford, Charolais or Angus, each weighing from 136 to 182 kg. Cattle were vaccinated, dewormed and acclimated for 2 weeks at the USDA-ARS Knipling-Bushland US Livestock Insects Research Laboratory (Kerrville, TX, USA) prior to shipment to the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL), a bio-secure 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 (AUPs). All procedures for handling and treating animals were approved by the Texas A&M University (TAMU) Institutional Animal Care and Use Committee (IACUC) (TAMU AUP 2019-0197 EX under PVP, referring to IACUC USDA-ARS approved AUP 2021-12). Ticks used in this study were obtained from colonies of *R. microplus* acaricide-susceptible Deutch strain established from ticks collected in 2001 from an outbreak in Webb County, Texas, USA [56]. Filial generations F65, F68 and F80 were used for the RNAi experiments performed on January 2018, December 2019 and July 2021, respectively.

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

Prior to RNA extraction, whole female ticks and tissues were disrupted using a Omni Bead Ruptor 12 homogenizer (Omni International, Inc., Waterbury, CT, USA) with equal proportions of 1.4- and 2.8-mm ceramic beads. Disruption of soft tissues, such as the female reproductive system, synganglion, Malpighian tubules and salivary glands, was for 1 min, and of the whole tick and carcass for 3 min, all at 5.65 Hz (m/s). Total RNA was extracted using the Zymo Quick-RNA™ Microprep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer’s specifications. Two DNase1 (Deoxyribonuclease 1) steps were conducted: (i) during RNA extraction 30 U of DNase1 was added to the sample prior to 15 min of incubation at room temperature (RT); and (ii) after solubilization of the sample in nuclease-free water (NF-water) 5 U of DNase1 was added and incubation followed for 15 min at RT. After DNase1 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. The reaction consisted of 150–200 ng of total RNA as template, 1 µl oligo-dT20 (50 µM) and 1 µl random hexamers (50 ng/µl) in 22 µl of final volume, and was performed using the SuperScript™ III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's
The stages of development were as follows: eggs, neolarvae, nymphs, and adults. All ticks collected for the analyses were unfed, equal to first instar larva on the host [59], nymphs and adults. The development stages were analyzed by qRT-PCR.

**Quantitative reverse-transcriptase PCR analyses for evaluation of expression and silencing**

Quantitative reverse-transcriptase PCR (qRT-PCR) was performed in a reaction volume of 10 µl containing 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 the qRT-PCR cycles consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. All oligonucleotide primers (Table 1) used for qRT-PCR were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

The reference genes selected for the qRT-PCR analysis had been previously tested on *R. microplus* on the same tissues and physiological conditions (3- to 5-day-old partially engorged females) [27, 29, 30]. These reference genes were elongation factor 1 alpha (*Rhimi-EF1A*; EW679365.1) and ribosomal protein S4 (*Rhimi-RPS4; CV436347*). The normalized relative quantity (NRQ) with respect to these reference genes was calculated for *Rhimi-CAP2bR* following the formulas in [58]. The NRQ values were subsequently utilized for estimation of fold change as ratios between the silenced ticks and controls (see section Tissue collection, RNA extraction, cDNA synthesis and qRT-PCR for evaluation of gene silencing).

**Rhimi-CAP2bR relative expression throughout different stages of development**

*Rhimi-CAP2bR* relative expression throughout different stages of development was analyzed by qRT-PCR. The development stages were as follows: eggs, neolarvae, equal to first instar larva on the host [59], nymphs and adults. All ticks collected for the analyses were unfed, flash frozen within 24 h of emergence and kept in 500 µl of RNAlater™ (Invitrogen, Thermo Fisher Scientific) at −80 °C until use. Nymphs and adults were obtained from two patches on a Hereford calf; each patch had been infested with approximately 250 mg of tick neolarvae. Engorged larvae were removed from the calf 6–7 days after infestation and allowed to molt in an environmental chamber at 25 ± 2 °C and a relative humidity (RH) of 95% [60]. Newly molted nymphs were placed on the animal and then removed as engorged nymphs 13–14 days post-infestation. Nymphs were placed in an environmental chamber maintained at the same temperature and RH as stated above until ecdysis. There were eight biological replicates (*n* = 8) per developmental stage and the replicates for each stage were as follows: (i) egg masses (approx. 100 mg [11.6 mg ± 0.9 mg] per replicate, mixed between 3 to 6 days after oviposition); (ii) neolarvae (10 whole bodies pooled per replicate); (iii) nymphs (5 whole bodies pooled per replicate); (iv) male (unmated, 2 whole bodies pooled per replicate); and (v) female (unmated, 1 whole body per replicate).

All ticks (F81) were kept under the same conditions as described in section Tick rearing and animal care. Total RNA extraction, cDNA synthesis, qRT-PCR conditions, reference genes and target gene (*Rhimi-CAP2bR*) were the same as those used for RNA isolation, cDNA synthesis and qRT-PCR analysis (see relevant sections), and for qRT-PCR analyses for evaluation of expression and silencing.

**Rhimi-CAP2bR gene silencing by RNAi**

*Synthesis of double-stranded RNAs for RNAi*

To obtain cDNA as template to synthesize double-stranded RNA (dsRNA) for silencing experiments, the sequence of the cloned cDNA was first amplified by PCR, followed by amplification of the 5′-untranslated region (UTR) by PCR and cloning. These two cDNA amplifications were performed with different primers and using independently synthesized cDNAs. The steps followed are detailed below.

To amplify the receptor cloned cDNA, total RNA was extracted from *R. microplus* Deutch strain whole unfed females, using the ZR Tissue & Insect RNA MicroPrep™ Kit (Zymo Research), following the manufacturer’s specifications. The extracted RNA was used for cDNA synthesis in a total reaction volume of 20 µl containing 1 µg of RNA as template and 1 µl oligo-dT20 (50 µM) using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific) and following the manufacturer’s specifications. Subsequently, *Rhimi-CAP2bR* cDNA sequence amplification was conducted by PCR, using the AmpliTaq Gold™ Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer’s specifications. For the PCR, specific primers were designed based on the sequence of the *Rhimi-CAP2bR* cloned cDNA KC614697.1 [25] (Table 1). The PCR template reaction was carried out using a dilution of the synthesized cDNA (1:10), at the following temperature-cycling parameters: 1 cycle at 95 °C for 5 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 1 cycle at 72 °C for 5 min. PCR products were cloned with the TOPO XL-2 PCR Cloning kit (Invitrogen, Thermo Fisher Scientific) and then sequenced. Correct PCR fragment
insertion was confirmed for clones #4 and #6, both carrying an insert of 1537 bp, which encompasses 349 bp of the 5′-UTR end of KC614697.1, the open reading frame and 349 bp of the 3′-UTR end (Additional file 1: Data S1).

To obtain a longer 5′-UTR sequence than that originally obtained (KC614697.1), the Rapid amplification of cDNA ends (RACE) SMARTer kit (Takara, Kusatsu, Shiga, Japan) was used, following the manufacturer’s instructions as laid down in the SMARTer RACE 5′/3′ Kit User Manual. To synthesize the 5′-RACE-Ready first-strand cDNA, total RNA was first extracted from *R. microplus* Deutch strain whole unfed females using the same methods and kit as described above. The reaction was carried out in a total reaction volume of 20 μl containing 1 μg total RNA; the temperature-cycling parameters for cdNA synthesis were: 1 cycle at 72°C for 3 min; 1 cycle at 42 °C for 2 min; 1 cycle at 42 °C for 90 min; and 1 cycle at 70 °C for 10 min. To extend the 5′-UTR region, a RACE PCR was run using 2.5 μl of 5′-RACE-Ready first-strand cDNA, the SMARTer RACE 5′/3′ Kit buffers and master mix and gene-specific primers and universal and nested primers (Table 1). The temperature-cycling parameters for the RACE PCR were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min; 25 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min. The obtained 5′-UTR-RACE PCR

### Table 1

| Primer name          | Oligo sequence 5′- 3′ | Notes                                |
|----------------------|------------------------|--------------------------------------|
| RmPerivis-200U19     | ATCTGCTGTGGCGCAGACAGT  | Rhimi-CAP$_{2b}$R PCR               |
| RmPerivis-1716L21    | GGCAGGTGGTGCGGATGCTG   | Rhimi-CAP$_{2b}$R PCR               |
| RmPerivis-388L18     | CCGTATGGTGCGGCTTGTC    | S′-RACE-PCR                          |
| RmPerivis-195L20     | GTCCGCAAGCCGAGTATCC    | S′-RACE-PCR                          |
| Long-Univ.-RACE     | CTAATAGCAGCTACTATAGAGGCAGATGTTA   | RACE-PCR                             |
| Short-Univ.-RACE    | CTAATACGAGCTACTATAGAGGCGGACGT   | RACE-PCR                             |
| RmPerivisR-680U20    | CCGCGCTTCAAGCTGACGTTG  | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-805L20    | CCGTCCACCATGACCTTTA    | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-680U20-T7 | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-805L20-T7 | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-956L20    | GTGAGGGTTTCCGGCTGAGAGA | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1109L20   | CCGTCCACATGTCGGCAACA   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-956U20-T7 | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1109L20-T7| AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1102U20   | TATCGGCTGCGCGCACTGGT  | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1200L20   | CAGCCCGCCAAGAATATCC   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1102U20-T7| AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| RmPerivisR-1200L20-T7| AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-CAP$_{2b}$R dsRNA synthesis   |
| Amp-fwd               | CGCTGTGTAAGTAAATATG   | beta-lactamase dsRNA synthesis [29]   |
| Amp-rev               | GCGGGAGAAACTAAGTAAATG | beta-lactamase dsRNA synthesis [29]   |
| Amp-T7                | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | beta-lactamase dsRNA synthesis [29]   |
| Amp-T7                | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | beta-lactamase dsRNA synthesis [29]   |
| BmAActin-1U20         | TCTCGGCTTCCGGGAAGACT  | Rhimi-ACTB dsRNA synthesis [29]      |
| BmAActin-285L18       | GGGGGAGGCAGGCTAGTCC   | Rhimi-ACTB dsRNA synthesis [29]      |
| BmAActin-1U20-T7      | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-ACTB dsRNA synthesis [29]      |
| BmAActin-285L18-T7    | AAAGCGCTTAAATACGACTCTAGATAGGCCGCTTGAGCTGTAGTCGATGTG   | Rhimi-ACTB dsRNA synthesis [29]      |
| RmCAP2b-qF1          | ATGGCGGCCTCCCATCAT    | Rhimi-CAP$_{2b}$R qRT-PCR [25]       |
| RmCAP2b-qR1          | AATGCGGCTTCCGGTAGTGC  | Rhimi-CAP$_{2b}$R qRT-PCR [25]       |
| BmELF1a-88-F         | CGTCTACAAAGTTGGCTGCTAGT | Rhimi-EF1A qRT-PCR [57]          |
| BmELF1a-196-R        | CTAGTAGCTTCCGGTAGTGC  | Rhimi-EF1A qRT-PCR [57]          |
| RmRPS4-qF1           | TCTACGCTTGAGCCTGACCA  | Rhimi-RPS4 qRT-PCR [27]             |
| RmRPS4-qR1           | ACGCGGCGAGCTCCTTACTC | Rhimi-RPS4 qRT-PCR [27]             |

cdNA Complementary DNA, dsDNA double-stranded DNA, qRT-PCR quantitative reverse-transcriptase PCR, RACE rapid amplification of cdNA ends, Rhimi-CAP$_{2b}$R *Rhipicephalus microplus* periviscerokinin receptor

* Sequences in italics were added to the primers and are not part of the tick cdNA sequences
fragment was also cloned with the TOPO XL-2 cloning kit and sequenced (Additional file 1: Data S1). This sequence was submitted to the GenBank database under accession number OP191701.

Target sequences for RNAi of 150–250 nucleotides (nt) in length were selected from the 5′-UTR of Rhimi-CAP2bR cloned cDNA KC614697.1, [25] and from the R. microplus periviscerokinin receptor 5′-UTR-RACE fragment extended sequence obtained as described above (Additional file 2: Data S2). After the release of the R. microplus genome, further analysis became possible to test the specificity of the designed Rhimi-CAP2bR microplus genome, further analysis became possible to test the specificity of the designed Rhimi-CAP2bR dsRNAs, to avoid potential off-target RNAi effects. To this end, for each Rhimi-CAP2bR 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) (Additional file 3: Data S3).

dsRNAs for the in vivo gene silencing experiments were synthesized following the manufacturer’s instructions, using the T7 RiboMAX™ Express RNAi system (Promega, Madison, WI, USA) and cDNA from whole unfed females as template. Oligonucleotide primer sequences used for dsRNA synthesis (Table 1) were designed using the Oligo v6.71 Primer analysis software www.oligo.net (Molecular Biology Insights, Inc., DBA Oligo, Inc., Colorado Springs, 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 of dsRNA synthesized was determined using a Nanodrop 1000 spectrophotometer using optical density A260/A280 ratios (Thermo Fisher Scientific). The dsRNA sequences for the negative (beta-lactamase) and positive [R. microplus beta-actin gene (Rhimi-ACTB)] dsRNA controls were successfully used for our recently published study [30]. The primers used for the synthesis of the control dsRNAs are listed in Table 1. The beta-lactamase gene (BLA) encodes a bacterial enzyme that is not present in ticks. The beta-actin gene (ACTB) encodes for a protein involved in cell motility, structure and integrity [61].

Experimental design and microinjection of ticks
For the RNAi experiments, unfed CFT adult females were collected at between 1 and 5 days after molt from nymphs and used for each treatment. Ticks from each treatment were held in round, cotton sleeves that were glued to the backs of calves [29, 62]. Each sleeve contained 35–40 females and 15–25 males to allow mating. The sleeves were distributed symmetrically on both sides of the animal, and each animal was considered to be a randomized block. Five calves were used for these RNAi experiments.

Five to seven independent RNAi experiments were performed per treatment, over three different dates: January 2018, December 2019 and July 2021. Each replicate consisted of dsRNA-injected ticks and non-injected ticks (as the negative control). Female ticks were microinjected with dsRNA specific for each treatment: (i) dsRNAs for Rhimi-CAP2bR, designated ds680-805 (196 bp), ds956-1109 (172 bp) and ds1102-1200 (132 bp); (ii) Rhimi-ACTB as positive controls (dsβ-act); and (iii) beta-lactamase as negative controls (dsβ-lac). For the first assay (January 2018), ticks were injected with 10^{11}–10^{12} dsRNA molecules/tick in 0.2 µl of NF-water. For the assays conducted in December 2019 and July 2021, dsRNA microinjections consisted of 0.2 µl of a solution prepared using NF-water and dsRNAs at concentrations ranging from 5.21 to 8.27 µg/µl, with the exception of dsRNA-Rhimi-ACTB, whose concentration ranged from 5.6 to 9.3 µg/µl (Additional file 4: Table S1). The methods described for tick micro-injection were used in all assays and have been previously described [63]. Injected ticks were transferred and held in cotton-stoppered glass vials in the laboratory under constant temperature (25 ± 2 °C) and 95% RH for 24 h [64]. Subsequently, live (obviously motile) injected and non-injected females were transferred to the sleeves on the calf.

The delay between the second and third replicate was due to the SARS-COVID-19 pandemic causing the closure of the facilities for animal experimentation at the USDA-ARS facility, Edinburg, Texas. Experiments described herein were conducted in parallel with those described in a previous publication [30].

Tissue collection, RNA extraction, cDNA synthesis and qRT-PCR for evaluation of gene silencing
To evaluate Rhimi-CAP2bR silencing after dsRNA injections, three tick females per treatment and replicate were collected on the third and fifth day after being placed on the animal. Females that fed for 3 days were individually kept in 250 µl of RNAlater™ (Invitrogen) at −80 °C until use. Whole ticks were used to test silencing at the organism level. In contrast, females that had been 5 days on the animal, were dissected under cold physiological saline and tissues were individually kept at −80 °C until RNA isolation. RNAlater™ was used to keep each carcass (250 µl), salivary glands, Malpighian tubules and the complete female reproductive system that included ovaries, uterus, vagina and associated glands (150 µl). Synganglion was immersed directly in 50 µl of Trizol™ reagent (Invitrogen, Thermo Fisher Scientific) to
improve RNA extraction, since the synganglion is a very small tissue that becomes fully transparent in RNAlater™ and cannot be visualized for recovery. Tissues selected to analyze the silencing efficiency were those in which a relative high expression for Rhimi-CAP2bR transcript had been previously reported in R. microplus for partially fed females of similar age [25].

To evaluate the effect of dsRNAs through the analysis of relative gene expression, we used the protocols for total RNA extraction, CDNA synthesis and qRT-PCR analysis and the sequences of the primers described in sections RNA isolation, cDNA synthesis and quantitative reverse-transcriptase PCR analysis and Quantitative reverse-transcriptase PCR analyses for evaluation of expression and silencing.

For the first experiment, performed January 2018, only whole ticks “3 days on the animal” were analyzed to verify gene silencing of ticks that had been injected with the three Rhimi-CAP2bR dsRNAs: dsRNA680-805, ds956-1109 and ds1102-1200. The cDNA samples for non-injected ticks, beta lactamase-injected-ticks and ticks injected with each of the mentioned dsRNAs were arranged on the same plate and run in duplicate. Three such 96-well plates were run. Two of the plates were used to analyze relative expression using primers for each reference gene, Rhimi-EF1A and Rhimi-RSP4, respectively, and the third plate was used to analyze relative expression of the gene of interest, Rhimi-CAP2bR, using primers for this gene.

For each of the other four replicates, December 2019 (1 replicate) and July 2021 (3 replicates), three 96-well plates were similarly loaded with cDNA samples for qRT-PCR analyses: two of these plates were used to analyze relative expression using primers for each reference gene, Rhimi-EF1A and Rhimi-RSP4, respectively, and the third plate was used to analyzed relative expression of the gene of interest, Rhimi-CAP2bR, using primers for this gene. All plates were prepared with the same arrangement: the specific tissue cDNAs for all treatments, that is cDNAs of non-injected ticks, Rhimi-CAP2bR dsRNA1102-1200-injected ticks and beta-lactamase dsRNA-injected ticks were loaded, as the statistical comparisons were among treatments. Each of the following tissues were analyzed: (i) whole ticks 3 days on the animal and (ii) tissues from ticks that had fed on animals for 5 days, including carcases, female reproductive system, synganglion, salivary glands and Malpighian tubules. In sum, three plates were prepared for each tissue.

The gene normalized relative quantity (NRQ) was calculated for Rhimi-CAP2bR following the protocol described in section Quantitative reverse-transcriptase PCR analyses for evaluation of expression and silencing. The relative transcript abundance of Rhimi-CAP2bR for each set was presented as a fold-change (FC) of beta-lactamase-injected ticks (FC=1).

**Phenotypic data collection**

To record daily tick feeding progression after ticks were placed on the animals, the sleeves were opened and ticks were photographed (Additional file 5: Table S2). The date of self-detachment from the host was recorded, and the period of time from attachment to self-detachment was designated “repletion period”. Self-detached ticks were removed, weighed, transferred to a cotton-stoppered glass vial and held at the laboratory maintenance conditions previously mentioned, with daily monitoring.

The date of the first oviposition was registered to calculate the pre-oviposition period, which extends from detachment to oviposition [60]. CFT females die after oviposition [59]; therefore, after the egg laying was finished, the dead female tick was removed from the vial and the egg masses were weighed. The egg mass incubation period was also registered, which corresponds to the period from egg laying to the first hatch. The percentage egg hatch per female (i.e. eggs that hatched per egg mass) was determined by visual estimation by the same experienced researcher (JPT) for the duration of each experiment, as previously described in [65].

Results for other phenotypic variables were recorded for each female, such as mortality, which was registered over the course of the whole experiment. The reproductive efficiency index (REI) was calculated as: (egg mass/replete female weight) × 100, according to [66]. Further, the number of females with no eggs was calculated from the number of females that survived and were placed in vials that did not lay eggs at all, and “females with no hatch,” which reflects the percentage of produced egg masses from which no egg hatching was observed at all. Finally, the overall time period from attachment to animal until hatching of the first egg for each female tick was also recorded as observation period.

As per the approved AUP that assures maintenance of animal health and taking into account an expected detachment time from the host for un.injected ticks to be between 7 and 9 days, an endpoint for the experiment of 2 weeks after tick attachment to the animals was chosen. Ticks that did not detach before this date were considered to be dead and were not included in subsequent analyses.

**Statistical analysis**

Statistical analyses of phenotypic traits and qRT-PCR assays were conducted using GraphPad Prism v6 software (GraphPad Software, San Diego, CA, USA), and the results were plotted using the same software. A one-way analysis of variance (ANOVA) followed by a Tukey’s HSD
test was performed to compare Rhimi-CAP2bR relative expression throughout all developmental stages, and to verify Rhimi-CAP2bR silencing in whole ticks and different female tissues. A Kruskal–Wallis ANOVA followed by a corrected Dunn’s multiple comparisons test was used to analyze phenotypic data. All results were presented as the mean ± standard error of the mean (SEM).

Results

Rhimi-CAP2bR expression throughout developmental stages

The qRT-PCR analyses detected the Rhimi-CAP2bR transcript throughout all developmental stages of R. microplus (Fig. 1). The lowest relative expression of Rhimi-CAP2bR was observed for eggs (Fig. 1), followed in decreasing relative expression for larvae, females and males, with Rhimi-CAP2bR relative abundance in these last three stages being similar and surpassing Rhimi-CAP2bR expression in eggs by a factor of 3.2–4.4 (n = 8 replicates; P < 0.05) (Fig. 1). The highest Rhimi-CAP2bR relative expression was observed for nymphs (Fig. 1), which surpassed Rhimi-CAP2bR expression in eggs by a factor of 6.8 (n = 8 replicates; P < 0.05) (Fig. 1). Relative abundance in eggs was used as the reference ratio (calibrator) for the FC calculation.

Rhimi-CAP2bR silencing

For the RNAi experiments, three Rhimi-CAP2bR dsRNAs, named ds680-805, ds956-1109 and ds1102-1200, were tested in vivo in the first assay performed on January 2018 (Fig. 2). BLASTn searches conducted to assess the risk of non-target effects only identified two sequences of ≤ 15 nt with identity to Rhimi-CAP2bR dsRNA956-1109; no such sequences were identified in the dsRNA1102-1200, which was then chosen for subsequent assays (Additional file 3: Data S3).

The present work was conducted in parallel with another study that had been published earlier [30], and the results for the negative controls [non-injected and beta-lactamase-injected females (dsβ-lac)] and positive controls (Rhimi-ACTB dsRNA-injected females [dsβ-act]) were shared among studies to minimize the number of calves used. Importantly, all of the RNAi treatments evaluated herein were in the same animals as the negative control treatments as a block, thereby conserving statistical validity. Silencing efficiencies of Rhimi-CAP2bR dsRNAs are reported with respect to dsβ-lac-injected ticks.

For the first RNAi experiment in January 2018, qRT-PCR analyses showed that the three tested dsRNAs, namely ds680-805, ds956-1109 and ds1102-1200, were equally significantly effective, producing respectively 73, 79 and 76% knockdown of Rhimi-CAP2bR mRNA in whole ticks (n = 3–9, P < 0.05; Fig. 2a). The two negative controls did not differ in terms of relative receptor transcript abundance, as expected (Fig. 2a). For subsequent experiments, performed December 2019 and July 2021, only ds1102-1200 was chosen for RNAi studies because it caused higher mortality than ds680-805, had a similar effect on mortality as ds956-1109 and, further, no identical sequence was found in the genome (Fig. 2b). Mortality after treatment with ds680-805 did not differ from the controls (Fig. 2b). qRT-PCR analyses showed that injections with ds1102-1200 caused a mRNA Rhimi-CAP2bR decrease of 74% (average for the 4 replicates) in whole ticks that were 3 days on the animal, consistent with the result of the first 2018 experiment (Fig. 3a). Females injected with ds1102-1200 that were dissected after 5 days on the animal also showed a decrease in Rhimi-CAP2bR transcript in the carcass, female reproductive system and synganglion of 81%, 95% and 75%, respectively (n = 12, P < 0.05; Fig. 3b–d). These females showed a decrease in Rhimi-CAP2bR transcript expression in salivary glands and Malpighian tubules of 81% and 77%, respectively (n = 12, P < 0.05; Fig. 3e, f). All tissues from silenced females were significantly different from the negative controls in relative receptor transcript abundance (Fig. 3).

Phenotypic changes associated to Rhimi-CAP2bR silencing

After Rhimi-CAP2bR silencing was verified by qRT-PCR analyses (Figs. 2a, 3), the phenotypic traits of the ticks under each treatment were evaluated, and results are summarized in Figs. 4 and 5. Tick pictures representing each treatment effect after 5, 7 and 9 days on the calves are shown in Fig. 4. These images reflect the RNAi effects on the phenotypic traits quantitatively evaluated in Fig. 5, and provide further visual evidence of the differential feeding progression among treatments. Many of the non-injected ticks (NI) and dsβ-lac-injected ticks fed to repletion between days 5 and 7, and by day 9 most of them had dropped from the animal (see vacant, yellow circles in Fig. 4). These records accurately reflect the significant Rhimi-CAP2bR dsRNA treatment effect in reducing female weight relative to the negative controls by the endpoint of the experiment, but these size differences resulted under a similar repletion period (Figs. 4, 5b, c). A detailed record of ticks feeding progression on calves is shown in Additional file 5: Table S2.

For the January 2018 RNAi experiment, female ticks injected with ds956-1109 and ds1102-1200 showed a significant increase in net mortality relative to the dsβ-lac-injected ticks (negative controls) of 31.79% and 23.25%, respectively (n = 30–92; P < 0.05), (Fig. 2b). Non-injected ticks and dsβ-lac-injected ticks had lower and similar mortalities (40% and 55%, respectively) than the Rhimi-CAP2bR dsRNA-treated ticks (Fig. 2b). For the
December 2019 and July 2021 RNAi experiments, where only ds1102-1200 was evaluated, Rhimi-CAP$_{2b}$$R$-silenced females showed a significant increased net mortality of 28% with respect to the dsβ-lac-injected ticks ($n$ = 140–216; $P < 0.05$) (Fig. 5a). Similarly, as found earlier, the negative control ticks had a similar and lower mortality than receptor-silenced ticks, 34% for non-injected ticks and 40% for dsβ-lac-injected ticks (Fig. 5a).

For the December 2019 and July 2021 RNAi experiments, additional phenotypic traits were analyzed. Rhimi-CAP$_{2b}$$R$-silenced females showed a significant decrease in net weight of 13% ($n$ = 75–135; $P < 0.05$) (Fig. 5c) and significant decrease in net weight of their egg masses of 15% ($n$ = 65–131; $P < 0.05$) (Fig. 5e), both relative to dsβ-lac-injected females. The egg incubation period was significantly longer by 1.6 days ($n$ = 59–120; $P < 0.05$) relative to dsβ-lac-injected females (Fig. 5h), and significant differences were not detected between the negative control treatments for both traits (Fig. 5c, e, h). However, for the overall observation period, encompassing the female feeding period until the day of first egg hatching, there was no difference between Rhimi-CAP$_{2b}$$R$-silenced ticks and dsβ-lac-injected females ($n$ = 56–120; $P > 0.05$) (Fig. 5k).

The egg hatching was also affected by Rhimi-CAP$_{2b}$$R$ silencing, and females injected with ds1102-1200 showed a net decrease of 20% egg hatching ($n$ = 58–108; $P < 0.05$) with respect to dsβ-lac-injected females (Fig. 5i).

Similarly, there were no differences between the negative control treatments for both traits.

Among the traits analyzed, there were no other phenotypic changes after Rhimi-CAP$_{2b}$$R$ silencing, in comparison to the negative controls. The repletion ($n$ = 71–135; $P > 0.05$) and pre-oviposition ($n$ = 64–131; $P > 0.05$) periods showed no difference between Rhimi-CAP$_{2b}$$R$-silenced ticks and negative controls (Fig. 5b, d). The REI, which reflects the percentage of conversion of female weight to egg mass ($n$ = 64–131; $P > 0.05$), showed no difference between Rhimi-CAP$_{2b}$$R$-silenced ticks and negative controls (Fig. 5f). Regarding the number of females with no eggs (live females that did not lay), no significant
Fig. 3  *Rhimi-CAP*$_{26}$R RNAi silencing using the dsRNA ds1102-1200. *Rhimi-CAP*$_{26}$R silencing was evaluated by qRT-PCR analyses of partially fed females from all treatments. a Whole tick, 3 days on animals, b carcass, c female reproductive system, d synganglion, e salivary glands, f Malpighian tubules. All tissues (b–e) were from female ticks 5 days on calves. Different lowercase letters above graph indicate significant difference at $P < 0.05$), based on statistical analysis using one-way ANOVA followed by a Tukey’s multiple comparisons test. Twelve ($n = 12$) biological replicates were analyzed per tissue and treatment.
statistical differences were observed ($n = 67–136; P > 0.05$) among the treatments (Fig. 5g). Finally, the number of egg masses that showed no hatch at all (females with no hatch) ($n = 64–133; P > 0.05$) was not statistically different between Rhimi-$\text{CAP}_{2bR}$-silenced ticks and negative controls (Fig. 5j).

**Discussion**

Periviscerokinins ($\text{CAP}_{2b}/\text{PVKs}$) are neuropeptides that elicit myotropic activity and diuretic control in insects [42–45]. In ticks, only the myotropic activity of the $\text{CAP}_{2b}/\text{PVKs}$ has been confirmed to date [54]. In the CFT, the $\text{CAP}_{2b}/\text{PVK}$ transcript receptor has been detected in the synganglion, salivary gland and Malpighian tubules and in the reproductive system of the female ticks [25].

We detected $\text{CAP}_{2b}/\text{PVK}$ receptor transcript expression in whole ticks in all developmental stages and in eggs, fulfilling the requirement of a broad developmental expression for a potential suitable target for tick control (Fig. 1). This expression pattern in ticks agrees with previous observations in insects, as transcript expression for the $\text{CAP}_{2b}/\text{PVK}$ receptor was observed in eggs.
of Halyomorpha halys [67] and Bombyx mori [68]. Also, expression of the CAP2b/PVK peptide precursor transcript was detected in eggs of H. halys [69], Solenopsis invicta [70] and Locusta migratoria [71]. One potential role of the periviscerokinin (CAP2b/PVKs) system in embryos of ticks could be control of myotropic activity, since muscle contraction was observed in embryos of the coleopteran insect Callosobruchus maculatus [72], but

Fig. 5 (See legend on previous page.)
no peptides associated with the regulation of this process have been identified to date. Another known function of the \textit{CAP2b}/PVK system in insects is diuretic; however, Malpighian tubules are under a process of cell proliferation and differentiation during embryonic development, and these are not fully functional until the larval stage (see, for example, [73, 74]); therefore, a function of the \textit{CAP2b}/PVK receptor in embryo Malpighian tubules is less likely.

Here, we used a proven RNAi protocol for the silencing of the CFT \textit{CAP2b}/PVK receptor (\textit{Rhimi-CAP2bR}) to investigate the \textit{CAP2b}/PVK signaling system in this tick species. This protocol was previously used successfully to silence two other tick GPCRs, namely the kinin and pyrokinin receptors [29, 30]. dsRNAs ranging from 132 to 196 bp were designed targeting the 5’-UTR region, since there is evidence of the advantage of choosing those regions over the coding sequence region, leading to higher efficacy and efficiency [75]. Possible off-target effects of the chosen \textit{Rhimi-CAP2bR} dsRNAs are highly unlikely because none was found for dsRNA1102-1200 and the maximum two contiguous identical sequences in \textit{Rhimi-CAP2bR} dsRNA956-1109 were 15 bp (Additional file 3: Data S3). These alignments showed that the similarities between the compared sequences were not enough to trigger off-target effects, according to previous studies that concluded that 19–21 nt of a contiguous identical sequence are required to produce significant silencing activity [75, 76]. Positive control ticks silenced for beta-actin were used in this experiment because they represent an extreme phenotypic and physiological effect of RNAi which allows relative comparisons of effects of different GPCRs against this extreme phenotype (see Figs. 2b, 4, 5a).

We will speculate next on the potential roles that the \textit{CAP2b}/PVK system may have in ticks, as suggested by qRT-PCR analyses, observed phenotypes upon silencing the \textit{Rhimi-CAP2bR} and the known PRX-amide peptide functions in insects. The following hypotheses will require testing in ticks.

After the \textit{Rhimi-CAP2bR} silencing, we observed a decreased female weight (Fig. 5c), as evidenced by a smaller size in photographs documenting the experiment (Fig. 4), which was obvious in comparisons on day 7 in dsβ-lac- versus \textit{Rhimi-CAP2bR} dsRNA-injected females. Our results suggest a direct or indirect role of the \textit{CAP2b}/PVK signaling system in the regulation of female feeding and growth in \textit{R. microplus}. However, no differences were observed on the duration of the repletion period between treated and control ticks (Fig. 5b). These results differ from previous observations on RNAi of GPCRs associated with female feeding in \textit{R. microplus}, in which after silencing of the kinin and pyrokinin receptors, there was a simultaneous decrease in female weight and extension of the repletion period [29, 30].

Ticks are pool feeders, and the feeding process involves penetration of the host skin by the hypostome, retraction of the cheliceral shafts and lateral movement of the cheliceral digits to introduce the feeding apparatus inside of the host [77]. Then, the blood is sucked into the food canal and pharynx by contraction of the dilator muscles of the pharynx, and relaxation of these muscles allows the blood to pass through the esophagus into the midgut [78]. Precise contraction of the feeding-related muscles represents a key aspect of this process. Recently, we demonstrated the myotropic activity of pyrokinins (PKs) in feeding-related tissues of two tick species, \textit{R. sanguineus} and \textit{I. scapularis} [32]. Further, myotropic activity was confirmed in the hindgut of \textit{I. scapularis} for \textit{CAP2b}/PVKs [54] utilizing the CAPA-PVK1 peptide PALIPFPRV as reported in [50]. The cDNA we previously cloned from \textit{R. microplus} includes PKs and two \textit{CAP2b}/PVKs [32, 52]; this co-expression could suggest a role of the \textit{CAP2b}/PVK signaling system on the myotropic activity of feeding-related tissues, as shown for PKs; however, the myotropic activity of \textit{CAP2b}/PVKs on feeding-related tissues has not yet been investigated. Alternatively or simultaneously, the silencing of the \textit{Rhimi-CAP2bR} could have diminished hindgut contractions, negatively affecting excretion [54].

Neupert et al. [50] co-immunolocalized kinin (LK) and \textit{CAP2b}/PVK in several neurons in the synganglion of ticks \textit{I. ricinus} and \textit{R. microplus}. The cheliceral ganglion integrates the supraesophageal region of the synganglion. The cheliceral nerve projects from the cheliceral ganglion and innervates the chelicerae [79]. Only LK-positive immunostaining was observed in the T1 neurons located in the anterior cheliceral ganglion [50]. Further, nerves from the supraesophageal area innervate salivary glands, the pharynx and the esophagus. Neuronal arborizations showing \textit{CAP2b}/PVK-positive staining were observed in the supraesophageal region [50]. Consequently, the \textit{CAP2b}/PVK signaling system could be associated with the feeding process in conjunction with the LK system. The \textit{CAP2b}/PVK receptor transcript has been found to be expressed in the salivary glands of \textit{R. microplus} [25]; the relative transcript expression level was similar to that in other tissues, such as the synganglion, ovary and Malpighian tubules [25]. Tick salivary secretions are injected into the feeding site to modulate the host’s hemostasis and immune response, allowing for a long feeding period [80]. This could suggest that the \textit{CAP2b}/PVK signaling system could be associated with the control of salivary gland function.

After the \textit{Rhimi-CAP2bR} silencing, the female reproductive output was affected through a decreased egg mass weight (Fig. 5e), a longer egg incubation period
(Fig. 5h) and a lower percentage of egg hatch, in comparison to the dsβ-lac-injected ticks (Fig. 5i). In ticks, the magnitude of female engorgement, in terms of quantity and quality of food intake, directly affects the development of the ovaries, egg maturation and the number of eggs produced [79]. The decrease in egg mass weight after Rhimi-CAP2bR silencing could be associated to a decreased blood intake (Figs. 4, 5c), but also with a reduction of CAP2b/PVK activity in the female reproductive tissues themselves, since Rhimi-CAP2bR transcript expression was observed in these tissues. Defects in feeding of silenced females, if resulting in a decreased heme acquisition, could explain a delayed incubation period and reduced egg hatch.

Heme and iron homeostasis are essential for normal tick feeding and reproduction [81–83]. In R. microplus, heme lipoproteins are present in the ovary [82] but also in the salivary glands [84], suggesting roles in reproduction and feeding. In R. sanguineus, silencing of ferritin 1 (FER1) and 2 (FER2), two proteins involved in iron metabolism, is associated with defects in feeding [81, 83] and egg hatching [81], respectively. Further, [81] found that depletion of FER2 from the tick hemolymph leads to a loss of FER1 expression in the salivary glands and ovaries after a blood ingestion. Moreover, vitellogenin (Vg) is responsible for heme binding and transport to the ovaries in hematophagous arthropods, including ticks [85, 86]. Consequently, the decrease in the reproductive performance observed here after Rhimi-CAP2bR silencing (Figs. 5E, H and I), could be associated to defects in both, vitellogenesis and normal heme/iron homeostasis.

In R. microplus, CAP2b/PVK receptor transcript expression was found in the female reproductive tissues [25]. In the coleopteran T. castaneum, silencing of CAP2b/PVK receptor led to a significant reduction in Vg accumulation in developing oocytes and a reduction of ~75% in egg production [87]. Further, in the whitefly Bemisia tabaci, egg production was significantly reduced after CAP2b/PVK receptor silencing [88]. In ticks, Hussein et al. [86] observed that silencing of VgR in R. microplus, was followed by decreased egg production, delayed embryonic development and reduced egg hatching. In the present study, the same three phenotypic results were observed for R. microplus females after Rhimi-CAP2bR silencing (Fig. 5e, h, i). Based on these insect reports and our RNAi observations in CFT, it would be worthy to investigate the relationship between tick Cap2b/PVKR and vitellogenesis regulation in ticks.

The C-terminal sequence WFGPRXa (X=L, I, M or V) is a highly conserved motif for the diapause hormones (DHs) in insects (with exceptions for some Paleoptera and Apterygota), and DHs are coded by two different genes: the CAPA gene encodes DH-1 and the PK/PBAN gene, DH-2 [34]. In Lepidoptera, DH initiates embryonic diapause in Bombyx mori [89, 90] and breaks pupal diapause in Heliothis and Helicoverpa spp. [91–94]. However, none of the predicted CAPA/PK peptides annotated for ticks, including R. microplus, carries the conserved motif mentioned above, specifically the WFG motif, as the PRXa C-terminus is conserved in all family members [32]. Several types of diapause have been observed in Prostriata and Metastriata ticks [95–97], including a delay of oogenesis in engorged females and a delay in the onset of embryogenesis in eggs [95]. Only a few studies have been conducted on hormonal control of diapause in ticks. In R. sanguineus and Dermacentor albipictus (Acari: Ixodidae), a breaking of larval diapause was observed after ecysteroid treatment [98, 99]. In the present study, we observed a delayed embryonic development after Rhimi-CAP2bR silencing (Fig. 5h); therefore, it would be important to determine the relationship between the CAP2b/PVK signaling system and ecysteroidogenesis, as it may appear that the CAP2b/PVK receptor loss of function could decrease ecysteroids.

In D. melanogaster, CAP2b/PVK silencing is associated with increased mortality under cold and/or desiccation stress conditions [100]. Silencing of the CAP2b/PVK receptor in the whitefly Bemisia tabaci led to a high mortality of approximately 30% [88], similar to the net 28% increase in mortality obtained in our study for Rhimi-CAP2bR silencing (Fig. 5a). These authors of the Bemisia tabaci study [88] suggested that mortality could be associated with a deregulation of the water/ionic balance. CAP2b/PVK transcript expression was detected in Malpighian tubules in R. microplus [27, 28], and myotropic activity of periviscerokinin was shown in the hindgut [54]; and in the present study the receptor transcript was significantly reduced by RNAi in these tissues (Fig. 3f). Thus, a CAP2b/PVK role in fluid homeostasis regulation in ticks can also be suggested.

**Conclusions**

Our results suggest that the CAP2b/PVK signaling system is significant in tick physiology, since loss of CAP2b/PVK receptor transcript expression was associated with reduced female weight, defects in reproduction and increased female mortality. These results indicate that the CAP2b/PVK system does not have a redundant function in ticks, or at least not a fully redundant function as there were significant effects. We had previously functionally characterized the CAP2b/PVK receptor in R. microplus [27]. Recently, after pyrokinin receptor and leuokokinin receptor silencing, we observed increased female mortality and decreased weight of eggs and females, similar to our observations in the present study; however, among
the three, only Rhimi-CAP2bR silencing caused a 20% reduction in egg hatching [29, 30]. Importantly, Rhimi-CAP2bR silencing was associated with a decrease in egg production which, combined with the reduction in egg hatching, will have a critical impact in reducing the numbers of the next generation.

In summary, our results support that the PRX-amide neuropeptide family may have pleiotropic functions in ticks and, accordingly, antagonists of these signaling systems could be explored for tick control.

Abbreviations
AUP: Animal use protocols; CAP2b/PVKs: Periviscerokinins; cDNA: Complementary deoxyribonucleic acid; CFTRL: Cattle fever tick research laboratory; CFT: Cattle fever tick; DH-1: Diapause hormone 1; DH-2: Diapause hormone 2; DNaše 1: Deoxyribonuclease 1; F.C: Fold-change; GPCR: G-protein-coupled receptor; NF-water: Nuclease-free water; nt: Nucleotides; PKs: Pyrokinins; PK/ PBAN/DH: Pyrokinin/pheromone biosynthesis activating neuropeptide/diapa- pause hormone; PKR: Pyrokinin receptor; PSOs: Perisympathetic organs; REI: Reproductive efficiency index; RH: Relative humidity; RNAi: RNA interference; SEM: Standard error of the mean; qRT-PCR: Quantitative reverse-transcriptase PCR; USDA-ARS: US Department of Agriculture-Agricultural Research Service; UTR: Untranslated region.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05457-7.

Additional file 1: Data S1. Nucleotide sequences of the Rhimi-CAP2bR (KC614697.1), two Rhimi-CAP2bR clones (clones #4 and #6) both identical to the original KC614697.1 cDNA, and a 5'-RACE PCR sequence obtained to extend the 5'-UTR region of the R. microplus periviscerokinin receptor. An alignment between the R. microplus genome (isolate Rmce-2018 chromosome 3, ASM1333972v1, NC_051167.1) and the R. microplus periviscerokinin receptor extended RACE 5'-UTR fragment (Accession number OP191701) is provided.

Additional file 2: Data S2. Nucleotide sequences of the Rhimi-CAP2bR (KC614697.1) and the extended Rhimi-CAP2bR 5'-UTR sequence. The dsRNAs used for Rhimi-CAP2bR silencing are displayed on these sequences. Further, an Emboss-Clustal Omega 1.2.4 multiple sequence alignment between KC614697.1 sequence and R. microplus periviscerokinin receptor RACE 5'-UTR fragment (R-5'CAP2bR) (Accession number OP191701) is provided.

Additional file 3: Data S3. NCBI-BLASTn searches to check for possible off-target effects of the Rhimi-CAP2bR dsRNA sequences. Only two short identical sequences to ds956-1109 were found to be ≤ 15 nt in length, which is not sufficient to cause off-target RNAi effects.

Additional file 4: Table S1. dsRNA treatments and respective concentrations used in each replicate of the RNAi experiment.

Additional file 5: Table S2. Pictures of ticks showing feeding progression from days 6 to 11 (December 2019), or from days 5 to 14 (July 2021) for all treatments.

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Author contributions
PVP and KBT conceptualized the experiments. JPW, KBT, KGS, JPT and PVP performed the experiments. JPW performed the statistical analyses and produced the figures. JPW and PVP wrote the manuscript. JPW, KBT, JPT and PVP reviewed and edited the manuscript. KHL 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
The data used and/or analyzed during the study are available from corresponding author on reasonable request. All relevant data are in the manuscript.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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