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The leucokinin-like peptide receptor from the cattle fever tick, *Rhipicephalus microplus*, is localized in the midgut periphery and receptor silencing with validated double-stranded RNAs causes a reproductive fitness cost

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ABSTRACT

The cattle fever tick, *Rhipicephalus microplus* (Canestrini) (Acari: Ixodidae), is a one-host tick that infests primarily cattle in tropical and sub-tropical regions of the world. This species transmits deadly cattle pathogens, especially *Babesia* spp., for which a recombinant vaccine is not available. Therefore, disease control depends on tick vector control. Although *R. microplus* was eradicated in the USA, tick populations in Mexico and South America have acquired resistance to many of the applied acaricides. Recent acaricide-resistant tick reintroductions detected in the U.S. underscore the need for novel tick control methods. The octopamine and tyramine/octopamine receptors, both G protein-coupled receptors (GPCR), are believed to be the main molecular targets of the acaricide amitraz. This provides the proof of principle that investigating tick GPCRs, especially those that are invertebrate-specific, may be a feasible strategy for discovering novel targets and subsequently new anti-tick compounds. The *R. microplus* leucokinin-like peptide receptor (LKR), also known as the myokinin- or kinin receptor, is such a GPCR. While the receptor was previously characterized in vitro, the function of the leucokinin signaling system in ticks remains unknown. In this work, the LKR was immunolocalized to the periphery of the female midgut and silenced through RNA interference (RNAi) in females. To optimize RNAi experiments, a dual-luciferase system was developed to determine the silencing efficiency of LKR-double stranded RNA (dsRNA) constructs prior to testing those in ticks placed on cattle. This assay identified two effective dsRNAs. Silencing of the LKR with these two validated dsRNA constructs was verified by quantitative real time PCR (qRT-PCR) of female tick dissected tissues. Silencing was significant in midguts and carcasses. Silencing caused decreases in weights of egg masses and in the percentages of eggs hatched per egg mass, as well as delays in time to oviposition and egg hatching. A role of the kinin receptor in tick reproduction is apparent.

1. Introduction

The cattle fever tick (CFT), *Rhipicephalus (Boophilus) microplus*, is the vector of pathogens causative of babesiosis and anaplasmosis, highly lethal diseases of cattle (reviewed by Aubry and Geale, 2011; Pérez de León et al., 2014). *Rhipicephalus microplus* populations infesting cattle in tropical and subtropical parts of the world

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have developed multiple acaricide resistance mechanisms that decrease the efficacy of almost all available acaricides, including amidines (amitraz), organophosphates, pyrethroids, fluazuron, and avermectins (Miller et al., 2013; Abbas et al., 2014; Reck et al., 2014; Rodríguez-Vivas et al., 2014, 2018; Vudriko et al., 2016; Klafke et al., 2017). Resistance to amitraz has been reported in eight countries in the Americas (Rodríguez-Vivas et al., 2018). *Rhipicephalus microplus* was declared eradicated in the United States in 1943, except in the Permanent Quarantine Zone (PQZ) located in south Texas along the USA-Mexico border (Giles et al., 2014). Eradication of CFT in the USA is maintained through monitoring programs, border checks, and enforced acaricide treatments (Pérez de León et al., 2012). The CFT is endemic to Mexico and is continually reintroduced into the USA by naturally ranging wildlife, including white-tailed deer and exotic ungulates (Pound et al., 2010; Cardenas-Canales et al., 2011; Busch et al., 2014; Wang et al., 2016; Foley et al., 2017; Lohmeyer et al., 2018). In 2017, 162 new infestations outside of the PQZ were reported in south Texas – the most since the 1976 surge in outbreaks (Lohmeyer et al., 2018). The extensive geographic range of this tick disease vector, global development of acaricide resistance in *R. microplus* populations, and the lack of effective vaccines against *Babesia* spp. demonstrates that novel approaches and targets for tick control are urgently needed for the global security of cattle herds.

G protein-coupled receptors (GPCRs) are cell surface membrane receptors for a number of endogenous ligands that are involved in regulating many physiological functions and, as such, have received renewed attention as druggable targets for the control of arthropod vectors (Pietrantonio et al., 2018). Support for this rationale is exemplified by the commercial acaricide amitraz which is thought to target octopamine (β-OAR) and tyramine (TAR1) GPCRs (Audsley and Down, 2015; Gross et al., 2015; Hauser et al., 2017; Pietrantonio et al., 2018). Beyond a few neuropeptides and GPCRs immunolocalized in the ticks *Ixodes scapularis* and *Ixodes ricinus*, very little is known about the physiological role of GPCRs in ticks (Neupert et al., 2005; Šimo et al., 2012; Šimo and Park, 2014).

Leucokinin-like peptides are synonymous with insect kinins or arthropod kinins, and their receptor is known as the leucokinin-like peptide receptor (LKR) and may also be referenced in the literature as the 'leucokinin receptor' or 'kinin receptor'. These arthropod kinin peptides are characterized by the conserved C-terminal core pentapeptide $FX^1X^2WGamide$, in which the second (X^1) and third (X^2) are variable amino acid positions, where X^1 = His, Asn, Ser, or Tyr and X^2 = Ser, Pro, or Ala (Xiong et al., 2018). An invertebrate-specific GPCR, the LKR, was the first neuropeptide receptor discovered in the Acari and the first LKR described in arthropods (Holmes et al., 2000; Mirabeau and Joly, 2013). The receptor transcript is expressed in all life stages of this tick, and is present in both sexes (Holmes et al., 2000).

Orthologue "kinin" receptors have been investigated in a number of other invertebrates (Holman et al., 1987; Schoofs et al., 1992; Seinsche et al., 2000; Radford et al., 2002; Kersch and Pietrantonio, 2011; Mirabeau and Joly, 2013). However, the kinin signaling system is not present in all insect orders and is thus considered part of the variable set of peptide hormones (Hauser et al., 2010). In insects, studies with kinins demonstrated that this signaling system is involved in a broad range of physiological processes. Initially, in vivo and in vitro studies showed that this receptor exhibited myotropic and diuretic functions (Hayes et al., 1989; Coast et al., 1990; Kersch and Pietrantonio, 2011; Bhatt et al., 2014). The insect kinin signaling system is now implicated in more diverse and complex insect processes including coordination of pre-ecdysis movements at the central level, tracheal air filling before ecdysis, feeding and meal size, inhibition of midgut digestive hormone release, circadian rhythms, increase in starvation stress tolerance, and the modulation of peripheral sugar taste perception (Wang et al., 1996; Harshini et al., 2002; Al-Anzi et al., 2010; Okusawa et al., 2014; Liu et al., 2015; Cannell et al., 2016; Cavey et al., 2016; Kucerova et al., 2016; Kwon et al., 2016; Lange et al., 2016; Zhang et al., 2017; Kim et al., 2018; Ohashi and Sakai, 2018). Despite these advances in insects, the function of the kinin signaling system is still unknown in ticks.

Functional characterization of the *R. microplus* LKR in a recombinant system determined that leucokinin peptides specifically activated the receptor at nanomolar concentrations (Holmes et al., 2003). Subsequently, the immunolocalization of tick kinin peptides in the synganglion of the tick *I. ricinus* demonstrated immunoreactivity in extensive nerve arborizations (Neupert et al., 2005). Moreover, the genome of the black legged tick, *I. scapularis*, revealed amplification in the number of kinin peptides encoded by a single gene in comparison with insects (up to 19 in the tick). The prediction of four putative leucokinin receptors in *I. scapularis* further suggested that this signaling system must be critical in ticks of the Family Ixodidae (Gulia-Nuss et al., 2016). The contributions of *R. microplus* LKR to overall fitness, including feeding and reproduction, were evaluated after RNA interference (RNAi)-induced silencing of the receptor. A dual-luciferase reporter system was developed to validate double stranded RNA (dsRNA) constructs in vitro prior to performing in vivo experiments with ticks on the bovine host (Tuckow and Temeyer, 2015). RNAi experiments were conducted in two different locations, USA and Mexico. After allowing ticks to feed on cattle and verifying silencing by quantitative real time PCR (qRT-PCR), the phenotypic characteristics of the silenced ticks and resultant egg masses were compared with those of control ticks.

Here, we document that antibodies developed against the receptor identified LKR expression in the periphery of the tick midgut, including the basal thin muscle layer, supporting a myotropic role for LKR, and perhaps also a role in feeding or midgut peristalsis and digestion. Importantly, in silenced ticks, qRT-PCR of the midgut demonstrated a significant decrease in receptor transcript. These ticks also presented abnormally light midgut coloration, potentially reflecting a defect in heme acquisition or metabolism upon decreasing kinin signaling. This study demonstrated in two geographic locations that LKR-silencing decreased the overall reproductive capabilities of *R. microplus*, an effect that apparently occurs through a direct or indirect action on midgut function.

2. Materials and methods

2.1. Animal care and use

All procedures for handling and treating animals were approved by the Texas A&M University Institutional Animal Care and Use Committee, USA, as described in Animal Use Protocol numbers 2003-0227 (Bovine antibody generation), 2007-215 (RNAi in Mexico), USDA/K-BUSLIRL/IACUC 2015-11 (RNAi at United States Department of Agriculture (USDA), USA) and 2015-0338EX (Texas A&M University (TAMU), USA – Institutional Animal Care and Use Committee (IACUC)).

2.2. Anti-leucokinin receptor polyclonal antibody production

Methodological details of the anti-LKR anti-peptide-antibody production in cattle, immunization schedule, and validation by ELISA and western blot are found in Supplementary data S1, Supplementary Tables S1 and S2, and Supplementary Fig. S1. Briefly, the leucokinin receptor amino acid sequence was analyzed for transmembrane region topology prediction and for antigenicity

to determine sequences to be used as antigens for anti-peptide polyclonal antibody production in cattle. For this, 13 overlapping peptides corresponding to the predicted extracellular LKR regions resulting from the aforementioned sequence analysis (Supplementary Fig. S1) were commercially synthesized and covalently bound as haptens to keyhole limpet hemocyanin (KLH). These conjugated peptides were injected with adjuvants in six Hereford calves and titers were evaluated by periodic blood extractions. Six calves were used as controls, only injected with the KLH and adjuvant. Complex statistical analyses demonstrated production of specific anti-LKR antibodies in sera. Further, these analyses identified that out of the 13 LKR antigens, six of the peptides were the most antigenic.

2.3. Immunohistochemistry

2.3.1. Tissue dissection and embedding for immunolocalization

The midguts of 5-day-fed female ticks were dissected in PBS. Tissues were fixed overnight in 10% formaldehyde at 4 °C and preserved in 70% ethanol. Tissues were serially dehydrated in 70, 95 and 100% ethanol. Dehydration steps were performed for 30 min each and were repeated twice. Ethanol was replaced with xylene and incubated twice, 30 min each, then sections were embedded in Paraplast-Xtra (Fisher Scientific, USA) at 60 °C for 4 h, embedded in blocks, and stored at 4 °C. Using a rotatory microtome, sections (12 μm thick) were cut and placed on Superfrost Plus™ slides (Fisher Scientific, USA) and dried for 2 days at 39 °C.

2.3.2. LKR immunolocalization

Wax from sections was removed by immersing the slides twice in xylene for 5 min each, then rehydrated serially in 100%, 95% and 70% ethanol for 10 min per step. Then, sections were equilibrated in water for 30 min at room temperature. After rinsing twice for 5 min per wash in PBS-T (PBS + 0.1% Tween-20), the slides were incubated in blocking solution (6% non-fat milk in PBS-T) at room temperature for 1 h. To detect the LKR, a mixed LKR-antiserum from four different calves obtained after the fourth immunization was used as the primary antibody. Slides were incubated overnight at 4 °C with either this antibody mix or with pre-immune sera, both diluted 1:40 in blocking solution. Rinses were performed in triplicate with PBS-T for 10 min per rinse. The slides were incubated in a 1:200 dilution of biotinylated goat anti-bovine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in blocking solution for 1 h. Sections were rinsed then incubated in a 1:200 dilution of Alexa Fluor 546 Streptavidin (Invitrogen, Carlsbad, CA, USA) for 1 h. Sections were rinsed again, then mounted in Vectashield Mounting medium with DAPI for nuclear staining (Vector Laboratories, Burlingame, CA, USA). Sections were imaged using the FV1000 Confocal microscope (Olympus America, Melville, NY, USA) in the Microscopy and Imaging Center (Texas A&M University) and analyzed with the Olympus FV10-ASW program (Olympus America, USA).

2.4. Leucokinin-like peptide receptor gene silencing by RNAi

2.4.1. In vitro RNAi evaluation of dsRNAs using the dual luciferase R. microplus LKR reporter plasmid

Due to the high cost, intensive labor, and restrictions for the use of large animals in tick research, it is critical to reduce the number of ineffective constructs to be tested in vivo. For this, the silencing efficiency of various LKR dsRNA sequences were tested first in vitro with the tick leucokinin-like peptide receptor mRNA. The dual luciferase reporter was constructed for *R. microplus* as described (Tuckow and Temeyer, 2015), and adapted for in vitro assessment of RNAi efficacy targeting transcripts of the *R. microplus* leucokinin-like peptide receptor. LKR cDNA partial sequences (positions 1-800 and 786-2354 derived from the LKR sequence; GenBank Accession number: AF228521.1) were amplified by PCR using primers (Table 1) and cloned into *Xma*I/*Sbf*I sites in the dual luc reporter plasmid using an InFusion® cloning kit (Clontech® Laboratories, Mountainview CA, USA). The newly constructed dual luciferase *R. microplus* LKR reporter plasmid was sequenced to verify its correct construction. Short dsRNAs corresponding to different receptor fragments were constructed (Table 2) as described below and transfected into *R. microplus* BmVIII cell cultures. Luciferase activities were measured 5 days post-transfection and the activity ratio was calculated (Tuckow and Temeyer, 2015). Three selected regions were considered suitable to proceed with in vivo experiments (Table 2).

Since the release of the *R. microplus* genome (Barrero et al., 2017), further analysis of specificity of LKR dsRNA constructs became possible. To this end, BLAST searches constrained to *R. microplus* were conducted to identify similar sequences in the genome that could lead to off target effects (Bastos et al., 2009). For each LKR dsRNA construct three different algorithms were used, megablast (highly similar sequences), discontiguous megablast (more dissimilar sequences) and blastn (somewhat similar sequences).

2.4.2. Synthesis of dsRNAs for RNAi

Target sequences for RNAi of 150-250 nucleotides (nt) were selected from the *R. microplus* LKR cDNA (GenBank Accession number: AF228521.1) primarily from the N-terminus and 5′- and 3′-untranslated (UTR) regions. dsRNAs for in vitro or in vivo gene silencing experiments were produced as per the manufacturer's instructions using the T7 RiboMAX™ Express RNAi system (Promega, Madison, WI, USA). Oligonucleotide primer sequences (Table 1) were selected using Oligo ver. 6.71 and adapted by addition of the T7 polymerase recognition sequence, as specified by the manufacturer for RNAi production (shown in Table 1). The sizes of the final constructs were checked by agarose gel electrophoresis and concentration was determined using a Nanodrop 1000 spectrophotometer using O.D. ratios (Fisher Scientific, USA). Constructs for dsRNA to serve as negative (β lactamase or EGFP) and positive (β actin or subolesin 4D8) controls in silencing experiments were similarly synthesized with primers listed in Table 1. Beta lactamase is a bacterial enzyme not present in ticks and for this reason was chosen as a negative control for dsRNA injections.

2.4.3. Cattle and ticks for RNAi experiments

Rhipicephalus microplus-naïve Hereford, Charolais or Angus cattle weighing approximately 136–182 kg were used. Cattle were vaccinated, de-wormed and acclimated for 2 weeks at the USDA-Agricultural Research Service (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, TX, USA. The cattle were used in tick production or for gene silencing experiments with *R. microplus*. The acaricide-susceptible Deutch strain of *R. microplus* ticks was obtained from ticks collected in 2001 from an outbreak in Webb County, TX, USA (Temeyer et al., 2007). This strain is permanently maintained at the CFTRL (Davey et al., 1980) and for these studies, filial generations F61, F63 and F68 were used. Additional RNAi experiments were performed at the University of Tamaulipas (Ciudad Victoria, Mexico) using the *R. microplus* acaricide-susceptible Media Joya strain originally characterized by the Centro Nacional de Servicios de Constatación en Salud Animal (CENAPA, Mexico) using European crossbred cattle.

Eight independent RNAi experiments were performed by placing injected ticks on cattle: three were conducted at the CFTRL in Texas, and five were at the University of Tamaulipas in Ciudad Victoria, Mexico. Eight calves were used for the experiments performed at the CFTRL, and five calves were used in the experiments

Table 1
Oligonucleotide primers for cloning, double-stranded RNA (dsRNA) synthesis and quantitative real time (qRT)-PCR.

Primer name	Primer sequence (5′ → 3′)[a]	Notes
BmLeucokinin-1U18*Xma*I-F	ACG*CCCGGG*CAGGTCGCTAAG	LKR 1-800 AF228521.1 cloning
BmLeucokinin-800L22-R	GCGGGAAAAAGTACTGCAGACA	LKR 1-800 AF228521.1 cloning
BmLeucokinin-786U22-F	AGTACTTTTTCCCGCTGCTCA	LKR 786-2352 AF228521.1 cloning
BmLeucokinin-2340L24-*Sbf*I-R	GGT*CCTGCAGG*TGCCTTCTAGGTA	LKR 786-2352 AF228521.1 cloning
Amp-T7	**TAATACGACTCACTATAGGG**CGCTGGTGAAAGTAAAATATG	β-Lactamase dsRNA synthesis
Amp-T7	**TAATACGACTCACTATAGG**GCCGGGAAGCTAGAGTAAGTA	β-Lactamase dsRNA synthesis
BmβActin-1U20-T7	**TAATACGACTCACTATAGGG**TCCTCGTCCCTGGAAGAAGTC	β-Actin dsRNA synthesis
BmβActin-285L18-T7	**TAATACGACTCACTATA**GGGGGAGCGATGATCTTG	β-Actin dsRNA synthesis
EGFP 241-33U18-T7	**TAATACGACTCACTATAGGG**CCCGAAGGCTACGTCCAG	EGFP dsRNA synthesis
EGFP 221-L20-T7	**TAATACGACTCACTATAGGG**TCTGCTTGTCGGCCATGATA	EGFP dsRNA synthesis
T7-P164	**TAATACGACTCACTATAGGG**ACGTAAACGGCCACAAGTTCAGCGTGTC	GFP dsRNA synthesis
P165-T7	**TAATACGACTCACTATAGGG**TCACGAACTCCAGCAGGACCATGTGATC	GFP dsRNA synthesis
RmSub-23U19-T7	**TAATACGACTCACTATAGGG**TGGCCATATTGAGGGACA	Subolesin dsRNA synthesis
RmSub-554L19-T7	**TAATACGACTCACTATAGGG**CACCTTCAAACCGCTTCTG	Subolesin dsRNA synthesis
BmLeukoR-6U15-T7	**TAATACGACTCACTATA**GGGCAGGTCGCTAAG	LKR 6-249 dsRNA synthesis
BmLeukoR-229L21-T7	**TAATACGACTCACTATAGGG**ACGGGAACCTGGTACAACTTG	LKR 6-249 dsRNA synthesis
BmLeukoR-1474U15-T7	**TAATACGACTCACTATAGG**GCCATGGCTCAAATG	LKR 1474-1680 dsRNA synthesis
BmLeukoR-1664L17-T7	**TAATACGACTCACTATAG**GGTAGAGCCCGACATTC	LKR 1474-1680 dsRNA synthesis
BmLeukoR-2032U17-T7	**TAATACGACTCACTATAGG**GCGCTTAAGGTGCTTTG	LKR 2032-2263 dsRNA synthesis
BmLeukoR-2247L17-T7	**TAATACGACTCACTATAGGG**CTAGGGCCTGTTTGCTC	LKR 2032-2263 dsRNA synthesis
BmLeukoR-2355U15-T7	**TAATACGACTCACTATAGG**GCGCGCTAGTCCAAA	LKR 2355-2516 dsRNA synthesis
BmLeukoR-2493L24-T7	**TAATACGACTCACTATAGGG**GGAAACGCACTAACTATATGTTCG	LKR 2355-2516 dsRNA synthesis
BmLKR-1146-F	GCGGTCATCGCTACAAGTCCC	LKR qRT-PCR
BmLKR-1260-R	GCGGAATTCTTGAAGCTCAGCC	LKR qRT-PCR
BmbA-1528-F	CAAACGGAGGTGGAGCTGTC	β-Actin qRT-PCR
BmbA-1629-R	GCTAGAATATGTGAGGGCGCGAC	β-Actin qRT-PCR
BmSub-1011-F	CGCAATGTTTGACTGGCTCATG	Subolesin qRT-PCR
BmSub-1192-R	GGCTCTCGAGTTGGGATGAGG	Subolesin qRT-PCR
BmELF1a-88-F	CGTCTACAAGATTGGTGGCATT	*elf1α* qRT-PCR Nijhof et al., (2009)
BmELF1a-196-R	CTCAGTGGTCAGGTTGGCAG	*elf1α* qRT-PCR Nijhof et al., (2009)
BmRPL4-511-F	AGGTTCCCCTGGTGGTGAG	*rpl4* qRT-PCR Nijhof et al., (2009)
BmRPL4-659-R	GTTCCTCATCTTTCCCTTGCC	*rpl4* qRT-PCR Nijhof et al., (2009)

[a] Sequences in bold were added to the primers and are not part of the tick cDNA sequences; sequences in italics indicate a restriction site for the enzyme indicated in the primer name. LKR, leucokinin-like peptide receptor.

Table 2
Luciferase activity ratios obtained from *Rhipicephalus microplus* BmVIII cells at 5 days post-transfection with double-stranded RNAs (dsRNAs).

Treatment set	Treatment applied to *R. microplus* leucokinin receptor dual luc expressing cells	Size of dsRNA (bp)	Luciferase ratio[a]	Silencing compared to no added dsRNA (%)[b]
Control	No dsRNA	–	0.651189	–
1	dsRNA LKR 6-249[c]	244	0.051349	92.1
2	dsRNA LKR 1474-1680[c]	207	−0.08952	113.7
3	dsRNA LKR 2355-2516	162	0.361266	44.5
4	dsRNA LKR 2032-2263[c]	232	0.029962	95.4

[a] Luciferase ratio steady-glo/nano-glo.
[b] Silencing = 1 − [luciferase ratio for dsRNA treatments ÷ luciferase ratio control value, no dsRNA].
[c] dsRNA was used for in vivo study.
RNAi, RNA interference.

performed at the University of Tamaulipas. Each calf could accommodate up to eight sleeves, four on each side of the animal, in a manner such that each animal was considered a randomized block (all treatments were randomly adjudicated to each of the sleeves). For the studies performed in Mexico, each sleeve contained 30 females (either injected with dsRNAs for the respective treatments or non-injected controls) and 15 males (simultaneously provided for mating). In the CFTRL studies, the sleeves contained 30–40 females and 15–35 males. Although two different technicians injected ticks in Texas and Mexico, respectively, the same person injected ticks for all experiments conducted in each respective location.

2.4.4. Microinjection of unfed adult R. microplus ticks

As RNAi has been effective in inducing specific gene silencing in *R. microplus* against several targets (Nijhof et al., 2007; Karim et al., 2008; Bastos et al., 2009; Kurscheid et al., 2009; Almazán et al., 2010; Lew-Tabor et al., 2011), female ticks were then microinjected with dsRNA specific for the LKR to investigate its function (Temeyer and Tuckow, 2016). Unfed female *R. microplus* were injected with 10^{11}–10^{12} dsRNA molecules/tick in 0.2–0.4 μl of water using the method previously described (Temeyer and Tuckow, 2016). After injection, ticks were moved to glass vials and maintained at 80 °F with 95% humidity for up to 1 day post-injection. Live, motile females were then transferred to the bovine host and held in labeled, round sleeves glued on the shaved backs of animals (Davey et al., 1980; Bastos et al., 2009).

2.4.5. In vivo data collection

Each day, sleeves were opened, ticks photographed, and any detached engorged ticks were removed, weighed and transferred to a cotton-stoppered glass vial to be held at 80 °F and 95% humidity. 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 oviposition (duration from detachment to oviposition). After oviposition, the dead female tick was removed and the egg masses were weighed. Egg masses were monitored to determine the incubation period (duration from egg laying to first hatch) and a visual estimation of the percentage of emergence (eggs that

hatched per egg mass) was performed by the same technician for the duration of each experiment as in Drummond et al. (1973). An overall view of the duration 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 (Bennett, 1974). Mortality was also recorded over the course of the experiments. Statistical analysis of all phenotypic data except mortality was performed using Graphpad Prism 6.0 and the Kruskal-Wallis one-way analysis of variance followed by Dunn's multiple comparisons test. Mortality was analyzed using χ^2 analysis followed by Fisher's Exact Test with pairwise comparisons using the Bonferroni correction, carried out in R (R Core Team, 2017) using the RVAideMemoire package (https://cran.rproject.org/web/packages/RVAideMemoire/index.html).

2.4.6. RNA extraction and cDNA synthesis

A subset of ticks ($n = 3$) from each sleeve were removed 5 days post-attachment to cattle (6 day post-injection) and dissected without surface sterilization under PBS by immobilizing the females dorsal side up. Midguts and carcasses devoid of obvious organs were separately kept in 100 µl of RNAlater™ (Invitrogen), refrigerated overnight, then stored at −80 °C until evaluation by qRT-PCR. Stored carcasses or whole ticks were disrupted using the Omni BeadRuptor 12 Bead Mill Homogenizer (Omni International, Inc., Waterbury, CT, USA) with 1.4 and 2.8 mm ceramic beads for 1–5 min at 5.65 m/s prior to RNA extraction. Total RNA was extracted from whole bodies and carcasses using the Zymo Quick-RNA™ Microprep kit (Zymo Research, Irvine, CA, USA). Extraction was performed following the manufacturer's protocol including the optional DNase step. For midguts, tissue disruption was done similarly as for carcasses but for only 1 min. Midgut mRNA was extracted using the Dynabeads® mRNA Direct kit (Thermo Fisher Scientific, USA). All RNA was treated subsequently with DNaseI using the TURBO DNA-free™ kit (Invitrogen) and evaluated for genomic contamination prior to cDNA synthesis. RNA was quantified spectrophotometrically using the Tecan Infinite M200 Pro plate reader (Tecan, Research Triangle Park, NC, USA). Synthesis of cDNA was performed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations using 200 ng of total RNA (whole ticks, carcasses) or 50 ng of mRNA (midguts) per 20 µL reaction. cDNA synthesis was performed using 1.5 µM each of oligo-dT_{20} and random hexamer primers. After synthesis, cDNA was used for qRT-PCR or stored at −20 °C until further use.

2.4.7. Quantification of gene silencing

Oligonucleotide primers for qRT-PCR were designed using the "primer3" tool (bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Sigma (Table 1). All primer pairs were subjected to Primer-BLAST (NCBI) constrained to *R. microplus* to confirm amplicon specificity prior to use in qRT-PCR. To evaluate silencing in the tissues from each independently collected tick, qRT-PCR was performed using 10 µL of reaction consisting of PowerUp SYBR™ Green PCR Master Mix (Applied Biosystems), 300 nM of each primer, and 10 ng of cDNA. All reactions were performed in duplicate. Real-time relative quantification was performed using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The conditions for the qRT-PCR cycles consisted of an initial denaturation step (5 min at 95 °C), followed by 40 cycles of 95 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s. Dissociation analysis was performed for each reaction, ranging from 95 to 60 °C, and no off-target products were detected. Analysis of qRT-PCR data was performed using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method, using the average of reference genes *Elf*1α and *Rpl*4 for normalization (Nijhof et al., 2009). Comparisons were made between the positive control (β-actin-silenced) or experimental sample (LKR-silenced), and β-lactamase-injected ticks (negative controls) to determine the relative transcript expression after silencing by RNAi (Livak and Schmittgen, 2001; Nijhof et al., 2009).

3. Results

3.1. Midgut localization of LKR

Using bovine anti-LKR antibodies from immunized cattle, fluorescent detection of LKR in the tick midgut was achieved (Fig. 1A and C, red signal; B shows the same tissue photographed with a DAPI filter only and at lower magnification). LKR localized only to the outer surface of the midgut in contact with the hemolymph, as indicated by the white arrow in Fig. 1C. LKR signal was not present at the luminal face of the midgut or in the digestive cells. In contrast, the low intensity red background signal in tissues treated with pre-immune sera (Fig. 1D and white arrow in 1F; E shows the same tissue at lower magnification and with a DAPI filter only) is non-specific and is similar in both the luminal and external midgut regions (Fig. 1D), and to the luminal region in LKR stained sections where the receptor signal is not present (Fig. 1A and D: compare the luminal face with the basal side of midgut tissue). Additional immunohistochemistry results from female tick midgut sections are shown in Supplementary Fig. S2. With respect to the generation of the bovine anti-LKR antibodies, cattle immunized with the cocktail of 13 peptides derived from the sequences of predicted extracellular loops of the *R. microplus* LKR (Supplementary Fig. S1, Supplementary Tables S1, S2) developed a significant specific immune response, as determined by statistical analyses of ELISA results. Antibodies to six of the 13 peptides developed a significant immune response by Bleed 4 at 122 days post priming compared with Bleed 0 (Supplementary Fig. S1B). Thus, these extracellular regions of LKR are immunogenic, as has been shown in similar receptors (Wieland et al., 1998). Further, sera from the fourth bleed detected the LKR protein in tick tissues by western blot (Supplementary Fig. S1C).

3.2. RNAi mediated silencing of LKR in R. microplus

In the first tick RNAi experiment, three dsRNA constructs designated LKR 6-249, LKR 1474-1680, and LKR 2032-2263 (locations on the LKR cDNA are shown in Fig. 2A) were tested. These three constructs were chosen because they efficiently silenced the LKR gene in the dual luciferase reporter assay using the tick cell line BmVIII (Table 2). To evaluate silencing efficiency in ticks, LKR- or β-actin-silenced females were compared with control dsRNA-β-lactamase-injected ticks (Fig. 2). At 5 day post-injection, ticks were dissected and the silencing efficiency of these constructs on tick carcasses was evaluated by qRT-PCR (Fig. 2B). Only LKR 6-249 and LKR 2032-2263 generated detectable receptor mRNA knockdown (71.0% and 69.7%, respectively) (Fig. 2B), while LKR 1474-1680 did not induce silencing and was, therefore, not used for further RNAi experiments (Fig. 2B). In addition, ticks injected with this construct were not as severely reduced in size as those injected with the other two (Supplementary Fig. S3). Fig. 2C shows the overall LKR gene silencing in *R. microplus* carcasses in two subsequent independent in vivo experiments. In these, LKR targeting produced an overall knockdown of 64.1%, when combining ticks injected with dsRNA LKR 6-249 and those injected with dsRNA LKR 2032-2263. Control β-actin-silenced ticks (ACTB) had an average silencing efficiency of 91.7% (Fig. 2C). Whole ticks at 3 days post-feeding were also evaluated for silencing efficiency by comparing them with the β-lactamase control-injected ticks ($n = 6$).

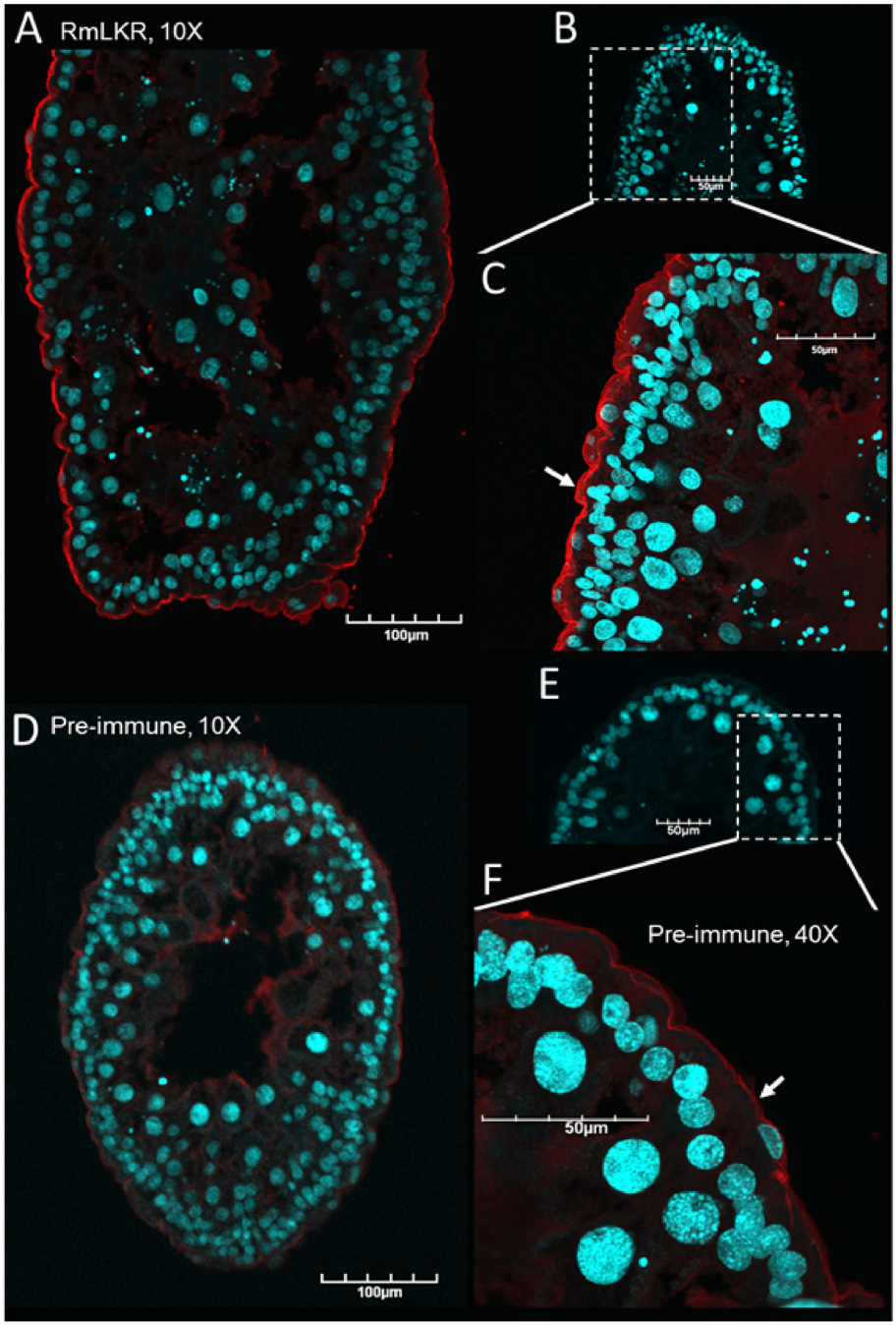

Fig. 1. Localization of the leucokinin-like peptide receptor (LKR) in the tick midgut. (A–C) Immunohistochemistry of the tick midgut shows fluorescent immunodetection of the *Rhipicephalus microplus* leucokinin-like receptor in the midgut using bovine anti-LKR antibodies. The receptor signal is in red (Alexa 546) and nuclei were detected with DAPI (blue). (A) In sectioned midguts, the anti-LKR antibody labels the outer midgut surface in contact with the hemolymph (10× objective). (C) LKR labeling is indicated by the white arrow (40× objective). (D-F) In midguts incubated with pre-immune sera, no receptor signal is present as highlighted by the white arrow. (B, E) Only the DAPI filter is featured, to indicate the location of tissues analyzed with both filters (red, DAPI) in C and F (white dashed boxes), respectively.

In the whole ticks, LKR silencing produced a knockdown of 61.8% ($n = 3$) and β-actin knockdown was 91.8% ($n = 2$) (data not shown). Therefore, two of the three LKR constructs validated in vitro in the BmVIII cell line were effective in silencing the LKR in live females with efficiencies of 64–71%.

3.3. Evaluation of LKR-silencing effects in ticks

After confirming silencing of LKR by qRT-PCR, phenotypic traits of the ticks injected with dsRNA 6-249 and dsRNA 2032-2263 were evaluated in both tick strains; results are summarized in Fig. 3, and details of the statistical analyses, including number of ticks injected per treatment are presented in Supplementary Tables S3–S5.

LKR-silenced female ticks of the Deutch strain evaluated in Texas did not differ in weight from the β-lactamase or non-injected ticks (Fig. 3A). In contrast, β-actin-silenced replete females weighed an average of 200 mg less than ticks from any other treatment used in this study (Fig. 3A, Supplementary Table S3). The repletion period of LKR-silenced ticks was intermediate between those of β-lactamase dsRNA-injected and non-injected ticks (Fig. 3B, Supplementary Table S4). β-actin-silenced ticks were not

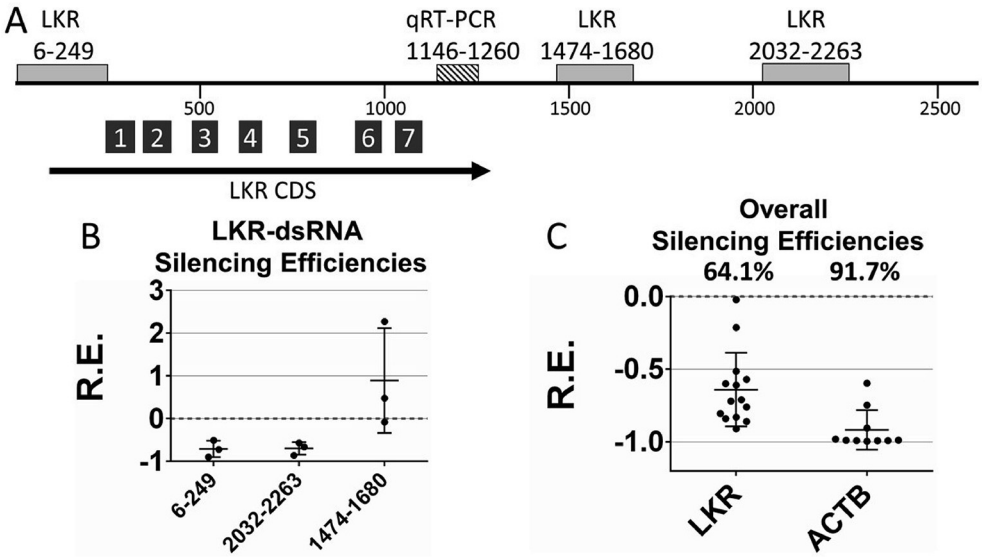

Fig. 2. RNA interference (RNAi) and expression of the leucokinin-like peptide receptor (LKR) in *Rhipicephalus microplus*. (A) Locations of double-stranded RNA (dsRNA) used in silencing experiments (gray) and for quantitative real time (qRT)-PCR detection of LKR transcripts (qRT-PCR, diagonal stripes). The coding region for LKR is depicted by the black arrow, and the seven transmembrane (TM) domains are shown in black. (B) First LKR RNAi experiment. Carcass-derived cDNA was used to evaluate three different LKR-targeting dsRNA constructs for silencing efficiency. The relative expression (R.E.) of the LKR transcript after silencing is compared between receptor-silenced ticks and those injected with the β-lactamase dsRNA construct. LKR 6-249 (71.0% average silencing efficiency) and LKR 2032-2263 (69.7% average silencing efficiency) were chosen for subsequent experiments, while LKR 1474-1680 did not produce a detectable decrease in LKR mRNA. For each treatment, $n = 3$. (C) Silencing efficiency in carcasses of ticks injected with chosen efficient LKR constructs (dsRNA for 6-249 and 2032-2263) or β- actin dsRNA (ACTB). Efficiencies are expressed as target gene expression (R.E.) in silenced ticks relative to the target expression in β-lactamase injected ticks (negative control). Each dot represents an individual carcass. Overall, LKR silencing (LKR; $n = 14$) averaged 64.1% knockdown and the positive control β-actin (ACTB; $n = 10$) averaged 91.7% knockdown. In both B and C, mean relative expression values (horizontal lines, center) are shown with their respective S.D.s (upper and lower lines).

included in the remaining analyses of the Deutch strain, as most females died and did not reach repletion or oviposit, as shown in previous studies (Karim et al., 2008; Temeyer et al., 2012). In LKR-silenced females of the Media Joya strain evaluated in Mexico, female weight decreased by approximately 30% (Fig. 3E, Supplementary Table S5), and the time from initiation of feeding to detaching was increased by 8.9% (Fig. 3F), compared with non-injected ticks and GFP-injected controls (statistical values are presented in Supplementary Table S5). Images from one replicate depicting the progression of feeding of *R. microplus* Media Joya ticks can be found in Fig. 3M. Images from one replicate of the experiments depicting the RNAi effect on the size of *R. microplus* Deutch strain can be found in Supplementary Fig. S3. In both experiments, the weights of β-actin-silenced ticks and subolesin-silenced ticks were significantly reduced (Fig. 3A, E). Mortality of β-actin-silenced ticks (positive silencing controls) was significantly higher than all other treatment groups (Supplementary Table S3). Mortality of Deutch strain LKR-silenced ticks exceeded that of the β-lactamase-injected controls by 19.07%, however, it was not different from that of non-injected controls ($P = 0.06$, Supplementary Table S3). Ticks injected with LKR construct 6-249 appeared significantly smaller at 5 days post-injection (Supplementary Fig. S3, LKR 5 day post-injection) than those injected with β-lactamase at 5 day post-injection (Supplementary Fig. S3, BLAC 5 day post-injection).

Egg masses were weighed and the reproductive efficiency index (percentage of conversion of female weight to egg mass) was calculated (Fig. 3, C–H). Egg masses derived from *R. microplus* Deutch strain LKR-silenced ticks weighed 28.3% less than egg masses from β-lactamase-injected females (Fig. 3C) and the reproductive efficiency index of LKR-silenced females was decreased by 24.2% compared with β-lactamase-injected controls (Fig. 3D, Supplementary Table S3). These results were corroborated by studies in the *R. microplus* Media Joya strain, in which LKR silencing reduced egg masses by nearly 40% (Fig. 3G) and decreased REI by 33.7%, both compared with GFP-injected control ticks (Fig. 3H, Supplementary Table S5). The pre-oviposition period for LKR-silenced Deutch ticks increased by 1.05 days compared with β-lactamase-injected ticks, a 45.1% increase (Fig. 3I, Supplementary Table S4), and the resulting eggs required an average of 2.4 additional days to hatch (Fig. 3J, Supplementary Table S4). Concerning hatching, an average of 36.7% of eggs hatched per egg mass from LKR-silenced ticks, while 56.9% of eggs per egg mass derived from β-lactamase-injected females hatched (Fig. 3K, Supplementary Table S3). Finally, the cumulative observation period that considered the number of days from the start of feeding to the first egg hatching was calculated for each treatment (Fig. 3L, Supplementary Table S4). LKR-silenced females of the Deutch strain had an average increase in cumulative observation period average of 2.4 days compared β-lactamase-injected females, a nearly 10% increase in duration from host attachment to egg hatching (Fig. 3L, Supplementary Table S4). We also determined that the overall reproductive period considering the time from female dropping from cattle to first egg hatching, was approximately 2 days longer in the LKR-silenced Deutch strain females (Supplementary Table S4).

The output of several BLAST searches indicated that LKR dsRNA off-target effects are highly unlikely. Both megablast and discontiguous megablast only returned similarity to the target *R. microplus* LKR (GenBank Accession number AF228521.1). The blastn algorithm (somewhat similar sequences) was the only search to detect off-target sequences with similarity to the LKR dsRNAs. Sequences with similarity to LKR dsRNA constructs longer than 13 bp are reported in Supplementary Table S6. It is generally accepted that dsRNAs of 15 bp (the maximum found in the blastn search) are too short to trigger meaningful off-target effects, as a sequence of 19 or more nucleotides of contiguous identical sequence is required to produce significant biological activity in RNAi-sensitive insect species (Bachman et al., 2013; reviewed by Christiaens et al., 2018). Further, no off-target gene was identified in common between the three dsRNA constructs used in this study

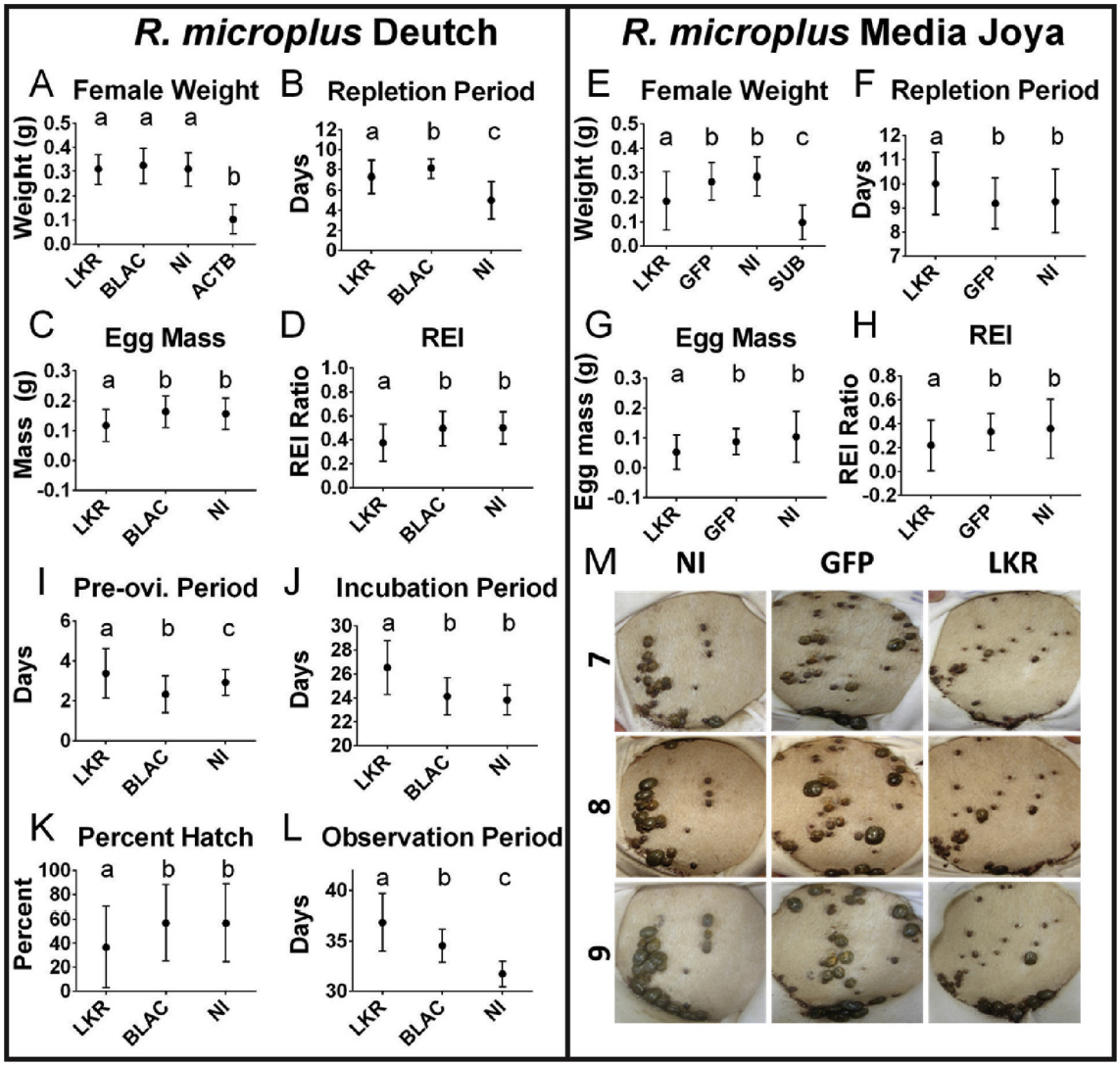

Fig. 3. Phenotypic evaluation of the leucokinin-like peptide receptor (LKR) silencing effects in two *Rhipicephalus microplus* tick strains. Female weight (A, E), repletion period (B, F), egg mass oviposited (C, G), reproductive efficiency index (REI) (D, H), preoviposition period (I), egg incubation period (J), percentage of egg hatching (K), and total observation period (L). (M) Photographs of open cotton sleeves showing shaved patches on cattle where the confined *R. microplus* female ticks (e. g. Media Joya strain) fed for 7, 8 or 9 days. On the *X* axes: LKR = leucokinin-like receptor dsRNA-injected ticks. Negative controls: BLAC = β-lactamase dsRNA-injected ticks; GFP = green fluorescent protein dsRNA-injected ticks, NI = non-injected ticks. Positive controls: ACTB = β-actin dsRNA-injected ticks, SUB = Subolesin dsRNA-injected ticks. Treatments sharing the same lowercase letter are not significantly different from each other ($P < 0.05$) as determined by the Kruskal–Wallis test followed by the Dunn's multiple comparisons test.

to evaluate LKR knockdown. Therefore, the results obtained with the aforementioned constructs are specific to the LKR silencing.

3.4. Phenotypic changes in the tick midgut

During tick dissections for qRT-PCR analyses performed 5 day post-injection, visible differences in the midguts were observed in LKR-silenced ticks. In LKR-silenced ticks, the midgut color ranged from dark brown (normal, Fig. 4A), to very light pink (Fig. 4B), mottled gray (Fig. 4C), or completely white in coloration (Fig. 4D). The latter was the most striking phenotype, which was never observed in any other treatments. Furthermore, sometimes the blind ends of midgut-diverticulum were conspicuously swollen (Fig. 4A, arrowhead). An uninjected control tick (Fig. 4E) showing normal coloration and an even width at the tips of midgut-diverticulum is shown for comparison. This tick in Fig. 4E and the β-actin-silenced tick (Fig. 4F) both show a dark midgut coloration. The dark brown color is present despite the extreme midgut phenotype of the β-actin-silenced tick, indicating that the light to white midgut phenotype is specific to LKR silencing. These compelling phenotypic changes in the midgut after RNAi are supported by the higher LKR silencing efficiency in tick midguts of 78.1% (Fig. 4G). This was higher than the silencing efficiency determined for the carcass (5 day post-injection), or whole, undissected ticks (3 day post-injection) - both of which produced LKR silencing of ~60% (Fig. 2C). Silencing of β-actin produced an average silencing efficiency of 86.6% (Fig. 4G), similar to the silencing produced in carcasses (5 day post-injection) and whole ticks (3 day post-injection) of ~91.0% (Fig. 2C).

4. Discussion

The study of the physiological role of the kinin signaling system has not yet been reported in any tick species. Before this work, only the functional characterization of the recombinant *R. microplus* kinin receptor and its use as a model to elucidate structure–activity relationships of designed kinin analogs were published (Holmes et al., 2000; Holmes et al., 2003; Taneja-Bageshwar et al., 2006;

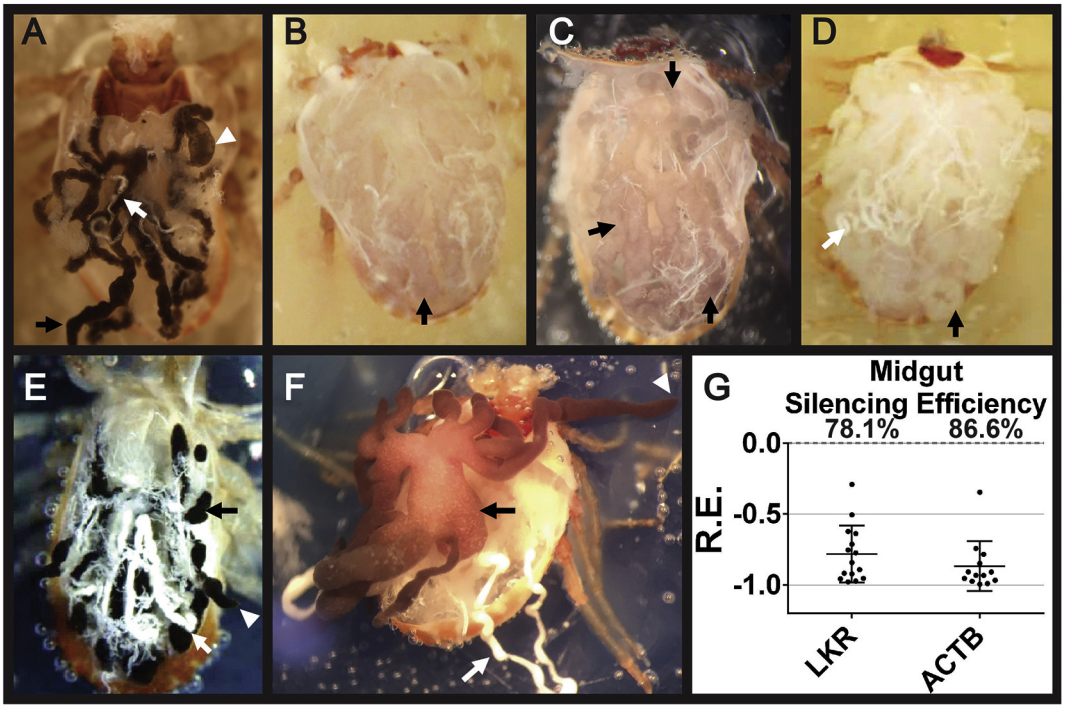

Fig. 4. Silencing of the leucokinin-like peptide receptor (LKR) in females of *Rhipicephalus microplus*. Photographs show dissected females (dorsal view) on the sixth day post injection (5 days post feeding). Black arrows indicate midguts, white arrowheads indicate tips of midgut-diverticulum, and white arrows indicate Malpighian tubules (where visible). (A–D) Leucokinin-receptor silenced ticks display variations of gut coloration, ranging from dark brown, pink, gray/pink to white. The phenotype of the LKR-silenced females is distinct and specific from the broadly disrupted actin-silenced female. Occasionally, swelling and a mottled appearance (A, white arrowhead) are seen near the ends of midgut-diverticulum in LKR-silenced ticks, which are not seen in controls (E and F, white arrowheads). (E) An untreated female shows the typical gut dark brown/maroon coloration. (F) β-actin-silenced females exhibit a swollen and deformed midgut. (G) Silencing of LKR in tick midguts ($n = 15$: double-stranded RNA (dsRNA) 6-249, $n = 9$, dsRNA 2032-2263, $n = 6$) and β-actin ($n = 13$) expressed as relative expression (R.E.) of target compared with β-lactamase-injected ticks ($n = 9$) Average LKR silencing in tick midguts was 78.1%, and average β-actin silencing was 86.6%. Mean relative expression values (horizontal lines, center) are shown with their respective S.D.s (upper and lower lines).

Taneja-Bageshwar et al., 2008; Lu et al., 2011; Xiong et al., 2018). Speculation on the important role of this signaling system in ticks had been derived from its amplification in the genome of the tick *I. scapularis* with respect to insects (Gulia-Nuss et al., 2016). In this tick, four different kinin receptor genes were predicted and 19 kinin peptides were identified in the kinin gene.

In contrast to our knowledge on ticks, insects where the kinin system is present normally have one receptor gene and a smaller number of predicted peptides. For example, three aedeskinins neuropeptides are present in the mosquito *Aedes aegypti* (Veenstra et al., 1997a; Pietrantonio et al., 2005). Due to the invertebrate specificity of the kinin signaling system and its significance in water balance and feeding in blood-feeding insect vectors, the tick kinin receptor (a "drugable" GPCR) is a potential candidate target for acaricide development (Pietrantonio et al., 2018). However, the normally low expression of GPCRs is a challenge when studying their function in non-model organisms with complex genomes, such as ticks. This difficulty was evident in analyses of *R. microplus* transcriptomes focusing specifically on GPCRs, where the LKR was not detectable (Guerrero et al., 2016; Pietrantonio et al., 2018). Additionally, the conservation in sequence of GPCRs, specifically in their transmembrane regions, makes them poor antigens as anti-tick vaccines and/or to obtain antibodies for receptor detection in immunoassays or histochemistry. Therefore, anti-peptide antibodies developed against GPCR loop sequences are required to ensure receptor detection specificity (Pietrantonio et al., 2005; Kwon et al., 2016).

As *R. microplus* is a "one-host tick" of cattle (all tick life stages live and feed on cattle), physiological studies on this tick are costly, of long duration, and in the USA are particularly limited by the reduced number of biosecure facilities authorized to house this species. In this study, to begin to elucidate the role of kinin neuropeptides in female ticks and thus potentially expand the repertoire of anti-tick targets, we addressed some of the above-mentioned challenges by: (i) developing specific anti-LKR-anti-peptide antibodies in cattle, (ii) using the dual luciferase assay in a tick cell line (*R. microplus* BmVIII) to validate in vitro dsRNA constructs for RNAi before in vivo testing, (iii) verifying LKR gene silencing using qRT-PCR, and finally, (iv) analyzing tick phenotypes after LKR silencing by (a) imaging dissected ticks and (b) measuring critical tick biological variables. The number of dsRNA targets tested on ticks in vivo can be decreased by selecting only the most efficacious dsRNA sequences in the dual-luciferase cell-based assay. This approach decreases the total number of animals needed and/or allowing for different dsRNA constructs to be evaluated simultaneously, as done here (Fig. 2).

In insects, the LKR contributes to various aspects of the feeding process, including hindgut contractions (Tanaka, 2016) and release of digestive enzymes in the gut (Harshini et al., 2002), further contributing to diuresis in blood-feeding mosquitoes (Veenstra et al., 1997b; Kersch and Pietrantonio, 2011). At the central level the LKR controls meal size, because silencing of the receptor decreases feeding frequency without altering total food ingested (Al-Anzi et al., 2010). Additionally, Zandawala et al. (2018) indicated that LKR silencing in *Drosophila* altered insulin-like peptide release and expression, thus further broadening the downstream physiological roles of LKR-signaling in regard to insect feeding behavior. For these reasons, we hypothesized that silencing the LKR would affect tick feeding. The immunolocalization of LKR in the external surface of the female midgut and the discoloration observed in

the midgut of silenced ticks (Fig. 4) supports a role for LKR in the physiology of the digestive system.

The observed distribution of LKR immunoreactivity resembles the midgut muscle layer reported for *R. microplus, R. sanguineus,* and *Amblyomma cajennense* (Agbede and Kemp, 1985; Caperucci et al., 2009; Remedio et al., 2013). The LKR localization at the outer surface of the midgut is likely occurring in the midgut muscle layer. Insect kinins were discovered for their myotropic activity in the hindgut, so this function and localization on enteric muscles appears to be conserved in ticks (Holman et al., 1986; Holman et al., 1990b,a; Holman et al., 1999; Kersch and Pietrantonio, 2011). The immunolocalization of LKR in the tick midgut (Fig. 1) does not exclude receptor protein expression in other tissues (i.e., synganglion) that could not be analyzed by histochemistry in this study.

Silencing the LKR gene mainly affected female reproductive output by decreasing the reproductive efficiency index (conversion), the weight of egg masses, and the percentage of eggs that hatched (Fig. 3). Further, LKR gene silencing increased the duration of the pre-oviposition period and egg incubation period (prior to hatching). We propose that the affected reproductive outputs and the overall results of this study are consistent with defects in feeding. There is a precedent in the fruit fly *Drosophila melanogaster* for decreased signaling of the leucokinin pathway, causing a lower frequency of larger meals without altering the total intake volume in the long term (Al-Anzi et al., 2010). *Rhipicephalus microplus* ticks use cement to attach to the host, and once attached, the female takes one prolonged meal that is characterized by two periods. These periods are the "slow phase" (~first 7 days in the adult with a fed:unfed weight ratio of 10:1) and the "rapid phase" (12–24 h in duration during which there is an additional 10-fold weight increase) (Kaufman, 1989). We documented through photographs that within the first 7–8 days after initiation of female feeding, the control ticks appeared larger than the LKR gene-silenced ticks, especially the Media Joya strain (Fig. 3M). We also observed that the LKR-silenced ticks of the Media Joya background were delayed in feeding to repletion compared with controls (Fig. 3F), while ticks from the Deutch strain were delayed with respect to non-injected ticks only (Fig. 3B).

The observation of a delay in achieving repletion may support a role for LKR during the slow phase of feeding, but perhaps also during the rapid phase, as female weights of the replete Media Joya strain also decreased (Fig. 3E, M). One plausible scenario in LKR gene-silenced ticks is that there is a delay or decrease in the normal blood volume ingested during the slow phase, followed by recovery of the lagging blood meal volume during the rapid engorgement phase (the 'big sip'), particularly in the Deutch strain (but not in Media Joya strain). This would result in the LKR-silenced females intaking the same blood volume by the end of feeding, irrespective of feeding duration. We suspect this because we did not observe differences in female weights in the Texas experiments (Deutch strain). More studies on the differing responses of these two tick strains may be needed, as they may be due to the strains themselves or the geographical and physical locations where the experiments were conducted.

The phenotypic effects observed in the midgut and the reduction in reproductive output are consistent with defects in feeding and heme acquisition. Heme is broadly important and functions as a prosthetic group for enzymes – particularly those that function in respiration and mediation of oxidative stress (Ponka, 1997; Hajdusek et al., 2016). Ticks are among the few organisms that lack a pathway for heme synthesis, and rather, rely solely on heme derived from the blood meal (Braz et al., 1999). Differing from other blood feeding arthropods, ticks digest red blood cells for heme acquisition and not strictly for amino acid uptake (Braz et al., 1999; Lara et al., 2005; Hajdusek et al., 2009; Hajdusek et al., 2016; Perner et al., 2016). In a previous study, ticks fed with serum but not provided with heme produced eggs, but those failed to hatch (Perner et al., 2016). The reproductive effects seen in our study may therefore result from a decrease in the number of red blood cells taken up during the blood meal, perhaps without altering the volume of plasma taken into the midgut. Alternatively, heme deficiency could occur through the inability to extract heme from red blood cells if enzyme availability is impaired.

When evaluating the role of heme in tick reproduction, Perner et al. (2016) observed a light gut phenotype in serum-fed ticks that resulted in termination of embryogenesis. This phenotype is similar to the gut phenotypes observed in the current study (Fig. 4). LKR-silencing may have induced a defect in digestion and nutrient uptake and utilization, specifically heme, which decreased tick egg production and hatching (Perner et al., 2016). These authors also showed that the amount of heme needed to restore successful reproduction was only one percent of the physiological heme concentration. Therefore, the partial silencing of LKR (~60% whole tick body, 78% midgut tissue specific; Fig. 4G) we observed could have allowed a minimally sufficient amount of heme to be available, thus enabling a portion of the embryos to develop normally and hatch, albeit in a delayed manner (Seixas et al., 2018).

There is recent evidence that the visual method of estimating egg hatching in *R. microplus* tends to overestimate the results of this variable, further supporting that the decrease observed in egg hatching is not only real but perhaps even more significant than reported herein (Figueiredo et al., 2018).

Repercussions of LKR silencing appear to occur beyond initial feeding for ticks, potentially by inhibiting digestion of the blood meal. To this end, the pre-oviposition and incubation periods were delayed, and the egg mass weights were decreased in LKR-silenced ticks compared with controls. The most likely explanation for these delays may be due to difficulties in muscular control during oviposition (Holman et al., 1987; Holman et al., 1990b,a; Schoofs et al., 1991; Coast, 1996). Due to the complex role of LKR in both the CNS and as a systemic neurohormone in peripheral signaling, other downstream LKR-signaling defects could also contribute to the observed delays in oviposition and egg incubation (Lundquist et al., 1993; Zhang et al., 2017). The potential mechanism of LKR silencing mediated defects may be multifactorial as RNAi silencing in *R. microplus* is passed transovarially to eggs and larvae (Kocan et al., 2007; Nijhof et al., 2007). Therefore, it is possible that direct LKR silencing could also have exerted a direct effect on the embryos (Fig. 3K).

The results of this study indicate that LKR plays a multifunctional role in the reproduction of *R. microplus.* Considering that LKR is a selective GPCR found only in invertebrates, this study advances the field of acarology by pointing to the need for further studies evaluating the role of LKR in tick physiology, heme metabolism, and reproduction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.11.006.

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