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Effect of Silencing subolesin and enolase impairs gene expression, engorgement and reproduction in *Haemaphysalis longicornis* (Acari: Ixodidae) ticks

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ABSTRACT

Importance: *Haemaphysalis longicornis* is an obligate blood-sucking ectoparasite that has gained attention due its role of transmitting medically and veterinary significant pathogens and it is the most common tick species in Republic of Korea. The preferred strategy for controlling ticks is a multi-antigenic vaccination. Testing the efficiency of a combination antigen is a promising method for creating a tick vaccine.
Objective: The aim of the current research was to analyze the role of subolesin and enolase in feeding and reproduction of *H. longicornis* by gene silencing.
Methods: In this study, we used RNA interference to silence salivary enolase and subolesin in *H. longicornis*. Unfed female ticks injected with double-stranded RNA targeting subolesin and enolase were attached and fed normally on the rabbit's ear. Real-time polymerase chain reaction was used to confirm the extent of knockdown.
Results: Ticks in the subolesin or enolase dsRNA groups showed knockdown rates of 80% and 60% respectively. Ticks in the combination dsRNA (subolesin and enolase) group showed an 80% knockdown. Knockdown of subolesin and enolase resulted in significant depletion in feeding, blood engorgement weight, attachment rate, and egg laying. Silencing of both resulted in a significant ($p < 0.05$) reduction in tick engorgement, egg laying, egg hatching (15%), and reproduction.
Conclusions and Relevance: Our results suggest that subolesin and enolase are an exciting target for future tick control strategies.

Keywords: Real-time PCR; RNA interference; vaccine; double-stranded RNA; knockdown; transcript

INTRODUCTION

Ticks are recognized as significant threats to human and veterinary public health, and they transmit the widest variety of infectious agents of all arthropod vectors due to their

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Conflict of Interest

The authors declare no conflicts of interest.

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excellent blood-sucking capacity [1]. Ticks take a maximum amount of nutrients and enough energy from blood for the subsequent off-host phase, causing a decrease in production and consequent economic losses [2].

Currently, chemical acaricides are the mainstay of tick management strategies. However, the environmental hazards associated with their use and the emergence of acaricide resistance make tick control difficult [3]. Nanotechnology has recently drawn attention because of its extensive usefulness and typically good environmental practices. Previous study showed that zinc oxide nanoparticles cause mortality in freshwater crustaceans and insects *Ceriodaphnia cornuta* [4]. Some repellents have been constructed from natural products. While these are eco-friendly, applying chemical repellents and/or utilizing regular acaricidal treatments are still the mainstays of tick bite prevention [5]. Research has focused on alternative tick management technologies, such as vaccines, as a workable strategy to provide a sustainable, safe, effective, and environmentally friendly option [6]. Therefore, anti-tick vaccination is a viable strategy for managing ticks and tick-borne pathogens (TBPs). Finding the appropriate antigen is essential for creating an anti-tick vaccine. RNA interference (RNAi) is a rapid method to silence gene expression, valuable for selecting potential vaccination candidates targeting different antigens [7].

Haemaphysalis longicornis, is extensively prevalent in the Asia-Pacific region that includes Korea, Japan, Australia, the Pacific Islands, and New Zealand, is one of the most significant tick species that poses the danger tick-borne infection [8]. Along with humans, generally, goats, cattle, sheep, cats, donkeys, pigs, *Bos mutus*, *Cervus elaphus*, *Mus musculus*, *Erinaceus europaeus*, *Mustela sibirica*, *Trichosurus vulpecula*, and various birds also frequently serve as hosts of *H. longicornis* [8,9].

H. longicornis has the ability to spread a wide range of diseases [10] and is implicated in the transmission of severe fever with thrombocytopenia syndrome virus (Bandavirus dabieense) in China [11]. *H. longicornis* can spread viruses, bacteria, and protozoa that can cause severe illness and even death in people worldwide. *Haemaphysalis spp.* transmit bacterial pathogens Rickettsia *spp.*, *Ehrlichia spp.*, *Anaplasma spp.*, *Francisella spp.*, *Coxiella spp.*, *and Borrelia spp.* respectively [12].

Tick salivary glands (SGs) play key physiological roles and function in TBP transmission. The crucial role that tick SGs play in producing physiologically active substances. These substances assist in obtaining a blood meal and contribute to the disease transmission process [13]. SG products foster an environment that is conducive for TBP transmission, survival, and proliferation within the vertebrate host [14]. A few SG components have been identified as being specifically implicated in disease transmission; however, several studies have indicated that ticks express different transcripts and proteins in pathogen infection [15]. To establish a tick control approach, identifying the genes responsible for the release of these bioactive chemicals and developing strategies to inhibit these genes may be useful.

A tick protective antigen known as enolase was recently identified in the *H. longicornis* (GenBank accession number ON871822). Enolase, a protein involved both as enzyme in glycolysis and gluconeogenesis, metabolic process [16]. While enolase is typically found in the cytosol [17] it can translocate to the cell surface in response to inflammatory signals [18]. On the cell surface, enolase functions as a plasminogen receptor [19,20] stimulating fibrinolysis and breakdown of the extracellular matrix. Fibrinolysis may be crucial for ticks to

break up any clots that may develop during feeding and to stop the ingested blood meal from clotting in the tick midgut. According to previous study, RNAi of the enolase gene disrupted in *Ornithodoros moubata* blood sucking and reproduction [21]. Thus, salivary enolase may be a viable antigen for the development of an anti-*O. moubata* vaccine.

Subolesin, a tick-protective antigen, was discovered in *Ixodes scapularis* [22]. Subolesin, a homolog of the akirins found in insects and vertebrates, is an intracellular protein. It impacts innate immune response, blood engorgement, digestion, reproduction and development and regulates gene expression [23]. Subolesin gene knockdown in *H. longicornis* by RNAi reduces gene expression from ticks and impacts on engorgement, egg mass, and egg mass to body weight ratio, reduced reproduction in females [23-25]. Gene silencing was observed in both eggs and larvae and recombinant subolesin reduced larval, nymphal, and adult *I. scapularis* infestations. In *Dermacentor variabilis* and *I. scapularis*, RNAi-mediated suppression of subolesin expression reduced fertility by 93% and 71%, respectively [24].

A multi-antigen anti-tick vaccines containing multiple antigens have a higher effectiveness rate than those with a single antigen [25]. This vaccine contained recombinant *Rhipicephalus microplus* Bm86 gut antigen. Two vaccines utilizing recombinant Bm86 were subsequently approved for use in Latin American nations (Gavac) and Australia (TickGARD). These vaccine reduce the quantity of engorging ticks, their weight, and their ability to reproduce [24].

Accordingly, the aim of the present work was the effects of salivary subolesin enolase gene knockdown by RNAi technology of *H. longicornis* ticks and their function in feeding and reproduction. This knowledge might be useful in choosing potential vaccination candidates for various antigens.

METHODS

Ticks rearing and feeding

The hard tick *H. longicornis* (Jeju strain) has been kept in an incubator at 25°C at 95% humidity for several generations at the Laboratory of Veterinary Parasitology, College of Veterinary Medicine, and Bio-Safety Research Institute at Jeonbuk National University in Iksan, Republic of Korea since 2003 [26]. Animals used in our experiments were treated under the ethical guidelines of Jeonbuk National University Animal Care and Use Committee (JBNU 2022-094).

SGs collection

Twenty unfed female and male ticks *H. longicornis* ticks were taped to the ears of specific pathogen-free (SPF) New Zealand White rabbits (Samtako, Korea). Female ticks that were partially engorged after five days of feeding in ears were taken out for SG collection. The removal ticks were keep at room temperature for 1 h. The ticks were washed in 70% ethanol and distilled water to avoid surface contamination. During SG collection, ticks were attached with glue to sterile slides with liquid paraffin (ventral side down). The dissection was performed using a dissecting microscope (Nikon SMZ-U, Japan) using a scalpel fitted with a no. 11 surgical blade. To remove midgut contamination, SGs were detached and washed three times in ice cold 1 × PBS (one time phosphate buffered saline solution) and immediately frozen with RNAlater (Ambion, Inc., USA) at –70°C.

RNA extraction and complementary DNA (cDNA) synthesis

Using a total RNA extraction kit (RiboExTM) and following the manufacturer's instructions, total RNA was extracted from the collected SGs (5 ticks already mentioned above). A NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, USA) was used to measure the concentration of RNA. The sample was then stored at –70°C. cDNA was synthesized using a WizScript cDNA Synthesis Kit (Wizbiosolutions Inc. Korea) in accordance with the manufacturer's instructions, using 1 μg of total RNA and an anchored oligo (dT)18 primer.

Reverse transcription polymerase chain reaction (RT-PCR) for detecting salivary enolase and subolesin

RT-PCR was performed using EzPCR HS 5x Master Mix with a master cycler gradient (Eppendorf, Germany) and (gene-specific primers; **Supplementary Table 1**). Actin cDNA was amplified as an internal control. Oligonucleotid primers were designed based on the sequences of subolesin, enolase, and actin GenBank Accessions Nos. EU289292.1, ON871822.1, and AY254898.1, respectively. A PCR cycle was used for the amplification. Amplification was performed using a PCR cycle profile, as follows: 95°C for 15 min, followed by 34 cycles at 95°C for 20 s, 60°C for 30 s and 72°C for 1 min, with a final extension of fifteen minutes at 72°C. and for subolesin 95°C for 15 min, followed by 34 cycles at 95°C for 20 s, 53°C for 30 s and 72°C for 1 min, with a final extension of fifteen minutes at 72°C.

Purification of PCR product and sequencing

PCR products were purified and evaluated using 1% agarose gel electrophoresis. PCR products were extracted from gels and purified using an EZ-Pure PCR Purification Kit ver. 2 (Enzynomics, Korea) in accordance with the manufacturer's instructions. The purified PCR products were sent for sequencing. The cDNA showed a sequence similarity of 97%–100% with *H. longicornis* enolase obtained from a NCBI nucleotide blast search (GenBank Accession No. ON871822.1). Sequence similarities of 98% were observed with *H. longicornis* enolase and 97%–98% were observed with *H. longicornis* subolesin respectively, using an NCBI nucleotide blast search (data not shown) (GenBank Accession No. EU289292.1).

Generation of double stranded RNA (dsRNA)

The PCR products of subolesin (218 bp) and enolase (194 bp) were joined to a T7 promoter sequence using T7 promoter-linked (at both the 5′ and 3′ ends) shown in **Supplementary Table 1**. A T7 promoter sequence was added, as described elsewhere [27].The PCR amplification profile was as follows: 95°C for 15 min, followed by six cycles at 95°C for 20 s, 63°C for 30 s, and 72°C for one minute and then 28 cycles at 95°C for 20 s, 77°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR bands were checked by 1% agarose gel electrophoresis. PCR products were purified using the EZ-Pure PCR Purification Kit ver. 2 (Enzynomics) in accordance with the manufacturer's instructions. dsRNA was synthesized from T7 linked DNA using the HIScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, Inc., UK) in accordance with the manufacturer's protocol. The concentration of dsRNA was determined using a NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific). The sample was aliquoted and kept at –70°C.

Injection of dsRNA

The four groups (n = 20/group) were made up of 80 mature, unfed female ticks. The injection dosage for the target gene was earlier studies [24]. Subolesin and enolase dsRNA injections were each given to two groups of ticks in a dose of 500 ng/tick. The third tick group received an injection of an equal mixture of subolesin and enolase dsRNA. The fourth

group (negative control) received an injection of injection buffer (10 mM Tris and 1 mM ethylenediaminetetraacetic acid, pH 7.4) as a control. Ticks were injected with dsRNA using a Hamilton 33-gauge needle as described elsewhere [28]. Survival was monitored overnight in a 25°C incubator with high humidity.

Four SPF rabbit's ears were used as test subjects, and the female ticks from each group were injected along with an equal amount of male (not injected) ticks. SGs from ten female ticks from each group were collected after five days of feeding to evaluate gene silencing by real-time PCR. After drop off the remaining ticks on their own. We kept record of attachment rate, feeding intervals, blood engorgement, egg mass, and hatching rate.

Gene silencing and expression analysis by real-time polymerase chain reaction

Total RNA was obtained from the SGs of female ticks that were injected and had fed for five days. Real-time PCR was used to measure gene expression using a One-step SYBR PrimeScript RT-PCR kit II (Clontech Laboratories, USA) with a Thermal Cycler Dice system (Takara, Japan). Primers are listed in **Supplementary Table 1**. Standard curve was constructed as a result, a slope value and a r-squared value and efficiency were derived; results shown in **Supplementary Data 1**. PCR amplification was carried out in accordance with the manufacturer's recommendations. Briefly, PCR amplification was conducted in the following three stages: stage 1 (reverse transcription, 42°C for 5 m, followed by 95°C for 10 s), stage 2 (PCR reaction repeats 40 cycles of 95°C for 5 s, 60°C for 30 s), and stage 3 (dissociation). Data were normalized with actin, as the internal control, and calculated using the ΔΔCt method, the percentage of knockdowns was calculated in the same manner as previously reported [29]. Assessment of the effects of knocking down subolesin and enolase calculation was made (showed in **Supplementary Data 2**) by using formulas drawn from earlier research on tick immunization, and these formulas were employed to make calculations as described elsewhere [30].

Statistical analysis

Graph Pad Prism 5 (Graph Pad Software, Inc., USA) was used for the statistical analysis, which included a Student's *t*-test (for unpaired and unequal variances). Data are expressed as the mean ± SE. *p* values of 0.05 or less were statistically significant.

RESULTS

Salivary enolase and subolesin detection using reverse transcription-PCR and sequencing

Enolase in the SGs of *H. longicornis* plays a vital role in blood engorgement. Enolase cDNA (1297 bp) was amplified with a gene specific primer (**Supplementary Table 1**). Purified PCR products were sequenced. A 247 bp of enolase in several organs was amplified and also checked in hemolymph (**Supplementary Fig. 1A**). *H. longicornis* has the subolesin gene in both its SGs and midgut, which is essential for blood engorgement. Subolesin cDNA (396 bp) was identified (**Supplementary Fig. 1B**) with a gene-specific primer (**Supplementary Table 1**).

Silencing of subolesin and enolase by RNAi

Gene silencing for enolase and subolesin was conducted (**Fig. 1**). Enolase dsRNA-injected ticks showed (fold-0.7) decreased expression by nearly 30%, after five days of feeding compared with the control (injection buffer) group (**Fig. 1**). Subolesin dsRNA-injected ticks also showed (fold-0.6) decreased expression by nearly 40%, compared with the control group. The ticks

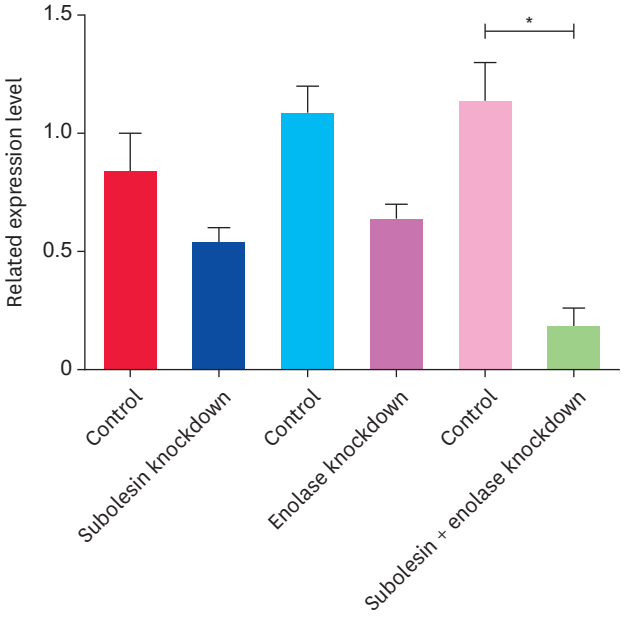

Fig. 1. Real-time polymerase chain reaction results indicated genes in the group treated with subolesin + enolase combinly shows significant decrease expression level following RNA interference than that of the gene subolesin and enolase separately treated with a single group. The data represent mean ± SD, normalized to the actin transcript level.
*$p < 0.05$, compared with the control group as determined by a Student's *t*-test.

group injected with both enolase plus subolesin dsRNA showed (fold-0.26) significantly decreased ($p < 0.05$) expression by nearly 74%, compared with the control group (**Fig. 1**).

Effects on feeding duration and engorgement

We next examined the effect of knockdown of subolesin and enolase on feeding. The mixture of subolesin and enolase dsRNA was injected into unfed females and observed in death and alive ticks 24 h after injection (**Fig. 2D**) and data were recorded attachment rate in rabbit ear at 24 h (**Fig. 2C**) were observed then allowed to feed until drop-off. Feeding duration at 11 days was longer compared with that of the control group. at 8 days (**Fig. 2A**), Engorgement weight (average: 58.21 mg) was significantly reduced compared with to the control group (average: 440 mg) ($p < 0.001$) (**Fig. 2B**). Ticks phenotypic changes during blood feeding period both dsRNA subolesin and enolase treated (**Fig. 3A-D**) and control ticks (**Fig. 3E-G**) (4^{th} day, 6^{th} day, 8^{th} day,10^{th} day) and changes in the SG can be observed as well (**Supplementary Fig. 2**).

Assessment of the effects of knocking down subolesin and enolase on survival, egg laying, and hatching

After dropping, ticks were weighed and then monitored for survival rate, egg laying and subsequent hatching to larvae at 25°C in incubator at 95% humidity. Reduction in hatching (81%), reduction in oviposition (75%), reduction in blood engorgement weight (86.77%) compared to control shown on (**Table 1**).

Effects of subolesin and enolase knockdown on reproduction

We next examined the effects on reproduction, egg mass, egg conversion ratio, and hatchability.

Subolesin and enolase dsRNA treated ticks after spontaneous drop down (**Fig. 4B**) compared to control (**Fig. 4A**). Due to apoptosis changes in egg mass (**Fig. 4D**), as compared to control

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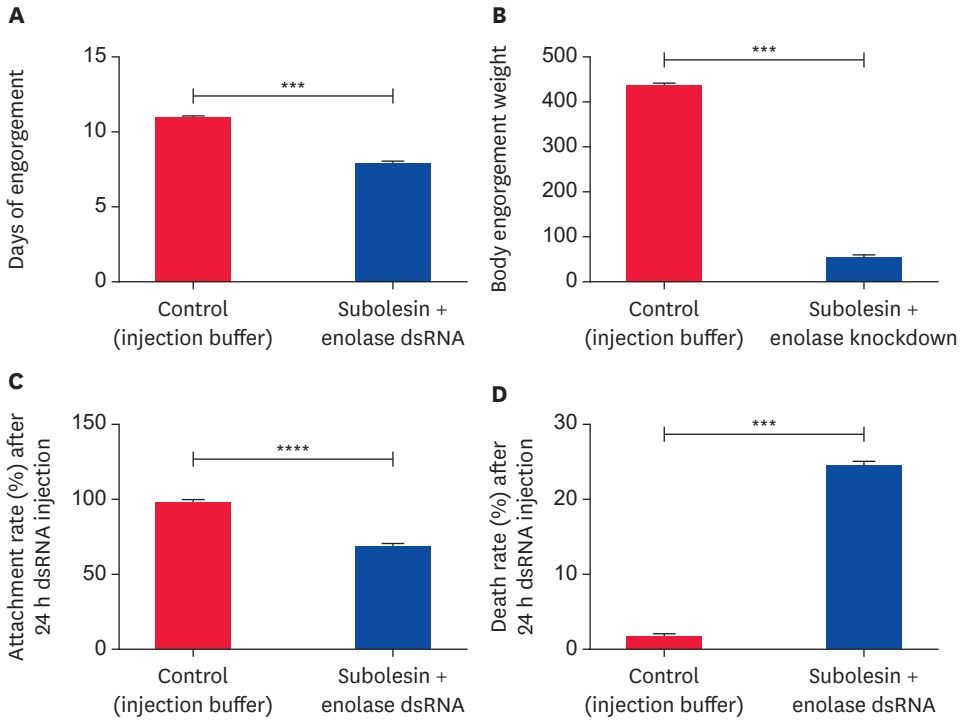

Fig. 2. Effects of subolesin and enolase dsRNA on tick engorgement and reproduction; (A) Group treated with Subolesin and enolase dsRNA. Significantly different compared to the control group (injection buffer); (B) Average engorgement weight in the group treated with Subolesin and Enolase dsRNA. Significantly different compared to the control group as determined by a Student's *t*-test; (C) Effect of Subolesin and Enolase dsRNA on tick attachment, highly significant compared to the control group; (D) Death rate of *Haemaphysalis longicornis* adult ticks 24 h after injection of Subolesin and Enolase dsRNA. Significantly different compared to the control group; Data are presented as median ± SD.
dsRNA, double stranded RNA.
***$p < 0.001$; ****$p < 0.0001$.

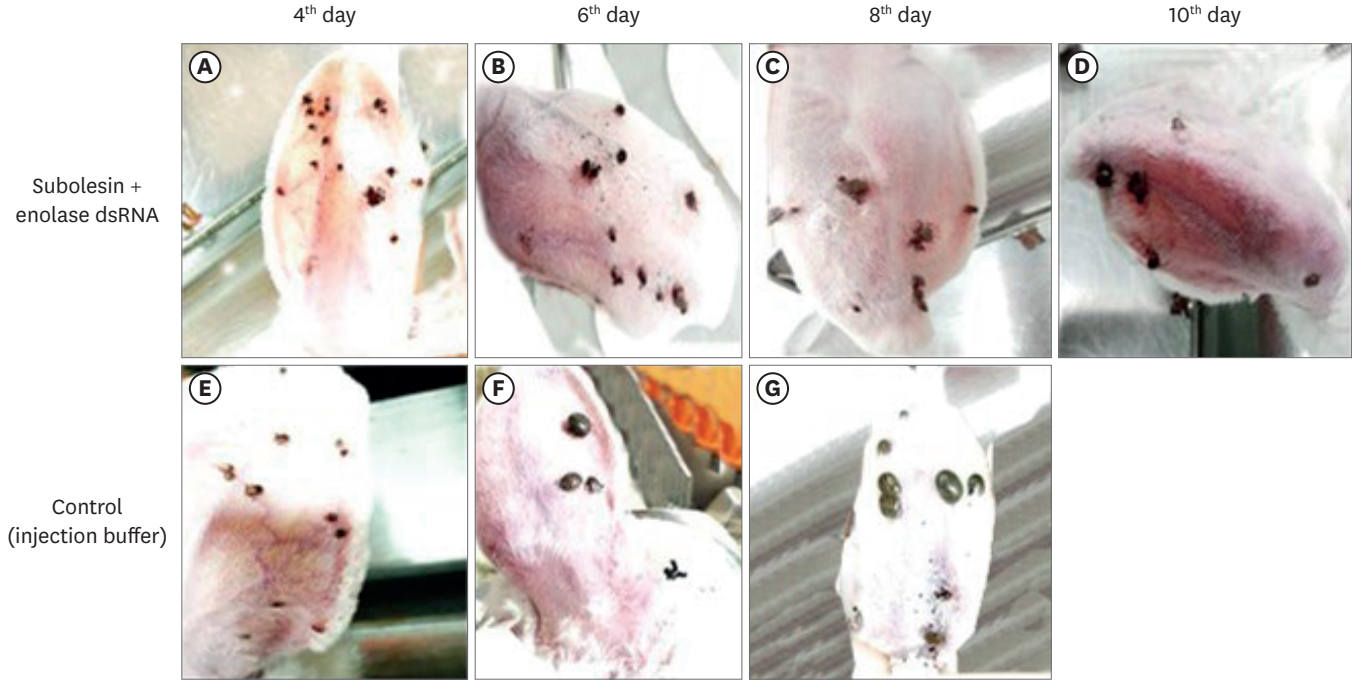

Fig. 3. Phenotypic changes of ticks after infestation in rabbit ear with dsRNA (subolesin + enolase) compared to control (injection buffer) injection. Changes in tick morphology during feeding several days after injecting subolesin + enolase dsRNA (A) 4th day; (B) 6th day; (C) 8th day; (D) 10th day; Compare to control group (Injection buffer); (E) 4th day; (F) 6th day; (G) 8th day.
dsRNA, double stranded RNA.

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Table 1. Assessment of the effects of knocking down subolesin and enolase formula and calculation

Experimental group	Percent of reduction[a,b]		
	Engorged weight (R_W)	Oviposition (R_O)	Hatched egg (R_H)
Subolesin + enolase dsRNA	86.77%* (0.058 ± 1.08)	75% (0.15 ± 0.51)	81% (15%)
Contriol (injection buffer)	(0.44 ± 0.01)	(0.39 ± 0.01)	(80%)

dsRNA, double stranded RNA.
[a]The percent reduction was calculated with respect to the control group: R_W, % reduction in engorged weight; R_O, % reduction in oviposition; R_H, % reduction in hatch. In parenthesis is shown the average ± SD for the tick weight after feeding. A Student's *t*-test with unequal variance was used to compare results between subolesin + enolase dsRNA treated and control groups (*$p < 0.05$).
[b]All calculation formula is shown on **Supplementary Data 2**.

egg mass (**Fig. 4C**), embryo development (**Fig. 4F**) was less than the control ticks (**Fig. 4E**) and changes in the SG can be observed as well (**Supplementary Fig. 2**). Combined silencing caused a significant retardation of egg mass (**Fig. 5A**) and egg conversion ratio (**Fig. 5B**) (average egg mass weight: 0.015 gm and 26% respectively) compared with those of the control group (390 mg and 89% respectively) ($p < 0.001$). Regarding hatchability, hatching rate was significantly reduced compared to controls (15% vs. 80%) (**Fig. 5C**). Therefore, our data indicates that the knockdown of subolesin and/or enolase plays a significant role in blood feeding and reproduction of *H. longicornis*.

DISCUSSION

The only source of nutrients *H. longicornis* ticks for their survival, development, and reproduction is blood. The SG plays a vital role in the consumption of blood, and its

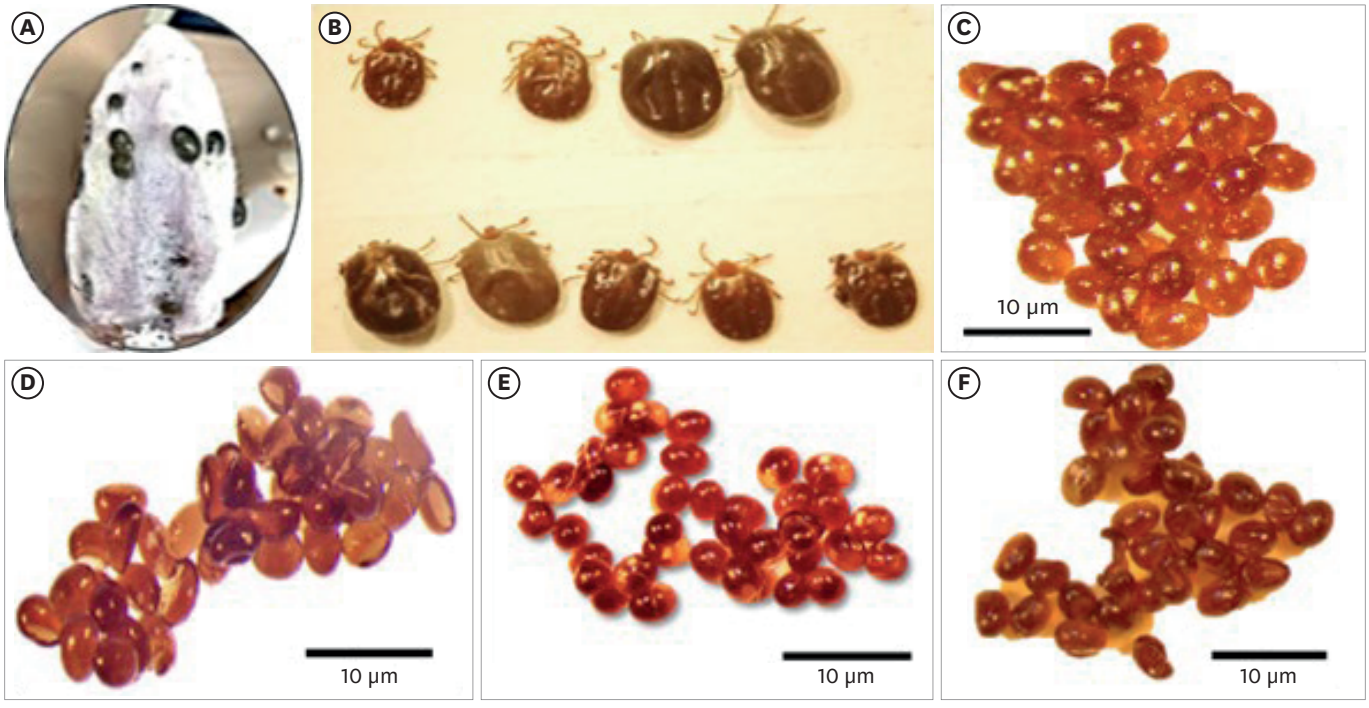

Fig. 4. Phenotypic changes in tick (*Haemaphysalis longicornis*) engorgement and egg morphology after subolesin + enolase dsRNA treatment. (A) Control ticks (Injection buffer); (B) Subolesin+ enolase dsRNA-treated ticks after spontaneous drop-down; (C) Control tick eggs; (D) Abnormal eggs of subolesin + enolase dsRNA-treated ticks; (E) Control tick eggs containing embryos; (F) Subolesin+ enolase dsRNA-treated ticks laid abnormal eggs (some have no embryos). Scale bar = (C-F) 10 μm.
dsRNA, double stranded RNA.

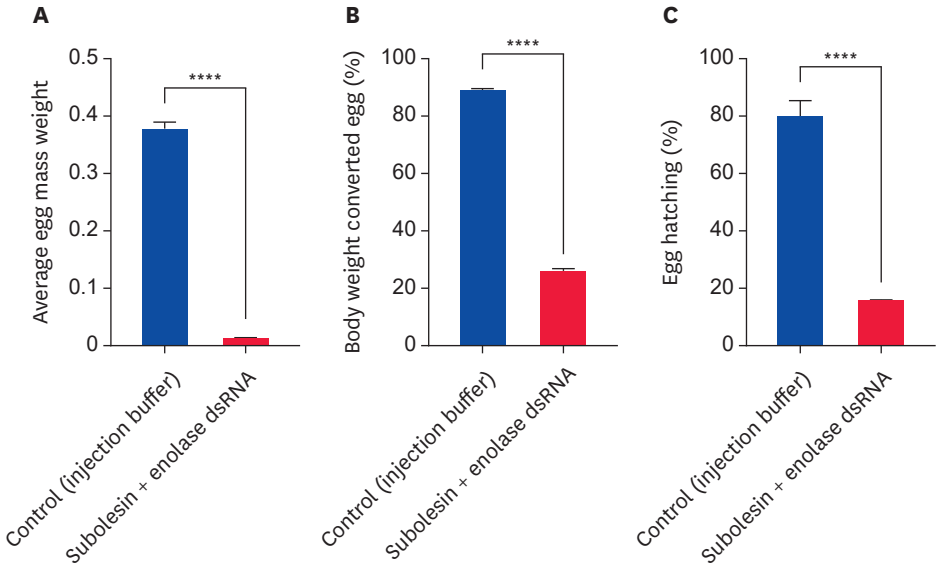

Fig. 5. Effects of subolesin+ enolase dsRNA on tick engorgement and reproduction. (A) Average egg mass weight in the treated group and the control group (injection buffer); (B) Average egg conversion ratio; (C) Average egg hatching ratio among treated group compared with the control group as determined by a Student's *t*-test. Data are presented as median ± SD.
dsRNA, double stranded RNA.
****$p < 0.0001$.

development begins when *Ixodid* ticks engage in bloodsucking [31]. Several functional proteins and peptides, including vasodilators, anticoagulants, inhibitors of platelet aggregation, and immunomodulators, proteinase inhibitors such as oxidation/detoxification proteins, and heme/iron metabolism-related proteins, are present in the saliva of ticks. Consequently, research into tick salivary genes is a prospective area for the creation of a tick control method. Therefore, a promising area of research in the developing of a tick control method is managing the genes that regulate tick saliva. Tick salivary gene silencing can be accomplished effectively by the reverse RNAi [32]. In previous studies there have been earlier reports of the Interacting effects of tick subolesin combination with Bm 86, Bm 91, and voraxin silencing in several tick species [23,33]. Previous studies showed that in *R. microplus*, *I. scapularis*, *R. haemaphysaloides, Dermacentor marginatus,* and *D. variabilis*, silencing of subolesin by RNAi caused the rate of larval engorgement, the rate of attachment and body weight of engorged nymphs, the rate of attachment and engorgement of adults, and the weight of the eggs per female tick were considerably reduced as well as an increase in mortality [34]. Subolesin functions in the digestion, reproduction, and development of ticks by acting as a tick- protective antigen. Subolesin orthologs could have a role in the regulation of developmental processes in other animals [23]. The impact of subolesin knockdown on tick infestation was examined in *Dermacentor variabilis* [35]. Previous study showed that subolesin knockdown in ticks by RNAi resulted in lowering the tick innate immunity that leads to pathogen infection [36]. subolesin knockdown impairs blood engorgement and consequent egg production. However, no study has been conducted regarding the combined effect of enolase and subolesin knockdown. The present study intended to determine whether subolesin and knockdown affected on feeding and reproduction in the *H. longicornis.*

Recently discovered salivary enolase, another significant salivary molecule, is crucial during the initial stages of blood feeding. Recombinant enolase immunization of mice reduced attachment and engorgement in *H. longicornis*, indicating their potential application in the

future as a component of a as part of a cocktail of vaccine antigens [37]. Enolase converts D-2-phosphoglycerate and phosphoenolpyruvate in the reversible manner in glycolysis and gluconeogenesis, two metabolic processes that are essential for cellular function [38]. RNAi experiments and immunization trials indicated that enolase could be also involved in the regulation of tick reproduction, suggesting new potential control strategies. As a conserved gene, enolase is important for tick blood feeding [39].

Enolase genes in the tick *Haemaphysalis flava* offer opportunity to create a possible antigen target for tick management. The putative enolase from *Ixodes ricinus* and the putative enolase from *Ornithodoros moubata* showed 83.3% and 85% amino acid similarity in the enolase, respectively [32]. According to our research enolase knockdown inhibits blood engorgement and consequent egg production. The combined impact of silencing by subolesin and enolase has not yet been studied. By targeting essential molecules within ticks, scientists aim to interfere with the ticks' ability to feed on hosts, reproduce, or transmit pathogens. This approach has the potential to provide an effective means of controlling tick populations and reducing the incidence of tick-borne diseases.

Overall, *R. microplus* infestations earlier study suggest that multi-antigenic molecule may be a promising approach for improving control efficacy against *R. microplus* and similar pathogens [40]. The most promising tick control method involves using protective antigens from *R. microplus* to create an antitick vaccination. The enolase shared an amino acid identity of 85.0% with that of *H. flava*, 81.1% with *I. ricinus*, and 81.3% with *O. moubata* [39]. knockdown of enolase by RNAi may affect tick reproduction, indicating that this may be a novel strategy for managing tick populations [41]. By describing the effect of loss of gene expression on tick physiology, RNAi enables research of gene function. RNAi in the tick species *Rhipicephalus sanguineus* to assess the roles of the genes encoding the tick protective antigens 4D8 and Rs86, a homolog of Bm86, in tick attachment, survival, feeding, and oviposition were all reduced when 4D8 was silenced. Silencing of the Rs86 gene similarly decreased tick weight and oviposition, however the impact was less pronounced [23]. *Amblyomma americanum* failure to engorge and lay eggs in females fed with males injected with a combination of subolesin and voraxin dsRNA [33].

In our present study, the silencing of subolesin and enolase by RNAi yielded a significant ($p < 0.05$) reduction of engorgement weight compared with that of the control group. Furthermore, subolesin dsRNA–injected ticks showed abrogated egg production. While enolase is expressed in all developmental stages, it is expressed the highest in the rapid blood feeding period of tick [42]. Silencing of enolase and subolesin in *H. longicornis* resulted in a significant reduction of body weight. Enolase and subolesin both play important roles in blood intake. To ascertain their roles in blood intake as well as their impact on egg production, we silenced both of these salivary antigens. Consequently, it might be a significant immuno-dominant protein whose inhibitory activity could reduce tick feeding and the spread of diseases caused by ticks. By targeting essential molecules within ticks, scientists aim to interfere with the ticks' ability to feed on hosts, reproduce, or transmit pathogens. This approach has the potential to provide an effective means of controlling tick populations and reducing the incidence of tick-borne diseases. These ticks also exhibited significant reductions of engorgement weight, egg mass, and egg conversion ratio compared with the control group ($p < 0.05$) (**Table 1**).

In the current study, enolase ds RNA-injected ticks showed a reduction of 30% enolase transcript level. These ticks also exhibited significant reductions of engorgement weight, egg mass, and egg conversion ratio compared with the control group ($p < 0.05$). Tick egg

development is hindered by interference with cholesterol metabolism, and the eggs become vulnerable to bacterial colonization [43].

In our present study a combined knockdown in our study significantly slowed down the weight of the engorgement, the mass of the eggs, and the egg conversion ratio. In conclusion, our findings imply that subolesin and enolase could be valuable components of a future vaccine antigen cocktail. we found that using multiple antigens are more effective than vaccines using just one antigens. This study was the first to show the effects of knocking down two different proteins subolesin and enolase, in a tick. This may lead to the use of as vaccine antigens. Subolesin and enolase research into a vaccine strategy will be conducted in the future. which will advance our knowledge of the topic and help us create tick control strategies. Therefore, our research results suggest that subolesin and enolase could be important components of a future vaccine antigen cocktail.

SUPPLEMENTARY MATERIALS

Supplementary Data 1
Calculation of enolase and subolesin PCR efficiency.

Supplementary Data 2
Detail formula and calculation effect of knock down subolesin and enolase.

Supplementary Table 1
Primer use in this study

Supplementary Fig. 1
Detection of salivary enolase subolesin by semi-quantitative RT-PCR. RT-PCR was performed using gene-RT-PCR. (A) Lane 1 shows a 100-bp plus DNA ladder; lane 2, salivary gland, enolase 247 bp; (B) Lane 1 indicates a 100 bp DNA ladder; lane 2, subolesin 396 bp; and lane 3, actin 540 bp.

Supplementary Fig. 2
Impact of subolesin + enolase dsRNA interference on salivary gland under microscope; (A) Salivary gland from a control tick; (B) Salivary gland from a subolesin + enolase dsRNA-treated tick showing a smaller size; (C) Showing inactive salivary gland. Scale bar = (A, B) 10 μm: (C) 100 μm.

REFERENCES

1. Wikel SK. Ticks and tick-borne infections: complex ecology, agents, and host interactions. Vet Sci. 2018;5(2):60. PUBMED | CROSSREF
2. Peter RJ, Van den Bossche P, Penzhorn BL, Sharp B. Tick, fly, and mosquito control--lessons from the past, solutions for the future. Vet Parasitol. 2005;132(3-4):205-215. PUBMED | CROSSREF
3. Rahman MK, Kim B, You M. Molecular cloning, expression and impact of ribosomal protein S-27 silencing in *Haemaphysalis longicornis* (Acari: Ixodidae). Exp Parasitol. 2020;209:107829. PUBMED | CROSSREF
4. Vijayakumar S, Vaseeharan B, Malaikozhundan B, Divya M, Abhinaya M, Gobi N, et al. Ecotoxicity of Musa paradisiaca leaf extract-coated ZnO nanoparticles to the freshwater microcrustacean *Ceriodaphnia cornuta*. Limnologica. 2017;67:1-6. CROSSREF

5. Benelli G, Pavela R. Repellence of essential oils and selected compounds against ticks-a systematic review. Acta Trop. 2018;179:47-54. PUBMED | CROSSREF
6. Bhowmick B, Han Q. Understanding tick biology and its implications in anti-tick and transmission blocking vaccines against tick-borne pathogens. Front Vet Sci. 2020;7:319. PUBMED | CROSSREF
7. Remil Linggatong G, Rika US, Masami M, Kozo F, Tetsuya T. RNA interference – a powerful functional analysis tool for studying tick biology and its control. In: Abdurakhmonov IY, editor. *RNA Interference*. Rijeka: IntechOpen; 2016.
8. Heath A. Biology, ecology and distribution of the tick, *Haemaphysalis longicornis* Neumann (Acari: Ixodidae) in New Zealand. N Z Vet J. 2016;64(1):10-20. PUBMED | CROSSREF
9. Teng KF, Jiang ZJ. *Economic Insect Fauna of China Fasc 39 Acari: Ixodidae*. Beijing: Science Press; 1991.
10. Zhuang L, Du J, Cui XM, Li H, Tang F, Zhang PH, et al. Identification of tick-borne pathogen diversity by metagenomic analysis in *Haemaphysalis longicornis* from Xinyang, China. Infect Dis Poverty. 2018;7(1):45. PUBMED | CROSSREF
11. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, et al. The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. J Infect Dis. 2014;209(6):816-827. PUBMED | CROSSREF
12. Kim M, Kim JY, Yi MH, Lee IY, Yong D, Jeon BY, et al. Microbiome of *Haemaphysalis longicornis* tick in Korea. Korean J Parasitol. 2021;59(5):489-496. PUBMED | CROSSREF
13. Šimo L, Kazimirova M, Richardson J, Bonnet SI. The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. Front Cell Infect Microbiol. 2017;7:281. PUBMED | CROSSREF
14. Kazimírová M, Jancinová V, Petríková M, Takác P, Labuda M, Nosál' R. An inhibitor of thrombin-stimulated blood platelet aggregation from the salivary glands of the hard tick *Amblyomma variegatum* (Acari: Ixodidae). Exp Appl Acarol. 2002;28(1-4):97-105. PUBMED | CROSSREF
15. Liu XY, de la Fuente J, Cote M, Galindo RC, Moutailler S, Vayssier-Taussat M, et al. IrSPI, a tick serine protease inhibitor involved in tick feeding and Bartonella henselae infection. PLoS Negl Trop Dis. 2014;8(7):e2993. PUBMED | CROSSREF
16. Avilán L, Gualdrón-López M, Quiñones W, González-González L, Hannaert V, Michels PA, et al. Enolase: a key player in the metabolism and a probable virulence factor of trypanosomatid parasites-perspectives for its use as a therapeutic target. Enzyme Res. 2011;2011:932549. PUBMED | CROSSREF
17. Piast M, Kustrzeba-Wójcicka I, Matusiewicz M, Banaś T. Molecular evolution of enolase. Acta Biochim Pol. 2005;52(2):507-513. PUBMED | CROSSREF
18. Haque A, Ray SK, Cox A, Banik NL. Neuron specific enolase: a promising therapeutic target in acute spinal cord injury. Metab Brain Dis. 2016;31(3):487-495. PUBMED | CROSSREF
19. de la Torre-Escudero E, Manzano-Román R, Pérez-Sánchez R, Siles-Lucas M, Oleaga A. Cloning and characterization of a plasminogen-binding surface-associated enolase from Schistosoma bovis. Vet Parasitol. 2010;173(1-2):76-84. PUBMED | CROSSREF
20. Díaz-Ramos A, Roig-Borrellas A, García-Melero A, López-Alemany R. α-Enolase, a multifunctional protein: its role on pathophysiological situations. J Biomed Biotechnol. 2012;2012:156795. PUBMED | CROSSREF
21. Pérez-Sánchez R, Oleaga A, Siles-Luca M, Díaz-Martín V, Torre-Escudero EDL, Hernández-González A, et al. Aplicación de ecualizadores de proteínas para la identificación de antígenos minoritarios de la saliva. Ornithodoros Moubata. 2010;5:143-145.
22. Jarmey JM, Riding GA, Pearson RD, McKenna RV, Willadsen P. Carboxydipeptidase from Boophilus microplus: a "concealed" antigen with similarity to angiotensin-converting enzyme. Insect Biochem Mol Biol. 1995;25(9):969-974. PUBMED | CROSSREF
23. de la Fuente J, Almazán C, Blas-Machado U, Naranjo V, Mangold AJ, Blouin EF, et al. The tick protective antigen, 4D8, is a conserved protein involved in modulation of tick blood ingestion and reproduction. Vaccine. 2006;24(19):4082-4095. PUBMED | CROSSREF
24. Kocan KM, Manzano-Roman R, de la Fuente J. Transovarial silencing of the subolesin gene in three-host ixodid tick species after injection of replete females with subolesin dsRNA. Parasitol Res. 2007;100(6):1411-1415. PUBMED | CROSSREF
25. Rahman MK, Saiful Islam M, You M. Impact of subolesin and cystatin knockdown by RNA interference in adult female *Haemaphysalis longicornis* (Acari: ixodidae) on blood engorgement and reproduction. Insects. 2018;9(2):9. PUBMED | CROSSREF
26. Tirloni L, Islam MS, Kim TK, Diedrich JK, Yates JR 3rd, Pinto AF, et al. Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study. Parasit Vectors. 2015;8(1):338. PUBMED | CROSSREF
27. Bullard RL, Williams J, Karim S. Temporal gene expression analysis and RNA silencing of single and multiple members of gene family in the lone star tick *Amblyomma americanum*. PLoS One. 2016;11(2):e0147966. PUBMED | CROSSREF

28. Kocan KM, Blouin E, de la Fuente J. RNA interference in ticks. J Vis Exp 2011;(47):2474. PUBMED

29. Haimes J, Kelley M. *Demonstration of a ΔΔCq Calculation Method to Compute Relative Gene Expression From qPCR Data*. Waltham: Thermo Scientific; 2010, p 1.

30. Rodríguez-Mallon A, Fernández E, Encinosa PE, Bello Y, Méndez-Pérez L, Ruiz LC, et al. A novel tick antigen shows high vaccine efficacy against the dog tick, *Rhipicephalus sanguineus*. Vaccine. 2012;30(10):1782-1789. PUBMED | CROSSREF

31. Ren S, Zhang B, Xue X, Wang X, Zhao H, Zhang X, et al. Salivary gland proteome analysis of developing adult female *Haemaphysalis longicornis* ticks: molecular motor and TCA cycle-related proteins play an important role throughout development. Parasit Vectors. 2019;12(1):613. PUBMED | CROSSREF

32. Lee SH, Li J, Moumouni PF, Okado K, Zheng W, Liu M, et al. Subolesin vaccination inhibits blood feeding and reproduction of *Haemaphysalis longicornis* in rabbits. Parasit Vectors. 2020;13(1):478. PUBMED | CROSSREF

33. Smith A, Guo X, de la Fuente J, Naranjo V, Kocan KM, Kaufman WR. The impact of RNA interference of the subolesin and voraxin genes in male Amblyomma hebraeum (Acari: ixodidae) on female engorgement and oviposition. Exp Appl Acarol. 2009;47(1):71-86. PUBMED | CROSSREF

34. Lu P, Zhou Y, Yu Y, Cao J, Zhang H, Gong H, et al. RNA interference and the vaccine effect of a subolesin homolog from the tick *Rhipicephalus haemaphysaloides*. Exp Appl Acarol. 2016;68(1):113-126. PUBMED | CROSSREF

35. Kocan KM, Zivkovic Z, Blouin EF, Naranjo V, Almazán C, Mitra R, et al. Silencing of genes involved in Anaplasma marginale-tick interactions affects the pathogen developmental cycle in *Dermacentor variabilis*. BMC Dev Biol. 2009;9(1):42. PUBMED | CROSSREF

36. Zivkovic Z, Torina A, Mitra R, Alongi A, Scimeca S, Kocan KM, et al. Subolesin expression in response to pathogen infection in ticks. BMC Immunol. 2010;11(1):7. PUBMED | CROSSREF

37. Haque MS, Islam MS, You MJ. Efficacy of recombinant enolase as a candidate vaccine against *Haemaphysalis longicornis* tick infestation in mice. Parasites Hosts Dis. 2023;61(4):439-448. PUBMED | CROSSREF

38. Díaz-Martín V, Manzano-Román R, Oleaga A, Encinas-Grandes A, Pérez-Sánchez R. Cloning and characterization of a plasminogen-binding enolase from the saliva of the argasid tick *Ornithodoros moubata*. Vet Parasitol. 2013;191(3-4):301-314. PUBMED | CROSSREF

39. Xu XL, Yang H. Molecular cloning and expression analysis of enolase in the *Rhipicephalus microplus* (Acari: ixodidae). J Med Entomol. 2021;58(6):2540-2546. PUBMED | CROSSREF

40. Parizi LF, Reck J Jr, Oldiges DP, Guizzo MG, Seixas A, Logullo C, et al. Multi-antigenic vaccine against the cattle tick *Rhipicephalus (Boophilus) microplus*: a field evaluation. Vaccine. 2012;30(48):6912-6917. PUBMED | CROSSREF

41. de la Fuente J, Kocan KM. The impact of RNA interference in tick research. Pathogens. 2022;11(8):827. PUBMED | CROSSREF

42. Xu XL, Cheng TY, Yang H. Enolase, a plasminogen receptor isolated from salivary gland transcriptome of the ixodid tick *Haemaphysalis flava*. Parasitol Res. 2016;115(5):1955-1964. PUBMED | CROSSREF

43. Xavier MA, Brust FR, Waldman J, Macedo AJ, Juliano MA, da Silva Vaz I Jr, et al. Interfering with cholesterol metabolism impairs tick embryo development and turns eggs susceptible to bacterial colonization. Ticks Tick Borne Dis. 2021;12(6):101790. PUBMED | CROSSREF