Parasitol Res (2007) 100:1411–1415
DOI 10.1007/s00436-007-0483-1

SHORT COMMUNICATION

# Transovarial silencing of the subolesin gene in three-host ixodid tick species after injection of replete females with subolesin dsRNA

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Received: 13 December 2006 / Accepted: 26 January 2007 / Published online: 14 February 2007


**Abstract** RNA interference (RNAi) has become the most powerful experimental tool for the study of gene function in ticks. Subolesin, initially called 4D8, was found to be protective against tick infestations when used as a vaccine and was shown to be highly conserved among ixodid tick species at the nucleotide and protein levels. RNAi caused systemic silencing of subolesin and demonstrated that this protein is involved in regulation of tick feeding, reproduction, and development. Recently, these results were extended to the one-host tick *Rhipicephalus* (*Boophilus*) *microplus* in which injection of dsRNA into replete females resulted in transovarial silencing of subolesin expression in eggs and larvae. Herein, we report transovarial silencing of subolesin by RNAi in the three-host ticks, *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis*. Silencing of subolesin expression by RNAi in these tick species also affected subolesin expression in eggs and larvae. Transovarial RNAi appears to be a common mechanism in ixodid ticks and provides a simple method for the rapid characterization of tick genes involved in oviposition, embryogenesis, and larval development.

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## Introduction

Ticks are ectoparasite vectors of wild and domestic animals and humans (Parola and Raoult 2001; Peter et al. 2005), and the control of tick infestations and tick-borne pathogens remains a challenge for human and animal health worldwide (de la Fuente and Kocan 2006; Willadsen 2006; Sonenshine et al. 2006). Some tick species such as *Rhipicephalus* (*Boophilus*) *microplus* complete their life cycle while feeding on a single host. For other tick species, including *Amblyomma americanum*, *Dermacentor variabilis*, and *Ixodes scapularis*, each stage (larvae, nymphs, and adults) feeds on a separate host. Control of three-host tick infestations is therefore difficult because of the need to target control measures to multiple hosts.

Molecular tools for characterization and manipulation of ticks are an important component of research toward development of novel tick control strategies. RNA interference (RNAi) is a nucleic acid-based reverse genetic approach that causes silencing of gene expression, thus contributing to the characterization of gene function and phenotypic effect (Fire et al. 1998). The genetic characterization of ticks by RNAi was first reported by Aljamali et al. (2002) and has rapidly become the most widely used gene-silencing technique in ticks and other organisms where alternative approaches for genetic manipulation are not available or are unreliable (de la Fuente and Kocan 2006).

Subolesin, initially called 4D8, was recently discovered in *I. scapularis* by use of expression library immunization in combination with sequence analysis of expressed sequence tags in a mouse model of larval infestations (Almazán et al. 2003). Subolesin was shown to be highly conserved at the nucleotide and protein levels among ixodid tick species (Almazán et al. 2005; de la Fuente et al. 2006a). RNAi experiments provided evidence that sub-

olesin is involved in the regulation of tick feeding, reproduction, and development (de la Fuente et al. 2005, 2006a–d; Nijhof et al. 2007). In addition, silencing of subolesin caused a reduction in *Anaplasma marginale* and *A. phagocytophilum* infections in *D. variabilis* and *I. scapularis*, respectively, thus reducing tick vector capacity (de la Fuente et al. 2006d).

Recently, Nijhof et al. (2007) demonstrated that RNAi of subolesin was transovarial in the one-host tick *R.* (*Boophilus*) *microplus* following injection of replete females with subolesin dsRNA. Subolesin gene silencing occurred in both eggs and larvae.

In this study, we report transovarial silencing of subolesin by RNAi in the three-host ticks, *A. americanum*, *D. variabilis*, and *I. scapularis*. Our studies and those of Nijhof et al. (2007) suggest that transovarial RNAi may be a common mechanism in ixodid ticks and provide a simple method for the rapid characterization of ixodid tick genes involved in oviposition, embryogenesis, and larval development.

## Materials and methods

*Ticks* *A. americanum*, *D. variabilis*, and *I. scapularis* adults were obtained from the laboratory colony maintained at the Oklahoma State University, Tick Rearing Facility. Off-host ticks were maintained in a 12-h light/12-h dark photoperiod at 22–25°C and 95% relative humidity. Female ticks were fed to repletion on cattle and, then, used for these studies. Cattle were housed at the Center for Veterinary Health Sciences with the approval and supervision of the Oklahoma State University Institutional Animal Care and Use Committee.

*Injection of replete female ticks with dsRNA* Subolesin species-specific dsRNA was prepared as reported previously using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA; de la Fuente et al. 2006a). Replete female ticks ($n$=3) of *A. americanum*, *D. variabilis*, and *I. scapularis* were injected in the left spiracle within 6 h post-repletion with 5 μl of dsRNA ($3\times10^{10}$–$1\times10^{11}$ molecules per microliter) while the ticks placed ventral side up on double sticky tape. The injections were done with a Hamilton syringe with a 1 in., 33-gauge needle. Ticks ($n$=2 for *A. americanum* and *I. scapularis* and $n$=1 for *D. variabilis*) were injected with an equal volume of injection buffer (10 mM Tris–HCl, pH 7.0, 1 mM ethylenediamine tetraacetic acid) to serve as controls.

*Analysis of tick oviposition and fertility* After injection, the replete females were held in a humidity chamber and allowed to oviposit. Eggs oviposited by each tick during the entire oviposition period were mixed and pooled for analysis. Oviposition was evaluated by determination of egg mass weights produced by individual ticks. Approximately one fourth of the egg mass was used for RNA extraction at 4–5 days post-oviposition. The remaining eggs were held in the humidity chamber until hatching for evaluation of fertility by calculation of the percent larvae that hatched from the eggs. Larvae were collected for RNA extraction immediately after hatching.

*Analysis of subolesin expression* Total RNA was isolated from pooled guts and salivary glands dissected from individual replete ticks 57 days after injection and from eggs and larvae using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. Subolesin expression was analyzed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using species-specific oligonucleotide primers for *A. americanum*, 4D8R5: 5′-GC TTGCGCAACATTAAAGCGAAC-3′ and 4D833: 5′-TT TGGTCGTACGTAAACTTGACAAATGTG-3′; *D. variabilis*, DV4D8RT5: 5′-CCAGCCTCTGTTCACCTTTC-3′ and DV4D8RT3: 5′-CCGCTTCTGAATTTGGTCAT-3′; and *I. scapularis*, IS4D8RT5: 5′-AGCAGCTCTGCTTCTCGT CT-3′ and IS4D8RT3: 5′-TCGTACTCGTCGCGTATC TG-3′. The tick 16S rRNA (approx. 215 bp) was analyzed using oligonucleotide primers T16S5 (5′-GACAAGAA GACCCTA-3′) and T16S3 (5′-ATCCAACATCGAGGT-3′). Control reactions were performed using the same procedures but without RNA added to control contamination of the PCR reaction. Real-time RT-PCR was performed with the RNA samples and gene-specific primers described above using the QuantiTec SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) and a Bio-Rad iCycler IQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer's recommendations. Amplification efficiencies were normalized against tick 16S rRNA using the comparative Ct method. Real-time RT-PCR experiments were repeated twice with similar results.

*Light microscopy studies of tick eggs* Samples of eggs were collected from individual eggs masses at 54 days after females were injected with subolesin dsRNA or buffer alone, placed on a microscope slide in a drop of immersion oil and cover-slipped. The eggs were examined in a light microscope and photographed with a 3-chip digital camera (Spot Diagnostics, Sterling Heights, MI, USA).

*Statistical analysis* The weight of replete ticks before injection, the egg mass weights, and tick fertility were compared between subolesin dsRNA and saline-injected *A. americanum* and *I. scapularis* ticks by Student's $t$-test ($P$=0.05).

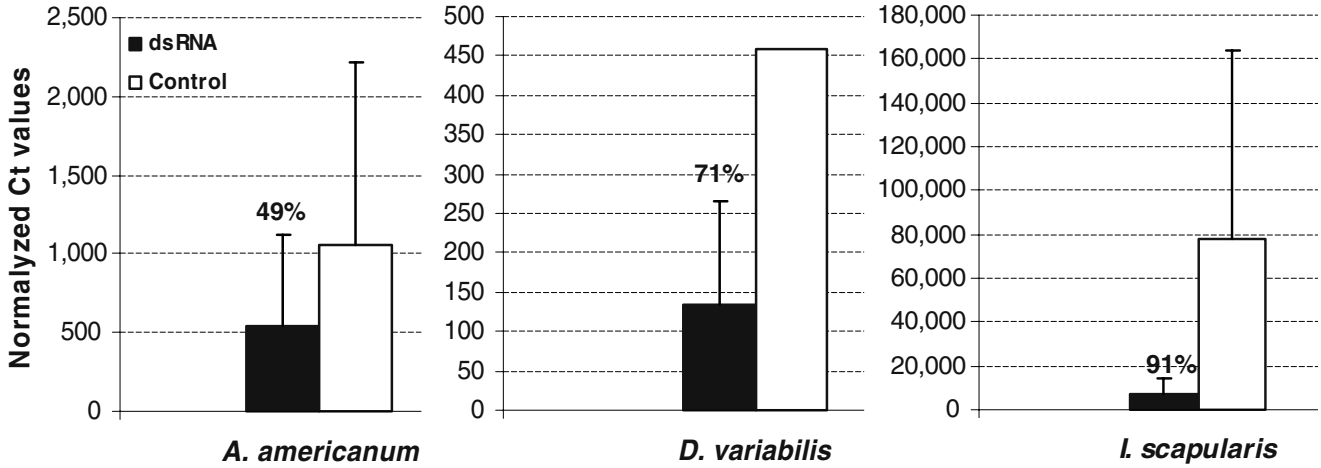


**Fig. 1** Analysis of subolesin expression after the injection of subolesin dsRNA into replete female ticks. Subolesin mRNA levels were determined by real-time RT-PCR in ticks 57 days after injection of species-specific subolesin dsRNA (*black bars*) or injection buffer alone (*white bars*). The percent of subolesin expression silencing (+SD) is indicated. Amplification efficiencies were normalized against tick 16S rRNA using the comparative Ct method

## Results and discussion

The experiments described here were done for the preliminary evaluation of the possibility of using transovarial RNAi in *A. americanum*, *D. variabilis*, and *I. scapularis* and to corroborate in three-host ticks the results of Nijhof et al. (2007) in experiments with *R.* (*Boophilus*) *microplus*. Therefore, the experiments were done with a small number of replete female ticks. However, for *A. americanum* and *I. scapularis*, the results were validated by statistical analysis.

The injection of replete *A. americanum*, *D. variabilis*, and *I. scapularis* females with species-specific subolesin dsRNA resulted in gene expression silencing of 49, 71, and 91%, respectively, as determined by real-time RT-PCR at 57 days after injection (Fig. 1). Subolesin expression was also reduced in *I. scapularis* and *D. variabilis* eggs (Fig. 2a) and larvae (Fig. 2b) derived from dsRNA-injected females. *A. americanum* females injected with subolesin dsRNA did not oviposit (Table 1), a result that we reported

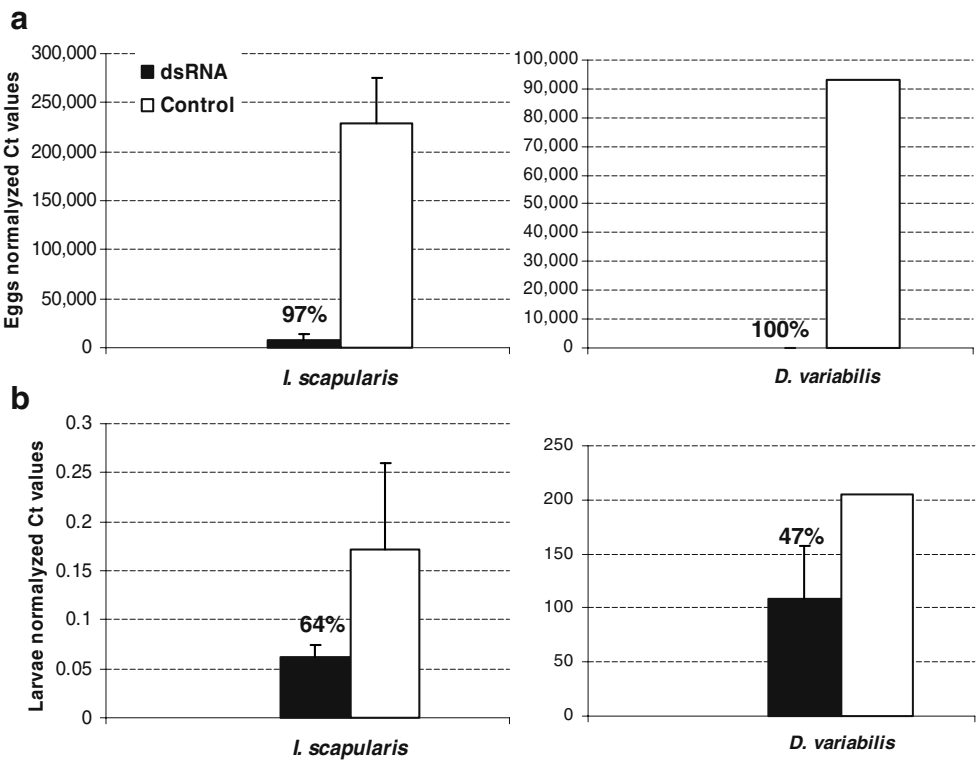


**Fig. 2** Analysis of subolesin expression in eggs and larvae after the injection of replete females with subolesin dsRNA. Subolesin mRNA levels were determined by real-time RT-PCR in tick eggs (**a**) and larvae (**b**) after injection of species-specific subolesin dsRNA (*black bars*) or injection buffer alone (*white bars*). The percent of subolesin expression silencing (+SD) is indicated. Amplification efficiencies were normalized against tick 16S rRNA using the comparative Ct method

**Table 1** Effect of subolesin silencing by RNAi on tick oviposition and fertility

| Biological parameter | *A. americanum* | | *D. variabilis* | | *I. scapularis* | |
|---|---|---|---|---|---|---|
| | dsRNA | Control | dsRNA | Control | dsRNA | Control |
| Tick weight before injection (g) | 0.45±0.25 | 0.33±0.01 | 0.50±0.07 | 0.54 | 0.28±0.03 | 0.21±0.13 |
| Egg mass weight/tick (mg) | 0±0* | 110±14 | 22±22 | 163 | 20±4** | 70±25 |
| Fertility (%) | No eggs laid | 94±6 | 5±7 | 70 | 27±15** | 92±4 |

Three replete female ticks per group were injected with subolesin dsRNA. Control ticks ($n$=2 for *A. americanum* and *I. scapularis* and $n$=1 for *D. variabilis*) were injected with an equal volume of injection buffer. Oviposition was evaluated by weighting the egg masses produced by each tick. Fertility was evaluated by calculating the percent of eggs that produced larvae. The weight of replete ticks before injection, the weight of egg masses and tick fertility were compared between subolesin dsRNA and saline injected *A. americanum* and *I. scapularis* ticks by Student's $t$-test (*$P$<0.005, **$P$<0.05).

previously when dsRNA was injected into unfed females (de la Fuente et al. 2006a).

The silencing of subolesin expression by RNAi affected oviposition, embryogenesis of the eggs, and larval hatching (Table 1 and Fig. 3). The effect of subolesin RNAi on oviposition apparently was not related to female weight differences because the weights of replete female before injection with buffer or subolesin dsRNA were similar (Table 1). Eggs oviposited by subolesin dsRNA-injected females did not develop or embryonate normally as compared with the controls (Fig. 3). These results were similar to those reported by Nijhof et al. (2007) in experiments with *R.* (*Boophilus*) *microplus* and corroborate that subolesin is involved in embryogenesis (de la Fuente et al. 2006a). Fertility was reduced by 93 and 71% in *D. variabilis* and *I. scapularis*, respectively, which most likely resulted from impaired embryogenesis (Table 1).

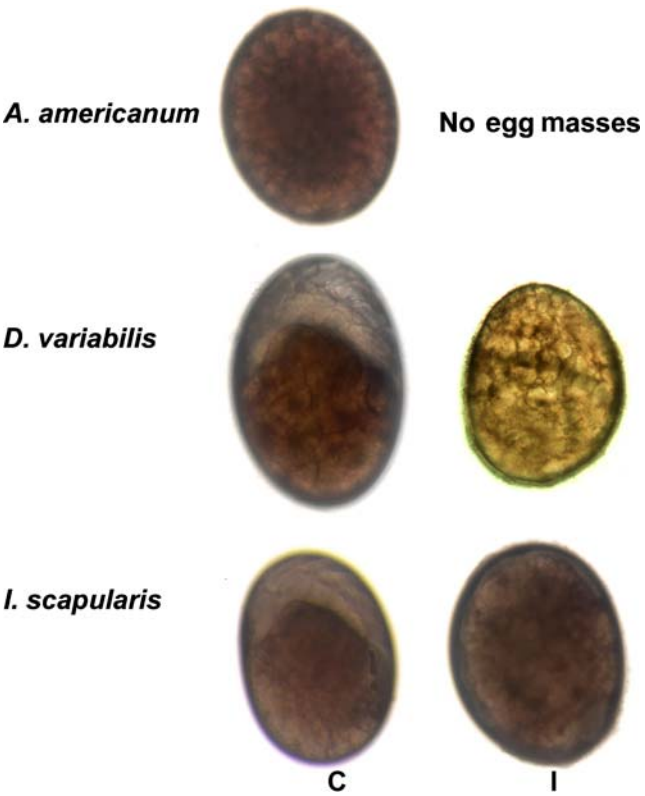


**Fig. 3** Tick eggs photographed at 54 days after females were injected with subolesin dsRNA or injection buffer alone. Eggs from the control females (*C*) were embryonated, whereas eggs from subolesin dsRNA injected females were undifferentiated (*I. scapularis*) or small and discolored (*D. variabilis*). *A. americanum* females injected with subolesin dsRNA did not oviposit

Previous experiments demonstrated systemic RNAi in ticks after injection of dsRNA into unfed ticks, and the resulting gene silencing occurred in multiple tick tissues (de la Fuente et al. 2006a,c; Nijhof et al. 2007). The results reported herein extend those of Nijhof et al. (2007) from the one-host tick *R.* (*Boophilus*) *microplus* to the three-host ticks *A. americanum*, *I. scapularis*, and *D. variabilis*, suggesting that the mechanism of transovarial RNAi may be common for all ixodid tick species. However, Nijhof et al. (2007) did not observe gene silencing in second generation adults. Furthermore, as shown herein, silencing of subolesin expression appears to be higher in the eggs than in the larvae (Fig. 2). Collectively, these results suggest that, although systemic and transovarial RNAi occurs in ticks, gene silencing is reduced as ticks undergo development, probably, as a result of slow dilution of gene silencing factors. Modification of gene expression in the nucleus, shown to be a mechanism of long-term gene silencing by RNAi in *Caenorhabditis elegans* (Vastenhouw et al. 2006), may therefore not occur in ticks.

RNAi has become the most powerful experimental tool for the study of gene function in ticks. Therefore, improved methods for RNAi in ticks would further enhance future applications for tick research. Transovarial RNAi by injection of replete females with dsRNA of the one-host tick *R.* (*Boophilus*) *microplus*, as established by Nijhof et al. (2007) and, subsequently, in three-host ticks reported in this research, provides a method for the rapid characterization of ixodid tick gene function in oviposition, embryogenesis, and larval development.

**Acknowledgments** This research was supported by the Oklahoma Agricultural Experiment Station (project 1669), the Sitlington Endowed Chair for Food Animal Research (K. M. Kocan, Oklahoma State University). Dr. Raúl Manzano-Roman was funded by Ministerio de Educación y Ciencia, Spain. These experiments comply with the current laws of the U.S.A.

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