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Gene Expression of Tissue-Specific Molecules in Ex vivo *Dermacentor variabilis* (Acari: Ixodidae) During Rickettsial Exposure

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Abstract

Ticks serve as both vectors and the reservoir hosts capable of transmitting spotted fever group *Rickettsia* by horizontal and vertical transmission. Persistent maintenance of *Rickettsia* species in tick populations is dependent on the specificity of the tick and *Rickettsia* relationship that limits vertical transmission of particular *Rickettsia* species, suggesting host-derived mechanisms of control. Tick-derived molecules are differentially expressed in a tissue-specific manner in response to rickettsial infection; however, little is known about tick response to specific rickettsial species. To test the hypothesis that tissue-specific tick-derived molecules are uniquely responsive to rickettsial infection, a bioassay to characterize the tick tissue-specific response to different rickettsial species was used. Whole organs of *Dermacentor variabilis* (Say) were exposed to either *Rickettsia montanensis* or *Rickettsia amblyommii*, two *Rickettsia* species common, or absent, in field-collected *D. variabilis*, respectively, for 1 and 12 h and harvested for quantitative real time-polymerase chain reaction assays of putative immune-like tick-derived factors. The results indicated that tick genes are differently expressed in a temporal and tissue-specific manner. Genes encoding glutathione S-transferase 1 (*dvgst1*) and Kunitz protease inhibitor (*dvkpi*) were highly expressed in midgut, and rickettsial exposure downregulated the expression of both genes. Two other genes encoding glutathione S-transferase 2 (*dvgst2*) and *β*-thymosin (*dvβ-thy*) were highly expressed in ovary, with *dvβ-thy* expression significantly downregulated in ovaries exposed to *R. montanensis*, but not *R. amblyommii*, at 12-h postexposure, suggesting a selective response. Deciphering the tissue-specific molecular interactions between tick and *Rickettsia* will enhance our understanding of the key mechanisms that mediate rickettsial infection in ticks.

Keywords

tissue-specific; tick immunity; backless tick; *Dermacentor variabilis*; *Rickettsia*

Tick-borne rickettsial diseases (TBRD) are caused by several species of bacteria within the genera *Anaplasma*, *Ehrlichia*, and *Rickettsia*. In the United States, human infection by

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Rickettsia spp. now accounts for ≈50% of the cases of TBRD reported in 2008 (Dumler 2010). The spotted fever group (SFG), *Rickettsia*, contains well-recognized rickettsial pathogens, such as *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever, as well as more recently recognized pathogens, including *Rickettsia parkeri* (Paddock et al. 2004). These rickettsial pathogens are transmitted to vertebrate host during tick bloodmeal acquisition. Often any feeding life cycle stage (larva, nymph, and adult) is capable of transmitting SFG *Rickettsia*, as the infection in ticks is also vertically maintained throughout the entire life cycle. Interestingly, intensive field surveys have coincided with increased recognition of a number of SFG *Rickettsia* in ticks that are not associated with human infection (Stromdahl et al. 2010). Some of these species were reported to function in an endosymbiotic manner (Simser et al. 2005), and their presence in tick populations may influence pathogen transmission by that population. For example, in *Dermacentor andersoni*, the vertically maintained rickettsial endosymbiont, *Rickettsia peacockii* (formerly East Side agent), influenced introduction of *R. rickettsii* into tick populations and limited transovarial transmission of *R. rickettsii* (Burgdorfer et al. 1981). Subsequently, laboratory experiments demonstrated that *Dermacentor variabilis* (Say) infected with *Rickettsia montanensis*, a *Rickettsia* species commonly associated with wild-caught *D. variabilis*, was refractory to secondary infection with *Rickettsia rhipicephali*, an SFG *Rickettsia* infrequently associated with *D. variabilis* (Macaluso et al. 2002). In addition, laboratory-infected *D. variabilis* were unable to maintain *R. rhipicephali* through multiple generations via transovarial transmission. Combined, these studies suggest that successful transovarial transmission of *Rickettsia* is dependent on the nature of the tick and *Rickettsia* relationship (Macaluso et al. 2002); however, contributing elements that mediate vector competence of ticks for rickettsial transmission are undetermined.

A molecular response has been described in SFG *Rickettsia* infections of ticks with a number of putative tick-derived immune molecules being identified in *R. montanensis*-infected *D. variabilis* (Macaluso et al. 2003, Mulenga et al. 2003, Macaluso et al. 2006). For example, glutathione S-transferase 1 and 2 genes (*dvgst1* and *dvgst2*) were identified from *R. montanensis*-infected *D. variabilis* using subtractive hybridization (Mulenga et al. 2003) and homolog cloning (Dreher-Lesnick et al. 2006). Transcription of *dvgst1* and *dvgst2* was upregulated during blood feeding, or *R. montanensis*-challenge, and downregulated when ticks were challenged with *Escherichia coli* (Mulenga et al. 2003, Dreher-Lesnick et al. 2006). More recently, *D. variabilis* Kunitz protease inhibitor (DvKPI) was identified as a novel antimicrobial molecule that limits rickettsial colonization in tick midgut during initial infection by an intimate association with rickettsiae (Ceraul et al. 2008, 2011). A *D. variabilis* mRNA sequence for *β*-thymosin (Dv*β*-Thy), an invertebrate-antimicrobial molecule (Gai et al. 2009, Schillaci et al. 2010), is available in GenBank; however, the function of *β*-thymosin (*β*-Thy) during rickettsial infection has not been characterized. Although most factors were identified in a model representing a commonly reported natural tick and *Rickettsia* pairing, little is known about the specific response of ticks to uncommon rickettsial infection.

Despite the sympatric distribution of multiple tick and SFG *Rickettsia* species, the persistent transmission of *Rickettsia* appears limited to species-specific tick and *Rickettsia* relationships. In contrast, cell culture systems (both tick and vertebrate derived) demonstrate a wide host range for many species of SFG *Rickettsia* (Kurtti et al. 2005, Baldridge et al. 2010), suggesting that infection is not a limitation of an individual rickettsial species' ability to infect a host cell. Thus, the underlying molecular mechanisms involved in successful rickettsial infection of ticks are potentially tick derived. Tissue-specific responses in ticks correlated to successful horizontal and vertical transmission of SFG *Rickettsia* in tick populations may be a critical element of successful transmission. Toward an understanding of this interaction, the objective of the current study was to quantify the differential tick

response to SFG *Rickettsia* in a temporal and tissue-specific manner. A quantitative real time-polymerase chain reaction (qRT-PCR) was developed to estimate expression of *D. variabilis* putative immune-like genes (*dvgst1*, *dvgst2*, *dvkpi*, and *dvβ-thy*) in a rickettsial infection bioassay using *Rickettsia* species common (*R. montanensis*) and absent (*Rickettsia amblyommii*) in *D. variabilis* in nature. Using a whole tick model, both tissue-specific and SFG *Rickettsia*-specific responses were identified.

Materials and Methods

Ticks

Rickettsia-free D. variabilis colony was routinely maintained using rats (*Rattus norvegicus*) for immature tick feeding and New Zealand White rabbits (*Oryctolagus cunniculus*) for adult feeding. All animals were handled according to the Old Dominion University's Institutional Animal Care and Use Committee.

Rickettsia

R. amblyommii strain Darkwater (provided by Dr. C. Paddock, CDC Atlanta, GA) and *R. montanensis* strain M5/6 were grown and maintained in Vero E6 cells in Dulbecco's modified medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT) at 34°C and 5% CO_2. For rickettsial purification, infected Vero E6 cells were detached using a cell scraper and transferred to Erlenmeyer flasks containing sterile 3-mm borosilicate glass beads (Sigma, St. Louis, MO). The infected cells were lysed by vortex at high speed for 3 min, and the cell lysate was filtered through a sterile two micron syringe filter (Whatman, Clifton, NJ). Viability of rickettsiae was determined using a *Bac*Light viability stain kit (Molecular Probes, Carlsbad, CA), and rickettsiae were counted using a Petroff–Hausser bacteria counting chamber (Kurtti et al. 2005, Sunyakumthorn et al. 2008).

Tissue-Specific Expression of Tick Immune Genes During Rickettsial Infection

To determine the tissue-specific expression of tick immune genes (*dvgst1*, *dvgst2*, *dvkpi*, and *dvβ-thy*) in unfed *D. variabilis*, five female ticks were dissected to collect salivary glands, midgut, and ovary. The tick tissues were kept in 100 μl RNA*Later* (Ambion, Austin, TX) at −20°C until RNA extraction.

An ex vivo bioassay of *D. variabilis* tissues (backless tick explant) was modified from previously described protocols (Bell 1980, Sunyakumthorn et al. 2012). Briefly, unfed female *D. variabilis* were cleaned with 70% ethanol and 10% benzalkonium chloride solution, and rinsed with sterile water three times. The ticks were placed on sterile filter paper in a biological safety cabinet. To remove the tick's dorsal cuticle, ticks were excised along the perimeter of alloscutum (Fig. 1). The backless ticks were incubated 34°C in 200 μl of complete L15B medium in a 96-well plate (Corning, Corning, NY). After 24 h, backless ticks were divided to three groups (10 ticks per group); the first group, unexposed, was incubated in 200 μl L15B medium, the second and third groups were exposed to *R. amblyommii* or *R. montanensis* (1.2×10^6 rickettsiae/μl), respectively. After 1- and 12-h postexposure (hpe), the tick tissues (five ticks per group per time point) were collected, and similar tissues were pooled into 100 μl RNA*Later* (Ambion, Austin, TX) and kept at −20°C until RNA extraction. Two independent experiments were performed.

RNA Extraction and qRT-PCR Assay

Total RNA was extracted using the RNasey Mini kit (Qiagen, Valencia, CA) and digested with (4 U per reaction) DNase Turbo (Ambion, Austin, TX). Total RNA (62.5 ng) was used

for cDNA synthesis in 25 μl total volume of iScript reverse transcription kit (Bio-Rad, Hercules, CA). A no-RT reaction (distilled water was added instead of reverse transcriptase) was included to confirm the absence of genomic DNA. PCR reaction reagents were mixed in 96-well plates containing 2 μl of cDNA template, 2X iTaq SYBR Green Supermix ROX (Bio-Rad, Hercules, CA), 100 μM each forward and reverse primers (Table 1) in a total volume of 35 μl per reaction. Ten microliters of each reaction mixture were transferred into three wells of 384-well plates and amplification occurred in an ABI 7900HT unit (Applied Biosystems, Foster City, CA) using SDS v2.3 software. *Actin* gene was used as internal control gene as previously described (Sunyakumthorn et al. 2012). Data for each sample were calculated as the difference in threshold cycle (C_T) value ($\Delta C_T = C_T$*dvactin* − C_T*tick immune gene*).

Statistical Analysis

The analysis of variance (ANOVA) was conducted using the SAS statistical package (version 9.2) GLM procedure. For tissue-specific gene expression, the relative expression of tick immune genes in different tick tissues was examined for potential differences. For the analysis of tick immune response during rickettsial infection, the time effect was absorbed from a model. When overall significance was found, Tukey's honestly significant difference (HSD) post hoc test was performed to determine the pairwise difference of means of main effects of relative gene expression among *R. amblyommii*- and *R. montanensis*-exposed and unexposed backless ticks. *P* value of <0.05 was considered significant.

Results

Tissue-Specific Expression of Tick Immune Genes During Rickettsial Infection

The initial analysis of tissue-specific gene expression of tick immune genes (*dvgst1*, *dvkpi*, *dvgst2*, and *dvβ-thy*) in unfed *D. variabilis* tissues including salivary glands, midgut, and ovary were performed before rickettsial exposure. The results demonstrated that expression of *dvgst1* was significantly higher in tick midgut compared with salivary glands, and that the expression of *dvkpi* was significantly greater in the midgut, compared with salivary glands and ovary. However, *dvgst2* and *dvβ-thy* expression was significantly higher in tick ovary, compared with other tissues (Fig. 2).

In the tick salivary glands at 1 and 12 hpe, the expression of *dvkpi*, *dvgst1*, *dvgst2*, and *dvβ-thy* was low, compared with midgut and ovary, and no significant difference in *dvkpi*, *dvgst1*, *dvgst2*, and *dvβ-thy* expression was demonstrated when ticks were exposed to rickettsiae.

In the tick midgut, *dvgst1* expression was decreased in *Rickettsia*-exposed ticks compared with unexposed ticks; however, the decrease is only significantly different at one hpe when *dvgst1* expression was reduced 80 and 78% in *R. amblyommii*- and *R. montanensis*-exposed ticks, respectively (Fig. 3A). In addition, the expression of *dvkpi* in midgut was significantly decreased in *R. amblyommii*- (72.5% decrease) and *R. montanensis*-exposed ticks (63.4% decrease) at one hpe, and likewise at 12 hpe *dvkpi* expression was significantly downregulated when exposed to *R. amblyommii* (83.8% decrease) and *R. montanensis* (86.2% decrease) (Fig. 3B). However, there was no difference in *dvkpi* and *dvgst1* expression between *R. amblyommii*-and *R. montanensis*-exposed ticks, and no difference in *dvgst2* and *dvβ-thy* gene expression by tick midgut was shown during rickettsial exposure.

In the tick ovary, at 1 hpe, there was no difference in *dvgst2* and *dvβ-thy* expression when exposed to *Rickettsia*. The expression of *dvgst2* and *dvβ-thy* was decreased at 12 hpe in *R. montanensis-exposed* ticks compared with unexposed ticks; however, a significant

difference was demonstrated only in *dvβ-thy* expression (Fig. 3C and D). Sixty percent of *dvβ-thy* expression was decreased when ticks were exposed to *R. montanensis* compared with unexposed ticks, and when compared with *R. amblyommii-exposed* ticks the *dvβ-thy* expression was 47% lower than in *R. montanensis-exposed* ticks. Although we were able to detect the *dvkpi* and *dvgst1* mRNA in tick ovary, a significant difference in expression postexposure and between rickettsial species was not observed in *Rickettsia*-exposed ticks, compared with unexposed ticks.

Discussion

Ticks serve as vectors and reservoirs for both pathogenic and nonpathogenic SFG *Rickettsia;* however, the role of tick immunity in vector competence for *Rickettsia* has not been well characterized. In this study, we determined tissue-specific expression of selected tick immune genes and the tick response to *R. amblyommii* and *R. montanensis*, which represented *Rickettsia* species absent and common in *D. variabilis*, respectively, in a tissue-specific manner using an ex vivo tick model (Sunyakumthorn et al. 2012). The results demonstrated differential expression of target genes in a temporal and tissue-specific manner, and in some cases, as a *Rickettsia* species-specific response.

Tick midgut is the first site of contact between tick internal organs and host blood that contain rickettsiae. Many tick molecules related to blood digestion and immunity are highly expressed in tick midgut including antimicrobial peptides, protease inhibitors, proteases, and lectins (Sonenshine and Hynes 2008). In this study, *dvkpi* and *dvgst1* expression was significantly downregulated in tick midguts during the exposure to either *R. amblyommii* or *R. montanensis*, with no specific differential expression of *dvkpi* and *dvgst1* between the different rickettsial species. This suggests that infection with either *Rickettsia* species can result in downregulated tick immune molecules in unfed tick midguts, and that the initial midgut response to rickettsiae is nonspecies specific when exposed to *R. amblyommii* or *R. montanensis*. Exposure to either *Rickettsia* species suppresses transcription of glutathione S-transferase and Kunitz protease inhibitor. In contrast, the previous study of *Rickettsia*-challenged ticks demonstrated that the expression of *dvgst1*was increased in *R. montanensis*-infected *D. variabilis* compared with uninfected ticks (Mulenga et al. 2003), and *dvkpi* expression was upregulated in *D. variabilis* challenged with *R. montanensis* for 72 h (Ceraul et al. 2008). The differences between the current results and these two previous studies may lie in the use of partially fed ticks in which *dvkpi* and *dvgst1*expression may be induced during bloodmeal acquisition. Many immune tick molecules are upregulated during bloodmeal acquisition, for example, defensin1 (Ceraul et al. 2007), defensin2 (Ceraul et al. 2007), lysozyme (Ceraul et al. 2007), and GST1 and GST2 (Dreher-Lesnick et al. 2006) in *D. variabilis*. As the current study sought to capture the early tissue response to rickettsial exposure, independent of a bloodmeal, newly molted unfed ticks were used in the bioassay. Further assessment of the tick response at the midgut interface should use actual animal models of rickettsial infection to mimic natural acquisition of rickettsiae. However, for most rickettsial species, including the two used in the current study, a viable animal model for rickettsial transmission or acquisition by ticks is not available.

Horizontal and vertical transmission of SFG *Rickettsia* by ticks requires infection of organs, associated with egg production and feeding, ovary and salivary glands, respectively. Combined field observations and laboratory studies suggest a specific association between SFG *Rickettsia* and their tick hosts (Macaluso et al. 2002, Ammerman et al. 2004, Smith et al. 2010, Stromdahl et al. 2010), which in-part may be influenced by a transmission organ-specific response. A survey to ascertain the molecular basis of specificity identified a number of tick-derived molecules from tick ovaries infected with *R. montanensis* using differential-display (Macaluso et al. 2003) and subtractive hybridization PCR (Mulenga et

al. 2003) including receptor or adhesion molecules, tick immune and stress response factors, and tick–host interaction molecules. To determine the specific immune response of tick ovaries during exposure to *Rickettsia* species commonly identified, or not identified, in *D. variabilis*, the gene expression of selected tick molecules was determined. During *R. montanensis* exposure, *dvβ-thy* expression was significantly downregulated compared with unexposed and an equivalent dose of *R. amblyommii*. Although not specifically tested in the current study, a suppressed immune response in ovary during *R. montanensis* infection may contribute to successful transovarial transmission of this *Rickettsia* by *D. variabilis*. Similar to the results of the current study, *Rickettsia* species-specific differential transcript regulation was demonstrated for *D. variabilis* α-catenin in *R. montanensis*-exposed ticks at 12 hpe, but not in *R. amblyommii-exposed* ticks (Sunyakumthorn et al. 2012). The specific mechanism by which these two genes are regulated during rickettsial exposure and how they directly influence rickettsial infection requires further characterization.

During bloodmeal acquisition, tick salivary glands facilitate blood feeding and likely enhance pathogen transmission to vertebrate hosts. As ticks feed, many tick-derived genes are upregulated to produce factors that enhance blood flow and counter the host immune response (Francischetti et al. 2009, Anatriello et al. 2010, Zivkovic et al. 2010). In the current study, expression of tick immune genes in salivary glands of unfed ticks before and after SFG *Rickettsia* exposure was examined. The results demonstrated the low expression of all four genes—*dvkpi*, *dvgst1*, *dvgst2*, and *dvβ-thy*—in salivary glands compared with midgut and ovary. In addition, in contrast to what was identified in the ovary samples, no differential expression was observed when exposed to either species of SFG *Rickettsia*. Previous examination of gene expression in partially fed *D. variabilis* chronically infected with *R. montanensis* demonstrated that mRNA expression of three putative tick proteins including Ena or vasodilator-stimulated protein (VASP), tubulin α-chain, and Cu^{2+}–transporting ATPase were upregulated in salivary glands, compared with uninfected ticks (Macaluso et al. 2003). The distinct pattern(s) of differential regulation of tick factors associated with acute and chronic rickettsial infection may provide a better understanding of maintenance of *Rickettsia* spp. by ticks and the ecology of tick-borne *Rickettsia*.

To study the tick tissue-specific response during rickettsial infection, a modified rickettsial infection bioassay using tick tissue culture of backless ticks (Bell 1980) and primary tick tissue culture (Mosqueda et al. 2008) was used. Both techniques allow for tissue-specific analysis during rickettsial infection; however, in our hands, the backless tick technique proved advantageous, as there was higher recovery of tick tissues and an absence of nonspecific microbial contamination. In summary, this study demonstrated that tick transcription in response to rickettsial exposure can be tissue-specific and differential depending on the *Rickettsia* species. Deciphering the orchestration of tick immune molecules during rickettsial infection of ticks may provide insight into the molecular constituents of vector competence and requires further study.

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Fig. 1.
Ex vivo tick tissue culture (backless tick explant). Unfed female D. variabilis were cleaned, air-dried, and transversely cut along the perimeter of alloscutum. Tick dorsal cuticle was removed, and backless ticks were placed in 96-well plates containing 200 μl complete L15B medium for 24 h before rickettsial exposure. (Online figure in color.)

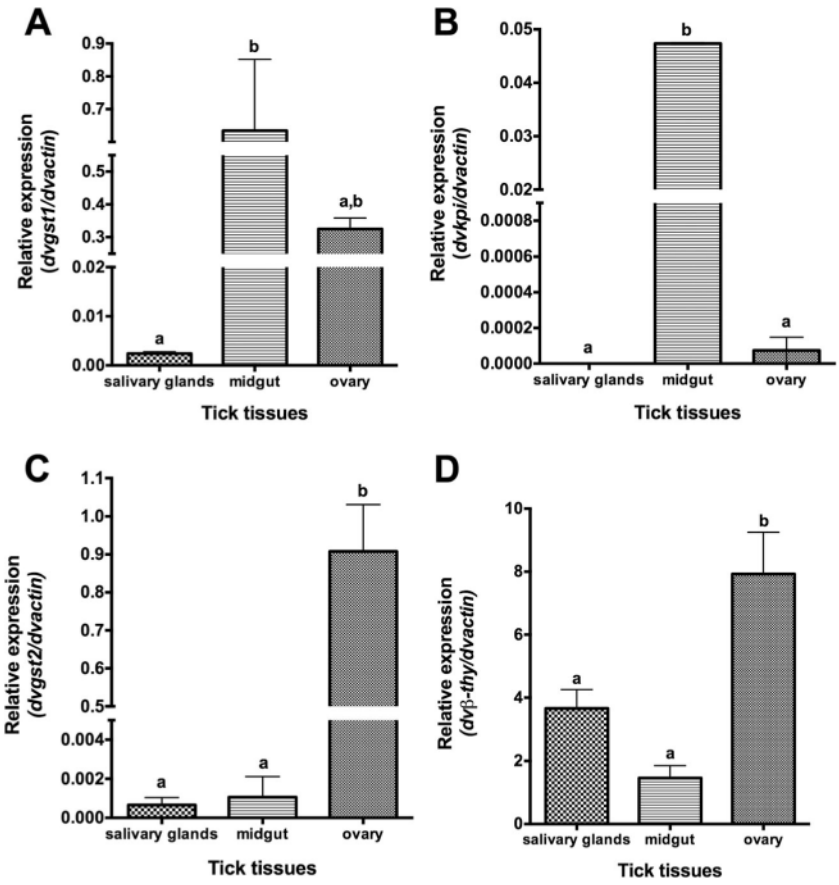

Fig. 2.

Tissue-specific expression of tick immune genes in unexposed ticks (A) *dvgst1*, (B) *dvkpi*, (C) *dvgst2*, and (D) *dvβ-thy*. Total RNA from unfed female *D. variabilis* tissues (salivary glands, midgut, and ovary) was subjected to qRT-PCR assay using specific primers. Transcription level of tick immune genes was normalized to *dvactin*. Data shown are mean relative expression. Error bar represents standard error of means, and the bars with same letter are not significantly different ($P < 0.05$).

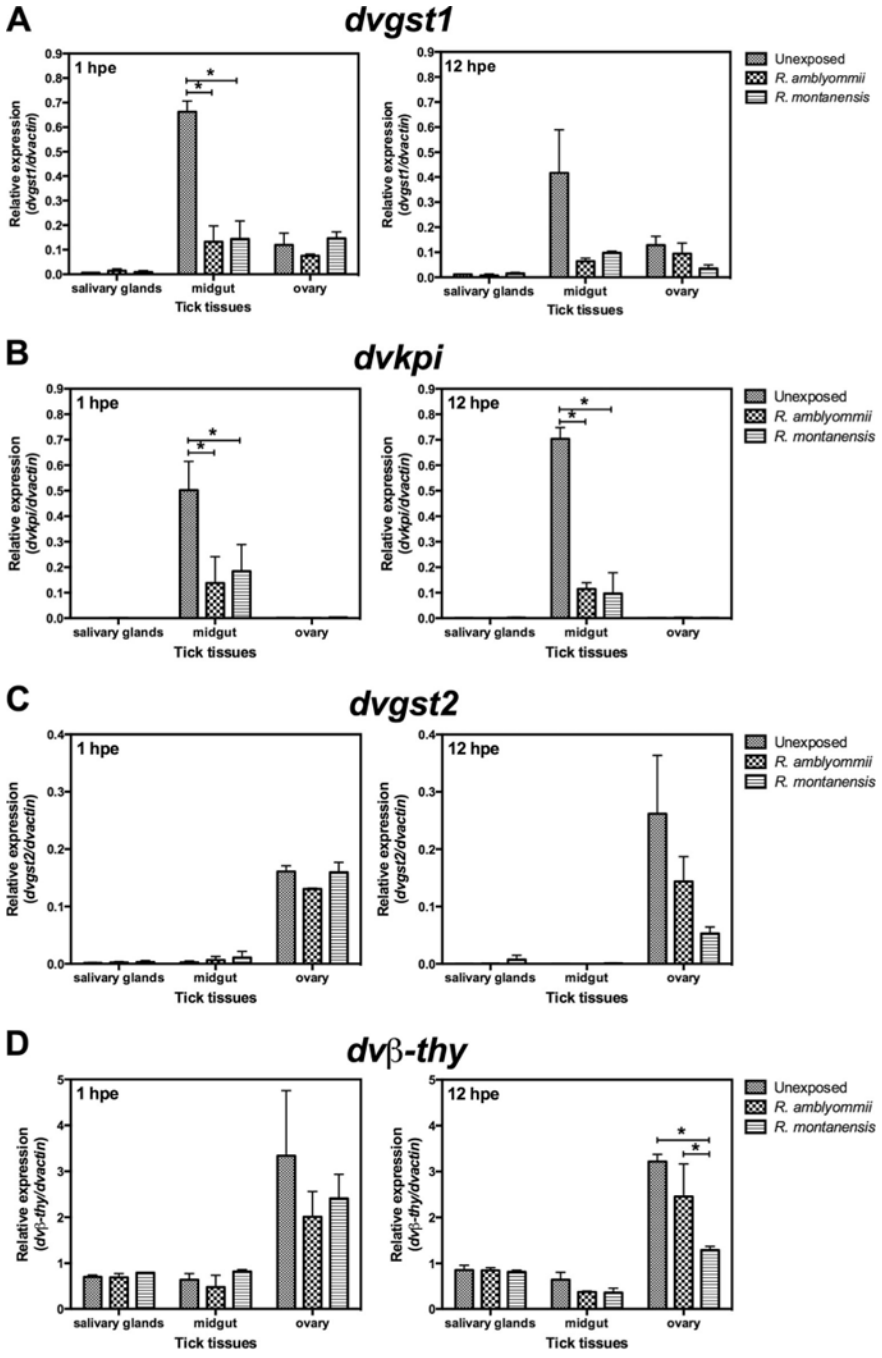

Fig. 3.

Tissue-specific response of tick immune genes in *Rickettsia* exposed ticks. (A) *dvgst1*, (B) *dvkpi*, (C) *dvgst2*, and (D) *dvβ-thy*. Backless ticks were exposed to *R. amblyommii* or *R. montanensis* for 1 and12 h, and tick tissues (salivary glands, midgut, and ovary) were dissected and subjected to qRT-PCR assay using specific primers. Transcription level of tick immune genes was normalized to *dvactin*. Data shown are mean relative expression. Error bar represents standard error of means, and the asterisk indicates significant difference ($P < 0.05$).

Table 1

Primers for qRT-PCR amplification of tick immune genes

Primer	Sequence (5′-3′)	Reference
DvKPIFor	CGAAGAATCAGAGTGCTGGAGAAC	Ceraul et al. 2007
DvKPIRev	CCGAGGTGGTTTTTAGGTCCTG	
DvGST1-416For	TATTTCCGGCCAAAGTGGTT	This study
DvGST1-590Rev	CCCAATCGCTACTCCCAGAG	
DvGST2-484For	AAGGCTGGAGCTCCTCATTG	This study
DvGST2-600Rev	ACAGGGTCCGCTGCAGTATT	
DvBthy-538For	CACAACCGATGCCAAGAGAA	This study
DvBthy-718Rev	GTTGATGAAAGGCTGCCACA	
DvActin-1424For	CTTTGTTTTCCCGAGCAGAG	Sunyakumthorn et al. 2012
DvActin-1572Rev	CCAGGGCAGTAGAAGACGAG	