2007

Tissue and Life Stage Distribution of a Defensin-Like Gene in the Lone Star Tick Amblyomma americanum

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Tissue and life stage distribution of a defensin-like gene in the Lone Star tick, *Amblyomma americanum*.

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Keywords: *Amblyomma*, defensin, innate immunity

Running title: *Amblyomma americanum* defensin gene

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Abstract. The transcript sequence of the *Amblyomma americanum* (Acari: Ixodidae) defensin, termed amercin (*amn)*, has been ascertained and a 219bp *amn* coding region identified. The gene encodes a 72 amino acid prepropeptide with a putative 37 amino acid mature peptide. Sequence comparisons with other tick defensins reveal *amn* to be 6bp shorter than the *Ixodes scapularis* Linnaeus (Acari: Ixodidae) and *Dermacentor variabilis* (Say) (Acari: Ixodidae) defensin sequences. The translated peptide would be two amino acids shorter than these other defensins with one amino acid less in the prepro- region and the other resulting in a shorter mature peptide. The amercin prepropeptide has 60.8% similarity with the *I. scapularis* prepropeptide and 59.5% similarity with the *D. variabilis* prepropeptide. The mature amercin peptide has 73.7% similarity with the mature *I. scapularis* peptide and 71.1% similarity with the *D. variabilis* peptide. In *A. americanum*, defensin transcript was found in the midgut, fat body, and salivary gland tissues as well as the hemocytes. Defensin transcript was also present in early stage eggs (less than 48hrs old), late stage eggs (approximately two weeks old), larvae, and nymphs of *A. americanum* and *I. scapularis*, both of which are vector competent for *Borrelia* spirochetes.

Introduction

The Lone Star tick, *Amblyomma americanum*, inhabits mainly the southeastern United States, although its territory extends as far north as Connecticut where it overlaps with that of the deer tick, *Ixodes scapularis*. These two ticks harbor similar pathogens which cause human illnesses with similar symptoms. *A. americanum* transmits *Ehrlichia chaffeensis*, which causes human monocytic ehrlichiosis (HME), while *I. scapularis* harbors the causative agent of human granulocytic ehrlichiosis (HGE) (Ijdo *et al.*, 2000), *Borrelia burgdorferi*, the causative agent of Lyme disease, and *Babesia microti*, the causative agent of the malaria-like human babesiosis (Sonenshine, 1993). *A. americanum* has recently been found to transmit *Borrelia lonestari*, the etiological agent of Southern tick-associated rash illness (STARI) (James *et al.*, 2001). This illness, although not yet well-characterized, has symptoms similar to Lyme Disease caused by *Borrelia burgdorferi* (Oliver *et al.*, 1998). Little research has been conducted on *B. lonestari* because it has only recently been cultured (Varela *et al.*, 2004). Conversely, the infection process of *B. burgdorferi* has been well studied and characterized.

Arthropods lack the highly developed adaptive immune system of vertebrates and rely, instead, on an innate immune system to combat invading pathogens. This innate immunity system consists of cellular and soluble responses. Cellular responses include phagocytosis, encapsulation, and nodulation which engulf or recruit pathogens in preparation for destruction. Typically these immune cells recognize pathogen-associated-molecular-patterns (PAMPs) such as lipopolysacharide, peptidoglycan, or other bacterial structural components. Soluble responses include the release of

antimicrobial peptides that exhibit cytotoxic effects on invading bacteria (Ceraul *et al.*, 2003).

Defensins are one family of antimicrobial peptides possessed by both vertebrates and invertebrates, which destroy invading bacteria by introducing voltage-dependent channels into bacterial membranes (Cociancich *et al.*, 1993; Saido-Sakanaka *et al.*, 1999). Defensin peptides are synthesized as prepropeptides, with the mature peptide usually ranging from 29 to 34 amino acids in length, and are approximately 4kDa in size (Cociancich *et al.*, 1993; Gillespie *et al.*, 1997). Insect and arthropod mature defensins possess six cysteine residues in their primary structure, which form three disulphide bonds. Disruption of the disulfide bridges results in loss of antimicrobial activity. The relative spacing of the cysteines and the placement of the disulphide bonds is conserved among the arthropod defensins (Cociancich *et al.*, 1993).

Within an organism antimicrobial peptides, including defensins, are expressed in tissues that closely contact invading microorganisms (Hancock and Scott, 2000). Most pathogenic bacteria, including *Borrelia* species, gain entry to the vector through the digestive system during the intake of a blood meal from an infected mammalian host. The *Borrelia* spirochetes then attach to the midgut epithelium (Matsuo *et al.*, 2004; Pal *et al.*, 2004). When an infected tick initiates another feeding, the spirochetes penetrate the midgut tissue and gain access to the hemolymph. They migrate through the hemolymph to the salivary glands where they can be transferred to the new host through salivary secretions. The hemolymph also allows the pathogens access to various tick tissues (Johns *et al.*, 2001a; Matsuo *et al.*, 2004) .

The main producer of defensin in insects is the fat body (Hoffmann, 1997), the site of PAMP recognition. Defensin is produced primarily in the hemocytes and fat body of *Boophilus microplus* (Fogaca *et al.*, 2004); defensin D of *Ornithodorus moubata* is also strongly expressed in the fat body (Nakajima *et al.*, 2002b). Isoforms A, B, and C of *O. moubata* are primarily expressed in the midgut. In *I. scapularis* and *Dermacentor variabilis*, defensin seems to be produced mainly by the hemocytes (Ceraul, 2005). It has been proposed that hemocytes from these ticks store defensin until needed. On challenge they then secrete the peptides into the hemolymph (Ceraul *et al.*, 2003). Due to both the role of the fat body in recognizing invading pathogens and the close contact *Borrelia* have with the midgut, hemocytes, and salivary glands, these tissues are prime sites for defensin expression.

We hypothesize that differences in defensin expression between competent and non-competent vectors for *Borrelia* allow only non-competent vectors to destroy invading spirochetes and prevent transmission to a new host. Tissue distribution and life stage distribution studies were performed on *A. americanum* and compared with *I. scapularis*, both competent vectors, to determine differences in defensin expression between these ticks and non-competent vectors.

Materials and Methods

Amblyomma americanum were supplied by Oklahoma State University, Stillwater, Oklahoma. *Ixodes scapularis* were collected from the Pine Ridge State Park, near Armonk, New York, U.S.A. Ticks were reared on New Zealand white rabbits (*Oryctologus cunniculus*) as described previously (Sonenshine, 1993). All animal

procedures were carried out in accordance with the Old Dominion University Institutional Animal Care and Use Committee approved protocol 04-001.

Whole tick tissue, which excluded cuticle and appendages, was collected under RNase-free conditions from *A. americanum* females fed for approximately one week. A messenger RNA extraction was performed with the QuickPrep micro mRNA Purification Kit as per the manufacturer's instructions (Amersham, Biosciences, Piscatasay, NJ, U.S.A). The mRNA was treated with DNA-free according to the manufacturer's instructions (Ambion, Austin, TX, U.S.A). Primers (Table 1) were obtained from IDT (Coralville, IA, U.S.A) if not supplied with a kit. Reverse transcription with an Oligo dT primer was performed with the Im-Prom II Reverse Transcriptase Kit (Promega, Madison, WI, U.S.A.) with a 5 min incubation at 25°C, 1 hr at 37°C, and 15 min at 70°C. Reverse-transcription polymerase chain reaction (RT-PCR) with Oligo dT and Is2b, a gene-specific primer corresponding to the beginning of a consensus sequence for mature defensin, was performed using Platinum Supermix HiFi (Invitrogen) with the following program: initial denaturation 1 min at 94°C; denaturation 1 min at 94°C; annealing 1 min at 42°C; extension 1 min at 72°C; 34 cycles of denaturation, annealing, and extension; final extension 10 min at 72°C. This 3' amplified transcript was cloned into pCR4-TOPO and transformed into TOP 10 chemically competent *Escherichia coli* cells (Invitrogen Corp., Carlsbad, CA, U.S.A.). Transformants were screened for presence of insert by PCR using M13F and M13R primers. Colonies containing an insert, as shown by gel electrophoresis, were inoculated in liquid broth and incubated with shaking at 37°C overnight. Plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System (Promega) according to kit protocol. Plasmid DNA

was then sequenced with an M13F or M13R primer on an ABI Prism 310 Genetic Analyzer using the Big Dye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A).

The 5' portion of the transcript was obtained with the GeneRacer Kit according to the manufacturer's instructions (Invitrogen). Reverse transcription was performed as described in the kit using Amb-GSP2, a primer designed from the previously determined 3' sequence. Using Platinum Supermix HiFi (Invitrogen), the following RT-PCR program was performed with a nested Amb-GSP1 primer and a GeneRacer primer provided with the kit: initial denaturation 2 min at 94°C; denaturation 1 min at 94°C; annealing 1 min at 58°C; extension 1 min at 72°C; 34 cycles of denaturation, annealing, and extension; final extension 10 min at 72°C. The PCR product was cloned, transformed, and PCR screened. Transformants with an insert were sequenced as above on an ABI Prism 3130xl Genetic Analyzer using Big Dye Terminator v3.3 Sequencing Kit (Applied Biosystems). Sequences obtained from the 3' and 5' regions were overlain to obtain a full transcript sequence; gene specific primers were generated corresponding to the beginning and end of the gene encoding region (Amb-ATG and Amb-TAG, respectively). RT-PCR was performed using these primers on the previously obtained RT product with the PCR program used to ascertain the 5' sequence. PCR products were cloned, transformed, PCR screened, and sequenced as above on the 3130xl Genetic Analyzer.

I. scapularis and *A. americanum* early stage eggs, late stage eggs, larvae, and nymphs were collected under RNase-free conditions into the extraction buffer of the mRNA extraction kit. Midgut, fat bodies, and salivary glands were collected under

RNase-free conditions from *A. americanum* females fed for approximately one week. Tissues were collected directly into extraction buffer provided in the mRNA extraction kit. Hemolymph from *A. americanum* fed females was collected into PBS buffer and centrifuged 20 min at 4,000rpm. The plasma was removed and the hemocytes resuspended in extraction buffer provided in the mRNA extraction kit. Extraction of mRNA from individual tissues and lifestages was performed with the QuickPrep micro mRNA Purification Kit as per the manufacturer's instructions (Amersham). After extraction, the mRNA was treated with DNA-Free (Ambion) according to the manufacturers' instructions and then ethanol precipitated. Reverse transcription reactions were performed on each tissue type and life stage using the Im-Prom II Reverse Transcriptase Kit (Promega), an Oligo dT primer, and the RT program used for original sequence acquisition. The following PCR program was performed on RT product using the gene specific primers Amb-ATG and Amb-TAG for *A. americanum* and Ix-ATG and Ix-TAG for *I. scapularis*: initial denaturation 1 min at 94°C; denaturation 1 min at 94°C; annealing 1 min at 58°C for *A. americanum* or 50°C for *I. scapularis*; extension 1 min at 72°C; 34 cycles of denaturation, annealing, and extension; final extension 10 min at 72°C. PCR products were cloned, transformed, PCR screened, and sequenced as described above on the 3130xl Genetic Analyzer. Sequence analysis and alignments were performed with the Vector NTI Suite (Invitrogen) of programs.

The *A. americanum* defensin sequence obtained from hemocytes is located in GenBank as accession number DQ864986.

Results and Discussion

The recent discovery of *A. americanum* as a competent vector of *B. lonestari* has stressed the need to gain a better understanding of the interactions between this tick and the etiological agent of Southern tick-associated rash illness (STARI). However, much is known about *B. burgdorferi*, the causative agent of Lyme Disease. This well-characterized spirochete is transmitted to humans when an infected *I. scapularis* tick in the nymph or adult stage feeds on a human. Interestingly, the tick *D. variabilis* is not a competent vector for *B. burgdorferi* because it destroys the spirochetes before they can be transmitted to a host (Johns *et al.*, 2001a; Johns *et al.*, 2001b). Upon injection of *B. burdorferi* into both tick species, intact bacteria were noted in the hemolymph and organs of *I. scapularis* but bacteria were lysed in *D. variabilis* (Johns *et al.*, 2001a). Additionaly, hemolymph of *D. variabilis* infected with *B. burgdorferi* caused lysis of cultured spirochetes. However, hemolymph of *I. scapularis* infected with *B. burgdorferi* did not destroy the bacteria. This led to the hypothesis that defensin remains unexpressed in *I. scapularis* but is expressed in *D. variabilis* (Johns *et al.*, 2001b). To determine if such defensin expression variations are responsible for differences in vector competence, defensin transcript distribution was examined in *A. americanum* and *I. scapularis* ticks, which are competent vectors for *Borrelia* species. The transcript distribution in these ticks was compared to that of the non-competent vector *D. variabilis*.

The full-length transcript sequence of the *A. americanum* defensin from hemocytes was determined by 3' RT-PCR and 5' Gene RACE procedures. A 219bp gene encoding region was identified for defensin, termed by the authors amercin *(amn)*.

The amercin gene encodes a 72 amino acid prepropeptide with a putative 37 amino acid mature peptide. The sequence contains six cysteine residues, which align with the conserved cysteines of other arthropod and insect defensins. Amercin displays similarity to other tick and insect defensins (Tables 2 and 3). Nucleotide similarities with amercin range from 42.6% to 71.6% for preprodefensins and from 48.7% to 71.8% for mature defensins. Amercin protein similarities are 26.2% to 71.6% for preprodefensins and 42.1% to 73.7% for mature defensins.

The amercin nucleotide sequence is six base pairs shorter than the defensin genes of *I. scapularis* and *D. variabilis*, termed scapularisin (*sln*) and varisin (*vsn*), respectively. As such, the amercin peptide is two amino acids shorter than that of scapularisin and varisin, with one amino acid less in the mature region and one less in the prepro- region. The amercin prepropeptide has 60.8% amino acid similarity with the scapularisin prepropeptide and 59.5% similarity with the varisin prepropeptide. The mature amercin peptide has 73.7% amino acid similarity with mature scapularisin and 71.1% similarity with mature varisin. At the nucleotide level, mature amercin has 71.8% similarity with scapularisin and 67.5% similarity with varisin. These results indicate that the novel defensin of *A. americanum* is more similar to the defensin of the competent vector, *I. scapularis*, than to the non-competent vector, *D. variabilis*.

Excluding *A. americanum*, *Amblyomma hebraeum* is the only tick of this genus in which defensin has been found. This tick, which inhabits the subtropics of Africa, possesses two defensin proteins. Interestingly, both defensins, termed Amblyomma defensin peptide-1 and Amblyomma defensin peptide-2, are anionic (Lai *et al.*, 2004). Anionic antimicrobial peptides have been noted in mammals (Fales-Williams *et al.*,

2002), but no other anionic defensins have been recognized in ticks (Lai *et al.*, 2004). The amercin prepropeptide shared 29.1% amino acid similarity with Amblyomma defensin peptide-1 and 26.2% amino acid similarity with Amblyomma defensin peptide-2. The mature amercin peptide was 44.7% and 42.1% similar to Amblyomma defensin peptide-1 and peptide-2, respectively. At the nucleotide level, mature amercin was 48.7% and 52.1% similar to Amblyomma defensin peptide-1 and peptide-2, respectively. Thus, amercin appears less closely related to the *A. hebraeum* defensins than to the *D. variabilis* and *I. scapularis* defensins.

Amercin varies in length and sequence from other tick defensins as well as other arthropod defensins including insects, mussels, and beetles. The defensin gene of the hard tick *B. microplus* encodes a 97 amino acid prepropeptide containing a 38 amino acid mature peptide (Fogaca *et al.*, 2004). The soft tick *O. moubata* possesses four defensin isoforms each with 73 amino acids in the prepropeptide which includes a 19 amino acid signal peptide, a 17 amino acid pro- region, and a 37 amino acid mature peptide (Nakajima *et al.*, 2001). The bumble bee *Bombus pascuorum* mature defensin is 51 amino acids in length. Bee defensins are typically longer than other insect defensins due in part to twelve additional highly conserved amino acids at the C-terminus (Rees *et al.*, 1997). The defensin transcript of the mosquito *Aedes aegypti* contains a putative 40 amino acid mature peptide (Cho *et al.*, 1996). The defensin signal peptide of the mussel *Mytilus galloprovincialis* is only 21 amino acids in length, and the mature region is 39 amino acids with a 21 amino acid C-terminal addition, the function of which is unknown (Mitta *et al.*, 1999). The mussel *Mytilus edulis* possesses two defensin isoforms which are 37 amino acids and 35 amino acids in length (Charlet

et al., 1996). The Coconut rhinoceros beetle, *Orycetes rhinoceros*, defensin peptide is 79 amino acids in length with a 43 amino acid mature peptide (Ishibashi *et al.*, 1999). The mature defensin of the beetle *Zophob utrutus* is also 43 amino acids in length (Bulet *et al.*, 1991).

As shown in figures 1 and 2, amercin and scapularisin transcript were detected in all investigated life stages: early stage eggs (i.e. less than 48 hours old), late stage eggs (approximately two weeks), larvae, nymphs, and adults. These results did not match life stage transcript distribution in *D. variabilis* which lacks defensin transcript in early stage eggs (Ceraul, 2005).

Amercin transcript was identified in hemocytes, midgut, fat body, and salivary gland tissues of *A. americanum* as shown in figure 3. These findings parallel defensin tissue distribution in both *I. scapularis* (Hynes, 2005) and *D. variabilis* (Sonenshine *et al.*, 2002; Ceraul, 2005) apart from the salivary glands of *D. variabilis* which have not been studied for the presence of defensin transcript. The similar, if not identical, tissue distribution between the non-competent and competent vectors indicates that destruction of *Borrelia* in non-competent vectors may rely upon either post-transcriptional regulation of defensin or activity of another immunity protein or suite of proteins.

The two defensin isoforms of the mussel *M. galloprovincialis*, termed myticins, are manufactured and stored in the hemocytes (Mitta *et al.*, 1999). In many insect and arthropod vectors, microbial challenge initiates release of defensin from the hemocytes into the hemolymph. Increased levels of defensin are detected in the hemolymph of *O. moubata* (Nakajima *et al.*, 2003), *A. gambiae* (Vizioli *et al.*, 2001), and *O. rhinoceros*

(Ishibashi *et al.*, 1999) following microbial challenge. We suspect this is what occurs in *D. variabilis* but not in *I. scapularis* or *A. americanum*.

In addition to inducible expression in the hemolymph, often both constitutive and inducible expression occurs in the midgut. Defensin isoform C of *A. aegypti* is the only defensin isoform found in the midgut. Expression of this isoform is solely constitutive and is not upregulated in response to challenge. Defensin is synthesized constitutively in the midgut of *A. gambiae* (Vizioli *et al.*, 2001) but is also upregulated when the mosquito feeds on blood infected with *Plasmodium berghei* (Hoffmann, 1997).

Ornithodoros defensins A, B, C, and D are expressed constitutively in the midgut and fat body. Defensins A, B, and C display higher levels of detection in the midgut, whereas defensin D displays a higher level of expression in the fat body (Nakajima *et al.*, 2002a). Microbial challenge results in increased defensin expression in the midgut of *O. moubata* (Nakajima *et al.*, 2003).

Defensin is expressed in the hemocytes and fat body of *B. microplus*. This expression pattern is closest to that of the *Ornithodoros* defensin D isoform (Fogaca *et al.*, 2004). In *I. ricinus*, a gene that shows similarity to defensin is expressed in the midgut but not in the salivary glands or hemolymph (Rudenko *et al.*, 2005). Defensin expression in the fat body of *I. ricinus* has not yet been examined.

Defensin is only one of many proteins involved in protecting disease vectors from invading microbial pathogens. These defensin transcript studies have shed more light on defensin, one key protein in the vast network of immune-related proteins of arthropods. Further studies are needed to determine the vector-microbe relationship between *A. americanum* and *Borrelia* spirochetes. The present findings have identified

a defensin gene in this tick and have elucidated defensin transcript distribution in *A. americanum*. Further research should focus on post-transcriptional regulation of defensin in competent and non-competent vectors of *Borrelia* species. These studies and studies of other immune-related proteins will be helpful in understanding vector competence differences between *A. americanum*, *I. scapularis*, and *D. variabilis*.

Acknowledgements. This work was supported by a grant from the National Science Foundation (IBN 0212901), which is gratefully acknowledged.

References Works Cited

Bulet, P., S. Cociancich, J. L. Dimarcq, J. Lambert, J. M. Reichhart, D. Hoffmann, C. Hetru and J. A. Hoffmann (1991). Insect immunity. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family. *Journal of Biological Chemistry,* **266**, 24520-24525.

Ceraul, S. M. (2005). Defensin in the ticks *Dermacentor variabilis* and *Ixodes scapularis*. PhD Dissertation. Department of Biological Sciences, Old Dominion University, Norfolk, VA, USA.

Ceraul, S. M., D. E. Sonenshine, R. E. Ratzlaff and W. L. Hynes (2003). An arthropod defensin expressed by the hemocytes of the American dog tick, *Dermacentor variabilis* (Acari: Ixodidae). *Insect Biochemistry and Molecular Biology,* **33**, 1099-1103.

Charlet, M., S. Chernysh, H. Philippe, C. Hetru, J. A. Hoffmann and P. Bulet (1996). Innate immunity. Isolation of several cysteine-rich antimicrobial peptides from the blood of a mollusc, *Mytilus edulis. Journal of Biological Chemistry,* **271**, 21808-21813.

Cho, W. L., Y. C. Fu, C. C. Chen and C. M. Ho (1996). Cloning and characterization of cDNAs encoding the antibacterial peptide, defensin A, from the mosquito, *Aedes aegypti. Insect Biochemistry and Molecular Biology,* **26**, 395-402.

Cociancich, S., A. Ghazi, C. Hetru, J. A. Hoffmann and L. Letellier (1993). Insect defensin, an inducible antibacterial peptide, forms voltage-dependent channels in *Micrococcus luteus. Journal of Biological Chemistry,* **268**, 19239-19245.

Fales-Williams, A. J., K. A. Brogden, E. Huffman, J. M. Gallup and M. R. Ackermann (2002). Cellular distribution of anionic antimicrobial peptide in normal lung and during acute pulmonary inflammation. *Veterinary Pathology,* **39**, 706-711.

Fogaca, A. C., D. M. Lorenzini, L. M. Kaku, E. Esteves, P. Bulet and S. Daffre (2004). Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation, structural characterization and tissue expression profile. *Developmental & Comparative Immunology,* **28**, 191-200.

Gillespie, J. P., M. R. Kanost and T. Trenczek (1997). Biological mediators of insect immunity. *Annual Reveiw of Entomology,* **42**, 611-643.

Hancock, R. E. and M. G. Scott (2000). The role of antimicrobial peptides in animal defenses. *Proceedings of the National Acadamy of Sciences U.S.A.,* **97**, 8856-8861.

Hoffmann, J. A. (1997). Immune responsiveness in vector insects. *Proceedings of the National Acadamy of Sciences U.S.A.,* **94**, 11152-11153.

Hynes, W. L., Ceraul, S.M., Todd, S.M., Seguin, K.C. & Sonenshine, D.E. (2005). A defensin-like gene expressed in the black-legged tick, *Ixodes scapularis. Medical and Veterinary Entomology,* **19**, 339-344.

Ijdo, J. W., C. Wu, L. A. Magnarelli, K. C. Stafford, 3rd, J. F. Anderson and E. Fikrig (2000). Detection of *Ehrlichia chaffeensis* DNA in *Amblyomma americanum* ticks in Connecticut and Rhode Island. *Journal of Clinical Microbiology,* **38**, 4655-4656.

Ishibashi, J., H. Saido-Sakanaka, J. Yang, A. Sagisaka and M. Yamakawa (1999). Purification, cDNA cloning and modification of a defensin from the coconut rhinoceros beetle, *Oryctes rhinoceros. European Journal of Biochemistry,* **266**, 616-623.

James, A. M., D. Liveris, G. P. Wormser, I. Schwartz, M. A. Montecalvo and B. J. Johnson (2001). *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. *Journal of Infectious Diseases,* **183**, 1810-1814.

Johns, R., J. Ohnishi, A. Broadwater, D. E. Sonenshine, A. M. De Silva and W. L. Hynes (2001a). Contrasts in tick innate immune responses to *Borrelia burgdorferi* challenge: immunotolerance in *Ixodes scapularis* versus immunocompetence in *Dermacentor variabilis* (Acari: Ixodidae). *Journal of Medical Entomology,* **38**, 99-107.

Johns, R., D. E. Sonenshine and W. L. Hynes (2001b). Identification of a defensin from the hemolymph of the American dog tick, *Dermacentor variabilis. Insect Biochemistry and Molecular Biology,* **31**, 857-865.

Lai, R., L. O. Lomas, J. Jonczy, P. C. Turner and H. H. Rees (2004). Two novel non-cationic defensin-like antimicrobial peptides from haemolymph of the female tick, *Amblyomma hebraeum*. *Biochemical Journal,* **379**, 681-685.

Matsuo, T., Y. Okoda, B. Badgar, N. Inoue, X. Xuan, D. Taylor and K. Fujisaki (2004). Fate of GFP-expressing *Escherichia coli* in the midgut and response to ingestion in a tick, *Ornithodoros moubata* (Acari: Argasidae). *Experimental Parasitology,* **108**, 67-73.

Mitta, G., F. Vandenbulcke, F. Hubert and P. Roch (1999). Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. *Journal of Cell Science,* **112** 4233-4242.

Nakajima, Y., H. Saido-Sakanaka, D. Taylor and M. Yamakawa (2003). Up-regulated humoral immune response in the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *Parasitology Research,* **91**, 476-481.

Nakajima, Y., D. Taylor and M. Yamakawa (2002a). Involvement of antibacterial peptide defensin in tick midgut defense. *Experimental and Applied Acarology,* **28**, 135-140.

Nakajima, Y., A. van der Goes van Naters-Yasui, D. Taylor and M. Yamakawa (2001). Two isoforms of a member of the arthropod defensin family from the soft tick, *Ornithodoros moubata* (Acari: Argasidae). *Insect Biochemistry and Molecular Biology,* **31**, 747-751.

Nakajima, Y., A. van der Goes van Naters-Yasui, D. Taylor and M. Yamakawa (2002b). Antibacterial peptide defensin is involved in midgut immunity of the soft tick, Ornithodoros moubata. *Insect Molecular Biology,* **11**, 611-618.

Oliver, J. H., Jr., T. M. Kollars, Jr., F. W. Chandler, Jr., A. M. James, E. J. Masters, R. S. Lane and L. O. Huey (1998). First isolation and cultivation of *Borrelia burgdorferi* sensu lato from Missouri. *Journal of Clinical Microbiology,* **36**, 1-5.

Pal, U., X. Li, T. Wang, R. R. Montgomery, N. Ramamoorthi, A. M. Desilva, F. Bao, X. Yang, M. Pypaert, D. Pradhan, F. S. Kantor, S. Telford, J. F. Anderson and E. Fikrig (2004). TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. *Cell,* **119**, 457-468.

Rees, J. A., M. Moniatte and P. Bulet (1997). Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). *Insect Biochemistry and Molecular Biology,* **27**, 413-422.

Rudenko, N., M. Golovchenko, M. J. Edwards and L. Grubhoffer (2005). Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection. *Journal of Medical Entomology,* **42**, 36-41.

Saido-Sakanaka, H., J. Ishibashi, A. Sagisaka, E. Momotani and M. Yamakawa (1999). Synthesis and characterization of bactericidal oligopeptides designed on the basis of an insect anti-bacterial peptide. *Biochemical Journal,* **338** 29-33.

Sonenshine, D. E. (1993). Biology of Ticks. New York, Oxford University Press, Inc.

Sonenshine, D. E., S. M. Ceraul, W. E. Hynes, K. R. Macaluso and A. F. Azad (2002). Expression of defensin-like peptides in tick hemolymph and midgut in response to challenge with *Borrelia burgdorferi*, *Escherichia coli* and *Bacillus subtilis*. *Experimental and Applied Acarology,* **28**, 127-134.

Varela, A. S., M. P. Luttrell, E. W. Howerth, V. A. Moore, W. R. Davidson, D. E. Stallknecht and S. E. Little (2004). First culture isolation of *Borrelia lonestari*, putative agent of southern tick-associated rash illness. *Journal of Clinical Microbiology,* **42**, 1163-1169.

Vizioli, J., A. M. Richman, S. Uttenweiler-Joseph, C. Blass and P. Bulet (2001). The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. *Insect Biochemistry and Molecular Biology,* **31**, 241-248.

Figure 1

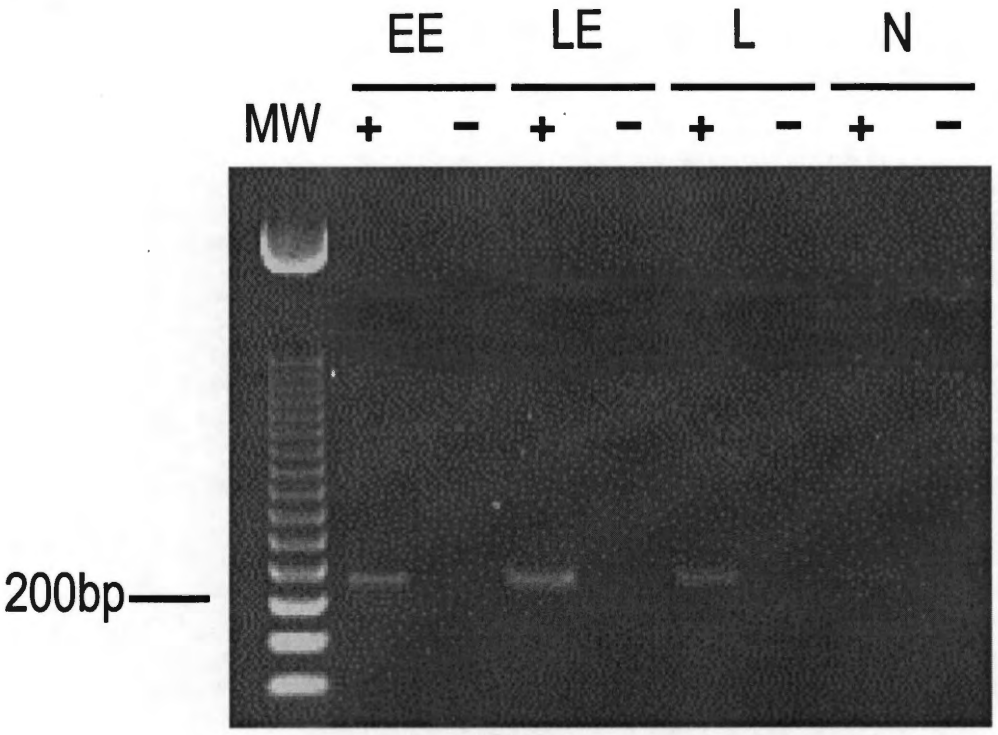

Figure 2

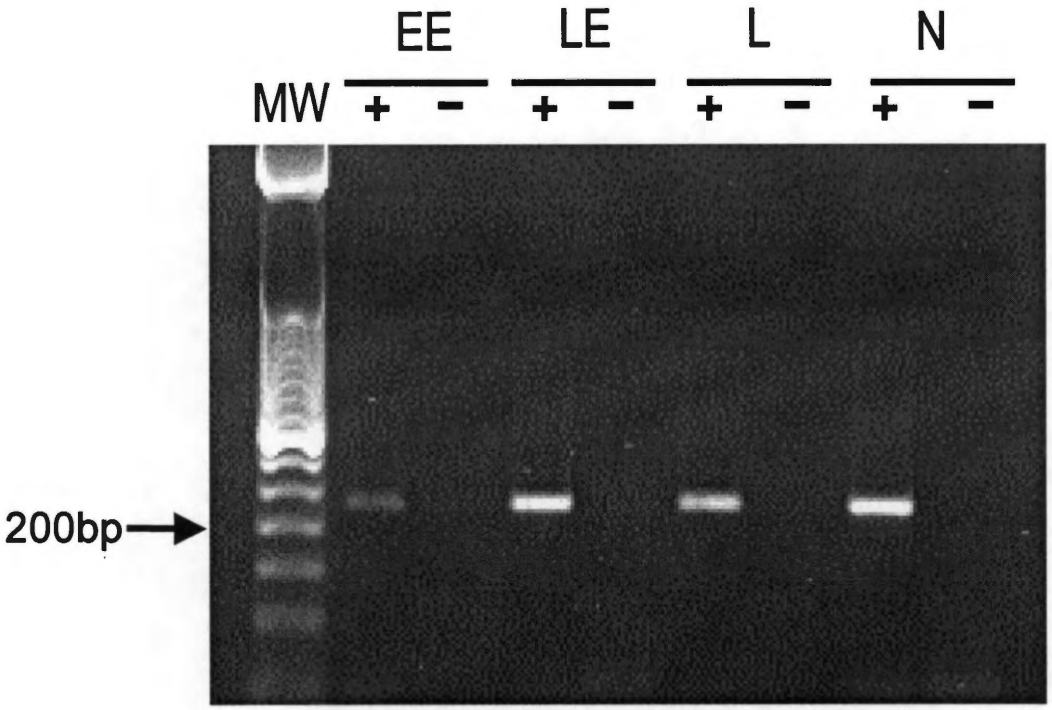

Figure 3

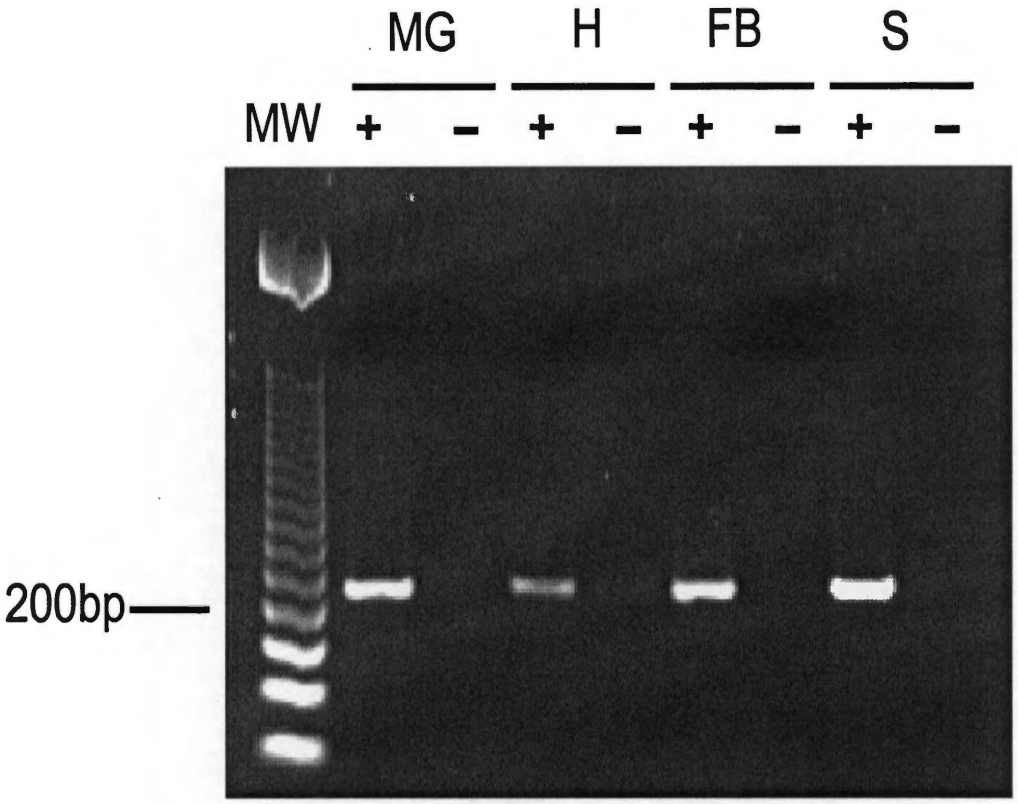

Fig.1. Defensin transcript presence in early stage eggs (EE), late stage eggs (LE), larvae (L), and nymphs (N) of *Amblyomma americanum* is demonstrated by presence of reverse transcription polymerase chain reaction (RT-PCR) product in the presence of reverse transcriptase (+) and lack of product in the absence of reverse transcriptase (-). The molecular weight marker is a 50bp ladder. Note that a PCR product for *A. americanum* nymphs was visible digitally but was not visible in print due to low band intensity.

Fig.2. Defensin transcript presence in early stage eggs (EE), late stage eggs (LE), larvae (L), and nymphs (N) of *Ixodes scapularis* is demonstrated by presence of reverse transcription polymerase chain reaction (RT-PCR) product in the presence of reverse transcriptase (+) and lack of product in the absence of reverse transcriptase (-). The molecular weight marker is a 50bp ladder.

Fig.3. Defensin transcript presence in the midgut (MG), hemocytes (H), fat body (FB), and salivary glands (S) of *Amblyomma americanum* is demonstrated by presence of reverse transcription polymerase chain reaction (RT-PCR) product in the presence of reverse transcriptase (+) and lack of product in the absence of reverse transcriptase (-). The molecular weight marker is a 50bp ladder.

Table 1: Primers used in this study

Primer Name	For/Rev	Sequence
Is2b	F	5' GGA TAC GGA TGC CCC TTC AAC C 3'
M13 F	F	5' CGC CAG GGT TTT CCC AGT CAC GAC 3'
M13 R	R	5' TCA CAC AGG AAA CAG CTA TGA C 3'
Amb-GSP1	R	5' CCG CCA CAA TAG CCC CCG CG 3'
Amb-GSP2	R	5' GTT GTA GCA AGT GCA GGT CGT 3'
Amb-ATg	F	5' ATG AAG GTC CTG GCC GTC GCA T 3'
Amb-TAg	R	5' CTA GTT GTA GCA AGT GCA GG 3'
Ix-ATG	F	5' ATG AGG GTC ATT GCT GTT AC 3'
Ix-TAG	R	5' CTA GTT GTG GTA GCA TGT GC 3'

Table 2: Nucleotide similarity of tick defensins

		Mature Defensin Nucleotide Similarity											
		A. americanum	*A. hebraeum* 1	*A. hebraeum* 2	*I. scapularis*	*D. variabilis*	*I. ricinus*	*B. microplus*	*H. longicornis*	*O. moubata* A	*O. moubata* B	*O. moubata* C	*O. moubata* D
Preprodefensin Nucleotide Similarity	*A. americanum*		48.7	52.1	71.8	67.5	64.0	67.5	70.9	66.7	67.5	66.1	65.0
	A. hebraeum 1	42.6		78.0	48.0	53.2	48.8	50.0	48.8	47.0	46.2	50.8	50.8
	A. hebraeum 2	43.0	62.4		52.0	54.4	48.4	50.8	48.8	48.4	47.6	48.4	47.6
	I. scapularis	67.1	45.3	46.0		72.6	59.8	76.1	76.9	61.7	62.5	61.7	60.8
	D. variabilis	61.3	44.1	43.1	66.5		62.5	86.3	81.2	65.8	65.0	65.8	65.8
	I. ricinus	61.8	44.4	44.1	58.4	57.1		61.6	56.4	63.5	63.5	64.3	60.9
	B. microplus	59.3	46.1	42.1	67.2	80.9	54.5		76.9	66.7	65.8	67.5	66.7
	H. longicornis	71.6	45.0	41.2	71.7	69.0	58.9	64.3		60.8	61.7	60.8	60.8
	O. moubata A	60.1	47.3	45.3	54.7	58.3	55.2	55.8	59.3		96.5	97.4	91.2
	O. moubata B	59.6	46.9	44.6	55.6	56.8	54.7	55.8	60.2	97.7		94.7	88.6
	O. moubata C	59.6	41.2	46.4	55.1	59.0	54.5	57.1	57.8	93.7	92.8		93.9
	O. moubata D	59.6	41.6	45.2	55.9	59.0	53.4	57.5	58.5	90.1	89.2	93.2	

Table 3: Amino acid similarity of tick defensins

		Mature Defensin Amino Acid Similarity												
		A. americanum	*A. hebraeum* 1	*A. hebraeum* 2	*I. scapularis*	*D. variabilis*	*I. ricinus*	*B. microplus*	*H. longicornis*	*O. moubata* A	*O. moubata* B	*O. moubata* C	*O. moubata* D	Dragonfly
Preprodefensin Amino Acid Similarity	*A. americanum*		44.7	42.1	73.7	71.1	55.3	73.7	71.1	71.1	71.1	71.1	65.8	63.2
	A. hebraeum 1	29.1		73.7	44.7	44.7	39.5	44.7	47.4	38.5	38.5	38.5	41.0	43.6
	A. hebraeum 2	26.2	46.4		42.1	42.1	36.8	42.1	42.1	38.5	38.5	38.5	35.9	38.5
	I. scapularis	60.8	30.2	23.8		78.9	57.9	86.8	81.6	59.0	59.0	59.0	59.0	66.7
	D. variabilis	59.5	25.0	27.4	62.2		50.0	89.5	92.1	59.0	61.5	59.0	56.4	66.7
	I. ricinus	55.3	26.2	22.6	55.3	46.1		55.3	55.5	51.3	53.8	51.3	48.7	51.3
	B. microplus	58.1	27.4	25.0	62.2	81.1	46.1		86.8	61.5	64.1	64.1	61.5	64.1
	H. longicornis	71.6	26.2	25.0	70.3	71.6	55.3	64.9		59.0	61.5	59.0	59.0	64.1
	O. moubata A	50.0	25.9	22.1	41.3	42.7	39.7	42.7	42.7		91.9	94.6	81.1	57.9
	O. moubata B	50.0	27.1	22.1	41.3	44.0	41.0	44.0	45.3	94.5		89.2	75.7	57.9
	O. moubata C	50.0	24.7	23.3	42.7	44.0	39.7	42.7	42.7	90.4	89.0		86.5	57.9
	O. moubata D	47.3	24.7	22.1	42.7	42.7	38.5	41.3	42.7	83.6	82.2	90.4		55.3
	Dragonfly	*	*	*	*	*	*	*	*	*	*	*	*	