BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF A TICK FEEDING

STIMULI RESPONSIVE *AMBLYOMMA AMERICANUM* ACIDIC CHITINASE IN

TICK FEEDING PHYSIOLOGY

A Thesis

by

TAE KWON KIM

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Chair of Committee,   Albert Mulenga
Committee Members,   Patricia Pietrantonio
                        Karen Snowden
Head of Department,   David Ragsdale

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ABSTRACT

Without successful feeding, ticks can neither cause damage to their host nor transmit disease agents. Thus, a deeper understanding of tick physiology as a means to discover weaknesses that can be targeted for tick control is needed. In a previous study, 40 genes were discovered that are differentially up regulated in *Amblyomma americanum* (*Aam*) (Linnaeus) females when exposed to feeding stimuli. The purpose of this study was to biologically and biochemically characterize one of the 40 candidate genes, a putative acidic chitinase (Ach), to understand its role(s) and significance in regulating tick feeding physiology. This research has shown that *A. americanum* expresses two putative *Aam*Ach isoforms [long (L) and short (S)], both of which are classified into the glycosyl hydrolase 18 (GH-18) family. Members of the GH-18 are involved in regulating multiple functions including nutrition processes in bacteria, morphogenesis in yeast and fungi, pathogen attack in plants, molting in insects, and inflammation and tissue remodeling in mammals. Spatial and temporal mRNA transcript analyses show that putative *Aam*Ach-L and *Aam*Ach-S are ubiquitously expressed, with highest transcript abundance post-attachment observed at 72h in all tissues, and at 96h in the salivary gland. *Pichia pastoris*-expressed recombinant *Aam*Ach-L was glycosylated, consistent with molecular analysis that predicted N- and O-linked glycosylation sites. Substrate hydrolysis analysis indicated that r*Aam*Ach-L does not apparently have chitinase activity. RNAi mediated silencing of *Aam*Ach mRNA apparently caused loosening or weakening of the tick cement plug at the feeding site. Animals injected with

*Aam*Ach-dsRNA bled around mouthparts, and were loosely attached on its rabbit host in that they easily detached with a light touch. The putative *Aam*Ach is therefore potentially involved in mediating tick attachment onto host skin. This thesis research advances our knowledge on the understanding of the molecular basis of tick feeding physiology and provides new information on the diverse physiological role of acidic chitinase-like proteins.

DEDICATION

This work is dedicated to my father, mother and brother who have supported me throughout the whole process. Without them this would not have been possible.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Ticks and Tick-Borne Diseases

Ticks are ectoparasites of major public and veterinary health importance. Ticks transmit various animal and human disease agents, including protozoa, bacteria, spirochaetes and viruses (Jongejan and Uilenberg, 1994). They are second to mosquitoes in terms of public health impact of pathogens they transmit, but are first in terms of the diversity of animal and human pathogens they transmit (Jongejan and Uilenberg, 2004; Sonenshine, 1993). Some tick species also inject toxins into animals and humans that cause paralysis (Kaire, 1966; Espinoza-Gomez et al., 2011). The lone star tick, *Amblyomma americanum,* the focus of this master's degree thesis research, is among important tick species of medical and veterinary health significance. *A. americanum,* which for many years was widely distributed in the southeastern United States (Mixson et. al., 2006) has also been found in the northeastern region as far as Maine (Keirans et al., 1998). This tick appears to be the most dominant tick species that bite humans in the Southern U.S. Felz et al., (1996) reported *A. americanum* in 83% of human tick infestations in the Southern states. *A. americanum* is involved in transmission of multiple animal and human disease agents including *Ehrlichia chaffensis*, the causative agent of human monocytic ehrlichiosis (Anderson et al., 1993), and *E. ewingii,* which also causes ehrlichiosis in humans, sometimes referred to as human granulocytic ehrlichiosis (Murphy et al., 1998; Buller et al., 1999; Wolf et al., 2000), *Francisella*

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tularensis (Tularemia) (Hopla et al., 1953; Taylor et al., 1991), a yet to be described disease agent that cause Lyme disease-like symptoms referred to as southern tick associated rash illness (STARI) (Armstrong et al., 2001; James et al., 2001), and an *E. ruminantium*-like organism referred to as the Panola Mountain *Ehrlichia* (PME) (Reeves et al., 2008; Yabsley et al., 2008). There is also evidence that *A. americanum* may transmit *Rickettsia amblyommii* to humans (Apperson et al., 2008). Of importance in veterinary health, *A. americanum* transmits *Theileria cervi* to deer (Laird et al., 1988), and *E. ewingii* to dogs (Little et al., 2010). There are reports of mortality in deer fawns that were attributed to a combination of heavy *A. americanum* infestation and *T. cervi* infections (Yabsley et al., 2005).

Tick Control and Alternatives

Tick control has been a costly ongoing battle globally. Current methods of tick control are primarily focused on the use of chemical acaricides. Although in the short term acaricide use can effectively control ticks, it does not provide a permanent solution because of numerous limitations such as ticks developing resistance to acaricides, environmental contamination, cost of developing new acaricides, and the inconvenience of application procedures. These limitations have necessitated the search for alternative novel tick control methods that will provide a permanent solution (de la Fuente and Kocan 2006; de la Fuente et al., 2007; Graf et al., 2004). Several alternative tick methods based on tick habitat modifications such as deforestation, landscape management, and rotational grazing have also been tried and proven inefficient

(Sonenshine, 1993). Research by Australian groups in the 1980s demonstrated that immunization of animals against tick feeding was a sustainable, environmental friendly alternative tick control method (Opdebeeck et al., 1988). To create successful anti-tick vaccines, our knowledge of the molecular mechanisms involved in tick feeding physiology must advance. This knowledge will result in discovery of "weak links" in tick biology that can be targeted for tick vaccine development.

Tick Feeding Behavioral Changes

Ticks are slow pool-feeding arthropods that accomplish feeding by lacerating tissue and small blood vessels in the skin to create a feeding lesion from which they imbibe host blood. A sequence of distinct physiological events occurs for a tick to successfully feed and reproduce (Walade and Rice, 1982) starting with the pre-appetence phase (PA), when newly hatched larvae or newly molted nymph and adult tick show no desire to feed (reviewed in Mulenga et al., 2007). Subsequently the tick attains appetence (Appetence phase, AP) and begins to display host-seeking behavior. Upon engaging the host, the tick transitions into the Pre-feeding phase (PF) when it selects the suitable feeding site on the animal, after which it transitions to the Preparatory feeding phase (PP). During the PP the tick secretes an adhesive substance, cement to attach onto host skin and create the feeding lesion. During this phase, the tick also secretes immunosuppressants that are thought to help Tick Borne Disease (TBD) agents to colonize the host (Piesman et al., 1987; Piesman et al., 1991; Katavolos et al., 1998; Ebel and Kramer 2004). Following the PP phase, the tick transitions into the Slow Feeding

phase (SF) when the tick is thought to maintain the feeding lesion, feed moderately, and undergo intermolt growth or neosomy (cuticular growth unrelated to the molt) to prepare for the rapid feeding phase (RP). In the RP phase the tick feeds to repletion and spontaneously detaches.

The tick feeding style is expected to trigger host defense mechanisms such as inflammation response, platelet aggregation, activate both the blood coagulation system and complement cascade (Chmelar et. al., 2011; Mulenga et al., 2007; Imamura et al., 2005; Nuttall and Labuda, 2004). Given that host response to tick feeding activity was expected to be swift, Mulenga et al., (2007) hypothesized that tick genes that regulate or facilitate the initial stages of tick feeding (attachment onto host skin and creating the feeding lesion) are expressed before or immediately around the AP or PF. Towards discovery of tick genes that regulate the initial stages of tick feeding, subtractive hybridization analysis was used to identify 40 *A. americanum* (*Aam*) genes that were differentially up regulated in ticks that were exposed to tick feeding stimuli (Mulenga et al., 2007). One of these 40 genes, a putative acidic chitinase was characterized in this thesis research.

Functional Roles of Chitinases

Chitinases are enzymes that hydrolyze the β-1,4 glycosidic linkages of N-acetylglucosamines primarily found in chitin. Chitin is an insoluble structural polysaccharide that is important as a supporting element in arthropod exoskeleton (Neville et al., 1976), cell wall of fungi and bacteria (Debono and Gordee, 1994; Gomaa,

2012), microfilarial sheath of parasitic nematodes (Araujo et al., 1993) and the lining of the digestive tracts of many arthropods (Souza-Neto et al., 2003; Zimoch et al., 2005; Khajuria et al., 2010). Arthropods development and morphogenesis rely on remodeling chitin and in the process requires chitin synthases and chitinases to control these processes (Merzendorfer and Zimoch, 2003). There are currently 133 different glycosyl hydrolase families according to the Carbohydrate-Active Enzymes database, cazy.org (Lombard et al., 2014). Chitinases are classified into the glycosyl hydrolase 18 or 19 family based on amino acid sequence similarity (Henrissat, 1991). At the time of this write up, according to the National Center for Biotechnology Information (NCBI) Conserved Domains (CDD) database (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), the GH-18 family includes chitolectins, chitotriosidase, chitobiase, hevamine, zymocin-alpha, narbonin, SI-CLP (stabilin-1 interacting chitinase-like protein), IDGF (imaginal disc growth factor), CFLE (cortical fragment-lytic enzyme) spore hydrolase, the type III and type V plant chitinases, and the endo-beta-N-acetylglucosaminidases.

Chitinases have widely different functions in many organisms. In fungi, chitinases play important biological and physiological roles such as in autolysis, nutrition, morphogenesis, and parasitism. It has been shown that the endochitinase of the dimorphic fungus *Benjaminiella poitrasii* significantly contributes to its morphological changes during the yeast-mycelium transition (Ghormade et al., 2000). Most bacterial chitinases are involved in degrading chitin to provide nitrogen and carbon (Patil et al., 2000). Chitinases play a major defensive role in all plants against attack by fungi and bacteria, and also against attack by insect pests (Gooday, 1999). They also play a role as

regulators of development and morphogenesis in plants (Grover, 2012). Insect chitinases are involved in cuticle turnover, digestion, and degradation of peritrophic membrane during molting and also as biopesticides (Kramer and Muthukristhnan, 1997; Merzendorfer and Zimoch, 2003; Rao et al., 2004). In nematodes, chitinase has been shown to play a role in development. In the filarial nematode *Acanthocheilonema viteae,* RNAi silencing of the chitinase inhibited molting of the L3 stage and hatching of microfilariae, alongside 50% mortality of female worms (Tachu et al., 2008). Chitinase in the microfilariae of parasitic nematode *Brugia malayi* is stored in the inner body, and it is secreted during the exsheathment of the microfilaria in the mosquito (Wu et al., 2008). In *Ascaris suum* the chitinase is involved in the initial molt and responsible for the digestion of eggshell during hatching (Geng et al., 2002). Mammals may not synthesize chitin, but the human genome encodes eight chitinases that are involved in T-cell mediated inflammation and asthma (Reese et al., 2007; Kawada et al., 2007). In mammals chitinases have also been shown to play a role in inflammation, tissue remodeling and injury (Lee et al., 2011). Available data show that chitinases also play an important role in tick physiology. In the tick, *Haemaphysalis longicornis,* immunization of rabbits with recombinant virus expressing a chitinase was used as a bioacaricide, which caused reduced feeding efficiency (You et al., 2003; Assenga et al., 2006), and prevented molting (You et al., 2009).

Significance of Research

This master's degree thesis research addresses an important problem, that is, to find effective antigens to develop vaccines against tick feeding to stop the spread of tick borne disease agents to animals and humans. The medical and veterinary impact of *A. americanum* as a vector of major human tick borne disease agents (Childs and Paddock, 2003) has made it imperative to develop novel tick strategies to stop the spread of tick borne diseases to the human population. Of significance to this research is that there is evidence of high conservation of key proteins, among *Amblyomma* spp. ticks (Mulenga et al., 2007, 2013). The implication of findings from this research could be extrapolated to other *Amblyomma* spp. of significant veterinary importance such as *A. variegatum*, the principal vector of *Erhlichia ruminantium*, which causes heart water, a destructive disease in domestic and wild ruminants (Esemu et al., 2013; Allsopp, 2010).

CHAPTER II

BIOINFORMATICS, TEMPORAL AND SPATIAL TRANSCRIPTION ANALYSES

OF PUTATIVE *AMBLYOMMA AMERICANUM* ACIDIC CHITINASE (*Aam*Ach)

Introduction

Recent advances in bioinformatics, genomics and proteomics have allowed discovery of putative candidate tick genes that regulate key tick physiological processes. The majority of these genes are of unknown functions, referred as hypothetical proteins in GenBank, while those that are provisionally identified are classified on basis of functional analysis data in other organisms (Juncker et al., 2009; Loewenstein et al., 2009). Two approaches, bioinformatics and transcriptional analyses are routinely used to gain insight on probable functions of candidate genes in tick physiology, and their relationship to the tick feeding process (Mulenga et al., 2008; 2009; Yamaji et al., 2009). Bioinformatics has been widely used to determine the relationship of candidate genes or protein of interest to predict its function with reference to tick feeding (Ibelli et al., 2013; Louw et al., 2013; Pellegrini et al., 1999). Multiple bioinformatics platforms such as ProtoParam to predict physiochemical properties of a protein (Gasteiger et al., 2005), ScanProsite to scan for annotated amino acid motifs (de Castro et al., 2006), and NCBI Blast to compare with other sequences in GenBank (Altschul et al., 1990) have been developed. These programs and other programs alike have been utilized to gauge probable function(s) of putative tick genes or proteins of interest (Mulenga et al., 2013; Ibelli et al., 2013; Tian et al., 2011). Limitations arise in the attempt to characterize

putative genes solely by bioinformatics analyses. This may provide clues to the relationship of genes, but it does not inform on the relationship of a candidate gene during the tick feeding process. To begin understanding the relationship of the candidate gene to tick feeding physiology, transcription profiling during specific periods of the tick feeding processes has been routinely applied (McNally et al., 2012; Mulenga et al, 2007; Rudenko et al., 2005). In this thesis research the author uses both bioinformatics and transcriptional analysis to gauge insight into probable functions of *Amblyomma americanum* putative acidic chitinase-like (*Aam*Ach) protein in tick physiology.

Materials and Methods

<u>Tick feeding, dissections, total RNA extractions and cDNA synthesis</u>

A. americanum ticks used in this thesis research were purchased from tick laboratories at Texas A&M University and Oklahoma State University (College Station, TX and Stillwater, OK, respectively). Routinely, ticks were fed on rabbits according to animal use protocols approved by Texas A&M University Institutional Animal Care and Use Committee (AUP 2011-189). To feed, *A. americanum* ticks were placed onto the outer part of the ear of specific pathogen free (SPF) New Zealand rabbits. Ticks were restricted onto the outer ears using orthopedic stockinet. The orthopedic stockinet was glued onto the rabbit ear with Kamar Adhesive (Kamar Products Inc., Zionsville, IN). Six male ticks were pre-fed for three days prior to introducing 15 female ticks on each ear stocking (total of 30 female ticks per rabbit). Using soft forceps, five female ticks were manually detached at 24, 48, 72, 96 and 120h post-attachment. Within the first

hour of detachment, ticks were prepared for dissections as described in Mulenga et al. (2013). Prior to dissecting, tick mouthparts were inspected to remove any remaining rabbit tissue and washed in RNase inhibitor diethylpyrocarbonate (DEPC) treated water. The tick was placed on a clean glass, ventral side down, and dissected using a sterile razor blade by carefully cutting the most extreme ends of the anterior, posterior and lateral portions along the leg line. Subsequently, the tick was placed on a sterile concave well slide covered with DEPC water and the dorsum was lifted and removed to expose all the tick organs using soft tissue forceps. Tick organs including, salivary glands (SG), midguts (MG), synganglion (SYN), Malpighian tubules (MT), and carcass (CA, the remnants after dissection) were isolated and placed in 1mL of the RNA extraction solution, Trizol (Life Technologies, Carlsbad, CA) and stored at -80° C until total RNA extraction.

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies) and re-suspended in DEPC treated water. Total RNA was quantified using a UV-VIS Spectrophotometer DU-640B (Beckman Coulter, Brea, CA). Total RNA (1µg) was used to synthesize cDNA using the qScript™ cDNA SuperMix according to the manufacturer's instructions (Quanta Bioscience, Inc, Gaithersburg, MD). The synthesized cDNA was quantified as above and stored in -20°C until use.

<u>Full-length *A. americanum* (*Aam*) Ach cDNA cloning, bioinformatics and phylogeny analyses</u>

The full-length cDNA sequence of putative *Aam*Ach cDNA was cloned using the Rapid Amplification of cDNA Ends (RACE) (ClonTech, Mountain View, CA) according to the manufacturer's protocol (cloning was done by Jenny Curran, a 2008 cohort REU-EXCITE student). RACE ready, 3′ and 5′ cDNA templates were synthesized using ~5ug 120h post-attached tick total RNA. In the first step an antisense primer amplified the 5′ fragment. Following sequencing of the 5′ fragment, a sense primer was designed at the extreme end of the 5′ ORF fragment to amplify the full-length *Aam*Ach cDNA in a 3′ RACE approach. PCR fragments were routinely cloned into the pGEM-T TA cloning vector (Promega, Madison, WI) and sequenced with T7 and SP6 promoter primers. DNA sequences were done at the Laboratory for Genome Technology at Texas A&M University (College Station, TX) for a fee-for-service. Sequences were analyzed, and the open reading frame was assembled using the MacVector program (MacVector Inc., Cary, NC).

<u>Bioinformatics and phylogeny analyses</u>

BlastX, BlastP, and NCBI protein database search engines were used to identify protein sequences in other organisms that showed identity to *Aam*Ach. The putative *Aam*Ach amino acid sequence was scanned on the SignalP Version 4.1 server (Petersen et al., 2011), and NetNGlyc 1.0 and NetOGlyc 4.0 servers (Steentoft et al., 2013) to determine presence of a signal peptide and putative N- and O-linked glycosylation sites.

To scan for annotated amino acid motifs, the putative *Aam*Ach amino acid sequence was scanned against entries in ScanProsite program (de Castro et al., 2006), and to gauge insight into physio-chemical properties using the ProtParam program (Gasteiger et al., 2005). Predicted isoeletric point (pI) and calculated molecular weights were determined using MacVector protein toolbox.

The top 19 GenBank sequence matches (Table 1) to putative *Aam*Ach from ticks, mosquitoes, helminths and mammals were downloaded and used to construct a guide phylogeny tree using MacVector software (MacVector Inc.). Based on initial blast results, *Aam*Ach was categorized as a GH-18 chitinase-like protein, therefore *Homo sapiens* (Accession # AAA35684.1) GH-18 chitobiase was used as an out-group in the phylogeny tree. Sequences were aligned using ClustalW and the phylogeny tree constructed using the Neighbor-joining method. To estimate bootstrap values, replications were set to 1000.

Validating if two putative *Aam*Ach forms are expressed

Sequence alignment analyses performed as the cloning experiments to obtain the full-length putative *Aam*Ach cDNA were carried out, revealed the possibility that *A. americanum* expressed a long (L) and short (S) forms, *Aam*Ach-L and *Aam*Ach-S. Preliminary pairwise sequence alignment analysis showed position 715-925 bp of *Aam*Ach -L is deleted in the *Aam*Ach −S.

To determine if both *Aam*Ach-L and *Aam*Ach-S mRNAs were expressed, respective specific forward $^{5'}$GAGGGGAGTCTGGAACGGAG$^{3'}$ and $^{5'}$CGCCCAAG

Table 1. Pairwise alignment showing percent identity and coverage of GH-18 chitinase-like proteins compared with *Aam*Ach- L.

Organism	Identity (%)	Query Coverage (%)	Accession #
*Aam*Ach- S	99	75	KF819830
R. sanguineus	83	88	ACX33152.1
I. ricinus	61	99	JAB70416.1
I. scapularis	55	78	XP_002407804.1
I. scapularis	48	93	XP_002407799.1
I. scapularis	48	78	XP_002407802.1
I. scapularis	42	94	XP_002407798.1
A. aegypti	39	84	AAB81849.1
A. gambiae	39	83	AEE44123.1
M. sexta	39	83	AAB53952
C. quinquefasciatus	38	84	XP_001863384.1
I. scapularis	36	94	XP_002399313.1
H. longicornis	36	90	BAC06447.1
M. musculus	35	84	NP_075675.2
H. sapiens	35	83	AAG10644.1
A. viteae	30	96	AAB68959.1
O. volvulus	30	95	AAB68960.1
W. bancrofti	30	84	AAF66988.1
B. malayi	30	83	AAA27854.1
B. pahangi	29	79	AAC47324.1
H. sapiens *	21	51	AAA35684.1

Percentage identity of amino acid sequences of 19 most similar sequences to *Aam*Ach- S (KF819830) and *Aam*Ach-L (KF819831) found in GenBank and compared using NCBI BLAST bl2seq. Included is a GH-18 family chitobiase from *Homo sapiens* (AAA35684.1) denoted by (*) used as the outgroup for phylogenetic tree.

CCCACGCCCTCGTC$^{3'}$ and the universal reverse $^{5'}$GAAGTCGTCCAGGTCGTTGT

TG$^{3'}$ PCR primers were designed as illustrated on Fig. 1A. These primers were used to

verify expression of *Aam*Ach-L and *Aam*Ach-S cDNA fragments in 3′ RACE ready

cDNA.

<u>Temporal and spatial transcription analyses during the first five days of tick post-</u>
<u>attachment</u>

To determine temporal and spatial transcription relationship to the tick feeding

process, *Aam*Ach-L and *Aam*Ach-S mRNA expression profiles were determined in

organs (SG, MG, SYN OV, and CA) that were dissected from ticks that were partially

fed for 24, 48, 72, 96, and 120h, using two-step semi-quantitative RT-PCR. For both

putative *Aam*Ach-L and *Aam*Ach–S, PCR cycling conditions were an initial denaturing

step at 94°C followed by 30 amplification cycles of 94°C for 30 s, 53°C for 30 s, 72°C

for 1 min, and a final extension step of 72°C for 5 min. Tick actin primers (For:

$^{5'}$GGACAGCTACGTGGGCGACGAGG$^{3'}$, Rev: $^{5'}$CGATTTCACGCTCAGCCGTGGT

GG$^{3'}$) were used for cDNA sample normalization (Chalaire et al., 2011). The actin

cycling conditions were an initial denaturing step at 94°C followed by 25 amplification

cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension step of 72°C

for 5 min. Routinely, PCR products were resolved using 2% agarose gel with 1μg/mL of

ethidium bromide and visualized using the FOTO/Convertible Transilluminator image

analyzer (Fotodyne Inc., Hartland, WI). To determine apparent transcript abundance,

A

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AamAchShort[KF819830]  702 CATTCCTGAAATTTCCAAGCACGTCGACTGGATCAACGCCCAAGCC--------------  747
AamAchLong[KF819831]   720 CATTCCTGAAATTTCCAAGCACGTCGACTGGATCAACGCCCAAGCCTACGACCTGAGGGG  779

AamAchShort[KF819830]  748 ------------------------------------------------------------  747
AamAchLong[KF819831]   780 AGTCTGGAACGGAGTTACCGATGTGCACACCCCACTCTACTCGAGATCCATTGATATCGG  839

AamAchShort[KF819830]  748 ------------------------------------------------------------  747
AamAchLong[KF819831]   840 TCCGCAGAAGACTCTGAATGTGAAAGACGGCCTCGCTCGCATCGTGAGCAGCGGTGCGCC  899

AamAchShort[KF819830]  748 ------------------------------------------------------------  747
AamAchLong[KF819831]   900 GAAGTCCAAGGTGGTGATGGGTATCGCTTTCTTCGGCCGGGGCTTCACGCTCCTCGACCC  959

AamAchShort[KF819830]  748 ----------------CACGCCCTCGTCAACCGCGACGTGCCTCCGCGCCCCGGACCCTT  791
AamAchLong[KF819831]   960 GGCACAGCACGGCCTCCACGCCCTCGTCAACCGCGACGTGCCTCCGCGCCCCGGACCCTT 1019

AamAchShort[KF819830]  792 TGTCAGGAGCAACAAGGTGTTCGCCTACTACGAGATCTGTCTCAACCTGAAGGGCAACTG  851
AamAchLong[KF819831]  1020 TGTCAGGAGCAACGAGGTGTTCGCCTACTACGAGATCTGTCTCAACCTGAAGGGCAACTG 1079

AamAchShort[KF819830]  852 GAAGCGCGAGTTCGACGAGGAGGGCAAGTGCCCGTACGTATACTACAGGGACCAGTGGAT  911
AamAchLong[KF819831]  1080 GAAGCGCGAGTTCGACGAGGAGGGCAAGTGCCCGTATGTATACTACAGGGACCAGTGGAT 1139

AamAchShort[KF819830]  912 CGGCTACGATGATGCTGTCAGCATTCGACACAAGATCAACTTCCTTCTGCAAGAGGGCTA  971
AamAchLong[KF819831]  1140 CGGCTACGATGATGCTGTCAGCATTCGACACAAGATCAACTTCCTTCTGCAAGAGGGCTA 1199

AamAchShort[KF819830]  972 CCGGGGCGTCTACGTGTTCAACAACGACCTGGACGACTTCCGAGGCTTCTGCGGCGAAAC 1031
AamAchLong[KF819831]  1200 CCGGGGTGTCTACGTGTTCAACAACGACCTGGACGACTTCCGAGGCTTCTGCGGCGAAAC 1259
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Fig. 1. Detection of long (L) and short (S) *Amblyommma americanum* (*Aam*) putative acidic chitinase (Ach) mRNA. (A) Pairwise sequence alignment of *Aam*Ach- L and S nucleotide sequences showing ~200bp gap denoted by broken line (------) that produce two in frame proteins [See Fig. 2]. Sequences were aligned using the ClustalW sequence alignment tool in MacVector DNA analysis software. The *Aam*Ach- L and S forward respective primers are in bold and highlighted with red (→) and blue (→), and a universal reverse primer in purple (←). (B) Shown below, PCR validation of two *Aam*Ach isoforms resolved on a 2% agarose gel containing 1 μg/mL ethidium bromide. B1 denotes the ~300 base pair (bp) band for *Aam*Ach- S, B2 denotes the ~500 bp band for *Am*Ach- L.

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B

Ladder (bp)   *Aam*Ach-S   *Aam*Ach-L

→ B2

→ B1
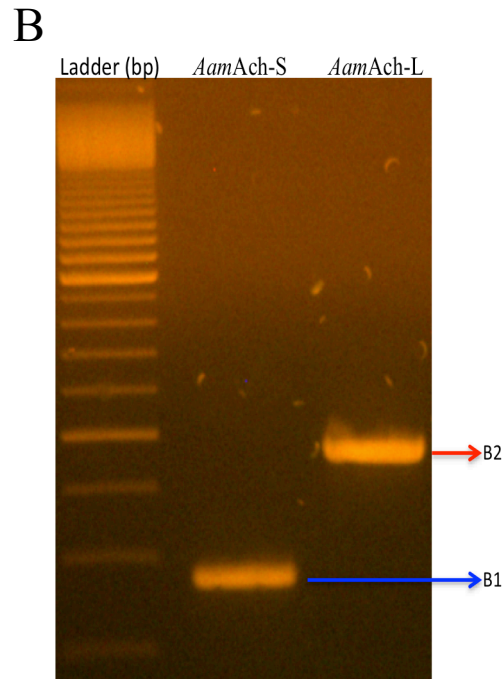
Fig. 1. Continued

PCR band densities were measured using the ImageJ software from the National Institute of Health (NIH, Bethesda, MD). To quantify transcript abundance, PCR band densities were normalized using the formula, $Y = V + [V (H-X)/X]$. Y is the normalized mRNA density, V is the observed putative *Aam*Ach PCR band density in individual samples, H is the highest tick actin PCR band density among tested samples, and X is the tick actin PCR band for the test tissue (Mulenga et al., 2008).

Results

Amblyomma americanum expresses long (L) and short (S) putative *Aam*Ach forms

RACE and sequence analysis identified a 1959 base pair (bp) (long, L, accession# KF819831) and 1718 bp (short, S, accession# KF819830) putative *Aam*Ach cDNA. Nucleic acid sequence alignment of *Aam*Ach-L and *Aam*Ach-S summarized in Fig. 1A shows a 212 bp deletion in *Aam*Ach-S. *Aam*Ach-L and *Aam*Ach-S full-length cDNA respectively contain 1332 and 1104 bp open reading frame (ORF) that encodes for a 443 (426 without signal peptide) and 367 amino acid residues proteins (Fig. 2). To determine if both *Aam*Ach-L and *Aam*Ach-S were expressed, specific forward and a universal reverse primers to respectively amplify ~500 and 300 bp, *Aam*Ach-L and *Aam*Ach-S cDNA fragments were designed (Fig. 1A). Fig. 1B shows that these primers amplified expected size *Aam*Ach-L and *Aam*Ach-S cDNA fragments confirming that the two putative *Aam*Ach types were expressed in *A. americanum*.

Putative *Aam*Ach- L and -S are chitinase-like proteins belonging to the GH-18 family

The ORF of both putative *Aam*Ach isoforms were subjected to amino acid motif scanning using ScanProsite Expasy server (de Castro et al., 2006), but retrieved no hits to any motifs (not shown). When scanned against entries in GenBank, both *Aam*Ach-L and *Aam*Ach-S yielded best matches to more than 20 hits to annotated chitinase and chitinase-like proteins in the glycosyl hydrolase-18 (GH-18) family with amino acid identity levels ranging from 40-85% (not shown). The GH-18 family is characterized by four signature amino acid sequence motifs, "FDG(L/F)DLDWE(Y/F)P",

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"K(F/V)M(V/L/I)AVGGW",   "M(S/T)YDL(R/H)G",   and   "GAM(T/V)WA(I/L)D"

(Arakane and Muthukrishnan, 2010). Visual inspection of both *Aam*Ach-L and *Aam*Ach-S amino acid sequences show that the GH-18 family consensus amino acid motifs (highlighted in purple) are conserved up to 55% (LDGVDMAWPFP), 67 % (KTLLSVGFW), 71% *Aam*Ach-L and 0% in *Aam*Ach-S (QAYDLRG), and 38% (GVYVRNND) shown in Fig. 2. Both *Aam*Ach-L and *Aam*Ach-S have (2 and 1, highlighted in red) and (25 and 6, highlighted in blue) putative N- and O-linked glycosylation sites (Fig. 2). A 17 amino acid signal peptide (indicated in Fig. 2) is predicted in *Aam*Ach-L but not in *Aam*Ach-S (Fig. 2).

Both *Aam*Ach- L and -S segregate with other blood feeding arthropods

At the time of this write up, the putative tick Ach when used as a query in GenBank retrieved eight tick GH-18 like chitinases, *Rhipicephalus sanguineus* (ACX33152.1), *Ixodes scapularis* (XP_002407804.1, XP_002407802.1, XP_002407799.1, XP_002407798.1, XP_002399313.1), *I. ricinus* (JAB70416.1), and *H. longicornis* (BAC06447.1). A phylogeny tree out-rooted from *Homo sapiens* GH-18 chitobiase (Accession#: AAA35684.1) was constructed using the Neighbor-joining method set to default parameters in the MacVector program. Sequences segregated into three clusters: a, b, and c, supported by 98, 79, and 88% bootstrap values respectively (Fig. 3). In cluster "a", both *Aam*Ach-L and *Aam*Ach-S amino acid sequences segregated together with annotated GH-18 chitinase sequences from GenBank of other blood

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AamAchShort[KF819830]    1 —MSYSATLALTSVCS—ACP———ADGMLRRSRVIAVTSARVRCPHPRRQPM—PRQAPPPGA   54
AamAchLong[KF819831]     1 MKLIALPLALLLACALGMPQQDGDATTPSTSSTSLSTSAASSVSTSQAPANATSSTTTGA   60

AamAchShort[KF819830]   55 ASSGRSPVVCYYYGWANTRPNPANYGVDDIPGDLCTHVNFAYAGVDPQTWELKSEVPEYE  114
AamAchLong[KF819831]    61 ASSGRSPVVCYYYGWANTRPNPANYGVDDIPGDLCTHVNFAYAGVDPQTWELKSEVPEYE  120
                                                                          *1
AamAchShort[KF819830]  115 GNRELFKNFTAIKTRYTQLKTLLSVGGWQHENGVFSAMAANSNRRALFIESVLRWMKEYN  174
AamAchLong[KF819831]   121 GNRELFKNFTAIKTRYTQLKTLLSVGGWQHENGVFSAMAANSNRRALFIESVLRWMKEYN  180
                              *2
AamAchShort[KF819830]  175 LDGVDMAWPFPGVSYRGGSPRDKENYASLIRELAGAFEGKGLLLTVVVPLPEEFLEAGYD  234
AamAchLong[KF819831]   181 LDGVDMAWPFPGVSYRGGSPRDKENYASLIRELAGAFEGKGLLLTVVVPLPEEFLEAGYD  240
                                     *3
AamAchShort[KF819830]  235 IPEISKHVDWINAQA—————————————————————————————————————————————  249
AamAchLong[KF819831]   241 IPEISKHVDWINAQAYDLRGVWNGVTDVHTPLYSRSIDIGPQKTLNVKDGLARIVSSGAP  300

AamAchShort[KF819830]  250 ———————————————————————HALVNRDVPPRPGPFVRSNKVFAYYEICLNLKGNW  284
AamAchLong[KF819831]   301 KSKVVMGIAFFGRGFTLLDPAQHGLHALVNRDVPPRPGPFVRSNEVFAYYEICLNLKGNW  360
                                                                        *4
AamAchShort[KF819830]  285 KREFDEEGKCPYVYYRDQWIGYDDAVSIRHKINFLLQEGYRGVYVFNNDLDDFRGFCGET  344
AamAchLong[KF819831]   361 KREFDEEGKCPYVYYRDQWIGYDDAVSIRHKINFLLQEGYRGVYVFNNDLDDFRGFCGET  420

AamAchShort[KF819830]  345 NILLKTIKEGLNGNKNEITASPA 367
AamAchLong[KF819831]   421 NILLKTIKEGLNGNKNEITASPA 443
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Fig. 2. Pairwise sequence alignment of *Aam*Ach- L and -S amino acid sequence showing the four consensus motifs of glycosyl hydrolase- 18 (GH-18) family. Sequences were aligned using the MUSCLE sequence alignment tool in MacVector DNA analysis software. The four consensus motifs of GH-18 family are in bold purple color and denoted with a (*) and a number. (*1) denotes the motif K(F/V)M(V/L/I)AVGGW, (*2) denotes the motif FDG(L/F)DLDWE(Y/F)P, and (*3) denotes the motif M(S/T)YDL(R/H)G and show 71 % identity with *Aam*Ach-L and is deleted in the *Aam*Ach –S. (*4) denotes the motif GAM(T/V)WA(I/L)D and shows ~67, 55, 38 % identity with respective *Aam*Ach-L and S. Prediction of (24 and 6) O-linked and (2 and 1) N-linked glycosylation sites in *Aam*Ach -L and S are represented by blue and red amino acids, respectively. Green colored amino acid represents both O-linked and N-linked glycosylation sites. A 17 amino acid signal peptide for *Aam*Ach- L is underlined and in bold.
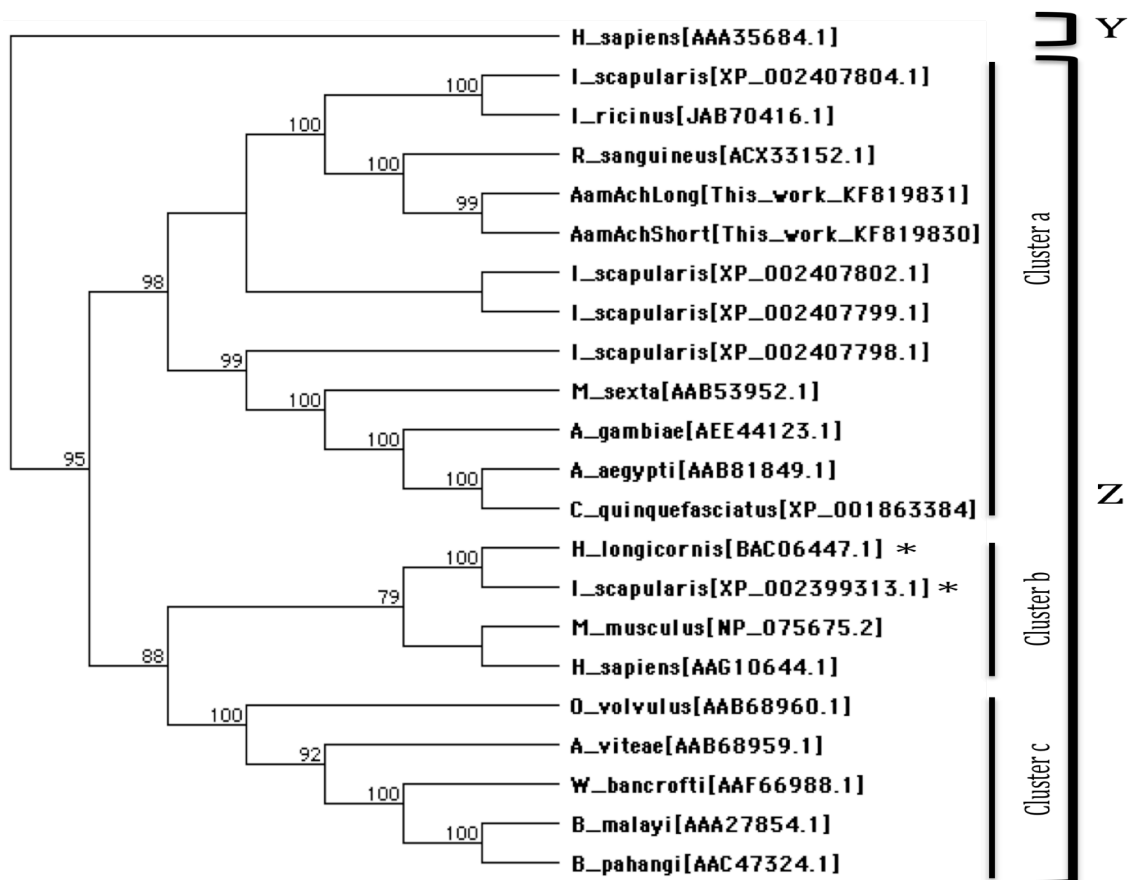
Fig. 3. Phylogenetic analysis of chitinases in GH-18 family against *Aam*Ach- L. Neighbor-joining guide phylogenetic tree showing the relationship of *Amblyomma americanum Aam*Ach- L and S putative proteins to glycosyl hydrolase (GH-18) chitinase proteins from ticks (n = 8), mosquitoes (n = 3), helminths (n = 5), mammals (n = 2), insect (n = 1) and a human GH-18 family chitobiase (n = 1) as the outgroup. The predicted putative *Aam*Ach –L and S was aligned with glycosyl hydrolase 18 chitinase and chitinase-like proteins sequences: *R_sanguineus* (ACX33152.1); *I_scapularis* (XP_002407804.1, XP_002407802.1, XP_002407799.1, XP002407798.1, XP_002399313.1); *I_ricinus* (JAB70416.1); *H_longicornis* (BAC06447.1), *C_quinquefasciatus* (XP_001863384); *A_aegypti* (AAB81849.1); *A_gambiae* (AEE44123.1); *M_sexta* (AAB53952.1); *B_pahangi* (AAC47324.1); *B_malayi*, (AAA27854.1); *A_viteae* (AAB68959.1); *O_volvulus* (AAB68960.1); *W_bancrofti* (AAF66988.1); *M_musculus* (NP075675.2); and *H_sapiens* (AAG10644.1, AAA35684.1), using ClustalW sequence alignment tool. The phylogenetic guide tree was constructed using the MacVector Neighbor-joining method with default parameters as described in materials and method section in chapter II. Cluster "a" consists of arthropods, Cluster "b" consists of mammals (except with [*], which are arthropods) and Cluster "c" consists of helminths. Y denotes the outgroup of GH-18 family chitobiase and Z denotes GH-18 chitinases and chitinase-like proteins. Numbers at each node represent bootstrap values that signify the level of confidence in the branch.

feeding arthropods including ticks: *R. sanguineus* (ACX33152.1), *I. scapularis* acid identity levels decreased to a respective 55 to 36% and 61% which is comparable to *Aam*Ach identity levels to mosquitoes (Table 1).

*Both Aam*Ach- L and -S *mRNA are ubiquitously expressed through 5 days post attachment*

Fig. 4 summarizes *Aam*Ach-L and *Aam*Ach-S transcription relationship to the tick feeding process through five days post-attachment. Semi-quantitative RT-PCR analyses revealed that both *Aam*Ach-L and *Aam*Ach-S mRNA were expressed during the first five days (Fig. 4A). Based on normalized mRNA transcript abundance, both forms of *Aam*Ach were highly abundant at the 72h post-attachment time point in all tick organs except the salivary gland, which peaked at the 96 h time point (Figs. 4 B & C).

Discussion

Up-regulation of the enzyme transcript for *Aam*Ach in response to tick feeding stimuli before ticks start to feed (Mulenga et al., 2007) encouraged the author to investigate the biological role(s) of putative *Aam*Ach during the tick feeding process. To begin understanding the role(s) of candidate genes in the tick feeding process, mRNA expression profiles were compared during the different stages of the tick feeding process. In this way, genes that are up regulated in response to feeding are thought to be associated with blood meal feeding (Mulenga et al., 2007, Aljamali et al., 2009;
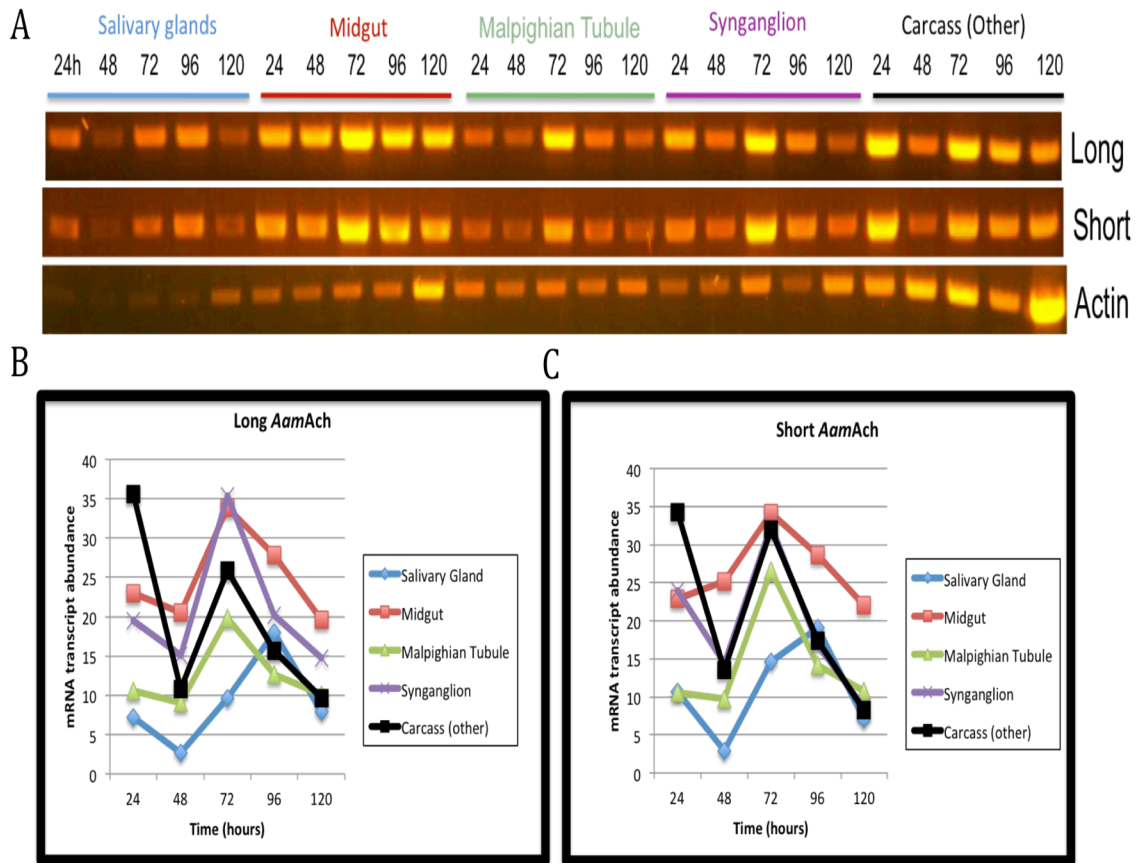
Fig. 4. Spatial and temporal mRNA expression profile in dissected tissues through 5 days post-attachment. (A) Five ticks detached from rabbits at 24, 48, 72, 96, and 120 h post-attachment were processed individually for total RNA extraction and subjected to two-step semi-quantitative RT-PCR to amplify the *Aam*Ach- L and S fragments and tick actin (load control). (B & C) Densities of *Aam*Ach-L and S (respectively) and tick actin PCR bands were determined using a web based ImageJ online program. Densities were normalized as described in the material and methods section using the following formula: $Y=V+[V(H–X)/X]$, where Y stands for the normalized mRNA density, V is the observed *Aam*Ach-L or S PCR band density in individual samples, H is the highest tick actin PCR band density among tested samples, and X is the tick actin PCR band density (Mulenga et al., 2008). Means and standard error of means (SEM) of the three PCR band densities were determined using the ImageJ analyzer program and the plotted in Microsoft Office Excel 2011. *Aam*Ach –L and S mRNA transcript was highest at 72 h post-attachment time point in all tissues except in salivary glands at 96 h. Labeling of tick organs are color coded: salivary glands (blue), midgut (red), Malpighian tubule (green), synganglion (purple), and remnants labeled carcass (black).

Mulenga and Khumthong, 2010a; Konnai et al., 2011) and those that are down-regulated are believed to play other roles in tick biology, but not associated with blood meal feeding (Aljamali et al., 2009; Umemiya et al., 2008). The observation that both *Aam*Ach-L and *Aam*Ach-S were up regulated in response to feeding for 72 h in all tissues and at 96 h in the salivary gland, may suggest that these two proteins may be associated with blood meal feeding events during the first four days of feeding. During this period the tick accomplishes attachment onto the host skin by secreting tick cement, create the feeding lesion, begin to transmit disease agents, and begins to grow new tissue and cuticle to prepare for imbibing huge volumes of blood (Sonenshine, 1993).

Chitinases are classified into the GH-18 or 19 family based on amino acid sequence similarity (Henrissat, 1991). In a previous study a GH-18 like family *H. longicornis* tick chitinase was shown to be involved in the hydrolysis of chitin and believe to be involved in controlling turnover and porosity of the chitinous peritrophic membrane (You et al., 2003). In this research two putative *Aam*Ach isoforms provisionally annotated as members of the GH-18 family were characterized. Most members of the GH-18 family are characterized by a multi-domain architecture including a signal peptide, Ser/Thr- rich linker regions that may be heavily glycosylated, one to five GH-18 catalytic domains, and none to five chitin binding domains (Arakane and Muthukrishnan, 2010; Huang et al., 2012). The Ser/Thr rich linker region is most likely the site of O-glycosylation and may contribute to immunogenicity and protein stability (Arakane et al., 2003). The chitin binding domains are sites where chitin may bind, which is then degraded. The absence of the domain does not affect the ability of

the enzyme to hydrolyze the soluble substrate, triacetylchitotriose, but abolishes its ability to hydrolyze insoluble chitin (Tjoelker et al., 2000). The observation that both *Aam*Ach-L and *Aam*Ach-S do not have the putative chitin-binding domain may suggest that the two proteins may not be involved in chitin hydrolysis. Native *Aam*Ach-L protein is likely to function in the extracellular space in that it is predicted to have a signal peptide, while the *Aam*Ach-S does not have a signal peptide and it is likely to function intracellularly.

Phylogeny analysis data revealed that *Aam*Ach-L and *Aam*Ach-S putative sequences are closely related to sequences in other blood feeding arthropods. It is interesting to note that similar to transcription profiling data in this research, *Anopheles gambiae* GH-18 chitinase (AEE44123.1) was up regulated in the foregut of blood fed females (Zhang et al., 2011). Tick acidic chitinases characterized here were also closely related to the helminth GH-18 chitinases. The worm known to cause brugian lymphatic filariasis, *Brugia malayi* (AAA27854), contained two active microfilarial GH-18 chitinases that may potentially be involved in degrading chitin structure in the microfilaria during development and transmission in its mosquito vector (Fuhrman et al., 1992). Data reviewed here show that there are active and inactive chitinase enzyme within the GH-18 family.

CHAPTER III

BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF *AMBLYOMMA*

AMERICANUM PUTATIVE ACIDIC CHITINASE

Introduction

In chapter II bioinformatics and transcriptional analyses revealed that putative *A. americanum* (*Aam*) acidic chitinase (Ach) is a member of the chitinase GH-18 family that is ubiquitously transcribed with transcript abundance being highest at the 72h post attachment time point in all tissues, except for the salivary gland where high expression was observed at the 96h time point. To begin understanding the role(s) of *Aam*Ach in tick feeding physiology, biochemical and biological characterization of *Aam*Ach will be discussed in this chapter. The Mulenga lab's interest is to find target antigens for anti-tick vaccine development. From this perspective *Aam*Ach-L, which is predicted to function in the extracellular environment was further characterized. The goals of this research in this chapter were two fold. The first goal was to determine if wild type and mutant recombinant *Aam*Ach-L were functional chitinases. The rationale to design and express recombinant mutant chitinase was motivated by preliminary data that showed that the wild type may not be a functional chitinase. The second goal was to determine if *Aam*Ach-L function was important to tick feeding success.

Biochemical characterization approaches have elucidated the possible functional role(s) of putative acidic chitinases in several organisms such as in insects (Merzendorfer and Zimoch, 2003) and in plants (Gooday, 1999). Biochemical

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function(s) of candidate acidic chitinases in multiple organisms were determined by characterizing recombinant proteins that were expressed in *E. coli* (Lan et al., 2006), insect cells (Huang et al., 2000; Zhu et al., 2008) or yeast cells (Li et al., 2010). Reported studies have used the ability to hydrolyze exochitinase substrates (4-nitrophenyl N,N′-diacetyl-β-D-chitobioside and 4-nitrophenyl N-acetyl-β-D-glucosaminide) and/ or endochitinase substrate (4-nitrophenyl β-D-N,N′,N″-triacetylchitotriose substrate) to determine the functional nature of chitinases. Usually, exochitinases progressively cleave off two subunits from the reducing or non-reducing ends of the chitin chain (Frandberg and Schnurer, 1994; Chernin et al., 1998). Endochitinases generally digest the β-1,4-linkages of the internal positions in N-acetyl-D-glucosamine polymers of chitin and cleave randomly within the chitin chain (Larsen et al., 2011).

The advent of RNA interference (i)-mediated gene silencing technology has provided opportunities to understand the role(s) and/or importance of candidate genes in tick physiology. As a naturally occurring process, RNAi was first demonstrated to efficiently turn-off gene expression in transparent worms, *Caenorhabditis elegans* (Fire et al., 1998). In experimental systems, the RNAi pathway is induced by the introduction of *in vitro* synthesized double stranded RNA (dsRNA) that is complementary to the gene of interest, into an organism or cell culture (Haley et al., 2003; Ovcharenko et al. 2005). Thereafter, the dsRNA is recognized as an exogenous material and the RNAi pathway is initiated with the presence of a Dicer enzyme which cleaves the dsRNA into smaller fragments called the small interfering RNA (siRNA) (Ketting et al., 2001; Macrae et al., 2006). The siRNA further unwinds to two strands, the parent and guide strands. The

parent strand is processed for degradation while the guide strand is associated with the multi-protein RNA-induced silencing complex (RISC). Post-transcriptional gene silencing occurs when the guide strand base pairs with its complementary mRNA sequence and induces cleavage by the Argonaute protein, which degrades the mRNA. In this way translation of the candidate protein is blocked. The general interpretation of RNAi silencing is that if the silenced target is important, there is some loss of fitness or performance by the organism and a phenotype would be measurable or observable.

Since the discovery of the RNAi pathway, researchers have utilized this tool to further elucidate the biological importance of target genes in their organism of interest. In the tick research community, RNAi was used to demonstrate the importance of candidate genes in hard ticks, *A. americanum* (Mulenga et al., 2013; Chalaire et al., 2011; Mulenga and Khumthong, 2010b; de la Fuente et al., 2010), *I. scapularis* (Dai et al., 2010; Kocan et al., 2007), *I. ricinus* (Sojka et al., 2012; Decrem et al., 2008), *D. variabilis* (Kocan et al., 2007), *R. microplus* (Kocan et al., 2007) and *R. haemaphysaloides* (Yu et al., 2013), and soft ticks *Ornithodoros moubata* (Diaz-Martin et al., 2013). Routinely the impact of RNAi silencing is investigated on tick feeding success, reproduction, viability of offspring and pathogen transmission and acquisition.

Materials and Methods

Expression and affinity purification of recombinant tick wild type and mutant

*Aam*Ach- L

Putative recombinant (r) wild type (Wt) and mutant (Mu) *Aam*Ach-L protein was expressed in the *Pichia pastoris* expression system as described in Mulenga et al. (2013). In preliminary experiments, Wt r*Aam*Ach-L did not show any chitinase activity. Thus, Mu r*Aam*Ach-L was expressed to attempt restoring function using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies Inc., Santa Barbara, CA). A mutant strand synthesis reaction was prepared to create a mutation of Proline to Glutamic Acid at amino acid position 189 by thermal cycling a sense ($^{5'}$GTGG ACATGGCCTGGGAGTTCCCAGGCGTCTCC$^{3'}$) and anti-sense ($^{5'}$GGAGAC GCCTGGGAACTCCCAGGCCATGTCCAC$^{3'}$) primers designed to contain a GAG in place of CCT at nucleotide positions 616 – 618, 100ng of *Aam*Ach-L/pPICZαA plasmid as template, and components from the kit. The thermal cycling conditions were an initial denaturing step at 95°C for 2 min followed by 18 amplification cycles of 95°C for 20 s, 60°C for 10 s, 68°C for 2 min, and a final step of 68°C for 5 min. The following reaction was digested with *Dpn* I enzyme, cloned and verified by sequencing.

An expression plasmid was constructed by sub-cloning the mature putative *Aam*Ach coding domain into pPICZαA *Kpn*I and *Not*I sites, using forward ($^{5'}$**GGTACC**ATGCCCCAGCAAGACGGGGATG$^{3'}$) and reverse ($^{5'}$**GCGGCCGC**AGCGGGTGATGCGGTAATCTCG$^{3'}$) primers with added restriction enzyme sites in bold. The pPICZαA-*Aam*Ach-L expression plasmid linearized with

PmeI was used to transform *Pichia pastoris* X-33 strain (Life Technologies) by electroporation using an ECM600 electroporator (BTX Harvard Apparatus Inc., Holliston, MA) with parameters set to 1.5kV, 25uF, and 186Ω. Transformed colonies were selected on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar plates with zeocin (100μg/μl), and then selected for methanol utilization on Minimal Methanol (MM) agar plates, both incubated at 28°C. The pPICZαA vector contains an AOX1 promoter for tightly regulated, methanol-induced expression. Positive transformants from PCR insert check were inoculated in Buffered Glycerol-complex Medium (BMGY) and grown overnight at 28°C with shaking (230-250 rpm). Subsequently the cells were used to inoculate Buffered Methanol-complex Medium (BMMY) to A_{600} of 1 after which protein expression was induced by adding methanol to 0.5% final concentration every 24h for five days. The pPICZαA plasmid contains a α-factor secretion signal that directs the recombinant protein for secretion in the media. To obtain the secreted recombinant protein, yeast culture spent media was centrifuged at 5000g for 10 min at room temperature. The recombinant protein in the supernatant was precipitated by ammonium sulfate saturation (525g/1L of media) with stirring overnight at 4°C. The precipitate was pelleted at 11,200g for 1-2 hrs at 4°C. The pellet was re-suspended in and dialyzed against phosphate buffered saline (PBS) pH 7.4.

To verify expression of *Aam*Ach-L, a routine western blot analysis was performed using the horseradish peroxidae (HRP)-labeled antibody to the C-terminus hexa histidine tag (Life Technologies) diluted to 1:5000 in 5% blocking buffer (5% Skim milk powder in PBS with 0.05% Tween). The positive signal was detected using metal

enhanced 3,3' Diaminobenzidine (DAB) chromogenic substrate kit (Thermo Scientific, Rockford, IL). Subsequently r*Aam*Ach-L was affinity purified under native conditions using Hi-Trap Chelating HP Columns (GE Healthcare). Affinity purified putative r*Aam*Ach-L was dialyzed against 1X PBS pH 7.4 for downstream assays. The expression protocol was repeated to express the mutant r*Aam*Ach chitinase. Routinely, affinity purified r*Aam*Ach-L was resolved on a 10% SDS-PAGE gel and visualized by silver staining to verify purity and background contamination. Protein in the samples was concentrated by either ammonium sulfate precipitation or by centrifugation using MicroSep Centrifugal Concentration Devices (Pall Corporation, Port Washington, NY).

N- and O- linked glycosylation assay

Bioinformatics analysis in chapter II predicted 2 and 24 N- and O-linked glycosylation sites in *AamA*ch-L. To determine if putative r*Aam*Ach was N-glycosylated and/or O-glycosylated, affinity purified r*Aam*Ach was treated with Protein Deglycosylation Mix following the manufacturer's instructions (New England Biolabs, Ipswich, MA). Deglycosylation was verified by Western Blot using antibody to C-terminus hexa histidine-tag (Life Technologies) and the positive signal was detected using HRP chromogenic substrate (Thermo Scientific).

Western blotting analysis

To determine if native putative *Aam*Ach-L is secreted by ticks into the host during the first 48 h of the post-attachment phase, affinity purified r*Aam*Ach-L was

subjected to routine western blotting analyses using antibodies developed against Tick Saliva Proteins (TSP) of *A. americanum*. These antibodies were produced by repeated tick infestations on rabbits every 48 h (Chalaire et al., 2011). Recombinant *Aam*Ach-L proteins were separated on a 10% SDS-PAGE gel and transferred onto a PVDF membrane by wet electroblotting. Subsequently, the electroblotted membrane was washed in PBS with 0.05% Tween and placed in 5% blocking buffer (5% Skim milk powder in PBS with 0.05% Tween) overnight on a rocker at 4°C. The following day, membranes were exposed to 48h TSP antibody and pre-immune serum (PI) both diluted to 1:50 – 1:1000 in 5% blocking buffer for 1 hr at room temperature, and then washed with PBS with 0.05% Tween five times for five minutes. The membranes were then exposed to a goat anti-rabbit IgG, HRP-conjugated secondary antibody (Millipore, Billerica, MA) diluted to 1:2000 for 1 hr at room temperature and washed as described above. The positive signal was detected using metal enhanced 3,3' Diaminobenzidine (DAB) chromogenic substrate kit (Thermo Scientific) and Microwell and/or Membrane HRP chemiluminescent substrate (SurModics, Eden Prairie, MN).

Our preliminary analysis showed that r*Aam*Ach-L non-specifically reacted with both the pre-immune (PI) serum and the antibody to 48h TSP. To validate if r*Aam*Ach non-specifically binds immunoglobulins, deglycosylated and glycosylated wild type (Wt) and mutant (Mu) protein samples were subjected to western blot analyses as described above using pre-immune sera of rabbit, chicken, and bovine (respective serums were collected from Lab Animal Resources and Research Facility, Poultry Science Department, and Rosenthal Slaughter House, all located at Texas A&M

University, College Station, TX). In all western blotting experiments, 1:50 – 1:1000 primary antibody and 1:2000 secondary antibody dilutions were used. Rabbit, chicken and bovine secondary antibodies used were a respective goat anti-rabbit IgG HRP-conjugated (Millipore, Billerica, MA), goat anti-chicken IgY HRP-conjugated (Santa Cruz Biotechnology, Inc., Dallas, TX) and goat anti-bovine IgG (H+L) HRP-conjugated (Jackson ImmunoResearch Lanoratories, Inc., West Grove, PA).

Chitinase substrate hydrolysis assay

To determine if Wt and Mu r*Aam*Ach were active chitinases, a chitinase substrate hydrolysis assay was performed using a commercial kit from Sigma (St. Louis, MO, Catalog # CS0980), according to manufacturer's instructions. r*Aam*Ach activity was assayed using substrates, nitrophenyl N,N′-diacetyl-β-D-chitobioside and 4-nitrophenyl N-acetyl-β-D-glucosaminide for exochitinase activity and 4-nitrophenyl β-D-N,N′,N″-triacetylchitotriose substrate for endochitinase activity. Prior to beginning the assay, standard and substrate solutions were equilibrated to 37°C by incubating for 10 min in a 37 °C water bath. The pre-warmed substrates 50μL (1mg/mL) were mixed with r*Am*Ach (1.91 μM and 0.477 μM), positive (0.8 μM and 0.2 μM) or negative (1.91 μM and 0.477 μM) controls in a 96 well plate. The standard reaction was placed into separate wells on the plate and the whole plate was mixed by shaking in the plate reader and incubated for 30 min at 37 °C. The reaction was stopped by addition of 100μL of stop solution (0.4 M sodium carbonate) and the optical density of each well was measured at A_{405} using the Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland) pre-warmed to 37 °C.

RNA interference (RNAi) mediated *Aam*Ach silencing

RNAi-mediated silencing was performed as previously published (Mulenga et. al., 2008). Double stranded RNA (dsRNA) was synthesized *in vitro* using the Megascript RNAi kit (Life Technologies) according to the manufacturer's protocol. To synthesize a 466 bp *Aam*Ach dsRNA target from postions 104 − 569 of *Aam*Ach −S and -L DNA sequences, templates for dsRNA synthesis were amplified using cloned *Aam*Ach plasmid DNA with specific primers with added T7 promoter sequence in bold (Forward: $5'$**TAATACGACTCACTATAGGG**CCACGTCAAGCACCACCACC$^{3'}$ and Reverse: $5'$**TAATACGACTCACTATAGGG**GCGTAGTTTTCCTTATCGCG$^{3'}$). Green fluorescent protein (GFP) PCR primers with T7 promoter sequence in bold (Forward: $5'$**TAATACGACTCACTATAGGG**ACGTAAACGGCCACAAGTTCAGCGTGTC$^{3'}$ and Reverse $5'$**TAATACGACTCACTATAGGG**TCACGAACTCCAGCAGGACC ATGTGATC$^{3'}$) were used to amplify the template for the control group. The purified dsRNA was eluted in nuclease free water. Two test groups of 15 female *A. americanum* ticks were injected with GFP-dsRNA injected control or *Aam*Ach dsRNA injected target. Ticks were injected with 0.5-1μL (~3 μg/μL) of dsRNA on the ventral side of the lower right coxa using a 33 guage/ 0.5 inches/ 45° angle beveled needle on a Model 701 Hamilton syringe (Hamilton Company, Reno, NV). Injected ticks were kept for 24h at room temperature in 85% humidity to recover before being placed on specific pathogen free (SPF) New Zealand rabbits to feed.

Tick feeding was performed as described in chapter II of this thesis. Ticks injected with *Aam*Ach-dsRNA (n = 15) and GFP-dsRNA (n = 15) were placed into a two-inch cotton stockinet tick containment cells attached on top of the rabbit ear to feed using the Kamar adhesive (Kamar Products Inc.). The effect of *Aam*Ach mRNA silencing on tick feeding success was assessed by investigating tick attachment and mortality rates, time to feed to repletion, engorgement mass (EM) as an index for amount of blood taken in by tick, and egg mass conversion ratio (EMCR) as measure of utilizing blood meal to produce eggs. Attachment rates were determined by daily counts of unattached ticks, and then subtracting from the total number of ticks that were placed on the animal less those that were found dead. Mortality rates were determined by calculating dead ticks as a fraction of the total number of ticks. EM was the weight of spontaneously detached ticks. To determine EMCR, ticks were allowed to lay eggs for 3-4 weeks at room temperature in 85% humidity. EMCR was determined by dividing egg mass over EM. Tick phenotypes during feeding were documented daily using the Canon EOS Rebel XS camera attached to a Canon Ultrasonic EF 100mm 1:2:8 USM Macro Lens (Canon USA Inc, Melville, NY).

To validate if injection of dsRNA caused disruption of *Aam*Ach mRNA, three GFP dsRNA and *Aam*Ach dsRNA injected female ticks were sampled at the 48h post-attachment time point by manual detachment. Ticks were processed individually for dissection of tick organs as described (Mulenga et al. 2013). Dissected organs, salivary glands (SG), midguts (MG), synganglion (SYN), Malpighian tubule (MT), ovary (OVR) and remnants labeled as carcass (CA), preserved in 200-400μL RNAlater (Life

Technologies) were processed for mRNA extraction using the Dynabead mRNA Direct Kit (Life Technologies). Concentration of mRNA was determined using the NanoQuant Plate in the Infinite M200 Pro plate reader (Tecan). Template cDNA was synthesized from ~200ng of mRNA using the qScript cDNA SuperMix following the manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). To quantify *Aam*Ach transcript abundance in treatment and control ticks, cDNA was subjected to quantitative real time reverse transcriptase (qRT-PCR) using the ABI7300 system (Life Technologies). Routinely ~50ng of cDNA, and 900μM of *Aam*Ach forward $^{5'}$GGGAACTGGCTGGTGCATT$^{3'}$ reverse $^{5'}$GAGCGGCACGACGACTGT$^{3'}$ primers were added to 25μL SYBR Green Master Mix (Life Technologies) in triplicate. For internal control, *A. americanum* 40S ribosomal protein S4 (accession number GAGD01011247.1) which is stably expressed in *Ixodes scapularis* during feeding (Koci et al., 2013) was used. Relative Quantification (RQ) of *Aam*Ach transcript was determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001) incorporated into the ABI 7300 System software (Life Technologies). To determine the apparent level of *Aam*Ach mRNA suppression, the formula, $S = 100 - (RQ^T/RQ^C \times 100)$ where S = mRNA suppression, RQ^T and RQ^C = RQ of tissues in *Aam*Ach-dsRNA and GFP-*Aam*Ach injected ticks respectively was used. For each tick organ, mRNA suppression was determined as the mean ± SD of two S values. The two S values were generated by independently comparing treatment RQ to two separate control tick RQs.

Results

Expression and affinity purification of wild type and mutant r*Aam*Ach in *Pichia pastoris*

Fig. 5 summarizes the analysis of daily protein expression and affinity purification of recombinant (r) wild type (Wt) and mutant (Mu) *Aam*Ach-L during a five-day pilot experiment. This analysis showed a progressive daily increase in both Wt and Mu r*Aam*Ach expression with the highest level at day five (Fig. 5A and 5C). A three-liter large-scale expression of Wt and Mu r*Am*Ach was precipitated by ammonium sulfate saturation and affinity purified using $NiCl_2^+$ charged column. Following purification, elution fractions with relatively pure r*Aam*Ach were pooled and concentrated using JumboSep™ centrifugal device filters (Pall Corporation). Concentrated proteins were subjected to SDS-PAGE on 10% acrylamide gel followed by silver staining to verify purity of Wt and Mu r*Aam*Ach as shown in Figs. 5B and 5D, respectively. The predicted molecular mass of both Wt and Mu r*Aam*Ach is approximately 61 kDa: comprised of ~11 kDa N-terminus region, ~47 kDa of mature r*Aam*Ach protein and ~3 kDa of C-terminal tag containing the hexa-histidine sequence. However when Wt and Mu r*Aam*Ach were electrophoresed on SDS-PAGE and visualized by silver staining, a band at about 70 kDa is observed (Figs. 5B and 5D). Sequence analysis in chapter I revealed that *Aam*Ach had 2 and 24 putative N- and O-linked glycosylation sites respectively. Thus, there is a possibility that the observed band shift from 61 kDa to 70kDa was due to post-translational glycosylation of r*Aam*Ach. When both Wt and Mu r*Aam*Ach were treated with a deglycosylation mix (New England
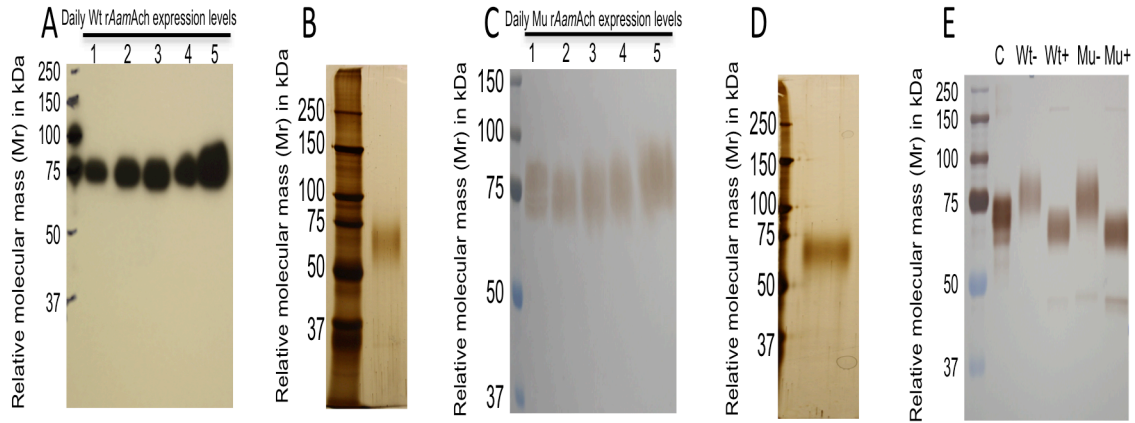
Fig. 5. Expression and affinity purification of recombinant (r) *Amblyomma americanum* **putative** *Aam***Ach expression in** *Pichia pastoris*. (A) Daily expression levels of wild type (Wt) putative r*Aam*Ach through 5 days of culture. Construction of the expression plasmid, induction and validation of expression levels using antibody to the C-terminus hexa-histidine tag and affinity purification of Wt putative r*Aam*Ach were performed as described in material and methods section in chapter III. Visualized by chemiluminescent detection. (B) Validation of affinity purification of Wt putative r*Aam*Ach using silver staining, was accomplished by combination of ammonium sulfate precipitation and affinity purification with NiCl^{2+} charged columns as described in material and methods section in chapter III. (C) Daily expression levels of mutant (Mu) putative r*Aam*Ach through 5 days of culture. Mu putative r*Aam*Ach was constructed as described for fig. 5A. Visualized by chromogenic detection. (D) Validation of affinity purification of Mu putative r*Aam*Ach using silver staining was performed as described in fig. 5B. (E) Validation of N- and/ or O-linked glycosylation post-translational modification of Wt and Mu putative r*Aam*Ach: 1μg of affinity purified Wt and Mu putative r*Aam*Ach was treated with a commercial deglycosylation mix described in material and methods section in chapter III. Deglycosylated Wt and Mu putative r*Aam*Ach was detected by western blot analysis using antibodies to the C-terminus hexa-histidine tag. The (C): positive hexa-histidine control, recombinant tick calreticulin, (Wt-): wild type putative r*Aam*Ach not treated with deglycosylation enzyme mix, (Wt+): wild type putative r*Aam*Ach treated with deglycosylation enzyme mix, (Mu-): Mutant putative r*Aam*Ach not treated with deglycosylation enzyme mix, (Mu+): Mutant putative r*Aam*Ach treated with deglycosylation enzyme mix.

Biolabs) that cleaves off both N- and O- linked glycans, there was a downshift in the molecular weight to about 61 kDa (Fig. 5E) confirming that r*Aam*Ach was glycosylated.

Both wild type and mutant (P189E) r*Aam*Ach-L apparently have no chitinase activity

To determine if *Aam*Ach had chitinolytic activity, Wt r*Aam*Ach-L was subjected to chitinase function assay using a colorimetric substrate Chitinase assay kit (Sigma). There was no apparent exo- or endo- chitinase activity of r*Aam*Ach with 4-nitrophenyl N,N′-diacetyl-β-D-chitobioside and 4-nitrophenyl N-acetyl-β-D-glucosaminide exochitinase substrates and 4-nitrophenyl β-D-N,N′,N″-triacetylchitotriose endochitinase substrate when compared to the chitinase from *Trichoderma viride* as a positive control which was provided in the kit (not shown). Likewise Mu (P189E, amino acid residue numbering is based on the *Aam*Ach- L, NCBI accession number: KF819831) r*Aam*Ach-L did not show activity.

Validation of RNAi-mediated silencing by qRT-PCR

The Mega script in vitro RNA synthesis kit (Life Technologies) was used to successfully synthesize and purify the target *Aam*Ach-dsRNA and control GFP-dsRNA (Fig. 6). Double stranded RNA quantitative real time RT-PCR expression analysis was used to confirm if the delivery of *Aam*Ach-dsRNA triggered silencing of *Aam*Ach mRNA as shown in Fig. 7. Fig. 7 show that *Aam*Ach mRNA was apparently suppressed by 77.5 - 91.2 % in the salivary gland, 79.9 - 96.6 % in the synganglion, 56.3 - 92.3 % in the midgut, and 0 - 91.2 % in Malpighian tubules. Silencing in the ovary and carcass
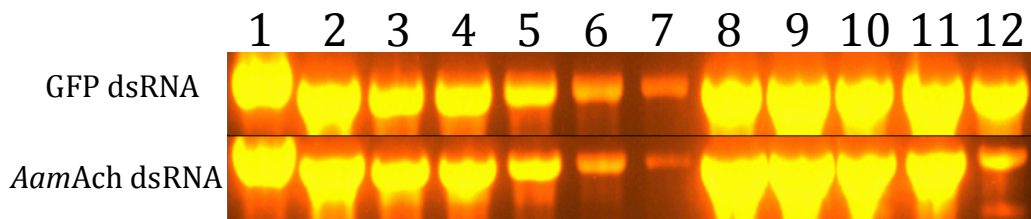
Fig. 6. Synthesis of *Aam*Ach and control GFP dsRNA. Synthesized and purified dsRNAs from *Aam*Ach (bottom panel) and control gene (GFP) resolved on a 2% agarose gel containing 1 µg/mL ethidium bromide. Lane 1 represents the dsRNA synthesis before digestion with DNase I and RNase. Lane 2 represents the dsRNA synthesis after digestion with DNase I and RNase. Lanes 3-7 show decreasing concentrations of purified dsRNA elutions. Lanes 8-12 represent decreasing concentrations of concentrated and purified dsRNA elutions.

were minimal, 32.2 - 54.3 % and 0 - 73 %. It is interesting to note that while in tick 1 *Aam*Ach mRNA was suppressed in the SG, SYN, MG, OVR, no silencing occurred in the MT and CA.

Putative *Aam*Ach may potentially be a key factor in the maintenance of a stable cement plug

Silencing of *Aam*Ach mRNA did not affect the ability of ticks to attach onto host skin and start feeding because 24 h after being placed on rabbits, 100% of both GFP-dsRNA and *Aam*Ach-dsRNA-injected ticks were attached (not shown). The tick RNAi-
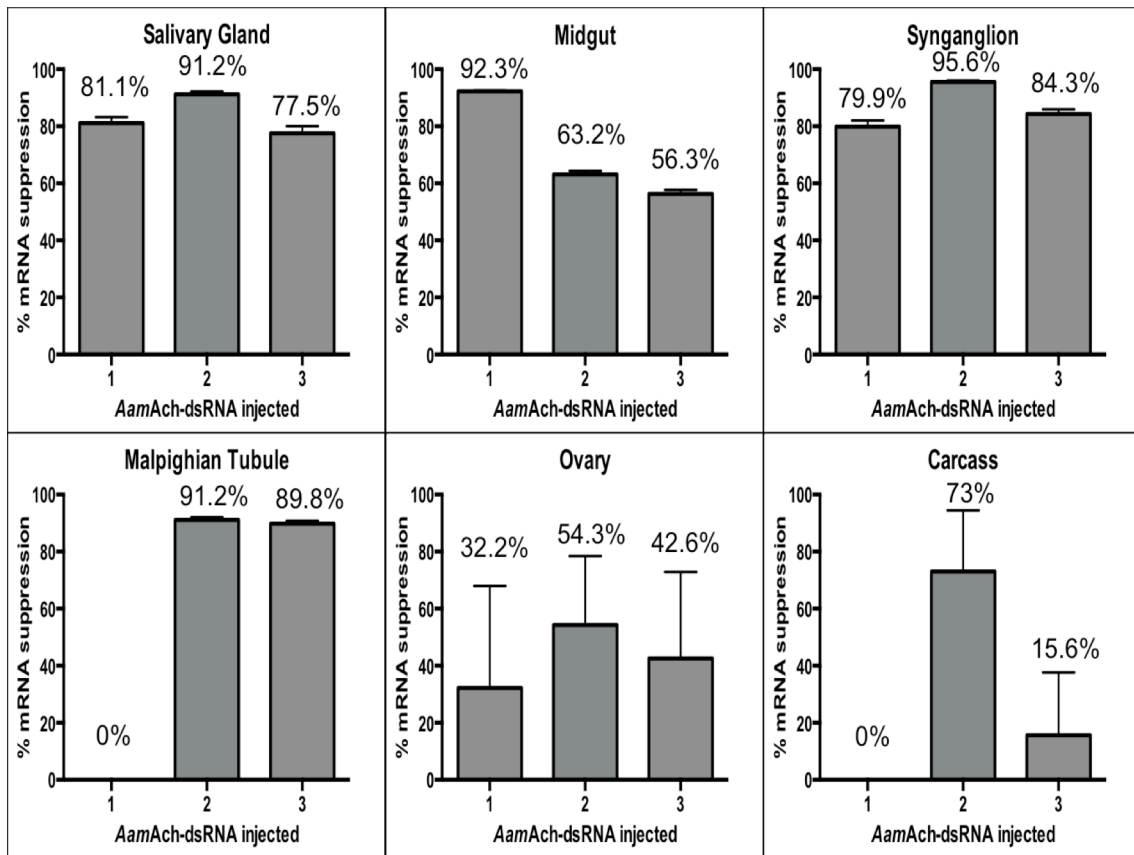
Fig. 7. Determining suppression level of *Amblyomma americanum Aam*Ch mRNA in tick feeding success. Validating the disruption of *Aam*Ch mRNA in *Aam*Ch-double stranded (ds) RNA injected ticks. Fifteen ticks were microinjected with 0.5–1 µL (2–3 µg/µL) of *Aam*Ch or GFP (control) dsRNA in nuclease free water. At 48 h post-attachment, three ticks per treatment of GFP-dsRNA injected control and *Aam*Ch-dsRNA injected ticks, were manually detached. Ticks were individually processed for mRNA extraction and then subjected to two-step quantitative (q) reverse transcriptase (RT)-PCR using *Aam*Ch PCR primers. A relative quantification (RQ) of gene expression study was performed using the ABI 7300 Software by the Comparative CT Method (ΔΔCt) to determine mRNA suppression levels of *Aam*Ch. To determine the apparent level of *Aam*Ch mRNA suppression, the following formula, $S = 100 - (RQ^T/RQ^C \times 100)$ where S = mRNA suppression, RQ^T and RQ^C = RQ of tissues of *Aam*Ch-dsRNA and GFP-*Aam*Ch injected ticks respectively, was used.

mediated silencing feeding phenotypes were documented every 24 hours with pictures. Fig. 8 presents documentation of *Aam*Ach-dsRNA and GFP-dsRNA injected ticks feeding on the rabbit host in two independent experiments. In both experiments there was leakage of blood around the tick mouthparts by day 13 post-attachment in *Aam*Ach-dsRNA injected ticks (indicated by red arrows). No leakage of blood was observed in GFP-dsRNA injected control ticks. In the first experiment, bleeding was so intense that by the 15th day, veterinarians at the Comparative Medicine Program (CMP) facility advised to halt the experiment due to the uncontrolled seepage of blood. Therefore, all the remaining ticks on the host were removed. In the second experiment bleeding had continued, but it was not as intense to stop the experiment when compared to first experiment. While manually detaching *Aam*Ach-dsRNA injected ticks, the author noticed that the ticks were weakly attached onto the host, in that the ticks would fall off the host with a gentle touch to the tick.

The effects of RNAi mediated silencing of *Aam*Ach on feeding efficiency and fecundity are apparent but not statistically

As an index to measure the amount of blood imbibed by ticks, engorgement mass (EM) of replete fed and spontaneously detached *Aam*Ach-dsRNA and GFP-dsRNA injected ticks were determined (Fig. 9A). EM of *Aam*Ach-dsRNA and GFP-dsRNA injected ticks ranged from 362.7 – 751.2 mg (n = 7) and 145.4 – 777.9 mg (n = 7), respectively. Although there is apparent difference between mean EM of *Aam*Ach-dsRNA (441.1± 92.27mg) GFP-dsRNA (504.2±53.66mg) injected ticks (Fig. 9B), an
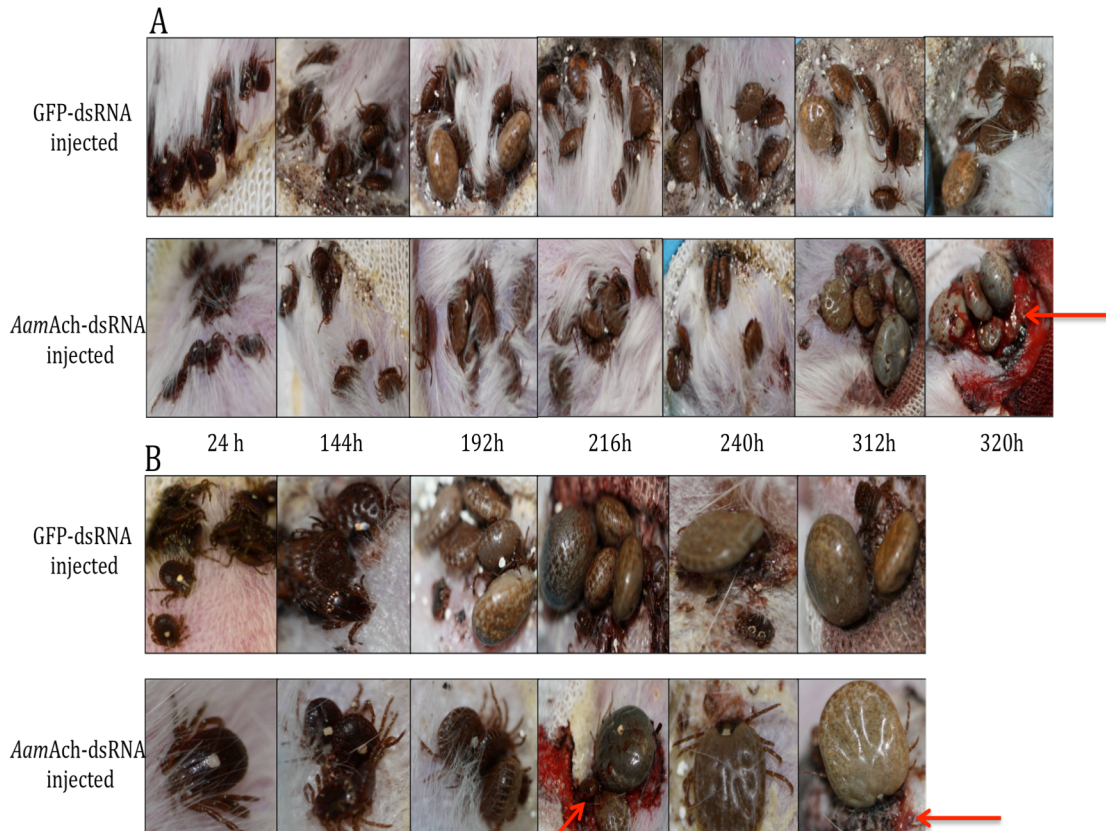
Fig. 8. Phenotype of *Aam*Ach-dsRNA injected ticks. Ticks were photographed every 24 hours post-attachment until the end of the experiment to note changes during tick feeding in putative *Aam*Ach-dsRNA injected ticks compared to control GFP-dsRNA injected ticks. The experiment was repeated twice: (A) Set of pictures from first trial that was stopped at 320h post-attachment due to excessive bleeding from host at tick feeding site and (B) set of pictures from second trial in which ticks completed feeding and tick organs were dissected and analyzed to measure suppression levels. Red arrows indicate the excess pool of blood around feeding site, not observed in control GFP-dsRNA injected ticks on both trials.

Fig. 9. Analysis of the effect of silencing *Amblyomma americanum* putative *Aam*Ach mRNA transcript on blood meal acquisition and fecundity. 15 unfed female ticks were injected with dsRNA pu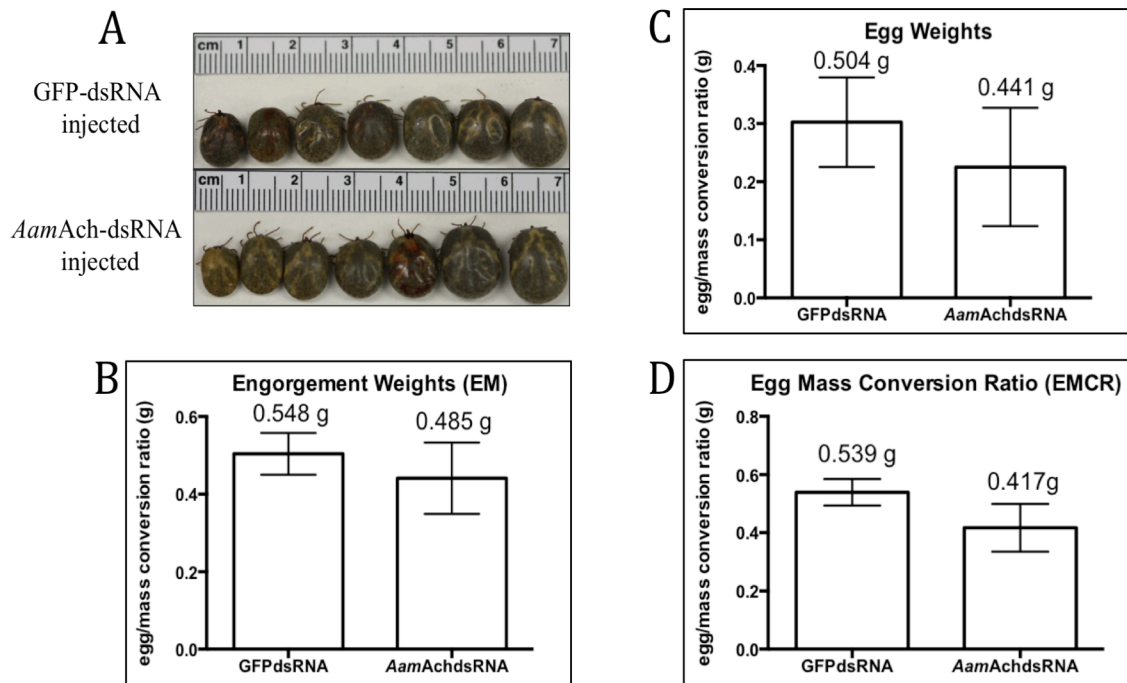tative *Aam*Ach and GFP. Each group of ticks were fed on rabbits and weighed after detachment. (A) Spontaneously detached ticks were photographed to document their phenotype. (B) Spontaneously detached ticks were individually weighed to determine the engorgement mass (EM). The EM means for each group were subjected to a unpaired student T-test to determine the statistical significance between treatments. Mean EM of treated and controls were not statistically different. Mean engorgement weight (grams) noted on top of respective bar. (C) After determining the EM, ticks were placed at room temperature for 30 days to lay eggs as described in materials and methods section in chapter III. Subsequently eggs were weighed (grams) to calculate the egg mass conversion ratio (EMCR: amount of blood meal converted for egg development). The egg weight means for each group were subjected to a unpaired student T-test to determine the statistical significance between treatments. Mean egg weights were not significantly different. Mean egg weights (grams) noted on top of respective bar. (D) Egg mass conversion ratio calculated by the egg weight divided by the EM. EMCR means for each group were subjected to unpaired student T-test to determine the statistical significance between treatments. ECMR between treatment groups was not significantly different. EMCR (grams) noted on top of respective bar.

unpaired student t-test showed that differences were not statistically significant (P = 0.5656).

To determine the effect of RNAi-mediated silencing on fecundity, ticks were allowed to oviposit for 30 days and egg clutches were weighed. As an index of the tick's ability to convert its blood meal to eggs, the egg mass conversion ratio (EMCR) was calculated by dividing the egg weight by EM. Calculated EMCR for GFP-dsRNA and *Aam*Ach RNA ranged from 594.9 – 449.1 mg (n=3) and 532.7 – 258.2 mg (n=3) respectively. While the EMCR of *Aam*Ach-dsRNA (417.0± 82.12 mg) injected ticks is apparently smaller than GFP-dsRNA injected ticks (539.3 ±45.52) (Fig. 9D), the observed difference is not statistically significant as revealed using an unpaired student t-test (P= 0.2626).

Discussion

Recent advances in genomics, proteomics, RNA sequencing and bioinformatics technologies have allowed for discovery of candidate genes that may be important to tick physiology. The majority of tick genes that are being discovered are of unknown functions (McNally et al., 2012; Mulenga et al., 2007; Ribeiro et al., 2006). The limited numbers of candidate tick genes that are annotated are based on sequence analysis compared to homologous genes that have been functionally characterized in other organisms. Whether or not annotated candidate genes perform the same functions in tick physiology is not known. In chapter II of this master's thesis research, the author determined that putative *Aam*Ach-L belongs in the GH-18 family chitinase and that the

mRNA transcript was ubiquitously expressed during the first five days of post-attachment. The next goal of the research was to understand whether or not *Aam*Ach-L was a functional GH-18 family chitinase. Therefore, to gauge insight into functional properties, r*Aam*Ach was successfully expressed in *Pichia pastoris*. Consistent with bioinformatics prediction of multiple putative N- and O-linked glycosylation sites in its sequence, r*Aam*Ach-L was significantly glycosylated. This meant that r*Aam*Ach-L was potentially well folded with appropriate posttranslational modifications, and thus functional analysis data could potentially reflect *in vivo* events. Based on substrate hydrolysis assays in this research, wild type r*Aam*Ach-L is apparently not an active chitinase. In GH-18 functional domain "FDG(L/F)DL**DWE**(Y/F)P", aspartic acid (D) and glutamic acid (E) (highlighted in bold) were thought to be important to activity of GH18 chitinases (Arakane and Muthukrishnan, 2010). Thus, the lack of chitinase activity by r*Aam*Ach-L may be explained by the fact that within the putative functional domain, "LDGVDM**AWP**FP", of *Aam*Ach amino acid sequence "D" is replaced by "A", while "E" is replaced by "P". In this research, site directed mutagenesis of "P" to "E" did not restore chitinase activity. This may suggest that restoration of function may require both "D" and "E" in the functional domain. The observation here is consistent with reported studies where GH-18 chitinase-like proteins in *Tribolium castaneum*, *Autographa californica*, and *Manduca sexta* that have mutations similar to *Aam*Ach were not functional chitinases (Arakane and Muthukrishnan, 2010; Thomas et al., 2002; Lu et al., 2002). It is also important to note that one tick chitinase (accession # BAC06447.1) reported in the phylogeny analysis in chapter II retains amino acid

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residues that are important for GH-18 chitinase function (You et al., 2003). This may imply that ticks encode both a functional and non-functional GH-18 like chitinases.

Another objective of this study was to investigate the significance of *Aam*Ach in tick feeding physiology. Although apparent silencing of *Aam*Ach was observed in the SG, MG, SYN and MT, it did not affect the ability of the ticks to attach, initiate feeding, and reproduce. Despite the lack of effect on other tick feeding parameters, observations that *Aam*Ach silencing apparently affected the cement plug were significant. Within 5-30 min of attaching onto the host, ticks secrete an amorphous substance called cement to anchor itself onto host skin (Sonenshine, 1993). Tick cement forms a plug that contains the tick-feeding site under host skin. In future research, it will be interesting to investigate if *Aam*Ach is part of the cement plug.

CHAPTER IV

CONCLUSION

Immunization of animals against tick infestation is a proven alternative to the acaricide-based strategies to control ticks and tick borne diseases (Willadsen, 2006). The major bottleneck towards global adoption of anti-tick immunization as a strategy to control ticks and tick borne diseases is hampered by lack of effective vaccine target antigens. The key pre-requisite towards development of anti-tick vaccines is a deeper understanding of the molecular basis of tick feeding physiology. While recent advances in genomics, proteomics and bioinformatics technologies have been streamlined towards the discovery of huge numbers of candidate genes, painstaking functional and biological analyses studies to understand the importance of candidate genes in tick feeding physiology remain to be done. This research has made a contribution towards understanding the role(s) and significance of putative acidic chitinases, here named as *Aam*Ach-L and *Aam*Ach-S in *A. americanum* tick feeding physiology.

Bioinformatics analysis in this research revealed that *Aam*Ach-L was likely to function in the extracellular environment, while *Aam*Ach-S was an intracellular protein. From the perspective of the long interest of the Mulenga laboratory to find target antigens for anti-tick vaccine development, *Aam*Ach-L is appealing in that it is likely to interact with the vertebrate host's immune response factors. It is important to note that experiments to verify whether or not *Aam*Ach-L was secreted into the host during tick feeding were inconclusive and remain to be investigated. Based on RNAi silencing data,

*Aam*Ach is apparently important during the tick attachment phase, as it appears to be important to maintenance of the tick cement plug. Ticks are long term blood feeding ecto-parasites that remain securely attached onto host skin for long periods of time, 4-7 days for larva and nymphs, and 10-14 days for adult ticks (Sonenshine 1993). Without the cement cone, ticks can be easily groomed off the host. From this perspective candidate proteins such as *Aam*Ach-L that are important to maintenance of tick cement stability could represent effective target antigens for tick vaccine development. It will also be interesting to find out if *Aam*Ach-L is a component of tick cement or if it is involved with up stream events that lead to formation of the tick cement plug.

Another notable contribution in this study is that for first time, it was demonstrated that RNAi silencing could be validated in individual tick organs from individual ticks. Most published studies have pooled tick organs together to validate silencing by either semi-quantitative or quantitative RT-PCR analyses (Aljamali et al., 2003; Karim et al., 2005; Chalaire et al., 2011; Mulenga et al, 2013). An important outcome of the approach in this thesis research was that silencing of target mRNA in individual organs was achieved to different levels. The cause of this cannot be explained by data in this thesis research. However these data could be used to explain the fact that the effectiveness of RNAi silencing is at best partial, and that target validation will require follow up anti-tick immunization experiments. It is interesting to note that in all three animals the highest mRNA suppression levels were observed in the salivary gland followed by synganglion, midgut, Malpighian tubules, carcass, and the ovary. Whether

or not this implies that the ovary is not easily accessible by dsRNA remains to be investigated.

The limitation of the RNAi silencing approach in this study was that the dsRNA construct targeted both the *Aam*Ach −S and −L, and it is most likely that the mRNA silencing observed here could represent suppression of both isoforms. Another limitation was that protein turnover could not be assessed due to lack of specific *Aam*Ach antibody. Regardless of the limitations, data presented here clearly show the potential role for putative acidic chitinases in maintaining the cement plug. In future experiments, it would also be interesting to investigate specific contributions of the *Aam*Ach −S and −L isoforms to stability of the cement plug.

In conclusion, this research has made a contribution towards understanding the molecular basis of the tick attachment phase. As alluded to earlier, putative *Aam*Ach was discovered in *A. americanum* ticks that were stimulated to feed. This research has revealed that putative *Aam*Ach-L may not be a functional chitinase, but it is likely important to maintaining the cement cone. Important questions that remain to be resolved are whether or not *Aam*Ach non-specifically binds IgG, and whether or not it is part of the cement cone.

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