EXPRESSION ANALYSIS AND TARGET VALIDATION OF GENES ENCODING CEMENT PROTEINS IN *Amblyomma americanum* TICK SALIVA

A Thesis

by

TAYLOR CHRISTIAN HOLLMANN

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Albert Mulenga
Committee Members,	Patricia Pietrantonio
	Maria Esteve-Gassent
Head of Department,	Ramesh Vemulapalli

May 2017

Major Subject: Biomedical Sciences

ABSTRACT

Without feeding success, ticks can neither cause damage nor transmit disease agents to their hosts. From this perspective a deeper understanding of tick feeding physiology has been advocated. Hard ticks such as *Amblyomma americanum* are adapted to complete feeding over several days. A combination of key biological adaptations allows the tick to complete feeding without the host detecting the presence of the tick. One of these adaptations is the deposition of the tick cement cone, which glues tick mouthparts into the host's skin tissue and prevents the host from grooming off the tick. Thus, understanding the molecular basis of how the tick cement cone is formed and deposited could lead to the discovery of important targets for development of methods against tick feeding and transmission of disease agents. One of the lines of research in the lab is to understand tick cement formation and deposition through discovery of the protein composition of the tick cement cone. Prior to this thesis research, a colleague in the lab utilized LC-MS/MS sequencing to identify 85 proteins in tick cement that was recovered from manually detached adult *Amblyomma americanum* ticks. This thesis determined the functional roles of glycine-rich proteins (n=13) of the 85 tick cement proteins in *A. americanum* tick feeding. This was motivated by published chemical analysis studies that demonstrated that the majority of proteins in the tick cement cone are characterized by high content of glycine amino acid residues. Data in this thesis research indicates that abundance of encoding mRNA of the glycine-rich proteins increase with blood meal feeding with majority of these proteins being associated with tick feeding events through

five days feeding. Of significant interest, the transcription of some of the encoding mRNAs coincided with the developmental period when ticks attained appetence, implying that these could represent the tick's molecular preparation to start feeding. Additionally, RNAi silencing of some of the encoding mRNAs affected tick attachment onto host skin as indicated by apparent inflammation and subsequent bleeding around tick attachment sites. In conclusion, this thesis has contributed to our understanding of the molecular basis of tick cement physiology.

DEDICATION

This work is dedicated to my family: my mother, Pam Hollmann, my father, Michael Hollmann, and my sister, Mikayla Hollmann, who have supported me throughout my education.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Mulenga, for all of the guidance and support he provided me in the pursuit of my master's degree. I must also thank my committee members, Dr. Pietrantonio and Dr. Esteve-Gassent, not only for their guidance and support throughout my project but also for being wonderful professors that helped increase my love for science.

I also have to thank the students and post-docs of the Mulenga lab for sharing their knowledge with me and for being great friends to me during my time in the lab.

I could not have achieved this goal without the help of the Department of Veterinary Pathobiology at Texas A&M University. The faculty, staff and students have all been excellent in providing a good environment for graduate studies.

Finally, I acknowledge my family and friends, especially Elizabeth Gino, Kat Stiegal, and Zachary Britten, for their unlimited support and encouragement.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Professor Albert Mulenga (advisor) and Professor Maria Esteve-Gassent of the Department of Veterinary Pathobiology and Professor Patricia Pietrantonio of the Department of Entomology

The LC-MS/MS data that provided the targets in this study was previously conducted by lab members, Tae Kim and Lucas Tirloni.

All other work conducted for the thesis was completed by the student.

Funding Sources

This work was made possible in part by funding from the National Institutes of Health under grants NIHR21 (R21AI119873) and NIHRO1 (R01AI093858), and the USAID Feed the future lab.

NOMENCLATURE

TBD	Tick-borne disease
RT-PCR	Reverse transcriptase- polymerase chain reaction
qRT-PCR	Quantitative real time- polymerase chain reaction
SG	Salivary glands
MG	Midgut
CA	Carcass, remaining after removal of SG and MG
UE	Unfed expressed
FI	Apparently feeding induced
UR	Upregulated in response to feeding
EM	Engorgement mass
EMCR	Egg mass conversion ratio

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

1.1 Ticks and Tick-Borne Diseases

Ticks are classified into three families, Argasidae, the soft ticks, Ixodidae, the hard ticks, and the least studied Nuttalliellidae (Sonenshine, 1993). The majority of information is based on hard and soft tick studies with information on the Nuttalliellidae family being limited to a few reports in Tanzania and Southern Africa (Mans et al., 2014; Bedford, 1931). Between soft and hard ticks, the latter has the highest impact on public and veterinary health. Hence the bulk of work on ticks has focused on understanding the biology of hard ticks, which will be subsequently referred to as 'tick.' The tick has four development stages: the egg stage, and the three feeding stages, larvae, nymph, and adult. The last three tick life stages take a blood meal once per life stage. Engorged larvae and nymphs undergo molting to develop into the next life stage, while engorged adult females lay eggs and then die.

The importance of ticks stems from their direct effects as pests and their function as vectors of several human and animal disease agents including *Ehrlichia, Theileria, Babesia, Borrelia, Rickettsia, Hepatazoon,* Heartland virus, and Bourbon virus (Prusinski et al., 2014; Shock et al., 2014; Dantas-Torres et al., 2012; Jongejan and Uilenberg, 2004; Buller et al., 1999). In the livestock industry, the cost of controlling tick infestations, managing tick borne disease infections and associated production losses amounts several millions of US dollars, especially in tropical and subtropical areas regions of the world

(Marsh et al., 2016; Malak et al., 2012; Kivaria et al., 2007; Mukhebi et al., 1995). For instance, a recent report in Brazil estimates losses due to *Rhipicephalus microplus* infestations and associated *Babesia* infections at 3.24 billion US dollars (Grisi et al., 2014). Even in the United States, a cattle fever tick outbreak would cost approximately 123 million dollars per year (Anderson et al., 2010). In public health, ticks are considered second only to mosquitoes in terms of health impact of the transmitted disease agents (Jongegan and Uilenburg, 2004). Since the description of *Borrelia burdorferi* as the spirochete causative agent of Lyme disease, there has been an increase in reported human tick-borne diseases (**TBD**) infections worldwide (Shapiro and Gerber, 2000). The World Health Organization (WHO,http://www.who.int/mediacentre/factsheets/fs387/en/) has listed 23 top vector borne diseases that collectively cause more than 1 million deaths. Of the 23 vector borne diseases, six are tick borne including, Crimean-Congo hemorrhagic fever, Lyme disease, Relapsing fever borreliosis (soft tick transmitted), Rickettsial diseases (spotted fever and Q fever), tick borne encephalitis, and tularemia. In the United States (US), the majority of human vector borne diseases are tick borne (Childs and Paddock, 2013; Shapiro and Gerber, 2000). The US Centers for Disease control listed 15 human tick-borne disease (TBD, Table 1.1.) agents that were recognized in the US at the time of drafting this thesis (CDC, https://www.cdc.gov/ticks/diseases/). *Amblyomma americanum* the focus of this dissertation research transmits five of the 15 human TBD agents in the US (Table 1.1.).

Table 1.1. Tick-borne disease (TBD) agents and medically important ticks in the US.

TBD	TBD agent	Vector tick
Anaplasmosis	*Anaplasma phagocytophilum*	*Ixodes scapularis* and *Ixodes pacificus*
Babesiosis	*Babesia microti*	*Ixodes scapularis*
Lyme disease	*Borrelia burgdorferi* and *Borrelia mayonii*	*Ixodes scapularis*
Borrelia miyamotoi infection	*Borrelia miyamotoi*	*Ixodes scapularis* and *Ixodes pacificus*
Colorado tick fever	Colorado tick fever virus	*Dermacentor andersoni*
Ehrlichiosis	*Ehrlichia chaffeensis, Ehrlichia ewingii*, and *Ehrlichia muris-like*	*Ambylomma americanum*
Heartland virus	Heartland virus	*Ambylomma americanum*
Powassan disease	Powassan virus	*Ixodes scapularis* and *Ixodes cookei*
Rickettsia parkeri rickettsiosis	*Rickettsia parkeri*	*Amblyomma maculatum*
Rocky Mountain spotted fever (RMSF)	*Rickettsia rickettsii*	*Dermacentor variabilis, Dermacentor andersoni* and *Rhipicephalus sanguineus*
STARI (Southern tick-associated rash illness)	not known	*Ambylomma americanum*
Tick-borne relapsing fever (TBRF)	*Borrelia* species	*Ornithodoros hermsi, Ornithodoros parkeri* and *Ornithodoros turicata*
Tularemia	*Francisella tularensis*	*Dermacentor variabilis, Dermacentor andersoni* and *Amblyomma americanum*
364D rickettsiosis	*Rickettsia phillipi*	*Dermacentor occidentalis*

The Lone Star tick, *A. americanum* is distributed in the southeastern part of the US with an expanding range to the northeast and Midwestern regions (Figure 1.1.). *A.*

americanum is a three host tick, meaning that each of the three blood feeding life stages (larva, nymph, and adult) feeds on a separate host. Potential hosts include raccoons, opossums, coyotes, deer, other small and medium sized mammals, and humans (Childs and Paddock, 2002; Kollars, 1993). *A. americanum* was considered only a nuisance species until the discovery that it was the primary vector for *Ehrlichia chaffeensis* and *E. ewingii*, which can affect humans, dogs, and horses (Childs and Paddock, 2002; Buller et al., 1999; Anderson et al., 1993). *A. americanum* has now been found to vector *Borrelia lonestari* which is the suspected causative agent of southern tick-associated rash illness (STARI), *Rickettsia amblyommii*, and *Francisella tularensis* in humans (Mani et al., 2015; Appearson et al., 2008; Varela et al., 2004; Childs and Paddock, 2002). Recently, Savage et al. (2013) pointed to the possible role of *A. americanum* in transmission of Heartland virus, previously reported as pathogenic for humans (McMullan et al., 2012). In veterinary health, *A. americanum* is a competent vector for *Cytauxzoon felis*, affecting domestic cats, and *Theileria cervi*, which affects white-tailed deer (Shock et al., 2014; Childs and Paddock, 2002).

Figure 1.1. Map of Lone Star Tick distribution in the United States.
(Centers for Disease Control and Prevention, 2011)

1.2. Prevention of Tick-Borne Disease Infections

In the absence of effective vaccines against the many TBD agents, TBD prevention targets the tick vectors by either preventing tick bites via tick repellants or killing the ticks using acaricides. Acaricide treatments are used most prominently to control ticks. However, though acaricides are effective in the short term, they do not offer permanent solutions. Acaricide contamination of the environment, tick resistance to acaricides, and the high cost, and labor intensive nature of acaricide application are just a few of the limitations of acaricide use (Willadsen 2006; Graf et al., 2004). Therefore this control method must be replaced or supplemented with methods to overcome these limitations. One potential strategy as an alternate or supplemental tick control strategy is the immunization of hosts against tick feeding as first described by Allen and Humpherys (1976). The first ever commercialized anti-tick vaccine, GAVAC/TickGARD, targets the midgut glycoprotein Bm86 (de la Fuente et al., 2007; Willadsen et al., 1995). The application of this vaccine reduced the need for acaricide use and disease incidence in cattle. However, a major limitation of this vaccine is that it is restricted to control of *Rhipicephalus microplus* genus only, which renders the vaccine useless against other tick species including *A. americanum* (Willadsen, 2004). The limited success of GAVAC/TickGARD has encouraged efforts to find better replacements. The major limitation toward development of anti-tick vaccines is the lack of effective target anti-tick vaccine antigens. With exception of a few cases when TBD infections occurred due to exposure to contaminated materials (Hutt et al., 2017; Mani et al., 2015), TBD agents requires successful tick feeding. From this perspective a deeper understanding of how

ticks feed is expected to provide for opportunities to develop innovative tick control methods (Kim et al., 2014; Mulenga et al., 2007; Mulenga et al., 2000).

1.3. Tick Feeding Physiology: The Importance of the Tick Cement Cone

Hard ticks such as *A. americanum* feed for long periods, 4-7 days for larvae and nymph, and 10-14 days for adult ticks (Soneshine, 2013; Kemp et al., 1982). To feed on blood, ticks penetrate host skin with chelicerae and insert their barbed hypostome, which helps anchor the tick into the skin. In order to stay attached onto host skin during the long feeding period, ticks secrete a cement-like adhesive substance into the skin called "tick cement". Tick cement anchors the tick mouthparts into the host skin to prevent removal by grooming, seals the wound around the mouthparts, and also protects the mouthparts from the host immune system (Alekseev et. al, 1995). At least two layers of the tick cement cone have been documented. The first layer, called the "cortex", starts to be secreted within five to thirty minutes the tick inserting its hypostome into host skin, and hardens within the first 24 h of the tick attaching (Bullard et al., 2016; Sonenshine, 1993; Kemp et al., 1982). The second layer, called the cortical layer, continues to be deposited through 96 h of tick feeding (Sonenshine, 1993; Kemp et al., 1982).

Early chemical analysis studies revealed that tick cement cones are composed primarily of protein, as well as of lipid and carbohydrate bound to the protein in the core and cortex, respectively (Kemp et al., 1982). These studies determined that the majority of proteins in tick cement are glycine-rich (Ribeiro et al., 2017; Kim et al., 2016; de Castro et al., 2016; Karim and Ribeiro, 2015; Tirloni et al., 2014; Jiang et al., 2014; Oliveira et

al., 2013; Maruyama et al., 2010; Mulenga et al., 2007; Ribeiro et al., 2006; Untalan et al., 2005; Valenzuela et al., 2002; Bishop et al., 2002). In nature, glycine-rich proteins have adhesive and tensile characteristics, and similar glycine-rich proteins are found in spider silk and insect egg glue (Xu et al., 2015; Vasanthavada et al., 2012; Maruyama SR et al., 2010; Li et al., 2008).

1.4. Significance of Research

Deposition of the tick cement cone precedes transmission of the majority of TBD agents, which occurs after the tick has fed for more than two days (Cook, 2014; Shkap et al., 2009; Hojgaard et al., 2008; Des Vignes et al., 2001; Kjemtrup and Conrad, 2000; Hodzic et al., 1998; Katavolos et al., 1998; Piesman et al., 1987). Thus, interfering with tick cement formation has the potential to prevent TBD infections. There is published evidence that host immunity to components of tick cement can reduce tick-feeding efficiency (Shapiro et al., 1989; Brown and Askenase, 1986). Studies have also shown that immunization of animals with putative recombinant tick cement proteins provoked strong anti-tick immunity that significantly affected tick-feeding performance (Havlikova et al., 2009; Bishop et al., 2002). For instance, Zhou et al., (2006) immunized rabbits against *Rhipicephalus haemaphysaloides* with recombinant glycine-rich protein and found significantly higher mortality of nymph, and lower attachment rate of adult and nymphal ticks. In a related study, rabbit immunity to an immuno-dominant *Haemaphysalis longicornis* 29-KDa putative tick cement protein significantly reduced engorgement weights in adult ticks and up to 40 and 56% mortality in larvae and nymphs (Mulenga et

al., 1999). In another study, immunization of Guinea pigs with the *Rhipicephalus appendiculatus* putative tick cement protein, 64TRP resulted in increased mortality rates and caused the midguts of some of the ticks to rupture (Trimnell et al., 2005; Trimnell et al., 2002). In a follow up study, Labuda et al., (2006) demonstrated that vaccinating mice with 64TRP blocked tick-borne encephalitic virus (TBEV) transmission to co-feeding nymphal ticks and protected mice from fatal infection with the virus. In a lone study, Bishop et al. (2002) describes vaccination of cattle with RIM36, a putative tick cement protein. They found a strong antibody response in the cattle, but no anti-tick vaccine efficacy was reported.

Despite its importance, molecular identities of the tick cement cone remain unknown. The majority of putative tick cement proteins in literature have been annotated based on glycine residue content via mass spectrometry analysis (Kim et al., 2016; Tirloni et al., 2014; Oliveira et al., 2013; Ribeiro et al., 2006; Untalan et al., 2005), Illumina sequencing (Ribeiro et al., 2017; de Castro et al., 2016; Karim and Ribeiro, 2015;), or other methods (Mulenga et al., 2007). However there is no evidence that majority of the currently annotated putative tick cement proteins are associated with tick cement formation.

Based on the evidence in this introduction, the working hypothesis for the thesis was that tick cement proteins are necessary for the formation of the cement cone that holds the tick mouthparts into the skin of the host; therefore, disrupting their formation would adversely affect tick feeding. This hypothesis was tested in two specific objectives: 1) To relate the transcription patterns of 13 putative tick cement genes to the *A. americanum* tick

feeding cycle. The hypothesis was that putative tick cement transcripts would expressed when ticks have attained appetence and during feeding only. 2) To determine the effect of RNAi silencing of putative tick cement transcripts on tick feeding success. The hypothesis was that disrupting candidate putative tick cement transcripts would adversely affect tick feeding.

CHAPTER II

TEMPORAL AND SPATIAL TRANSCRIPTION ANALYSIS OF GLYCINE-RICH PUTATIVE AMBLYOMMA AMERICANUM TICK CEMENT PROTEINS

2.1. Introduction

Without feeding success, ticks can neither cause damage nor transmit disease agents to their hosts. From this perspective a deeper understanding of tick feeding physiology has been advocated (Dantas-Torres et al., 2012; Mulenga et al., 2007, de la Fuente et al., 2007; Willadsen, 2006; Willadsen, 2004). Hard ticks such as *Amblyomma americanum* are adapted to complete feeding over several days. A combination of key biological adaptations allows the tick to complete feeding without the vertebrate host grooming it off. One of the principal adaptations that allow the tick to complete feeding is the deposition of the tick cement cone (Bullard et al., 2016; Alekseev et al., 1995; Kemp et al., 1982). The tick cement cone glues the tick mouthparts to the host's skin tissue and prevents the host from grooming off the tick. Thus, understanding the molecular basis of how the tick cement cone is formed and deposited could lead to discovery of important targets for development of methods against tick feeding and transmission of disease agents. Therefore, one of the goals in the lab is to understand tick cement formation through discovery of the protein composition in tick cement cone. Prior to this thesis research, a colleague in the lab had conducted LC-MS/MS sequencing of proteins in tick cement that was recovered from the mouthparts of manually detached adult *A. americanum* ticks. In this second chapter of this thesis research, the goal was to gauge

insight into functional roles of glycine-rich proteins that were identified in the LC-MS/MS analysis of *A. americanum* tick feeding. The rationale is based on the findings that the majority of proteins in the tick cement cone are characterized by high content of glycine amino acid residues (Ribeiro et al., 2017; Kim et al., 2016; de Castro et al., 2016; Karim and Ribeiro, 2015; Tirloni et al., 2014; Jiang et al., 2014; Oliveira et al., 2013; Maruyama et al., 2010; Mulenga et al., 2007; Ribeiro et al., 2006; Untalan et al., 2005; Valenzuela et al., 2002; Bishop et al., 2002).

Semi- quantitative reverse transcriptase-PCR or quantitative real time-PCR transcription analysis represents the principal approach used to relate expression profiles of tick genes to the tick feeding process (Mulenga et al., 2008; 2009; de Castro et al., 2016; Kim et al., 2016). Routinely, total RNA is extracted from dissected organs salivary glands (SG), midgut (MG), and carcass (CA, tick remnant after removal of SG and MG of unfed and those that are partially fed for different periods of time. In this way, the up and down regulation of genes during the different stages of tick feeding can be determined. In this study, the author adopted similar approaches to describe transcription profiles of selected *A. americanum* tick cement glycine-rich proteins.

2.2. Materials and Methods

2.2.1. Tick feeding, dissections, and total RNA extraction and cDNA synthesis

Ticks used in this study were obtained from the National Institute of Health the Biodefense and Emerging Infections Research Resources Repository (BEI Resources) or purchased from Oklahoma State University tick lab. Nymph and adult *A. americanum*

ticks were fed on rabbits according to animal use protocol (AUP) 2014-0311. To feed on rabbits, ticks were confined to feed on top of rabbit ears using stockinetts glued onto the ears with Kamar Adhesive (Kamer Inc, Zionsville, IN). To feed nymphs on rabbits, the cotton stockinet tick containment cell was lined with pantyhose material to prevent nymphs from escaping. Larvae and nymphs were also fed on chickens as per AUP 2014-0311. Larvae and nymphs were fed on chickens confined to a cage. Ticks that dropped off the chicken were collected in a container surrounding the chicken cage.

Adult ticks partially fed on rabbits for 24, 48, 72, 96, and 120 h were manually detached and processed for dissection of salivary glands (SG), midguts (MG), and carcass (CA, tick remnant after removal of SG and MG) as described (Kim et al., 2014). Ticks for dissection were washed in diethylpyrocarbonate (DEPC) treated water and then placed on a clean glass slide ventral side down. Subsequently, a sterile razor blade was used to trim mouthparts around the basis capituli and the lateral edges around the tick. The ticks were placed on a concave well slide with DEPC water and the dorsum was lifted and removed to expose the tick organs. Dissected tick organs were stored in Trizol at -80°C.

To extract total RNA, tick organs, SG, MG, and CA pooled from five ticks fed for 24, 48, 72, 96, and 120 h were placed in 1 mL of Trizol (Life Technologies, Waltham, Massachusetts) total RNA extraction reagent. For engorged whole larvae and nymphs, ticks were minced in 1 mL Trizol with soft tissue scissors. Both dissected tick organs and whole ticks were homogenized using a tissue dissmembranator (Thermo Fisher Scientific, Waltham, Massachusetts). Samples were processed for total RNA extraction using the Trizol reagent according to the protocol provided by the manufacturer. Template cDNA

was synthesized from up to 500 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, Massachusetts).

2.2.2. Transcription analysis in unfed and partially fed adult ticks during feeding

Transcription analysis was done using two approaches, qualitative reverse transcription-polymerase chain reaction (**RT-PCR**) and quantitative real time polymerase chain reaction (**qRT-PCR**) analysis. In the first approach, qualitative RT-PCR was conducted to validate transcription of glycine-rich protein encoding transcripts as previously described (Mulenga et al., 2008). Tick feeding time points examined in nymphs were unfed, and those that were partially fed for 24, 48, and 72 h. In female adult ticks, transcription analysis was done in dissected SG, MG, and CA of unfed ticks and those that were partially fed for 24, 48, 72, 96, and 120 h. All qualitative RT-PCR reactions were conducted using the C1000 Touch Thermal Cycler (BioRad, Hercules, CA) according to the following cycling conditions: initial denaturation at 95°C 2 min, and 35 cycles; denaturation at 95°C 30 s, annealing (Table 2.1.) for 30 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. All targets were under 1 kilo base pair and thus extension time was set to 1 min. All qualitative RT-PCR reactions followed the recipe of GoTaq2X 5 µL, Forward primer 10 µM 0.5 µL, Reverse primer 10 µM 0.5 µL, water 3 µL, cDNA 1 µL. All PCR products were resolved on a 1-2% gel (depending on expected size of transcript product) with 1 µL of Ethidium bromide (10mg/mL) and photographed using the BioRad ChemiDoc MP Imaging System (Hercules, CA). Actin

was considered the control gene for expression, using primers described elsewhere (Chalaire et al 2011).

Table 2.1. Qualitative RT-PCR Primers.

I.D.	Expected Size (bp)	Forward primers 5'-3'	Reverse Primers 3'-5'	Annealing Temp. (°C)
Aam-179667	769	CCAGTGCGGCTGATGTCTTGC	GTAACGACACGAACGTCGCTG	58
Aam-16245	200	TGGCGGTTACTTTGGTCGTA	GACCAGACAAGCGTCCGTAG	53
Aam-178006	710	CCCTGGAGGATTCAGGCACTTC	TGGGTGCGGAACTGGGTGTG	56
Aam-177101	258	TACTTCGGGTTTCACCTTCCGTG	GCATAGAAGCCACCTTGGGAAC	55
Aam-177916	570	TGGTGGTGAAGACGGTCAG	GAGCCTCCATCATATCCACTGC	53
Aam-177785	822	ACTGGTCTTCTCGGAGGCTAC	GCTTCCATAGAGACCTGAGACG	53
Aam-326	762	GAATGGGATCAGCGGGTGGTTC	CTCCTTGCCTCCTGTACAAACG	54
Aam-177792	755	ACTGGTCTTCTCGGAGGCTAC	CCGAATGATCCCCAAGGACC	56
Clust_1237-2-1	842	CTTTGACAGTGGCAGCGGAAG	TCGTTCTACTGAGCGTCTCTCC	60
Aam-10038	200	TGCAGGTTTTGGTGGC	CTTGGAACCCAACCGT	48
Aam-181586	477	AAGCGTATTACGGTGGCTTG	GACCACCGAGACGTCCAAAG	52
Aam-42453	234	CTCCCAGGCTTGAGGAACC	AGGATCACTTAGAGGGCTTCCA	53
Contig_99147	181	GGTGGTGACTTAGGAGGCAG	GCTGGAGGAACTGTCGGTAG	53

Following confirmation of transcription, qRT-PCR analysis was done in three biological replicates of dissected SG that were pooled from five unfed ticks and those that were partially fed for 24-120 h as described above. In the first step, optimal concentrations of qRT-PCR primers (Table 2.2.) was determined. All reactions were performed using the BioRad CFX96 Real-Time System (Hercules, CA). The continuous amplification program consisted of 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min followed by one cycle of melt curve from 65°C to 95°C, increment of 0.5°C every 5 sec. Ribosomal protein S4 (*rps4*) was used as an internal

reference gene. This reference gene was selected based on the data gathered by Koči (2013) and has been used as a stable *A. americanum* reference gene by colleagues in the lab (Kim et al., 2014).

Table 2.2. Quantitative RT-PCR primers.

I.D.	Forward primers 5'-3'	Reverse Primers 3'-5'	Annealing Temp. (°C)	Primer concentration
Aam-179667	ACTCGGAGGTCCTTTGACAC	CAGTGGATGGGTCAGTAACG	55	150/150
Aam-16245	TATGGAGGATACCCGAGCTT	AACCGCCATAACCTGGATAC	60	150/150
Aam-178006	TGGAGGATTCAGGCACTTC	GGATCGGCGTTGGCACTAG	60	150/150
Aam-177101	AAACCAAAGCTGTTGCCAAG	TGGAGAATCAGGGCATAGAG	60	300/300
Aam-177916	CGTAAACCATTTGTAGGCAA	TCGCAATACCAACAGCAGAG	60	300/300
Aam-177785	GGCTCATTTGGACATTTGG	AATCCGCTTCCATACAAACC	55	150/150
Aam-326	CTCCAACCACCGCCGCC	GCTTAGGTGGAGGTTCG	-	-
Aam-177792	TGGTTTCCCTGGAGTTTACG	CGAAAGGTCCGTAGGTATGG	60	150/150
Clust_1237-2-1	GTGGAAGTGGTCAAGGAGGA	CTGATTCCCGATAGTGATGG	60	300/300
Aam-179800	GCTCCTTTGCTTATGGTTCG	GTCCGAAAGATTCAATGCGT	60	300/300
Aam-181586	GCCGCCCAAGCCACCGAG	GGTGGCTTGTATGGCGGAGG	55	300/300
Aam-42453	GGCGTAATCATGGTCATAG	GGCTCGTATGTTGTGTGG	60	300/300
Contig_99147	GAGGAACTGTCGGTAGCTTG	GGAAGTGGAGGAATTGGCAG	60	300/300

2.2.3. Transcription analysis during the "pre-appetence to appetence" transition

Newly molted ticks transition from the "pre-appetence phase" when they show little to no desire to feed, to the "appetence phase" when they show an increased desire to feed within 7-21 days after ecdysis (Walade and Rice, 1982). In the preliminary qualitative RT-PCR analysis three (Aam-16245, Aam-179667, Aam-178006) of the glycine-rich protein-encoding transcripts showed high expression in both unfed and partially fed ticks. The author was curious to determine when the three tick cement genes were transcribed

during the pre-appetence or appetence phase. Therefore, qRT-PCR analysis of the three transcripts was done during molting and post-ecdysis. Larvae and nymphs that engorged on chickens were sampled at Days 0 (the day the engorged larvae/nymph dropped off the host), 3, 7, 11, 14, 21 and 28 during molting, one week and two weeks post-ecdysis (around Days 35 and 42). Total RNA extraction, cDNA synthesis, and qRT-PCR were done using the same procedure outlined above.

2.2.4. Statistical analysis

The relative quantification (RQ) of transcript abundance in the biological replicates were calculated using the formula $2^{-\Delta\Delta C_T}$ as described by Livak and Schmittgen, (2001) and adopted by our lab (Kim et al., 2014). Using the C_T values of the three biological replicates, the ΔC_T values were determined by the formula C_T (Target) – C_T (RPS4) per time point. Subsequently, the $\Delta\Delta C_T$ values were determined using the formula ΔC_T (replicate) – C_T (average of replicates per tissue) per time point for each individual replicate. The fold change were calculated for each replicate in each tissue by using the formula $2^{-\Delta\Delta C_T}$. Data were graphed as average of the three biological replicates using the ANOVA function of the PRISM 6 software (Graphpad Software, La Jolla, CA).

2.3. Results

2.3.1. Candidate glycine-rich protein encoding transcripts are expressed in multiple tick organs

Qualitative RT-PCR analysis summarized in Figure 2.1. confirmed that encoding transcripts of glycine-rich tick cement proteins were expressed in multiple tick organs during tick feeding. Transcripts of all glycine-rich proteins investigated in this thesis were expressed in the SG, MG, and CA except for three (Clust_1237-2-1, Aam-42453, Aam-177785) and two (Aam-177792, Aam-177785) transcripts that were not detected in MG and CA respectively. Based on apparent abundance in SG of unfed ticks, targets were grouped into three categories including: (i) unfed expressed (UE), for those that that were apparently strongly expressed in both unfed and partially fed tick SG, (ii) up regulated in response to feeding (UR) for those that were weakly expressed unfed tick SG when compared to partially fed tick SG, and (iii) feeding induced (FI), for those transcripts that were not detectable in unfed tick SG (Figure 2.1.). All UE targets were also expressed in nymphs during feeding as shown in Figure 2.2.

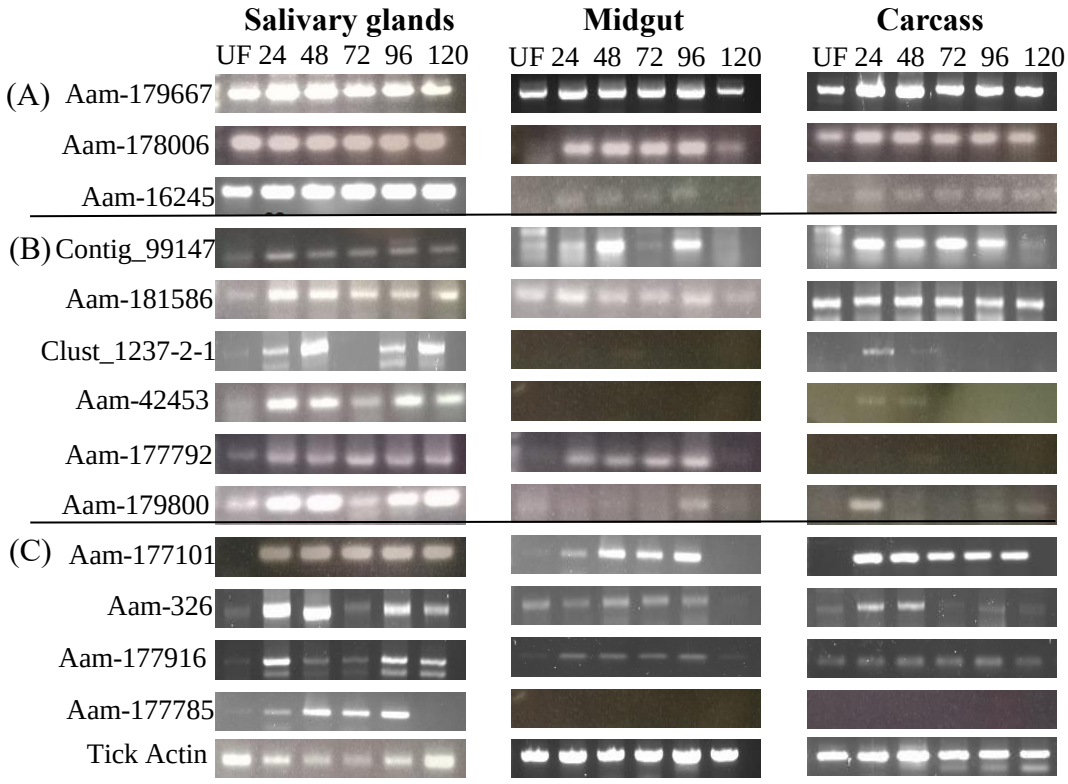

Figure 2.1. Qualitative RT-PCR expression analysis of mRNA transcripts during tick feeding (unfed through 120 hours of feeding) with tick actin control. Analysis completed using the primers listed in Table 2.1. Template cDNA made from total RNA extracted from dissected tick salivary gland (SG), midgut (MG), and carcass (CA) (remnants after removal of SG and MG). Transcripts grouped according to mRNA expression in salivary glands: (A) Unfed expressed (UE) are expressed in unfed ticks and through feeding. (B) Up-regulated in response to feeding (UR). (C) Apparently feeding induced expression (FI).

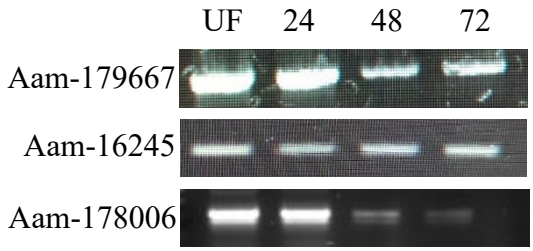

Figure 2.2. Qualitative RT-PCR expression analysis of UE mRNA transcripts during nymph tick feeding (unfed through 72 h of feeding) with tick actin control. Analysis completed using the primers listed in Table 2.1. Template cDNA made from total RNA extracted from whole nymph ticks that were manually detached from the host at the selected time points.

Following validation of transcription in SG during feeding (Figure 2.1.), a much more sensitive approach, qRT-PCR analysis was used to accurately relate mRNA expression profiles in SG to the tick feeding process. As shown in Figure 2.3., qRT-PCR revealed four expression profiles as indicated by transcript abundance in SG of unfed ticks compared to levels in SG of partially fed ticks. The first profile (Figure 2.3. A-D) includes transcripts for which mRNA abundance in unfed SG increased by more than 20 folds by the 24 h feeding time point and remained at this level through the 120 h feeding time point. Likewise transcript abundance in unfed SG for clust_320-51-1 increased by more than 100 folds by the 24 h time point and remained at that level through 120 h of feeding (Figure 2.3. E). The second profile involves Aam-177101, clust_1237-5-1, contig_99147, Aam-177785, and Aam-177916 for which transcript abundance in unfed SG respectively increased by more than 5, 100, 200, and 300 folds at the 24 h time point before it declined to near steady state levels by the 120 h feeding time point (Figure 2.3. F-J). In the third profile, Aam-179800 transcript level was at steady state through the 96 h feeding time point before increasing by more than 100 folds at the 120 h time point (Figure 2.3. K).

Lastly in the fourth profile, mRNA abundance in unfed tick SG for Aam-42453 decreased by two folds by the 24 to 72 h time points before it increased by two folds at 96 h and declined by 3 folds at the 120 h time point (Figure 2.3. L).

2.3.2. Unfed expressed (UE) glycine-rich genes are not expressed during molting but are induced after ecdysis

In preliminary qualitative RT-PCR analysis, transcripts of Aam-179667, Aam-178006, and Aam-16245, which were classified as unfed expressed, were strongly expressed in SG of unfed ticks (Figure 2.1.). To determine time points during tick feeding cycle when the three genes are transcribed, qRT-PCR analysis was conducted in cDNA of engorged larvae (Figure 2.4. A-F) and nymph (Figure 2.4. G-L) ticks that were sampled at zero, (sampled immediately after engorging), 3, 7, 11, and 21 days during molting, as newly ecdysed, and one and two weeks old nymph and adult ticks. This analysis revealed that all three genes were expressed at low steady state during molting. Figure 2.4. G-L show that transcription of the three genes increased immediately after ecdysis and significantly increased in adults at one week post-ecdysis. While the three genes were lowly expressed in larvae immediately after engorgement, the transcription profile was similar to that observed in molting nymphs with slight differences in that increased mRNA expression started at day 14 during molting and ramped up at day 5 post-ecdysis (Figure 2.4. A-F).

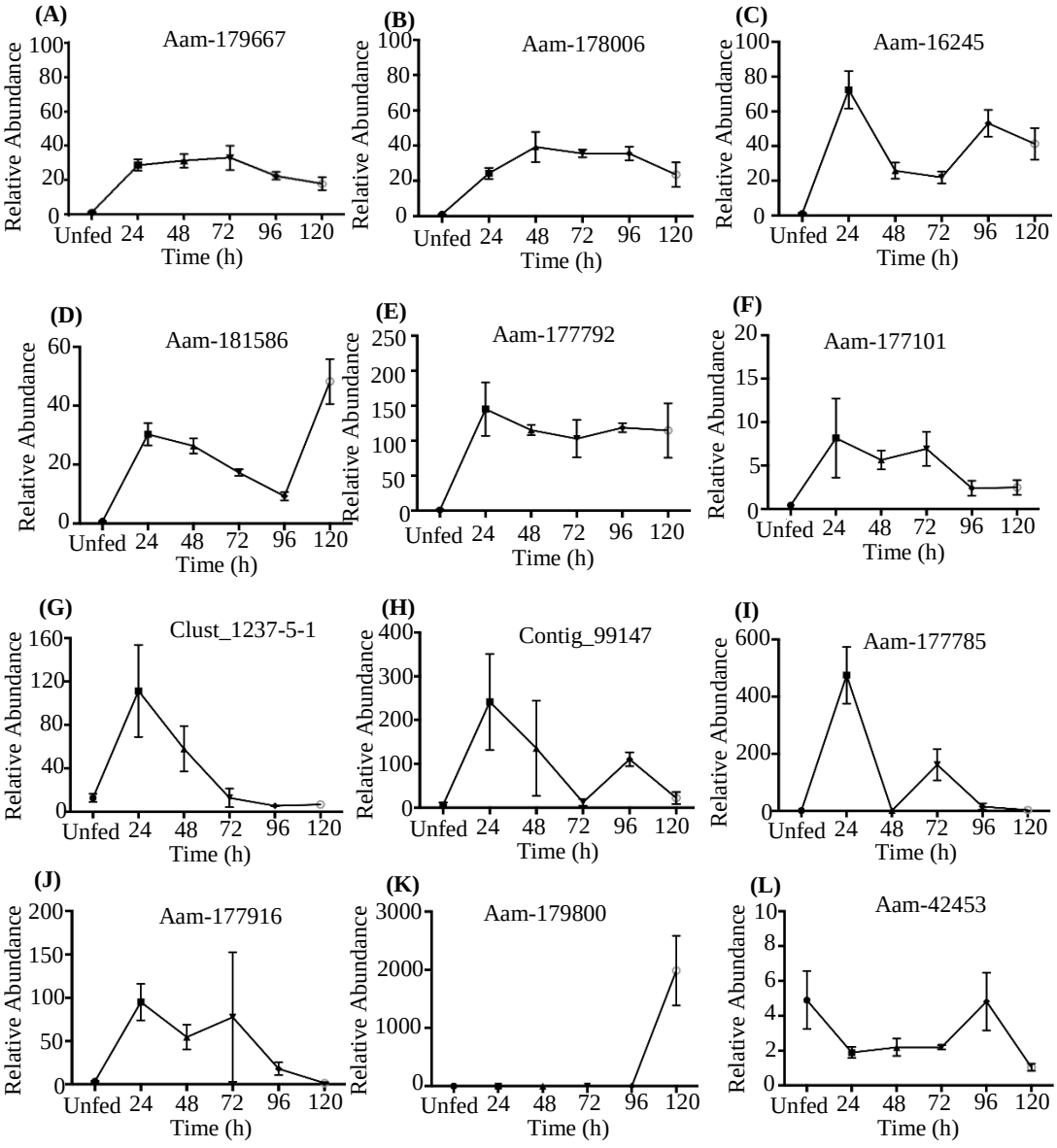

Figure 2.3. Expression analysis of putative tick cement protein mRNA transcript expression in salivary glands during tick feeding. Analysis conducted using the primers listed in Table 2.1. Template cDNA was the same as used for the qualitative analysis. mRNA relative abundance was calculated using the formula $2^{-\Delta\Delta C}{}_{T}$. Average relative abundance with standard error mean per time point as compared to the stable *Amblyomma americanum* reference gene rpS4 in tick salivary glands (n=3 per time point). *Expression of Aam-326 could not be determined.

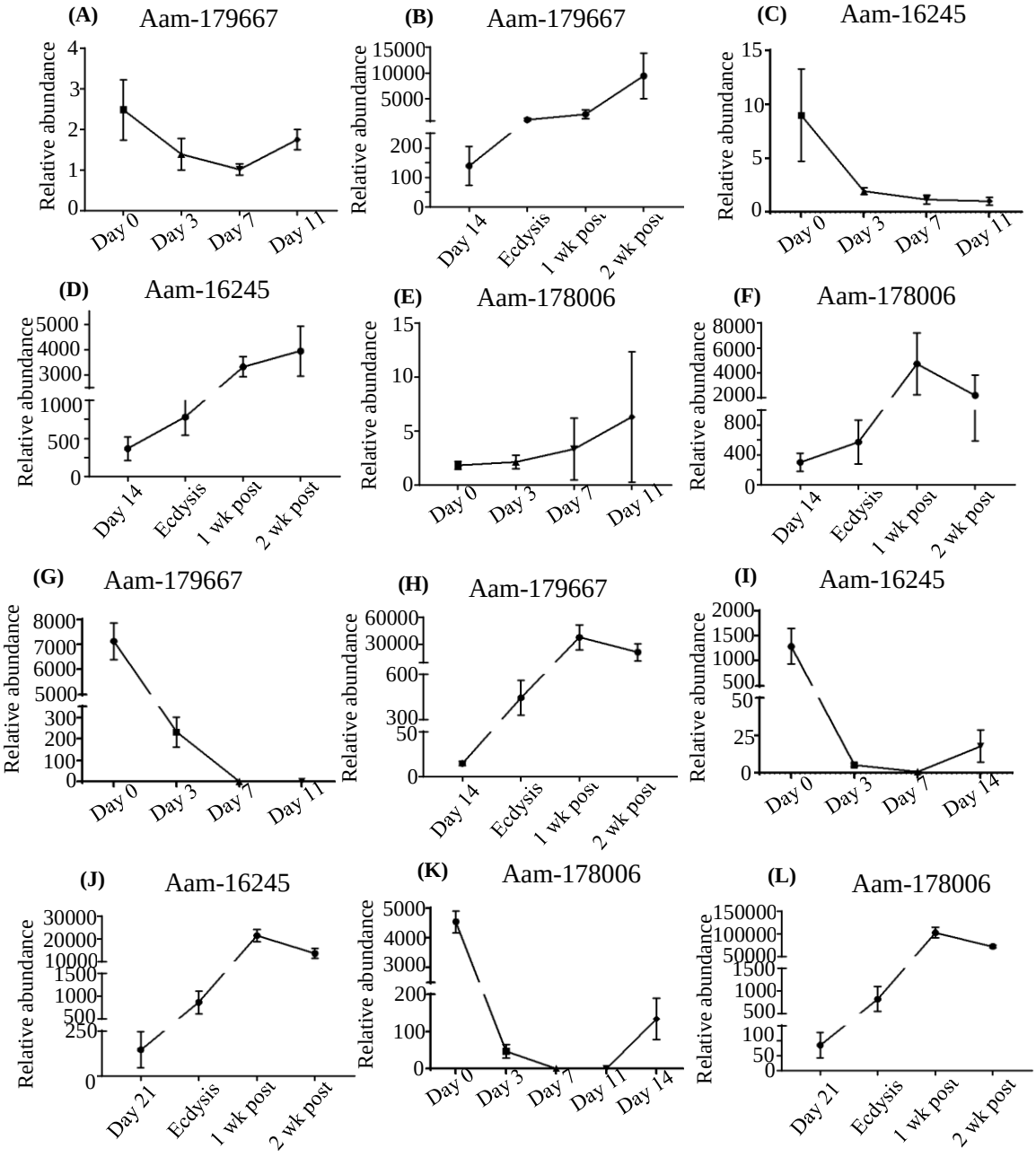

Figure 2.4. Quantitative RT-PCR analysis of UE mRNA transcript expression in larvae and nymphs during molting and post-ecdysis. Larvae and nymph ticks were placed on chickens and allowed to feed to repletion. Once ticks were engorged and dropped off of the host, the ticks were collected and sampled at the time points. Three whole ticks were sampled per time point. cDNA was created from extracted total RNA, and the mean expression with standard error mean per target as compared to the stable reference gene rpS4 was calculated.

2.4. Discussion

The second chapter of this thesis documents the transcriptional relationship of the thirteen candidate glycine-rich proteins to the tick feeding process. As noted in the first chapter of this thesis, components of the cortex layer of the tick cement cone are deposited within 5-30 min of the tick inserting its hypostome in host skin, while the outer layers continue to be secreted through the 96 h tick feeding time point (Sonenshine, 1993; Kemp et al., 1982). There is evidence that some of the early tick cement proteins are expressed and stored in the granular type II acini of the tick salivary glands before the tick engages its host to start feeding. This is based on observations that the size of the type II acini significantly shrinks after emptying its contents by the third day of feeding (Obenchain and Galun, 2013; Sauer et al., 1995). On this basis one may suspect that encoding transcripts of some the early tick cement proteins could be degraded once the tick cement proteins are expressed. Based on these observations, there was a possibility that some of the transcripts that encoded some of the 13 glycine-rich tick cement proteins studied here might not be expressed during feeding. However, transcription profile data demonstrates that encoding transcripts of all glycine-rich tick cement proteins studied in this thesis were expressed during tick feeding.

Transcription analysis in cDNA of ticks at different feeding stages as used in this research is among methods that are routinely used to gauge insight into functions of tick genes in tick feeding physiology (Kim et al., 2014; Chalaire et al., 2011; Maruyama et al., 2010). The tick feeding process is composed of a series of behavioral and physiological changes beginning with appetence and finishing with engorgement (Walade and Rice,

1982). After the tick attains appetence and engages the host, the act of feeding commences and can be classified into three steps: the preparatory feeding phase (PFP), Slow Feeding Phase (SFP), and Rapid Feeding Phase (RFP). The PFP during which the tick creates the feeding lesion, begins to deposit the cortex layer of the tick cement cone, and adheres onto host skin occurs during the first 24-36 h of feeding (Alekseev et. al, 1995; Sonenshine, 1993; Kemp et al., 1982). This is followed by the SFP during which the tick takes low amounts of blood and starts to transmit disease agents (Cook 2014; Shkap et al., 2009; Hojgaard et al., 2008; Des Vignes et al., 2001; Kjemtrup and Conrad, 2000; Hodzic et al., 1998; Katavolos et al., 1998; Piesman et al., 1987) may last from 7-10 days in adult ticks after the PFP. The final stage is the RFP during which the tick consumes large volumes of blood and lasts for 24 h. On this basis tick glycine-rich tick cement proteins for which transcript abundance was highest in unfed or 24 h fed tick SG but decreased as feeding progressed could be associated tick feeding functions related to the PFP. On the other hand those tick cement genes that were up regulated throughout feeding could have been involved with other biological functions that transcend the tick feeding process. The observations that tick cement transcripts in this study were expressed in multiple tick organs could point to the importance of glycine-rich proteins beyond tick cement formation; however, the focus of the study was on expression in SG, and MG expression was not further investigated. There is evidence in other organisms of glycine-rich peptides performing multiple functions including antimicrobial activity (Rahman et al., 2017; Carlier et al., 2015). One of the functions of tick salivary secretions is to keep the tick-feeding site free of antimicrobial contamination (Alekseev et al., 1995). Whether or not

some of the glycine-rich proteins discussed here, have antimicrobial functions remain to be investigated.

Another interesting observation in this study is that unfed expressed glycine-rich genes were lowly expressed during molting but were strongly induced after ecdysis. This was particularly interesting in engorged nymphs where transcript abundance in young adult ticks was up regulated by more than 3000 fold at one-week post ecdysis. It is noteworthy that this expression pattern coincides with tick attainment of appetence or desire to seek the host, which has been observed to occur at least one week after ecdysis (Anderson et al., 1998; Davey. 1987; Tukahirwa, 1976; Gladney et al., 1970). Published studies have demonstrated that newly ecdysed ticks require a physical maturation phase before they attain appetence (physiological readiness to feed), respond to feeding stimuli, and seek a blood meal source (Anderson et al., 1998; Davey. 1987; Tukahirwa, 1976; Gladney et al., 1970). Recent studies have identified ecdysis-related hormones in ticks including bursicon, which signals hardening of the tick exoskeleton (Egekwu et al., 2014; Roller et al., 2010). Therefore, the transcription of the targets' mRNA's may be induced by the presence of ecdysis-related hormones and the existence of the mRNA transcripts may be an indication of the tick's readiness to feed.

The tick feeding style of lacerating host tissue and then sucking up host blood that bleeds into the wounded area (commonly known as the tick feeding site) is thought to stimulate host tissue repair responses that are aimed at stopping further blood loss. However, ticks ensure a full blood meal by secreting a cocktail of enzymes that disarm the host's tissue repair response (Valenzuela et al., 2005; Ribeiro, 1995; Ribeiro, 1989;

Ribeiro et al., 1985). The host's tissue repair response to tick feeding activity is expected to be swift, and, logically, must be swiftly countered by the tick. On this basis, genes encoding proteins such as the unfed expressed tick cement proteins in this study that are expressed upon tick attainment of appetence could represent the tick's preparation to start feeding. In conclusion, this second chapter of this thesis has set a foundation for further studies into functional roles of glycine-rich tick cement proteins in *A. americanum* tick feeding.

CHAPTER III

DETERMINE THE IMPORTANCE OF PUTATIVE TICK CEMENT PROTEINS IN AMBLYOMMA AMERICANUM TICK ATTACHMENT AND FEEDING

3.1. Introduction

Expression of transcripts encoding candidate glycine-rich tick cement proteins in this study was confirmed in the second chapter of this thesis. The goal of this third chapter was to determine the significance of the thirteen tick cement proteins in *A. americanum* tick feeding success using the RNA interference (RNAi) mediated post-transcriptional gene silencing technology. In the tick research community, the RNAi silencing method has been widely used to determine the importance of target genes in hard ticks such as, *I. scapularis* (Kim et al., 2014; Aljamali et al., 2003), *A. americanum* (Kim et al., 2016; Mulenga et al., 2013; Chalaire et al., 2011; Kocan et al., 2007), and the soft ticks, *Ornithodoros moubata* (Diaz-Martin et al., 2013). The RNAi pathway is a naturally occurring mechanism in which the presence of double stranded RNA (dsRNA) induces degradation of cognate mRNA (Berstein et al., 2000). RNAi is initiated by the injection of dsRNA that correlates with the target mRNA (Haley et al., 2003). The dsRNA is cleaved into small interfering RNA (siRNA) by the dicer enzyme of the RNase III family (Ketting et al., 2001; Macrae et al., 2006). The siRNA direct RNA-induced silencing complexes (RISCs) to the target mRNA which is then destroyed, inhibiting further translation. The general understanding of RNAi is that if a target is biologically important, then silencing the target should result in a loss of fitness or observable phenotype change.

3.2. Materials and Methods

3.2.1. Combinatorial RNAi silencing analysis

Given the high number of candidate glycine-rich proteins, targets were disrupted as a group using combinatorial RNAi silencing as described (Kim et al., 2014). Three combinatorial (co) double stranded (ds) RNA were designed according to transcriptional profiles that were established in the second chapter of this thesis. These included unfed expressed (UE, three targets), the apparently feeding induced (FI, four targets), and apparently upregulated in response to feeding (UR, six targets) group.

Template DNA for co-dsRNA synthesis was designed in-house and custom synthesized at Life Technologies (Thermo Fisher Scientific, Waltham, Massachusetts). To design template composite DNA for the three co-dsRNA, I selected and spliced unique ~200 base pairs (bp) of each candidate tick cement transcript using MacVector (MacVector Inc., Apex, North Carolina). To identify unique sequence regions, candidate tick cement sequences were searched against *A. americanum* entries in GenBank using BLASTN and portions of sequences that did not show similarity to other sequences were selected. The rationale was to avoid non-target effects of RNAi silencing. The custom synthesized co-dsRNA were ligated into pMX plasmid.

Following custom synthesis, plasmids containing co-dsRNA templates were re-solubilized in 20 µL of water and transformed into DH5α competent cells using routine heat shock methods. To transform the plasmid, 1µL of plasmid was mixed with 20 µL of DH5α competent cells. After 20 min of incubation, the cells were incubated in a water bath at 42°C for 30 sec and then placed on ice for two minutes. At the end of the two

minutes, 250 µL of warm SOC broth were added and the mixture was incubated with shaking at 37°C for one hour. Transformants were selected on LB Agar plates with Ampicillin and grown overnight at 37°C. Inserts were checked by PCR using plasmid specific primers to further validate positive transformants. Bacteria colonies confirmed to contain the insert were used to inoculate 7 mL of SOB with Ampicillin and incubated overnight at 37°C with shaking. To purify plasmids, overnight cultures were subjected to a mini-prep using the Promega Wizard Plus SV Minipreps DNA Purification System (Fitchburg, Wisconsin). To confirm co-dsRNA template DNA sequences, purified plasmids were prepared for sequencing using BigDye and plasmid specific primers. Sequencing was done at Gene Technologies Laboratory at Texas A&M University.

The Ambion Megascript RNAi Kit (Thermo Fisher Scientific, Waltham, Massachusetts) was used to synthesize co-dsRNA according to manufacturer's instructions. Approximately 2 µg of purified plasmid DNA was used as template for co-dsRNA synthesis using primers with added T7 promoter sequence (Table 3.1.). Green fluorescent protein (GFP) primers with added T7 promoter were used to amplify the template for synthesis of control dsRNA.

Table 3.1. Primers for co-dsRNA synthesis.

UE	Forward	TAAACGACTCACTATAGGGACATCTGTCTCCACCGCACCCG
	Reverse	TAAACGACTCACTATAGGGGGTGCGGAACTGGGTGTGGGA
UR	Forward	TAAACGACTCACTATAGGGGCTTGTACGGAGGCTACCCAGGAG
	Reverse	TAAACGACTCACTATAGGGCTTAGGAAGTGGAGGAATTGGC
FI	Forward	TAAACGACTCACTATAGGGGAGATCTGGGCTGCTGGTAT
	Reverse	TAAACGACTCACTATAGGGGTGTTGAGAGAGATCCTAG

3.2.2. Determine the timeline to depletion of mRNA of UE tick glycine-rich cement proteins that predated co-dsRNA injection

Qualitative RT-PCR analysis in the first chapter of this thesis determined that mRNA of UE tick cement proteins was strongly expressed in unfed ticks. Based on previous experience in the lab, it has been observed that the conventional approach of incubating ticks that are injected with dsRNA for 24 h before feeding to evaluate the effects of RNAi silencing did not provide for sufficient time to deplete target mRNA that predated induction of RNAi silencing. Without this step, there was a likelihood that pre-existing mRNA would mask the effects of RNAi silencing. The plan was to determine the timeline at which UE tick cement transcripts were significantly depleted in unfed ticks. Subsequently this timeline was used to plan the study to analyze the effect of RNAi silencing of UE tick cement transcripts on tick feeding.

Female ticks (n = 20) were injected with ~0.5-1 µL (3-5 µg/µL) of co-dsRNA or GFP dsRNA (control) behind the fourth coxae using 33 gauge Hamilton needles (0.50”

45° #7803-05) (Reno, Nevada) or with pulled 10 µL glass capillary. Three ticks each were sampled at 3, 7, 14, 21, and 28 days post co-dsRNA injection and individually processed for total RNA extraction and cDNA synthesis as described in chapter two. Individual tick cDNA was subjected to qRT-PCR to validate disruption of target mRNA as biological replicates. qRT-PCR (Table 3.2.) primers used were designed outside sequence regions that were used to design dsRNA. The rationale of this approach was to avoid amplifying the injected dsRNA. All reactions were performed on the BioRad CFX96 Real-Time System (Hercules, CA) according to this procedure: one step at 50°C for 2 min**,** 95°C for 10 min, 40 cycles at 95°C for 15 sec, 60°C for 1 min followed by one cycle of a melt curve from 65°C to 95°C with increments of 0.5°C every 5 sec.

Table 3.2. Quantitative RT-PCR primers for validation of mRNA silencing.

I.D.	Forward primers 5'-3'	Reverse Primers 3'-5'	Annealing Temp. (°C)
Aam-179667	ACTCGGAGGTCCTTTGACAC	CAGTGGATGGGTCAGTAACG	55
Aam-16245	TATGGAGGATACCCGAGCTT	AACCGCCATAACCTGGATAC	60
Aam-178006	GACAAGGAGAGGACCCATGT	GTCACGTCGGTCGTAGTTGT	55
Aam-177101	AAACCAAAGCTGTTGCCAAG	TGGAGAATCAGGGCATAGAG	60
Aam-177916	CGTAAACCATTTGTAGGCAA	TCGCAATACCAACAGCAGAG	60
Aam-177785	GGCTCATTTGGACATTTGG	AATCCGCTTCCATACAAACC	55
Aam-17792	TGGTTTCCCTGGAGTTTACG	CGAAAGGTCCGTAGGTATGG	60
Clust_1237-2-1	GTGGAAGTGGTCAAGGAGGA	CTGATTCCCGATAGTGATGG	60
Aam-179800	GCTCCTTTGCTTATGGTTCG	GTCCGAAAGATTCAATGCGT	60
Aam-181586	GCCGCCCAAGCCACCGAG	GGTGGCTTGTATGGCGGAGG	55
Aam-42453	GGCGTAATCATGGTCATAG	GGCTCGTATGTTGTGTGG	60
Contig_99147	GAGGAACTGTCGGTAGCTTG	GGAAGTGGAGGAATTGGCAG	60

The Ct values of the each of the biological replicates per time point were determined by averaging the technical replicate values. Relative quantification (RQ) of tick cement target transcripts was determined as described in chapter two. Target mRNA suppression was calculated using the formula, $S = 1 - (RQ^T/RQ^C \times 100)$ where S = mRNA suppression, RQ^T and RQ^C = RQ of tissues in co-dsRNA injected and GFP-dsRNA injected ticks, respectively as described (Kim et al., 2014). Treatment and control values were compared to determine statistical significance using ANOVA in the PRISM software package (Graphpad Softare, La Jolla, CA).

3.2.3. Assessment of effects of RNAi silencing on adult tick feeding

Unfed adult female ticks that had attained appetence (n = 20 each) were injected with UE, or FI, or UR co-dsRNA, or GFP-dsRNA (control) as described above. All groups were incubated at 25°C with relative humidity set to 85% for the three weeks before feeding on rabbits to assess the effects of RNAi silencing. The three-week incubation period was empirically determined in section 3.2.2. (above) as the period when up to 100% of UE mRNA that predated dsRNA injection was suppressed. The rationale to incubate FI and UR co-dsRNA injected ticks for three weeks was to allow for the use of the same GFP-dsRNA injected control ticks for all treatments. Following incubation, ticks were fed on rabbits as described in chapter two. To confirm mRNA suppression during feeding, three ticks per treatment were sampled at 24 h post attachment and individually processed for validation of RNAi silencing as described above.

The effects of RNAi silencing on tick feeding success was evaluated by assessing the following tick feeding parameters: tick attachment rate onto host skin, time to feed to repletion, mortality rates, engorgement weight (EW, index for amount of imbibed blood), and fecundity as measured by egg mass conversion ratio (ECMR, weight of egg batch divided by EW), and ability or inability to lay eggs. The attachment rate was calculated as the percent of unattached ticks at 24 h after ticks were placed on the rabbit over the total number of ticks. Likewise mortality rate was determined as the percent of ticks that died during feeding over the total number of ticks. The EW was determined by weighing engorged ticks that spontaneously detached from the host. Following detachment, engorged ticks were incubated at 25°C with relative humidity set to 85% to lay eggs. Subsequently the number of ticks that did not lay eggs was documented and the egg batch weighed. Throughout feeding tick physical phenotypes were recorded photographically.

3.2.4. Effects of RNAi silencing on tick feeding when co-dsRNA is injected into newly-ecdysed ticks

qRT-PCR expression analysis data in chapter two demonstrated that expression of UE transcripts was induced at one week post-ecdysis, but not during the molting process. The implication was that UE tick cement proteins are expressed after ticks have ecdysed. Thus, the goal was to inject UE co-dsRNA into newly ecdysed adult ticks before UE tick cement mRNA was transcribed. The assumption was that injecting ticks right before the mRNA was expressed would trigger the RNAi silencing machinery that would disrupt UE mRNA as soon as it was expressed and in this way prevent UE protein translation.

At the time of drafting this write up, there were no reports of whether or not the RNAi silencing pathway was active in molting ticks or newly ecdysed adult ticks. Thus, prior to RNAi silencing, the expression of the dicer was determined to validate the activity of the RNAi pathway in ecdysing and newly ecdysed ticks. The dicer is an RNase that is involved in pre- and post-transcriptional modification, specifically the cleavage of double stranded RNA (Bernstein et al., 2001). Therefore, expression of the dicer enzyme mRNA indicated that the RNAi silencing pathway was active. The cDNA samples of molting and newly ecdysed ticks were subjected to qualitative RT-PCR using dicer PCR primers (For: $^{5\prime}$GTGGGTGACTACGTGCTCAA$^{3\prime}$, Rev: $^{5\prime}$GACCTCCTGACACTCGATGA$^{3\prime}$).

Preliminary analysis revealed that injection of UE co-dsRNA into newly ecdysed adult females caused between 80-100% suppression UE mRNA at 14 days post co-dsRNA injection (described above). Thus, newly ecdysed (one day post ecdysis) female ticks (n = 20) were injected with UE co-dsRNA and incubated for 14 days before assessing the effects of RNAi silencing. Following incubation ticks were placed on SPF New Zealand white rabbit ears to feed. Effects of RNAi silencing were assessed as described in section 2.3.

3.3. Results

3.3.1. Injection with combinatorial (co) dsRNA triggers mRNA suppression of individual targets in the construct

Figure 3.1. demonstrates that injection of co-dsRNA successfully induced the RNAi silencing pathway to specifically disrupt individual components in the construct. In

the second chapter of this thesis, qualitative RT-PCR demonstrated that mRNA of UE targets was strongly expressed in unfed ticks. On this basis, the author reasoned that there was a possibility that target proteins translated from mRNA that predated induction of RNAi silencing pathway could mask the effects of RNAi silencing at the start of tick feeding. To attempt to prevent this, unfed ticks were injected with UE co-dsRNA and disruption of target mRNA was monitored as summarized in Figure 3.1. As shown in Figure 3.1., mRNA encoding for two of the three UE tick cement proteins, Aam-179667 and Aam-16245 were depleted by up to 90% after one and three weeks following injection of UE co-dsRNA. Disruption of the third target, in the UE co-dsRNA construct, Aam-178006 was not conclusive.

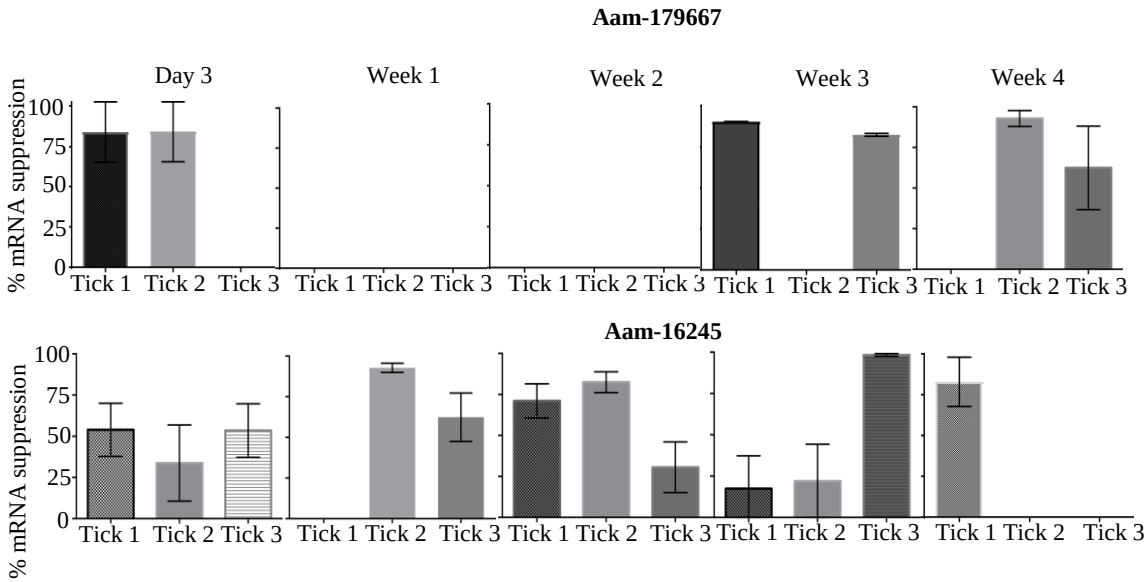

Figure 3.1. Percent mRNA suppression in adult ticks injected with UE co-dsRNA. Twenty adult female ticks were injected with ~1µg UE co-dsRNA and twenty were injected with ~1µg dsGFP as a control. Three ticks were sampled per time point and the percent suppression was calculated. Average percent suppression with standard error mean shown per time point. Suppression of Aam-178006 could not be verified.

3.3.2. Disruption of UE and FI but not UR mRNA caused bleeding around tick mouthparts.

Based on data in Figure 3.1., it was determined that most of mRNA of UE tick cement proteins could be depleted if co-dsRNA injected ticks were incubated for three weeks before feeding them on rabbits to assess the effects of RNAi silencing. In order to minimize the number of control animals used, FI and UR co-dsRNA as well as GFP-dsRNA injected ticks were also incubated for three weeks before assessing the effects of RNAi silencing. This allowed the use of one rabbit ear to feed GFP-dsRNA control ticks. Following the three weeks incubation period, ticks were fed on rabbits to assess the effects of RNAi silencing. Prior to assessing the effects of RNAi silencing, three ticks each were sampled at 24 h post attachment to validate disruption of cognate mRNA in co-dsRNA injected ticks (Figure 3.2.). As shown in Figure 3.2,, injection of UE co-dsRNA triggered suppression of mRNA of all three UE tick cement proteins, Aam-179667, Aam-178006, and Aam-16245 mRNA between 70-90%, 60-90%, and 27-96% respectively. For UR co-dsRNA injected ticks, suppression of Aam-177792 and Aam-42453 mRNA, which ranged between 31-92%, and 21-74%, and all targets in FI co-dsRNA injected ticks that were inconclusive (not shown).

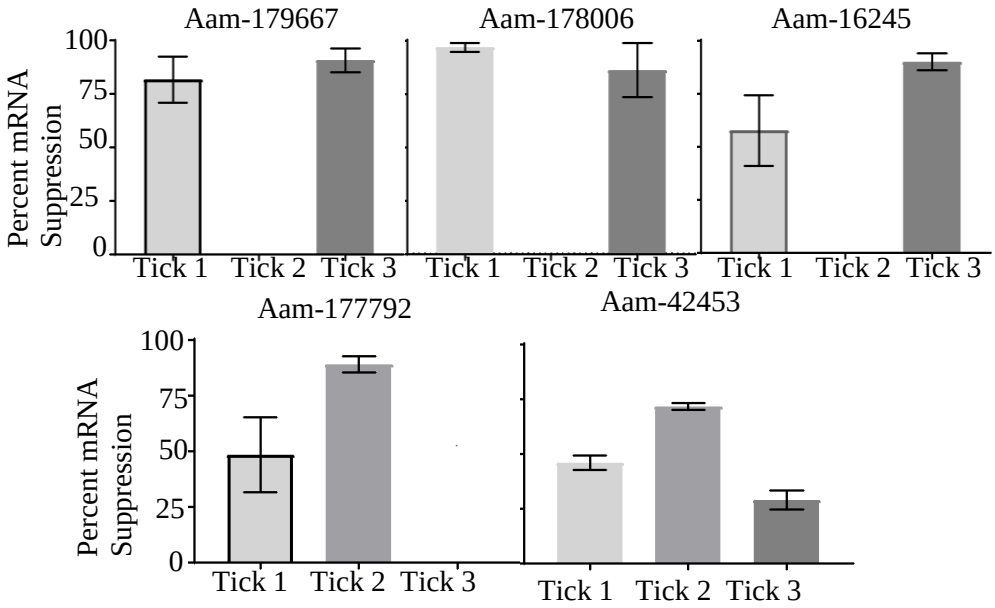

Figure 3.2. Validation of silencing by quantitative RT-PCR round one. Three ticks were sampled at 24 h post-attachment to determine the percent suppression of target mRNA. UE, UR, and FI target validation. Suppression of UR targets Contig_99147, Aam-181586, Clust_1237-2-1, and Aam-10038, and FI targets could not be verified.

Analysis of tick attachment at 24 h after ticks were placed on the rabbit revealed that that RNAi silencing of either cluster genes did not affect the ability of ticks to attach onto skin and start feeding (results not shown). Ticks were allowed to feed to repletion and physical phenotypes documented pictorially at 24 h intervals (Figure 3.3.). At day four, post tick attachment, reddening an indication of inflammation, was observed around attachment sites of UE and FI co-dsRNA injected ticks (not shown). Interestingly, reddened tick attachment sites started to bleed at days seven post attachment (Figure 3.3. A, asterisks marked). Leakage of blood became so intense such that on day nine, veterinarians at Texas A & M University Comparative Medicine Program (CMP) advised

to halt the experiment for the wellbeing of the animal. Blood leakage also appeared around the mouthparts of FI injected ticks at day nine and peaked around day 11, but it was not so intense as to cease tick feeding (asterisks marked in Figure 3.3. A). In order to validate whether or not observed bleeding was caused by disruption of UE and FI tick cement protein transcripts or by tick overcrowding, the experiment was repeated with fewer ticks (n =15 compared to n = 20 in round one). It was interesting to note that, similar to observations in Figure 3.3. A, reddening and subsequent pooling and leakage of blood was observed in the second round of RNAi silencing starting from days eight and ten post attachment respectively (Figure 3.3. B, indicated by black asterisks). However bleeding was not as intense as in the first round and thus the experiment was not stopped. It's important to note that dried blood was observed on control ticks at day 8, however it was not continuous bleeding.

3.3.3. Disruption of UR and FI but not UE tick cement genes reduced blood meal size

Analysis of time taken by ticks to complete feeding was apparently not affected by disruption of mRNA of tick cement proteins in both first and second rounds of RNAi silencing (not shown). Spontaneously detached ticks were individually weighed and photographed as a group to document phenotypes of engorged ticks (Figure 3.4. A, and 3.4. B). Figures 3.4. C and 3.4. D summarizes engorgement mass (EM) as an index for amount of blood taken by the tick. In the first round of RNAi silencing, EMs ranged from 0.265-0.813, 0.183-0.500, 0.232-0.796, and 0.145-0.560 g for GFP, UE, FI and UR dsRNA injected ticks (Figure 3.4. C). In the second round of RNAi silencing, EM of GFP,

UE and FI dsRNA injected ticks respectively ranged from 0.24-0.73, 0.14-0.64, and 0.05-0.38 g (Figure 3.4. D). One-way ANOVA determined that there were significant differences among mean EMs in both first [$F(3, 45) = 3.44$, $P = 0.0245$) and second [$F(2, 29) = 14.26$, $P < 0.0001$]. However Tukey's multiple comparison tests determined that the first round mean EM of UE (0.37 ± 0.041), FI (0.49 ± 0.051), UR (0.35 ± 0.047 g) co-dsRNA injected ticks were not significantly different from GFP-dsRNA injected ticks (0.53 ± 0.043 g) although apparently smaller. In the second round of RNAi silencing, the mean EM of FI (0.17 ± 0.032) was significantly different from GFP dsRNA injected ticks (0.49 ± 0.052) but UE co-dsRNA injected ticks (0.46 ± 0.054 g) were not.

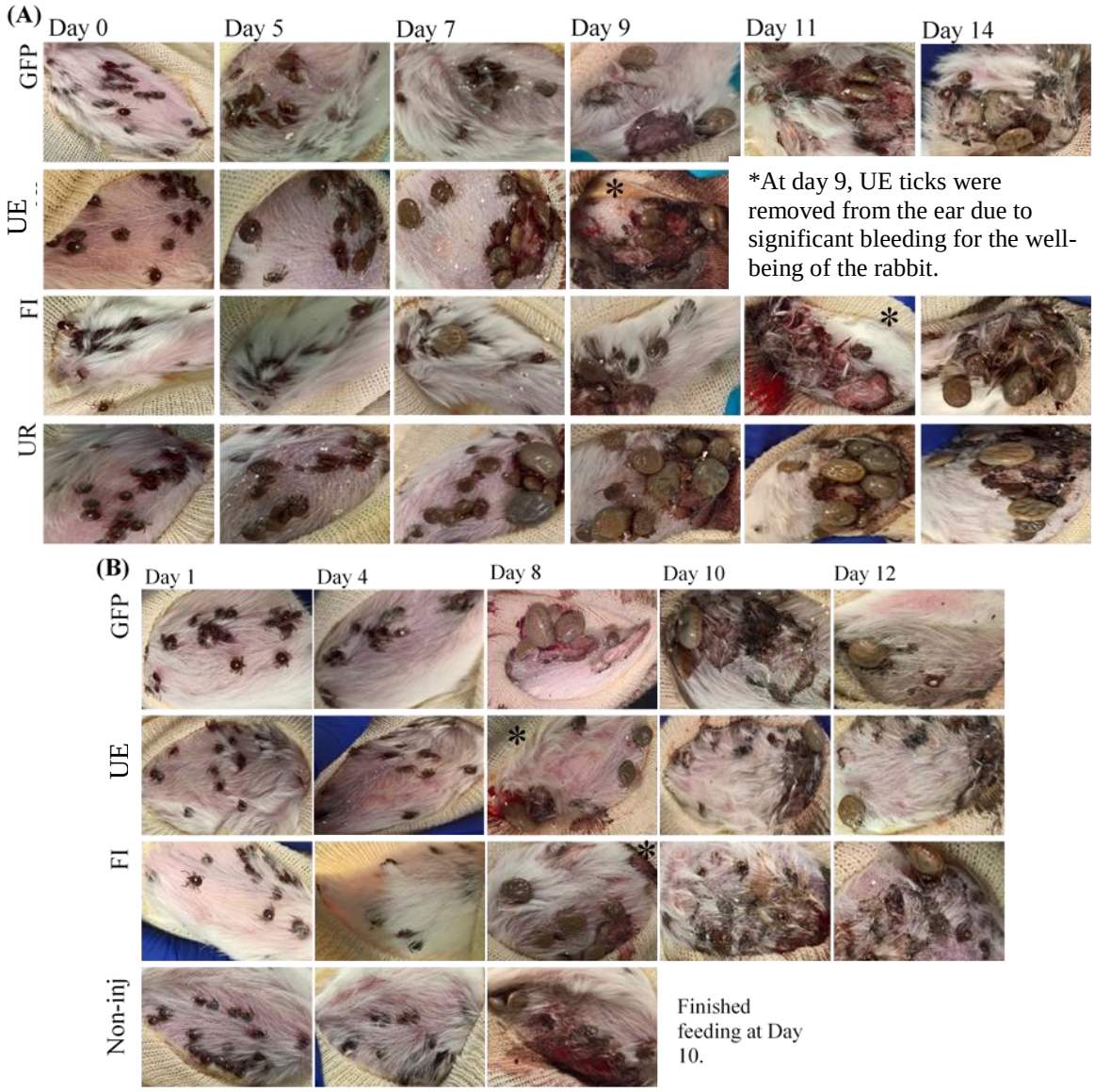

Figure 3.3. Phenotypes during tick feeding. Twenty ticks per target were injected with ~1µg dsRNA (ds-GFP and UE, FI, and UR co-dsRNA) and were maintained at 25°C with relative humidity set to 85% for three weeks. After three weeks, the ticks were placed on rabbit ears and allowed to attempt to feed. (A) Round one. (B) Round two. Bleeding indicated by asterisks.

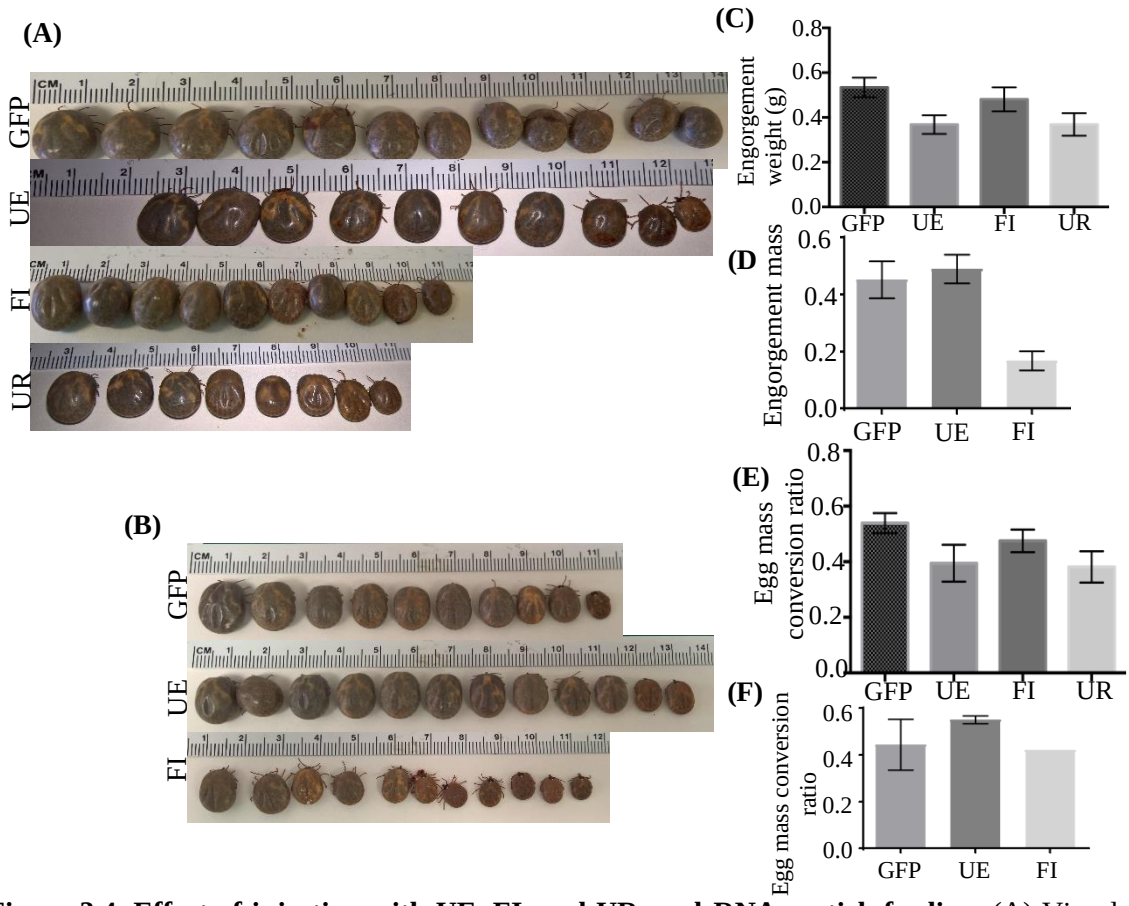

Figure 3.4. Effect of injection with UE, FI, and UR co-dsRNA on tick feeding. (A) Visual comparison of engorged tick groups; round one. (B) Visual comparison of engorged tick groups, round 2. (C) Round one average engorgement weight (an index for amount of imbibed blood). GFP n=14, UE n=10, FI n=14, UR n=10. (D) Round two average engorgement weight GFP n=10, UE n=11, FI n=9. (E) Egg mass conversion ration; round one. (F) Egg mass conversion ratio; round two.

3.3.4. Disruption of UE and UR but not FI tick cement genes affected fecundity.

To determine the effect of RNAi silencing on fecundity, ticks were allowed to oviposit and egg and the egg mass conversion ratio (ECMR, tick's ability to convert its blood meal to eggs) determined by dividing the weight egg clutch by EM. Tukey's multiple

comparison test determined that mean EMCR of UE and UR co-dsRNA injected ticks but not FI injected were significantly different from GFP dsRNA injected ticks (Figure 3.4. E). EMCR data in round two of RNAi silencing was not significant (Figure 3.4. F).

3.3.5. Injection of UE co-dsRNA into day zero newly ecdysed adult ticks prevents accumulation of transcripts.

In the second chapter of this thesis research qRT-PCR analysis revealed that mRNA of UE tick cement genes was lowly expressed in molting nymphs, but increased expression in newly ecdysed going up 3000 fold in one week old ticks. Thus, I reasoned that, if newly ecdysed ticks were injected with UE co-dsRNA, this would prevent accumulation of transcripts. Prior to conducting RNAi silencing in newly ecdysed ticks expression of the dicer gene using the qualitative RT-PCR confirmed that the RNAi silencing pathway was active in newly ecdysed ticks (Figure 3.5.). Figure 3.6. demonstrates that injection of UE co-dsRNA into newly ecdysed ticks prevented accumulation of UE transcripts by up to 100% in one to two weeks old ticks as revealed by qRT-PCR analysis. Subsequently, two groups of newly ecdysed adult ticks: groups A (n = 11) and B (n = 6) were injected with UE co-dsRNA or GFP dsRNA and incubated for up to two weeks before feeding on rabbits to assess the effects of RNAi silencing. Three ticks were sampled to confirm silencing (Figure 3.7.). It was interesting to note that, although much fewer ticks were used, reddening and subsequent bleeding as in Figure 3.3. A and 3.3. B was observed around mouthparts of both group A and B ticks that were injected with UE co-dsRNA but not GFP dsRNA treated (Figure 3.8. A and 3.8. B). On

the underside of one singly attached female tick, a huge expanding hematoma was observed (not shown). The EM for round one UE and GFP dsRNA injected ticks ranged from 0.1592-0.6749g (n=6) and 0.0977-0.7749 g (n=5) respectively. An unpaired t-test revealed that there was no significant difference in EM of treatment and control ticks (Figure 3.9. A). In round two, the EM of UE and GFP dsRNA injected ticks ranged from 0.1541-0.4526g (n=3) and 0.0872-0.671g (n=3) respectively. An unpaired t-test determined that there was no significant difference in the EM of treatment and control ticks (Figure 3.9. B). However, as the GFP dsRNA injected tick with an EM of 0.0872 appears to be an outlier, removing that tick from the analysis resulted in a significant difference between the EM of the treatment and control ticks.

Figure 3.5. Dicer present in ticks the day they ecdysed into adults and one week post-ecdysis. Qualitative RT-PCR was conducted using cDNA from ticks the day they ecdysed into adults and one week post-ecdysis.

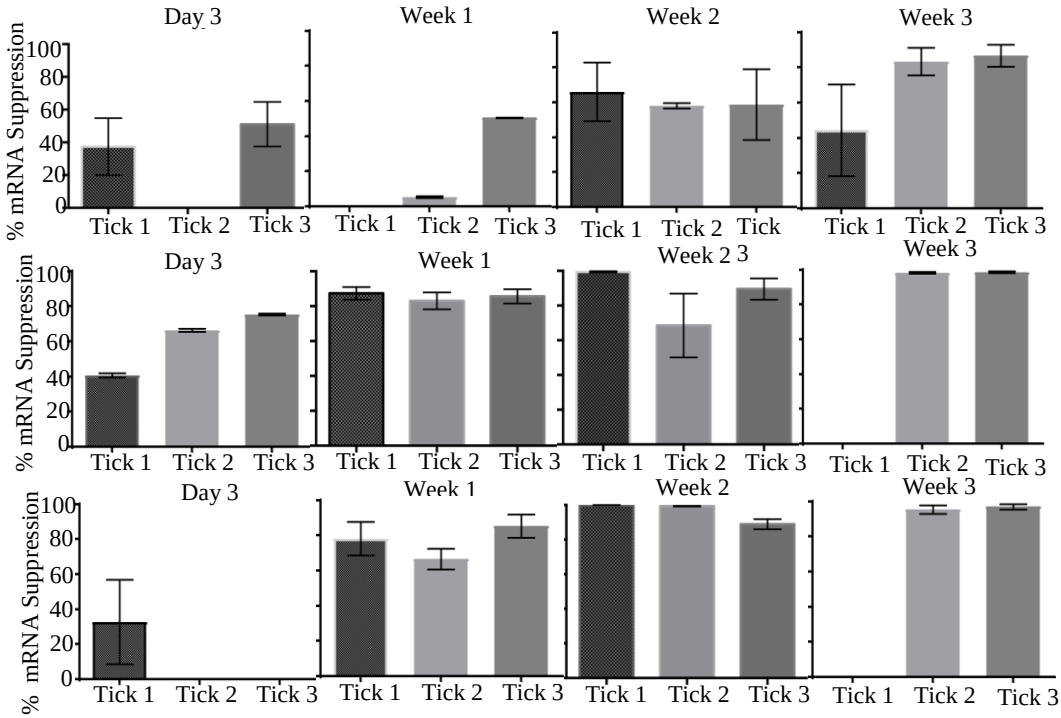

Figure 3.6. Percent mRNA suppression in adult ticks injected with co-dsRNA one day post ecdysis. Twenty newly ecdysed female ticks each were injected with ds-GPF or UE co-dsRNA. Three ticks per target were sampled per time point. Average percent suppression with standard error mean were calculated per tick per time point. (A) Aam-179667 (B) Aam-178006 (C) Aam-16245

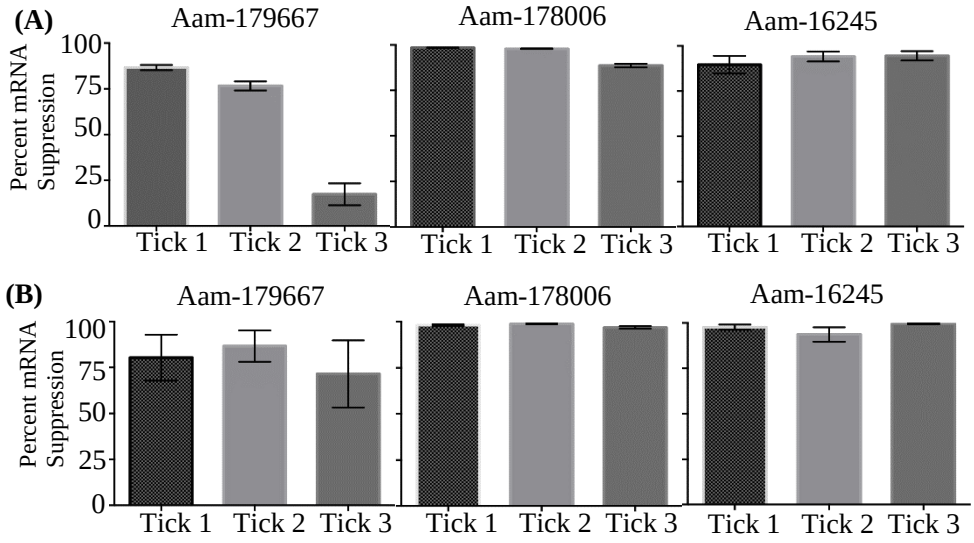

Figure 3.7. Validation of percent mRNA suppression in adult ticks injected with co-dsRNA one day post-ecdysis. Three ticks per target were sampled. Average percent suppression with standard error mean were calculated per tick per time point. (A) Round one. (B) Round two.

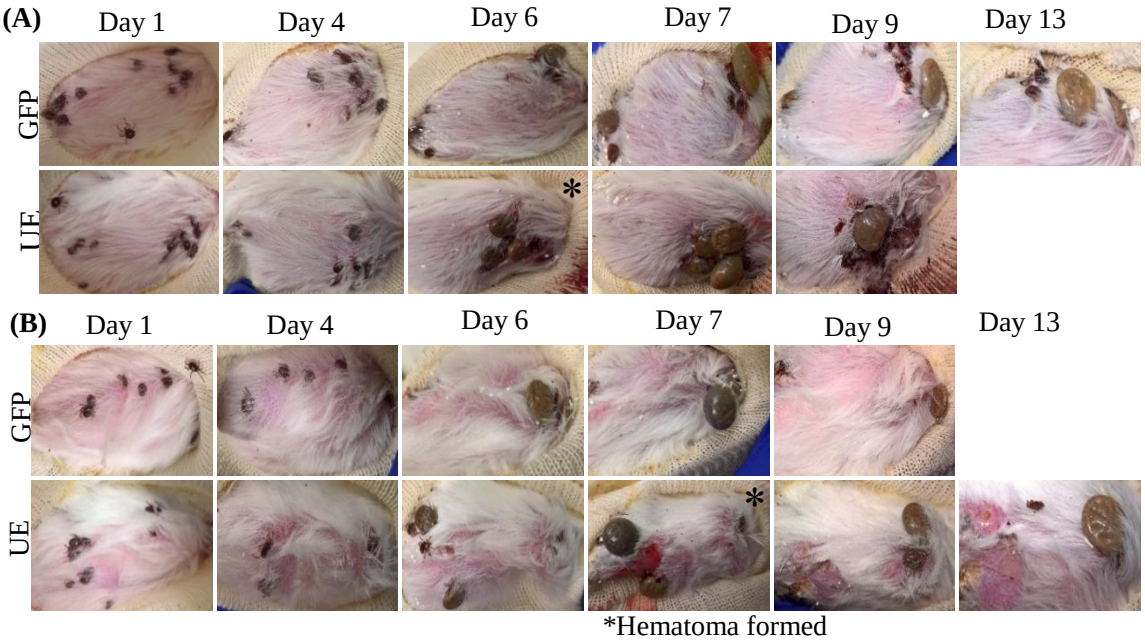

Figure 3.8. Effect of RNAi on tick feeding in co-dsRNA injected newly ecdysed ticks. Ticks were injected with ~1µg dsRNA (ds-GFP and UE co-dsRNA) and were maintained at 25°C with relative humidity set to 85% for one week. After the time had elapsed, the ticks were placed on rabbit ears and allowed to attempt to feed. Photographic documentation of tick feeding progression. (A) Round one. (B) Round two.

To determine the effect of RNAi mediated silencing on fecundity, ticks were allowed to oviposit and egg clutches were weighed. Egg mass (EgM) of round one UE and GFP dsRNA injected ticks ranged from 0.0974-0.424 g and 0.0193-0.482 g respectively. An unpaired t-test confirmed no significant difference (Figure 3.9. C). EgM of round two UE and GFP dsRNA injected ticks was similarly not significantly different with ranges of 0.0691-0.2352 g and 0.0058-0.4081 g respectively (Figure 3.9. D). However, removal of the same GFP dsRNA injected tick as in the EM analysis resulted in

significant difference between UE and GFP dsRNA injected ticks. 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. An unpaired t-test of the EMCR of UE and GFP ticks in round one and two did not find significant difference between treatment and control ticks (Figure 3.9. E and 3.9. F). However, removal of the same GFP dsRNA injected tick as in the EM and EgM analysis of round two resulted in significant difference between UE and GFP injected ticks.

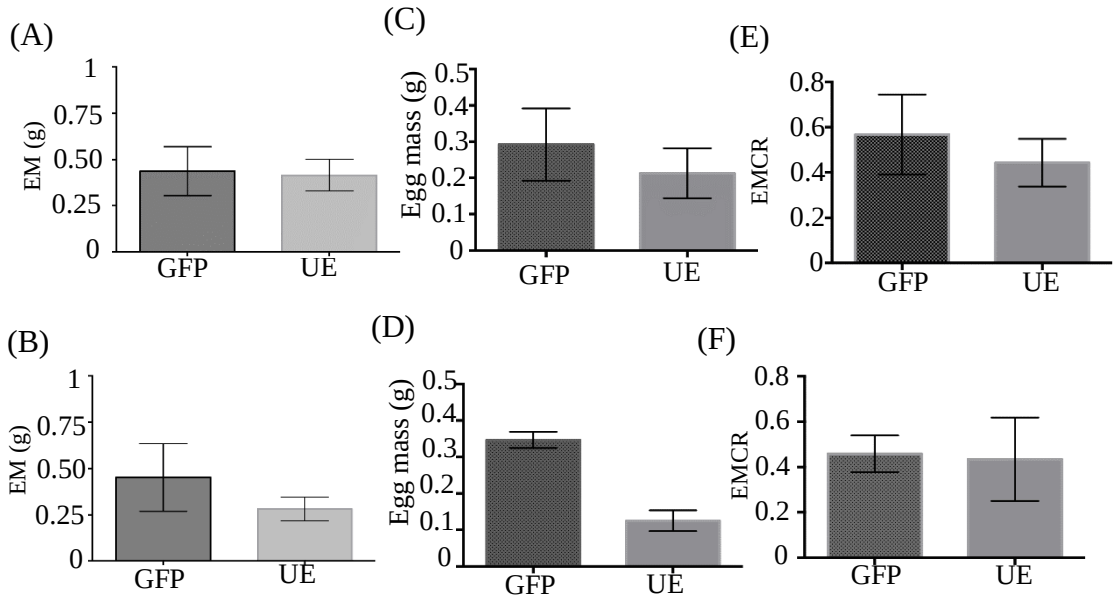

Figure 3.9. Analysis of RNAi on tick engorgement and fecundity. (A) Average engorgement weight (an index for amount of imbibed blood); round one. (B) Average engorgement weight; round two. (C) Egg mass (g); round one. (D) Egg mass (g); round two. (E) Egg mass conversion ratio; round one. (F) Egg mass conversion ratio; round two.

3.4. Discussion

The RNAi silencing technology has been widely utilized to determine functional roles of tick genes in tick feeding physiology (Aljamali et al., 2003), specifically *A. americanum* (Kim et al., 2016; Mulenga et al., 2013; Chalaire et al., 2011; Kocan et al., 2007). In the majority of reported RNAi silencing studies, single tick genes were targeted (Kim et al., 2016; Mulenga and Khumthong 2010). In few reported studies, double stranded RNA based on conserved sequence domains were synthesized to target multiple targets in the same gene family (Tchurikov et al., 2016; Kim et al., 2014). Similar to this thesis, the tick glycine-rich protein family is large (Ribeiro et al., 2017; Kim et al., 2016; de Castro et al., 2016; Karim and Ribeiro, 2015; Tirloni et al., 2014; Jiang et al., 2014; Oliveira et al., 2013; Maruyama et al., 2010; Mulenga et al., 2007; Ribeiro et al., 2006; Untalan et al., 2005; Valenzuela et al., 2002; Bishop et al., 2002) and there has been no consensus of a conserved region that can be used in RNAi silencing to disrupt multiple glycine-rich proteins at once. The high number of glycine-rich tick cement proteins and the lack of a consensus sequence domain that can be targeted in a single dsRNA construct to disrupt multiple members present a challenge in studies to validate the significance of these proteins in tick feeding. In this thesis research, the combinatorial RNAi silencing analysis approach (Herrera-Carrillo and Berkhout, 2015; Bakhetia et al., 2008) was for the first time used in tick research to successfully disrupt multiple glycine-rich protein encoding mRNAs. This approach allowed for determining the significance of tick cement genes in tick feeding in clusters that were assembled based on transcriptional pattern similarity.

Given the functional role of the tick cement cone to securely anchor the tick on host skin (Sonenshine, 1991; Kemp et al., 1982), it is logical to expect that disruption of tick cement genes as was observed in this research could result in ticks in failing to attach onto host skin and initiate tick feeding. However, data in this research revealed that despite the observed disruption of glycine-rich tick cement genes studied here did not affect the ability of ticks to attach and initiate feeding. The tick cement cone is deposited in two layers: the first, called the cortex, is secreted within five to thirty minutes of attachment; the second, called the cortical layer, continues to be deposited through 96 hours of feeding (Sonenshine, 1991; Kemp et al., 1982). The cortex layer initiates formation of the tick cement cone while the second cortical layer strengthens the tick cement cone. Tick cement proteins studied here were identified from a fully formed tick cement cone, and whether or not they are part of the cortex or cortical layer is unknown. It is potentially possible that disruption of cortex layer tick cement proteins results in ticks failing to attach while disruption of cortical layer could result in a destabilized tick cement cone. Thus, the observation that disruption of both the unfed expressed (UE, expressed in unfed that are ready to feed) and feeding induced (FI, induced with feeding) resulted in bleeding around tick mouthparts was interesting. Whether or not the observed bleeding was due to a weak tick cement cone could not be ascertained based on data here.

The limitation of RNAi silencing is that functions of a target protein that is translated prior to injection of dsRNA will not be affected by RNAi silencing. The consequence of this in tick research could be that the effects of RNAi silencing at the start of the tick feeding process could be masked. However this may not be the case for UE tick

genes in that injection of UE co-dsRNA into newly ecdysed ticks blocked the accumulation of UE transcripts. Although western blotting data is needed for validation, it is potentially possible that UE proteins did not form. Despite this effect, injection of UE co-dsRNA into newly ecdysed ticks did not prevent tick attachment, which may indicate that genes here may not be associated with formation of the cortex layer of the tick cement cone.

Another interesting observation was that observed bleeding was preceded by reddening signaling inflammation around tick attachments sites of UE and FI dsRNA injected ticks. Thus, it is potentially possible that the observed bleeding around mouthparts of UE and FI dsRNA injected ticks could be due to an intense inflammation response rather than a weakened tick cement cone. Additionally, as there is no *A. americanum* genome, there may have been downstream affects due to silencing these targets. It is important to note that the bleeding was not detected around mouthparts of all UE and FI dsRNA injected ticks; this could be explained by the fact suppression of target mRNA was achieved at different levels. Apart from the observed bleeding around mouthparts, the effect of RNAi silencing on other tick feeding parameters were modest. Although disruption of both FI and the up regulated with feeding (UR) cluster resulted in smaller blood meals, fecundity was not affected. In conclusion, UE and FI glycine-rich tick cement proteins encoding genes characterized here could be associated with tick cement deposition while UR genes are likely associated with other functions.

CHAPTER IV

CONCLUSION

Vaccination of animals against ticks is a proven sustainable alternative tick control method that does not entail the harmful after-effects of acaricide use (Willadsen 2006; Graf et al., 2004). However, effective target anti-tick vaccine antigens must first be identified through a better understanding of tick feeding physiology. Successful prolonged tick attachment relies on the deposition of the tick cement cone which acts as anchor to hold the tick mouthparts into host skin (Soneshine, 2013; Alekseev et. al, 1995; Kemp et al., 1982). Deposition of the tick cement cone precedes transmission of the majority of tick borne disease (TBD) agents, which occurs after the tick has fed for more than two days (Cook, 2014; Shkap et al., 2009; Hojgaard et al., 2008; Des Vignes et al., 2001; Kjemtrup and Conrad, 2000; Hodzic et al., 1998; Katavolos et al., 1998; Piesman et al., 1987). Thus, many studies have been undertaken to investigate tick cement proteins. Chemical analysis of tick cement revealed that its protein composition is majority glycine-rich proteins (Ribeiro et al., 2017; Kim et al., 2016; de Castro et al., 2016; Karim and Ribeiro, 2015; Tirloni et al., 2014; Jiang et al., 2014; Oliveira et al., 2013; Maruyama et al., 2010; Mulenga et al., 2007; Ribeiro et al., 2006; Untalan et al., 2005; Valenzuela et al., 2002; Bishop et al., 2002). There is published evidence that host immunity to components of tick cement can reduce tick-feeding efficiency and may prevent TBD transmission (Havlikova et al., 2009; Zhou et al., 2006; Trimnell et al., 2005; Trimnell et al., 2002; Bishop et al., 2002; Shapiro et al., 1989; Brown and Askenase, 1986). This thesis

research has contributed to understanding functional roles and significance in tick feeding of glycine-rich tick cement proteins (n=13) that were directly identified from the tick cement cone.

Transcription profiles of the targets show that all of them are present during tick feeding. Glycine-rich tick cement proteins for which transcript abundance was highest in unfed or 24 h fed tick SG but decreased as feeding progressed could be associated with early stage tick feeding functions during the preparatory feeding phase and initial tick attachment. On the other hand tick cement genes that were up-regulated throughout feeding could be involved with later cement deposition or other biological functions that transcend the tick feeding process. Another interesting observation in this study is that unfed expressed targets Aam-179667, Aam-16245, and Aam-178006 were expressed lowly during molting in larva or nymphs, but were strongly induced after ecdysis. This expression pattern mirrors the time period to tick attainment of appetence, which occurs approximately one week after ecdysis (Walade and Rice, 2013; Mulenga et al., 2007). Therefore, the presence of the mRNA transcripts may be an indication of the tick's readiness to feed.

RNAi mediated silencing was used to determine the role of the targets in tick attachment and feeding. Silencing of the UE transcripts weakened the attachment as evidenced by bleeding around the tick mouthparts. The bleeding was so significant in round one that the ticks were manually removed for the well-being of the animal. Bleeding was also seen around the mouthparts of FI injected ticks, although it was not as significant as the UE ticks. Blood leakage around the tick mouthparts indicates that the wound was

not properly sealed upon tick insertion of the hypostome. However, since the transcripts were targeted in clusters, it was impossible to determine the effect of silencing targets individually. Additionally, a major limitation of RNAi mediated silencing is that it cannot deplete proteins that have already been translated. In an attempt to counteract that limitation, ticks were injected with dsRNA before the mRNA transcript was transcribed. This led to close to 100% suppression of the UE transcripts.

In conclusion, this study has contributed towards understanding the molecular basis of tick attachment and tick cement. Further studies should be conducted to better appreciate the roles of glycine-rich proteins in tick attachment. Glycine-rich proteins in nature have similar adhesive characteristics, such as spider web silk and egg glue (Xu et al., 2015; Vasanthavada et al., 2012; Li et al., 2008). Collagen, which is involved in mammal wound healing is also glycine-rich. Therefore, it would be interesting to investigate the potential role of salivary gland secreted tick cement glycine-rich proteins in healing the wound produced by the tick mouthparts.

REFERENCES

Alekseev, A.N., Burenkova, L.A., Podboronov, V.M., and Chunikhin, S.P., 1995. Bacteriocidal qualities of ixodid tick (acarina: Ixodidae) salivary cement plugs and their changes under the influence of a viral tick-borne pathogen. J. Med. Entomol. 32, 578-582.

Aljamali, M.N., Bior, A.D., Sauer, J.R., and Essenberg, R.C., 2003. RNA interference in ticks: A study using histamine binding protein dsRNA in the female tick *Amblyomma americanum*. Insect Mol. Biol. 12, 299-305.

Aljamali, M.N., Hern, L., Kupfer, D., Downard, S., So, S., Roe, B.A., Sauer, J.R., and Essenberg, R.C., 2009. Transcriptome analysis of the salivary glands of the female tick *Amblyomma americanum* (acari: Ixodidae). Insect Mol. Biol. 18, 129-154.

Aljamali, M.N., Ramakrishnan, V.G., Weng, H., Tucker, J.S., Sauer, J.R., and Essenberg, R.C., 2009. Microarray analysis of gene expression changes in feeding female and male lone star ticks, *Amblyomma americanum* (L). Arch. Insect Biochem. Physiol. 71, 236-253.

Allen, J.R., and Humphreys, S.J., 1979. Immunisation of guinea pigs and cattle against ticks. Nature 280, 491-493.

Anderson, B.E., Sims, K.G., Olson, J.G., Childs, J.E., Piesman, J.F., Happ, C.M., Maupin, G.O., and Johnson, B.J., 1993. *Amblyomma americanum*: A potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. 49, 239-244.

Anderson, R.B., Scrimgeour, G.J., and Kaufman, W.R., 1998. Responses of the tick, *Amblyomma hebraeum* (acari: Ixodidae), to carbon dioxide. Exp. Appl. Acarol. 22, 667-681.

Apperson, C.S., Engber, B., Nicholson, W.L., Mead, D.G., Engel, J., Yabsley, M.J., Dail, K., Johnson, J., and Watson, D.W., 2008. Tick-borne diseases in North Carolina: Is "*Rickettsia amblyommii*" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector Borne Zoonotic Dis 8, 597–606.

Bakhetia, M., Urwin, P.E., and Atkinson, H.J., 2008. Characterisation by RNAi of pioneer genes expressed in the dorsal pharyngeal gland cell of *Heterodera glycines* and the effects of combinatorial RNAi. Int. J. Parasitol. 38, 1589-1597.

Bedford, G.A.,H., 1931. *Nuttalliella namaqua*, a new genus and species of tick. Parasitology 23, 230-232.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363-366.
Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., Morzaria, S., Musoke, A., and Nene, V., 2002. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833-842.

Bowman, A.S., and Sauer, J.R., 2004. Tick salivary glands: Function, physiology and future. Parasitology 129 Suppl, S67-81.

Brown, S.J., and Askenase, P.W., 1986. *Amblyomma americanum*: Physiochemical isolation of a protein derived from the tick salivary gland that is capable of inducing immune resistance in guinea pigs. Exp. Parasitol. 62, 40-50.

Browning, R., Adamson, S., and Karim, S., 2012. Choice of a stable set of reference genes for qRT-PCR analysis in *Amblyomma maculatum* (acari: Ixodidae). J. Med. Entomol. 49, 1339-1346.

Bullard, R., Allen, P., Chao, C., Douglas, J., Das, P., Morgan, S.E., Ching, W., and Karim, S., 2016. Structural characterization of tick cement cones collected from in vivo and artificial membrane blood-fed lone star ticks (*Amblyomma americanum*). Ticks and Tick-borne Diseases 7, 880-892.

Buller, R.S., Arens, M., Hmiel, S.P., Paddock, C.D., Sumner, J.W., Rikhisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F.A., Liddell, A.M., Schmulewitz, N., and Storch, G.A., 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. 341, 148-155.

Burgdorfer, W., 1991. Lyme borreliosis: Ten years after discovery of the etiologic agent, *Borrelia burgdorferi*. Infection 19, 257-262.

Carlier, L., Joanne, P., Khemtémourian, L., Lacombe, C., Nicolas, P., El Amri, C., and Lequin, O., 2015. Investigating the role of GXXXG motifs in helical folding and self-association of plasticins, Gly/Leu-rich antimicrobial peptides. Biophys. Chem. 196, 40-52.

Chalaire, K.C., Kim, T.K., Garcia-Rodriguez, H., and Mulenga, A., 2011. *Amblyomma americanum* (L.) (acari: Ixodidae) tick salivary gland serine protease inhibitor (serpin) 6 is secreted into tick saliva during tick feeding. J. Exp. Biol. 214, 665-673.

Childs, J.E., and Paddock, C.D., 2003. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu. Rev. Entomol. 48, 307-337.

Chinery, W.A., 1973. The nature and origin of the "cement" substance at the site of attachment and feeding of adult *Haemaphysalis spinigera* (ixodidae). J. Med. Entomol. 10, 355-362.

Centers for Disease Control and Prevention. Approximate Distribution of the Lone Star Tick. Centers for Disease Control and Prevention, 02 Aug. 2011. Web. 26 Apr. 2017.

Cook, M.J., 2014. Lyme borreliosis: A review of data on transmission time after tick attachment. Int. J. Gen. Med. 8, 1-8.

Dantas-Torres, F., Chomel, B.B., and Otranto, D., 2012. Ticks and tick-borne diseases: A one health perspective. Trends Parasitol. 28, 437-446.

Davey, R.B., 1987. Effect of age of *Boophilus microplus* larvae (acari: Ixodidae) on attachment to cattle. J. Med. Entomol. 24, 118-120.

Davidson, W.R., Siefken, D.A., and Creekmore, L.H., 1994. Seasonal and annual abundance of *Amblyomma americanum* (acari: Ixodidae) in central Georgia. J. Med. Entomol. 31, 67-71.

de Castro, M.H., de Klerk, D., Pienaar, R., Latif, A.A., Rees, D.J., and Mans, B.J., 2016. De novo assembly and annotation of the salivary gland transcriptome of *Rhipicephalus appendiculatus* male and female ticks during blood feeding. Ticks Tick Borne Dis. 7, 536-548.

de la Fuente, J., Almazan, C., Canales, M., Perez de la Lastra, J.M., Kocan, K.M., and Willadsen, P., 2007. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim. Health. Res. Rev. 8, 23-28.

de la Fuente, J., Manzano-Roman, R., Naranjo, V., Kocan, K.M., Zivkovic, Z., Blouin, E.F., Canales, M., Almazan, C., Galindo, R.C., Step, D.L., and Villar, M., 2010. Identification of protective antigens by RNA interference for control of the lone star tick, *Amblyomma americanum.* Vaccine. 28, 1786-1795.

des Vignes, F., Piesman, J., Heffernan, R., Schulze, T.L., Stafford, K.C.,3rd, and Fish, D., 2001. Effect of tick removal on transmission of B*orrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. J. Infect. Dis. 183, 773-778.

Diaz-Martin, V., Manzano-Roman, R., Oleaga, A., Encinas-Grandes, A., and Perez-Sanchez, R., 2013. Cloning and characterization of a plasminogen-binding enolase from the saliva of the argasid tick *Ornithodoros moubata*. Vet. Parasitol. 191, 301-314.

Egekwu, N., Sonenshine, D.E., Bissinger, B.W., and Roe, R.M., 2014. Transcriptome of the female synganglion of the black-legged tick *Ixodes scapularis* (acari: Ixodidae) with

comparison between illumina and 454 systems. PLoS One 9, e102667. doi:10.1371/journal.pone.0102667.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Fitak, R.R., Kelly, D.J., Daniels, M.K., Jiang, J., Richards, A.L., and Fuerst, P.A., 2014. The prevalence of rickettsial and ehrlichial organisms in *Amblyomma americanum* ticks collected from Ohio and surrounding areas between 2000 and 2010. Ticks Tick Borne Dis. 5, 797-800.

Francischetti, I. M. ., Sá-Nunes, A., Mans, B. J., Santos, I. M., & Ribeiro, J. M. C., 2009. The role of saliva in tick feeding. Frontiers in Bioscience : A Journal and Virtual Library 2051–2088.

Gladney, W.J., Drummond, R.O., Whetstone, T.M., and Ernst, S.E., 1970. Effect of age on the attachment rate of the parasitic stages of the lone star tick, *Amblyomma americanum* (linnaeus) (acarina: Ixodidae), in the laboratory. J. Med. Entomol. 7, 92-95.

Gladney, W.J., Drummond, R.O., Whetstone, T.M., and Ernst, S.E., 1970. Effect of age on the attachment rate of the parasitic stages of the lone star tick, *Amblyomma americanum* (linnaeus) (acarina: Ixodidae), in the laboratory. J. Med. Entomol. 7, 92-95.

Graf, J.F., Gogolewski, R., Leach-Bing, N., Sabatini, G.A., Molento, M.B., Bordin, E.L., and Arantes, G.J., 2004. Tick control: An industry point of view. Parasitology 129 Suppl, S427-42.

Gratz, N.G., 1999. Emerging and resurging vector-borne diseases. Annu. Rev. Entomol. 44, 51-75.

Grisi, L., Leite, R.C., Martins, J.R., Barros, A.T., Andreotti, R., Cancado, P.H., Leon, A.A., Pereira, J.B., and Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in brazil. Rev. Bras. Parasitol. Vet. 23, 150-156.

Guilfoile, P.G., and Packila, M., 2004. Identification of four genes expressed by feeding female *Ixodes scapularis*, including three with sequence similarity to previously recognized genes. Exp. Appl. Acarol. 32, 103-110.

Haley, B., Tang, G., and Zamore, P.D., 2003. In vitro analysis of RNA interference in *Drosophila melanogaster*. Methods 30, 330-336.

Havlikova, S., Roller, L., Koci, J., Trimnell, A.R., Kazimirova, M., Klempa, B., and Nuttall, P.A., 2009. Functional role of 64P, the candidate transmission-blocking vaccine antigen from the tick, *Rhipicephalus appendiculatus*. Int. J. Parasitol. 39, 1485-1494.

Herrera-Carrillo, E., and Berkhout, B., 2015. The impact of HIV-1 genetic diversity on the efficacy of a combinatorial RNAi-based gene therapy. Gene Ther. 22, 485-495.

Hodzic, E., Fish, D., Maretzki, C.M., De Silva, A.M., Feng, S., and Barthold, S.W., 1998. Acquisition and transmission of the agent of human granulocytic ehrlichiosis by *Ixodes scapularis* ticks. J. Clin. Microbiol. 36, 3574-3578.

Hojgaard, A., Eisen, R.J., and Piesman, J., 2008. Transmission dynamics of *Borrelia burgdorferi s.s*. during the key third day of feeding by nymphal *Ixodes scapularis* (acari: Ixodidae). J. Med. Entomol. 45, 732-736.

Hutt, J.A., Lovchik, J.A., Dekonenko, A., Hahn, A.C., and Wu, T.H., 2017. The natural history of pneumonic tularemia in female fischer 344 rats after inhalational exposure to aerosolized *Francisella tularensis* subspecies tularensis strain SCHU S4. Am. J. Pathol. 187, 252-267.

Ibelli, A.M., Kim, T.K., Hill, C.C., Lewis, L.A., Bakshi, M., Miller, S., Porter, L., and Mulenga, A., 2014. A blood meal-induced *Ixodes scapularis* tick saliva serpin inhibits trypsin and thrombin, and interferes with platelet aggregation and blood clotting. Int. J. Parasitol. 44, 369-379.

Jiang, X., Gao, J., Wang, W., Xu, M., Li, W., Qi, M., Yang, C., Ji, L., Zhang, D., Luo, J., and Yin, H., 2014. Molecular characterization of an alanine-, proline-, glycine-, threonine-, and serine-rich protein of the hard tick *Haemaphysalis qinghaiensis* and its effect as a vaccine against tick infestation in sheep. Ticks Tick Borne Dis. 5, 14-20.

Jongejan, F., and Uilenburg, G., 2004. The global importance of ticks. Parasitology 129, S3–S14.

Karim, S., and Ribeiro, J.M., 2015. An insight into the sialome of the lone star tick, *Amblyomma americanum*, with a glimpse on its time dependent gene expression. PLoS One 10, e0131292.

Katavolos, P., Armstrong, P.M., Dawson, J.E., and Telford, S.R.,3rd, 1998. Duration of tick attachment required for transmission of granulocytic ehrlichiosis. J. Infect. Dis. 177, 1422-1425.

Kazimirova, M., and Stibraniova, I., 2013. Tick salivary compounds: Their role in modulation of host defences and pathogen transmission. Front. Cell. Infect. Microbiol. 3, 43.

Kemp, D.H., Stone, B.F., and Binnington, K.C., 1982. Tick attachment and feeding: Role of the mouthparts, feeding apparatus, salivary gland and the host response. In: Frederick

D Obenchain, Rachel Galun (Ed.), Physiology of ticks. Pergamon Press, Oxford, England, pp. 119-168.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H., 2001. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 15, 2654-2659.

Kim, T.K., Curran, J., and Mulenga, A., 2014. Dual silencing of long and short *Amblyomma americanum* acidic chitinase forms weakens the tick cement cone stability. J. Exp. Biol. 217, 3493-3503.

Kim, T.K., Radulovic, Z., and Mulenga, A., 2016. Target validation of highly conserved *Amblyomma americanum* tick saliva serine protease inhibitor 19. Ticks Tick Borne Dis. 7, 405-414.

Kim, T.K., Tirloni, L., Pinto, A.F., Moresco, J., Yates, J.R.,3rd, da Silva Vaz, I.,Jr, and Mulenga, A., 2016. *Ixodes scapularis* tick saliva proteins sequentially secreted every 24 h during blood feeding. PLoS Negl Trop. Dis. 10, e0004323.

Kivaria, F.M., Ruheta, M.R., Mkonyi, P.A., and Malamsha, P.C., 2007. Epidemiological aspects and economic impact of bovine theileriosis (east coast fever) and its control: A preliminary assessment with special reference to kibaha district, Tanzania. Vet. J. 173, 384-390.

Kjemtrup, A.M., and Conrad, P.A., 2000. Human babesiosis: An emerging tick-borne disease. Int. J. Parasitol. 30, 1323-1337.

Kocan, K.M., Manzano-Roman, R., and de la Fuente, J., 2007. Transovarial silencing of the subolesin gene in three-host ixodid tick species after injection of replete females with subolesin dsRNA. Parasitol. Res. 100, 1411-1415.

Koci, J., Simo, L., and Park, Y., 2013. Validation of internal reference genes for real-time quantitative polymerase chain reaction studies in the tick, *Ixodes scapularis* (acari: Ixodidae). J. Med. Entomol. 50, 79-84.

Kollars, T.M.,Jr, 1993. Ticks (acari: Ixodidae) infesting medium-sized wild mammals in southwestern Tennessee. J. Med. Entomol. 30, 896-900.

Krolak, J.M., Ownby, C.L., and Sauer, J.R., 1982. Alveolar structure of salivary glands of the lone star tick, *Amblyomma americanum* (L.): Unfed females. J. Parasitol. 68, 61-82.

Labuda, M., Trimnell, A.R., Lickova, M., Kazimirova, M., Davies, G.M., Lissina, O., Hails, R.S., and Nuttall, P.A., 2006. An antivector vaccine protects against a lethal vector-borne pathogen. PLoS Pathog. 2, e27.

Lambson, B., Nene, V., Obura, M., Shah, T., Pandit, P., Ole-Moiyoi, O., Delroux, K., Welburn, S., Skilton, R., de Villiers, E., and Bishop, R., 2005. Identification of candidate sialome components expressed in ixodid tick salivary glands using secretion signal complementation in mammalian cells. Insect Mol. Biol. 14, 403-414.

Lew-Tabor, A.E., Moolhuijzen, P.M., Vance, M.E., Kurscheid, S., Valle, M.R., Jarrett, S., Minchin, C.M., Jackson, L.A., Jonsson, N.N., Bellgard, M.I., and Guerrero, F.D., 2010. Suppressive subtractive hybridization analysis of R*hipicephalus (boophilus) microplus* larval and adult transcript expression during attachment and feeding. Vet. Parasitol. 167, 304-320.

Li, D., Huson, M.G., and Graham, L.D., 2008. Proteinaceous adhesive secretions from insects, and in particular the egg attachment glue of *Opodiphthera sp*. moths. Arch. Insect Biochem. Physiol. 69, 85-105.

Livak, K.J., and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25, 402-408.

Lunardi, C., Nanni, L., Tiso, M., Mingari, M.C., Bason, C., Oliveri, M., Keller, B., Millo, R., De Sandre, G., Corrocher, R., and Puccetti, A., 2000. Glycine-rich cell wall proteins act as specific antigen targets in autoimmune and food allergic disorders. Int. Immunol. 12, 647-657.

Macrae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., and Doudna, J.A., 2006. Structural basis for double-stranded RNA processing by dicer. Science 311, 195-198.

Malak, A.K., Mpoke, L., Banak, J., Muriuki, S., Skilton, R.A., Odongo, D., Sunter, J., and Kiara, H., 2012. Prevalence of livestock diseases and their impact on livelihoods in central equatoria state, southern Sudan. Prev. Vet. Med. 104, 216-223.

Mani, R.J., Metcalf, J.A., and Clinkenbeard, K.D., 2015. *Amblyomma americanum* as a bridging vector for human infection with *Francisella tularensis*. PLoS One 10, e0130513.

Mans, B.,J., de Klerk, D.,G., Pienaar, ,Ronel, and Latif, A.,A., 2014. The host preferences of *Nuttalliella namaqua* (ixodoidea: Nuttalliellidae): A generalist approach to surviving multiple host-switches. Experimental & applied acarology 62, 233-240.

Mans, B.J., and Neitz, A.W.H., 2004. Adaptation of ticks to a blood-feeding environment: Evolution from a functional perspective. Insect Biochem. Mol. Biol. 34, 1-17.

Marsh, T.L., Yoder, J., Deboch, T., McElwain, T.F., and Palmer, G.H., 2016. Livestock vaccinations translate into increased human capital and school attendance by girls. Science Advances 2, - e1601410.

Maruyama, S.R., Anatriello, E., Anderson, J.M., Ribeiro, J.M., Brandao, L.G., Valenzuela, J.G., Ferreira, B.R., Garcia, G.R., Szabo, M.P., Patel, S., Bishop, R., and de Miranda-Santos, I.K., 2010. The expression of genes coding for distinct types of glycine-rich proteins varies according to the biology of three metastriate ticks, *Rhipicephalus (boophilus) microplus*, *Rhipicephalus sanguineus* and *Amblyomma cajennense*. BMC Genomics 11, 363-2164-11-363.

McMullan, L.K., Folk, S.M., Kelly, A.J., MacNeil, A., Goldsmith, C.S., Metcalfe, M.G., Batten, B.C., Albariño, C.G., Zaki, S.R., Rollin, P.E., Nicholson, W.L., and Nichol, S.T., 2012. A new phlebovirus associated with severe febrile illness in missouri. N. Engl. J. Med. 367, 834-841.

McSwain, J.L., Essenberg, R.C., and Sauer, J.R., 1982. Protein changes in the salivary glands of the female lone star tick, *Amblyomma americanum*, during feeding. J. Parasitol. 68, 100-106.

Mukhebi, A.W., Kariuki, D.P., Mussukuya, E., Mullins, G., Ngumi, P.N., Thorpe, W., and Perry, B.D., 1995. Assessing the economic impact of immunisation against east coast fever: A case study in coast province, kenya. Vet. Rec. 137, 17-22.

Mulenga, A., Blandon, M., and Khumthong, R., 2007. The molecular basis of the *Amblyomma americanum* tick attachment phase. Exp. Appl. Acarol. 41, 267-287.

Mulenga, A., Khumthong, R., and Blandon, M.A., 2007. Molecular and expression analysis of a family of the *Amblyomma americanum* tick lospins. J. Exp. Biol. 210, 3188-3198.

Mulenga, A., Khumthong, R., Chalaire, K.C., Strey, O., and Teel, P., 2008. Molecular and biological characterization of the *Amblyomma americanum* organic anion transporter polypeptide. J. Exp. Biol. 211, 3401-3408.

Mulenga, A., Kim, T.K., and Ibelli, A.M., 2013. Deorphanization and target validation of cross-tick species conserved novel *Amblyomma americanum* tick saliva protein. Int. J. Parasitol. 43, 439-451.

Mulenga, A., Sugimoto, C., and Onuma, M., 2000. Issues in tick vaccine development: Identification and characterization of potential candidate vaccine antigens. Microbes Infect. 2, 1353-1361.

Mulenga, A., Sugimoto, C., Sako, Y., Ohashi, K., Musoke, A., Shubash, M., and Onuma, M., 1999. Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated 29-kilodalton protein and its effect as a vaccine against tick infestation in rabbits. Infect. Immun. 67, 1652-1658.

Mulenga, A., and Khumthong, R., 2010. Silencing of three *Amblyomma americanum* (L.) insulin-like growth factor binding protein-related proteins prevents ticks from feeding to repletion. J. Exp. Biol. 213, 1153-1161.

Oaks, J.F., McSwain, J.L., Bantle, J.A., Essenberg, R.C., and Sauer, J.R., 1991. Putative new expression of genes in ixodid tick salivary gland development during feeding. J. Parasitol. 77, 378-383.

Obenchain, F.D., and Galun, R., 2013. Physiology of ticks. Elsevier Science, Kent.
Olds, C.L., Mwaura, S., Odongo, D.O., Scoles, G.A., Bishop, R., and Daubenberger, C., 2016. Induction of humoral immune response to multiple recombinant *Rhipicephalus appendiculatus* antigens and their effect on tick feeding success and pathogen transmission. Parasit. Vectors 9, 484-016-1774-0.

Oliveira, C.J., Anatriello, E., de Miranda-Santos, I.K., Francischetti, I.M., Sa-Nunes, A., Ferreira, B.R., and Ribeiro, J.M., 2013. Proteome of *Rhipicephalus sanguineus* tick saliva induced by the secretagogues pilocarpine and dopamine. Ticks Tick Borne Dis. 4, 469-477.

Piesman, J., Mather, T.N., Sinsky, R.J., and Spielman, A., 1987. Duration of tick attachment and *Borrelia burgdorferi* transmission. J. Clin. Microbiol. 25, 557-558.

Prusinski, M.A., Kokas, J.E., Hukey, K.T., Kogut, S.J., Lee, J., and Backenson, P.B., 2014. Prevalence of *Borrelia burgdorferi* (spirochaetales: Spirochaetaceae), *Anaplasma phagocytophilum* (rickettsiales: Anaplasmataceae), and *Babesia microti* (piroplasmida: Babesiidae) in *Ixodes scapularis* (acari: Ixodidae) collected from recreational lands in the hudson valley region, New York state. J. Med. Entomol. 51, 226-236.

Rahman, M.S., Choi, Y.H., Choi, Y.S., and Yoo, J.C., 2017. Glycin-rich antimicrobial peptide YD1 from *B. amyloliquefaciens*, induced morphological alteration in and showed affinity for plasmid DNA of E. coli. AMB Express 7, 8-016-0315-8. Epub 2017 Jan 3.

Ribeiro, J.M., 1989. Role of saliva in tick/host interactions. Exp. Appl. Acarol. 7, 15-20.

Ribeiro, J.M., 1995. Blood-feeding arthropods: Live syringes or invertebrate pharmacologists? Infect. Agents Dis. 4, 143-152.

Ribeiro, J.M., Alarcon-Chaidez, F., Francischetti, I.M., Mans, B.J., Mather, T.N., Valenzuela, J.G., and Wikel, S.K., 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. Insect Biochem. Mol. Biol. 36, 111-129.

Ribeiro, J.M., Labruna, M.B., Mans, B.J., Maruyama, S.R., Francischetti, I.M., Barizon, G.C., and de Miranda Santos, I.K., 2012. The sialotranscriptome of *Antricola delacruzi* female ticks is compatible with non-hematophagous behavior and an alternative source of food. Insect Biochem. Mol. Biol. 42, 332-342.

Ribeiro, J.M., Makoul, G.T., Levine, J., Robinson, D.R., and Spielman, A., 1985. Antihemostatic, antiinflammatory, and immunosuppressive properties of the saliva of a tick, *Ixodes dammini*. J. Exp. Med. 161, 332-344.

Ribeiro, J.M., Slovak, M., and Francischetti, I.M., 2017. An insight into the sialome of *Hyalomma excavatum*. Ticks Tick Borne Dis. 8, 201-207.

Roller, L., Žitňanová, I., Dai, L., Å imo, L., Park, Y., Satake, H., Tanaka, Y., Adams, M.E., and Žitňan, D. 2010. The ecdysis triggering hormone signaling in arthropods. Peptides 31, 429-441.

Sauer, J.R., McSwain, J.L., Bowman, A.S., and Essenberg, R.C., 1995. Tick salivary gland physiology. Annu. Rev. Entomol. 40, 245-267.

Sauer, J.R., McSwain, J.L., Tucker, J.S., Shelby, K.S., Williams, J.P., and Essenberg, R.C., 1989. Protein phosphorylation and control of tick salivary gland function. Exp. Appl. Acarol. 7, 81-94.

Savage, H.M., Godsey, M.S.,Jr, Lambert, A., Panella, N.A., Burkhalter, K.L., Harmon, J.R., Lash, R.R., Ashley, D.C., and Nicholson, W.L., 2013. First detection of heartland virus (bunyaviridae: Phlebovirus) from field collected arthropods. Am. J. Trop. Med. Hyg. 89, 445-452.

Shapiro, E.D., and Gerber, M.A., 2000. Lyme disease. Clin. Infect. Dis. 31, 533-542.

Shapiro, S.Z., Voigt, W.P., and Ellis, J.A., 1989. Acquired resistance to ixodid ticks induced by tick cement antigen. Exp. Appl. Acarol. 7, 33-41.

Shkap, V., Kocan, K., Molad, T., Mazuz, M., Leibovich, B., Krigel, Y., Michoytchenko, A., Blouin, E., de la Fuente, J., Samish, M., Mtshali, M., Zweygarth, E., Fleiderovich, E.L., and Fish, L., 2009. Experimental transmission of field *Anaplasma marginale* and the *A. centrale* vaccine strain by *Hyalomma excavatum*, *Rhipicephalus sanguineus* and *Rhipicephalus (boophilus) annulatus* ticks. Vet. Microbiol. 134, 254-260.

Shock, B.C., Moncayo, A., Cohen, S., Mitchell, E.A., Williamson, P.C., Lopez, G., Garrison, L.E., and Yabsley, M.J., 2014. Diversity of piroplasms detected in blood-fed and questing ticks from several states in the United States. Ticks Tick Borne Dis. 5, 373-380.

Tchurikov, N.A., Fedoseeva, D.M., Gashnikova, N.M., Sosin, D.V., Gorbacheva, M.A., Alembekov, I.R., Chechetkin, V.R., Kravatsky, Y.V., and Kretova, O.V., 2016. Conserved sequences in the current strains of HIV-1 subtype A in Russia are effectively targeted by artificial RNAi in vitro. Gene 583, 78-83.

Tirloni, L., Reck, J., Terra, R.M., Martins, J.R., Mulenga, A., Sherman, N.E., Fox, J.W., Yates, J.R.,3rd, Termignoni, C., Pinto, A.F., and Vaz Ida, S.,Jr, 2014. Proteomic analysis of cattle tick *Rhipicephalus (boophilus) microplus* saliva: A comparison between partially and fully engorged females. PLoS One 9, e94831.

Trimnell, A.R., Davies, G.M., Lissina, O., Hails, R.S., and Nuttall, P.A., 2005. A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. Vaccine 23, 4329-4341.

Trimnell, A.R., Hails, R.S., and Nuttall, P.A., 2002. Dual action ectoparasite vaccine targeting ‘exposed’ and ‘concealed’ antigens. Vaccine 20, 3560-3568.

Tukahirwa, E.M., 1976. The feeding behaviour of larvae, nymphs and adults of *Rhipicephalus appendiculatus*. Parasitology 72, 65-74.

Untalan, P.M., Guerrero, F.D., Haines, L.R., and Pearson, T.W., 2005. Proteome analysis of abundantly expressed proteins from unfed larvae of the cattle tick, *Boophilus microplus*. Insect Biochem. Mol. Biol. 35, 141-151.

Valenzuela, J.G., Francischetti, I.M., Pham, V.M., Garfield, M.K., Mather, T.N., and Ribeiro, J.M., 2002. Exploring the sialome of the tick *Ixodes scapularis*. J. Exp. Biol. 205, 2843-2864.

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

Vasanthavada, K., Hu, X., Tuton-Blasingame, T., Hsia, Y., Sampath, S., Pacheco, R., Freeark, J., Falick, A.M., Tang, S., Fong, J., Kohler, K., La Mattina-Hawkins, C., and Vierra, C., 2012. Spider glue proteins have distinct architectures compared with traditional spidroin family members. J. Biol. Chem. 287, 35986-35999.

Walade, S.M., and Rice, M.J., 2013. The sensory basis of tick feeding behaviour. The sensory basis of tick feeding behaviour. Physiology of ticks. Pergamon Press, pp. 71-112.

Wang, H., Henbest, P.J., and Nuttall, P.A., 1999. Successful interrupted feeding of adult *Rhipicephalus appendiculatus* (ixodidae) is accompanied by reprogramming of salivary gland protein expression. Parasitology 119 (Pt 2), 143-149.

Willadsen, P., 2006. Vaccination against ectoparasites. Parasitology S2-S25.

Willadsen, P., 2004. Anti-tick vaccines. Parasitology 129 Suppl, S367-87.

Willadsen, P., Bird, P., Cobon, G.S., and Hungerford, J., 1995. Commercialisation of a recombinant vaccine against *Boophilus microplus.* Parasitology 110 Suppl, S43-50.

Xu, D., Shi, X., Thompson, F., Weber, W.S., Mou, Q., and Yarger, J.L., 2015. Protein secondary structure of green lynx spider dragline silk investigated by solid-state NMR and X-ray diffraction. Int. J. Biol. Macromol. 81, 171-179.

Zhou, J., Gong, H., Zhou, Y., Xuan, X., and Fujisaki, K., 2006. Identification of a glycine-rich protein from the tick *Rhipicephalus haemaphysaloides* and evaluation of its vaccine potential against tick feeding. Parasitol. Res. 100, 77-84.