Characterization of Glycine Rich Proteins From the Salivary Glands of the Lone Star Tick Amblyomma americanum

Rebekah Lynn Bullard
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CHARACTERIZATION OF GLYCINE RICH PROTEINS FROM THE SALIVARY GLANDS OF THE LONE STAR TICK *AMBLYOMMA AMERICANUM*

by

Rebekah Lynn Bullard

A Dissertation
Submitted to the Graduate School and the Department of Biological Sciences at The University of Southern Mississippi in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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ABSTRACT

CHARACTERIZATION OF GLYCINE RICH PROTEINS FROM THE SALIVARY GLANDS OF THE LONE STAR TICK AMBYLOMMA AMERICANUM

by Rebekah Lynn Bullard

May 2016

Ticks are blood sucking arthropods that feed on living hosts for up to three weeks. The ticks secrete a multitude of pharmacologically active proteins into the host during feeding which allow the tick to avoid the host immune response, establish a blood pool, and form a firm attachment. The firm attachment is facilitated by the formation of a cement cone which surrounds the tick mouthparts and intertwine between the host skin layers. In this study, gene expression of 44 A. americanum genes was measured throughout the bloodmeal to reveal the differential expression of these genes. Each of the genes tested exhibits a differential expression; however, at least one gene of each family is expressed throughout feeding. To further study the tick cement cones, an in vitro feeding system was developed which feeds nymph and adult ticks through a silicone membrane for an extended period of time better mimicking in vivo feeding than other in vitro methods. This allows the cement cone to form with no interference from skin host and hair. The cement cones were then collected and structurally compared to in vivo fed cement cones. Structural differences were identified; however, in vitro feeding allows for cone collection much earlier in the feeding process. A GRP was identified from both in vivo and in vitro fed cones, and a subset of GRPs were studied using RNA interference. The GRPs studied did not
have any role in cement cone formation; however, there was significant change in microbial presence in the salivary glands. Inconsistent gene expression data in knock down ticks led to identification of differential expression during the stress response.
ACKNOWLEDGMENTS

I would first like to thank my mentor, Dr. Shahid Karim, who has guided me through this process and has always been supportive of my ideas. I would also like to thank my committee members, Dr. YanLin Guo, Dr. Sarah Morgan, Dr. Vijay Rangachari, and Dr. Glen Shearer, for their assistance in holding my work to the highest standard. Lastly, I would like to thank my fellow lab mates who have worked with me to understand new procedures, new concepts, and always holding me responsible.
DEDICATION

I would like to give special thanks to my husband who has supported me through all the times I felt discouraged and ready to quit. It is with his love and support that I have been able to accomplish this work. I would also like to thank my family for never giving up on me and being ready for anything I needed.
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Tick Classification

Ticks are obligate hematophagous arthropods of the class Arachnida, subclass Acari, and suborder Ixodida. They are documented ecto-parasites in all parts of the world and in terms of medical and veterinary importance are second only to mosquitoes in the transmission of bacteria, viruses, protozoa, and other parasites. Ticks are also responsible for significant livestock loss in many countries. Along with disease transmission, ticks are also responsible for conditions such as southern tick associated rash illness (STARI), Red Meat Allergy, and tick paralysis. Ticks can be primarily classified as hard or soft ticks.

Ticks are classified into three families which are distinguishable by the presence or absence of the scutum, a hard plate which covers the whole dorsal portion of males and half of the dorsal side of females.\(^1\) The presence of the scutum along the entire dorsal side limits the ability of the body to expand during the blood meal however as only half of the female dorsal side is covered by the scutum the female is able to expand up to 100X her original weight during the bloodmeal.\(^2\)

Soft ticks belong to the family Argasidae. This family consists of 183 species divided into four genera: Argas, Carios, Ornithodoros, and Otobius.\(^1,3\) Soft ticks have no scutum present along the dorsal side. The leathery surface extends anteriorly over the mouthparts preventing them from being visible from
the dorsal side. Soft ticks differ from hard ticks in their feeding pattern. Soft ticks are able to fully engorge in a matter of hours compared to days or weeks for hard ticks. This family of ticks is largely distributed across Old World countries in which animal husbandry accounts for high percentages of economy. Infestations of ticks on ruminants can lead to decreased weight, lowered milk production, or anemia. Argaside ticks transmit many diseases such as human tick borne relapsing fever, viral encephalitis, African Swine fever virus, fowl spirochetosis, anaplasmosis-like infections, and epizootic bovine abortion. Members of the soft tick family reside in tropical and subtropical zones as they are able to withstand high temperatures, as well as into the arid regions of Central Asia and Africa due to unique oilskin and cement components of the cuticle which make them highly resistant to desiccation. Ticks of this family also undergo more than one instar during the nymphal life stage.

Hard ticks belong to the family Ixodidae which contains 683 species divided into 12 genera: *Ixodes, Dermacentor, Rhipicephalus, Haemaphysalis, Hyalomma, Amblyomma, Anomalohimalaya, Bothriocroton, Cosmiomma, Nosoma, Margaropus, and Rhipicentor*. Ticks belonging to Ixodidae can also be classified based on the location of the anal groove on the ventral surface. *Ixodes* is the only genus of Ixodidae which are classified as Prostriata with the anal groove located anteriorly to the anus. The remaining 11 genera of Ixodidae ticks are classified as Metastriata in which the anal groove is located posteriorly to the anus. Unlike soft ticks which have multiple molts for each life stage, ixodid ticks undergo only one molt between each stage with no instar stages.
The third family, Nuttalliellidae, contains only one species, *Nuttalliella namaqua*. This species has only been found in eastern and southern Africa.\textsuperscript{1,3} This family of ticks displays morphological characteristics of both soft and hard ticks. Similar to hard ticks, the head is located apical to the body, and a pseudo-scutum is present. However, the Nuttalliellidae family also contains soft tick characteristics such as fewer denticles on the hypostome and a leathery integument.\textsuperscript{5}

Ixodidae ticks can also be classified regarding the length of the mouthparts. Ticks which possess small or short mouthparts are termed Brevirostrata. Ticks which possess long mouthparts are termed Longirostrata. Although ticks of both long and short mouthparts secrete a proteinaceous matrix into the host to assist in attachment, it is believed that Brevirostrata ticks secrete more of this matrix to compensate for their shorter mouthparts. It has also been demonstrated that Longirostrata ticks saliva contains a more diverse mixture of pharmacologically active molecules as the wound on the host is larger therefore requiring a stronger defense against the host immune response.\textsuperscript{6}

*Tick Life cycles.* Soft ticks and hard ticks differ in feeding and life cycle. Soft ticks are able to imbibe a large amount of blood in a short amount of time due to the absence of a scutum. A soft tick is able to detach and reattach multiple times on the same host before fully engorging. While soft ticks are similar to hard ticks in life stages (larvae, nymphs, and adults), they are dissimilar in that soft ticks may go through up to nine instars during the nymphal stage.\textsuperscript{4} Because adult feeding may take place in transiently, oviposition also is different in that soft ticks
lay only a few hundred eggs compared to ixodid ticks which can lay several thousand. The eggs hatch into six-legged larvae which are sexually indistinguishable (Figure 1.1). The larvae feed typically on a small mammal or ground dwelling bird for a period of 2.5 to 8 days. After engorgement, the larva will detach from the host and molt into an eight-legged nymph. This nymph may seek out a new host to feed on and will again feed for 2.5 to 8 days until engorgement. The nymph will detach from the host and will molt into a sexually dimorphic male or female adult. Male ticks will only feed for a few days while female ticks will feed for 5-12 days dependent on species, mating, and temperature. After the female tick engorges, she will drop off of the host and lay her eggs among the brush.

Figure 1.1. Life Cycle of 1-, 2-, and 3-host ticks.

Different species of tick may feed on one, two, or three hosts during its life cycle. It is necessary for each life stage to feed before molting into the next stage. One host ticks, such as *Rhipicephalus microplus*, will seek out a host as a newly hatched larva and will go through all feeding stages on that one host. In
the case of one host ticks, the host is typically ungulates or hoofed animals. Two host ticks seek out a host as a larvae and will molt into a nymph on that host. The fed nymph will fall off the host and molt into a male or female adult who will feed on a large mammal. The adult will drop off the host after engorgement and will lay eggs if female. Examples of one host and two host ticks include species of the *Rhipicephalus*, *Dermacentor*, and *Hyalomma* genera. In the case of three host ticks, the tick will fall off the host for each molt and will seek out a new host for each life stage. Larvae and nymphs typically feed on small rodents or ground dwelling birds while adults may feed on larger mammals such as cattle or deer. Examples of three host ticks include members of the *Amblyomma*, *Anomalohimalaya*, *Bothriocroton*, *Haemaphysalis*, and *Ixodes* genera.

Anatomy of Tick mouthparts and salivary glands. The tick mouthparts consist of hypostome, a pair of chelicerae, and a pair of palps all connected to the basis capitulum. Characterization of the mouthparts from the tick *Ixodes ricinus* confirmed that the hypostome is fixed immovably to the basis capitulum. Along of dorsal surface of the hypostome a shallow basin is flanked on either side by denticles which resemble serrations along the hypostome. The chelicerae sit next to either side of the hypostome and protect the hypostome. The chelicerae are able to retract fully into the basis capitulum during hypostome insertion. The palps vary greatly in size and importance depending on the species of tick. In general, palps consist of four segments. In *Amblyomma*, the second palp segment is at least twice as long as the third segment. In many
ixodid species, the fourth segment is recessed into segment three and may possess setae near the tip.\textsuperscript{1,3}

*Figure 1.2. Location and changes seen in tick salivary glands during feeding (Modified from Binnington, 1978\textsuperscript{10}).*

The salivary glands of ticks are also important to note here. Salivary glands come in pairs and contain three types of acini (Figure 1.2). Type I acini are the smallest of the three acini types and are positioned along the main duct of the salivary glands.\textsuperscript{10} Type I acini contain four cell types: pyramidal, central, peritubular, and constrictor.\textsuperscript{11} Type I acini are agranular and considered to be necessary in maintaining osmoregulation during periods of fasting.\textsuperscript{12} This type of acini undergo very little change throughout the feeding cycle\textsuperscript{12} further suggesting their function is osmoregulatory opposed to secretory. Type II acini contain six types of heavily granulated cells.\textsuperscript{11,12} The number and density of the secretory granules vary by cell type. The granules stain with a periodic acid-Schiff reaction which stains carbohydrates. A-cells from acini II and D-cells of acini III (discussed more below) are believed to secrete components responsible for the tick cement cone visible after attachment.\textsuperscript{12} Interestingly, the type II acini of *Ixodes holocyclus*
contains only two cells types. This detail becomes more intriguing when combined with the fact that *I. holocyclus* does not form a cement cone to assist in attachment. The final type of acini present in female salivary glands is Type III. While type II acini do undergo a change during the feeding cycle, the changes to type III acini are rapid and significant. Type III acini contain three types of granular cells. Prior to blood-feeding, the cells accumulate large numbers of the granules. Markedly, at the onset of feeding, d-cells of acini III reduce the number of granules in each cell. It is hypothesized that these secretions are responsible for cement cone formation in the early stages of feeding. Proteins isolated from *Rhipicephalus appendiculatus* cement cones have been localized to the e-cells of acini III. Changes in acini II and III were evident after 12 hours post infestation, and changes were significant after 48 hours including enlarged rough endoplasmic reticulum which indicate increased protein production.

*Amblyomma Genus*

Within the family Ixodidae, the subfamily Amblyomminae contains the genus *Amblyomma* and previous genus *Aponomma*. The genus Amblyomma includes *A. maculatum, A. americanum, A. variegatum, and A. hebraeum* along with 125 other species. Ticks of the Amblyomma family are medium sized, generally have long mouthparts, ornate scutum, and have long antenna. The genus is distributed worldwide mainly in humid tropical or subtropical regions. Ticks of the *Amblyomma* genus which are important to the medical and veterinary communities include *A. maculatum, A. hebraeum, A. variegatum* and *A. americanum*. 
The Gulf Coast tick (*A. maculatum*) is prevalent in the southeastern United States along the Gulf Coast up into the Mid-Atlantic states.\textsuperscript{3,15} This species is also found in Central American countries\textsuperscript{3} which puts migratory birds at risk for infestation.\textsuperscript{16} *A. maculatum* feeds on a range of small mammals and birds in the immature stages and on large mammals such as deer or ruminants as an adult.\textsuperscript{3} Infestation of *A. maculatum* can create large bite sites and loss of useable cattle hides as well as lead to secondary infections. *A. maculatum* is also a known vector for pathogens such as *Rickettsia parkeri* (the etiological agent of a Rocky Mountain spotted fever like disease), *Ehrlichia ruminantium* (Heartwater), and an emerging *Ehrlichia* species currently known as Panola Mountain Ehrlichia.

*A. hebraeum* is commonly known as the bont tick and is indigenous to southern Africa.\textsuperscript{3} The bont tick is a known vector for *Ehrlichia ruminantium* and *Rickettsia africae*. *E. ruminantium* is a zoonotic disease which causes heartwater in ruminants. Heartwater has up to a 90% mortality rate in some African regions.\textsuperscript{17} This disease has devastating effects on livestock production. *R. africae* is the known agent of African tick bite fever.\textsuperscript{3}

*Amblyomma americanum*. *Amblyomma americanum*, commonly named the Lone Star Tick, are endogenous to the southern United States. A collection of tick survey data prior to 1985 reveals that this tick is found primarily in the southern states and along the Atlantic coast (Figure 1.3). Through the last 30 years, their habitat has expanded into the Mid-Atlantic and New England states as well as into the Plain States (Figure 1.3). The expansion of the Lone Star Tick in such a small amount of time demonstrates the resilience and ability of this tick
to invade the continental United States. *A. americanum* females can be distinguished from other tick species by the presence of a white spot at the bottom of the scutum. A. *americanum* is known to vector bacteria (*Ehrlichia chaffeensis*, *E. ewingii*, and *Francisella tularensis*), and viruses (Heart Land Virus). *A. americanum* is an aggressive blood sucking arthropod and has very little specificity for a host.

![Figure 1.3. Comparison of the distribution of *A. americanum* from 1986 and 2015.](image)

The Lone Star Tick, *Amblyomma americanum* hatches from the egg as a six-legged larva (Figure 1.4). Shortly after hatching, the larva feeds on a small rodent such as a mouse or squirrel. After the larva has engorged, it repletes off the host and molts from a larva into an eight-legged nymph. The nymph then finds a small rodent or larger vertebrate as a host. After engorging, the nymph
repletes off the host and molts into an eight-legged, sexually dimorphic adult. Adults typically feed on larger vertebrates such as deer or cattle. Adults feed and mate on the host. After the females engorge, they replete off the host and undergo oviposition.\(^1\) *A. americanum* appear in the early spring and remain active throughout the spring and summer till autumn. Total population numbers generally peak in the early summer and decrease in population by the end of July. Larvae emerge and peak later in the season compared to nymphs and adults becoming active from June through October typically peaking in August.

*Figure 1.4. Life Stages of *A. americanum*\(^1\).*

*Amblyomma americanum* are capable of transmitting many pathogenic agents of public health and veterinary importance. *Ehrlichia chaffeensis*, the causative agent of Human Monocytic Ehrlichiosis (HME), is an obligate intracellular gram-negative bacterium which replicates inside the mononuclear phagocytes of the human host. *E. ewingii* is the etiological agent of Human Ewingii Ehrlichiosis. *E. ewingii* is also an obligate intracellular gram-negative bacterium however it replicates within the granulocytes of the host. HME is of
significant importance as 42% of cases require hospitalization and up to 17% of patients develop life-threatening complications. *F. tularensis* can be transmitted via various mechanisms; however, when transmission by an arthropod vector causes the glandular and ulceroglandular forms of tularemia at the bite site.

Heartland virus is a newly recognized phlebovirus virus first discovered in 2009. To date, only eight cases have been documented. Patients suffering from Heartland virus typically present with fever, thrombocytopenia, and leucopenia and often complain of fatigue and anorexia. However, to date, no work has been published characterizing the virus. A virus recently discovered in *A. americanum*, Tacaribe virus, belongs to the * Arenaviridae* family of viruses and is classified as a single stranded RNA virus. Tacaribe virus was first discovered in bats in the 1950s as well as few mosquitos; however, a survey of other small mammals and mosquitos in the area was negative for the virus. Nearly 70 years later, this virus has been isolated from 11.2% of *A. americanum* ticks collected. Some members of this family of virus are known to cause hemorrhagic fever in humans; however, no known clinical manifestation of Tacaribe virus to date.19

*A. americanum* is also responsible for medical conditions such as STARI and Red Meat Allergy. STARI presences as a red erythema migrans rash typically seen in Lyme disease however, the rash does not respond to antibiotics.20 Symptoms of STARI are similar to Lyme disease: fever, headache, myalgia, and/or arthralgia. While the symptoms are the same, the severity is much less.20 *A. americanum* ticks are also associated with allergic reactions to red meat. Patients who develop this allergy have antibodies against a sugar
galactose-α-1,3-galactose or α-gal.\textsuperscript{21} This sugar is commonly present in non-primate mammals. The distribution for red meat allergy closely follows the distribution for Rocky Mountain spotted fever like illnesses indicating a similar vector may be the source.\textsuperscript{21} However, very little is known about how the allergen passes from the tick into the host.

**Tick Blood Feeding**

The feeding behavior of the tick consists of nine major steps\textsuperscript{1,2}:

1. Appentence - hunting or seeking a host
2. Engagement - adherence to the skin or fur of the host
3. Exploration - searching on the skin for a suitable attachment site
4. Penetration - insertion of mouthparts into the epidermis and dermis
5. Attachment - feeding site established
6. Ingestion - uptake of blood and other fluids
7. Engorgement - partial or complete meals of blood taken
8. Detachment - withdrawal of the mouthparts
9. Repletion - tick drops off the host

These steps are often divided into three major stages: Questing and Attachment (steps 1-5), Early Feeding or slow feeding phase (step 6), and Late Feeding or fast feeding stage (steps 7-9). The time frame in which these stages occur often differ based on species and host however the characteristics of each stage are the same.
**Questing and Attachment**

Ticks are prepared to feed shortly after emerging from the egg and need a bloodmeal at each stage before molting into the next life stage. The first steps of feeding require finding and attaching to a host. Finding a host can occur by two mechanisms: questing or hunting. Ticks which use “questing” as their primary strategy for finding a host will often climb to the top of tall grass, bushes, or shrubbery and extend their front legs which have claws capable of grasping onto the hair, fur, or clothing of a passing host. The tick then searches the host for a suitable attachment site. Some species of ticks “hunt” for their host by using sensory organs to sense for CO₂, host pheromones, or other olfactory triggers and “chase” the host by tracking. Once the tick has found its host, it must find an attachment site. Because ticks remain attached to the host for 7-21 days in some cases, it is necessary for the tick to find a site where it can remain undetected and undisturbed for an extended period of time. Sites which are warm with soft or thin skin or between folds of skin are often the preferred sites for attachment.

As the tick attaches to the host, it first pierces the skin with its hypostome. The hypostome is a serrated organ of the mouth which cuts through the skin to access the blood of the host. Once the hypostome is buried within the skin of the host, the tick secretes multiple pharmacologically active compounds through the saliva. These compounds help maintain the attachment by evading the host immune system and preventing any immune response, particularly wound healing. There have been many sialotranscriptomes of tick salivary glands that examine the gene expression through multiple stages of feeding. Looking at the
recently generated unfed sialotranscriptome of *A. americanum* genes which are upregulated include metalloproteases, serpins (serine protease inhibitors), lipocalins, glycine rich proteins, and ixodegrins (tick specific disintegrins).^22^ These proteins work together to prevent clot formation, wound healing, activation of the host immune system, and formation of the cement cone.

*Slow Feeding*

During the early feeding stage, between 0 and 120 hours post attachment, the tick secretes additional pharmacologically active compound through the saliva which prevent blood clotting, delay wound healing, and evade the immune system. During this stage, the tick is producing a blood pool within the host. By preventing the host from activating platelet aggregation and fibrin activation, the blood forms a small pool just under the skin of the host from which the tick starts its feeding. During the slow feeding stage, the female ticks may uptake a significant amount of "non-blood" versus blood.^23^ The blood taken in is enough to start mating rituals of the ticks as unfed adults will not mate.^23^ Also during this time, the midgut epithelium develops for the intercellular digestion of the bloodmeal.^23^

*Fast Feeding*

After approximately 120 hours after attachment, the tick begins the late feeding or fast feeding stage of the bloodmeal. During this time, the tick rapidly takes up blood from the blood pool while continuing to secrete pharmacologically active compounds through the saliva which suppresses host wound healing, blood clotting, and host immune responses such as itching, redness, and pain.
This stage of feeding allows the female ticks to engorge on the bloodmeal increasing in size sometimes more than 100X their original body weight. In order for the tick to obtain the most nutritious bloodmeal possible, the red blood cells are concentrated in the midgut and excess water is secreted back into the host through the salivary glands.\textsuperscript{23} During this stage, nutrients are used to grow the cuticle which will rapidly expand in the last 24-48 hours of feeding.\textsuperscript{23} Once the bloodmeal is complete, the ticks replete and fall off the host. The females then begin oviposition during which eggs are laid within two to three weeks.

\textbf{Biological Significance of the Tick Cement Cone during Attachment}

During the attachment phase, the tick must establish a firm attachment by secreting multiple proteins and other compounds. Some of these compounds solidify once inside the host to form a proteinaceous matrix termed the cement cone/cement glue. While the primary component of the cement cone is protein, the cone also contains compounds such as lipids and carbohydrates.\textsuperscript{13,24–26} It is speculated that this cement cone helps protect the hypostome while in the host as well as assist in long-term attachment of the tick to the host. Two types of cement have been found: early cement which is secreted only minutes after attachment and hardens rapidly, and late cement which is secreted after 24 hours on the host and hardens gradually.\textsuperscript{13,24,27} If the cement cone is essential for long-term tick attachment and feeding, utilizing these proteins in vaccines could help prevent tick feeding and disease transmission from these arthropods.
Components of Cement Cones from Other Tick Species

Studies comparing the genes of salivary gland tissues from female *Rhipicephalus sanguineus*, *Rhipicephalus microplus*, and *Amblyomma cajennense* show that a significant amount (6.19%, 3.51%, and 3.82% respectively) of glycine rich proteins (GRPs) are present in the salivary glands.\(^{28}\) The higher incidence of GRPs in *R. sanguineus* (a Brevirostrata one host tick) compared to *R. microplus* (a Brevirostrata multi-host tick) and *A. cajennense* (a Longirostrata multi-host tick) may be the result of repeated exposure of *R. sanguineus* to the host's immune system.\(^{28}\) Phylogenetic analysis of GRPs found in *R. microplus*, *R. sanguineus*, and *A. cajennense* showed the contigs separated into two different clads: those which show homology with spider silk proteins, and those which show homology with cement-like proteins of ticks. It is possible that these two distinct types of GRPs have distinct roles to play during attachment.\(^{28}\) Analysis of five to seven day fed *R. sanguineus* saliva by gel electrophoresis, and LC-MS/MS showed only two secreted glycine-rich proteins. This result serves as evidence to the hypothesis that GRPs are localized to the cement cone and not secreted into the host.\(^{29}\)

A study of two probable cement proteins of *Haemaphysalis longicornis*, hlim2 and hlim3, showed no homology of hlim2 to other proteins but it did have a 34.6% glycine and 14.5% serine content. The sequence of hlim3 showed homology with two other *H. longicornis* salivary gland proteins. The sequence of hlim3 also contains YPG repeats in the tyrosine-rich domain of the protein and a PXP repeat in the C-terminal proline-rich region.\(^{30}\) Analysis of the transcriptome
from *A. americanum* reveals that these repeats are also present in GRPs from *A. americanum*. It was also observed that mice vaccinated against hlim3 had a significantly lower attachment rate of *H. longicornis* at 24 hours post infestation compared to the controls.\(^{30}\)

Anti-sera from cattle raised against *R. appendiculatus* infected with *Theileria parva* was used to detect immunogenic proteins present in the salivary glands of *R. appendiculatus*. This anti-sera detected a protein of approximately 36kDa. Analysis of both the nucleotide and amino acid sequence showed a homology with genes from the patent ‘Tissue cement proteins from *Rhipicephalus appendiculatus*’. Further examination of the protein revealed it was rich in glycine (24.5%), proline (11.4%), leucine (12.5%), and serine (13.2%) however cysteines were found to be absent in the sequence.\(^{13}\) The sequences to be studied in this work have been found to contain 19.8-30.7% glycine (all four proteins) and 10.3-19.0% serine (three proteins) along with at least one cysteine in each protein.

Additionally from *R. appendiculatus*, a protein named 64P has been characterized and identified in the salivary glands and cement cone.\(^{31}\) The similarity of 64P to the proteins of the surrounding tissues prevents its recognition by the host immune system assisting in the protective properties of the cement cone. The primary sequence of this protein reveals multiple repeats of GGYG. The abundance of glycine allows the protein to adopt a variety of confirmations and the increased presences of tyrosine assists in the aggregation of these
proteins by cross-linking of the tyrosine residues. The identified properties of 64P make it a good candidate for vaccine development.\textsuperscript{31}

The only proteins confirmed to be present in the cement cone have been glycine rich proteins. Examination of the proteins present in tick salivary glands reveals an overabundance of glycine rich proteins. This family of proteins have also been identified in many tick species from all classifications: hard and soft ticks, short and long mouthparts. The number of GRPs identified in each tick species differs dependent on the degree of research performed on that species. Table 1.1 shows the number of glycine proteins that have been deposited onto the NCBI database for many species of ticks. \textit{Ixodes ricinus} and \textit{Rhipicephalus pulchellus} have the most GRPs identified than any of the other species listed. This is likely due to the amount of research of these two tick species and not an overabundance of GRPs alone.

Table 1.1

\textit{Glycine-Rich proteins identified in tick species}

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of GRPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Ixodes ricinus}</td>
<td>126</td>
</tr>
<tr>
<td>\textit{Rhipicephalus pulchellus}</td>
<td>126</td>
</tr>
<tr>
<td>\textit{Ixodes scapularis}</td>
<td>37</td>
</tr>
<tr>
<td>\textit{Amblyomma cajennense}</td>
<td>9</td>
</tr>
<tr>
<td>\textit{Hyalomma marginatum}</td>
<td>7</td>
</tr>
<tr>
<td>\textit{Rhipicephalus microplus}</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 1.1 (continued).

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of GRPs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amblyomma maculatum</em></td>
<td>4</td>
</tr>
<tr>
<td><em>Rhipicephalus sanguineus</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Rhipicephalus haemaphysaloides</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Haemaphysalis qinghaiensis</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Ornithodoros parkeri</em></td>
<td>1</td>
</tr>
</tbody>
</table>

Components of Other Cement/Glue Secretions

Many other insects use a proteinaceous glue like matrix in egg sac attachment, pupal attachment, and in binding of the silk threads of webs and cocoons. However, the composition of these protein based glues can differ greatly. *Drosophila* pupa glue contains many glycosylated proteins and threonine rich regions within the protein sequences. Egg casings from mantids are largely α helical structures. Sericins from *Bombyx mori* are serine rich proteins and are responsible for binding of the silk of the cocoon.\(^{32}\)

Comparison of GRPs from ticks to other proteins found homology with Flagelliform silk protein identified from various spider species.\(^{28}\) A study of the genes located within the salivary glands of *R. microplus*, *R. sanguineus*, and *A. cajennense* found that, respectively, 23, 17, and 8 transcripts were found to have sequence similarity to the silk of true spiders.\(^{28}\)
Barnacles such as *Amphibalanus amphirite* produce a cement substance which allows the barnacle to attach to its substrate. Proteins compromise a significant proportion of the barnacle’s cement. The remaining components include carbohydrates and lipids. The granules present in the salivary gland acini react strongly in a periodic acid-Schiff reaction indicating a presence of carbohydrates. Due to the reduction in the number of granules shortly after attachment, it is hypothesized that these carbohydrates may makeup part of the cement cone similar to the presence of carbohydrates and lipids in barnacle cement. However, barnacle cement is cysteine rich whereas the GRPs studied in this work contain only a few cysteines. Analysis of the amino acid sequence of two cement proteins show more than 14% cysteine and a high incidence of histidine at 16.8%. A 20kDa cement protein isolated from the barnacle *Megabarnalu rosa*, Mrcp20k, contains multiple repeated cysteine domains. The peptide sequence of this protein also contains a significant percentage of charged amino acids such as aspartate (11.5%), glutamate (10.4%), and histidine (10.4%).

Analysis of multiple glue types from various species of glue producing insects showed a high presence of glycine in many of the proteins identified. Glues and adhesive films were collected from egg masses or directly from the accessory glands of multiple insects: *Lucilia cuprina* (Australian sheep blowfly), *Opodiphthera eucalypti* (Emperor gum moth), *Opodiphthera helena* (Helena gum moth), Shield bug, Planthopper, Spittle bug, *Propylea quatuordecimpunctata* (14-spotted ladybird beetle), *Hygrotechuis conformis* (large spotted ladybird beetle).
Of all the adhesives tested, each species contained at least one (if not multiple) proteins which were classified as glycine rich with 11-36% glycine (27/33 proteins). Remarkably, egg glue from *O. eucalypti* contains eight proteins which have high glycine content (14%-36%). Other amino acids also overrepresented in these proteins include serine (12-33% in 17 proteins), alanine (12-17% in 2 proteins).\(^{32}\) Strength and tensile tests of the adhesives showed a wide range of results as well as varying degrees of solubility, color, and consistency.\(^{32}\)

Glycine Rich Proteins

*Properties of GRPs*

Proteins which have high glycine content are typical of vertebrate extracellular matrix proteins such as keratin and collagen. The glycine within the three dimensional structure is able to adopt a wide range of chain conformations.\(^{13}\) Spider silk contains a significant proportion of glycine in the amino acid sequence of the spidroins. The core region of spider silk holds very little three dimensional structure while in solution. This lack of intrinsic structure enables the protein to form insoluble cross-β fibrils in a cross linking fashion.\(^{34}\) These silk proteins are able to self-assemble into these cross-β fibrils although the glycine/proline rich region may remain unstructured during this self-assembly.\(^{34}\) The major proteins found in dragline silk, MaSp1 and MaSp2, contain almost 60% glycine and alanine residues. The regions of the protein which are high in alanine are able to form the β-sheet structures which enable the proteins to link together to form long chains. The areas which are higher in
glycine have a more flexible range of confirmations that they can adopt, which improves the silk’s flexibility.

GRPs have been extensively studied in plants in which they are divided into two groups: signal peptide present which targets the proteins for the cellular membrane or RNA binding in which a RNA binding motif is present. Proteins which are classified as RNA binding often have other domains such as oleosin conserved domains, cold shock domain, CCHC zinc-fingers, C-rich carboxy terminus, amphiphilic α helix, H-rich, P-rich, and T-rich sequences. GRPs which do not contain an RNA binding motif are often characterized based on glycine repeats found in the sequence. Common repeats include: GGXXXGG, GXGX, and GGGX. A GRP from the French bean contains the GGX tripeptide repeat. This protein was shown to be localized to the cell wall strongly indicating a structural role. A tobacco GRP which contains a GGXXXGG repeat is localized to the vascular tissue and increases the amount of callose deposition through the cell wall. A list of characteristics found in the GRPs studied in this work is listed in Appendix A.

Very little information is available concerning secondary or tertiary structure of GRPs likely due to the lack of pure protein from natural or recombinant sources. Two secondary structures which have identified from GRPs are glycine loops and β-sheets. Glycine loops are regions with high flexibility. This flexibility allows for a Velcro type interaction with other glycine loops. It is also suggested that GRPs may exist as β-sheets in which non-
glycine rich amino acids are localized to one side of the sheet. However, computer modeling indicates that a β-sheet structure is unlikely.  

**Function**

GRPs have been identified in many organisms and have a wide variety of functions. These functions fit into three major categories (Figure 1.5). GRPs can play a role in abiotic functions such as stress mediation. In plants and other invertebrates such as snow fleas, an upregulation of GRPs has been noted when the organism is exposed to extreme temperatures or when injured. Additionally, GRPs have been shown to exhibit biotic functions such as regulation of circadian rhythms and regulation of gene expression by binding RNA. Other functions include antimicrobial activity identified in insects, and it is possible the tick GRPs play a role in maintaining the microbial community in the tick. GRPs also play a role in the formation of spider web silks and tick cement cones. Below is a more detailed review of GRPs.

**Structural Function.** The most common form of structural GRPs are represented by silk generated from spiders and silkworms. Dragline silk, formed by web forming spiders, is the focus of spider silk research as it exhibits high tensile strength properties and is characteristically much stronger than other forms of silk.  

Silk is primarily composed of proteins however, there have been some reports of lipids or glycoproteins within the silk fiber. The silk is very prone to self-assembly into the cross β-structure however, the spider is capable of keeping the proteins soluble and disordered until needed. Due to the self-assembly nature of spider silk proteins, recombinantly expressed proteins often
adopt the anti-parallel β-sheet structure which minimizes solubility. Dragline and flagelliform silk from spiders are able to absorb large amounts of energy, in order to stop the prey, without breaking showcasing the extreme elasticity and tensile strength of these fibers. A common glycine repeat found in spider silk is GGX which can also be found in structural GRPs of plants as discussed previously. Other repeats include GPGGX and GA repeats. Proline can often be hydroxylated which is commonly seen in GRPs. GRPs with GA repeats are often suggested to form the β-sheet structures seen in many fibrillary proteins.

Other sources of GRPs with structure based functions include the oberhautchen layer of snake epidermis. The β-proteins of the snake epidermis do contain cysteines (6-11.5%) which likely contribute to the firm cross-linking of these proteins. The expression of GRPs and cysteine-rich GRPs in the epidermis is increases through the tissue layer transition from β-cells to the harden outer oberhautchen cells.

**Figure 1.5.** Characterization of the functions of GRPs.
Pharmacological Functions. In insects, anti-microbial peptides (AMP) are part of the humoral immune system and are stored in the hemolymph where the majority of immune response would be needed. AMPs are typically low molecular weight proteins with a positive charge and work in a broad-spectrum antimicrobial activity. AMPs can be classified into five groups: cecropin, insect defensins, lysozymes, proline-rich proteins, and glycine-rich proteins. Here, a focus on glycine-rich proteins will be presented.

The most common examples of glycine rich AMP in insects are attacins. Attacins isolated from the moth *Hyalophora cecropia* were found to contain glycine at a frequency of 9.5-10.2% in all six isoforms. Attacins act on growing bacteria cells in a bacteriostatic fashion preventing the cells from growing but not destroying the cell. When attacin E is added to an *E. coli* culture exposed to penicillin G, the effect of penicillin G is increased indicating that the attacin E may act in such a way as to interfere with the outer membrane of the cell making it more permeable. While attacins are the most common glycine rich AMP, none have been characterized in ticks.

Testing of GRP AMPs from the plant *Coffea canephora* against fungal contaminants showed a decrease in growth of both *Candida albicans* and *Candida tropicalis* by 90.9% and 59%, respectively. These yeasts were then analyzed for colony formation after incubation with Cc-GRP (GRP AMP isolated from *C. canephora*) and showed a decrease of 95.8% and 45.1% in *C. albicans* and *C. tropicalis* growth respectively. These yeasts were also studied using a fluorescent dye to measure the permeability of the cell wall. The dye was visible
within the cell indicating that the Cc-GRP is capable of increasing the
permeability of the yeast cell wall.\textsuperscript{44}

Another source of anti-microbial GRPs is amphibians such as Plasticins in
South-American hylid frogs.\textsuperscript{45} Plasticins are much smaller than many of the
GRPs found in ticks typically only 23-29 residues long but the GXXXG motif of
these plasticins is very specific.\textsuperscript{46} This repeated motif is found in an \( \alpha \)-helix which
lays in the cell membrane. The \( \alpha \)-helix positions the glycines to the membrane
surface.\textsuperscript{46} These proteins can be anti-microbial or hemolytic depending on the
identity of the remaining residues.\textsuperscript{46} The hemolytic activity indicates these
proteins are able to disrupt the cell membranes of neighboring cells.

GRPs from plants and invertebrates, such as the silkworm \textit{Bombyx mori},
have been indicated in stress responses. A review of plant GRPs\textsuperscript{35} compares the
GRPs expressed in multiple plant species when introduced to stressors such as
lack of water, cold temperatures, wounds, and pathogens. However, very little is
known about the mechanisms or functions of the GRPs when induced by a
stressor. Four GRPs were identified from \textit{B. mori} mouthparts which contained
secretory signal peptides.\textsuperscript{47} One of these GRPs was found to be upregulated
when the silkworm was subjected to starvation stress. While the diets of ticks and
silkworms are vastly different (blood versus vegetation), this GRPs is unlikely to
have a structural function giving further evidence to the possibility that
invertebrate GRPs are functional proteins.

Another common stressor is cold temperatures. Anti-freeze proteins have
been identified in snow fleas and \textit{I. scapularis}. The anti-freeze protein identified
in snow fleas is glycine rich\textsuperscript{48} however, the protein identified in \textit{I. scapularis} is not. It is possible many anti-freeze proteins are present, some rich in glycine and some without. The overexpression of \textit{I. scapularis} antifreeze protein in \textit{Drosophila melanogaster} increases the cold tolerance of the flies.\textsuperscript{49} A study of \textit{I. scapularis} ticks infected with \textit{Anaplasma phagocytophilum} revealed an increase of anti-freeze proteins which allowed the infected ticks to survive harsher cold conditions.\textsuperscript{50} This increase in anti-freeze protein production allows the ticks to remain active during the cold months increasing the probability of transmitting the pathogen.\textsuperscript{50}

During tick feeding, the tick must be able to prevent blood clot formation in order to continue taking up the blood meal. Typically, after an injury to the skin, the host undergoes multiple processes to resume hemostasis such as blood coagulation, platelet aggregation, and vasoconstriction.\textsuperscript{51} Addition of dietary glycine into rats yielded increased bleeding times by more than 300 seconds. Platelet aggregation was measured both \textit{in vivo} and \textit{in vitro}. The results showed a decrease of platelet aggregation by 40\% and 50\%, respectively.\textsuperscript{52}

Plants have multiple RNA-binding glycine rich proteins. Among this class of proteins are a special type which confer RNA-binding activity on the N-terminal portion of the protein yet are rich in glycine along the C-terminal region.\textsuperscript{53} Analysis of one of these RNA-binding GRPs reveals that while the N terminus are highly structured while the glycine rich C-terminus remains largely unstructured.\textsuperscript{53} However, not all GRPs are capable of interacting with RNA. A RNA-binding motif must also be present most commonly a RNA recognition motif.
or a cold shock domain. Proteins which contain RNA recognition motifs are commonly involved in pre-mRNA processing however, proteins with the cold shock domain are capable of interacting with both RNA and DNA.

The act of hemostasis involves two processes; primary hemostasis which leads to the formation of a "platelet plug" which initially stops any more blood from exiting the vessel, and coagulation cascades which create a fibrin network throughout the clot. Two types of collagen, I and III, are known to interact with platelets and induce activation. However, a collagen receptor glycoprotein VI has also been identified as having an anti-thrombotic mechanism. Pretreatment of platelets with glycine increased the influx of calcium into the platelets when activated with thrombin thereby increasing the platelet response to thrombin.

A study in rats pretreated with dietary glycine prior to partial removal of the liver (as performed in live donor liver transplantations) showed a significant decrease in liver injury after surgery. Each subject was tested for levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin which are indicators of liver cell damage. In each case, the addition of dietary glycine decreased the amount of these molecules 12 hours after surgery.

Statement of Problem and Hypothesis

Tick feeding takes place over many days and is facilitated by the plethora of salivary proteins secreted into the host. A. americanum has become an invasive species by spreading further northward and westward in the last 30 years. Tick control methods have focused on broad control by spraying acarides
and treating livestock and pets with repellant. However, this does not protect wide animals which can maintain the tick population or outdoorsmen who spend many hours in wooded areas professionally or recreationally. Focused control methods that target specific tick proteins are being studied but are many years from being developed. Many of the proteins under investigation interfere with the tick’s ability to feed or to lay eggs. Here, I have selected a class of proteins which may be involved in all of the feeding stages: attachment, slow feeding, and fast feeding. This work focuses on Glycine Rich Proteins and their role in tick attachment. It is hypothesized that GRPs are involved in prolonged tick attachment and assist the tick in the feeding success. Information regarding tick attachment will be gathered by comparing cement cones from ticks which have been fed using live animal hosts and ticks fed using an in vitro feeding system. This feeding system will allow for the cement cones to be collected in a higher success rate than with ticks fed on live animals. To ensure the cones formed by in vitro fed ticks are a suitable model for cement cone development a comparative study of the structure and composition of in vivo and in vitro fed cement cones is performed. To further elucidate the role of GRPs in cement cone development, the effect of gene depletion is measured on tick attachment, feeding, and other functions.
CHAPTER II

TEMPORAL GENE EXPRESSION OF MULTIPLE GENE FAMILIES IDENTIFIED FROM THE SIALOTRANSCRIPTOME OF A. AMERICANUM¹

Abstract

Saliva is an integral factor in the feeding success of veterinary and medically important ticks. Therefore, the characterization of the proteins present in tick saliva is an important area of tick research. Here, we confirmed previously generated sialotranscriptome data using quantitative real-time PCR. We analyzed the temporal expression of seven housekeeping genes and 44 differentially expressed salivary molecules selected from a previously published Amblyomma americanum sialotranscriptome including six protease inhibitor domain containing proteins, seven lipocalins, 9 glycine rich proteins, seven tick specific genes with unknown function, five immunity related proteins, 3 miscellaneous genes, and six metalloproteases. Separate reference genes were selected for the salivary glands and midgut from among the seven housekeeping genes, to normalize the transcriptional expression of differentially expressed genes. The salivary gland reference gene, ubiquitin, was used to normalize the expression of 44 salivary genes. Unsurprisingly, each gene family was expressed throughout the blood meal, but the expression of specific genes differed at each time point. These data provide insight into the expression and functions of tick salivary proteins expressed while feeding on its host.

¹ This chapter has been published as part of Bullard et al., (2016) PLoS One. 149
Introduction

*Amblyomma americanum*, the Lone Star tick, is widespread across the entire eastern seaboard westward as far as central Texas and has begun to invade up into the central plains.\(^{59-64}\) *A. americanum* has increased in its geographical location, likely because of an association with white tailed deer (*Odocoileus virginianus*), the keystone host, yet it should be noted the lone star tick has been found on a wide variety of host including humans, cattle, horses, dogs, and cats.\(^{64-66}\) The non-specific and aggressive nature of *A. americanum* ticks are of significant importance in the veterinary and medical communities. The Lone Star tick is a vector of diseases such as Spotted Fever Group Rickettsiosis, Human Monocytic Ehrlichiosis (HME), Southern Tick Associated Rash Illness (STARI), Theileriosis, Tularemia, Heartland Virus, and newly discovered, in this tick, Tacribe Virus (for a review, see \(^{19,59,65-67}\)). In addition to these diseases, *A. americanum* has been associated with delayed anaphylaxis to red meat and has become the first recorded example of an ectoparasite causing a food allergy.\(^{68,69}\)

Tick feeding requires the insertion of the hypostome into the host’s skin and the formation of a blood pool beneath the dermis at the bite site. This blood pool remains fluid throughout the tick feeding which may take several days, during which the female will engorge and grow by more than 100x her original mass.\(^2\) In order to maintain the blood pool the tick salivary glands secrete an abundance of pharmacologically active compounds into the host through the saliva.\(^{70}\) Most piercing injuries similar to tick bites would elicit such a strong
hemostasis and inflammatory response the host would be extremely aware of the foreign object. However, in the case of ticks, attachment on the host for multiple days or weeks dependent on species is required for the uptake of a full blood meal. A variety of sialomes from multiple species examine the hypothetical protein composition of tick saliva. The molecules and proteins secreted into the host through the saliva are responsible for: 1) preventing clot formation for the formation of the blood pool, 2) blocking host immune signaling molecules to prevent immunity cascades, 3) preventing inflammatory responses to reduce swelling, erythema, and localized pyrexia, and 4) transmission of pathogens from the tick salivary glands to the host. The proteins produced by the salivary glands, which are secreted into the host through the saliva, come into direct contact with the host and have the most effect on the tick feeding. Although there are similar proteins found in the midguts, a focus on the salivary proteins increases the knowledge of how the tick is able to interact with the host. Overall, tick saliva is composed of many redundant proteins such as multi-gene families that are differentially regulated suggesting a role in evasion of immune defenses according to the vertebrate host. Many researchers have interest in the clinical application of these tick proteins, as they could assist in medical conditions such as auto-immune disease, vascular symptoms, or blood clotting disorders. Yet even with an increase in knowledge in “big data”, the functional characterization of these biomolecules is still needed.

Sialotranscriptomes are currently the standard technique used to identify proteins predicted to be in the salivary glands of ticks. This technique uses cDNA
isolated from the salivary glands of unfed, partially fed, and/or fully fed females as a sequencing template. By using multiple feeding points, any transcriptional variation can be observed. However, it is important to validate this data using a more targeted approach such as quantitative real time PCR (qRT-PCR). QRT-PCR utilizes the stable expression of a gene, commonly one involved in general cellular housekeeping to normalize the expression of the genes of interest.

Due to the central role the housekeeping gene plays in the determination of transcriptional gene expression, it is vitally important to select a properly stabilized gene. Within tick research, it has been shown that the housekeeping gene used for a reference can differ between tick species and tick tissues. A study of the various life stages of *Rhipicephalus microplus* and *Rhipicephalus appendiculatus* revealed Elongation Factor 1A as the most suitable reference gene when comparing two algorithms (geNorm and NormFinder). Although the geNorm analysis showed the lowest variation using six reference genes, the amount of sample required for six genes is prohibitive in tick research. A similar study using *Ixodes scapularis* examined the expression levels of multiple housekeeping genes in two different tissues throughout the blood meal. Expression of the selected housekeeping genes within the *I. scapularis* tissues showed little variation throughout the bloodmeal with two ribosomal proteins having the least amount of variation in both synganglia and salivary glands. Additional comparison of the genes between the tissues also show the ribosomal proteins to have the lowest variation. Previous work from this lab using the Gulf Coast tick, *Amblyomma maculatum* validated Actin as the most suitable gene.
across tick life stages and throughout the adult blood meal. This study also compared the expression levels of one gene of interest, VAMP ½, using each of the tested housekeeping genes. It was discovered that the expression of VAMP ½ varied greatly (up to 70%) dependent on the housekeeping gene. The variation of housekeeping genes just among Ixodidae ticks species demonstrates the need for validation in every individual tick species. Here, we evaluated the transcriptional stability of seven housekeeping genes throughout the blood meal in two separate tissues (salivary gland and midgut). The data provides new insight into the need for reference gene verification for individual tissue types.

The *A. americanum* sialotranscriptome was used to identify multiple genes which are differentially expressed throughout the bloodmeal. The work presented here serves as a validation of the expression measured in RNA-Seq as well as gives a more detailed look at the wide range transcriptional expression of selected genes throughout the bloodmeal. These genes were assessed over eight feeding time points (unfed, 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs, 144 hrs, and 168 hrs post infestation) to identify individual gene expression levels in the salivary glands of female *A. americanum*.

Methods

**Ticks and Ethical Statement**

Ticks were purchased from the Oklahoma State University Tick Rearing Facility. Adult male and female *Amblyomma americanum* were kept according to standard practices at room temperature (25°C) with approximately 90% relative humidity for a photoperiod of 14h light/10h dark. Ticks were fed on a sheep and
approximately 20-25 female ticks were removed throughout the blood meal at 24 hour intervals (24hr-168hr). All animal experiments were carried out according to approved IACUC protocols (10042001; renewed 15101501) from the University of Southern Mississippi and in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Efforts were made to minimize pain and distress of the animal.

**Tick tissue dissection**

Unfed female ticks and partially fed female ticks from time points including (unfed, 24hr, 48hr, 72hr, 96hr, 120hr, 144hr, and 168hr) removed from the sheep were dissected and the salivary glands and midguts removed and cleaned in ice cold M199 buffer. Salivary glands and midguts from each time point were pooled together according to tissue type and stored in RNAlater (Life Technologies, Carlsbad NM) at -80°C until used.78

**Transcription gene expression analysis**

*bRNA isolation and cDNA synthesis.* Frozen tick tissues were placed on ice to thaw and RNAlater was carefully removed with precision pipetting. RNA was isolated from the time point pooled salivary glands and midguts using illustra RNAspin Mini kit (GE Healthcare Lifesciences) protocols. RNA concentration was measured using a Nanodrop spectrophotometer and stored at -80°C or used immediately. To synthesize cDNA, 2µg of RNA was added to a 20 µl reaction using the iScript cDNA synthesis kit (Bio-Rad). The reverse transcription reaction is then heated in a Bio-Rad thermocycler under the following conditions: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and hold at 10°C. The
resultant cDNA was diluted to a working concentration of 25 ng/µl with nuclease free water and stored at -20°C until used.

**Analysis of Housekeeping Gene.** PCR of each gene was performed to verify product size and specificity of the primers. Thermocycling conditions for PCR amplification include: 95°C for 5 minutes; 35 cycles of 95°C for 5 seconds and 60°C for 30 seconds; followed by 72°C for 7 minutes and a 10°C hold. PCR product purity and size was analyzed using a 2% Agarose gel stained with SYBR Safe (Invitrogen) and visualized using a GelDoc system (Bio-Rad). The PCR products were purified using QIAquick PCR Purification Kit from Qiagen (Qiagen, CA). Standard curves were made for each of the genes using a Bio-Rad CFX 96 Real Time System fitted to a C1000 Thermal Cycler under the same thermocycling conditions listed for the PCR. Each of the seven genes was then assessed in all eight blood meal samples for salivary glands and midguts separately. Data from each of these samples was then used to determine the expression stability by using three algorithms (BestKeeper, NormFinder, and ΔΔCt).

**Quantitative Real-Time PCR.** A list of all genes tested can be found in Appendix A. qRT-PCR was performed within the guidelines of Bio-Rad protocols provided with iTaq Universal SYBR Green Supermix. Briefly, 50 ng of cDNA was added to a 20 µl qRT-PCR reaction using SYBR Green supermix with 300 nM of each gene specific primer. The samples were subjected to the following thermocycling conditions: 95°C for 30 sec; 35 cycles of 95°C for 5 sec, and 60°C for 30 sec with a fluorescence reading after each cycle; followed by a melt curve
from 65°C to 95°C in 0.5°C increments. Each reaction was performed in triplicate along with no template controls. Primers used for gene expression validation can be found in Appendix A. Gene expression validation was performed using Ubiquitin as the reference gene.

Results

Reference genes differ by tissue type

The first aim of this work was to determine the suitability and stability of various housekeeping genes in tick salivary glands and midguts for the purpose of normalization of the transcriptional gene expression. Here, salivary glands and midguts were analyzed separately to determine any differences in time-dependent housekeeping gene expression profiles upon blood feeding. Comparison of each gene from each statistical method is provided in Table 2.1 and 2.2 for salivary glands and midguts, respectively.

Figure 2.1. Temporal Gene Expression of Housekeeping Genes. Raw Ct values of seven *A. americanum* housekeeping genes from tick salivary glands (A) and midguts (B) RNA throughout the bloodmeal (unfed-168hr). Each gene was amplified in each sample and the point at which the amplification curve crosses the threshold is considered the Ct.
Table 2.1

Expression Stability of candidate reference genes for tick salivary glands.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Bestkeeper Gene</th>
<th>ΔΔCt Gene</th>
<th>NormFinder Gene</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ubiquitin</td>
<td>Ubiquitin</td>
<td>Actin</td>
<td>0.068</td>
</tr>
<tr>
<td>2</td>
<td>Actin</td>
<td>Actin</td>
<td>Calreticulin</td>
<td>0.069</td>
</tr>
<tr>
<td>3</td>
<td>GAPDH</td>
<td>Histone H3</td>
<td>Ubiquitin</td>
<td>0.072</td>
</tr>
<tr>
<td>4</td>
<td>Calreticulin</td>
<td>GAPDH</td>
<td>Heat Shock Protein</td>
<td>0.134</td>
</tr>
<tr>
<td>5</td>
<td>Heat Shock Protein</td>
<td>Calreticulin</td>
<td>GST</td>
<td>0.140</td>
</tr>
<tr>
<td>6</td>
<td>Histone H3</td>
<td>Heat Shock Protein</td>
<td>Histone H3</td>
<td>0.169</td>
</tr>
<tr>
<td>7</td>
<td>GST</td>
<td>GST</td>
<td>20.83</td>
<td></td>
</tr>
</tbody>
</table>

Ubiquitin in the salivary gland samples exhibits Ct values with minimal variation (Fig 2.1) as well as ranking first according to BestKeeper and the ΔΔCt method. Using the same methods as above Histone H3 proved to be the most stable gene in midgut samples according to BestKeeper and the ΔΔCt method.

Table 2.2

Expression Stability of candidate reference genes for tick midguts.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Bestkeeper Gene</th>
<th>ΔΔCt Gene</th>
<th>NormFinder Gene</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Histone H3</td>
<td>Histone H3</td>
<td>Calreticulin</td>
<td>0.058</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH</td>
<td>GAPDH</td>
<td>Heat Shock Protein</td>
<td>0.088</td>
</tr>
</tbody>
</table>

38
Temporal Gene Expression analysis of Amblyomma americanum genes

After the most stable reference gene for *A. americanum* salivary glands was determined, the temporal gene expression of 44 salivary genes was measured (Appendix A). Genes were selected based on the differential expression observed in the *A. americanum* sialotranscriptome.\textsuperscript{22} The work presented here serves as a validation of data collected using RNA-Seq\textsuperscript{22} as well as expands on that data by measuring the expression of these genes during each 24 hour time frame as opposed to combined time points. *A. americanum* genes that did not show amplification across any time point may not have amplified due to folding and protection of the mRNA or primer inefficiency. This could likely be due to inefficient primer binding as the sequences may be forming secondary structures which prevent proper amplification. It is also possible that changes in feeding seasons between the samples collected for transcriptome...
analysis and for qRT-PCR validation resulted in some temporal specific genes which were present during only one of the collection times.

*Protease Inhibitor Domain.* Two protein families which contain protease inhibitor domains were selected from the sialotranscriptome for further analysis (Fig 2.2). Four genes which contain at least one Kunitz protease inhibitor domain show a range of expression profiles. Gene Aam-36184 (Fig 2.2A) is most highly expressed prior to the 48 hour time point as well as Aam-41471 (Fig 2.2B) albeit at a lower level. Gene AamerSigP-40989 (Fig 2.2C) exhibits a unique expression profile throughout the blood meal, with expression localized during (24-48 and 72 hr). Last in the Kunitz superfamily genes, Aam-35414 (Fig 2.2D) is differentially expressed during the latter time points of tick feeding, with the highest expression throughout 120-168hr.

**Figure 2.2.** Temporal gene expression of genes encoding for protease inhibitor domains. Six genes from two protein families are represented. Genes were quantified using qRT-PCR and normalized using Ubiquitin.
The second family selected for further analysis includes proteins which contain TIL II domains, *trypsin inhibitor-like domain*. These two genes (AamerSigP-41415 and AamerSipP-26789, Fig 2.2E-F) are both expressed before 48 hours and the highest levels of expression are measured at 24 hours after attachment.

**Lipocalins.** The gene expression of seven lipocalins indicate this family may play a vital role throughout the blood meal (Figure 2.3). Aam-41264, Aam41091, AamerSigP-40605 and AamerSigP-12055 (Figure 2.3A-D) are more highly expressed through the unfed and 48 hour time points. The represented lipocalins which are more highly expressed during the later time points include AamerSigP-33384 and Aam-41375 (Fig 2.3E-F). Interestingly, gene AamerSigP-18604 (Data not shown) showed no expression throughout the blood meal as measured by qRT-PCR.

*Figure 2.3. Temporal gene expression of six lipocalins. AamerSigP-18604 shows no expression by qRT-PCR against any time point.*

**Glycine Rich Proteins.** The glycine rich protein (GRP) family is a diverse class of proteins characterized only by the predominance of glycine residues Due
to the variability of this class, the functions of many GRPs have not been elucidated. The expression profiles of nine GRPs show a range of differential expression throughout the bloodmeal (Fig 2.4). Gene Aam-41235 (Fig 2.4A) exhibits differential expression in the 48 hour time point with little to no expression throughout the remaining time points. AamerSigP-34358, Aam-40766, and AamerSigP-39259 (Fig 2.4B-D) show the highest levels of expression at 24 hours after attachment and taper off throughout the remaining blood meal. After 96 hours, AamerSigP-34358 expression levels drop drastically, while Aam-40766 expression continues to taper off from the 24 hour level. AamerSigP-41913 (Fig 2.4E) is similar to the previously mentioned genes however expression becomes minimal after 24 hours. AamerSigP-41539 (Fig 2.4F) is unique among the GRPs selected for this study. The expression of AamerSigP-41539 is mostly exclusive to the unfed salivary glands. Aam-41540 (Fig 2.4G) also shows a distinctive profile by being expressed primarily between 24 hours and 96 hours. Aam-3099 and Aam-36909 (Fig 2.4H-I) exhibit different profiles (late expression and consistently expressed, respectively) however, transcript levels of Aam-41540, Aam-3099, and Aam-36909 remain centered around 1.0 normalized expression units indicating none of them are significantly expressed differentially throughout feeding.
Figure 2.4. Temporal gene expression of glycine rich protein genes. Genes were quantified using qRT-PCR and normalized using Ubiquitin.

Tick Specific Family: Unknown Function. Genes which belong to this family are homologous to other tick proteins although no function has yet been elucidated. The expression of these genes is sporadic both within genes and throughout the family (Fig 2.5). Two genes (AamerSigP-40930 and AamerSigP-41354 Fig 2.5A-B) demonstrate higher expression levels during the late feeding time points. This could indicate possible functions specific to the late feeding mechanisms or possibly antigenic variation of a conserved function. AamerSigP-41425 (Fig 2.5C) is primarily expressed during the mid-time points 48-72 hours. During this time frame, the tick prepares for the fast-feeding stage. Three genes including (AamerSigP35954, AamerSigP-15297, and AamerSigP-39321, Fig 2.5D-F) exhibit sporadic expression throughout the course of the blood meal.
Lastly gene AamerSigP-22563 (Data not shown) reveals no expression as measured by qRT-PCR.

![Temporal gene expression of tick specific genes with unknown function. AamerSigp-22563 exhibits no expression when measured with qRT-PCR.](image)

**Figure 2.5.** Temporal gene expression of tick specific genes with unknown function. AamerSigp-22563 exhibits no expression when measured with qRT-PCR.

*Immunity Related Proteins.* Other proteins which were selected from the transcriptome include those involved in tick immunity. The expression of an evasin gene Aam-12127 (Fig 2.6A) displays its highest level of expression before 48 hours of feeding. Another evasin gene AamerSigP-41992 showed no expression according to qRT-PCR (Data not shown). Along with evasins, another immunity related peptide, defensin was tested (Fig 2.6B). This gene, Aam-31196 is primarily expressed at 48 hours during the blood meal. Also listed as an immunity related peptide are genes Aam-6020 and Aam-40185 (Fig 2.6C-D) which belong to the 5.3kDa peptide family.
Figure 2.6. Temporal gene expression of immunity related proteins. Of note, gene AamerSigP-41992 showed no amplification during qRT-PCR.

**Miscellaneous Proteins.** This group of genes includes a variety of protein families including novel salivary protein, cytoskeletal protein, protein modification, and extracellular matrix proteins (Fig 2.7). As noted in multiple families above, one of the genes (Aam-5252, Data not shown) showed no expression as measured by qRT-PCR. The remaining genes show a range of expression profiles, including early expression (Aam-22013, Fig 2.7A), mid-time point expression (Am-40687, Fig 2.7B) as well as late expression (AmerSigP-25878, Fig 2.7C).

Figure 2.7. Temporal gene expression of a miscellaneous pool of protein families. The genes were quantified using qRT-PCR and normalized using Ubiquitin.
**Metalloprotease Family.** Metalloproteases (MPs) selected from the sialotranscriptome can be further divided into neprolysin-like and reprolysin-like (members of the M13 family) genes. (Fig 2.8). For the neprolysin-like class, a single gene was selected from the sialotranscriptome, AamerSigP-41953 (Fig 2.8A). This gene showed highest levels of expression at 120 and 144 hrs.

![Graphs showing gene expression](image)

*Figure 2.8. Temporal gene expression of metalloproteases. Metalloproteases from two protein families were quantified using qRT-PCR and normalized using Ubiquitin.*

Within the reprolysin-like class, AamerSigP-41680 (Fig 2.8C) shows expression throughout the course of the blood meal. AamerSigP-20700 (Fig 2.8D) exhibits its highest expression levels early during tick feeding (24-48 hours). The remaining three reprolysin genes (Aam-41579, AamerSigP-35996, and Aam-41580, Fig 2.8E-G) are more highly expressed after 48 hours and throughout the remainder of tick feeding.
Discussion

*Tissue specific reference gene selection*

Other studies have typically examined expression stability of a single time point throughout all tissues or compared combined tissues from multiple time points.\(^{74–76}\) NormFinder requires a standard curve for the calculation of number of copies in each of the time point samples.\(^{79}\) The number of copies for each of the genes throughout the blood meal is then used to calculate a stability value. The correlation between stability value and suitability for a reference gene is inversely related. BestKeeper, unlike NormFinder, uses raw data instead of relative quantities. BestKeeper identifies the most stable reference gene as the gene with the lowest standard deviation (SD) value and the highest coefficient of correlation with the BestKeeper index (uses geometric mean of candidate C\(_{t}\) value).\(^ {80}\) The \(\Delta\Delta C_t\) method compares the Ct values of the tested sample against a reference.\(^ {81}\) In this case, unfed tissue samples are used as the reference against which the remaining samples are compared. The use of unfed tissues as the reference gives a baseline of expression before the stressors and increase metabolism of feeding are present. After calculation of the \(\Delta\Delta C_t\), the values can be converted into the amount of target gene using the following equation:

\[
\text{Amount of target} = 2(−\Delta\Delta C_t)
\]

After the amount of target is determined the standard deviation can be calculated. The sample with the smallest standard deviation represents the gene with the most consistent expression levels. Each gene was ranked based on their suitability as a reference gene as seen in Tables 2.1 and 2.2 for salivary glands.
and midguts, respectively. Ubiquitin in the salivary gland samples exhibit Ct values with minimal variation (Fig 2.1) as well as ranking first according to BestKeeper and the ΔΔCt method. Using the same methods as above Histone H3 proved to be the most stable gene in midgut samples according to BestKeeper and the ΔΔCt method. It is important to note that the genes tested exhibited differing expression profiles in each tissue. Previously, reference gene determination looked at the expression in multiple tissues together while the results presented here indicate that reference genes should be selected for each tissue type separately. In light of these results, qRT-PCR may be better normalized when stable genes are selected according to each tissue types as well as any developmental stages.

Analysis of the temporal gene expression in A. americanum salivary glands

Genes were selected based on the differential expression observed in the A. americanum sialotranscriptome. The work presented here serves as a validation of data collected using RNA-Seq as well as expands on that data by measuring the expression of these genes during each 24 hour time frame as opposed to combined time points.

Protease Inhibitor domain. Kunitz-domain proteins are one of the most represented protease inhibitor families identified in sialome studies. Kunitz containing peptides have been described in several ticks including Ixodes scapularis, Rhipicephalus appendiculatus, and Ornithodoros moubata. I. scapularis Kunitz containing peptides have been shown to be closely related to tick-derived protease inhibitor (TdPI) and a strong inhibitor of human skin β-
tryptase. In the literature, around 15 single Kunitz-domain peptides from ticks have been functionally characterized. These proteins can vary in the number of cysteines motifs (normally ≈ 6 Cys residues), and in *I. scapularis* are organized into three groups (I, II, and III) based on their Cys motif.

An *I. scapularis* protein, Ixolaris, has been recombinantly expressed and is a known inhibitor of FVIIa. This effectively inhibits the blood coagulation pathway which is advantageous to the tick in terms of the formation of the liquid blood pool. In *Haemaphysalis longicornis* a unique Kunitz domain containing inhibitor, haemaphysalin, is responsible for interrupting the kallikrein-kinin system. This system signals for additional blood coagulation compounds as well as members of the inflammatory pathway. The involvement of Kunitz domain containing protease inhibitors on a known inflammatory signaling system indicates that the protein family may play a part in other aspects of tick feeding.

These peptides have shown bacteriostatic activity in the tick *Dermacentor variabilis*. A kunitz type serine protease demonstrated its ability to limit rickettsial colonization within the midgut. Also, *Rhipicephalus microplus* has demonstrated differential protein expression of a kunitz type serine protease inhibitor in ovarian response to *Babesia bovis* infection.

The TIL proteins typically have five disulfides bonds formed by 10 cysteines. Recently, the subtilisin inhibitor with a TIL II domain identified in *R. microplus* demonstrated an antimicrobial property suggesting that these polypeptides may also be involved in antimicrobial functions.
Lipocalins. In tick saliva, lipocalin proteins are believed to play a role in the immune response and cell homeostasis. Although lipocalins may not play a direct role on the initial feeding mechanism of the tick, they are vitally important to the ability of the tick to avoid detection of the host’s immune system\textsuperscript{94,95} and remain attached for the duration of the blood meal. They are often characterized by the diverse functions and low molecular weight.\textsuperscript{96} The family as a whole has little sequence identity however there are conserved motifs throughout the sequence which are used to identify members of the family. Lipocalins characteristically contain an eight –stranded antiparallel β-sheet which forms a β-barrel. They have been previously identified in other soft and hard ticks such as *Ornithodoros moubata*\textsuperscript{97}, *Rhipicephalus appendiculatus*\textsuperscript{98}, and *Dermacentor reticulatus*\textsuperscript{99}. A study of lipocalins in *Ixodes ricinus* male and female ticks shows a similar variation in expression between sex and feeding stage.\textsuperscript{100} Lipocalins reduce the histamine from the host at the feeding site and reduce or prevent the histamine-mediated cutaneous inflammation/host response. Lipocalins are also well characterized in other functions such as ligand binding (retinoids and various steroids), receptor binding (possible transport mechanism for steroids), and pheromone activities (odorant binding properties).

Glycine Rich Proteins. Because little information is known regarding the function of GRPs, a wide range of expression profiles is not unexpected. Members of the GRP family are not characterized by function, and so multiple functions could be represented in the few GRPs selected here. Without additional information regarding the functions of these proteins, it is difficult to compare the
gene expression profiles to others of the same classification. Different functions during the blood meal would require varied expression. It is also possible that some of the genes selected here are members of the same class and have similar or redundant functions.

A significant proportion of research of GRPs has been performed on spiders and plants. In plants, GRPs have been characterized by the presence of glycine repeats and conserved domains within the protein. The repeats characterized in plants include GGX, GGXXXGG, GXGX, general glycine rich, and GGX/GXGX. Plant GRPs have also been characterized to be involved in cell walls, RNA binding, and cold shock. However, in ticks, little is known regarding function so classification generally involves the identification of glycine repeats.

GRPs in ticks may hold many functions but are typically attributed to the formation of a proteinaceous matrix formed around the tick mouthparts known as the cement cone. This cone is believed to help anchor the tick into the host dermis as well as protect the mouthparts from the host immune responses. The firm attachment of the tick to the host allows for uninterrupted access to the bloodmeal. Two GRPs have been investigated for their vaccine potential. A study of 64P, a GRP found in *R. appendiculatus*, used antibody staining to verify the presence of 64P within cement cones imbedded into hamster skin. This same study examined the expression of 64P during blood feeding. They found the most elevated levels of expression in the first three days of feeding after which expression returned to unfed levels or lower. Research of a second GRP in the
same species, RIM36, measured the antigenicity of the protein against antisera collected from tick infested cattle. Using antisera, RIM36 was localized in the salivary glands and also identified in the cement lysate verifying its role in the cement structure.\textsuperscript{13}

\textit{Tick Specific Family: Unknown Function.} The 8.9kDa protein family is characterized by the presence of an 8.9-kDa domain within the protein structure. In this study, genes AamerSigP-40930, AamerSigP-41425, and AamerSigP-22563 represent the 8.9 kDa family. This family of proteins can often be divided into three groups based on the commonality of sequences: 1) prostriates and metastriates, 2) metastriates only, and 3) more than one 8.9 kDa domain within the structure.\textsuperscript{51} No functional role has been attributed to this class of proteins.

The Ixoderin family of proteins is representing genes AamerSigP-35954 and AamerSigP-15297. Ixoderins, also known as ficolins, are oligomeric lectins that have both collagen and fibrinogen like domains; they are often considered to be a binding complex for viral, bacterial, and protozoan pathogens (specifically the glycosylated components of pathogens), activating complement molecules by the lectin pathway and are therefore understood to be important in innate immunity.\textsuperscript{101,102} Lectins have previously been found in soft ticks as membrane and soluble proteins and their coding sequence have high homology with Ixoderins.\textsuperscript{102} Although the exact function of Ixoderins is unknown in ticks, invertebrate and arthropod lectins are considered analogues to immunoglobulins and tick lectins have been implicated to participate in the transmission of pathogens to the host.\textsuperscript{103} Due to the nature of sample collection (salivary gland
tissues versus saliva), it is possible that these molecules are utilized by the salivary glands themselves and are not secreted into the host. The implicated functions in innate immunity would assist in tick feeding by reducing the pathogen load within the tick and therefore the saliva. This reduction in pathogen load would reduce the immunological assault on the host immune system. However, this has not be experimentally explored.

*Immunity Related Proteins.* Evasins bind chemokines, particularly those involved in pro-inflammatory responses, as a way to avert the host immune response. Using cross linking assays, the specificity of some of the evasin groups have been elucidated. Evasin-1 has been found to inhibit the binding of CCL3 and CCL4 to the respective receptors\textsuperscript{103} while evasin-3 has been shown to inhibit the binding of CXCL8 to its receptor CXCR1 and prevent neutrophil influx during an inflammatory event.\textsuperscript{104} The inability of host chemokines to bind to the host receptors prevents the host from signaling a pro-inflammatory response against the tick bite. It has also been shown that different species maintain distinct chemokine binding profiles. This may in part be due to the size of the mouthparts and the engorgement weight. A study of *A. variegatum, I. ricinus, D. reticulatus,* and *R. appendiculatus* measured the reactivity of tick nymphal proteins to human and murine chemokines. The study showed *A. variegatum* protein extracts to contain a larger repertoire of chemokine binding molecules possibly due to more extensive damage to the host from the larger mouthparts.\textsuperscript{105}
Defensins are considered to be the most well-defined class of antimicrobial peptides in arthropods. Defensin proteins are typically produced as preproproteins\textsuperscript{106} meaning the mature peptide is translated along with two extra sequences which are cleaved off at different points of the maturation process. Defensins are small peptides with mature molecular weights typically less than 9kDa. Homology within the defensin class is low however, a conserved pattern of cysteines (typically six in insects) is necessary for proper protein folding.

Insect defensins have been identified in many different classes of insects. These proteins are primarily active against Gram-positive bacteria.\textsuperscript{107} Defensins can be located in any of the tick tissues however, defensins localized only to the salivary glands, such as DefMT2 and DefMT5 of *Ixodes ricinus*,\textsuperscript{108} are likely secreted out into the host through the saliva and may assist tick feeding by managing microbial contamination from the puncture site to reduce host immune response.

*Metalloprotease Family.* The function of neprolysin metalloproteases have previously been associated with the degradation of bioactive peptides in mammalian systems including peptides with a role in catabolism of pro-inflammatory peptides such as tachykinins and atrial natriuretic peptide family.\textsuperscript{109} This has been established in neprolysin knocked out mice which exhibited peripheral hyperglasia instead of analgesia.\textsuperscript{110} Tick saliva has been associated with the ability to degrade bradykinin,\textsuperscript{111} a pro-inflammatory peptide, suggesting that these salivary M13 metalloproteases may play a role in their degradation. Neprolysin-like MP’s have been found in abundance in the genome of both
Drosophila melanogaster and C. elegans (as reviewed by\textsuperscript{112,113}). Within invertebrates, neprolysin and neprolysin-like peptides show a role in reproduction health.\textsuperscript{112}

These expression profiles indicate that the reprolysin MPs are abundantly expressed as a family in the salivary glands during the entire blood meal, although different genes were expressed at different points of the bloodmeal. Analysis of the sequences obtained from the RNA-seq demonstrates theoretical signal peptides, suggesting that they are secreted into the tick saliva and act on components of the extracellular matrix.\textsuperscript{114,115}

These metalloproteases are a Zn$^{2+}$ dependent family of enzymes, secreted as proenzymes and require activation for proteolytic activity.\textsuperscript{109} Each member of this family contains the zinc binding motif: HEXXHXXGXXHD. Other hard tick species also have genes encoding metalloproteases such as I. scapularis\textsuperscript{82} and Haemaphysalis longicornis.\textsuperscript{116} Tick MPs have been associated with fibrinolytic and gelatinase activity as well as disaggregation of platelets.\textsuperscript{114,117} The ability of tick reprolysin metalloproteases to degrade fibrin and fibrinogen demonstrates its role in maintaining the blood pool.\textsuperscript{114,118} All of these functions have been associated with the ticks’ ability to maintain a feeding cavity and suggest these MPs are directly interacting with components of the host extracellular matrix.\textsuperscript{116,119} Ticks are not alone in the use of MPs to interfere with blood clotting. Both snake and spider venom has been identified to contain MPs with activity associated with disturbing homeostasis\textsuperscript{120} which has also been the target of snake venom antisera. Additionally, metalloproteases have been
associated with the dissemination of *Borrelia* spirochetes in human cells, as both MMP-9 and MMP-1 are up regulated in infected cells.121 Borrelia spirochetes upregulate matrix MPs in human tissues to increase their movement across the tissues linking the roles of MPs to pathogen transmission.121
CHAPTER III

DEVELOPMENT OF AN ARTIFICIAL MEMBRANE FEEDING

ASSAY FOR A. AMERICANUM

Abstract

Ticks require access to a bloodmeal to progress through the life stages. In nature, this bloodmeal is acquired from a living host over the course of 2-3 weeks. Under laboratory conditions, ticks are batch fed using research animals or when only low levels of feeding are required, using capillary tubes filled with host blood. Recently, membrane feeding systems have been developed that use a range of membrane materials. Here, a silicone membrane is used as a skin mimic to feed A. americanum nymphs and adults. Ticks can be routinely fed to a partially fed state, and feeding can be monitored as the tick passes from one feeding phase to the next.

Introduction

Ticks act as a vector for more types of pathogenic organisms than any other hematophagous arthropod and are thus considered one of the most costly and medically significant arthropods to humans and livestock. Amblyomma americanum, the lone star tick, is a competent vector for a number of disease-causing agents of varying severity including: Ehrlichia chaffeensis, E. ewingii, and STARI (Southern Tick Associated Rash Illness) as well as non-pathogenic conditions such as delayed hypersensitivity to alpha-galactose, a sugar commonly found in red meat. The survival of these pathogens rely on the survival and life cycle of its vector, A. americanum. For a hard tick to successfully
feed on its host, it must secrete hundreds to thousands of bioactive molecules through the saliva, as well as the potential pathogens that utilize the tick salivary glands and saliva for transmission into a vertebrate host. The understanding of these interactions between host-pathogen-vector is vital for development of anti-tick vaccinations and preventing tick-borne diseases.

Tick research gives rise to ethical issues with performing experiments using live animals because it is potentially dangerous to the animal. However, due to ethical limitations and funding restrictions, new artificial methods are being developed. A number of artificial feeding techniques have been employed for tick feeding. The various methods include feeding the ticks with capillary tubes, artificial membrane systems, and animal skin membrane systems. Capillary feeding consists of a fine glass capillary tube containing blood or serum to be positioned over the mouth parts of the tick. It has been reported that the ticks are able to become partially fed, but are unable to feed to repletion. The most successful trials using capillary feeding have required ticks to feed on a live animal before or after being fed with the capillary tube to increase probability that engorgement will occur. In infection studies, ticks are typically fed on an animal until partially engorged, forcibly removed and fed the pathogen by capillary feeding, and the placed back onto the animal to continue feeding until repletion.

Skin membranes have been made using skin of several different species of animals which allow the tick to feed on blood. These membranes contain natural stimuli such as hair and pheromones, which allow for engorgement within
a comparable time period. Skin membranes work similarly to artificial membranes. However, skin membranes deteriorate quickly making it impractical to gather the needed materials.\textsuperscript{124} Skin membranes also do not reduce the animal costs or the ethical concerns surrounding tick research.

There have been various artificial membranes composed of different substances and structures that have been altered to accommodate to different species of ticks. The majority of the membranes used are primarily made of silicone or a rubber material known as Baudruche.\textsuperscript{122} For membrane feeding, it is imperative that there is a reinforcement on the membrane and stimuli to entice the tick to attach and feed successfully.\textsuperscript{125} The tick exposed blood has to be changed every 12 hours to continue the mimicry of a live animal in the artificial system.

Use of animal feeding models has drawbacks such as ethical and monetary issues, however, the use of an artificial feeding system allows for increased control over the feeding system and allows for better isolation of tick saliva and the testing of antibodies and acaricidal compounds. While artificial feeding has previously been successful, it is still a difficult task to support long feeding periods and find appropriate stimuli to induce tick attachment. One important aspect of tick attachment that is difficult to study in \textit{in vivo} fed ticks and cannot be studied in capillary fed ticks is the formation of the cement cone. The components of the cement cone are secreted from the salivary glands at the onset of attachment and mixed with the tick saliva for the next few days. As the cement cone grows it fills the bite site cavity to prevent leaking of the liquid blood.
pool. The cement cone is burrowed into the host and is difficult to collect as it must be removed simultaneously with the tick or forcibly removed with forceps after the tick has been removed causing high levels of pain and discomfort to the animal. Using artificial membrane feeding, the ticks remain attached for a longer length of time allowing the formation of the cement cone and the cones are clearly visible on the other side of the silicone membrane.\textsuperscript{126} The production of the cement cones in artificial membrane feeding will increase the amount of cones available for further research.

Methods

Tick Rearing and Dissection

Ticks purchased from the tick rearing facility at Oklahoma State University were maintained at \( \approx 25^\circ \text{C} \) with 80-90\% relative humidity and were subjected to a long photoperiod of 14h light – 10h dark as recommended by Patrick and Hair.\textsuperscript{127} Ticks were fed on either artificial membranes as described below or on a naïve sheep and dissected for the partially fed tissue collection. The partially fed ticks were dissected into cold M199 buffer and midgut, salivary gland, and carcasses were removed. The detached tissues were stored in RNALater (Invitrogen, Carlsbad, CA) at \(-80^\circ \text{C}\).

Preparation of Silicone Membrane

The artificial system is composed of a reinforced, silicone membrane attached to an acrylic chamber. The silicone solution coating the membrane is composed of 10g silicone glue (Elastosil E4, Wacker, Germany), 3.3mL silicone oil (Sigma-Aldrich), and 31mL or 26mL Toluene (Sigma-Aldrich) for A.
Americanum and A. maculatum, respectively. The membrane consists of a 3cm X 3cm lens paper coated with the above silicone solution and reinforced by a fiberglass mesh. The A. americanum membranes are only coated with the silicone solution in one fluid motion on one side of the lens paper and are hung from a clothesline to dry for 48 hrs (Figure 3.1A). Fiberglass mesh wiring is glued to the non-coated side of the A. americanum silicone membrane, and the reinforced membrane is glued to the acrylic chamber (Figure 3.1B). The system is left to dry for 24 hours, and the excess membrane around the acrylic chamber is removed. The chambers fit directly into the wells of a 6-well plate (Figure 3.1C).

Figure 3.1. Assembly of membrane feeding apparatus. (A) Silicone coated lens paper is hung from a suspended string to dry. (B) Silicone membranes reinforced with fiberglass mesh are glued to the acrylic chambers. (C) The completed chambers fit into a 6-well micro well plate.

Artificial Feeding of Ticks

Bovine blood is collected every 1-2 weeks from a local abattoir and defibrinated manually by immediately stirring the blood with 5mL pipette tips after it is removed from the animal, and disposing of the clots that accumulate around the pipette. The defibrinated blood is transferred into conical tubes and stored at
4°C. Prior to each feeding, 3-4 mL of the blood is warmed to 37°C. The warm blood was placed into a well of a six-welled plate and the artificial apparatus is placed into the blood (Figure 3.2A). Fifteen female *A. americanum* and five males were placed in the apparatus (Figure 3.2B) with a small amount of animal hair from the local PetSmart and a cotton stopper to close the chamber. The amount of ticks placed in the assembled chamber differs among species due to size and aggressiveness. The artificial feeding system was stored in a 37°C to simulate a summer environment and maintain blood temperature. The blood was exchanged every 12 hours, and the membrane was rinsed with a penicillin-streptomycin (pen-strep) solution to remove any excess blood, and prevent bacterial growth due to the constant warm and moist environment, before being placed into the fresh blood.\(^{125}\) The antibiotic solution consists of 1X PBS with 100 units/mL of pen-strep. During transmission studies, 1-2mL of the tick exposed blood was collected before the blood is changed.

*Figure 3.2. A. americanum* ticks feed in a membrane feeding apparatus. (A) Three milliliters of prewarmed blood are added to a well of a 6-well plate. (B) Ticks are added to the chamber of the feeding apparatus which is closed with a cotton stopper and placed into the well with the prewarmed blood. (C) Attached ticks are visible through the acrylic which allows the ticks to be monitored throughout blood feeding.
Results

Attachment and feeding of A. americanum ticks

Ticks were placed inside the acrylic chamber and inspected every 24 hours to gage attachment success. During the first inspection, it is common to find a majority of the tick have climbed to the cotton stopper and these ticks are placed back to the bottom of the chamber underneath the animal hair to force interaction with the membrane. Ticks which are not attached after 48 hours are removed from the chamber as they are unlikely to attach after that point. After 48 hours, the attached ticks are monitored daily for changes in female size indicating successful feeding. The underside of the membrane is examined at each cleaning to verify tick attachment and to search for the presence of fungal contamination or cement cones which can be removed as experimental design dictates (Figure 3.3).

Figure 3.3. Hypostomes and cement cones on the underside of the membrane feeding apparatus. (A) Six hypostomes are visible piercing through the surface of the membrane. (B) The same region of the membrane from another angle shows the ticks in the chamber attached to the membrane.

By checking the membrane each day, cement cone growth can be monitored and documented. As seen in Figure 3.3, the ticks cluster together when feeding. Because of this, tick attachment does not always happen at straight through the skin or membrane, often times happening at an angle where
the hypostome takes a longer path to the blood supply. The two ticks visible through the chamber wall are a good example of this. The tick to the right has attached directly through the membrane, and the hypostome is protruding through the membrane more than the surrounding hypostomes. The tick to the left has attached at an angle most likely because of crowding. The hypostome of this tick does not protrude through the membrane as extensively as the first tick. It would be interesting to correlate the angle of attachment and consequently the amount of hypostome present on the other side of the membrane with the engorgement size and engorgement time to determine if there is any change in the ability to feed.

The daily observation of the attached ticks revealed some differences between in vivo and membrane fed ticks. Ticks which are fed on live animals attach within the first 24 hours; however, tick which are fed using the in vitro feeding system show a delayed attachment with the highest attachment rates after 48 hours. Along with the increased attachment period, there is also a decrease in total tick attachment. Feeding of ticks on live animals during the proper seasons often results in attachment rates close to 90%. However, when ticks are fed using the membrane feeding system, attachment rates range between 50-75%.

_A. americanum_ fed on live animals reach an engorged state after 12 days of feeding. The size of female ticks changes slowly during the first 5-7 days with drastic changes taking place in the final 3-4 days of feeding. Ticks fed using an in vitro feeding system require a longer feeding period before reaching the fast

64
feeding stage (Figure 3.4). The weights of ticks removed from membrane feeding are consistently lower than ticks fed for identical amounts of time on live animals. *In vivo* fed ticks that match the weights of membrane fed ticks have an average feeding time 2 days lesser than the membrane fed ticks (Table 3.1).

![Tick feeding progression](image)

*Figure 3.4* shows the feeding progression of ticks using artificial membrane feeding. Pictures were taken to document changes in tick size as feeding continued. Ticks have been feeding for (A) 2 days, (B) 4 days, (C) 6 days, (D) 8 days, (E) 10 days, and (F) 24 days. (G) Attached tick can be seen through the chamber wall and the hypostome is visible through the membrane. (H) Close examination of the hypostome shows a small cement cone that has been secreted from the tick.

The ticks fed using artificial membrane feeding can be examined in lab using a dissecting microscope so changes in tick size can be more clearly distinguished. Ticks that are fed *in vivo* can only be examined with a microscope after being removed from the host. The progress of feeding from 2-10 days (Figure 3.4A-E) shows that it takes more than 10 days for the tick to enter the fast feeding stage. Figure 3.4F shows a tick that has been attached to the membrane for 24 days. After 15 days of feeding changes in size slowed. Eggs were visible underneath the cuticle however, the tick was unable to detach. The
tick was forcibly removed and placed in a humid chamber for ovipositioning however, no eggs were deposited.

Figure 3.5. *A. americanum* nymphs fed using an artificial membrane feeding apparatus. (A) Engorged nymphal ticks can be seen attached to the membrane ready to drop off. (B) Comparison of unfed and fully engorged nymph ticks.

Table 3.1

*Comparison of feeding weights, feeding times, and attachment time of in vivo and membrane fed ticks.*

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Mean Weight (mg)</th>
<th>Days Feeding</th>
<th>Adjusted Days Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vivo</em> Fed</td>
<td>23.6</td>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td>Membrane Fed</td>
<td>21.6</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

Nymphal ticks were also fed using the artificial membrane feeding system (Figure 3.5). In laboratory settings, nymphs are commonly fed on small mammals such as hamsters or mice. Nymphs fed in the artificial membrane feeding system were weighed and stored in a plastic vial inside a high humidity chamber to monitor the molting process. The nympha
ticks that fed to engorgement weights higher than 10.0 mg (one exception of 7.30 mg) molted into female adult ticks.
(Table 3.2). Of the 11 nymphs that successfully fed and molted, seven molted into adult females.

Table 3.2

*Sex of molted adult ticks and the weights of nympha1 ticks fed using an artificial membrane feeding system.*

<table>
<thead>
<tr>
<th>Nymph engorgement weights of female adults (mg)</th>
<th>Nymph engorgement weights of male adults (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.7</td>
<td>8.9</td>
</tr>
<tr>
<td>12.2</td>
<td>7.3</td>
</tr>
<tr>
<td>10.9</td>
<td>6.6</td>
</tr>
<tr>
<td>10.5</td>
<td>5.5</td>
</tr>
<tr>
<td>10.3</td>
<td>--</td>
</tr>
<tr>
<td>10.0</td>
<td>--</td>
</tr>
<tr>
<td>7.30</td>
<td>--</td>
</tr>
</tbody>
</table>

Discussion

*Amblyomma americanum males and females are able to feed on an artificial silicone membrane*

Tick attachment requires a complex relationship between the tick and the host. The tick requires sensing molecules from the host skin such as CO₂, heat, and pheromones. These signaling molecules allow the tick to identify a suitable site for attachment for the bloodmeal. In the absence of these signals attachment is difficult as the tick is unable to distinguish the viability of the host. To combat
this potential problem, other groups using artificial feeding have made their own "host pheromone perfume" by dissolving bovine hair in organic solvents to be sprayed onto the membranes before gluing them to the chamber. While this does result in a more successful attachment rate, it is time consuming, volatile organic solvents fumes are noxious, and can be hazardous without proper training. Here, animal hair collected from a local pet grooming facility is used to cover the membrane to mimic both scent and protection the tick may encounter while on a host. The absence of the appropriate signaling molecules is likely the cause for increased attachment time.

*In vivo* fed *A. americanum* reach full engorgement after 12 days of feeding. Two distinct feeding stages are necessary for engorgement: slow feeding and fast feeding. During the slow feeding stage, the tick focuses on the establishment and maintenance of the blood pool underneath the skin of the host. Blood is taken in slowly during this stage and any differences in size are small. Once the blood pool is fully established, the tick enters a fast feeding stage where the majority of the blood meal is taken in. The fast feeding stage may last only two or three days and changes in tick size are substantial.

However, when ticks are fed using an artificial membrane system, the blood pool is already established. While one would assume that the pre-established blood pool would reduce the time needed for the slow feeding stage, it actually increases the time spent in this phase. Standard blood contains a multitude of clotting factors and other signaling factors which the tick must combat during the slow feeding. The absence of these factors in the defibrinated blood may
interfere with the tick’s natural rhythm of gene expression. Measurement of the tick’s weight during membrane feeding is consistently two or more days behind the weights of \textit{in vivo} fed ticks. Ticks which have been fed artificially can take more than 10 days to enter a faster feeding stage and up to 18 days before full engorgement is reached.

\textit{Membrane feeding may not be a viable option for the maintenance of a lab colony}

Nymphal ticks were fed using the artificial membrane feeding system and allowed to molt into adult sexually dimorphic ticks. Although there were ticks which were able to successfully fed, the total attachment rate was much less than 50\%. In addition to the poor attachment rate, the number of nymphal ticks that were able to feed to engorgement was greatly reduced, only 11 of the 25 attached. The reduced attachment rate combined with the decrease in feeding success makes the use of membrane feeding systems under the guidelines set here unreliable for maintenance of the lab colony. This is further indicated by the lack of ovipositioning of engorged female ticks. As the tick reaches full engorgement, the cuticle expands and the eggs become visible which verified the ability of the ticks to mate while feeding in the apparatus. Female ticks enter the fast feeding stage after mating at which point the ovaries begin to develop rapidly. The females’ engorgement and the visibility of the eggs beneath the surface of the cuticle are necessary steps for the development and laying of the eggs. However, female ticks which reached engorgement using the artificial membrane feeding system were unable to lay the eggs clearly visible under the
cuticle. It is unclear if this inability of the ticks to complete ovipositioning comes from a change in the digestion and utilization of the bloodmeal or from the change in environment from *in vivo* to membrane feeding.

_Future utilization of artificial membrane feeding in tick research_

The field of tick research is hindered by the complications of the tick-host interaction. Tick attachment is a complicated process that likely requires the back and forth signaling between the host and the tick. Identifying the tick’s role in the attachment process is difficult as the host is an integral aspect of attachment. Artificial membrane feeding is the only method of *in vivo* feeding that allows the ticks to attach to a substrate. Ticks fed using capillary feeding can only imbibe a small volume of blood and therefore does not feed for the amount of time needed to measure cement cone formation. The use of artificial membranes increases the time that the ticks can feed. This allows for the formation of cement cones which can then be collected and studied.\textsuperscript{126,128} Another benefit to artificial membrane feeding is the closed feeding system. As the tick secretes saliva into the host, it is quickly disseminated through the bite site and then circulated through the host. Membrane feeding allows for the saliva and its components to be collected in the remaining blood pool and the components can be investigated. This is especially helpful when studying the dissemination of pathogens from the tick to the host. In order to identify the pathogen in the host blood, the pathogen must multiply in the host to reach a detection threshold. The time required to meet this threshold makes it difficult to determine the time of transmission from the tick. Using an artificial membrane system, the blood can be
sampled to measure pathogen transmission much sooner. Artificial membrane feeding can also be used to study the acquisition of a tick-borne pathogen.\textsuperscript{129} Pathogen can be added to the blood which is then fed to the tick. As the ticks feed, they can be forcibly removed from the membrane to test for the presence of the pathogen. This is especially useful for tick-borne pathogens which do not have a suitable animal model such as \textit{Ehrlichia chaffeensis}. 
CHAPTER IV
COMPARATIVE STUDY OF A. AMERICANUM CEMENT CONES FROM IN VIVO AND ARTIFICIAL MEMBRANE FEEDING SYSTEMS

Abstract

The Lone Star tick, Amblyomma americanum, is endemic to the southeastern United States and capable of transmitting pathogenic and non-pathogenic diseases. To remain firmly attached to the host, the tick secretes a proteinaceous matrix termed the cement cone which hardens around the tick’s mouthparts to assist in the attachment of the tick as well as to protect the mouthparts from the host immune system. Cement cones collected from ticks on a host are commonly contaminated with host skin and hair making analysis of the cone difficult. To reduce the contamination found in the cement cone, an artificial membrane feeding system has been developed. Cones collected from in vivo and membrane fed ticks are analyzed to determine changes in the cone morphology. Structural analysis techniques such as light microscopy, scanning electron microscopy, and FT-IR are performed to monitor any changes in the morphology or composition of the cone. Additionally, proteomic analysis using LC-MS/MS yields further information regarding the composition of the cones collected from each feeding type. General inspection of the cones reveals similar structural components. Closer examination using SEM exposes two distinct cone structures. This is further verified using FT-IR which exhibits varying secondary structure profiles between the two cone types. Proteomic analysis of the cones identified both secreted and non-secreted tick proteins. Identification of the tick
cement cone proteins opens new avenues of tick research for control of these parasites. In addition, the properties of the cement cone and therefore the cement proteins could be used for biomedical applications such as skin adhesive or bone mimics.

Introduction

In order to reach the nutritious bloodmeal, ticks must penetrate the host’s skin with the hypostome and is assisted by the presence of recurved teeth along the hypostome surface. Adult Ixodid ticks stay attached to the host for 7-21 days dependent on the species, and consequently firm attachment to the host by way of a cement cone is required. Tick feeding is divided into three general stages: attachment, slow feeding, and fast feeding.\textsuperscript{2,24} The attachment phase has multiple steps including identification of a vertebrate host, penetration of the barbed mouthparts deep into the dermis, encasement of the hypostome in a narrow secreted cement cone for a stealthy but secure attachment and to provide a conducive environment for the injection of pathogenic microbes during the bloodmeal.\textsuperscript{2} The cement cone protects the mouthpart from the host immune system while also anchoring the tick into the host dermis. The proteinaceous matrix of the cement cone is secreted by both longirostrata (long mouthparts) and brevirostrata (short mouthparts) tick species.\textsuperscript{24}

Ticks secrete two types of cement, a primary “core” cement and a secondary “cortex” cement. The primary secretion, or the core, has been noted as early as 5-30 minutes after attachment.\textsuperscript{24,27} The core cement hardens almost instantaneously once in place, while the cortex cement secretes from the tick for
multiple days and has a graduate hardening process. Although both the core and cortex cement are predominantly proteinaceous, the core cement also contains lipids, while the cortex cement has more carbohydrates than lipids.

The relative quantities of amino acids in the cement cone revealed that small amino acids such as glycine, serine, and leucine were the most abundant, followed by tyrosine, an amino acid known for its cross-linking properties. Although tyrosine is present, there is currently no evidence regarding its role in the protein structure or the aggregation of proteins for the formation of the cement cone.

The majority of research regarding the cement cone structure comes from the study of tick bite site biopsies. Using common histological stains, hematoxylin and eosin, the cement cone can be identified by its bright pink color. Differences in the core and cortex cement are clearly visible after histology staining and can give some insight into the formation of the cement and how feeding takes place through the cement. The core cement lies close to the hypostome and forms a tapered tube in which the hypostome fits. Transverse cuts of the biopsy indicate that the primary cement is almost perfectly circular, indicating that full coverage of the hypostome is important in preventing host detection of the hypostome. The cortex cement is layered on the exterior of the core cement, coming into direct contact with the host skin. The strands of the fully cured cortex cement are intertwined with the surrounding skin tissue allowing for a more secure attachment.
Adhesive protein secretions are produced in many other invertebrates for structures such as egg casing adhesives, spider silk, and barnacle adhesive. Analysis of the protein composition of these other invertebrate adhesive secretions revealed over-representation of small amino acids, glycine and serine being the most prevalent\textsuperscript{32} similar to the amino acid composition found in tick cement proteins. Insect egg casing adhesives can be found as either hydrogel with high elasticity and tack or as a glue which becomes tough and dry through the evaporation of the solvent.\textsuperscript{32} Proteomic analysis of the egg casing adhesives revealed mostly large molecular weight proteins (more than 75 kDa) except for shield bugs which contain proteins spanning the entire range of molecular weights according to SDS-PAGE.\textsuperscript{32} Various spider silk formulations are used as a cocoon to protect the spider from the environment during development, adhesive to secure the spider webbing and egg casings to the substrate, and webbing to provide safety and assist in capturing food.\textsuperscript{38} The most commonly studied spider silk is dragline silk which has high tensile strength and is commonly comprised of only a few proteins. These proteins self-assemble into crystalline repeats which are bound together by disulfide bond formation, glycosylation, or cation interactions.\textsuperscript{38} Barnacles also secrete an adhesive cement which is used to firmly adhere to their substrate however, barnacle cement differs from the other adhesives listed here as the protein composition of barnacle cement contains cysteine repeats\textsuperscript{33} rather than a large number of glycine repeats. However, the two-cement process of barnacles\textsuperscript{131} mimics the two-cement composition of tick cement.
The focus of tick cement cone research has shifted in the last 40 years. Original research of the topic focused on identifying structural characteristics of the cone and using histological staining to uncover bits of information regarding its composition.\textsuperscript{24–26} As molecular biology based techniques improved, the focus began to shift towards understanding the proteins responsible for the makeup of the cement cone and how to exploit these proteins for vaccine development.\textsuperscript{13,22,24,28,31,132,133} However, structural and proteomic research of the cement cone has been largely lacking in the last 10-15 years due to the difficulty in collecting cement cones which remain embedded in the skin of the host. Another complication in cement cone research is the solubility of the cement cone. The curation process for the cement formation results in an extremely hard cone which is difficult to solubilize. Solubilization has been most successful in hot acids or bases,\textsuperscript{24} however at these extreme conditions analysis is impossible. The use of molecular techniques and the development of an artificial membrane feeding system allow the tick research community to circumvent this problem. Common artificial feeding methods involve feeding the tick via a capillary tube fitted on its mouthparts; however, this does not simulate a natural feeding environment, and feeding cannot proceed for multiple days as with \textit{in vivo} feeding. To better replicate natural feeding, membranes are now being used to simulate skin and the ticks feed on a blood pool supplied underneath. This method has been used to feed multiple tick species with varying mouthpart sizes.\textsuperscript{125,134–136} Previous studies using \textit{Amblyomma hebraeum} have shown cement cones produced on the underside of the membrane to be easily
collectable without the inference of the host dermis. Here, we utilized an artificial membrane feeding system to farm cement cones from adult female *Amblyomma americanum* ticks. These cones are compared to cones which have been collected from *in vivo* fed *A. americanum* ticks in terms of surface structure, protein structure, and protein composition.

**Methods**

**Materials**

All common laboratory supplies and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri) or Fisher Scientific (Grand Island, New York) unless otherwise specified.

**Tick rearing**

Adult *A. americanum* ticks were purchased from the Tick Rearing Facility at Oklahoma State University and maintained at the University of Southern Mississippi according to established methods. The adult ticks were maintained at room temperature with 90% relative humidity and a long light cycle of 14 hours light 10 hours dark. All animal work was conducted according to the approved protocol by the institutional Animal Use and Care Committee (IACUC) of the University of Southern Mississippi (protocol# 15101501). All efforts were made to minimize animal suffering.

**Tick feeding**

*In vivo feeding.* A total of 50 female and 25 male ticks were placed onto the back of a sheep and enclosed in a sock glued to the sheep. Ticks were manually removed at regular intervals using forceps and inspected for cement
cone formation. Pictures of intact cement cones were taken with a Dino Light camera. Cones were carefully removed from the tick mouthparts using sharp point forceps and stored dry at -80°C until analyzed.

*In vitro feeding.* A total of 15 females and 5 males were placed into an artificial feeding chamber. Animal hair collected from a local pet grooming facility was then placed on top the silicone membrane to simulate host fur and animal scent to the membrane. Using a cotton stopper, the total volume of the chamber was reduced to force the ticks to interact with the membrane. Blood was collected from a local abattoir who regularly slaughters bovine and porcine animals. Blood is defibrinated by manual agitation using a plastic stirring rod and clots were removed. Defibrinated blood was stored at 4 °C as 25 mL aliquots until used and 3-4 mL aliquots were pre-warmed to 37 °C, added to a single well of a 6-well plate and the feeding chamber was placed into the well so that the membrane comes into direct contact with the blood. To maintain the optimal temperature for feeding, the feeding system was placed in a 37 °C incubator. The blood was changed twice daily and the membrane was rinsed with a PBS solution with 2% Penicillin/Streptomycin at each blood change.\(^{137}\) The outside of the silicone membrane was examined daily for the presence of cement cones, and the cones were removed with sharp pointed forceps during the course of blood feeding. Cones from *in vitro* fed ticks are placed in 1.5 mL microcentrifuge tubes and placed at room temperature for 24 hours to dry the cones and are then stored at -80 °C.
Scanning electron microscopic analysis

Cement cone samples were oriented and mounted onto standard aluminum scanning electron microscopy (SEM) mounts with carbon conductive adhesive tabs and were coated with a thin deposit of silver using a Quorum Emitech K550X (East Sussex, United Kingdom) sputter coater to remove any charging that may occur on the surface of the sample. Electron micrographs were captured with an FEI Quanta 200 (Hillsboro, Oregon) environmental scanning electron microscope (ESEM) operating in high vacuum mode at an accelerating voltage range of 10kV to 20 kV.

Fourier Transform Infrared Spectroscopy – Attenuated Total Reflectance

The Fourier Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR) spectra were obtained for in vivo fed and membrane fed collected cones. More than 300 scans were taken to increase the signal to noise ratio. Spectra were deconvoluted using Fityk 0.9.8 software. Gaussian equations were applied to the Amide II peak until the deconvoluted spectrum matched the original curve. Secondary peaks were identified and peak wavelengths matched to known secondary structures.

Proteome

Cement cones collected from in vivo and membrane fed ticks were grouped accordingly, and each group was placed in a 1.5 mL microcentrifuge tube with 250 μL of 8M urea. The tubes were secured to a vortex and allowed to vortex overnight. The samples were centrifuged briefly on a small tabletop centrifuge to sedimentate the remaining insoluble cement fragments, and the
supernatant was removed. The supernatant was mixed in a 1:1 ratio with reducing Laemmli sample buffer (Bio-rad, Hercules, California) and subjected to SDS-PAGE using AnykD gels (Bio-Rad). After electrophoresis, the gel was stained using Gel Code Blue overnight and destained with 3-5 washes of 20 mL water. Gel images were obtained using Bio-Rad Versa Doc white light transillumination.

Selected bands were manually excised from the gels and washed with 100 mM ammonium bicarbonate buffer (pH 8.5) and 100% acetonitrile mix (v/v, 1:1) until the color disappeared. The gel slices were washed with HPLC grade water followed by 100% acetonitrile, and then dried in a speedvac. The proteins were digested with trypsin (0.5 mg/mL, Promega, Madison, WI, USA) using a 50:1 ratio (protein: trypsin) overnight at 37°C. Hydrophilic peptides were eluted using Nanopure water, followed by the elution of hydrophobic peptides with 50% acetonitrile with 5% trifluoroacetic acid. The eluted peptides were dried in a speedvac and resuspended in water/acetonitrile (50:50) and 0.1% formic acid to a final peptide concentration of 1 mg/mL. The digested samples were analyzed on a LTQ Vello mass spectrometer (Thermo Electron) with in-line HPLC.

Data analysis

Protein identification was performed using the Sequest algorithm in the Protein Discoverer v. 1.4 (Thermo Electron, Woburn, MA, USA) and the tick database containing 3500 tick specific polypeptides. The identified peptides were further evaluated using the charge state versus cross-correlation number (Xcorr). The criteria for positive identification of peptides were Xcorr >1.5 for
singly charged ions, Xcorr >2.0 for doubly charged ions, and Xcorr >2.5 for triply charged ions. Only the best peptides were considered. To positively identify a protein, one tryptic peptides had to detect in all three analysis, or at least two different peptides had to be detected in a single analysis. Nonspecific matches (i.e., false positive matches) were eliminated by searching with publically available rabbit and sheep databases on NCBI (to eliminate any host proteins) and a reversed protein sequence database generated from *Amblyomma americanum* sialome sequence.\textsuperscript{22}

**Results**

*Description of cement cone*

In Figure 4.1, two female ticks are shown, which have been removed from a host sheep. The cement cone is visible from the dorsal and ventral views (Figure 4.1A and 4.1B, respectively), and the hypostome is visible through the cone. The tip of the cone is tinted a slight red color indicating that it may have been in direct contact with blood. The cone appears to be relatively smooth with a pointed tip. Ticks fed using an artificial membrane feeding system also form a cement cone that surrounds the mouthparts (Figure 4.2). Inspection of these cones can take place much earlier than *in vivo* fed ticks (Figure 4.2A), and the same cement cone can be observed the course of the bloodmeal. The general morphological characteristics of the cone remain regardless of feeding type. The cone is slightly transparent with the mouthparts still visible through the cement (Figure 4.2B) as seen in the *in vivo* fed cones (Figure 4.1), and the cement cone base is wide across the surface of the membrane. However, there is a lack of
structural definition in the membrane fed cement cones as observed when removing the cones from the tick’s mouthparts.

![Figure 4.1](image1.png)

*Figure 4.1:* *In vivo* fed adult female *Amblyomma americanum* ticks removed from a sheep during feeding. The cement cone is visibly attached to the hypostome of the right tick from both the dorsal (A) and ventral (B) views. The cone located on the hypostome of the tick on the right covers the hypostome for the purpose of tick attachment and protection of the hypostome.

![Figure 4.2](image2.png)

*Figure 4.2.* Membrane fed ticks with visible hypostomes. The external view of the artificial membrane feeding apparatus membrane (A) allows for the visualization of the cement cone (B) as the cone is forming. Ticks can be removed from the membrane feeding apparatus with cones intact (C) and without contamination for further analysis.

When the cone is grasped with the forceps, there is more give to the cone indicating that the cone has not completed the hardening process. To better preserve the microstructures on the cement cone surface, ticks can also be
removed from the membrane by cutting the membrane away and the cones can be visualized on the mouthparts (Figure 4.2C) and removed the same as *in vivo* fed cones. By removing cement cones in this way differences in cement cone size and shape can clearly be seen. Often the cones are flat and widespread on the tick mouthparts (Figure 4.2C).

*Comparison of SEM images*

Cement cones collected from both *in vivo* (Figure 4.3) and membrane (Figure 4.4) fed ticks were subjected to SEM for further analysis of the cone surface. Figure 4.3 examines two *in vivo* cones (Figure 4.3A-D and 4.3E-H). Each *in vivo* cone is approximately 250 µm at the base and extends between 300-700 µm. The outer surface has regions which appear relatively smooth near the tip of the cone (blue arrow) as well as highly textured regions near the base. In Figure 4.3B the cone is positioned such that the opening in which the mouthparts fit is visible. In this view, the layering of the cone can easily be seen. The layering of the protein secretions causes a basket weaving structure consisting of both sheet like arrangements and fiber arrangements (Figure 4.3C). Interestingly, this view also shows the presence of small circular indentations aligned along the inner edge of the cone opening (yellow arrow). Close examination of the outer regions of the cone shows an overall smooth appearance with extensive flaking near the edges. Similar structures can be found in the second cone (Figures 4.3E-H). Images 4.3F and 4.3G exhibit the same sheet and fiber basket weaving as seen in Figure 4.3B. In addition to these assemblies, Figure 4.3G also reveals clusters of clumps similar to cauliflower.
The inner edge of the mouthpart opening visualized in Figure 4.3H also contains the indentations (yellow arrow) as seen in Figure 4.3C, although the definition is decreased.

*Figure 4.3.* Scanning Electron Microscopy (SEM) images for cones collected from *in vivo* sheep fed ticks after +5 days of feeding. These cones contain multiple morphological characteristics such as fibers (B), layers (C and G), flaking (D) and indentions (C and H). The blue arrows indicate the tip of the cement cone which comes in contact with the bloodmeal. The yellow arrows identifies the areas of hypostome indentions.

Figures 4.4A-D shows the structural morphology of a membrane fed cone collected 24 hours after attachment and compared to a membrane fed cone 7 days post-attachment (Figures 4.4E-H). The 24 hour membrane cone is much smaller than the *in vivo* fed cones, measuring 250µm across and approximately 100µm long (Figure 4.4A). The overall topography of the cone exhibits large mounds (Figure 4.4A), which are not present in any of the cones collected from later time points. Closer examination of the cone reveals two distinct textures (Figure 4.4B), a remarkably smooth surface located closest to the base (red arrow) and a more porous region near the tip of the cone (blue arrow). Close
examination of the cone base (Figure 4.4C) shows areas which were previously heavily textured prior to the full curation of the primary cement. We believe that this cement cone collected at 24 hours after attachment gives a detailed look into the cured and uncured (Figure 4.4C and 4.4D, respectively) primary cement.

Figure 4.4. Scanning Electron Microscopy (SEM) of cement cones collected from membrane fed ticks. Comparison of a cone collected from a tick which had only been feeding 24 hours (A-D) and shows different morphology than later fed cones (E-H). In these cones, a distinction can be made between primary (D) and secondary (C) cement. The base of the cement cone is indicated with red arrow. Blue arrow identifies the tip of the cement cone. Also, the layering of the recurved teeth on the hypostome is visible along the inner walls of the cone (G and H) and indicated by yellow arrows.

As the feeding progresses, the cement additional layers of cortex cement adds bulk to the cement cone. In Figure 4.4E, the cone is turned upward so that the location of the hypostome can be clearly seen. This cone has a smooth micro surface with a varied topography of the cone as a whole. The outer surface (Figure 4.4F) displays a smooth surface similar to that seen in Figure 4.4C. However, irregular features are observed on the surface, which maybe the result of contamination from immune cells found in the blood or a fungal contaminant
resulting from the constant moist conditions. Inspection of the inner surface of the cone, where the hypostome would have been located (Figure 4.4G), reveals a pattern of indentions (yellow arrow). Close examination (Figure 4.4H) of this area shows that the indentions (yellow arrow) are formed in rows down the length of the cone opening. Interestingly, the fibrous/basket weaving formations seen in \textit{in vivo} formed cement cones (Figure 4.3) are completely absent from \textit{in vitro} cones.

\textit{Comparison of Fourier Transform Infrared Spectroscopy (FTIR) images}

In order to obtain information regarding the protein composition of the cement cones, FTIR-ATR was used to determine the secondary structures of the proteins present on the outer surface of the cone. Using this method, it was determined that all cement cones (\textit{in vivo} and membrane fed) contain to some extent \(\beta\)-sheet structures and most of the cement cones contain \(\beta\) turns (Figures 4.5 and 4.6, respectively). The cones collected from \textit{in vivo} fed ticks also possess proteins in a helical confirmation (\(3_{10}\) helix in Figure 4.5A and \(\alpha\) helix in Figure 4.5B). In each \textit{in vivo} cone, \(\beta\) sheet structures represent over half of the total structures found (Figure 4.5A-B). The percent of helical structures present varies between the \textit{in vivo} fed cones, with more helix formation in the lab fed cement cone (Figure 4.5A) compared to the field collected cement cones (Figure 4.5B). The remaining secondary structure identified in Figure 4.5B corresponds to spectra from \(\beta\) turns.
Figure 4.5. FTIR spectra with Gaussian deconvolution for the identification of protein secondary structure. *In vivo* cones contain a mixture of β sheet and helical structural components.

Cones collected from *in vitro* fed ticks have a markedly different spectrum from *in vivo* fed cones. Each of the membrane fed cones (Figures 4.6A-C) contains only two secondary structures. Analysis of a cement cone collected from a membrane fed female tick after just 72 hours of feeding reveals almost exclusively random coil structures with a minor portion of the spectra assigned to β-sheet structures (Figure 4.6A). The remaining two cones collected from membrane fed ticks (female after seven days of feeding and male after five days of feeding) show less variation in their structural analysis (Figure 4.6B and C, respectively). These cones contain predominantly β sheet structures (Figure 4.6B-C). The remaining structures of the cement cones analyzed in Figures 4.6B-C consists of β-turns.
Figure 4.6. FTIR spectra with Gaussian deconvolution of membrane fed cement cones. Cones collected later in feeding contain primarily β sheet structures while a cone which was collected during the first 3 days of feeding is primarily random coil in structure.

Comparative list of identified proteins

The solubilization of tick cement cones in 8M urea does not dissolve the cone completely but does allow for the investigation of the soluble fraction of the cement cone. SDS-PAGE analysis of the soluble proteins shows multiple proteins ranging from 10-250 kDa (Figure 4.7). To identify these proteins, the bands were subjected to trypsin digestion in gel segments as indicated by the boxes in Figure 4.7. The digested protein fragments were subjected to LC-MS/MS and mapped using a previously generated transcriptome from the salivary glands of *Amblyomma americanum*.\(^{22}\)

The proteins identified in the segments SF1-SF9 from *in vivo* cones show interesting results (Table 4.1). Only seven *A. americanum* proteins were identified from only four gel segments (SF2, SF3, SF4, and SF9). Four of these proteins are considered intracellular proteins with functions necessary for cell maintenance (Putative deSUMOylating isopeptidase, putative cytochrome p450, putative ribosomal protein I6, and putative Histone H2B). The remaining proteins
identified from the cement cones include a glycine rich protein of nearly 70 kDa, a metalloprotease, and a hypothetical secreted protein with no known function. Also identified from the cement cones were host proteins. *In vivo* cones often have skin and hair embedded within the cement structure and it is common for proteomic analysis of *in vivo* cones to identify more host proteins than tick proteins. Cones that were collected from membrane fed ticks resulted in the identification of many more proteins than those found in *in vivo* cones. From the eight segments (AF1-AF8) excised from Lane 3 of Figure 4.7 from a membrane fed cone, six of these segments (AF2, AF4-AF8) led to the identification of a mixture of 26 secreted and non-secreted proteins (Table 4.2). Identified secreted proteins include glycine rich proteins, serine protease inhibitors, metalloproteases, and unclassified secreted proteins.

*Figure 4.7. SDS-PAGE of cement soluble fraction.* The cones were dissolved in 8M urea overnight and subsequently run on a SDS-PAGE. The bands were excised as indicated by the boxes and digested using trypsin. Lane 1 – *in vivo* fed cone, Lane 2 – molecular weight marker (from bottom: 10 kDa, 15 kDa, 20 kDa, 25 kDa, 37 kDa, 50 kDa, 75 kDa, 100 kDa, 150 kDa, 250 kDa), Lane 3 – membrane fed cone.
Table 4.1

Proteins identified from cement cones collected from in vivo fed ticks

<table>
<thead>
<tr>
<th>Gel Slice</th>
<th>Protein ID</th>
<th>Peptide Sequences</th>
<th>% Coverage</th>
<th>MW (kDa); pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF2</td>
<td>Putative deSUMOylating isopeptidase 2, partial GI: 759085692</td>
<td>YHLMNK</td>
<td>3.53</td>
<td>19.0; 8.19</td>
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<td></td>
<td>Putative cytochrome p450 4w1, partial GI: 759084668</td>
<td>GRKLpK</td>
<td>11.93</td>
<td>12.7; 9.99</td>
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<tr>
<td>SF3</td>
<td>Putative Glycine-rich secreted cement protein, partial GI: 759090220</td>
<td>YPGLSGLYGR</td>
<td>4.25</td>
<td>69.2; 9.41</td>
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<tr>
<td>SF4</td>
<td>Putative ribosomal protein I6 GI: 759086918</td>
<td>TGLLMVTGpYGINGcPLRR</td>
<td>13.12</td>
<td>32.1; 11.05</td>
</tr>
<tr>
<td></td>
<td>AamerSigP-2853</td>
<td>TWWSRWLSRDIFIAVVIAS MSATFSWLWR</td>
<td>27.62</td>
<td>12.2; 10.46</td>
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<tr>
<td>SF9</td>
<td>Putative secreted metalloprotease, partial GI: 759089918</td>
<td>LLGYLCVMVNSANLRYQDT VAPRVK</td>
<td>10.78</td>
<td>26.0; 7.02</td>
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<td>Putative Histone h2b, partial GI: 759084736</td>
<td>LLLPGELAK</td>
<td>7.89</td>
<td>12.8; 10.54</td>
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Table 4.2

Proteins identified from cement cone collected from membrane fed ticks

<table>
<thead>
<tr>
<th>Gel Slice</th>
<th>Protein ID</th>
<th>Peptide sequences</th>
<th>% Coverage</th>
<th>MW (kDa); pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF2</td>
<td>Serine protease inhibitor</td>
<td>Lidtpvdlalpk Llslklidtpvdalpk MtilllpR</td>
<td>15.75</td>
<td>15.9; 6.96</td>
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<tr>
<td></td>
<td>Putative RNA recognition motif 1, partial</td>
<td>VATSRAIR KPRLIVR</td>
<td>9.68</td>
<td>17.1; 9.99</td>
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<tr>
<td></td>
<td>Hypothetical protein, partial</td>
<td>Elfdeiwtlrr</td>
<td>7.14</td>
<td>18.4; 4.48</td>
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<tr>
<td></td>
<td>Putative p1 ap, partial</td>
<td>VgcpmxxxlSARIIQCYA TRMHFYAR KSakmtDCSdCHATLvaa SDVppaalTELr</td>
<td>17.56</td>
<td>37.5; 8.35</td>
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<tr>
<td>AF4</td>
<td>Putative tick metalloprotease, partial</td>
<td>MEGLVGpRHRIEPLSVse KLIVLVLVLTVPTKGL EQpMLVYpRlleer</td>
<td>24.37</td>
<td>22.5; 6.07</td>
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<td></td>
<td>Putative metallopeptidase, partial</td>
<td>IDGEKSIIQNPTEAQRK</td>
<td>8.37</td>
<td>22.8; 5.54</td>
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<td>Hypothetical protein, partial</td>
<td>Lfakqqgnaqalspal Tgkr</td>
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<td>Serine protease inhibitor, partial</td>
<td>RslaifvpAPSSNLAALEK VSAAKHLAVFRAGHR</td>
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<td>28.5; 6.38</td>
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Table 4.2 (continued).

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<th>Gel Slice</th>
<th>Protein ID</th>
<th>Peptide sequences</th>
<th>% Coverage</th>
<th>MW (kDa); pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF4</td>
<td>Serine protease inhibitor, partial GI:805449067</td>
<td>RAQPPpVEFRVEHp TGGKIPK</td>
<td>5.71</td>
<td>42.4; 9.20</td>
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<td>AF5</td>
<td>Hypothetical protein GI:759090180</td>
<td>ENLVANTVAGPALLDTAATT VR</td>
<td>5.58</td>
<td>39.1; 9.19</td>
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<td>Putative coiled-coil domain-containing protein, partial GI:759087600</td>
<td>TEKLIQFTKDEPK</td>
<td>2.62</td>
<td>52.3; 8.91</td>
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<tr>
<td></td>
<td>Putative tick metalloprotease, partial GI:759089956</td>
<td>QLNVSNSFEEK</td>
<td>4.98</td>
<td>27.0; 5.08</td>
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<tr>
<td>AF6</td>
<td>Putative cement protein RIM36, partial GI:196476756</td>
<td>VITDPSTGLPIAQAVYIGIVR</td>
<td>12.65</td>
<td>16.8; 9.41</td>
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<td>Putative purine nucleoside phosphorylase, partial GI:759086528</td>
<td>VFGLSLISNECISNYDTQQV ANHEEVLETGQKRK</td>
<td>14.47</td>
<td>26.2; 8.76</td>
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<td>Putative secreted protein precursor, partial GI:759089510</td>
<td>NARDYEcNNHHEENYCPGQ SpLQCKGNGVCVCDR</td>
<td>18.78</td>
<td>21.0; 8.40</td>
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<tr>
<td>AF7</td>
<td>Hypothetical protein GI:759090180</td>
<td>ENLVANTVAGPALLDTAATT VR</td>
<td>5.58</td>
<td>39.1; 9.19</td>
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</table>
Table 4.2 (continued).

<table>
<thead>
<tr>
<th>Gel Slice</th>
<th>Protein ID</th>
<th>Peptide sequences</th>
<th>% Coverage</th>
<th>MW (kDa); pI</th>
</tr>
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<td>AF8</td>
<td>Putative DNA replication licensing factor mcm4 component GI:759087732</td>
<td>KAIACLLFGGSVKR ETVPNVPIKPGLEGYALPR ITAIGVYSIK KGPQE KNSDRAGQPKCPVDpFFIVp DK HLASSPNYERIAKSIpSIYG FADVK GEIQRpGpRLSATAAEK</td>
<td>15.85</td>
<td>82.1; 7.91</td>
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<td>Putative transcription factor a mitochondrial, partial GI:759085292</td>
<td>KPRSPRSAYAFFCIEAR</td>
<td>11.64</td>
<td>17.1; 8.95</td>
</tr>
<tr>
<td>Hypothetical protein GI:759088860</td>
<td>mRARSVAVFSLLLHSTpS QK</td>
<td>17.05</td>
<td>14.0; 10.29</td>
<td></td>
</tr>
<tr>
<td>Putative methylthioadenosine phosphorylase mtap, partial GI:759086166</td>
<td>QLQiPHHEAGTVVTIEGPR</td>
<td>9.09</td>
<td>22.9; 8.15</td>
<td></td>
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</tbody>
</table>

**Discussion**

*Cement cones from both feeding systems have similar characteristics*

The tick hypostome has recurved teeth which are believed to assist the tick in piercing the host skin and initial attachment to the host. The firm attachment of the tick to the host is necessary for the tick to maintain access to the bloodmeal. Soon after attachment, the tick begins to secrete a highly...
proteinaceous saliva into the host. Some components of this saliva begin to aggregate and form a hardened cement surrounding the mouthparts, referred to as a cement cone. While fluid, this cement intertwines itself between the layers of the host’s skin and fits firmly within the bite cavity. As the tick is forcibly removed, the cement cone is often left buried in the skin of the host as seen with the left tick presented in Figure 4.1. The cone extends passed the hypostome in both in vivo and membrane fed ticks (Figure 4.1A and figure 4.2B, respectively).

Upon examination of the cement cone, it appears as a solid matrix which completely encompasses the tick mouthparts. However, a solid cone surrounding the hypostome would cause great difficulty in uptake of the bloodmeal as the tick would not have any direct contact with the blood pool. This indicates that there must be some opening of the cone to allow for the uptake of the bloodmeal.

Histological staining of tick bite site biopsies has revealed an opening at the tip of the cone. This opening was present through the entirety of the cone so that the blood was drawn into the cone and then taken into the tick by its hypostome. This way, the hypostome never comes into direct contact with the in vivo blood pool reducing the chances of immune system activation. Our finding of cone discoloration near the tip of the in vivo cone supports the histology data. Many of the proteins previously identified from solubilized cement cones are host contaminate proteins even when the cone has been rinsed with PBS and ethanol. The presence of these proteins after washing is a direct consequence of the blood present within the center of the cone.
One significant difference observed between the two cones types is the changes in the curing process. Although the exact curing processes is not understood, *in vivo* fed ticks use air flow as a means of drying the cement. This allows for both the primary and secondary cement to harden albeit at different rates. However, due to the lack of a dermis in the membrane system, air flow to the cement is minimal which causes the membrane fed cones to remain soft. The cone is still intact indicating some level of aggregation or cross-linking involved in the curation process. In order to collect accurate structural data from the membrane fed cement cones, great care is taken to remove the cone from the hypostome and the cone is placed into a 1.5mL tube and the drying process is allowed to finish at room temperature for at least 24 hours before long term storage.

It is important to note that during the cone removal process there is significant pulling of the hypostome. This stress on the hypostome would occur in nature when a tick is attached to an unsuitable location for feeding. It would be advantageous for the tick to quickly detach from the host to prevent possible death by being pressed against a hard surface or by being pulled apart. When the hypostome is pulled during the cone removal, the tick secretes a few microliters of saliva which is able to completely dissolve the cement cone in less than one minute. The components of this secretion have not yet been elucidated due to the small volume present. This quick solubilization is remarkable as the cones are otherwise insoluble in anything other than hot acids. The identification of the compounds present in the secretion could have many applications in the
biomedical field such as dissolution of blood clots and plaques. It has not yet been elucidated if the tick dissolves the cone using this secretion once the tick has fed to repletion or if the cone is left embedded in the skin of the host.

**SEM reveals differences at the cone surface**

The skin of a host can be millimeters in thickness and the tick hypostome must reach through the full thickness to reach the bloodmeal. *In vivo* cones are embedded in the host skin and therefore create an interlocking network of host tissue and cement. Impressions of this network are recognizable by SEM of *in vivo* fed cones (Figure 4.3). The layering and texture surrounding these cones could easily allow for a more secure attachment to the host. The membranes of the membrane feeding system are typically less than one millimeter, and therefore the cone is present solely on the outer surface of the membrane removing the rigid surrounding matrix of the skin. This explains the lack of layering and fibrous textures easily visualized in the *in vivo* cones.

Along the inner surfaces of the cones examined in this work, the impressions left by the hypostome teeth are clearly visible (yellow arrows in Figures 4.3 and 4.4). It is a reasonable conclusion that the indentions found are caused by these structures on the hypostome. When the cement cone is forcibly removed from the mouthparts, the hypostome remains imbedded in the cement and the teeth can be seen extending out of the cement (Data not shown). As the tick is feeding the hypostome is filled with fluid, either saliva or blood. Because of the presence of this extra fluid, the hypostome is slightly swollen making it fit directly into the sides of the cement. As the tick finishes feeding, the
hypostome no longer contains the same amount of fluid and therefore is smaller in size. This allows for the easy removal of the hypostome from the bite lesion.  

Identification of two types of cement from membrane fed cones

Membrane feeding of the ticks allows for the examination and collection of the cement cone at a much earlier point in tick feeding when compared to \textit{in vivo} feeding giving us a unique look at the early stages of cone development. In Figure 4.4, a cone collected approximately 24 hours after attachment contains cement at two different stages of the curation process. The smooth region of the cone has likely been present longer and represents cement that has cured for a longer time. It is believed that a primary cement is secreted in the early hours of feeding that cures very quickly followed by a secondary cement secreted after the initial attachment and has a longer curing time.\textsuperscript{13} It is also important to note here that although air is necessary for the complete curation of the cones as noted above, more than just air is required. If the hardening of the cement was based purely on contact with air, the 24 hour cone which was allowed to dry in a 1.5 mL microcentrifuge tube would exhibit the same level of curation throughout. However, this is not the case. This implies that there are some components which are secreted after the laying of the initial cement components that allow for the full hardening of the cone.

One hypothesis of cement cone hardening is sclerotization which is the same process as the hardening of the tick scutum. It is known that insect cuticle hardens by a sclerotizing process which is believed to take place by protein cross-linking and dehydration. In insects, cuticle proteins are cross-linked
together at tyrosine positions through interactions with quinone compounds such as catechols.\textsuperscript{141} Although a literature search for quinones or catechols in ticks yields no results related to the sclerotizing of the cuticle or the hardening of the cement cone this is likely due to a decreased interest of the cement cone and the tick cuticle in the last few years. Previous proteomic analysis of \textit{in vivo} fed cement cones from our lab (data not shown) has also found tick tissue transglutaminase in the cement cone. Tissue transglutaminase is able to cross-link proteins at the glutamine residues.

\textit{Secondary structures of cement proteins exhibit minor differences}

Early research noted that the cone is only soluble in hot acidic or alkali solutions however this makes proteomic analysis difficult. This lack of proteomic data has hindered cement research and many current conclusions about tick cement are purely speculative. The commonalities of tick glycine rich proteins and spider silk GRPs is a prime example of this.\textsuperscript{28} Spider GRPs are known to be extremely long in length with 10-20 amino acid long repeats.\textsuperscript{142} However, tick GRPs are smaller and repeats are typically 3-7 amino acids long or the repeats are lacking.\textsuperscript{28} To gather more information about the proteins present on the surface of the cement cone face, FTIR-ATR is used to determine protein secondary structure. The spectra for two \textit{in vivo} fed cones (Figure 4.5) show similar structural components, although the amounts in which these secondary structures are found differ. The lack of helical structures in the membrane collected cones is likely due to the cone not having contact with the host dermis. Typical host skin contains many proteins which are helical in nature to give the
skin its elasticity. The interlocking nature of the cement cone with the layers of
the host skin would allow for skin cells and extracellular proteins to become
imbedded within the cured cone. The fact that these structures are missing from
the membrane fed cones goes to show that this method does allow for the study
of tick cement cones without interfering host proteins. Comparison of the later fed
membrane fed cones also shows that there is no significant difference between
the secondary structures found in cones from male and female ticks. The
presence of a full sclerotized scutum covering their backs prevents males from
expanding during feeding as females do, and therefore they feed intermittently on
the host taking in multiple small blood meals from the same host. The shorter
feeding time of males also contributes to the difficult task of collecting cement
cones from in vivo sources. Proteins of the male salivary glands have only
recently begun to be investigated.

Along with time comparable cones from in vivo and membrane fed
sources, a cone removed from an artificial membrane fed female was removed
after just 3 days of feeding. This cone did not show a predominance of β-sheet
structures as seen with both in vivo and longer feeding membrane cones but
rather contained almost exclusively random coil structures. As seen with early
cones in the SEM (Figure 4.4A-D), there is a curing process that occurs, which
hardens the cone. Although it is not currently known how this process occurs, the
data shown here indicates that a conformational shift in the proteins may play a
role in this hardening process. This needs to be further investigated by
measuring the secondary structures of cones collected at multiple time points of the feeding.

*Proteins of in vivo and membrane fed cement cones contain the same protein families*

Identification of proteins from the cement cone is a difficult process. To date, the only proteins known to be present in the cement cone include GRPs isolated from two different tick species (*Rhipicephalus appendiculatus* and *Haemaphysalis longicornis*) using immunochemistry and chitinase which was experimentally proven using RNA interference. However, direct solubilization of the cone for proteomic studies typically reveals more host proteins than tick proteins. The interlocking nature of the cement cone with the host dermis makes the collection of a cone free of host proteins impossible. Membrane feeding removes the host dermis and therefore allows for a cement cone completely free of host skin cells.

When investigating the protein composition of cement cones, a large focus is placed on finding the structural components. However, in the comparative proteomes listed here (Tables 4.1 and 4.2) there are few proteins which would serve as a scaffolding for the cement. Proteomic analysis of the cement cone is complicated due to the solubility of the cone and the lack of genomic information of ticks. The cones used here were solubilized in 8M urea to allow for solubilization as well as analysis using SDS-PAGE. Other solubilization methods such as acids effectively prevent the protein from being analyzed under physiological conditions. After SDS-PAGE, the proteins are subjected to
digestion using trypsin and the fragments are analyzed using LC-MS/MS.

However, the peptide fragments can only be mapped using databases such as the A. americanum sialotranscriptome\textsuperscript{22} or the non-redundant NCBI database. While the parameters are optimized for identifying most tick proteins, the lack of genomic and proteomic data often results in peptides being matched with other organisms such as the host (especially in cases where the protein is highly conserved) or not at all.

Proteomic analysis of the \textit{in vivo} cement cones results in only seven A. americanum proteins identified from four gel regions. These proteins include both intracellular proteins such as cytochrome p450, ribosomal proteins, and histone components. The most logical explanation for the presence of these proteins are residual tick hypostome cells embedded in the cement. Proteins identified from the cement cone which are either secreted or have enzymatic function which could be used to the benefit of the tick include deSUMOylating isopeptidase, glycine rich proteins, and a metalloprotease. DeSUMOylating isopeptidases are involved in the removal of small ubiquitin-like modifiers which can affect cellular processes and act as gene expression modifiers.\textsuperscript{144} The presence of this protein in the cement cone may be the result of residual tick cells, or it may be secreted from the salivary glands into the host to modulate the protein functionality of host immune proteins. A glycine rich protein was also identified in the \textit{in vivo} cement cone proteome. GRPs are a class of proteins which contain more than 20\% glycine in the primary sequence. These proteins are the major component of spider silks\textsuperscript{38,142} and are also found abundant in plant cell walls.\textsuperscript{35} It is
hypothesized that the GRPs present in the cement cone give the cone its strength and insoluble characteristics. The third protein identified from the in vivo cement cone proteome which has probable function is a metalloprotease. Metalloproteases assist in the prevention of blood clot formation throughout the bloodmeal. The presences of this protein in the cement cone could be a remnant of the saliva secreted from the tick during feeding. It is also possible that the metalloprotease is necessary at the location of the cement cone to prevent blood clotting specifically at the cone by the activation of platelets by collagen like proteins in the cement.

The proteome assembled from membrane fed cones revealed many more A. americanum proteins (26 proteins from six gel sections). The membrane fed cones also contained intracellular proteins such as nucleoside phosphorylase, DNA replication factors, transcription factors, and Golgi membrane proteins. As mentioned above, these intracellular proteins are the result of tick hypostome cells embedded into the cement of the cone. Other proteins found in the membrane fed cones include metalloproteases, serine protease inhibitors, multiple hypothetical proteins with unknown functions, and a RIM36 like glycine rich protein. Thrombin is a serine protease found in the blood which is necessary to the formation of blood clots. The inhibition of thrombin by the protease inhibitors likely reduces the amount of clot formation around the cement cone. There is a high incidence of hypothetical proteins in the membrane fed cement cone. These proteins have been identified as RNA transcripts from the sialotranscriptome generated for A. americanum. However, these proteins have
not been characterized in any other organism and so it is not possible to predict a function for these proteins.

The presence of functional proteins within the cement cone has been investigated previously. Tick tissues, saliva, and cement cones were collected from *R. appendiculatus* and the lysozyme activity of the tissues was measured.\textsuperscript{145} Although the activity did not differ greatly throughout the samples, there was a distinct difference between the cones of female and male ticks. There was also a significant increase of the lysozyme activity in cones collected from ticks infected with tick-borne encephalitis virus.\textsuperscript{145} This added activity found in the cone itself leaves open the possibility for many other functions embedded in the cone. The presence of proteins with known enzymatic functions within the cement cone structure supports the idea that the cone may play more than just a structural role. It should be noted that the cross-linking of the scaffolding proteins would likely make them impenetrable to the solubilization techniques used here. The interaction of 8M urea with the cement cone is likely to only occur with proteins which are present along the surface or that are not tightly bound to each other. To further investigate these proteins, the cross-linking nature of the scaffolding would need to be disrupted.
CHAPTER V
ELUCIDATION OF THE FUNCTIONAL CHARACTERISTICS OF GLYCINE RICH PROTEINS OF THE LONE STAR TICK A. AMERICANUM

Abstract

Tick feeding requires the secretion of a multitude of pharmacologically active proteins which are responsible for the formation of the cement cone, the establishment of the blood pool, and the prevention of the host immune system activation. Glycine rich proteins are found in many organisms and can function in a variety of cellular processes and structures. The exact role of GRPs in the salivary glands of ticks has not been fully elucidated. It has been suggested that GRPs purely play a role in the formation of the cement cone; however, new evidence is opening an emerging line of study into other roles the GRPs may be involved in. GRPs were depleted in *A. americanum* adult females however, no change was detected. To further investigate GRP function, the expression of GRPs was measured after exposure to multiple types of stress. This caused an increase in AaGRP-B, AaGRP-C, AaGRP-D, and AaGRP-H as high as 826-fold.

Introduction

The feeding success of ticks requires the secretion of pharmacologically active proteins and molecules into the host. These molecules interact with the host immune system to assist the tick in establishing a liquid blood pool. Previous data presented here and other publications have confirmed the proteins secreted through the saliva are not constitutively expressed throughout the entirety of the bloodmeal but rather differentially expressed. These proteins are responsible
for mediating the host response by preventing clot formation, wound healing, immune system activation, and inflammatory cascades.\textsuperscript{51}

In addition to the modulation of the host immune system, some of the secreted proteins accumulate around the tick mouthparts and harden to form a cement cone which is described in detail in the previous chapter. Proteomic analysis of these cones has identified multiple protein families including functional and structural proteins. One class of proteins found in the cement cone, glycine-rich proteins, has documented roles of physiological functions and structural characteristics.

GRPs are the primary component of spider silks and also found in insect egg casings and adhesives. Structural GRPs have a higher incidence of glycine up to 60-70\% in the case of spider silks. A high prevalence of glycine in the protein would decrease the solubility of the protein in an aqueous environment; however, these proteins are able to remain soluble in the cytosol and in the lumen of silk glands. Once the silk, egg casing, or adhesive is secreted from the insect, the protein becomes hydrophobic and hardens into a silk or film. The hydrophobic nature of these secretions ensures the structural integrity of the spider web, the egg case, or the adhesive which adheres the egg case to its substrate. Hydrophobicity is also one of the contributing factors to the aggregations and assembly of these proteins into macromolecules.

In other organisms, GRPs have physiological functions that play a variety of roles. In plants, GRPs have been identified as stress response related proteins. These GRPs are upregulated in response to multiple stressors including
injury, extreme temperature, pathogen invasion, and lack of water. GRPs can also play a role in regulating gene expression as RNA binding proteins. It is currently unknown if the GRPs which are upregulated during the stress response play a role in mediating the stress directly or indirectly by interfering with the gene expression.

In this study, two GRPs are knocked down to elucidate their function in tick feeding. After evaluation of the feeding phenotypes of the knockdown ticks, further analysis of GRP expression is performed.

Methods

Ticks and Ethical Statement

Ticks were purchased from the Oklahoma State University Tick Rearing Facility. Adult male and female *Amblyomma americanum* were kept according to standard practices\(^7\) at room temperature (25°C) with approximately 90% relative humidity for a photoperiod of 14h light/10h dark. Ticks were fed on a sheep and approximately 20-25 female ticks were removed after five days of feeding. All animal experiments were carried out according to approved IACUC protocols (10042001; renewed 15101501) from the University of Southern Mississippi and in strict accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Tick tissue dissection

Partially fed female ticks removed from the sheep were dissected and the salivary glands and midguts removed and cleaned in ice cold M199 buffer. Salivary glands and midguts from each time point were pooled together
according to tissue type and stored in RNALater (Life Technologies, Carlsbad NM) at -80°C until used.78

Transcription gene expression analysis

RNA isolation and cDNA synthesis. Frozen tick tissues were placed on ice to thaw and RNALater was carefully removed with precision pipetting. RNA was isolated from the time point pooled salivary glands and midguts using illustra RNAspin Mini kit (GE Healthcare Lifesciences) protocols. RNA concentration was measured using a Nanodrop spectrophotometer and stored at -80°C or used immediately. To synthesize cDNA, 2µg of RNA was added to a 20 µl reaction using the iScript cDNA synthesis kit (Bio-Rad). The reverse transcription reaction is then heated in a Bio-Rad thermocycler under the following conditions: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and hold at 10°C. The resultant cDNA was diluted to a working concentration of 25 ng/µl with nuclease free water and stored at -20°C until used.

Quantitative Real-Time PCR. A list of all genes tested can be found in Table 1. qRT-PCR was performed within the guidelines of Bio-Rad protocols provided with iTaq Universal SYBR Green Supermix. Briefly, 50 ng of cDNA was added to a 20 µl qRT-PCR reaction using SYBR Green supermix with 300 nM of each gene specific primer. The samples were subjected to the following thermocycling conditions: 95°C for 30 sec; 35 cycles of 95°C for 5 sec and 60°C for 30 sec with a fluorescence reading after each cycle; followed by a melt curve from 65°C to 95°C in 0.5°C increments. Each reaction was performed in triplicate along with no template controls. Primers used for gene expression validation can
be found in Appendix 1. Gene expression validation was performed using Ubiquitin as the reference gene.

*dsRNA Synthesis & Tick Injections.* The gene of interest was amplified using PCR with gene specific primers and purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). Gene specific T7 promoter sequences were added to the 5’ and 3’ end of the purified product using PCR and were purified. The purified T7 PCR products was confirmed by sequencing and transcribed into dsRNA using the T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA). The dsRNA produced was purified via ethanol precipitation and the concentration was measured using a Nanodrop spectrophotometer and was analyzed on a 2% Agarose gel. Unfed females were injected with 500ng of the purified dsRNA using a 31-gauge needle and were maintained at 37°C with 90% humidity overnight. The ticks were placed onto the feeding source (*in vivo* or *in vitro* source). The ticks were forcibly removed at different time points to determine the expression.

*Stress Exposure*

*Cold stress.* Fifteen female ticks were placed in 4°C for multiple months to mimic winter conditions. GRP expression was measured in both dissected salivary glands (10 pairs of glands pooled) and in crushed whole ticks (three individual ticks).

*Heat Stress.* Fifteen female ticks were placed in a 37°C incubator with high humidity conditions for 4 days. GRP expression was measured in crushed whole ticks (three individual ticks). Remaining ticks were to be used for pooled
salivary gland samples; however, high temperature caused desiccation of tick tissues preventing proper dissection.

**Injury Stress.** Fifteen female ticks were injuried by piercing the cuticle or pulling a hind leg from the tick. The ticks were placed in a humid environment and room temperature to recover from the injury. GRP expression was measured in both dissected salivary glands (10 pairs of salivary glands pooled) and in crushed whole ticks (three individual ticks).

**Oxidative Stress.** Fifteen female ticks were injected with 10mM Paraquat to induce a high oxidative environment in the tick tissues and allowed to recover over a 48 hour period. Ten of the ticks were dissected and the salivary glands pooled and tested for GRP expression.

**Pathogen Stress.** Nymphal ticks were experimentally infected with *E. chaffeensis* by dipping. The nymphs were immediately fed on a host and allowed to molt into adults. Female infected ticks were fed on a sheep and pulled after 5 days. The ticks were dissected and the individual salivary glands were tested for GRP expression.

**Results**

*Bioinformatic analysis of GRP sequences*

GRPs are characterized purely by the overabundance of glycine in the protein’s sequence. This complicates typical bioinformatic analyses which would predict structure and function by comparing the unknown protein’s sequence to other well described proteins. Because GRPs are not divided based on structure or function the protein sequence provides little information. Sequence identity is
insignificant as the sheer number of glycine residues are the most homologous residues. To determine if the GRPs selected from the *A. americanum* sialotranscriptome\(^{22}\) the tripeptide and penta-peptide repeats commonly found in GRPs were used as a way to group the proteins (Table 5.1). When compared in this way, a possible pattern begins to emerge. GRPs with fewer amino acids typically have the GGX repeat whereas

Table 5.1

*Protein characteristics and tripeptide repeats of nine AaGRPS.*

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of Amino Acids</th>
<th>Molecular Weight (kDa)</th>
<th>Significant Amino Acid Prevalence</th>
<th>Significant Amino Acid Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aam-41235 (A)</td>
<td>107</td>
<td>10.0</td>
<td>22.4% Gly 22.4% Ser 15.0% Ala</td>
<td>GGX</td>
</tr>
<tr>
<td>AamerSigP-34358 (B)</td>
<td>205</td>
<td>18.7</td>
<td>30.7% Gly 17.1% Ser 13.2% Pro</td>
<td>GGX/GXG, PISGGSGGVRLP GQSGSKPG</td>
</tr>
<tr>
<td>Aam-40766 (C)</td>
<td>310</td>
<td>29.4</td>
<td>27.7% Gly 19% Ser 11% Ala</td>
<td>GGX/GXG</td>
</tr>
<tr>
<td>AamerSigP-39259 (D)</td>
<td>54</td>
<td>51.7</td>
<td>22.2% Gly 16.7% Ser 13.0% Ala</td>
<td>GGX/GXG</td>
</tr>
<tr>
<td>AamerSigP-41913 (E)</td>
<td>116</td>
<td>11.8</td>
<td>19.8% Gly</td>
<td>GGX</td>
</tr>
<tr>
<td>AamerSigP-41539 (F)</td>
<td>116</td>
<td>11.6</td>
<td>24.1% Gly 13.8% Ala 10.3% Ser</td>
<td>GXG/GGX</td>
</tr>
<tr>
<td>Aam-41540 (G)</td>
<td>222</td>
<td>22.6</td>
<td>20.3% Gly 13.1% Ala</td>
<td>GGX</td>
</tr>
<tr>
<td>Aam-36909 (H)</td>
<td>325</td>
<td>Undetermined</td>
<td>29.2% Gly 20.6% Ser</td>
<td>GGX/GXG</td>
</tr>
</tbody>
</table>
proteins with more than 200 amino acids have a near equal mixture of GGX and GXG repeats. While GXXXG repeats are found in these proteins, they are not more abundant than the tripeptide repeats listed. This pattern does not hold true when looking at GRPs from other organisms or even other ticks of the same species so this information may not be a usable criterion for identifying GRP classes.

The names assigned to the GRPs was produced according to the identification of a signal peptide and the identification of the peptide during the generation of the sialotranscriptome. To simplify the names repeated here, the gene names have been replaced with AaGRP signifying that these proteins are glycine rich proteins from *A. americanum* and each is assigned a letter A-I. The letters are listed in Table 5.1 and also correspond to the location of the gene in the previous study Figure 2.4. This new nomenclature will be used from here for the identification of the GRPs.

*Expression of GRPs after the depletion of a GRP transcript*

The injection of dsRNA-AaGRP-F effectively depleted AaGRP-F transcripts by >95% (data not shown). Although the GRP transcript was significantly reduced, there was no measurable change in the ability of the tick to attach to the host, maintain a firm attachment, or ability to feed successfully (Figure 5.1). The other GRPs previously investigated were measured in the AaGRP-F deficient ticks to evaluate the presence of compensatory mechanisms (Figure 5.2). Two GRPs, AaGRP-D and AaGRP-H, are significantly over expressed when AaGRP-F is depleted (Figure 5.2). Interestingly, the depletion of AaGRP-F also results in
the significant depletion of AaGRP-G, and AaGRP-I. There is no significant change in AaGRP-B or AaGRP-C (Figure 5.2).

![Graph](image1)

**Figure 5.1.** Female engorgement weight of ticks depleted of AaGRP-F transcripts. Weights of each female tick is plotted. The average weight is noted by the red square.

![Graph](image2)

**Figure 5.2.** Gene expression of multiple GRPs after the depletion of AaGRP-F. The depletion of AaGRP-F leads to changes in the expression of five other GRPs. GRPs AaGRP-F, AaGRP-I, and AaGRP-G are significantly reduced or not present. One gene, AaGRP-B, is not significantly changed while genes AaGRP-H and AaGRP-D are significantly upregulated.

Similar methods were used to elucidate the function of an additional GRP, AaGRP-C. The depletion of AaGRP-C transcripts was verified using qRT-PCR.
and function was monitored as feeding progressed. As seen with AaGRP-F, the depletion of AaGRP-C yielded no significant changes in attachment or engorgement weight (Figure 5.3). The expression of the other GRPs was measured to identify compensatory mechanisms (Figure 5.4). The depletion of AaGRP-C resulted in the reduction of five other GRPs (AaGRP-A, E, F, G, I). The other GRPs tested showed no significant change in expression levels.

Figure 5.3. Female engorgement weights of AaGRP-C deficient ticks. Each of the weights from fully fed females are plotted. The average of each group is indicated by a red square.

None of the knockdown ticks were collected with cement cones attached. However, it is important to note that of all the ticks fed, less than 10 cones were able to be collected making it difficult to determine if the lack of cement cones from knockdown ticks is due to a change in phenotype or just by chance none were collected. It was noticed during data analysis of all datasets (including those not shown here) that GRPs were upregulated even in non-relevant dsRNA injections such as LacZ or GFP. During RNAi, the ticks are subjected to injections and high humid heat conditions. Previous research in plants has shown
the increased presence of GRPs during wound healing. A tick GRP responsible for wound healing would explain these results. A look into the expression of GRPs during stress conditions and other known GRP functions were then performed.

![Gene expression of AaGRPs after the depletion of AaGRP-C. The depletion of AaGRP-C results in the depletion of transcripts of genes AaGRP-A, AaGRP-E, AaGRP-F, AaGRP-G, and AaGRP-I. The remaining genes show no significant change.](image)

**Figure 5.4.** Gene expression of AaGRPs after the depletion of AaGRP-C. The depletion of AaGRP-C results in the depletion of transcripts of genes AaGRP-A, AaGRP-E, AaGRP-F, AaGRP-G, and AaGRP-I. The remaining genes show no significant change.

*Prevalence of microbial community present after the depletion of a GRP*

The lack of measurable attachment/feeding difference of the GRP depleted ticks indicates that AaGRP-F and AaGRP-C may serve a pharmacological function as opposed to a structural function. In insects, GRPs play a role in reducing microbial colonization. To determine if tick GRPs may play a role in maintaining bacterial communities, the total bacterial load was calculated by measuring 16S rRNA in the tissue. In the no treatment control ticks, after 5 days of feeding, there were approximately 20 16S rRNA molecules for every 10,000 tick Ubiquitin (Figure 5.5). However, in the case of AaGRP-F deficient ticks, this increases to 400 16S rRNA molecules for every 10,000 ubiquitin. This 20-fold
increase would signify that AaGRP-F is at least partially responsible for maintaining the microbial community within the tick salivary glands (Figure 5.5). The mechanism of this role is not understood. The GRP may be acting on the bacteria directly interfering with growth or AaGRP-F could be a component of an antimicrobial pathway. In contrast to the significant bacterial growth in AaGRP-F depleted ticks, when AaGRP-C is knocked down, the change in 16S rRNA is only 2-fold (Figure 5.6). It should be noted that the AaGRP-F ticks partially fed for 5 days while the AaGRP-C ticks were partially fed for 8 days. This could have an effect on the magnitude difference between the two studies.

![Figure 5.5. Prevalence of microbial communities in AaGRP-F depleted ticks. Microbial load is normalized using Ubiquitin transcript levels. After the depletion of AaGRP-F, the total bacterial load increases 20 fold.](image)

Figure 5.6. Prevalence of microbial communities in AaGRP-C depleted ticks. Microbial load is normalized using Ubiquitin transcript levels. After the depletion of AaGRP-C 16S rRNA doubles in the tissues.

Effect of abiotic stress on GRP transcript levels

Following the same reasoning for testing the anti-microbial activity, the GRPs were monitored under various stress conditions to evaluate their similarity to plant GRPs. The ticks were exposed to low temperatures, high temperatures, and injury were used to identify stress related GRPs (Figure 5.7). The GRP expression profile after cold exposure shows the differential expression of many GRPs. The cold temperature decreases the metabolic rate of the tick which down regulates many proteins. The decreased expression of AaGRP-A (4-fold), AaGRP-H (2-fold), AaGRP-I (9-fold), and the complete depletion of AaGRP-G transcripts are likely due to this decrease in metabolic rate or the GRPs are not expressed in the unfed time stages. The temperature however does cause an increase in AaGRP-B (29-fold), AaGRP-C (2-fold), and AaGRP-D (7-fold). An increase in the temperature also affects the GRP expression profile. When the ticks are exposed to high temperatures, there is a decrease in AaGRP-A (20-fold), AaGRP-F (2-fold), and AaGRP-I (31-fold). There is an increase in many of
the other GRPs including AaGRP-B (43-fold), AaGRP-C (11-fold), AaGRP-D (23-fold), and AaGRP-H (5-fold). The GRP expression of injured ticks was also measured. These salivary glands did have a decrease in GRPs such as AaGRP-A (10-fold), AaGRP-H (11-fold), and AaGRP-I (13-fold). The expression of AaGRP-B and AaGRP-D are again upregulated as the other stress conditions however the fold change is much higher (83-, and 347-fold, respectively).

![Figure 5.7. Gene expression of GRPs in whole crushed ticks after exposure to stress. Ticks were exposed to (A) cold temperature, (B) injury, and (C) high temperature stress.](image)

There is also a slight increase in expression of AaGRP-C (3-fold). The increase of bacteria after the depletion of AaGRP-F also indicates that GRPs are important for the maintenance of the bacterial community. The GRPs overexpressed during the other stress conditions were the only genes tested
here due to small sample size. Interestingly, the GRPs which are upregulated during cold, heat, and injury are down regulated between 3-fold and 3000-fold except for AaGRP-B which is upregulated between 6- and 12-fold.

The gene expression in the salivary glands show a similar change when exposed to stress (Figure 5.8). the salivary glands of ticks exposed to the low temperature have an increase of AaGRP-B (6.6-fold), AaGRP-D (3-fold), and AaGRP-H (8-fold). There is a significant decrease in AaGRP-C (6-fold) however no change is seen in AaGRP-I. AaGRP-A did not amplify in the salivary glands, and AaGRP-E, -F, and –G were not tested due to limited sample availability. Similar results are seen in salivary glands after an injury has occurred to the tick. A decrease in AaGRP-C (8-fold) and the non-amplification of AaGRP-A and AaGRP-I show a change in the salivary glands from preparing for attachment and blood feeding to dealing with the trauma of the injury. Although the salivary glands are not directly injured by the removal of one of the hind legs, there is an increase in AaGRP-B (88-fold), AaGRP-D (34-fold), and a slight increase of AaGRP-H (2.4-fold). As the tick is feeding, it encounters a wide range of reactive oxygen species which can cause oxidative stress in the tick. The GRP expression after injection with paraquat is similar to that of cold and injured ticks. There is no amplification in AaGRP-A or AaGRP-I, a decrease in AaGRP-C (17-fold) and an increase in AaGRP-B (24-fold), AaGRP-D (8-fold), and AaGRP-H (3-fold).
Figure 5.8. Gene expression of GRPs in the salivary glands after exposure to stress. Ticks were exposed to (A) cold temperatures, (B) injury, (C) oxidative stress, and (D) pathogen infection.

Discussion

Depletion of GRPs does not result in a change in feeding phenotype

The injection of dsRNA causes an immunological reaction in the ticks against a presumed viral attack. The dsRNA is digested into siRNAs and then used as a template for the search and digestion of homologous sequences. The RNase enzymes are unable to distinguish the difference between “viral” dsRNA and tick RNA to be translated into protein. The digestion of tick RNA diminishes the transcripts available for translation thereby reducing the amount of available protein. It was hypothesized that the reduction of GRP transcripts would interfere with cement cone formation thereby making attachment difficult both at the initial
attachment and throughout the prolonged bloodmeal. This however was not the case as well as no change in bloodmeal uptake (Figure 5.1 and 5.3). While removing the ticks from the host, care was taken to ensure maximum cone retrieval; however, it was not possible to collect cones from each test condition at the same point of feeding. This is a complication of this area of research and therefore cannot be considered indicative of changes in the cement cone formation without much more investigation.

The tick genome is incredibly large and contains multiple copies of structurally and functionally similar proteins. As presented in chapter 2, these proteins are not expressed all at one time or all throughout the bloodmeal but rather during small time frames of the bloodmeal. This could be evolutionarily designed so that the tick is able to switch through all of the available genes to prevent detection from the host immune system. This becomes more complicated when applying this concept to GRPs. Because of the way GRPs are identified, it is possible (although not probable) that each of the nine GRPs chosen here have nine different functions. This would not fit into the switching hypothesis as the expression of one GRP would not compensate for another.

The GRR expression in AaGRP-F depleted ticks, shows the upregulation of three AaGRPs (AaGRP-B, AaGRP-D, and AaGRP-H, Figure 5.2). However, when these results are compared to the gene expression in ticks suffering from injury stress, the same genes are upregulated. It is unlikely that the changes in the gene expression are a response from the tick to compensate for the loss of
function from AaGRP-F but rather a response to the injection injury during the delivery of the double stranded RNA.

*Depletion of AaGRP-F causes an increase in bacterial community in the salivary glands*

There is a class of GRPs with antimicrobial activities in insects. Glycine rich antimicrobial peptides (GR-AMPs) are typically small peptides and can act on the microbes in a bacteriostatic fashion. Gloverins, an anti-microbial peptide isolated from *Hyalophora Gloveri*, contains 18% glycine and has no significant similarities to known antimicrobial peptides. Antimicrobial peptides can range from only a few dozen residues to a few hundred as gloverins and attacins.\textsuperscript{146} Based on this criterion, the AaGRPs could serve as antimicrobial peptides. Another family of glycine rich antimicrobial peptides are plasticins from South American hylid frogs.\textsuperscript{147} Plasticins are able to disrupt the membrane mimetic environments\textsuperscript{46} such as a cell membrane of evading pathogens.

The increase in 16S rRNA is used as a measure of bacterial growth in the salivary glands. However, each bacteria cell contains more than 1 copy of 16S rRNA and so the increase cannot be taken as a true increase of bacteria. In the case of AaGRP-C depleted ticks, there is an 2-fold increase of 16S rRNA molecules (Figure 5.6). This increase could be due to an increase in bacteria or to an increase of protein production in the bacteria or a mixture of both situations. The 20-fold increase of 16S rRNA in AaGRP-F depleted ticks (Figure 5.5) however cannot be attributed solely to an increase in protein production. This high of an increase must be due, at least in part, to an increase in bacteria cells.
**GRPs are differentially expressed during times of abiotic stress**

In plants, GRPs are well documented to exhibit differential expression during multiple stress conditions. In Arabidopsis, a glycine rich domain protein (AtGRDP2) which is expressed throughout plant development and when AtGRDP2 is functionally not present there is a decrease in plant growth. The over expression of the protein results in increased growth and increased tolerance to stress conditions such as increase salinity. GRPs have also been differentially expressed in *Bombyx mori* after periods of starvation, although the exact mechanism involved has not yet been identified. To explore the role of AaGRPs in the stress response, ticks were exposed to various stress conditions such as cold temperature, hot temperature, oxidative stress, injury, and pathogen invasion (Figures 5.7 and 5.8). When the gene expression of stressed salivary glands is compared to stressed whole ticks, it becomes apparent that some of the genes are differentially expressed in different tissues. This is most evident in the expression of AaGRP-C and AaGRP-H. In stressed salivary glands (Figure 5.7), AaGRP-C is down regulated in each of the conditions. However, when the whole tick is used, AaGRP-C is upregulated. It is possible that this protein is utilized by other tissues of the tick and is not necessary for feeding. Contrary to this, AaGRP-H is down regulated in cold stress and injury stress of whole ticks (Figure 5.8) but is upregulated in both of those stress conditions in the salivary glands. AaGRP-H may play a role in a cellular process in the salivary glands which is necessary for proper function but is not present to any large extent in the
other tissues. More work is required to determine the mechanism of stress mediation.
CHAPTER VI

CONCLUSIONS

Tick salivary glands are responsible for the production of the proteins which are needed for a successful feeding. The proteins are differentially expressed throughout the blood meal. It is currently unknown if these proteins are expressed sequentially as the tick progresses from one feeding stage to another or if the tick receives multiple signaling factors from the host which induce the expression of each protein. The proteins have a range of functions including maintaining a liquid pool, mediating the host immune response, and the formation of a cement cone. The formation of this cone is necessary for the proper attachment which will persist throughout the entirety of the feeding process. Recent studies of the cement cones have focused on the identification of probable cement proteins from sialotranscriptomes and the subsequent identification of that protein in the cement cone using sera from GRP vaccinated animals. This however does not prove the presence of GRPs in the cement cone just the presence of the epitope. These methods do not use pure cones but rather skin biopsies of the tick bite site and also do not look at the cone as a whole as GRPs have only been found on the cone surface.

Here we have adapted a feeding system which allows ticks to feed on animal blood using a silicone membrane instead of a live host. Other in vitro feeding methods such as capillary feeding has been unsuccessful in feeding the ticks to repletion. The use of a silicone in vitro feeding system allows for the feeding of nymphs and adult ticks to repletion. While the system does allow for
full engorgement, we have not been successful with the ovipositioning of fully fed female adult ticks. This method may not be suitable for the maintenance of a lab colony it is useful for producing partially fed and fully fed adults for further study. The attachment of the ticks to a thin silicone membrane also allows for the observation of cement cone formation. The cones can be visualized and documented on the underside of the membrane and collected without contamination from host skin or hair.

Investigation of the in vitro fed cement cones reveals some changes in surface structure which could be used to determine the curation process of the ticks. Comparison of the cones under magnification show no distinct differences in the appearance of the cones. The structures of the cones are further investigated using SEM to examine the surface topography. Using SEM, a difference can be distinguished between the two feeding types. The formation of the cone in in vivo feeding takes places within the dermal layers of the host which are likely responsible for many of the morphological changes seen here. Another aspect of cement cone research is to determine the proteins which are responsible for the cone’s composition. To do this, we utilized FTIR-ATR to determine the secondary structures found on the cone’s surface. Each of the cones contained a significant proportion of β-sheet structures. In each of the in vivo cones, a helical structure is also present. It is possible that these helical structures are due to proteins synthesized by the host skin. One final evaluation compared the proteins identified from each cone type. Cones collected from in vivo fed ticks yielded fewer identifiable tick-specific proteins than cones collected
from *in vitro* fed ticks. The presence of host skin cells on the *in vivo* cones are more abundant and the protein sequence database for mammals is more developed making them easier to identify. The study of cement cones collected from *in vitro* fed ticks is an important advancement for the proteomic study of cement cones. *In vitro* feeding systems also yields an opportunity to collect and study cement cones much earlier than *in vivo* feeding allows.

Proteomic analysis of the *in vitro* and *in vivo* fed cement cones revealed a common protein family, glycine rich proteins. A subset of GRPs were selected form a lab generated sialotranscriptome and it was hypothesized that some of these GRPs would be involved with cement cone formation. However, gene depletion of two GRPs by RNAi revealed that identifying the GRPs responsible for cement cone formation will be much more difficult than depleting a few proteins. The redundant nature of the tick genome allows for many levels of compensatory mechanisms. While the gene expression of GRPs after AaGRP-F depletion seems to reveal one such compensatory mechanism, further investigation of the GRP functions revealed that this is a response to the injection injury not to the depletion of AaGRP-F. The identification of the effect of stress on GRP expression renders routine studies impossible. An attempt was made at knocking down AaGRP-B which is upregulated during the early feeding as well as during stress. The injection of the double stranded RNA increased the transcripts to such a level that the “depleted” ticks contained more transcripts than the no treatment controls. Therefore, to determine the functions of GRPs, other methods must be developed which do not induce a stress on the tick.
The finding that GRPs are involved in the stress response of the ticks leads to the question of why these genes are over expressed during the feeding stages. Typically feeding is not considered a stress on the tick as it is a necessary process. A new hypothesis has emerged from the data presented here that individual GRPs may play multiple roles during different stages of the tick life cycle. The GRPs may function as antimicrobial or serve any of the other necessary functions for blood feeding when a tick is attached to the host but during times of molting, fasting, or overwintering the same GRPs may be repurposed for stress mediation. More work is required to fully elucidate the functions of the proteins, and recombinant protein expression will be key to that process.
### APPENDIX A

### PRIMER LIST

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<td>Reverse Primer 5'→3'</td>
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<td>Immunity Related Proteins</td>
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REFERENCES

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