Spring 5-2016

Painless Hematophagy: The Functional Role of Novel Tick Metalloproteases in Pain Suppression

Joseph W. Jelinski
University of Southern Mississippi

The University of Southern Mississippi

Painless Hematophagy: The Functional Role of Novel Tick Metalloproteases in Pain Suppression

by

Joseph Jelinski

A Thesis
Submitted to the Honors College of
The University of Southern Mississippi
in Partial Fulfillment
of the Requirement for the Degree of
Bachelor of Science
in the Department of Biological Sciences

May 2016

Approved by:

Shahid Karim, Ph.D., Thesis Advisor
Associate Professor of Biology

Shiao Wang, Ph.D., Interim Chair
Department of Biological Sciences

Ellen Weinauer, Ph.D., Dean
Honors College

Abstract

Ticks secrete a plethora of pharmacologically active molecules in their saliva while feeding. These allow the tick to feed upon a host over prolonged periods of time in an itch free and painless attachment. The exact mechanism of pain suppression by the tick has barely been investigated. In this study, two angiotensin converting enzymes (ACEs), members of the metalloprotease family, are identified as potentially responsible for the degradation of pro-inflammatory peptides. It is hypothesized that these tick ACEs block the function of bradykinin through degradation of the peptide, contributing to the tick's ability to conduct a pain and itch-free host attachment for prolonged periods of time. To elucidate the functions of the target tick molecules, the time dependent and tissue specific transcriptional gene expression has been investigated by qRT-PCR. Furthermore, an RNAi knockdown approach was used to assess the ability of saliva to degrade bradykinin in an *in vitro* assay. To measure degradation in ACE deficient saliva, HPLC was used to monitor bradykinin breakdown. Upon knockdown of the target genes, the ticks showed significantly reduced blood intake and the vertebrate host became highly agitated. It was observed through a HPLC of the saliva that the knockdown produced a markedly different pattern of degradation. This data, both quantitative and qualitative, shows that these genes have functional roles for bradykinin degradation and tick feeding. Therefore, these highly conserved genes are attractive targets for tick control measures, or as a novel source of analgesics for medical application.

Key Words: Pain response, Metalloprotease, Angiotensin-Converting Enzymes, Bradykinin, Amblyomma Maculatum

Dedication

Jaclyn Williams, Rebekah Bullard, Virginia Meyers,

Gary Crispell, Khem Budachetri, and Deepak Kumar,

I could not have asked for better graduate advisors and lab partners.

I will never forget the way you lent a helping hand every time I stumbled.

Acknowledgements

As I graduate, I would like to give my warmest thanks to my thesis advisor, Dr. Shahid Karim, for his constant efforts to push me into becoming a better scientist. This experiment would never have been possible without his vision and indomitable spirit. Thank you for the opportunity, and for so much more.

Also, I want to give my appreciation to the faculty at the Honor's College. They believed in my efforts every step of the way, and showed me doors of opportunity I would never have seen by myself. Without their counseling I would not be where I am today.

Table of Contents

List of Figures

Chapter 1: Problem Statement

In my experiment I will study two recently identified metalloproteases in the saliva of the Gulf Coast tick, *Amblyomma maculatum*.[1] These ticks are an emerging problem in the United States, as they are competent vectors for a variety of pathogens, including *Rickettsia parkeri, Ehrlichia ruminantium*, and *Leptospirosis Pomona*.[2] These are transmitted when the tick feeds off of its host, usually mammals or ground birds. However, in most cases the tick needs to be attached to the host for several hours before the pathogen is transmitted. How does the tick feed for that long without the host noticing and removing it? Normally, when being cut or burned, a short peptide signal called bradykinin is released into the injured area. This hormone binds to the B1 and B2 nociceptors on nerve cells, triggering inflammation and creating the sensation of pain.[3]

Ticks secrete a variety of pharmacologically active factors in their saliva, most of which have effects to circumvent the host immune system. Their effects range from preventing platelet clotting to disrupting immune response. An additional proposed function of the saliva is to degrade pro-inflammatory peptides involved in the host's pain response, preventing the host organism from becoming aware of the tick. In a recent experiment, two new metalloproteases were identified in tick sialotranscriptome projects, angiotensin converting enzymes (ACE) in *A. maculatum*. Metalloproteases are so named because their hydrolysis mechanism utilizes a divalent metal cation, usually zinc, to activate the water molecules. As of now, only three families of metalloproteases have been identified in ticks: Angiotensin-converting enzymes (ACE), Reprolysin/ADAM, and Neprolysins. One of the proposed functions of metalloproteases in invertebrates is to prevent coagulation. This is done by inhibiting platelet aggregation and integrin dependent cell adhesion by interacting with the

beta1 and beta3 integrin family receptors.[3] Neprolysins, such as dipeptidyl peptidase, have been reported to destroy the pain producing peptide bradykinin, and knockdown of neprolysins in mammals has been shown to cause heightened sensitivity to pain.[4] As neprolysins have a similar proposed function to angiotensin-converting enzymes, I hypothesized that these tick metalloproteases block the function of bradykinin through degradation, contributing to the tick's ability to conduct a pain and itch-free attachment of the host for an extended period of time. To elucidate the functions of the target tick molecules, the two genes in question will be knocked down via RNAi. The ticks will then be evaluated for their ability to feed and for their saliva to degrade bradykinin, one of the pro-inflammatory peptides associated with pain.

Chapter 2: Literature Review

Interspecies Relationships

All over the world, groups of organisms have evolved together over thousands of years. In this time, many species begin to form relationships, or show symbiosis. This symbiosis is used to affect a variety of factors, such as shelter, nutrient intake, or pest relief. In many participants, their symbiotic mechanism has become so important that it has reshaped every aspect of their physiology and life cycle. There are three primary forms of symbiotic relationship. The first is mutualism, so called because the relationship provides a mutual benefit between both organisms involved. A classic example is mycorrhizae, a fungus that affects the roots of many plant species. In this case, the fungus receives the products of the plant's photosynthesis, such as sugar, while the plant enjoys a massively increased root surface area, allowing it to grow much more effectively. The second relationship is called commensalism. This occurs when one organism benefits, though the other is unaffected. This is the hardest to prove, because often the other organism will be determined to experience some slight advantage or drawback. The third relationship is parasitism, which occurs when one organism, the parasite, benefits while the other, the host, is harmed. It is important to note that, unlike predation, parasitism does not often result in the death of the host. This is because the parasite requires something from the living host, usually a resource for their metabolism. This class of relationship, parasitism, is where my research takes place.

There are two kinds of parasite. The first is the endoparasite, or the parasite that lives inside the host while benefiting from it. This group can include many large organisms, including the well-known tapeworm. It also includes non-Animalia parasites, such as the fungal ring-worm, and even encompasses pathogenic bacteria, viruses, and other microbes.

These organisms will usually live their entire lives inside of the host, entering as eggs and exiting only at the point of death. Often, their entire physiology is formed around infecting their host species, which is usually highly specific. The second type of parasite is the ectoparasite. This type of parasite is free living, and never truly enters the host's body. They often act by finding and tracking a potential host, then feeding on the host over long periods of time. This attachment is to obtain a metabolic requirement from the host, usually through draining its blood. Because of their free living nature, the ectoparasites are far less host specific, and can afflict far more species.

Because of their ability to feed off of multiple hosts in a single life cycle, ectoparasites are often crucial participants in the life cycles of endoparasites. This is especially true for pathogenic bacteria and microbes, which are transmitted from host to host through an ectoparasite vector. In this situation, the ectoparasite shuttle is called a vector for the disease. Arguably the most important vector for disease is the mosquito, which can carry a large number of deadly pathogens, including malaria, yellow fever, and West Nile Virus. Second to the mosquito, ticks are primary transmitters of disease causing agents, and are capable of transmitting a greater variety of pathogens than mosquitos.[5]

There are two primary species of tick in the United States Gulf Coast region, *Amblyomma americanum*, the Lone Star Tick, and *Amblyomma maculatum*, the Gulf Coast Tick, which my experiment focuses on. These prevalent arachnid parasites constantly feed off a wide variety of hosts. The white-tailed deer is the primary host of adult ticks, though other species, such as squirrels and foxes, also serve as hosts.[6] Cotton rats and northern bobwhite quail serve as important vertebrate hosts for immature stages of *A. maculatum*.[7] In Mississippi, mammals such as raccoons, opossums, cottontail rabbits, and white-tail deer have been shown to have antibodies to spotted fever group rickettsia. This is evidence of their exposure to the

pathogen and, therefore, tick parasitism.[7] In attaching to cattle, they cause serious damages through bites and blood loss. Despite modern pesticide methods, management and treatment of major tick borne disease (TBD) agents (babesioses, theillerioses, anaplasmoses and Heartwater) continue to be a major source of significant monetary loss to the livestock industry.[8] However, the parasitic activity will often transfer into attachment of humans, and the ticks can carry many zoonotic pathogens.

Zoonotic pathogens are diseases which reside in animal reservoir species and may spill into human populations, and are emerging at an unprecedented rate. Several of the most rapidly emerging vector-borne zoonotic pathogens in the US are transmitted by the Gulf Coast Tick, *Amblyomma maculatum* Ticks will acquire the pathogens by 2 primary modes of transmission: vertical transmission, where the pathogen is passed from female ticks to their eggs, and horizontal transmission, where the pathogen is acquired by feeding on an already infected animal host.[6] These diseases include *Rickettsia parkeri, Hepatozoon americanum, Leptospira pomona, Ehrlichia ruminantium,* and tick paralysis.[2]

Diseases Carried by A.maculatum

Rickettsiosis is caused by *Rickettsia parkeri*, a member of spotted fever group rickettsiae (SFGR). This recently recognized tick borne disease was first reported in a human in 2004.[7] This is surprising, as *R. parkeri* was first described in the US in 1939, when it was isolated from *A. maculatum* in Texas.[9] Therefore, it was observed solely in livestock and other mammals for the past 70 years. *Rickettsia parkeri* is an alpha-proteobacteria of Rickettsiaceae. Like other SFGR, it requires a host, either vertebrate or invertebrate, for both proliferation and survival.[7] *A.maculatum* is a known carrier of rickettsial species, which pass from the tick's midguts to the salivary glands, and then into the host via the tick saliva.[10] A recent study reported 28% of

unfed *A. maculatum* ticks from Florida and Mississippi to be infected with *R.parkeri*, representing a range of 11% - 40% in the individual counties sampled.[7]

Hepatozoon americanum is the agent of American canine hepatozoonosis throughout the southern United States, and is primarily carried by *A. maculatum*. This disease is exhibited through leukocytosis and periosteal bone proliferation.[11] Interesting, dogs can acquire the infection by consuming and ingesting either nymphal or adult *A. maculatum* ticks that had been infected by feeding from a previous host. In addition, this disease is puzzling, as canines are not the favored hosts of the Gulf Coast tick.[11] However, examination of the overlapped ranges of the tick and the disease show that the disease distribution mirrors that of *A. maculatum* throughout the southern states.[11]

Leptospira pomona is the cause of leptospirosis in livestock, and it has been shown that the Gulf Coast tick is capable of transmitting this disease.[12] Leptospirosis is a bacterial disease capable of affecting humans and animals. Although some infected individuals are asymptomatic carriers of the disease, the disease can lead to kidney damage, meningitis, liver failure, respiratory distress, and even death.[13] In 1957, Burgdorfer was able to demonstrate the ability of *A. maculatum* to transmit *Leptospira Pomona* by feeding the ticks concentrated infected blood, then feeding the ticks upon uninfected guinea pigs. The guinea pigs then became ill with the infection, proof of transmission.[12] However, the role of Gulf Coast ticks in a natural transmission of the disease is still unknown.[12]

Ehrlichia ruminantium, formerly known as *Cowdria ruminantium*, is the causative agent of Heartwater disease in ruminants. Heartwater is an economically important disease of Africa and the Caribbean with potentially fatal effects.[14] This pathogen infects multiple ruminants, including cattle, deer, and sheep, and is transmitted by *Amblyomma* tick species.[14] Although Heartwater has not yet been observed in the United States, the US contains multiple target

species, and *Amblyomma maculatum* has been determined as a strong potential vector for *Ehrlichia ruminantium*, which could quickly spread the disease throughout the Southeastern US.[15]

Tick paralysis is a rare effect caused by toxins released within the tick saliva during feeding.[16] This paralysis has been observed in both humans and dogs. While victims will usually recover within 24-48 hours of tick removal and detachment, the tick is not always detected.[16] This prolonged tick attachment and symptom progression can lead to flaccid paraplegia and arreflexia, eventually progressing to the respiratory system and even death.[16]

Nearly all tick borne disease agents require successful tick feeding to be transmitted. So, avoidance of tick bites through suppression of tick vector populations is the method of choice.[17]

Parasite Attachment

Parasites can attach through a number of ways. Endoparasites, such as the tapeworm, are ingested as eggs, then use a large number of hooks and suction cups to attach to the host's intestinal tract. Ectoparasites, however, will bite through the outside skin of the host in order to access the blood. The feeding of the ectoparasite is commonly for one of two purposes. The first is that the blood meal provides the energy required for the tick to molt or metamorphose into the next stage of its life. The second is that the female must take in a massive amount of blood for the energy required to generate thousands of viable eggs. However, this simple method can exhibit wide variation. For example, mosquitos will only rest on the host for a few moments before beginning to feed, and only a small amount of their mouthparts actually penetrate the host. This "flying syringe" method allows for the mosquito to feed quickly and escape. On the other hand, tick feeding involves multiple behavioral

changes that begin with a period of starvation and end with satiation. There are multiple phases of this feeding behavior: (1) attainment of appetence, where they acquire a desire to attach for nutrients; (2) engagement with the host, find a way to land or crawl upon the host's body unnoticed; (3) exploration of the host for a suitable feeding site, usually to find a point on the host's skin that is thin to allow easy access to the host's capillaries; (4) penetration and(5) attachment onto host skin and establishing the feeding site by biting into the host and using their specialized mouthparts to anchor themselves; (6) ingestion of host blood, causing (7) engorgement.[18] Once a feeding location is found, the tick will burrow its head into the host's skin, creating a blood pit, where the tick stays entrenched over the course of several days while it feeds. When completed, the tick will (8) detach from the host by pulling out of the anchored feeding site, and (9) disengage from the host and drop off.[18]

While ticks do feed for long periods of time, the feeding is not uniform throughout. Adult ticks feed for 5 to 12 days. For the first 24 hours, there is little increase in the weight of the tick. The period of slow feeding varies with the species of tick, but it will usually last a few days. In the days following, though, the tick will slowly gain in weight, followed by a dramatic amount of uptake in the final 24 hours.[5] Within this timeframe, the female ticks will initiate a stage known as fast feeding. This causes the ticks to engorge, the females swelling with ingested blood to over 100 times their original weight.[5] Interestingly, transmission of pathogens, such as Lyme disease, is observed primarily in this stage of feeding, as opposed to the initiation of feeding.[5] What this means in actual volume can vary with tick species. The adult brown dog tick will feed to an average of 0.55 ml, while the adult Lone Star tick feeds until about 0.81 ml blood is collected. In nymphal ticks, the females gain nearly twice the weight of the males.[19] Although the brown dog tick takes in less blood than the other ticks, it has been shown that several hundred ticks per dog, which is not uncommon, can cause death

via blood loss.[19] It is only after fast feeding is completed that the females have the requisite energy stores to generate eggs, and so the females will not detach from the host until this stage is complete, potentially causing great harm to the host through both exsanguination and disease transfer .

Overcoming Host Defense

The host organism has a complex system of defenses against internal invaders. Leukocytes capture and digest bacterial cells, rendering them inert. Large invaders are encased in a cyst, isolated from the rest of the system. When attacked by an ectoparasite, the host body does secrete immune agents, such as antibodies, antigen-presenting cells, and T lymphocytes. These, though usually unable to halt attachment completely, do inhibit the tick's feeding. After being targeted multiple times, the host's immune system may combat the tick, resulting in reduced ingestion, decreased weight, prolonged feeding, reduced egg production, molting inhibition, and death. However, ticks are usually able to evade these defenses, and, in order to halt their feeding completely, the host body relies on the physical removal of the attacker.[5]

For this, one simple thing is required, localized pain. Normally, when the skin is damaged, such as by a cut or a burn, injured cells and recruited inflammatory cells release multiple inflammatory mediators, including cytokines, growth factors, chemokines, prostanoids, amines, purines, protons, and kinins. These mediators reduce the threshold of nociceptor peripheral terminals, a phenomenon known as peripheral sensitization.[3] In addition, the precursor molecule known as kininogen is broken down by protease kallikreins and kininases, releasing kinins, such as bradykinin, into the injured area.[3] This alerts the brain to the site of the injury, and so further steps can be taken to rectify the problem and treat the wound.

Ticks imbibing the blood must be able to circumvent the natural defenses of their host organism. This is done through the secretion of their saliva, which has a complex composition and hundreds of proteins that are expressed throughout feeding. Proteins are large molecules made up of hundreds of amino acids, and their structure dictates their ability to perform highly specific tasks. Saliva of some ticks includes anticoagulants, antihistamines, and prostaglandins, which may facilitate feeding.[5] The saliva also includes antihemostatic, anti-inflammatory, and immunosuppressive properties.[20] In tick saliva, the roles of these bioactive molecules includes preventing clotting by platelets and halting innate immune responses. This confirms the similar traits that the tick has with other blood feeders, such as tsetse flies and mosquitos.[20]

Platelet aggregation provides the main hemostatic obstacle to hematophagous organisms, because the duration of blood secretion by damaged capillaries depends mainly upon platelet function. The individual platelet molecules are capable of binding together, or aggregating, and the binding of hundreds of molecules forms a platelet plug. This, as the name suggests, works as a physical obstacle at the wound site, preventing further blood loss. Important stimuli that induce platelet aggregation include ADP (released by injured cells), collagen fibrils (exposed in subendothelial tissues), thrombin (produced post coagulation cascade), and PAF (the platelet aggregating factor released by leukocytes). Platelet derived factors will also contribute to thrombin formation and clotting, providing rigidity to the platelet plug.[20]

A proposed function of the saliva addressed in this research relies on a secretory protein that may degrade pro-inflammatory peptides, such as bradykinin, which are involved in the host's pain response. This degradation acts to prevent the host organism becoming aware of the tick's presence. In this experiment, two metalloproteases will be investigated. Proteases are proteins that break down other structures composed of amino acids, such as

peptides or other proteins through a process called hydrolysis. Hydrolysis is a standard reaction in which a molecule is divided concurrently with an H_2O molecule, adding H and OH to the split ends. Metalloproteases are so named because their hydrolysis utilizes a divalent metal cation, usually zinc^{2+}, to activate the water molecules. As of now, the only families of metalloproteases identified in ticks are Angiotensin-converting enzymes (ACE), Reprolysin/ADAM, and Neprolysins.

Bradykinin Activation and Degradation

Kinins are small peptide hormones that mediate a variety of cardiovascular effects, such as vasodilation, release of endothelial autacoids, natriuresis, myocardial preconditioning, and inhibition of cell proliferation.[21] These actions have acquired therapeutic significance due to the development of angiotensin I-converting enzyme (ACE) inhibitors, hypertension medications, that prevent kinin degradation by ACE, therefore increasing the total amount of kinins in the body.[21] Bradykinin is a short peptide hormone, a chemical signal between cells, that is made up of only nine amino acids. Among more than a dozen host agents involved in inflammation, bradykinin plays at least two important roles: (1) it is a mediator of pain and (2) it increases capillary permeability, leading to edema (an excess of watery fluid in tissue). Both reactions are associated with tick rejection. Pain leads to increased grooming of the host, leading to the tick to be detected and removed.[22] Bradykinin is formed in vertebrate tissues after the activation of coagulation factor XII, which is a large molecule normally found only in the intravascular compartment. Upon vascular injury and contact with sub-endothelial components, factor XII is activated and initiates the clotting pathway by activating fXI. Activated factor XII also activates prekallikrein to kallikrein, which then acts on kininogen to release bradykinin.[22] The bradykinin peptide acts on constantly expressed G-protein-coupled

receptor (GPCRs) B2 and injury-induced receptor B1. These receptors are expressed on primary sensory neuron peripheral terminals, and contribute to peripheral sensitization.[3] This triggers inflammation to increase transport of healing factors to the injured region and also provides the sensation of pain. This has been confirmed by injection of intrathecal bradykinin, producing mechanical and thermal hyperalgaesia, or pain sensitivity, that peaks at 75 minutes, and slowly decreases over 4 hours.[3]

Two main enzymes destroy bradykinin in vertebrates: kininase I, a plasma carboxypeptidase with specificity to basic terminal amino acids, and kininase II, which is mainly found in tissues. Kininase II was actually shown to be identical with angiotensin-converting enzymes (ACE) in later experiments.[22] ACE is a metalloenzyme that acts by removing two amino acids at a time from various peptides.[22] In human blood plasma, bradykinin is initially cleaved at the 7–8 and 8–9 amino acid positions by the actions of the two major kininases ACE and CPN. These two enzymes are almost exclusively responsible for bradykinin degradation in human plasma, and their individual contributions have recently been determined .[21]

ACE's primary action is to generate the physiologically active peptide Ang II by cleaving the c-terminal dipeptide His-Leu from Ang I.[23] The binding of Ang II to the Ang II type 1 receptor mediates regional blood flow and other processes. It may also be involved in platelet activation and aggregation.[24] *In vitro*, ACE2 cleaves Ang I to form Ang (1-9) and Ang II to form Ang (1-7), which has effects opposing those of Ang II.[24] ACE also cleaves other peptides, such as bradykinin.[23] Past studies have demonstrated that ACE represents more than half of the total kininase activity of rats. Additional kininase activity involves the enzymes APP and CPN, which also contribute significantly to kinin hydrolysis. The total kinin degradation activity of past experiments corresponds to a half-life of 31s, calculated for 10 μM BK in

undiluted plasma. The relative contribution of ACE is consistent with earlier findings that attributed 46.8 and 50% of rat plasma kininase activity to this enzyme.[21]

There are two forms of ACE in humans: the somatic, or body, ACE and another form located in the testes. Somatic ACE is composed of two highly homologous domains (N and C domains), both of which contain the Zn^{2+} binding motif HExxH found in other Zn^{2+} metalloproteases. Both domains bind the Zn^{2+} and are catalytically active.[23] The majority of the protein is extracellular, connected to a short intracellular C-terminal tail by a hydrophobic sequence that bridges the plasma membrane.[23] ACE secreted from insects, such as Drosophila, lack the hydrophobic C-terminal region, allowing them to be soluble in the blood stream.[23]

One of the functions of metalloproteases in invertebrates is to prevent coagulation. This is done by inhibiting both the clumping of platelets and the integrin dependent cell adhesion by interacting with the beta1 and beta3 integrin family receptors. Neprolysins, such as dipeptidyl peptidase, are zinc metallopeptidases with a single HEXXH active-site motif with a preference for cleaving on the N-terminal side of hydrophobic residues, with primarily endopeptidase activities.[24] This has been reported to destroy the pain producing peptide bradykinin, and knockdown of neprolysins in mammals has been shown to cause heightened sensitivity to pain.[4] I hypothesized that two tick genes, Am 23738 and Am 930, code for salivary metalloproteases whose purpose is to defeat the host's attempts to signal danger through a pain response, contributing to the tick's ability to feed without fear of interference from an agitated host. If these metalloproteases do have significant function in the degradation of bradykinin, then this could open up a new resource for pharmaceutical painkillers, and have a wide variety of future applications, such as vaccinations against ticks, allowing the host to be aware of the tick bites.

My hypothesis was tested by the knockdown of the two tick metalloprotease genes. The genes to be knocked down depended on their ability to be amplified via PCR, which is crucial for the formation of the double stranded RNA for the knockdown. The efficacy of the knockdown was evaluated in two methods. The first is the production rate of proteins at different predetermined time points in the tick feeding. This information, gathered via qRT-PCR, was compared against the qRT-PCR data of normal type *A. maculatum* salivary glands. This determined whether or not the knockdown of the genes was a success. The change in the ability of the saliva to degrade bradykinin was assessed via HPLC. This method measured the production and rate of the degraded bradykinin fragments by exposure to the tick saliva. This was compared to the bradykinin degradation from control tick saliva to determine whether the knockdown was successful in affecting the tick's ability to degrade bradykinin. Overall, the knockdown tick salivary glands were examined to determine protein expression, whether the ability to degrade bradykinin was affected, and then to further elucidate the roles these metalloproteases play in the tick saliva.

Chapter 3: Experimental Method

Source of ticks

Amblyomma maculatum ticks were purchased from the Oklahoma State University's tick rearing facility. Prior to infestation on the sheep, all adult unfed ticks were kept at 24 – 26°C, 90% RH, for a 14/10 light/dark cycle. All ticks were fed on sheep and then removed at differing time points from the host, in accordance with the IACUC approved protocol at The University of Southern Mississippi. Replete ticks were collected as they detached on their own over multiple days, until day 14 of feeding, when all remaining ticks were collected.

Saliva Collection

Ticks were placed dorsal side down on a strip of tape. Capillary tubes were placed over their mouthparts to collect saliva, and roughly 1% pilocarpine solution was injected into the ticks' abdomens to induce salivation. The saliva collected in this method is the substrate upon which future tests will be based.

Transcriptional Gene Expression

Total RNA was extracted from the tick saliva for time points. Total RNA of the saliva was reverse transcribed to cDNA using the premade iScript kit from BioRad, CA, USA, followed by qRT-PCR analysis for genes Am 930 and Am23738. Am 930 and Am23738 analysis used actin as the normalizing gene.

RNA Extractions, cDNA Synthesis, and qRT-PCR for Differential Expression

Using the RNAspin Mini RNA isolation kit (GE, Healthcare, NJ, USA), total RNA was purified from pooled salivary glands and midguts from partially fed adult female *A. maculatum*, with slight modifications made for the tick RNA extractions. The total RNA was then analyzed with the Nanodrop spectrophotometer and stored at -80ºC. The total RNA is reverse transcribed according to the manufacturer's protocol (Invitrogen) to form hour specific cDNA for each gene in question. PCR amplification was performed using gene specific primers purchased for the purpose. The PCR product was then analyzed on a 2% Agarose electrophoresis gel and visualized using a GelDoc system (Bio-Rad). The positive samples were PCR purified and submitted to Eurofins MWG Operon for sequencing using gene specific primers. Once successful sequencing was obtained, sequences were searched for in multiple databases, including NCBI BLAST and Expasy. Approximately 2,000 ng of total RNA were reverse transcribed using concentrations of obtained cDNA used for qRT-PCR gene expression in approximately 25ng/ul and 150 nM concentrations for gene specific primers for each reaction. All samples were run in triplicate with a non-template control (NTC). The C1000 Thermal Cycler for qRT-PCR was run under conditions: 10 mins at 95C followed by 35 cycles of 15 seconds at 95, 30 sec at 60C, and 30 sec at 72C with a fluorescence read at the end of this 30 second step.

These steps isolate the RNA coding sequences present at the time of saliva collection in the salivary glands and midguts of the tick. These tissues are examined because the salivary glands produce the bulk of the salivary proteins, while the midguts are a reservoir for diseases and other factors that can exit with the saliva. These RNA sequences signal for the proteins that the DNA is coding for at that moment. RNA is only produced from the portions of the DNA that is active at that point in time. Because of this, the RNA can be reverse transcribed

to form the cDNA of that time point. This molecule, formed from the RNA, represents all of the coding sequences of DNA at that time. The greater the amount of RNA coded for, the greater that gene is represented in the cDNA. This allows qRT-PCR to be used to analyze the production of different proteins at specific timepoints by analyzing RNA production at specific timepoints. The genes to be measured via qRT-PCR are dependent upon gene specific primers, which will allow production of only the RNA of the selected gene.

Reverse Phase HPLC

RP-HPLC was performed using a CM400 pump and SM4100 dual-wavelength detector set at 220 nm from ThermoSeparation Products. A 15 cm x 2.1 mm Macrosphere octadecyl column was perfused with (0.25 ml/min) with the indicated mixture of acetonitrile and water containing TFA (0.05% v/v). Kininase assays were analyzed using 10mM Hepes, 150mM NaCl, and 20 uM bradykinin. The reaction vial was 150 ml and incubated for 5 minutes at 37^{o}C. Reaction was started by adding 2-5 ul of *A. maculatum* saliva.

This reaction is used to determine the ability of the saliva to degrade bradykinin. This is done by adding the tick saliva to the solution containing bradykinin. The HPLC instrument measures the amount of time it takes for molecules to move through the column, or elute. The rate of elution depends upon the size and charge of the molecules present. This is used to determine if the bradykinin is actually degraded by comparing the amount and times of molecule dilution with and without the addition of the tick saliva.

Double-stranded RNA Formation and RNAi Gene Knockdown

Gene sequences for Am 930 and Am 23738 were amplified using PCR amplification. The PCR products were examined via 2% Agarose gel electrophoresis. The PCR products were sent off for sequencing, to ensure that the desired products were being produced. Once the product had been identified as the proper product, it was again amplified through PCR, using a gene-specific primer that adds a T7 viral tag to the ends of the product. These tags anneal between the PCR products, ligating the strands together to form double-stranded RNA products. These were injected into live ticks for RNAi knockdown of the genes. Mutated and control ticks were fed upon sheep hosts before being removed at pre-determined time points, in accordance with IACUC protocol. Mutated and non-mutated ticks were weighed to assess difference in blood meal uptake, and their saliva was collected and examined once again through the steps described above to ensure successful RNAi gene knockdown.

The purpose of this step is to form the double-stranded RNA of the specific tick genes. This was injected into the tick, causing the tick cells to break down not only the double stranded RNA, but also the matching single stranded RNA. This effectively removes the RNA of the specified gene from the tick, causing the proteins corresponding to those RNA to never be produced. As these genes code for the tick metalloproteases, it halts production of those metalloproteases, hypothetically causing the tick to be unable to break down the bradykinin. The repeated tests with the new mutated tick salivary glands make it known whether the genes were successfully knocked down, and effect on the tick saliva's ability to break down bradykinin. If both the gene is successfully knocked down and the saliva becomes unable to degrade bradykinin, then the function of the proteins in question will be positively determined, allowing future experiments to take place.

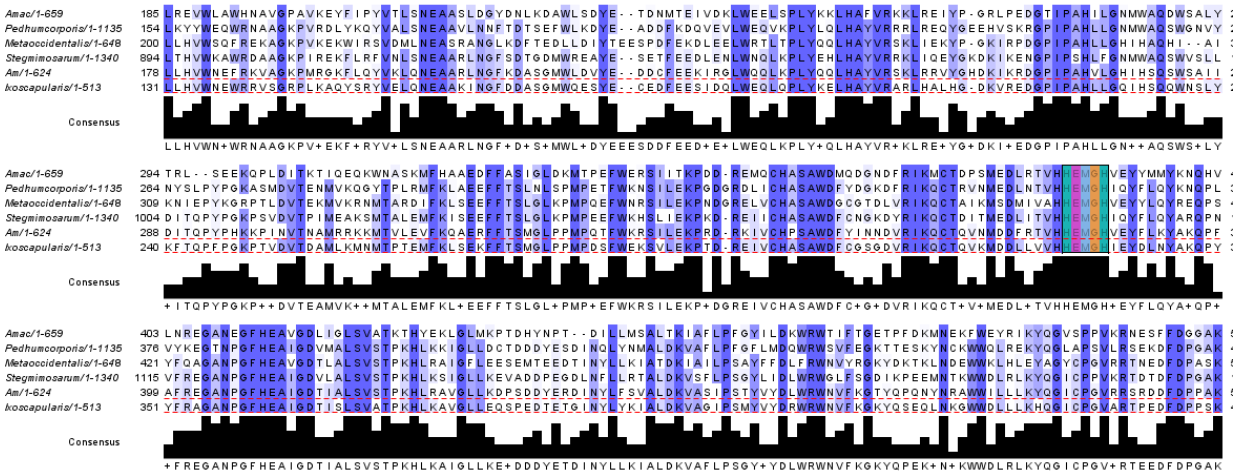
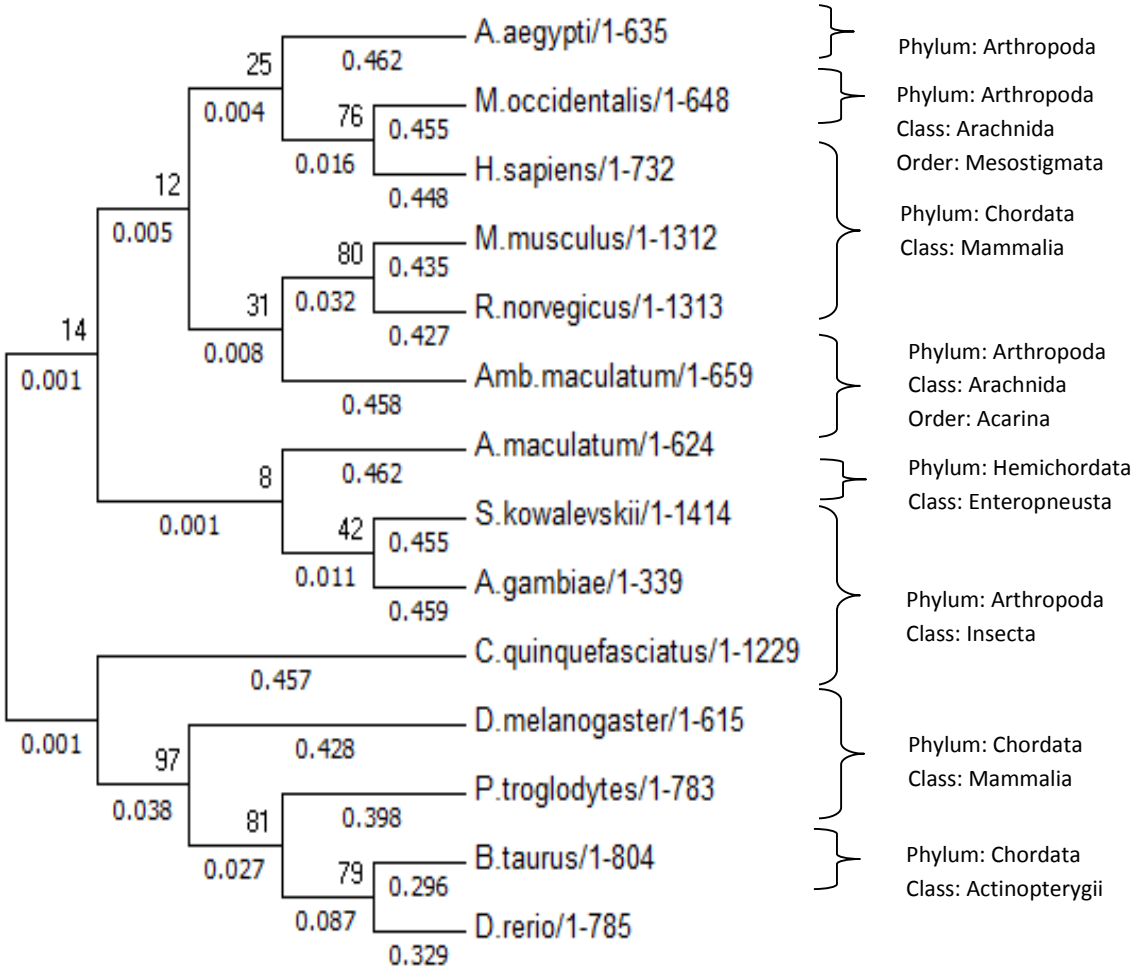


Figure 1-Bioinformatics Analysis

Bioinformatics Analysis

Sequences of *A. maculatum 23738* and *930* proteins were run through the BLAST program to find arthropods with generally homologous sequences. Then, using corresponding arthropods and insects (*Ixodes scapularis, Metaseiulus occidentalis, Stegodyphus mimosarum,* and *Pediculus humanus corpus),* a multiple sequence alignment was made using Clustal and Jalview programs to determine sequence conservation of the Angiotensin-Converting Enzyme. *S. mimosarium* had sequence identity of 54%, *I. scapularis* of 63%, *M. occidentalis* of 54%, and *P. humanus corpus* of 49%. Highlighted sequences of pink, light blue, orange, and green show the zinc-binding (active sites) of the enzyme. This shows overall sequence homology for the ACE amongst the arthropod and insect species.



F

Phylogenetic Tree

Sequences for A. maculatum genes 930 and 738 were compared through BLAST, and were compared to multiple metalloprotease sequences across multiple clades with Mega6 to find create a phylogenetic tree of 14 proteins. Of the sequences examined, the two *Amblyomma maculatum* metalloproteases were found to be most closely related to angiotensin converting enzymes from *S. kowalevskii, A. gambiae*, *R. norvegicus*, and *M. musculus*. Bootstrap values greater than 70 are accepted as reliable data. Branch length reveals evolutionary closeness, showing that the A. maculatum proteins, though on the same branch, are not closely related to nearby proteins.

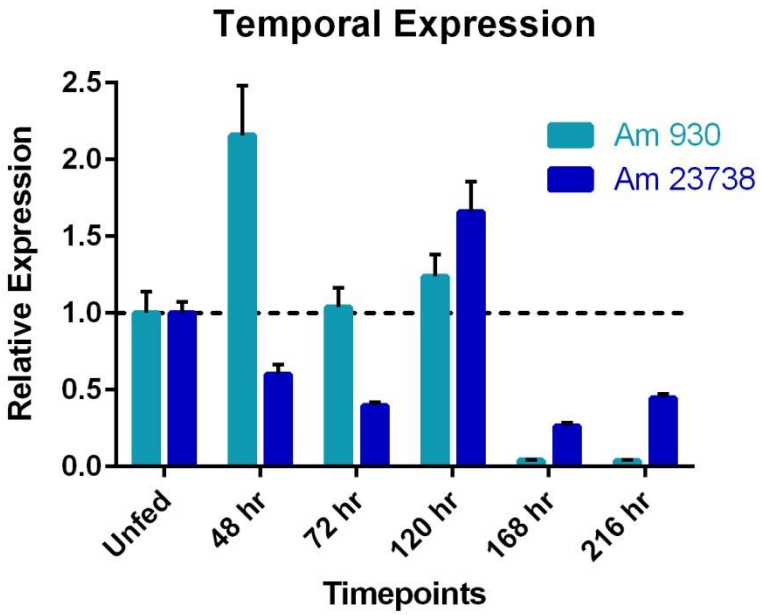


Figure 3A: Temporal Gene Expression

Temporal Gene Expression

cDNA was extracted from the salivary glands from unfed wild-type ticks, as well as ticks partially fed at 48hr, 72hr, 120hr, 168hr, and 216hr. Actin was used as the normalizing gene, and unfed tick samples were used as control samples. The expression of *Am 930* peaks at 48hr before it drops in expression for 72 and 120hr. After 120hr, *Am 930* expression drops to negligible levels. *Am 23738* expression, except for a 1.66 peak at 120hr, remains below 0.6 relative expression throughout feeding.

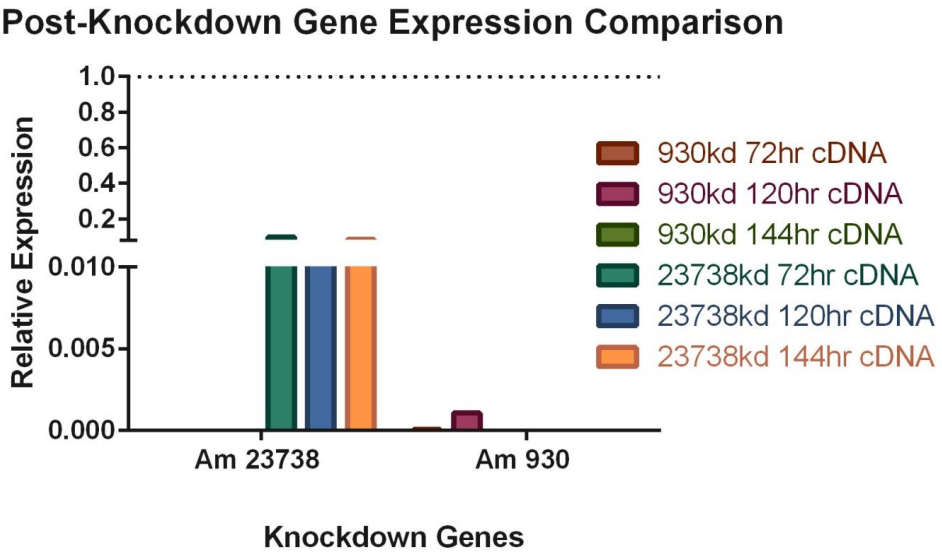


Figure 3B: Expression of Knockdown Genes

Expression of Knockdown Genes

cDNA was made from salivary glands extracted from *A. maculatum* ticks knocked down for *Am 930*, *Am 23738*, or *LacZ* for a control. Actin was used as the normalizing gene. Salivary glands were extracted at the 72hr, 120hr, and 144hr timepoints after the ticks were applied to host sheep. No expression of *Am 930* was detected in *Am 930* knocked down ticks at 144hr, and a maximum of 0.001 expression at 120hrs. Small amounts of *Am 23738* expression, between 0.047 and 0.099, was detected in *Am 23738* knocked down ticks at every time point. Expressions were compared to *LacZ* controls to find that, overall, a significant knockdown of expression was observed in both tick genes.

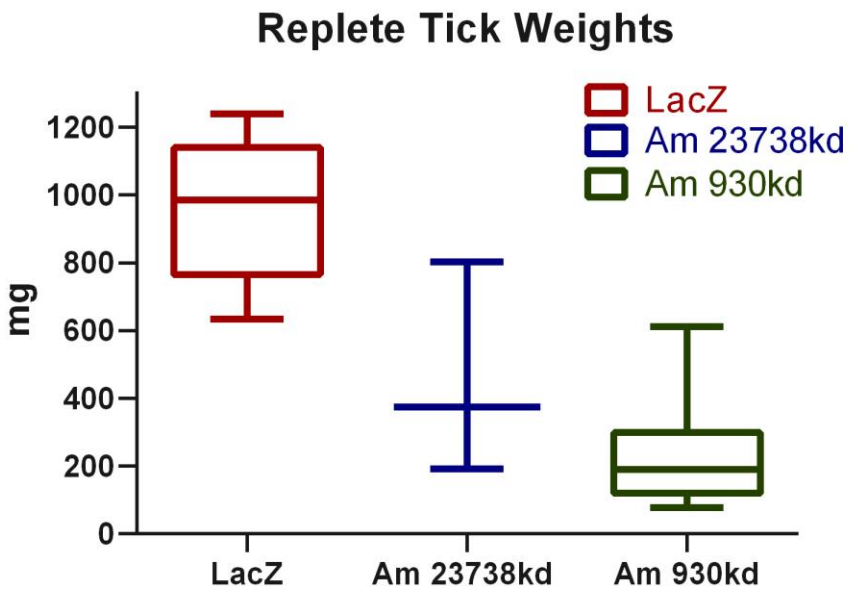


Figure 4: Replete Tick Weights

Replete Tick Weights

Ticks from each group were allowed to feed to repletion, and their final weights were measured. *LacZ* control ticks weighed an average of 959mg while Am 23738kd ticks weighed 456mg, and *Am 930* ticks showed the smallest replete weights, with an average of 241mg. LacZ controls had n = 7, Am 23738kd had n = 3, and Am 930kd had n = 9.

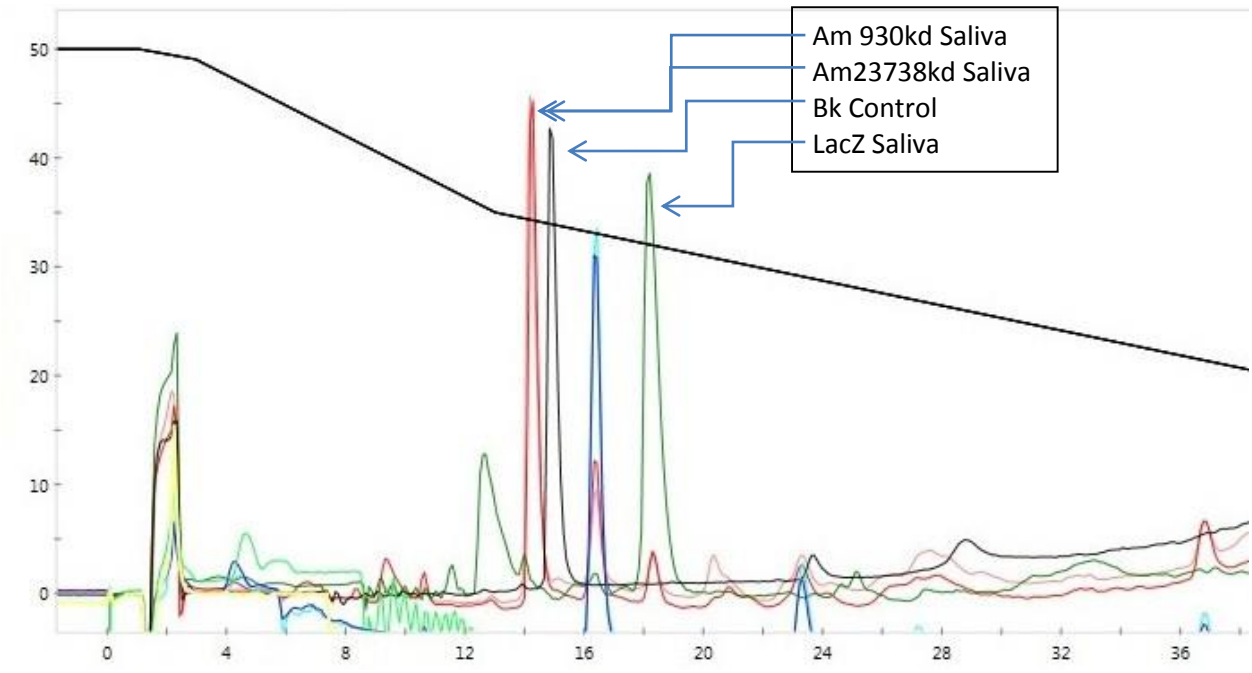


Figure 5: Changes in Bradykinin Degradation

Changes in Bradykinin Degradation

Sample absorbance was observed at 220λ. Control bradykinin was run through an HPLC to give the black peak seen at 15min. *LacZ* control saliva produced the green peaks seen at approximately 13min, 18min, and 57min. Both *Am 23738* and *Am 930* knockdown saliva produced peaks at approximately 14 minutes and 62 min, overlapping to form the red peak. Both experimental knockdowns also showed a blue peak at 16 min at 280λ. This change in degradation from the *LacZ* saliva to the knockdown salivas show that both genes produce proteins that have nearly identical bradykinin degrading functions.

Host Distress

The host sheep that the knock down ticks were fed upon showed distinctly increased distress as compared to other experimental host sheep. This forced the caretaker to give the sheep 3 doses of Prednisone in comparison to other host sheep, which received none.

Chapter 5: Discussion

Amblyomma maculatum, the Gulf Coast tick, is a hematophagous ectoparasite capable of feeding upon a wide variety of hosts. In order for it to feed for extended periods of time, it excretes hundreds of proteins through its saliva to undermine the host's immune response. In this study, the genes Am 23738 and Am 930 were investigated to determine their impact upon tick feeding. These genes were hypothesized to produce angiotensin converting enzymes which degrade bradykinin, a peptide hormone that leads to inflammation and the sensation of pain. In this work, I was able to determine that the genes Am 23738 and Am 930 not only code for angiotensin converting enzymes, but also confirm that they have a critical role upon the tick's ability to successfully feed upon a host.

The first step was to confirm that these genes do indeed code for ACEs. To accomplish this, I compared the experimental protein sequences to those of ACEs from 4 different arthropods that are known to participate in hematophagy to some degree, a tick, a mite, a spider, and a louse. As seen in figure 1, there is high sequence homology seen between the different sequences, and complete uniformity is seen at the active site sequence of HEMGH. This evidence confirms that the experimental sequences are indeed angiotensin-converting enzymes.

Once it was determined that the gene products were indeed ACEs, their importance throughout tick feeding had to be determined. This was accomplished through temporal gene expression of unfed and partially fed ticks. Salivary glands of ticks were collected at multiple timepoints, from which mRNA was extracted and reverse transcribed into cDNA. Through qRT-PCR of these samples I was able to determine the relative expression of the genes throughout feeding. Am 930 showed expression equal to or upregulated from unfed ticks

throughout the first 120 hours of feeding. In particular, the expression levels at 48 hours was over twice that of unfed ticks. However, Am 930 expression dropped dramatically after the 120 hour mark, to show almost no expression for the remainder of timepoints measured. In contrast, Am 23738 was downregulated throughout the feeding, with the sole exception of the 120 hour timepoint. There, Am 23738 was upregulated to over 1.5x that of the unfed tick. This selective expression at different timepoints could be evidence of a process known as sialome switching. In these cases, the organism will present strong shifts between the expression levels of different genes in order to form a different phenotypic behavior. These shifts could be in response to either environmental cues or to an internal timeline. In this case, the dramatic change likely served as a safeguard against the host's potential ability to overcome the effects of the Am 930 protein, allowing the tick to feed for a longer period of time.

Now that it was determined that the target genes were showing changes in expression during tick feeding, their impact upon the tick's feeding ability needed to be determined. This was accomplished through performing an RNAi knockdown of the target genes, then feeding the ticks upon a host over the course of two weeks. From this stage, three pieces of evidence were acquired from the ticks: their weight, their salivary glands, and, in later stages, their saliva. The salivary glands were used to confirm that the knockdown had been a success, and showed that both genes presented only a reduced expression in the feeding ticks. Am 23738 showed expression of less than 20% its normal level, while Am 930's expression was hardly detectable at less than 0.5% of normal expression levels. It was necessary to confirm these changes, as the effects upon the tick weights were astonishing. Among ticks fed to repletion, 7 were collected from LacZ controls, 3 from Am 23738 knockdown ticks, and 9 from Am930 knockdown ticks. The average weight of the LacZ controls was 959mg, while the average weights of the Am 23738 knockdown ticks were 456mg, and Am 930 knockdown ticks were

241mg. This incredible drop in tick weights, especially in combination with the consistently low Am 930kd tick weights, demonstrates the necessity of these proteins in tick feeding.

However, it is still necessary to ascertain whether these proteins have their purported effect of degrading bradykinin. The feeding of knockdown ticks provided qualitative data that they did. This was due to a marked increase in distress in the host sheep during the feeding of knockdown ticks, which forced the caretaker to give it three injections with the painkiller Prednazone. This effect was unobserved in the LacZ ticks and other ticks being fed for other experiments. Though this observation was promising, it needed to be examined more closely. This was done through the collection of tick saliva, which was used to digest bradykinin peptides, before analyzed through R-HPLC. This provided peaks for the LacZ control saliva degradation, as well as for both knockdown saliva samples. Interestingly, both knockdown saliva degradation peaks, while distinct from the control degradation peak, were perfectly overlapped in multiple regions. This gives clear evidence that these two proteins not only degrade bradykinin, but they degrade bradykinin in a nearly identical fashion, as would be expected from both being angiotensin-converting enzymes. Despite this, the knockdown degradation peaks remained distinct from the bradykinin control peak, implying that there are other degradative factors at work in the tick saliva.

In summary, tick blood feeding induced increased expression of Am 930 for the first 120 hours, as well as a peak in Am 23738 expression at 120 hours. These genes appear to code for angiotensin-converting enzymes, which possess the ability to degrade bradykinin. I observed that a knockdown of the target genes resulted in increased host distress, as well as significantly reduced replete tick weight. Saliva of ticks was used to determine that the two target genes not only functioned in the saliva to degrade bradykinin, but also that they functioned in nearly identical fashion. By removing this effect, the ability of the tick to feed is

severely compromised. The results from this research are a foundation for future research studies investigating the potential to create a vaccine response against tick infestation in humans and livestock.

Bibliography

1: Karim S, Ribeiro JMC (2015) An insight into the sialotranscriptome of the Lone Star tick, Amblyomma americanum, with a glimpse on its time dependent gene expression. *PLoS One*. 2011;6(12):e28525. doi: 10.1371/journal.pone.0028525. Epub 2011 Dec 21. PubMed PMID: 22216098; PubMed Central PMCID: PMC3244413

2: http://entnemdept.ufl.edu/creatures/URBAN/MEDICAL/Gulf_coast_tick.htm

3: Wang et al (2005) Bradykinin Produces Pain Hypersensitivity by Potentiating Spinal Cord Glutamatergic Synaptic Transmission. *Journal of Neuroscience*. 25:7986-7992

4: Rice GI, Thomas DA, Grant PJ, AJ Turner, Hooper NM (2004). Evaluation of angiotensin -converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. *The Biochemical Journal.* 51:45–51.

5: Anderson and Magnarelli (2008) Biology of Ticks. *Infectious Disease Clinics of North America.* 22: 195 – 215.

6: Allan et al (2010) Blood Meal Analysis to Identify Reservoir Hosts for Amblyomma americanum Ticks. *Emerging Infectious Diseases*. 16: 433 – 440

7: Moraru et al (2013) Evidence of Antibodies to Spotted Fever Group Rickettsiae in Small Mammals and Quail from Mississippi. *Vector Borne and Zoonotic Diseases*. 13: 1-5

8. Jongejan and Uillenberg (2004) The Global Importance of Ticks. *Parasitology* 129 Suppl., S3-S14.

9: Silveira et al (2015) Rickettsial Infection in Animals, Humans, and Ticks in Pauliceia, Brazil. *Zoonoses and Public Health*. 1-9

10: Browning and Karim (2013) RNA Interference Mediated Depletion of NSF and SNAP-25 Results in the Inhibition of Blood Feeding of the Gulf-Coast Tick, Amblyomma Maculatum. *Insect Molecular Biology*. 22: 245-257.

11: Ewing and Panciera (2003) American Canine Hepatozoonosis. *Clinical Microbiology Review*. 16 (4): 688-97.

12: Burgdorfer (1957) Artificial Feeding of Ixodid Ticks for Studies on the Transmission of Disease Agents. *Journal of Infectious Diseases*. 100 (3) 212-4

13: http://www.cdc.gov/leptospirosis/

14: Peter et al (2000) Detection of the Agent of Heartwater, Cowdria ruminantium, in Amblyomma Ticks by PCR: Validation and Application of the Assay to Field Ticks. *Journal of Clinical Microbiology*. 38 (4) 1539-1544.

15: Camus and Barre (1992) The Role of Amblyomma Variegatum in the Transmission of Heartwater with Special Reference to Guadeloupe. *Annals of the New York Academy of Sciences*. 653: 33-41.

16: Espinoza-Gomez et al (2011) Tick Paralysis Caused by Amblyomma Maculatum on the Mexican Pacific Coast. *Vector Borne Zoonotic Diseases*. 11 (7) 945-6.

17: Mulenga and Khumthong (2010) Silencing of Three Amblyomma americanum (L.) Insulin-like Growth Factor Binding Protein-related Proteins Prevents Ticks from Feeding to Repletion. *Journal of Experimental Biology*. 213: 1153 – 1161.

18: Walade and Rice (*(1982)*. The sensory basis of tick feeding behavior. *In Physiology of Ticks. Oxford UK: Pergamon*

17: Koch and Sauer (1984) Quantity of Blood Ingested by Four Species of Hard Ticks (Acari: Ixodidae) Fed on Domestic Dogs. *Ann. Entomology Society of America*. 77: 142-146.

20: Ribeiro et al (1985) Antihemostatic, Antiinflammatory, and Immunosuppressive Properties of the Saliva of a Tick, Ixodes Dammini. *Journal of Experimental Medicine*. 161 332 – 344

21: Dendorfer et al (2001) Pathways of Bradykinin Degradation in Blood and Plasma of Normotensive and Hypertensive Rats. *American Journal of Physiology*. 280: 2182-2188.

22: Ribeiro and Mather (1998) Ixodes scapularis: Salivary Kininase Activity is a Metal Dipeptidyl Carboxypeptidase. *Experimental Parasitology*. 89, 213 – 221

23: Cornell et al (1995) Cloning and Expression of an Evolutionary Conserved Single-domain Angiotensin Converting Enzyme from Drosophila melanogaster. *The Journal of Biological Chemistry*. 270: 13613-13619

24: Rice et al (2004) Evaluation of Angiotensin – Converting Enzyme (ACE), its Homologue ACE2 and Neprilysin in Angiotensin Peptide Metabolism. *Biochemistry Society*. 45 – 51.

Appendix A: IACUC Approval

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	**15101501 (Replaces 10042001)**
PROJECT TITLE:	**Tick Sialome**
PROPOSED PROJECT DATES:	**10/2015 - 09/2018**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Shahid Karim**
DEPARTMENT:	**Biological Sciences**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATION DATE:	**September 30, 2018**

Frank Moore, PhD
IACUC Chair

10/01/2015
Date