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Alpha-Gal Syndrome: Molecular Characterization of Amblyomma americanum α-D-galactosidase in α-gal Metabolism and Onset of Red Meat Allergy

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Alpha-Gal Syndrome: Molecular Characterization of *Amblyomma americanum* α-D-galactosidase in α-gal Metabolism and Onset of Red Meat Allergy

by

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Abstract

Tick-borne red meat allergy is an IgE-mediated delayed hypersensitivity reaction, increasingly widespread in tick endemic areas in the United States of America, and worldwide. Bites from the lone-star tick (*Amblyomma americanum*) are believed to be involved as the source of the sensitization of humans to the oligosaccharide galactose-α-1,3-galactose (α-gal), which is found in most mammal-derived food products, including gelatin, broths, and red meat. The purpose of this study is to functionally characterize the lone-star tick α-D-galactosidase (AGS) enzyme and assess its role in α-gal metabolism. This enzyme cleaves terminal α-galactose moieties from glycoproteins and glycolipids. Hence, I hypothesized that AGS is involved in the carbohydrate homeostasis in the tick vector of Alpha-Gal Syndrome. A reverse genetic approach was utilized to characterize the functional role of α-D-galactosidase in carbohydrate metabolism, and to discover its link to red meat allergy. My results from AGS gene silencing revealed a significant increase in tick weight, supporting a critical functional role in energy utilization. The silencing of AGS induced the decreased expression of downstream genes in the tick galactose metabolism pathway. Western blotting and N-glycan analysis revealed that AGS-silenced ticks ultimately expressed less α-gal epitopes due to the reduction of available UDP-galactose. Western blotting and basophil-activation experiments revealed that AGS plays a role in the tick α-gal expression and host response to tick saliva. Additional immunological assays need to be conducted to further elucidate the role of α-D-galactosidase in tick-host interactions and the possible involvement in the emergent Red Meat Allergy.

Keywords: α-gal, tick, red meat allergy, hypersensitivity, α-D-galactosidase, silencing

Dedication

I would like to first thank my advisor Dr. Shahid Karim for his relentless support, mentorship and guidance. Your compassion, expertise and work ethic have been so inspiring to me. You have pushed me to be the best researcher that I can possibly be. It has been an absolute honor and privilege working in your lab. Next, I would like to thank my mentor/trainer, Gary Crispell. Even though you were my mentor, you felt more like a big brother to me. You equipped me with the skills and tools I needed to succeed in the lab. When you left the lab, I promised to try my best to carry on your impact. I hope I was able to fulfill that promise.

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List of Abbreviations

AGS	α-D-galactosidase
α-gal	galactose-α-1,3-galactose
β-1,4-GT	β-1,4-galactosyltransferase
CDC	Centers for Disease Control and Prevention
GALK	galactokinase
GALT	galactose-1-phosphate uridyltransferase
STT3A	Aam SigP-24522 putative dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit STT3A
Am. americanum	*Amblyomma americanum*
KD	Knockdown
KO	Knockout
IU	International Unit
EGFR	epidermal growth factor receptor
IgE	Immunoglobulin E

Chapter 1: Introduction and Literature Review

General Timeline Leading to Alpha-Gal Syndrome Discovery

Until 2008, the association between tick bites and the novel delayed hypersensitivity to red meat was unclear and yet to be discovered. Key events and patterns ultimately led to the discovery of the emerging tick-borne, α-gal induced red meat allergy. In 2007, severe reactions to the cancer drug cetuximab in Tennessee and North Carolina patients were reported by O'Neil et al. (2007). Cetuximab is a chimeric mouse-human monoclonal IgG antibody against the epidermal growth factor receptor (EGFR), and is used to treat patients with EGFR-positive head-and-neck cancer, and colon and rectal cancer. In patients treated with cetuximab in clinical trials, immediate hypersensitivity reactions were observed after the first dose at a significant 22% frequency. Moreover, patients with prior allergic history were more prone to a hypersensitivity response after the initial infusion of cetuximab.

The specific epitope responsible for the hypersensitivity reactions seen in cetuximab treated patients was revealed in 2008 when Chung et al. discovered Immunoglobulin E (IgE) antibodies specific against the oligosaccharide galactose-α-1,3-galactose (α-gal) which is found in the Fab region of the cetuximab heavy chain (Chung et al., 2008). This finding preceded the α-gal association between tick-induced red meat hypersensitivity and cetuximab-induced hypersensitivity and helped incite the investigations linking anti-α-gal-IgE and reported reactions to red meat, which had been reported as early as 1989 (Steinke et al., 2015).

The cetuximab-induced hypersensitivity prevalence in southeastern regions of the United States was noteworthy to researchers. The pattern that ultimately led to focusing on the potential involvement of ticks in causing α-gal allergy was the observation that cases of Rocky Mountain Spotted Fever correlate with the geographical prevalence of reported cases of cetuximab hypersensitivity and red meat hypersensitivity. Furthermore, Rocky Mountain Spotted Fever is reported predominantly in the southeastern regions of the United States in correlation with the geographical distribution of its vector, lone-star tick. In 2011, Commins et al. confirmed the growing evidence that ticks are involved in red meat sensitization (Commins et al., 2011). After reporting anti-α-gal-IgE mediated delayed anaphylaxis to red meat by utilizing ImmunoCAP IgE assay, the group subsequently demonstrated that patients with a history of tick bites circulate serum IgE antibodies specific against the α-gal epitope and do so often in markedly high titers of ≥ 100 IU/mL (international unit per milliliter). Furthermore, IgE antibodies in response to whole body lone-star tick extracts correlated with the presence of anti-α-gal-IgE, thus confirming the presence of immunogenic α-gal in lone-star ticks. In 2014, Commins et al. corroborated the delayed nature of red meat sensitization via open food challenge tests (Commins et al., 2014). Clinical symptoms appeared 3-6 hours after red meat consumption and correlated with basophil activation, giving insight into the novel and unique immunology of Alpha-Gal Syndrome (Figure 1). Sensitivity can develop in adulthood and symptoms include urticaria (hives), nausea, swelling of the face and anaphylaxis.

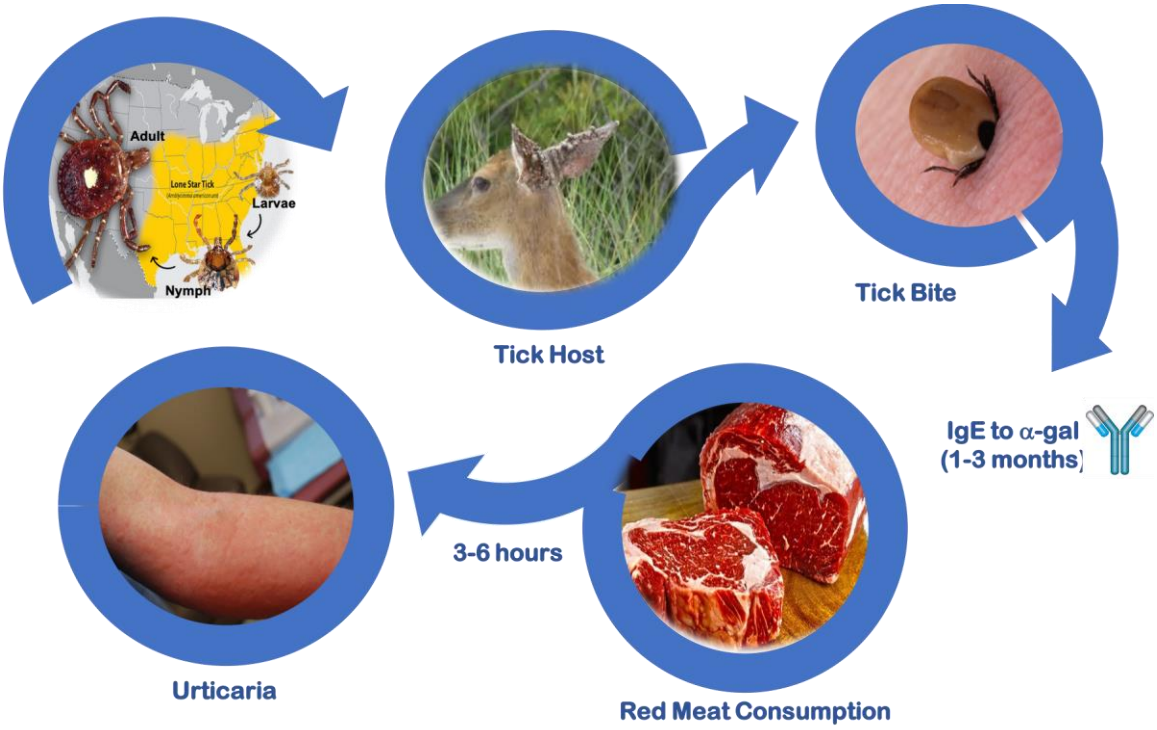


Figure 1: Red Meat Allergy Acquisition Flowchart – The lone-star tick larva, nymph or adult feeds on a reservoir host such as a deer, then bites its human host, transmitting the α-gal antigen. The human host subsequently produces IgE antibodies against α-gal, then within 3-6 hours after consuming red meat, symptoms of hypersensitivity arise such as urticaria, nausea and anaphylaxis.

Prevalence and Management of Alpha-Gal Syndrome

The exact prevalence of individuals diagnosed with Alpha-Gal Syndrome is unknown. Limitations in α-gal surveillance exists such that it is difficult to monitor the precise number of confirmed Alpha-Gal Syndrome cases. Difficulties in surveillance are due to a neglect to account for Alpha-Gal Syndrome as a food allergy (Pattanaik et al., 2018), likely due to its recent emergence and limitation of diagnostic testing (Levin et al., 2019; Wilson et al., 2019). The most common way for clinics to test for Alpha-Gal Syndrome is to carry out blood tests for anti-α-gal-IgE. A positive blood test along with a history of tick bites and allergic responses to red meat often leads to diagnosis.

Interestingly, data from the Centers for Disease Control and Prevention (CDC) and Viracor Eurofins Clinical Diagnostics presented at the 2020 Vector Week conference (https://vectorweek2020.com) shows that in the United States, two dozen known cases of Alpha-Gal Syndrome in 2009 has risen quickly to over 34,000 total positives. The ubiquitous nature of mammal-derived products makes it difficult to manage Alpha-Gal Syndrome. Mammal-derived products that could potentially serve as triggers include red meats (beef, pork, lamb, etc.), dairy, gelatin-products, pig-skin derived footballs, leather derived from non-primate mammals, cat dander and heart valves surgically replaced with those from non-primate mammals, to name a few (Commins, 2016). The increasing prevalence of this novel food allergy is also reported in France, Germany, Sweden, among other European countries, Australia and Brazil. In the United States, the only tick implicated in Alpha-Gal Syndrome is the lone-star tick. Overseas, other ticks possibly implicated include *Ixodes holocyclus* in Australia, and *Ixodes ricinus*, *Rhipicephalus bursa*, and *Hyalomma marginatum* in Europe.

Function of Lone-star Tick Saliva in Initiation of Alpha-Gal Syndrome

Ticks are ectoparasites that have the capacity to transmit a multitude of pathogenic microbes, macromolecules and other compounds that cause disease. In the United States, Alpha-Gal Syndrome is induced by the bite of *Amblyomma americanum* (lone-star tick). Additional tick bites have been shown to sustain or increase the titer of anti-α-gal-IgE in Alpha-Gal Syndrome patients (Commins et al., 2011; Levin et al., 2019); whereas avoidance of tick bites has led to eradication of Alpha-Gal Syndrome and renewed tolerance to red meat in some cases (Commins, 2016). The lone-star tick

salivary glands have been found to contain different glycoforms of α-gal. Interestingly, the tick that acts as a vector for Lyme Disease, *Ixodes scapularis* (deer tick), also contains α-gal (Crispell et al., 2019), but has not yet been recognized as a causative agent of Alpha-Gal Syndrome.

It is not yet well understood how much α-gal titer is requisite for the onset of IgE response against α-gal, and the source of the tick α-gal is unknown. A recent study found that α-gal IgE sensitized basophils were activated by lone-star tick saliva regardless of blood meal status (Commins et al., 2019). This finding suggests that the tick innately expresses the α-gal epitope and does not acquire it from feeding on a reservoir host

Tick saliva is essential in tick pathogen and antigen transmission to the tick host. Its immunomodulatory functions are well studied and possibly contribute to the human endogenous response to α-gal. When ticks attach to the host and begin feeding, the tick secretes an abundance of molecules that contribute to the tick's biological success, host evasion and vector competence. Bioactive compounds that assist in blood feeding inhibit the hosts' blood clotting, platelet aggregation, vasoconstriction, as well as pain and itching (Valenzuela, 2004; Karim et al., 2011; Karim and Ribeiro 2015). Immunomodulatory and antimicrobial peptides have also been found in tick saliva, and RNAseq has revealed that salivary particle proteins are differentially expressed during different timepoints of feeding (Karim & Ribeiro, 2015). The lone-star tick has been found to contain salivary molecules implicated in circumvention and alteration of host defense mechanisms, such as prostaglandins, which are involved in mediating vasodilation (Bowman et al., 1995; Karim and Ribeiro 2015), and glycine-rich proteins,

involved in the formation of the cement cone and tick stress response (Bullard et al., 2019). Moreover, tick salivary structures such as salivary acini have been found to be involved in the prevention of desiccation by absorbing moisture from the environment (Gaede & Knülle, 1997).

Ticks have the capacity to attach and feed on a multitude of hosts including goats, sheep, deer, rodents and most importantly, humans (Mlera and Bloom, 2018). Many ixodid tick species secrete a cement cone substance that serves to enhance attachment. During feeding, ticks concurrently secrete saliva while ingesting the blood of their host. Pathogens and etiological agents have developed mechanisms to remain viable within the tick saliva prior to transmission (Šimo et al., 2017).

The tick saliva acts as a vehicle for the transmission of pathogens and other molecules. In regard to pathogens, there are two prevalent ways that transmission can occur: 1.) The tick acquires a pathogen from a host during feeding, becomes a reservoir and transmits the pathogen to a new host; and 2.) an essential, commensal tick microbe is transmitted to the host and becomes an etiological agent to its new reservoir. Moreover, the tick saliva has been implicated in various disease processes. For example, *Borrelia burgdorferi*, the etiological agent of Lyme Disease, has been isolated from the saliva of *Ixodes scapularis* (Ewing et al., 1994), and *Anaplasma phagocytophilum*, the etiological agent of the zoonotic disease Anaplasmosis, has been detected in the saliva of *Ixodes ricinus* ticks (Lejal et al., 2019).

For Alpha-Gal Syndrome, saliva and salivary glands (the organ that secretes saliva) serve as paradigms to test α-gal expression in the lone-star tick. *Amblyomma*

americanum likely uses salivary glands as a means to transmit α-gal, ultimately causing delayed hypersensitivity to red meat. According to tick-borne pathogen transmission mechanisms, pathogens occupy the midgut prior to affecting the salivary glands (Šimo et al., 2017). The mechanism of transmission of the α–gal antigen is yet to be fully elucidated. The interplay between tick organs in regard to α-gal development, synthesis and transmission needs to be further studied.

Unique Amblyomma americanum Salivary Factors Possibly Facilitate Onset of Alpha-Gal Syndrome

Surveying potential salivary factors unique to *Amblyomma americanum* can potentially elucidate target candidates for vaccine development against Alpha-Gal Syndrome. Linking a salivary molecule with direct synthesis of α-gal or glycosylation of proteins to form the α-gal allergen would be a significant discovery in the fields of medical entomology and allergology. It is the goal of this study to decipher the interplay between lone-star tick galactose metabolism genes and α-gal expression. Comparing the lone-star tick to closely related tick species can help us better understand α-gal and its immunogenicity. The Gulf Coast tick (*Amblyomma maculatum*) is associated with transmitting *Rickettsia parkeri* and causes Rickettsiosis. Although part of the same genus as *Amblyomma americanum*, the Gulf Coast tick does not express any α-gal-containing antigens (Crispell et al., 2019). Conducting a complete comparative genome analysis of *Amblyomma americanum* and its "sister" tick that cannot serve as a vector for α-gal, *Amblyomma maculatum*, could potentially facilitate the identification of key genes and

salivary factors that play a role in α-gal expression and can ultimately offer more clues to solve the mystery of how the lone-star tick bite causes the atypical Red Meat Allergy.

α-Gal – Exploring its Structure and Metabolism

Unlike other food allergies, Alpha-Gal Syndrome coincides with an immune response directed towards an oligossacharide epitope rather than the commonly immunogenic protein epitope. The structure of α-gal is similar to the structure of blood antigen B (fucosylated Galα1, 3Gal epitope), which has interestingly been shown to correlate with a tolerance and protection against α-gal in humans with blood antigen B group (Cabezas-Cruz, Mateos-Hernández, Alberdi, et al., 2017; Brestoff et al., 2018). The structure of α-gal is demonstrated by Galα1-3Galβ1-(3)4GlcNAc-R and consists of two galactose units N-linked in an alpha 1-3 configuration. The galactose units are bound to an N-acetylglucosamine as part of a glycosolated protein or glycolipid assembly (Figure 2) (Crispell et al., 2019; Chung et al., 2008).

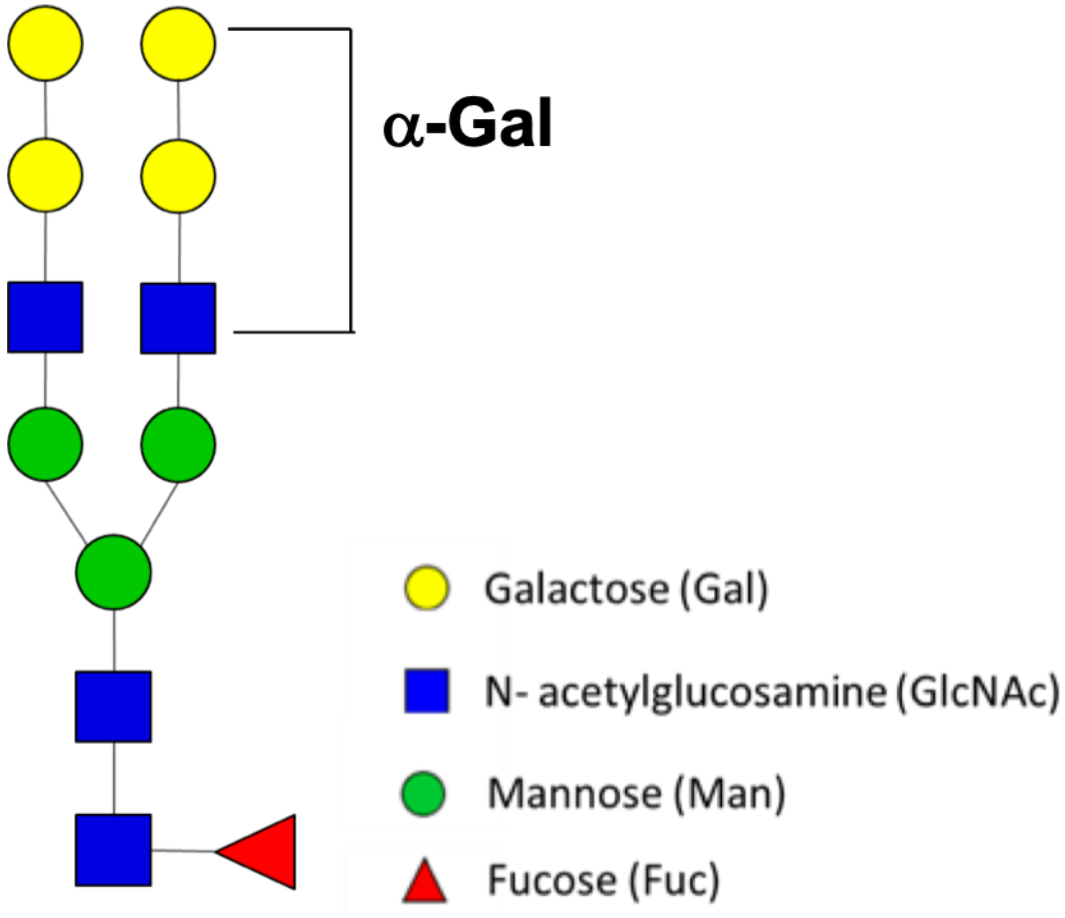


Figure 2: The Structure of α-gal consists of two galactose units linked to an N-acetylglucosamine (GlcNAc) conjugated to a glycoprotein or glycolipid.

The synthesis of α-gal expression in *Amblyomma americanum* has been an area of recent focus. Enzymes that have been proposed to play a role in tick α–gal expression include various galactosyltransferases and glucosidases (Crispell et al., 2019). Other glycosylation mechanisms have also been postulated to mediate α-gal synthesis. A recent study suggests that a combination of xylosylation and fucosylation mechanisms occurs in α-gal moieties and plays a role in N-glycan maturation (Park et al., 2020). Furthermore, glycosylation has been found to correlate with an increase in α-galactosyl residues as observed in the cancer drug cetuximab (Qian et al., 2007). Studies on the metabolomics

of *Amblyomma americanum* are limited. The tick galactose metabolism pathway can provide insight into the tick's α–gal synthesis and expression. The Leloir pathway involves the metabolism of α-D-galactose and can potentially be used as a paradigm of α-gal metabolism (Figure 3). The pathway consists of the following steps: Galactokinase phosphorylates α-D-galactose to galactose 1-phosphate. Then, galactose 1-phosphate is converted to UDP-galactose and D-glucose 1-phosphate by galactose-1-phosphate uridylyltransferase via utilization of UDP-glucose as the uridine diphosphate source. Lastly, D-glucose 1-phosphate is converted to D-glucose 6-phosphate by phosphoglucomutase. D-glucose 6-phosphate can then be channeled into the glycolysis pathway to produce energy. In this study, the Leloir metabolic pathway component α-D-galactose will be manipulated by altering the expression of the enzyme α-D-galactosidase (AGS), which cleaves the terminal α-D-galactose residue from α-gal.

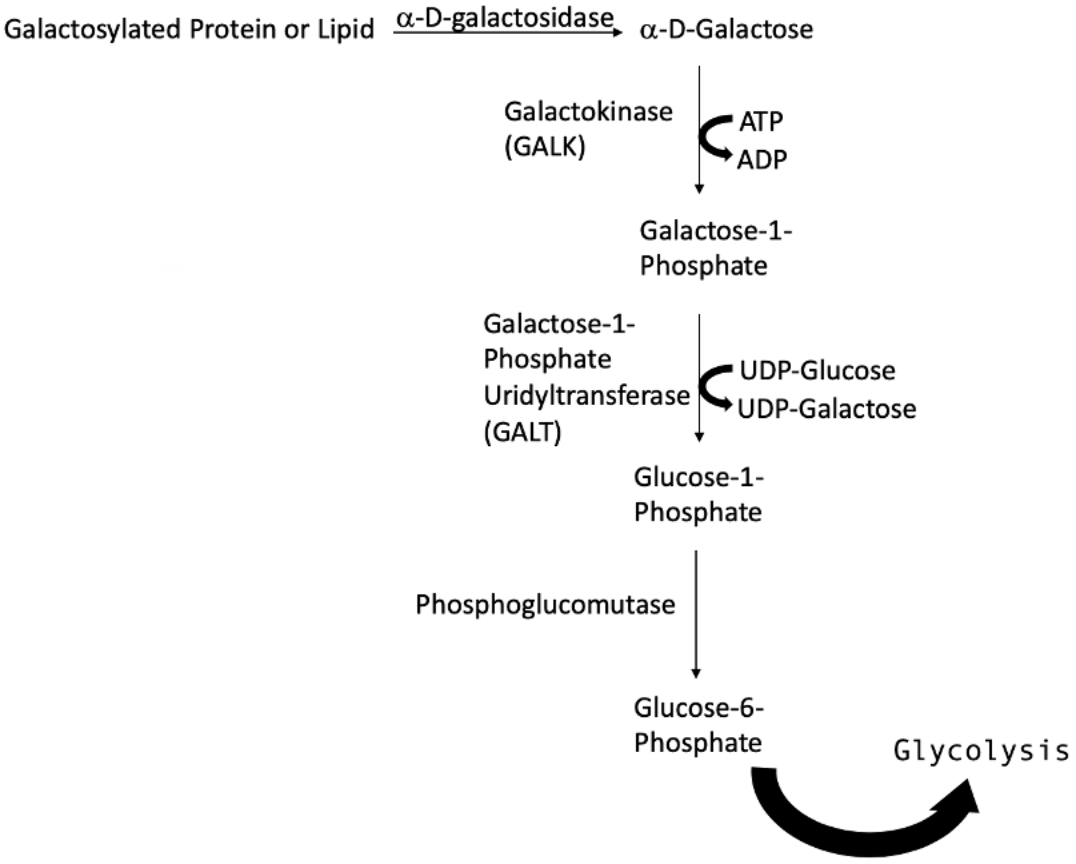


Figure 3 – Proposed Galactose Metabolism Pathway of *Amblyomma americanum*. Intermediate enzymes of the pathway have been identified in the tick genome.

Evolutionary Foundation of anti-α-Gal IgE and Possible Microbiome Role

Antibodies specific against α-gal account for about 1% of circulating antibodies in human sera (Galili et al., 1984). Humans and all other Old-World primates, including apes and Old-World monkeys, do not have the gene necessary to synthesize the α-gal epitope. Unlike Old-World primates, all non-primate mammals such as pigs, cows and sheep express the α–gal epitope (Galili et al., 1987). It is postulated that a significant evolutionary occurrence (e.g. an α-gal associated pathogenic outbreak) caused all Old-

World primates to lose the ability to produce α–gal. Approximately 125-140 million years ago, all mammals including humans (*Homo sapiens)* had the ability to make α-gal. It was not until 20-30 million years ago when the inactivation of the gene α-1,3-galactosyltransferase (α-1,3-GT) in humans coincided with the production of specific antibodies against α-gal (anti-gal), and humans along with apes and Old World monkeys no longer being able to make α-gal (Galili et al., 1987) (Figure 4). The distinguishing factor between α-gal viable organisms (e.g. pigs, lamb) and α-gal inviable organisms (e.g. humans, apes) is the gene α-1,3-GT. α-1,3-GT encodes for the enzyme that catalyzes the transfer of the residual α-D-galactose moiety to galactoproteins/galactolipids. α-1,3-GT is the common denominator in α-gal producing non-primate mammals. This element gives credence to the notion that α-1,3-GT plays an essential role in α-gal expression in non-primate mammals, and that the inactivation of α-1,3-GT in humans ultimately triggered the production of Anti-Gal.

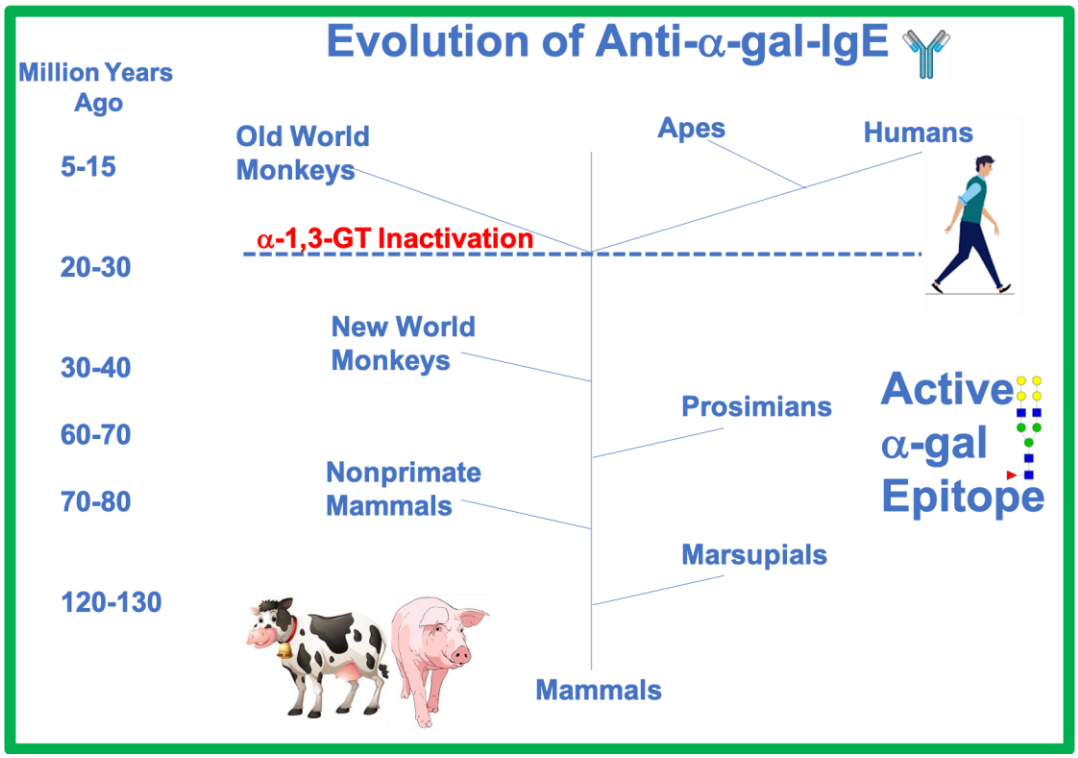


Figure 4: Evolutionary Tree Leading to Loss of α-gal in Old World Monkeys, Apes and Humans. 20-30 million years ago, the inactivation of α-1,3-GT coincided with the production of Anti-α-gal-IgE in Old World monkeys, apes and humans.

Proposing the purpose of the evolutionary selection against α-gal in humans is important to understanding the foundation of Anti-Gal. Thus far, Anti-Gal has been associated with xenotransplantation denial (Galili, 2001), tick-borne delayed hypersensitivity to red meats (Commins et al., 2014), and protection against malaria and other pathogens. The latter of which has been studied in a mouse model by Yilmaz et al.(2014). The malaria etiological agent *Plasmodium spp*. and the human gut pathobiont *E. coli* O86:B7 both express the α-gal epitope. The group found that anti-α-gal antibodies confer protection against malaria transmission in α1,3GT-deficient mice and humans. It is postulated that an α-gal associated pathogenic microbial outbreak of sorts could have applied selective pressure for the inactivation of α-1,3-GT and the production of anti-α-

gal. The malaria association with anti-α-gal should prompt additional research aiming to connect microbiome and Alpha-Gal Syndrome – α-gal tagged microbes emitted from lone-star ticks during blood feeding potentially mediate onset of Alpha-Gal Syndrome. Moreover, Schwarzer et al. (2019) recently discovered that germ-free, α-gal knockout (KO) mice do not develop food allergies despite higher levels of circulating IgE compared to conventional mice and observed an association between the host microbiome and mast cell viability. The microbial communities in ticks have been found to play a role in their vector competence. For example: *Rickettsia spp.* is involved in pathogen transmission and exclusion; *Coxiella spp.* is involved in pathogen transmission and reproduction and survival (de la Fuente et al., 2017*)*; and *Candidatus Midichloria mitochondrii* plays a synergistic role with *Rickettsia parkeri* to facilitate rickettsial colonization in the Gulf Coast tick vector (Budachetri et al., 2018). However, existing studies lack information about the possible lone-star tick microbial community effect on α-gal transmission. Though yet to be fully elucidated, lone-star tick whole microbiome genome sequencing could reveal microbial expression of α-gal, or lack thereof, and could reveal microbial genes potentially involved in α-gal metabolism and onset of Alpha-Gal Syndrome.

Immune Response to α-Gal Sensitization

The IgE associated sensitization to α-gal is the subject of growing research interest. As previously alluded to, unlike other allergies, Red Meat Allergy is induced by an IgE-mediated hypersensitivity response to an oligosaccharide antigen rather than the normally immunogenic protein antigen. Carbohydrates are abundant in metabolic

pathways, the normal gut flora and overall physiology of vast organisms. However, the α-gal oligosaccharide is immunogenic following tick bite – i.e. the human immune system recognizes α-gal as a non-self (foreign) molecule, thus inducing an immune response. Humans have naturally occurring IgG antibodies circulating in the sera that recognizes α-gal. However, these IgG antibodies are innocuous and do not correlate with disease or the symptoms seen in Red Meat Allergy. The IgG antibodies against α-gal are associated with xenotransplantation rejection in regard to incorporation of pig organs in humans (Galili, 2001). Humans have ubiquitous naturally occurring IgM, IgA, IgD and IgG antibodies against α-gal. The production of IgE antibodies following tick bites poses the question of how exactly tick bites and tick salivary factors lead to anti-gal IgE proliferation. Postulated explanations for the immunology of Alpha-Gal Syndrome have included the following:

1.) Immune Response comparable to a Th2 response leading to IgG1 and IgE in addition to IgG2 (Rispens et al., 2013)

2.) Tick salivary factors such as prostaglandins have immunomodulatory properties that trigger the type 2 immune response and induce class switch recombination (CSR) from pre-existing B cell clones producing anti-α-Gal IgM and/or IgG antibodies to B cells producing anti-α-Gal IgE (Cabezas-Cruz, et al., 2017; Wilson et al., 2017; Oliveira et al., 2011). Most likely, an abundance of tick salivary factors are involved in the pathogenesis of α-gal. For example, lectins recognize allergen-associated carbohydrates and bind carbohydrate moieties on mast cells (Barrett et al., 2011).

The prolonged feeding of ticks and constant injection of α-gal epitopes could potentially induce a class switch to the anti-gal IgE associated with delayed hypersensitivity to red meat from the harmless anti-gal IgG associated with gut microbiota. Moreover, prolonged and consecutive tick bites could induce a type 2 immune response. A recent study has illuminated the initiation of IL-3 + CD4+ memory T cells, basophil recruitment and induction of anti-α-Gal IgE in response to consumption of red meat in α1,3GT-KO mice (Chandrasekhar et al., 2019). It has recently been demonstrated that anti-α-gal-IgE binding α-gal containing glycolipids mediate the activation of basophils, which function as allergic effector cells (Iweala et al., 2020). Furthermore, it has been reported that fattier mammalian meats, characterized by higher lipid content, correlate with more severe hypersensitivity responses in Alpha-Gal Syndrome patients (Steinke et al., 2016). Taken together, these two studies suggest that α-gal moieties attached specifically to glycolipids serve as important antigens in Alpha-Gal Syndrome onset.

Two mechanisms might explain the production of anti-α-Gal IgE Abs after tick bites. The first mechanism proposes that the α-gal antigen on tick salivary proteins is presented to antigen-presenting cells and B-lymphocytes in the context of Th2 cell-mediated immunity. The second mechanism is based on the possibility that tick salivary factors including lectins, phospholipases and/or prostaglandin E2 triggers immunoglobulin class switching to anti-α-Gal IgE-producing B cells from preexisting mature B cells clones producing anti-α-Gal IgM and/or IgG.

Implication of a-D-galactosidase Silencing

Complete genome sequencing of the lone-star tick is still ongoing and α-1,3-galactosyltransferase is not identified in the lone-star tick. A recent study conducted using *Ixodes scapularis* as the model organism revealed that tick galactosyltransferases are associated with endogenous α-gal synthesis (Cabezas-Cruz et al., 2018). In this study, I identified and targeted a key gene α-D-galactosidase that I suspected to be involved in the lone-star tick's ability to vector α-gal due to its modification of the α-gal epitope and its role in freeing up a UDP-galactose that can subsequently galactosylate other moieties. α-D-galactosidase was identified by an immuno-proteome approach as a molecule potentially involved in tick α-gal metabolism. As aforementioned, α-D-galactosidase is an enzyme that functions to clip the terminal UDP-galactose residue from α-gal (Calhoun et al., 1985). No previous study has been conducted to determine the effect α-D-galactosidase has on the tick's overall ability to synthesize α-gal. In this study, I was able to find a correlation between tick α-D-galactosidase expression and tick α-gal expression. Furthermore, I also found an association between RNAi of α-D-galactosidase and downregulation of galactose metabolism related genes including β-1,4-galactosyltransferase. Better understanding of tick genetic factors involved in the emerging hypersensitivity reaction to α-gal can possibly facilitate the identification of feasible target molecules for vaccination and treatment. Further information regarding the existing knowledge gap in pertinence to Alpha-Gal Syndrome is displayed in Figure 5.

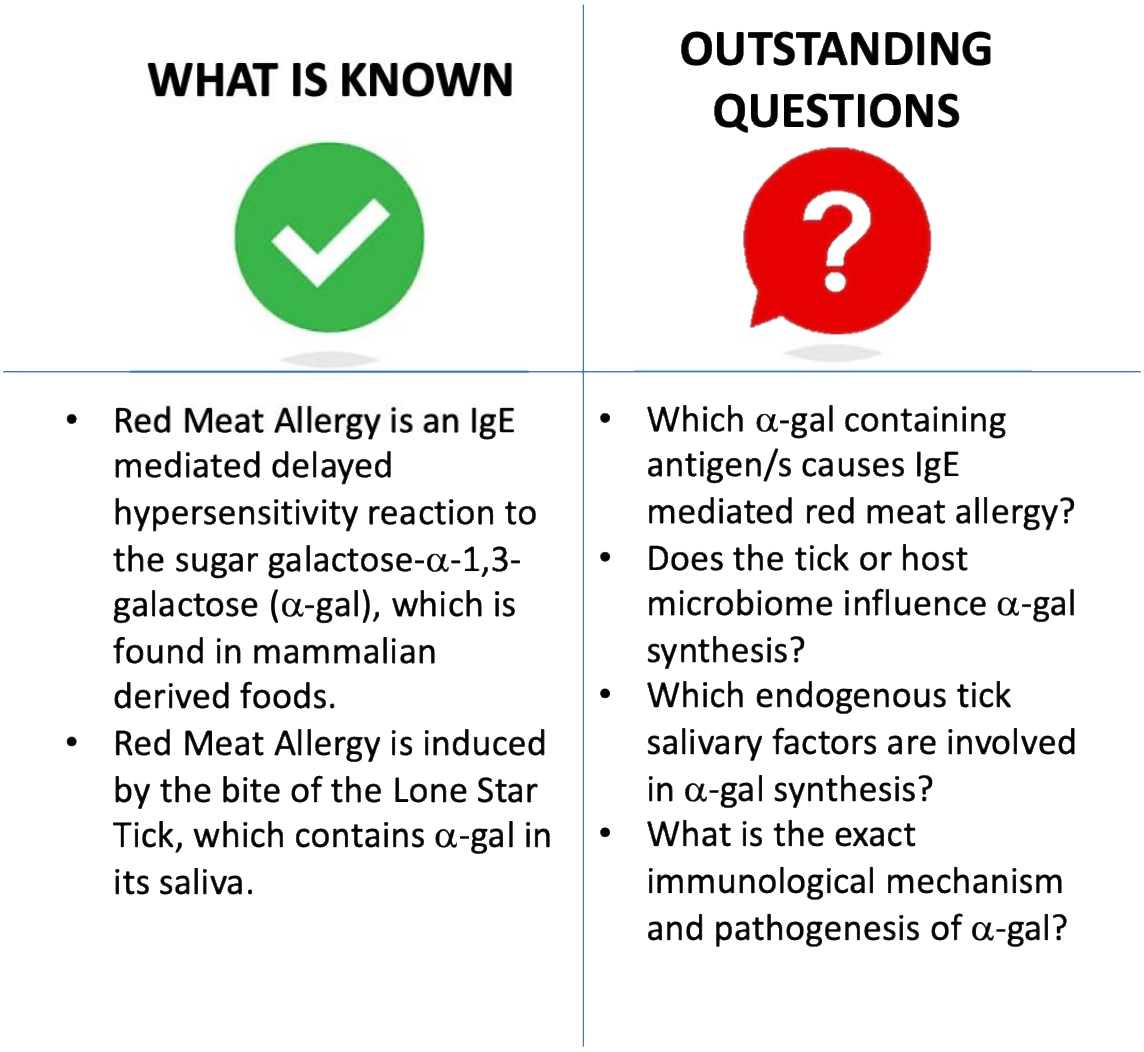


Figure 5: The Fundamental Concepts of Red Meat Allergy ("What is Known") and the Outstanding Questions. The question that this study covered is closely related to the third bulleted question, "Which endogenous tick salivary factors are involved in α-gal synthesis?" with the focus being on the specific tick salivary factor and gene, α-D-galactosidase.

Chapter 2: Materials and Methods

Ethics statement

All experiments involving animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. Ticks were reared at The University of Southern Mississippi according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Southern Mississippi (protocol #15101501.1).

Materials

Common laboratory supplies and chemicals were procured through Bio-Rad (Hercules, CA, USA), Sigma-Aldrich (St. Louis, MO, USA), and Fisher Scientific (Grand Island, NY, USA), unless specifically noted.

Ticks

Adult unfed lone-star ticks (*Amblyomma americanum*) were purchased from Oklahoma State University's tick rearing facility (Stillwater, OK, USA) and maintained at the University of Southern Mississippi following an established protocol (Patrick & Hair, 1975). Adult ticks were maintained at room temperature at approximately 90% humidity with a photoperiod of 14 hours of light and 10 hours of darkness prior to infestation on sheep. The adult ticks were fed on sheep for time intervals between 1 and 11 days for tissue collection, depending on the experimental plan.

dsRNA Synthesis

Double-stranded RNA was synthesized for AGS T7 and GFP T7 using a reverse genetic approach (Figure 6) (Karim & Adamson, 2012). 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 product 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.

Injecting Ticks with dsRNA

Unfed females were injected with approximately 1000 ng of the purified dsRNA using a 31-gauge needle and were maintained at 37˚C with 90% humidity overnight. After injection with dsRNA, the ticks are then fed on sheep in accordance with Institutional Animal Care and Use Committee (IACUC) of the University of Southern Mississippi (protocol #15101501.1). The ticks were forcibly removed at different time points (5, 7 and 9 days post infestation) of the bloodmeal. Following tick removal, I weighed the ticks on a lab scale (in milligrams) to assess tick phenotype in the different timepoints of the bloodmeal. I also monitored and ensured successful bloodfeeding and engorgement rate during the bloodmeal, and subsequently determined the temporal gene and protein expression. This method follows the method previously established (Karim & Adamson, 2012)

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 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 reverse transcribed 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 with nuclease free water to a working concentration of 25 ng/µl and stored at -20°C until used. This method was derived from methods previously established in other studies (Adamson et al., 2013; Bullard et al., 2016; Karim & Adamson, 2012).

Temporal gene expression

Partially fed female ticks removed from the sheep were dissected and the salivary glands removed and cleaned in ice cold M-199 buffer (Crispell et al., 2019). 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 (Karim & Adamson, 2012). cDNA was synthesized and diluted to a working concentration of 25 ng/µl as previously described (Adamson et al., 2013; Bullard et al., 2016; Karim & Adamson, 2012). 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 the AGS T7 primer (Supplemental Table 1). Gene expression was normalized with β-Actin unfed ticks.

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 non-template controls. Primers used for gene expression validation can be found in Supplemental Table 1. Gene expression was normalized against the housekeeping genes Beta-actin and histone (EZ000248.1; GI:759084459) as described (Bullard et l., 2016).

Quantification of total bacterial load

The total bacterial load in tick tissues was determined using the method described previously in other studies (Budachetri and Karim, 2015.; Narasimhan et al., 2014; Budachetri et al., 2018). The 25 µl volume reaction mixture contained 25 ng of tissue cDNA, 200 µM 16S rRNA gene primer and iTaq Universal SYBR Green Supermix (Bio-Rad). qPCR assay was conducted using the following conditions: 94 °C for 5 min followed by 35 cycles at 94°C for 30s, 60°C for 30s and 72°C for 30s. A standard curve

was used to determine the copy number of each gene. The bacterial copy number was normalized against *A. americanum* actin. All samples were run in triplicate.

Protein Extraction

Proteins were solubilized from dissected pooled tick salivary glands and midgut tissues (n = 5 ticks) in 100 uL of protein extraction buffer composed of 0.5 M Tris-HCl, pH 8.0, 0.3 M NaCl, and 10% glycerol. Tissues were then treated with 1% HALT protease inhibitor cocktail. The tissues were crushed using pestles and sonicated using a Bioruptor Pico (Diagenode, Denville, NJ, USA) sonication device for 10 full cycles of 30 s pulse/30 s rest at 4°C. Tissue lysates were centrifuged at 5,000 x g for 10 min at 4°C and the supernatants were collected. The protein concentrations were then quantified using the Bradford method (Bradford, 1976) and protein was stored at −80°C.

SDS-PAGE and Immunoblotting

SDS-Polyacylamide Gel Electropheresis and Immunoblotting, Indirect Basophil Activation Test and N-glycome analysis were carried out using the methods described previously (Crispell et al., 2019). Proteins extracted from the midguts (15 ug) and salivary glands (15 ug) were fractionated on a Mini-PROTEAN TGX Any kD, 4–20% gel (Bio-Rad) using SDS-PAGE and were then transferred onto nitrocellulose membrane in a Transblot cell (Bio-Rad). The transfer buffer consisted of 25 mM Tris-HCl and 192 mM glycine in 20% methanol. Blocking of nonspecific protein binding sites was executed with 5% BSA in a TBS and Tween-20 solution, and the membranes were incubated with α-galactose (M86) monoclonal IgM antibodies (Enzo Life Sciences,

Farmingdale, NY, USA) at a dilution of 1:10 using an iBind western device (Life Technologies, Camarillo, CA, USA). The antigen-antibody complexes were visualized using a secondary horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Sigma-Aldrich) at a dilution of 1:10,000, and were detected with SuperSignal chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA) using a Bio-Rad ChemiDox XRS.

Indirect Basophil Activation Test (Crispell et al., 2019)

Peripheral blood mononuclear cells (PBMCs) taken from a healthy, non-α-gal allergic donor (α-gal sIgE <0.10) were isolated using a Ficoll–Paque gradient (GE Healthcare, Chicago, IL, USA). Endogenous IgE was stripped from basophils within the PBMC fraction by incubating the cells with cold lactic acid buffer (13.4 mM lactic acid, 140 mM NaCl, 5 mM KCl) for 15 min. Basophils were sensitized with plasma from α-gal allergic and non-allergic subjects overnight in RPMI 1,640 cell culture media (Corning CellGro, Manassas, VA, USA) in the presence of IL-3 (1 ng/mL, R&D Systems, Minneapolis, MN, USA) at 37°C and 5% CO2.

PBMCs were subsequently stimulated for 30 min with RPMI media, cetuximab (10 μg), rabbit anti-human IgE (1 μg; Bethyl Laboratories Inc., Montgomery, TX, USA), saliva from Am. americanum (10 μg), or partially-fed salivary gland extracts from *Am. americanum* (50 μg), *Am. maculatum* (50 μg), or *Ix. scapularis* (50 μg). Stimulation reactions were stopped with 20 mM EDTA and PBMCs stained with fluorescently-labeled antibodies against CD123 (BioLegend, San Diego, CA, USA), human lineage 1

(CD3, CD14, CD16, CD19, CD20, CD56, BD Biosciences, San Jose, CA, USA), HLA-DR, CD63 (eBiosciences ThermoFisher, Waltham, MA, USA), and CD203c (IOTest Beckman Coulter, Marseille, France) in flow cytometry staining buffer with 0.02% NaN3. Samples were acquired on a CyAN ADP flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo v10 software (FlowJo LLC, Ashland, OR, USA). Data analysis was performed using Prism version 7.03 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney U-tests were used to compare the frequency of CD63+ basophils detected following stimulation with various compounds. A p-value < 0.05 was considered significant.

N-Glycome Analysis

N-linked glycans were released from 30 μL of *Am. americanum* saliva with an estimated protein concentration of 200 μg, after being reduced, alkylated, and then digested with trypsin in Tris-HCl buffer overnight. After protease digestion, the sample was passed through a C18 sep pak cartridge, washed with 5% v/v acetic acid, and the glycopeptides were eluted with a blend of isopropanol in 5% v/v acetic acid, before being dried by SpeedVac. The dried glycopeptide eluate was treated with a combination of PNGase A (Sigma) and PNGase F (New England Biolabs, Ipswitch, MA, USA) to release the N-linked glycans. The digest was then passed through a C18 sep pak cartridge to recover the N-glycans. The N-linked glycans were then permethylated for structural characterization by mass spectrometry. Briefly, the dried eluate was dissolved with dimethyl sulfoxide and methylated with NaOH and methyl iodide. The reaction was quenched with water and per-O-methylated carbohydrates were extracted with methylene chloride and dried

under N2. The permethylated glycans were reconstituted in 1:1 MeOH:H2O containing 1 mM NaOH, then introduced to the mass spectrometer (Thermo Fusion Tribrid Orbitrap) with direct infusion at a flow rate of 0.5 μL/min. Full MS spectra, as well as an automated "TopN" MS/MS program of the top 300 peaks, were collected and fragmented with collision-induced fragmentation. These fragmentation data were used to confirm a Hex-Hex-HexNAc signature, both with a diagnostic fragment, as well as expected neutral losses.

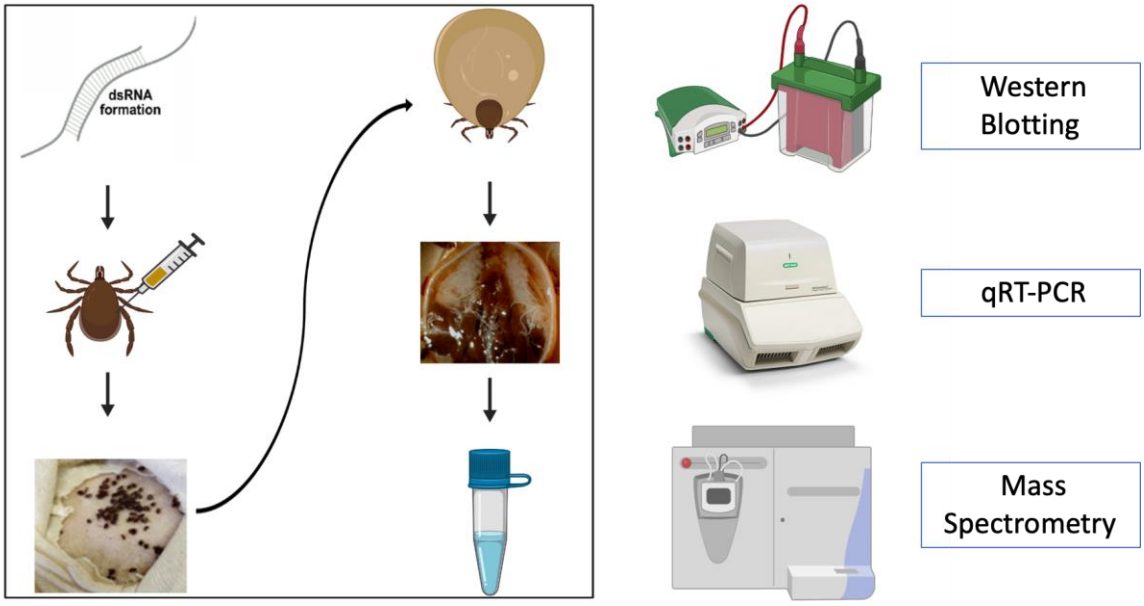


Figure 6: Methodology – the methodology of this study consisted of synthesizing dsRNA specific for AGS and GFP, injecting it into the tick, feeding the tick on sheep for various timepoints in order to monitor phenotype throughout the bloodmeal, dissecting the tick for its salivary glands and midgut tissues, extracting RNA and protein and conducting assays including western blotting, qRT-PCR and Mass Spectrometry. Indirect Basophil Activation was also conducted.

Chapter 3: Results

AGS silencing and tick phenotype

Weight of partially blood fed both AGS-KD and GFP-KD ticks revealed a key phenotypic difference between my two treatment groups. 5dpi ticks treated with dsAGS engorged faster than ticks injected with dsGFP irrelevant control particulary in the late fast-feeding phase (Figure 7). In 5dpi tick weights, the mean for AGS-KD ticks was 22.0 mg compared to 12.4 mg in the GFP control ticks; In 7dpi tick weights, the mean for AGS-KD ticks was 28.6 mg compared to 21.6 mg in the GFP control ticks. In 9dpi tick weights, the mean for AGS-KD ticks was 244.1 mg compared to 99.2 mg in the GFP control ticks. In 10dpi tick weights, the mean for AGS-KD ticks was 653.2 mg compared to 430.2 mg in the GFP control ticks.

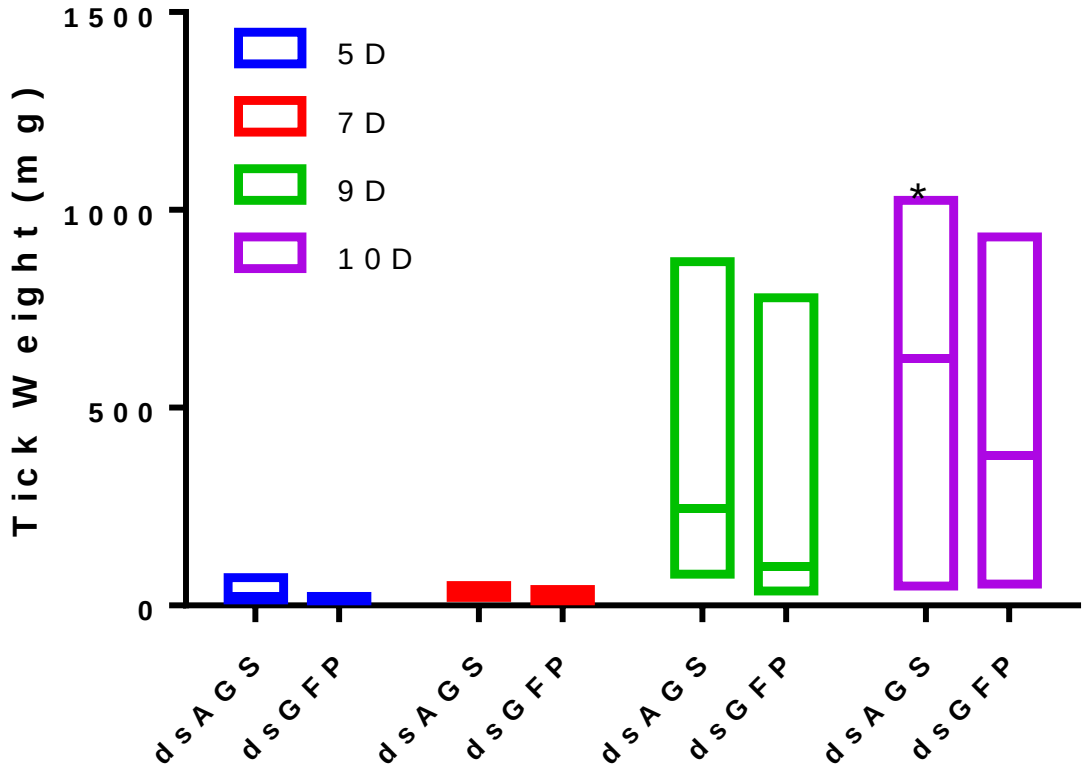


Figure 7: Time-dependent Engorgement Weights of Ticks Treated with dsRNA. Tick weights were graphed at 4 time-points during the bloodmeal after treatment with dsAGS or dsGFP double-stranded RNA. The bars represent the the range of tick weights and the horizontal line indicates average tick weight. Two-way ANOVA determined that there was a significant weight gain average in *dsAGS* injected ticks. (*p=0.023).

Time dependent gene expression of a-D-galactosidase in tick salivary glands

Time dependent transcriptional gene expression in *Am. americanum* salivary glands revealed that expression of alpha-D-galactosidase increases by approximately 2 fold after tick attachment to the host during the slow feeding phase up to 5 days post-infestation (dpi), but is downregulated by approximately 2 fold at 7dpi and 10 dpi, during the rapid feeding phase (Figure 8).

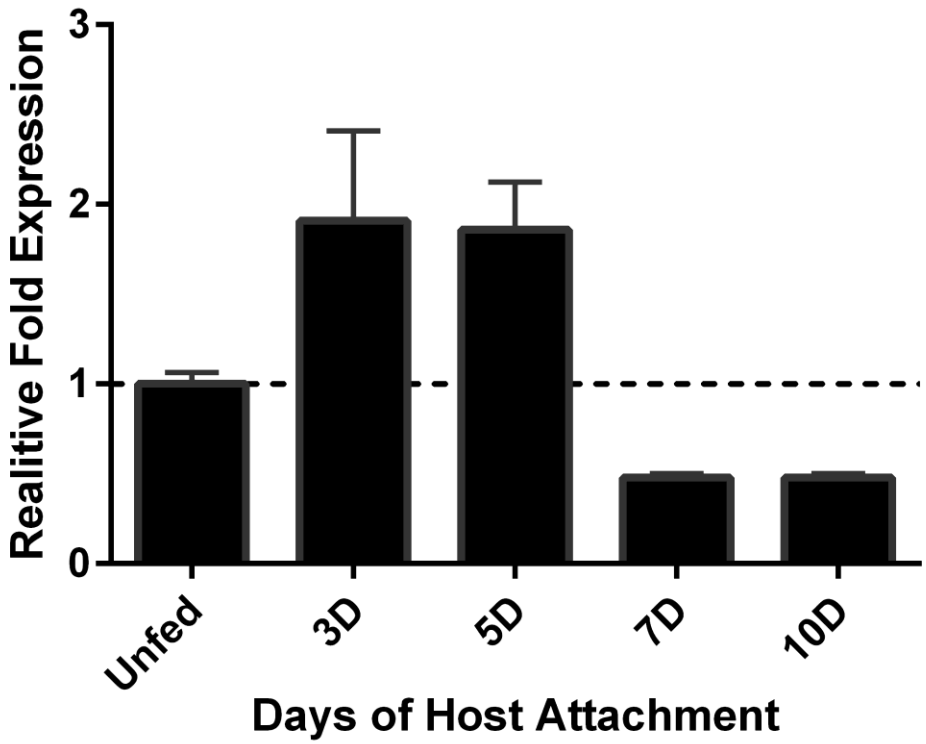


Figure 8: Time-dependent Transcriptional Gene Expression in Salivary Glands. Expression of α–D-galactosidase transcriptional expression was assessed at various time-

points (3dpi, 5dpi, 7dpi, 10dpi) during the tick bloodmeal. Expression data were normalized with β-*Actin* unfed ticks (dashed line).

α-*D-galactosidase silencing and impact on galactose related genes involved in Leloir pathway*

α-D-galactosidase dsRNA injections led to the downregulation of α-D-galactosidase gene expression in both midguts and salivary glands (Figure 9). My transcriptional gene expression results revealed that we were able to successfully silence the AGS transcript in partially fed ticks (5dpi) by using the reverse genetic approach as previously discussed by Karim & Adamson (2012). AGS was downregulated by approximately 85% in the salivary glands and 65% in the midguts. Silencing of dsAGS led to the significant downregulation of galectin (50%) and a significant upregulation of β–tubulin in the midgut (2 fold increase) and salivary gland tissues (2.5 fold increase). In the salivary glands, there was a significant downregulation of β-1,4-galactosyltransferase (β-1,4-GT) of approximately 2 fold and galactose-1-phosphate uridylyltransferase (GALT) by approximately 2 fold, and a non-significant decrease in galectin. The dsAGS also induced significant upregulation of β–tubulin in the salivary gland tissues (2.5 fold increase) and midgut tissues (2 fold increase).

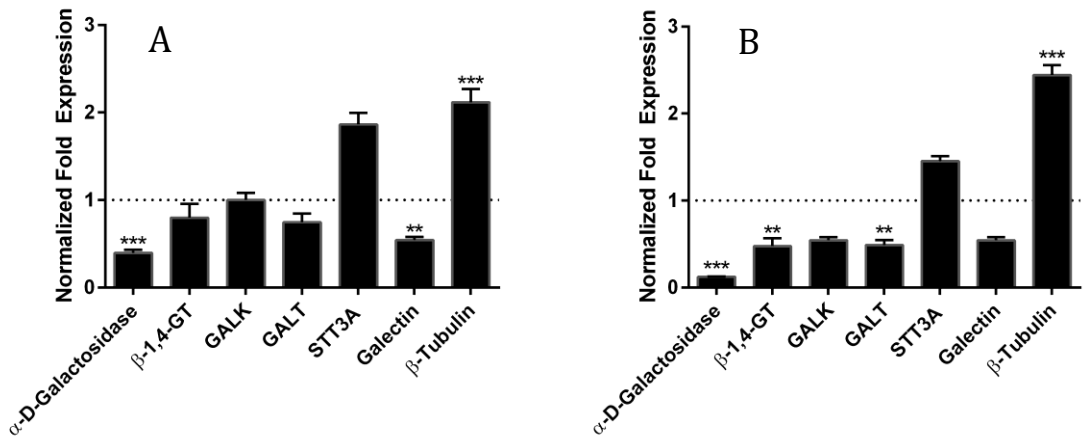


Figure 9: Transcriptional Gene Expression in dsAGS-treated *Am. americanum* 5dpi midgut tissues (A) and salivary glands (B). Target genes were α–D-galactosidase, β-1,4-galactosyltransferase (β-1,4-GT), galactokinase (GALK), galactose-1-phosphate uridyltransferase (GALT), Aam SigP-24522 putative dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A (STT3A), Galectin and β-Tubulin; *Histone H3* and β-*Actin* as housekeeping genes, ** p<0.01, *** p<0.001

Bacterial load of ticks injected with dsRNA

16S rRNA bacterial load quantification was reduced more than 4-fold in the salivary gland tissues of *Am. americanum* ticks that received dsAGS injections, as compared with dsGFP irrelevant control injected ticks (Figure 10).

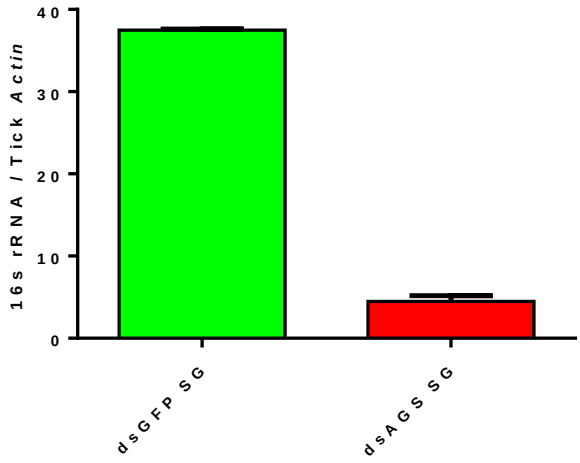


Figure 10: Bacterial Load Quantification in the salivary glands of 5dpi dsGFP treated and dsAGS treated *Am. americanum* ticks utilizing lone-star tick cDNA and 16S rRNA, normalized with β−Actin.

Detection and quantification of α-gal in dsAGS and dsGFP injected salivary glands

Salivary glands from 5 dpi, 7dpi, and 9 dpi *Amb. americanum* ticks injected with dsAGS and dsGFP irrelevant control RNA were assessed using immunoblotting with an anti-α-gal antibody (Figure 11). Densitometry analysis was conducted to determine the relative abundance of α−gal in dsAGS injected tick protein against dsGFP control protein. There was over an 80% reduction in alpha-gal in dsAGS salivary gland protein when compared to the dsGFP control. There was a reduction in α-gal of more than 30% in the 7 dpi dsAGS injected tick salivary glands, but the 9 dpi salivary glands contained ~10% more α-gal than the dsGFP irrelevant control (Figure 11). The accompanying Figure 12 represents the density of the dsAGS bands in relation to the density of the dsGFP bands. The image was generated via the ImageJ software (NIH).

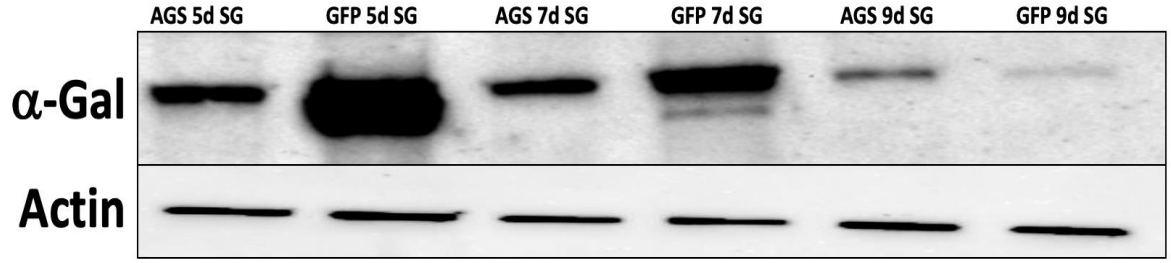


Figure 11: Identification of α-Gal and actin in the adult female salivary glands (SG) of *Amblyomma americanum* during the blood meal. Western blot using monoclonal actin antibody**.**

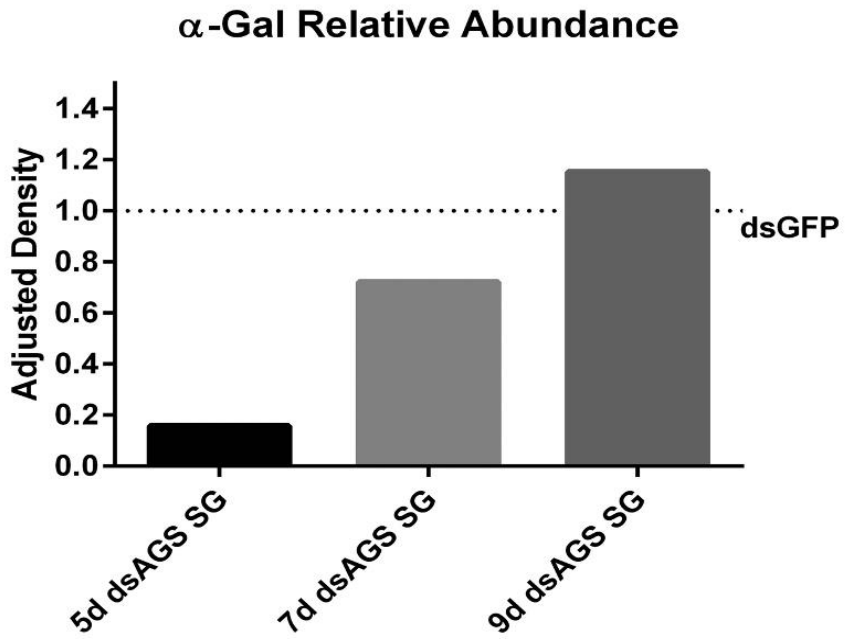
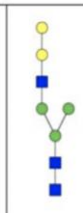
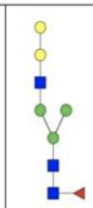
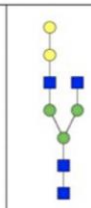
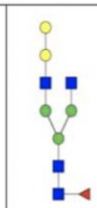
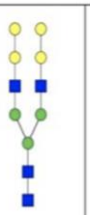
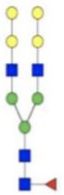
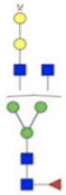


Figure 12: Adjusted Density and α-gal Relative Abundance in the salivary glands of dsAGS injected ticks along the blood meal. The densities of detected α-Gal in Figure 11 were normalized against β-actin.

Reduction in α-gal containing glycoforms in Alpha-D-Galactosidase silencing experiments.

Amblyomma americanum ticks were fed for 5 days on the sheep host, salivary glands were dissected, proteins were extracted, and N-glycan analysis was performed. Results indicate that 24.02% of all dsGFP control tick salivary gland N-glycans contain α–gal moieties, but the N-glycans in the dsAGS treated salivary glands that contain α-gal were significantly reduced to just 2.81%. Among these data, the α-gal containing glycoforms at m/z of 2478 and 2723 were absent in the dsAGS treated salivary glands, and significantly reduced at m/z 2652 and 2897 as compared to control (Figure 13). These results strengthen the hypothesis that tick alpha-D-galactosidase is involved in the synthesis or transfer of α-gal to tick salivary glycoproteins.

									TOTAL % Alpha-Gal
m/z	1825	1999	2070	2244	2478	2652	2723	2897	
SG-Control		0.25%	NO	0.00%	2.10%	8.94%	4.43%	8.30%	24.02%
SG-KD	0.11%	0.51%	NO	0.41%		1.23%		0.46%	2.81%

Greyed out boxes indicate that this mass was not detected in that sample.
"Unknown" = the MS/MS fragmentation was ambiguous,
"NO" = MS/MS fragmentation resulted in 486.23 ion
"YES" = MS/MS fragmentation resulted in 690.33 ion
YES/MIX" = MS/MS fragmentation resulted in both ions

Figure 13: N-Glycan Analysis on 5dpi lone-star tick salivary gland protein extracts. The mass to charge ration (m/z) signifies different α–gal glycoforms. Total % Alpha-Gal indicates the percentage sum of different α-gal glycoforms detected in dsGFP-treated lone-star ticks (SG-Control) and dsAGS-treated lone-star ticks (SG-KD).

Basophil Activation Test Results in AGS knockdown tick salivary glands

Frequency of CD63+-activated donor basophils decreases when PBMCs are sensitized with AGS silenced 5dpi *Am. americanum* salivary gland protein extract in comparison to the nontreatment 5dpi *A. americanum* salivary glands, cetuximab and Anti-IgE positive control (Figure 14). The results suggest a correlation between tick AGS depletion and potential reduction in the host immune response.

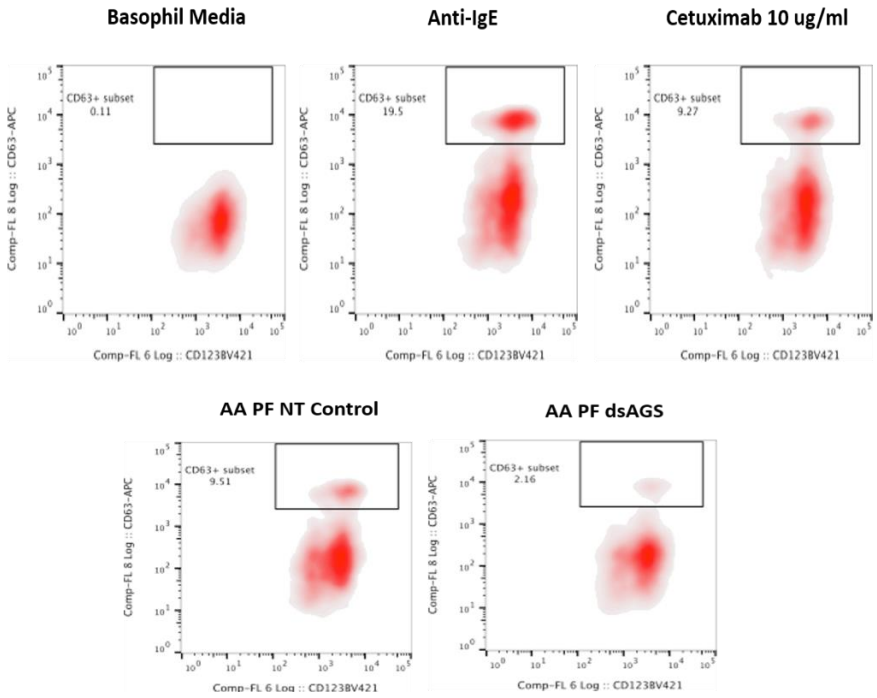


Figure 14: CD63+ Basophil Activation in Basophil Media (-) control, Anti-IgE (+) control, Cetuximab, salivary glands of *Amb. americanum* ticks partially fed nontreatment control and partially fed ticks injected with dsAGS.

Chapter 4: Discussion and Conclusion

The lone-star tick (*Amblyomma americanum)* has not been shown to express α-1,3-galactosyltransferase (α-1,3-GT), the enzyme that is required for non-primate mammals to synthesize α-gal. Nonetheless, previous studies have revealed that the *Am. americanum* salivary glands contain α-galactose antigens particularly in the salivary secretory vesicles of the salivary acini (Crispell et al., 2019). The key inquiry that this poses is the question of how *Am. americanum* expresses the α-galactose moiety that has been shown to cause delayed hypersensitivity to red meats whereas other ticks do not. In anticipation of elucidating the vector competency for α-gal in the lone-star tick, this study aimed to examine the functional role of the α-D-galactosidase glycoside hydrolase that is found in the *Am. americanum* genome and other galactose-acting tick genes including β-1,4-galactosyltransferase (β-1,4-GT), galactokinase (GALK), galactose-1-phosphate uridyltransferase (GALT), Aam SigP-24522 putative dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A (STT3A), Galectin and β-Tubulin to ultimately elucidate α-D-galactosidase's association with α-gal expression in the lone-star tick.

Weight of partially blood fed both AGS-KD and GFP-KD ticks revealed a key phenotypic difference between my two treatment groups. Ticks with depleted AGS expression engorged at a significantly faster rate than their GFP counterparts. I speculate that this observed phenotypic difference is due to the tick compensating for losing a key galactose metabolizing molecule. As my results demonstrate, AGS silencing impedes the tick's Leloir galactose metabolism pathway by downregulating the expression of key intermediate enzymes, galactokinase (GALK) and galactose-1-phosphate

uridyltransferase (GALT), and correlates with differential expression of other galactose-modifying genes including β,1-4,galactosyltransferase, galectin and STT3A. The Leloir pathway ultimately channels into glycolysis and produces ATP. Although ATP was not quantified, I speculate that the downregulation of Leloir pathway genes along with reduction in α-gal led to the tick producing less energy and thus having to compensate by ingesting high quantity of blood in the bloodmeal (blood contains galactose moieties which help determine blood group types) (Raven & Johnson, 1995). My results demonstrate that AGS likely plays a role in the tick's energy utilization.

Temporal gene expression in *Am americanum* salivary glands show that AGS is upregulated in the tick slow-feeding phase and is downregulated in the fast-feeding phase. Previous studies indicate that salivary particles are expressed during different timepoints of feeding in order to facilitate and contribute to the tick's biological success and vector competence (e.g., Karim & Ribeiro, 2015). Bioactive compounds that assist in blood feeding inhibit the hosts' blood clotting, platelet aggregation, vasoconstriction, as well as pain and itching. Immunomodulatory and antimicrobial peptides in tick saliva are expressed in a time-dependent manner. The temporal gene expression in *Am americanum* provides us with a glimpse of α-D-galactosidase's possible contribution to tick hematophagy; the blood meal initially upregulates the expression of AGS in tick salivary glands in correlation with data that α-gal expression increases with the blood meal (Crispell et al., 2019). I speculate that the tick needs α-D-galactosidase to cleave host galactose moieties more during the beginning of blood feeding to promote galactose metabolism and to reduce the viscosity of host blood. When AGS is silenced, two intermediate Leloir galactose metabolism genes (GALK and GALT) are also

downregulated, suggesting that the collective action of several genes maintains the carbohydrate homeostasis within the tick vector. The beginning of blood-feeding provides the tick with energy. The tick begins metabolizing the blood in the early stages of the blood meal and does not use as much AGS to metabolize blood later in the blood meal. The tick possibly conserves energy by expressing AGS less during the later stages of blood-feeding. When the tick is fast-feeding and nearing full-engorgement, AGS is downregulated. The temporal gene expression provides preliminary and supplementary data for other time-dependent data in this study.

The qRT-PCR assay provides evidence of the interplay in *Am. americanum* galactose-metabolism genes. By silencing AGS, other important galactose-metabolism related genes were subsequently differentially expressed. β-1,4-galactosyltransferase, which functions to transfer the penultimate galactose moiety to the α-gal, was significantly downregulated in the salivary glands and slightly downregulated in the midguts. GALT, another galactosyltransferase variant and an intermediate enzyme of the Leloir galactose metabolism pathway, was also found to be significantly downregulated in the salivary gland and slightly downregulated in the midguts. Dolichyl-diphosphooligosaccharide--protein glycosyltransferase (STT3A), also another galactosyltransferase variant, was upregulated in both the salivary glands and the midguts, although insignificantly. Galactokinase, an intermediate phosphorylating enzyme of the Leloir galactose metabolism pathway, was downregulated in the salivary glands and similarly expressed to the control in the midguts. Galectin, which binds galactose-binding proteins, was downregulated in the salivary glands and significantly downregulated in the midguts. Interestingly, galectin also functions as an immune gene

that recognizes allergen-associated galactose moieties & binds carbohydrate moieties on mast cells. Galectin has been shown to recognize α-gal in association with human monocyte activation via pig endothelial cells (Jin et al., 2006). The downregulation of galectin via AGS silencing could be of immunological relevance and mitigate α-gal sensitization. Conducting immunological assays after galectin knockdown could further elucidate galectin's role in Alpha-Gal Syndrome onset.

As previously alluded to, α,1-3,GT, the gene responsible for α-gal expression in non-primate mammals, is not expressed in the genome of *A. americanum*, suggesting that the tick's α-gal synthesis pathway involves other genes and enzymes. A study recently found that galactosyltransferases, including β-1,4,galactosyltransferase (β-1,4,GALT), are likely involved in the tick's α-gal synthesis pathway (Cabezas-Cruz et al., 2018). My data provides evidence that there is a correlation between AGS expression and β-1,4,GALT expression as well as expression of other galactosyltransferase variants. I speculate that the STT3A gene expression was upregulated (insignificantly) due to a compensatory mechanism used by the tick to offset the downregulation of the other galactosyltransferases. My results also provide evidence that there is a correlation between galectin and AGS expression. Galectin binds and "catches" α–gal moieties; reducing α–gal abundance via AGS knockdown corresponds with reduced transcriptional expression of the α-gal binding gene. Galactokinase and galactosyltransferase, two intermediate enzymes in the Leloir pathway, were both significantly downregulated in tick salivary glands, suggesting that AGS impedes the tick's galactose metabolism pathway. In the Leloir pathway, α-D-galactose is directly phosphorylated by galactokinase and the resulting product is acted upon by galactosyltransferase.

α−galactose moieties were reduced based on my N-glycomics (Figure 13) and immunoblotting data (Figure 11), so it would be feasible to see downregulation in the galactokinase and galactosyltransferase gene expression since there is less of the starting product to be metabolized. Lastly, β-tubulin was significantly upregulated in both salivary glands and midguts. β-tubulin has an abundance of documented functions including functions in microtubule formation, cell shape and structure. The upregulation in β-tubulin corresponds with the upregulation in structure I observed in the tick phenotype analysis in which the AGS-KD tick engorged at a much faster rate than the GFP irrelevant control.

Overall, the differential gene expression observed by silencing AGS shows that AGS is an important molecule in the tick's galactose metabolism pathway and is possibly clinically significant due to its association with the α-gal antigen. To get a clearer picture of the effect of AGS, it would be beneficial to completely knockout the AGS transcript. My RNAi approach led to only 85% downregulation, which although significant, is not complete knockout. Residual AGS gene expression could have possibly affected my results. CRISPR/Cas systems could potentially aid in gene knockout in the tick organism to better elucidate the role of tick genes. It would also be useful to knockdown other genes of interest to further evaluate the potential endogenous synthesis of α-gal. RNAi of tick galactosyltransferases, galectin and β-tubulin followed by gene expression analysis and α-gal expression analysis along with Mass Spectrometry and other immunological assays would be effective to revealing the impact of tick salivary factors to α-gal synthesis. There is most likely a combination of tick genetic and salivary factors that contribute to α-gal expression. RNA-sequencing following AGS-KD would reveal the

detailed effect of AGS on the tick galactose metabolism pathway gene expression. Although effective, quantitative real time PCR only gives a glimpse into gene expression.

Identification of α-Gal in the adult female salivary glands (SG) of *Am americanum* during the blood meal by western blotting revealed significant reduction in α-gal in AGS silenced ticks. However, at 9dpi, the α-gal expression in AGS-KD salivary glands was slightly higher than the control. I speculate that this is due to the dsRNA becoming highly diluted at this point of the bloodmeal, and also due to dsRNA becoming less effective over time and possibly degrading in the tick (e.g., Wang & Carmichael, 2004). My immunoblotting results were validated in my N-glycome analysis. The significant 21.21% difference in α−gal containing N-glycans between the AGS depleted lone-star tick salivary gland extract and the GFP control lone-star tick salivary gland extract further reinforces the conclusion that AGS is significantly involved in the tick's ability to synthesize and express the α-gal antigen. The observed α-gal reduction is possibly due to downstream effects of AGS silencing. The downregulation of AGS hinders the freeing up of galactose moieties that could glycosylate other molecules to form α-gal.

Indirect Basophil Activation Test tests the activation of basophils which is a type of white blood cell associated with allergic reactions. CD63 surface proteins are expressed whenever basophils are activated, and fluorescent labeled CD63 antibodies detect activation. The basophil activation assay results reveal a distinct difference in basophil activation in dsAGS treated partially fed ticks and partially fed nontreatment ticks and the positive controls. I found that the frequency of CD63+ basophils was significantly decreased following stimulation with AGS-silenced tick salivary gland

samples from *Am. americanum.* Basophil media served as the negative control and produced no basophil activation. Basophils were highly activated by cetuximab, Anti-IgE and *A. americanum* partially-fed nontreatment control. *Am. americanum* partially fed nontreatment control is expected to produce regular amounts of α-gal, whereas AGS-KD *Am. americanum* has a significant reduction of α-gal. Comparing the basophil activation in both of these samples demonstrates that *Am. americanum* likely induces α-gal-specific IgE mediated hypersensitivity reactions in humans and that AGS silencing in the tick reduces the risk of developing red meat allergy in humans. It should be noted, however, that variation of basophil activity in different human donors could lead to misrepresentation of data.

The microbial load quantification assay using 16S rRNA primer and Real Time PCR indicated that AGS-KD salivary glands have a significantly lower presence of bacterial community compared to GFP-KD salivary glands. This provides credence to the notion that the tick microbiome possibly plays a role in the tick's α-gal synthesis. The source of α-gal could be derived from bacterial galactosyltransferase enzymes used in cell wall biosynthesis or other bacterial genes (Hamadeh et al., 1996). Microbiome sequencing could reveal the bacteria that is most involved in tick galactose gene expression and α-gal expression.

Overall my findings (Figure 15) reveal that:

1.) AGS is involved in tick energy utilization.

2.) AGS plays a role in the tick's galactose metabolism pathway.

3.) AGS is implicated in tick α-gal synthesis.

4.) AGS is associated with the tick microbiome and

5.) AGS silencing decreases the frequency of CD63+ basophils.

It is my hope that my findings have contributed to the finding of a potential treatment and vaccine target (i.e. dsAGS) for Alpha-Gal Syndrome. It is apparent that the *Am. americanum* tick salivary factor AGS plays some important role in the α-gal synthesis pathway. Reducing this AGS molecule hinders the tick's ability to serve as a vector for α-gal, which is found ubiquitously in mammal-derived products. Moreover, AGS silencing could potentially be used as a form of biological control of the α-gal antigen in ticks. Further investigations utilizing an AGS inhibitor could reveal the precise functional role of AGS in the onset of the emerging public health hazard, Alpha-Gal Syndrome.

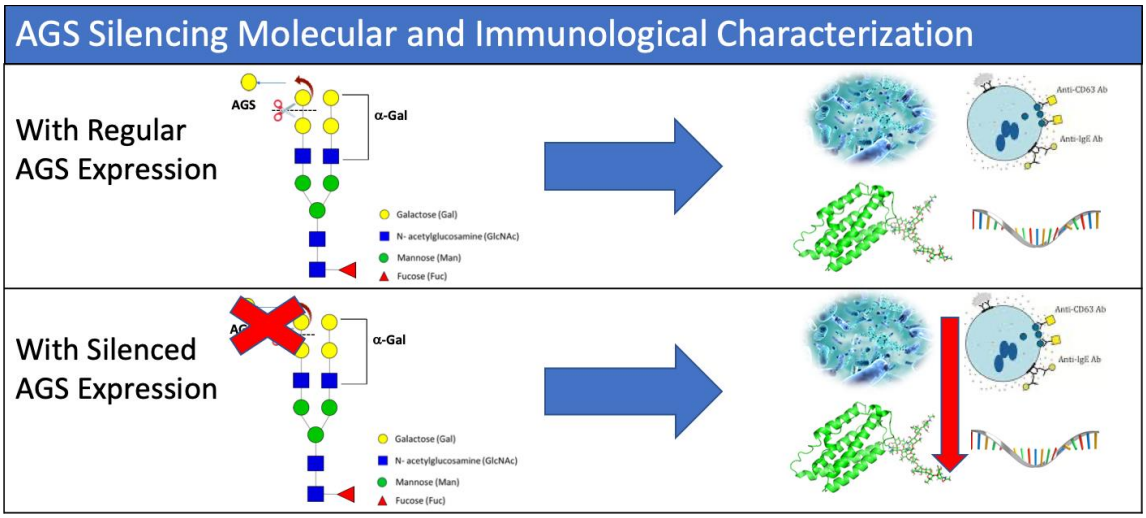


Figure 15: Results Infographic – Silencing of AGS had deleterious effects on bacterial load, galactose related gene expression, basophil activation and α-gal expression.

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Gene	Accession	Forward primer 5'-3'	Reverse primer 5'-3'	Size (bp)
Aa β-Actin	EZ000248.1	TGGTATCCTCACCCTGAAGTA	ACGCAGCTCGTTGTAGAAG	100
Aa Histone H3	GI:759084459	GAAGCCAGTGAGGCATACTT	GCTGGATATCCTTTGGCATGA	104
Aam-37720 Galectin	N/A	AACGGGCACTACTACCTACA	AAGATGCCAGCAGCACAT	93
Aam-23951 β-1,4-N-acetylgalactosaminyl transferase	N/A	TCCAGTGCTTCGTGTTCC	TTTCTCGTGACGGACATGTG	100
Aam-33934 putative dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit stt3a	N/A	CCACGCCACCCGACAAGAAG	CACGATGGAGGGCGACGAGTA	161
Aam SigP-24522 putative dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit stt3a	N/A	AGACTCTATTCTTTGGGGCAGTGACT	GCAAGTCAAAGAAGAAGGAGAACCACG	207
Aam-4310 galactokinase	N/A	GCAAGAACACGAAACACCTG	CAAATGTCCTTGAACTGCCAC	97
T7 AamerSigP-37433 α−D-galactosidase	T7	GTAATACGACTCACTATAGGGAGTTGGTCTGTTTCTTGCTTTTC	GTAATACGACTCACTATAGGGTACCCATCTTCAACGAGGTGATCT	193
L4440 GFP	N/A	GTCTTGTAGTTCCCGTCATCTT	AGCCAACACTTGTCACTACTT	154
Beta-Tubulin	N/A	GGTAGAGAACACGGATGAAACCTACTGTATTGA	TGACAGCCAGTTTGCGAAGGTC	96

Supplementary Table 1 – List of genes, accession numbers, primers, and base size used in this study. The following genes were used for transcriptional expression. T7 primer listed was used for RNA interference experiment.

Appendix A: IACUC Approval

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI
INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.5997 | 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:	**15101501.1 (Replaces 15101501)**
PROJECT TITLE:	**Tick Sialome**
PROPOSED PROJECT DATES:	**10/2018 – 09/2020**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Shahid Karim**
DEPARTMENT:	**School of Biological, Environmental, and Earth Sciences**
FUNDING AGENCY/SPONSOR:	**USDA NIFA/NIH/USAID**
IACUC COMMITTEE ACTION:	**Designated Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2020**

Jake Schaefer, PhD
IACUC Chair

Date: October 25, 2018