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INVESTIGATING THE INVOLVEMENT OF THE TICK VECTOR IN THE INDUCTION OF ALPHA-GALACTOSE HYPERSENSITIVITY (ALPHA-GAL SYNDROME, RED MEAT ALLERGY) IN THE UNITED STATES.

Gary Crispell

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by

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ABSTRACT

Alpha-gal syndrome (AGS or sometimes called red meat allergy) is a result of the development of specific IgE antibodies to the oligosaccharide galactose-α-1,3-galactose (α-gal) after a person has had exposure to tick bites. This dissertation investigates four common tick species found in North America: the lone-star tick (*Amblyomma americanum*), the Gulf-Coast tick (*Amblyomma maculatum*), the American dog tick (*Dermacentor variabilis*), and the black-legged tick (*Ixodes scapularis*) for the presence of α-gal by utilizing a combination of immunoproteomic approaches and carbohydrate analysis techniques.

Anti-α-gal IgM antibodies (M86) were used in immunoblotting to detect α-gal in the saliva and salivary glands of both *Am. americanum* and *Ix. scapularis*, while *Am. maculatum* and *De. variabilis* were found to lack α-gal. Incubation of *Am. americanum* partially-fed salivary gland protein extracts with PNGase F confirmed the deglycosylation of N-linked α-gal containing glycans from tick salivary glycoproteins. Immunolocalization of α-gal moieties to the tick salivary secretory vesicles of the salivary acini by confocal microscopy also confirmed the likelihood of a secretory nature of α-gal-containing antigens by ticks. *Am. americanum* ticks were fed mechanically-defibrinated human blood (lacks α-gal) using a silicone membrane system in an attempt to determine the source of the α-gal. N-linked glycan analysis revealed that *Am. americanum* and *Ix. scapularis* contain α-gal in their saliva and salivary glands, but *Am. maculatum* contains no detectable quantity of α-gal. Consistent with the N-glycan profiling and analysis, salivary samples from *Am. americanum* and *Ix. scapularis* stimulated activation of basophils that were primed with

plasma from α-gal allergic subjects. Proteomic data generated from these experiments offered multiple potential targets for further investigation and RNAi gene silencing.

Together, these data support the theory that bites from only some tick species may specifically create an enhanced risk for the development of α-gal-specific IgE and hypersensitivity reactions in humans. The findings within this research have paved the way for future α-gal research in the tick, however, the exact mechanism by which ticks sensitize a host to α-gal continues to remain unknown.

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Latoyia Downs, Jonathan Linder, Paige Allen, and Faizan Tahir you are truly great friends

DEDICATION

I would like to dedicate this dissertation to my family. Firstly, I would like to dedicate this to my loving wife Fanny Crispell, who has been by my side since long before I ever thought to pursue an education. She pulled more than her share of the weight for many years to ensure that I was able to complete my degrees. To my daughters, Kara Bella and Andrea Mariajoy, and my sons, Bruce Wayne and Grayson Carlos, I'm grateful for your understanding and support of my pursuit of higher education even though it meant I had to miss so much time with you all. I love you all and I look forward to spending much more time together. To my mother, Joy, and my brother, Tyler, thank you for all of time that we were able to spend together in Hattiesburg.

Secondly, I would like to dedicate this dissertation to the family that I lost while attending graduate school. Douglas Bruce Crispell, my uncle that raised me like a father. Terry Wayne Howard, my step-father that also raised me. My brother-in-law Jesse Guzman that lost his life too young. My dog and companion, Major, thank you for being there for Tyler and me when we needed you. I hope that you all are looking down and watching me with pride.

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CHAPTER I - BACKGROUND AND SIGNIFICANCE

1.1 Ticks

Ticks are obligate blood-feeding ectoparasitic arthropods that can be found worldwide and can quest for and feed on a variety of different hosts, including mammals, reptiles, birds, and amphibians. Ticks are well-known to be disease-transmitting arthropods that commonly attach to animal hosts with their mouthparts and engorge on host blood to acquire nutrition. Ticks can be categorized as one of two families, hard-bodied ticks (Ixodidae), and soft-bodied ticks (Argasidae). Focus throughout the following discussions will be on hard-bodied ticks, more specifically, *Amblyomma americanum*.

The CDC reports that ticks are responsible for transmitting various pathogens that cause such diseases as anaplasmosis, babesiosis, *Borrelia miyamotoi* Disease, Colorado Tick Fever, ehrlichiosis, Heartland and Bourbon Virus Diseases, Lyme Disease, Powassan Virus Disease, Rocky Mountain Spotted Fever, *Rickettsia parkeri* Rickettsiosis, Tickborne Relapsing Fever, Tularemia, and various other tickborne diseases globally (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2020). In recent years, Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) (Yu et al., 2011) and Yezo virus (YEZV) (Kodama et al., 2021) have been identified in Asia with reports of severe disease and high mortality rates.

1.2 *Amblyomma americanum*: The Lone Star Tick

Amblyomma americanum, commonly referred to as the “Lone Star Tick”, is often found in the southeastern United States, but ranges from the southern states of Texas to

Florida and to the northern States of Wisconsin, Iowa, Maine and all of the states in-between (**Figure 1.1**).

Figure 1.1 *Geographic distribution of Am. americanum in the United States.*

Figure from (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), 2020)

Am. americanum are typically found in low-density woodlands with seasonal activity from early spring until fall. Adults will typically begin to become active and quest for hosts from late February until late July, depending on the latitude, with the height of activity from April to June. Nymphs can also be active at this time if they have overwintered as unfed nymphs or engorged larva. Larva that have not fed will typically not survive an overwintering event (Burgdorfer, 1969). Adult *Am. americanum* are sexually dimorphic, and the females can be easily identified by a white "dot" on their dorsal side (**Figure 1.2**).

Figure 1.2 *Image of adult female Am. americanum.*

Adult unfed female *Am. americanum* are easily distinguished from other tick species due to the unique whiteish-brown spot on their dorsal side. Image adapted from (Crispell et al., 2019).

In the United States, this tick species has been known to transmit *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Francisella tularensis,* Bourbon virus, and Heartland virus (Dumler & Bakken, 1995; Mixson et al., 2006; Molins et al., 2017; Petersen et al., 2009; Savage et al., 2013, 2018). The lone-star tick has also been suggested to be uniquely involved in the development of Alpha-gal Syndrome (AGS), a delayed-type hypersensitivity to an oligosaccharide, Galα1-3Galβ1-4GlcNac-R or galactose-alpha-1,3-galactose, which is commonly referred to as "alpha-gal" in the United States (Commins et al., 2011; Commins & Platts-Mills, 2013a, 2013b).

1.3 *Amblyomma americanum* Life Cycle

Amblyomma americanum ticks have three life stages after hatching from the egg, and each utilizes a different host to imbibe blood (**Figure 1.3**). Larva are sexually amorphic and six-legged, and under laboratory-controlled conditions typically feed for 4-9 days to repletion. After repletion, they molt for 21-26 days into sexually amorphic eight-legged nymphs. Nymphs need to feed on another host for 3-8 days, followed by a molting to the

adult stage of 28-39 days. The emerging adults are sexually dimorphic; the males tend to have less mass than the female ticks from the same species (Bouzek et al., 2013). Adult *Am. americanum* ticks mate on the host, and each male is capable of inseminating multiple females (Gladney & Drummond, 1970). Female *Am. americanum* will feed until repletion on the host for approximately 10-15 days, followed by a gestation period of 14-21 days. After egg clutches are laid, the incubation period of the eggs are on average 50-61 days (Troughton & Levin, 2007).

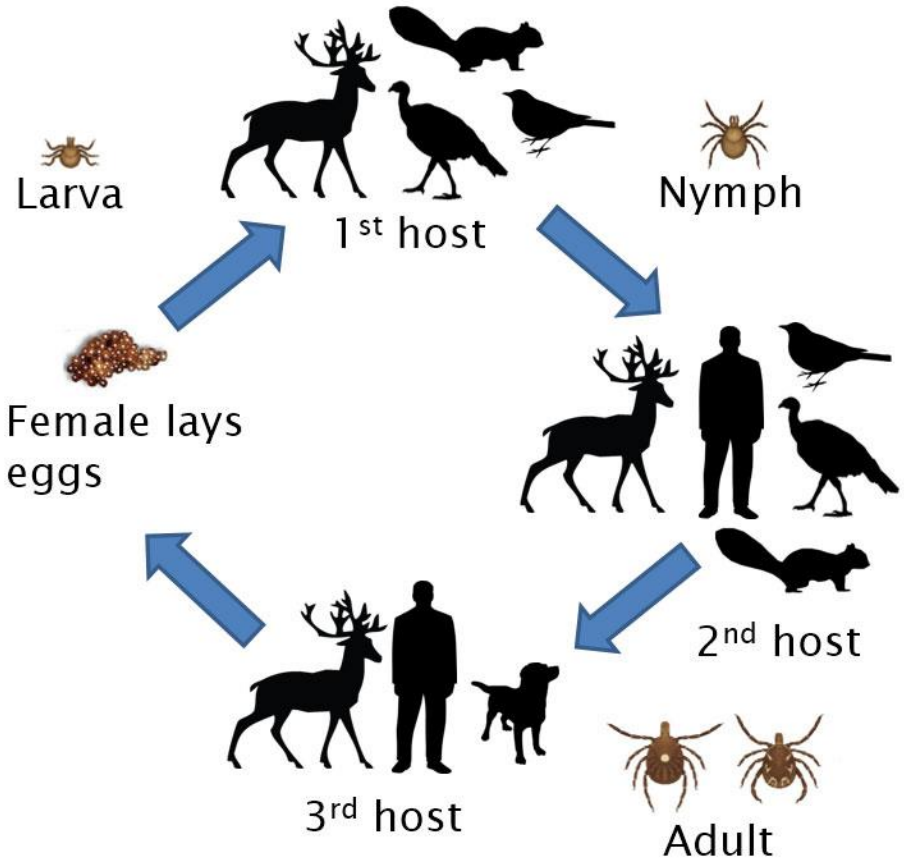


Figure 1.3 *Image broadly depicting lifecycle of Am. Americanum ticks.*

Figure depicts the life-cycle of *Am. americanum* ticks from eggs hatching to larva, feeding, molting to nymphs, feeding again, molting into sexually dimorphic adults, feeding on the third host, and then female ticks laying eggs. Image from (Occi et al., 2018).

1.4 *Amblyomma americanum* Feeding

Amblyomma americanum are aggressive generalist feeders that opportunistically feed at all life stages on many types of hosts, including humans (Goddard & Varela-Stokes, 2009). Questing *Am. americanum* nymphs have been found in greater numbers by tick-drags in leaf litter during the daytime hours with the highest ambient temperature (Schulze & Jordan, 2003), however under laboratory conditions, there has been no observed preference to diurnal or nocturnal questing (Huang et al., 2021). It has been shown that all life stages of *Am. americanum* are attracted to the emission of CO_2 gas (Wilson et al., 1972).

Adult female *Am. americanum* ticks under laboratory conditions for experiments that were conducted at the University of Southern Mississippi typically attached to a host and imbibed blood for up to 12 days to reach repletion. In my experiments, their unfed weight changes from approximately 10-15 milligrams before the blood-meal to weighing over 825 milligrams once they have reached repletion, but engorged weights may vary due to a variety of environmental conditions including season, temperature, location, host type, or injection with dsRNA. A historical study from Oklahoma State University demonstrated that the mean final weight for females was 642.7 ± 29.6 mg after 13.8 ± 0.27 days (Hume et al., 1985), which supports that engorged weights might vary depending on conditions.

When *Am. americanum* females find a host, they will penetrate the epidermis with their barbed hypostome, and will begin to secrete a proteinaceous cement to form a cone around the hypostome, which aids in their ability to remain attached to the host (Bullard et al., 2016; Hollmann et al., 2018). During attachment, the adult female ticks have noticeable morphological changes to accommodate the large volumes of nutrients that they parasitize

from their host. Most noticeably, when feeding on a host there is a notable change from being subjectively hard and flat during the slow-feeding stage (SFS) to becoming softer and more round once the fast-feeding stage (FFS) is initiated (**Figure 1.4**). During this time, the female engorged ticks become much more susceptible to damage from animals laying down or piercing by forceps as the tick body expands and the dorsal shield (scutum) covers a smaller percentage of total surface area.

While ticks remain attached to the host during prolonged periods of feeding, there is a constant exchange of host blood and tick saliva. It has been shown that *Am. americanum* uptake host immunoglobulins into the midgut and hemolymph, where they can still be found in unfed adults 4-5 months after molting (Jasinskas et al., 2000).

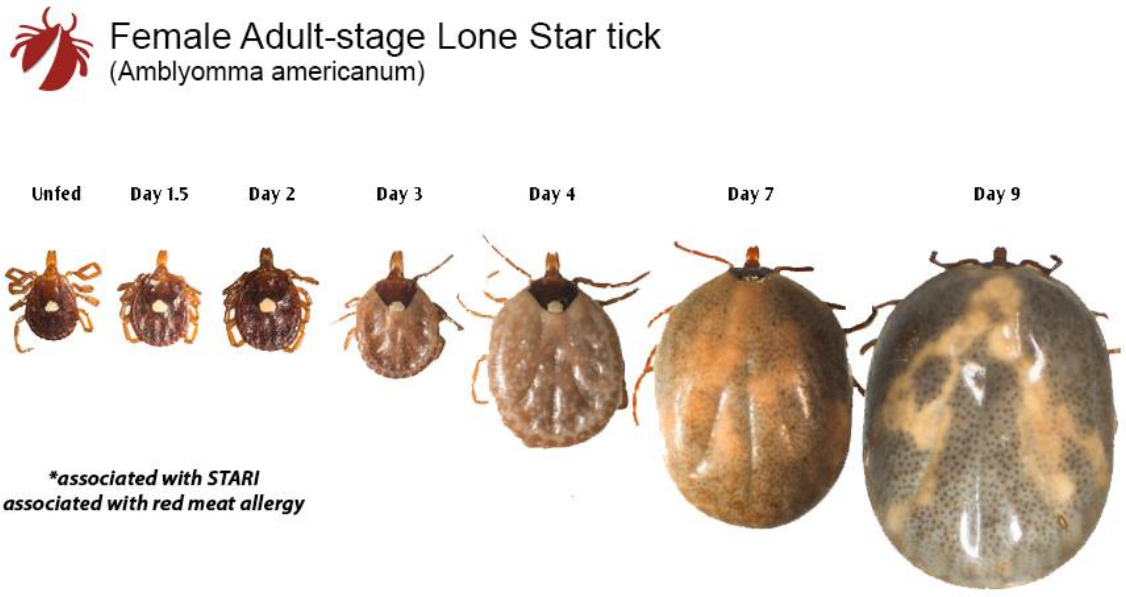


Figure 1.4 *Image showing growth of adult female Am. americanum ticks during the feeding cycle.*

Female ticks can be seen growing larger as the blood-meal progresses. Image taken from (TickEncounter (The Univeristy of Rhode Island), 2022)

1.5 Tick Salivary Glands and Saliva

After *Am. americanum* attaches to a host and initiates the blood-meal, the salivary glands steadily grow through the fast-feeding stage until the tick reaches repletion. It was shown that the salivary glands of ixodid ticks begin to rapidly degrade within hours of repletion, and there is a delayed degradation when prematurely removed from the host (Harris & Kaufman, 1981). There are dynamic changes occurring to the protein profiles at all stages of attachment and feeding. Previous research has shown that there is "sialome switching" of genes expressed at various stages of the tick blood-meal, which could play a role in successful feeding and combating the host immune system (Karim & Ribeiro, 2015). This sialome switching phenomenon likely supports the earlier research that shows that the salivary gland protein profile of *Am. americanum* changes greatly over the course of the tick's bloodmeal (McSwain et al., 1982). The salivary glands produce and secrete saliva, and contained within it are a wide array of molecules including cement proteins, histamine and immunoglobulin binding proteins, anti-microbial peptides, proteins for anti-coagulation and analgesia, and other pharmacologically active compounds to successfully reach repletion. The salivary glands produce the saliva that enters the host's body throughout attachment, which can contain not only bacteria, fungi, and viruses, but also contain a plethora of foreign antigens that could be recognized by an immune system and elicit an immune response. Tick saliva itself contains proteins such as metalloproteinases that are typically found in various venoms (Bullard et al., 2016). The fact that tick saliva can potentially be considered venom is an often argued possibly because of the lack of localized reactions, or that the research focus is on the pathogens contained and transmitted within the saliva, but studies have shown that saliva from the tick *Ixodes holocyclus* in

Australia contains a cytotoxin that is capable of causing a condition known as "tick-paralysis" (Hall-Mendelin et al., 2011; Rodriguez-Valle et al., 2018).

1.6 Tick Carbohydrate Metabolism and Capture

Research has shown that some hard-ticks use the conventional methods of glucose metabolism including glycolysis, the pentose-phosphate pathway, and gluconeogenesis (Moraes et al., 2007). Carbohydrates, often in the form of glycogen, are stored with lipids and proteins in tick fat bodies. The fat bodies will synthesize storage proteins and trehalose, which will then be found in the hemolymph.

There are many various genera and species of ticks world-wide that have not had many scientific studies performed on them. Also, annotated genomes do not currently exist for most tick species. This presents a problem for researchers that want to study a tick species that is a non-model organism, as data resources will be limited or assumptions might be made based on more well studied and documented organisms like *Ixodes scapularis*, *Ixodes ricinus*, *Rhipicephalus microplus*, or *Haemaphysalis longicornis*.

Lectins, or haemagglutinins, are proteins that have carbohydrate binding sites, which are proteins that have carbohydrate binding sites located in various tissues of ticks. Each lectin/agglutinin has a different binding specificity in carbohydrate affinity. Lectins can serve in enhancing the blood meal and to assist in self/non-self recognition against bacteria or fungi that might be encountered during the blood meal (Grubhoffer et al., 2004). A novel galectin, *OmGalec*, found in the argasid tick, *Ornithodoros moubata*, was shown to have detectable binding affinity towards the Galili antigen pentose glycolipid (Galα1-

3,Galβ1-4GlcNAcβ1-3Galβ1-4Glc), however, this data was not discussed in the results or discussion of the manuscript (X. Huang et al., 2007a).

Midgut α-L-fucosidase activity in *Amblyomma cajennense* was described and characterized earlier (Moreti et al., 2013). The authors noticed that the enzyme cleaved α-L-fucosyl linkages solely at the non-reducing ends of fucoidan. The authors speculate that the secreted digestive enzyme plays a role in removing fucose residues from the glycans of glycolipids and glycoproteins to aid in the digestion process. They also speculated that it might play a protective role in bacterial and fungal infection, as they contain fucosylated compounds that aid in the relationship between host and parasite. It is important to recognize that ticks are equipped with glucosidase, galactosidase, fucosidase, and other carbohydrate cleaving and enzymes that can be used to aid in the metabolism and biosynthesis of various glycan structures. Human blood groups (A/B/O) involve the use of an α(1,2)fucosyltransferase (GDP-Fuc:Galβ 2-α-L-fucosyltransferase) to produce α1,2 fucosylations on blood group antigens (Lowe, 1993). Ticks could potentially cleave these core α1,2-fucosylations from human B-type blood with a fucosidase, leaving us with the alpha-gal antigen.

Ixodid ticks are the only arthropods known to be unable to synthesize heme (Braz et al., 1999; Donohue et al., 2009). It is believed that to alleviate this; ticks have developed multiple hemelipoglyco-carrier proteins (CP) that can bind, transport, and store host-blood heme (Gudderra et al., 2002; Donohue et al., 2008), most of which are still poorly understood. One of these carrier proteins is hemelipoglycoprotein (HLGP), which is one of the most abundant that has carbohydrate-binding specificity. A study showed that there

was strong binding affinity of *Dermacentor marginatus* HLGP towards galactose compared to other monosaccharides (Dupejova et al., 2011).

1.7 Glycosylation of Proteins

A broad understanding of the post-translational modifications that occur to proteins that glycans are attached to, modified on, and cleaved from are important concepts needed to be able to determine how various glycan configurations exist on glycoproteins. Oligosaccharides are assembled within the rough endoplasmic reticulum and are bound to a dolichyl phosphate lipid carrier as depicted in **Figure 1.5**. To summarize the figure drawn by Kornfeld & Kornfeld, 1985, the addition of each carbohydrate molecule occurs stepwise beginning with the addition of two GlcNAc species using UDP-GlcNAc, followed by nine mannose residues using GDP-mannose, and three glucose molecules are transferred on to the oligosaccharide chain. The transfer of the oligosaccharide from the lipid-linked donor to an asparagine residue is catalyzed by a complex, N-oligosaccharyl transferase (OST) containing dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunits (STT3A and STT3B). Terminal glucose residues are trimmed from the N-linked core glycans on the newly synthesized glycoproteins in the endoplasmic reticulum by α-glucosidase I followed by α-glucosidase II (Trombetta et al., 1996). Terminal mannose residues are then hydrolyzed by endoplasmic reticulum mannosyl-oligosaccharide 1,2-alpha-mannosidase, which trims the $Man_9GlcNAc_2$ to $Man_8GlcNAc_2$ (Tremblay & Herscovics, 1999). Proteins are then transported in an anti-retrograde direction from the ER to the cisternae of the Golgi apparatus through a set of transitional elements that bud at

transitional regions of the ER into vesicles, a concept that is still not fully understood. (Jamieson & Palade, 1968).

Inside the cis-Golgi, the core N-linked glycan structure is again trimmed by Golgi α-mannosidase I before being trafficked to the trans-Golgi. The structure is then elongated by adding an N-acetylglucosamine residue to the terminal mannose to the core glycan structure (Kornfeld & Kornfeld, 1985). The trans Golgi is the final Golgi compartment where post-translational modification of glycoproteins occurs. In mammals, the trans Golgi houses enzymes such as the α(1-3)galactosyltransferase family, which are responsible for catalyzing the biochemical reaction that transfers a UDP-galactose to N-acetyllactosamine, resulting in the terminal alpha-gal cap on N-glycans (Blanken & Van den Eijnden, 1985; Taylor et al., 2003).

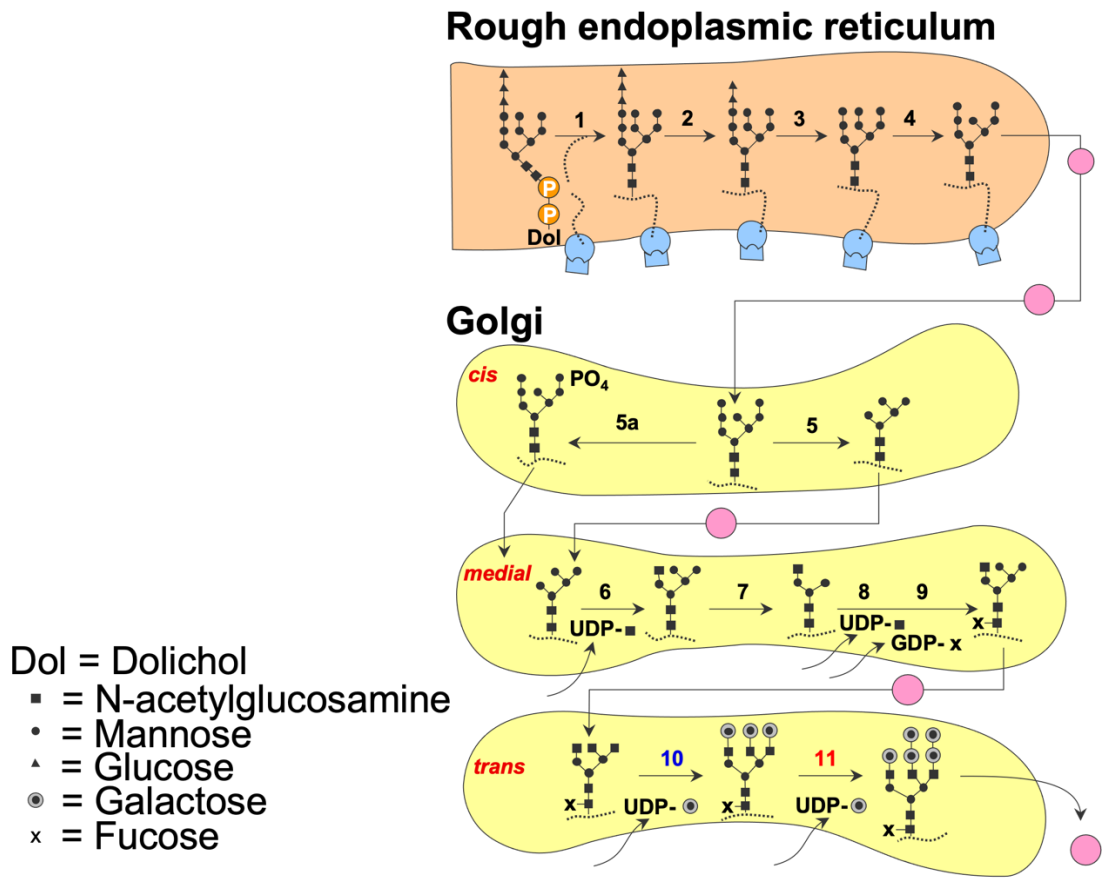


Figure 1.5 *Schematic pathway of oligosaccharide processing of N-linked glycoproteins in the Rough Endoplasmic Reticulum and Golgi network.*

The above figure depicts the processing pathway of freshly synthesized glycoproteins through the endoplasmic reticulum and the Golgi network (Figure made using Motifolio Molecular Cell Biology Illustration Toolkit). Enzymes used to modify glycan structures are (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II, (4) endoplasmic reticulum α-1,2-mannosidase, (5) Golgi α-mannosidase, (5a) N-acetylglucosaminylphosphotransferase, (6) N-acetylglucosaminyltransferase I, (7) Golgi α-mannosidase II, (8) N-acetylglucosaminyltransferase II, (9) fucosyltransferase, (10) β-1,4-galactosyltransferase, and (11) α-1,3-galactosyltransferase.

1.8 α-Galactose Structure/Variations/Blood Group Antigens

The alpha-galactosyl (α-gal) epitope is identified as a terminal galactose-alpha-1,3-galactose residue found at the end of a glycan chain. This epitope can be found on mammalian N-linked glycoproteins and O-linked glycolipids (Stults et al., 1989). The epitope can be found on many various antennary structures of N-linked glycans, and acts

as a terminal cap, but once capped, carbohydrates cannot be added to the glycan chain. α-gal is synthesized by adding an alpha-linked galactose to N-acetyllactosamine through an enzymatic reaction involving α-1,3-galactosyltransferase and uridine diphosphate galactose (UDP-gal) in the trans-network of the Golgi apparatus. Another carbohydrate, N-acetylneuraminic acid, can also be added to N-acetyllactosamine as a terminal cap of a glycan structure. α-gal moieties, in many various glycan configurations, can be readily found on the glycoproteins of non-primate mammals (**Figure 1.6**).

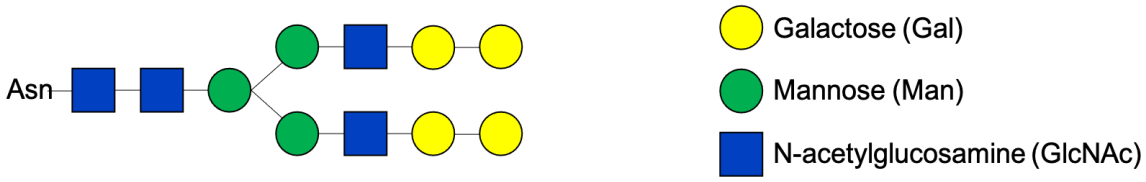


Figure 1.6 *Alpha-gal containing biantennary N-Glycan structure.*

N-Glycans can be found linked to asparagine residues at glycosylation sites on the surfaces of glycoproteins. This is an example of a non-fucosylated bi-antennary containing two terminal alpha-gal structures.

Human blood group B is similar to the α-galactosyl epitope, except the human blood group antigen is fucosylated at the α-1,2 position (**Figure 1.7**), which prevents antibodies that specifically target the α-gal epitope from binding at that location, which would result in an autoimmunity situation (Galili, 1989). It was speculated that aging erythrocyte cells of group B blood can express cryptic α-gal epitopes that will be bound by anti-gal antibodies and would lead to cell lysis (Galili, 1988).

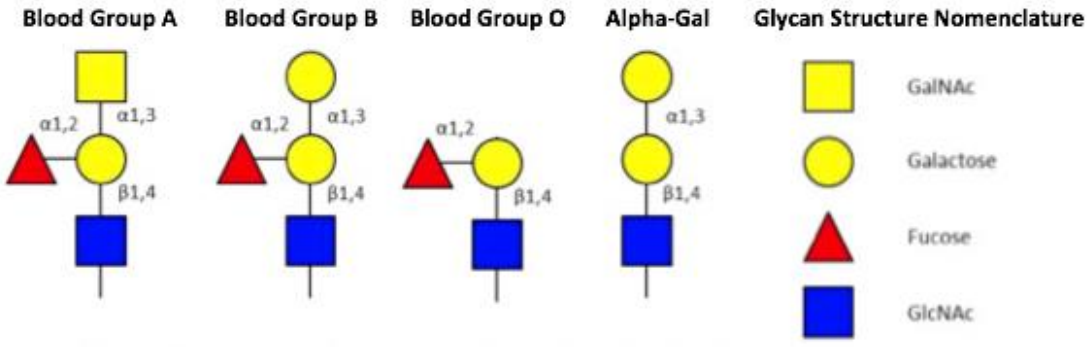


Figure 1.7 *Glycan structures of human blood group antigens and alpha-galactosyl epitopes.*

Images depicts blood groups A, B, and O and the alpha-gal epitope (Image modified from Platts-Mills et al., 2015).

1.9 Anti -Gal Antibody and Alpha-Galactose History in Xenotransplantation

Uri Galili's studies in the 1980's found that in humans, ~1% of all circulating IgG antibodies were specific to α-galctosyl epitopes, Galα1-3Gal-R or Galα1-4Gal-R (Galili et al., 1984). Later discoveries included that the anti-Gal antibody would bind to various bacteria using a variety of antibody binding assays, including *Serratia minnesota*, the capsule of *Escherichia coli* O86, and the lipopolysaccharide (LPS) of *Klebsiella pnuemoniae* strain 18022. They also discovered that the anti-Gal antibody titer would be increased after protozoan infections of *Trypanosoma cruzi* and *Leishmania mexicana* (Galili et al., 1988). Later research uncovered that the natural anti-Gal antibody found in old-world monkeys and humans would reject pig xenografts due to recognition of Galα1-3Galβ1-4GlcNac-R epitopes on the pig cells (Uri Galili, 1993; Sandrin et al., 1993). The natural Anti-Gal antibody exists in humans with all blood types, but humans also have Anti-Gal A and Anti-Gal B antibodies (**Figure 1.8**). Together, they are responsible for

ABO incompatibility and xenograft rejection of mammalian sourced organs or tissues in xenotransplantation (Uri Galili, 2006).

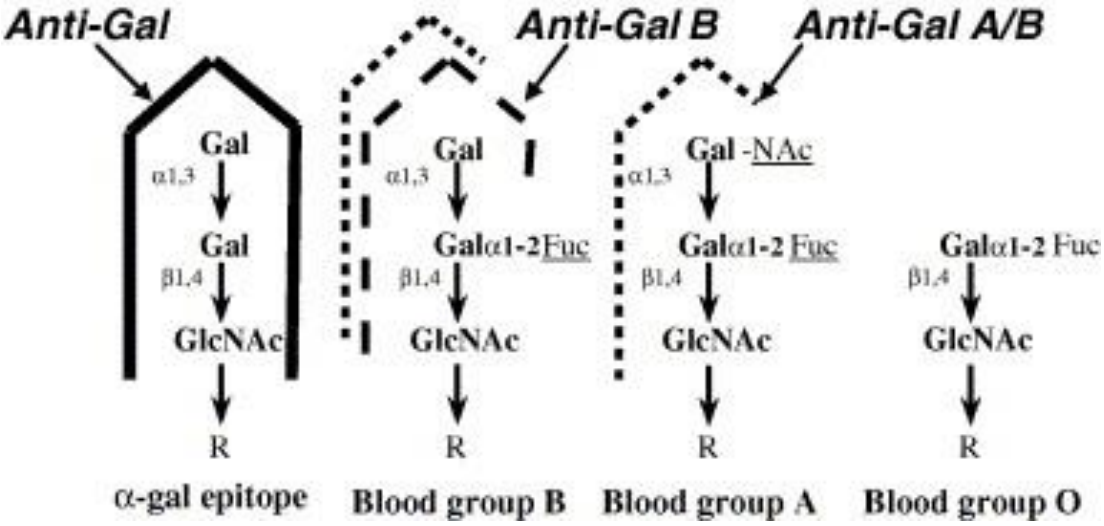


Figure 1.8 *Binding of anti-gal antibodies to human blood group antigens.*

Gal – galactose, Fuc – fucose, GlcNAc – N-acetylglucosamine, Gal-NAc – N-acetylgalactosamine. (Figure from Galili, 2006.)

1.10 Alpha-Gal Syndrome (AGS) Red Meat Allergy (Hypersensitivity)

The initial discovery of AGS involves Cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR) used to treat several types of cancers, including metastatic colorectal cancer and squamous-cell carcinoma. Cetuximab has been linked with inducing rapid-onset and often severe hypersensitivity reactions following infusions in certain patients in the Southeastern United States. Surveillance of IgE antibodies against Cetuximab from patients with or with and without hypersensitivity reactions to Cetuximab, and control participants showed that the control participants from Tennessee had elevated IgE levels versus participants from California and Massachusetts (Chung et al., 2008; Commins et al., 2009; O'Neil et al., 2007). Cetuximab is a chimeric antibody therapeutic produced in the SP2/0 murine myeloma cell line that expresses a gene for α-1,3-

galactosyltransferase. Cetuximab also contains multiple glycosylations on the Fc region at Asn88 and on the heavy chain of IgG_1 on the chimeric antibody at Asn299 (Qian et al., 2007), and possibly at the glycosylation site of V_H at Asn43 as depicted in **Figure 1.9** (Chinuki & Morita, 2019). The α-gal-containing glycosylations on the cetuximab Fab binding domain permit the cross-linking of IgE on mast cells (Chung et al., 2008). Natural exposure to α-gal in the diet can promote the production of anti-α-gal IgE in some, and it is known that catarrhine mammals have a pseudogene for *GGTA1*, which codes for α-1,3-galactosyltransferase, and at least of 1% of all circulating IgG in the body is specific for α-gal (Galili et al., 1984; Koike et al., 2007). However, Cetuximab produced in Chinese hamster ovary (CHO) cells lack α-1,3-galactosyltransferase and thus will not induce an IgE response (Qian et al., 2007; Restelli & Butler, 2002). A recent discovery is that patients with AGS might also suffer anaphylaxis during the treatment of envenomation from Crotalidae pit vipers using sheep-derived Crotalidae-polyvalent Fab antivenom (CroFab) treated with α-galactosidase (Straesser et al., 2021). Interestingly, more than half of all AGS patients that were part of a study in Sweden were found to also be sensitized to wasp venom, but to proteins rather than cross-reactive carbohydrate determinants (Kiewiet et al., 2022).

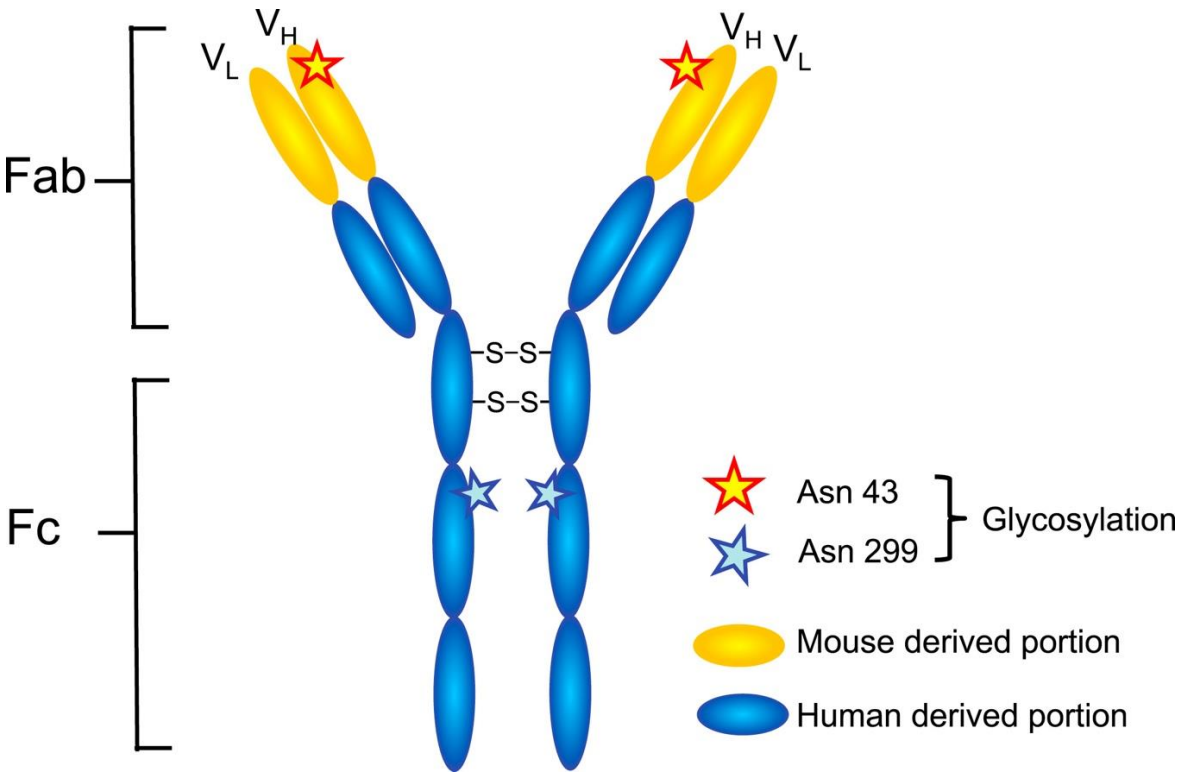


Figure 1.9 *Glycosylation sites of Cetuximab containing α-gal.*

The glycosylation sites on the structure of the Cetuximab antibody that contain galactose-α-1,3-galactose. Figure from (Chinuki & Morita, 2019).

Alpha-Gal Syndrome was originally classified as an idiopathic hypersensitivity reaction characterized by the presentation of angioedema, urticaria, and anaphylaxis. Patients typically reported consumption of red-meat products 3-6 hours prior to symptoms, with cofactors such as exercise and alcohol affecting the time to onset of reaction. Typical skin prick assays with commercial meat extracts followed by sera sIgE testing confirmed that α-gal was the instigator of the episodes (Commins et al., 2009; Fischer et al., 2014). Many patients reported that they had issues tolerating red meat-products in the past. Sometimes, cessation of red-meat products alone was not enough to prevent recurrences of

the reactions. Still, some other mammalian-derived food products, such as gelatin, need also be avoided (Commins et al., 2016).

1.11 Alpha-Gal Syndrome or "Red Meat Allergy" Caused by Ticks?

There have been reports from around the world that implicate various tick species involved with the onset of Alpha-Gal Syndrome (AGS). The first documented account was by a physician in Australia that believed there was a connection between carbohydrate cross-reactivity after ingestion of red-meat products and bites from *Ixodes holocyclus* ticks (Van Nunen et al., 2009). A short time later, another group began to notice a similar phenomenon in populations in the Southeastern United States, but this time, *Am. Americanum* was the center of attention (Commins et al., 2011; Commins & Platts-Mills, 2009, 2013a, 2013b). *Am. americanum* larval or "seed" ticks and nymph and adult stage ticks are known to attach to human hosts (Duckworth et al., 1985; Jones, 1981). The group's initial research began to link hypersensitivity reactions to a monoclonal antibody, Cetuximab (Commins et al., 2009), in patient populations with increased risk for tick bites in the Southeastern United States. Later, there were reports of similar reactions to Cetuximab and ingestion of mammalian meat products in Sweden (Jacquenet et al., 2009; Nuñez et al., 2011). Another research group in Sweden reports a detectable amount of α-galactosyl epitopes found in the gastrointestinal tract of a hard tick, *Ixodes ricinus*, using an anti-gal antibody (Hamsten et al., 2013). In Japan, *Haemaphysalis longicornis* and *Amblyomma testudinarium* have also been suspected of being a primary instigator in inducing hypersensitivity reactions in humans (Chinuki et al., 2015; Fujiwara & Araki, 2019; Hashizume et al., 2018; Kondo et al., 2017).

The exact mechanism by which ticks potentially sensitize humans to α-gal leading to Alpha-Gal Syndrome or delayed-type hypersensitivity reactions is still not fully understood. It is believed that sensitization to α-gal is caused by multiple typical innate and adaptive immune cells including memory B cells, dendritic cells, CD4+, CD27+, and Th2 cells, but there might still be an unknown underlying mechanism involved in sensitization (Carson et al., 2022; Chandrasekhar et al., 2020). Still, there are many groups globally that continue working towards finding the answers.

CHAPTER II – SIGNIFICANCE AND HYPOTHESIS

2.1 Significance

Alpha-gal Syndrome (AGS), which can be potentially life-threatening, has become increasingly prevalent in the southeastern United States since it was first reported over a decade ago (Commins & Platts-Mills, 2009). Delayed-type hypersensitivity reactions including urticaria and anaphylaxis occur hours after ingesting most types of mammalian-derived food products (Commins et al., 2009). Many of the reports of AGS onset occur in patients that have a history of tick bites (Commins et al., 2011). Most aspects of tick involvement or the biochemical mechanisms at play are still unknown, and little research has been published on North American ticks to determine the exact cause of the onset of AGS in patients with a history of tick bites. Before this research began, it was unknown if any North American ticks contained α-gal in their saliva, salivary gland tissues, or midgut tissues. Discovery of α-gal containing tick biomarkers and an understanding of the mechanisms involved with the onset of AGS have the potential of paving a way forward for the clinical management of patients with AGS.

2.2 Hypothesis

I believe that tick bites can mechanically introduce α-gal-containing glycoproteins to a human host during the process of blood-feeding, resulting in sensitization to the α-gal containing glycans and hypersensitivity reactions upon future exposure to the antigen.

CHAPTER III - MATERIALS AND METHODS*

3.1 Ethics statement

All animal experiments were conducted according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. The protocol of tick blood-feeding on the sheep was approved by the Institutional Animal Care and Use Committee of the University of Southern Mississippi (protocol # 15101501) (**Appendix A**). All efforts were made to minimize animal suffering.

3.2 Materials

Unless otherwise specified, all common laboratory supplies and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Bio-Rad (Hercules, CA, USA), or Fisher Scientific (Grand Island, NY, USA).

3.3 Ticks and other animals

The Lone Star tick (*Amblyomma americanum*), Gulf-Coast tick (*Amblyomma maculatum*), the American Dog tick (*Dermacentor variabili*s), and the Black-legged tick (*Ixodes scapularis*) were maintained at the University of Southern Mississippi according to the established methods (Patrick CD, 1975). Unfed adult ticks were purchased from Oklahoma State University's tick rearing facility (Stillwater, OK, USA). Adult ticks were kept at room temperature with approximately 90% relative humidity under a photoperiod of 14 hours of light and 10 hours of darkness before infestation on sheep. Adult ticks were

* This chapter contains previously published work (Crispell et al., 2019).

blood-fed on sheep and removed at various intervals between 1 and 11 days, depending upon the experimental protocol.

3.4 Artificial membrane feeding system used to feed human blood to *Am. americanum* ticks

A silicone membrane-based artificial feeding system was used to feed human blood to ticks using an original developed method (Kröber & Guerin, 2007) with slight modifications. Briefly, membranes were constructed by covering lens paper in a silicone oil solution and formed by sweeping the paper across the surface of the oil (Bullard et al., 2016). Each membrane was allowed to dry for a minimum of 48 hours to ensure that the silicone cured properly. After curing, a fiberglass mesh was glued to the surface of the membranes using silicone adhesive and then adhered to the acrylic chambers. The silicone cured for a minimum of 24 hours and the seal was verified by soaking the chamber in 70% ethanol for 20 minutes. A total of 10 virgin females and 4 males were placed into each feeding chamber, along with sheep's wool on the surface of the silicone membrane to compensate for the host odor. Defibrinated whole human blood (Bioreclamation IVT, NY, USA) was purchased for artificial feeding of human blood to ticks in this study. Human blood was stored at 4°C until utilized when 3-4 mL aliquots were warmed to 37°C, and then added to a single well of a 6-well plate. The feeding chamber was placed into the well in a way that the membrane came into direct contact with the human blood. Each chamber was plugged using a cotton stopper to isolate the ticks to that area. To maintain the optimal temperature for feeding, the feeding system was placed in a 37°C incubator. The blood was replaced twice daily, and the membrane was rinsed with 1X PBS containing a 5% Pen/Strep

antibiotic mix. The six-well plate was also cleaned with water and the antibiotic solution at each blood change. The ticks were observed multiple times daily for interactions with the membrane. The inner surface of the chamber was also checked daily to visualize any changes in tick attachment rates, tick size increase, and to monitor feeding or tick mortality.

3.5 Tick tissue dissections and saliva collection

The unfed and partially blood-fed female adult ticks were dissected within four hours after removal from the sheep as described previously (Karim et al., 2002; Karim et al., 2011). Tick tissues were dissected, and washed gentle with ice-cold M-199 buffer (J F Morgan et al., 1950; Joseph F. Morgan et al., 1955). Tissues were stored immediately after dissection at -80°C in 0.15M Tris-HCl, pH 8.0, containing 0.3M NaCl, 10% glycerol, and 1% protease inhibitor cocktail (Amresco, OH, USA). Tick saliva was collected by inducing partially blood fed female *Am. americanum* to salivate into capillary tubes using the modified pilocarpine induction method as described previously (Ribeiro et al., 2004; Valenzuela et al., 2000). The saliva was stored immediately at -80°C until downstream western blot analysis.

3.6 Protein extraction

Proteins were solubilized from dissected pooled tick salivary glands and midgut tissues (n=5 ticks) in a protein extraction buffer consisting of 0.5 M Tris-HCl, pH 8.0, 0.3 M NaCl, and 10% glycerol, and 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. Homogenates were centrifuged at 5000 x g for 10 min at 4°C and the supernatants were collected. Protein concentrations were estimated using the Bradford method (Bradford, 1976), and protein was stored at −80°C.

3.7 SDS-PAGE and Western Blotting

Extracted proteins from the midguts (15 µg), salivary glands (15 µg), and saliva (10 µg) were fractionated on a Mini-PROTEAN TGX Any kD, 7.5%, or 4%–20% gels (Bio-Rad) using SDS-PAGE and were then transferred onto nitrocellulose membrane in a Transblot cell (Bio-Rad). The transfer buffer comprised 25 mM Tris-HCl and 192 mM glycine in 20% methanol. Nonspecific protein binding sites were blocked 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) 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. Membranes were incubated overnight at 4°C with human serum samples at a dilution of 1:200 in TBST with 5% BSA, and an anti-IgE antibody was used to detect bound antibodies.

3.8 Deglycosylation of *Am. americanum* salivary proteins

Peptide-N-glycosidase F (PNGase F) was used for the deglycosylation of tick salivary glands and saliva glycoproteins as per the manufacturer's instructions. Briefly, the

tick protein samples (150 µg) were incubated with PNGase (15 units) at 37°C for 3 h, then the reactions were stopped by heating to 100°C for 5 min. Deglycosylation efficacy of α-gal was assessed by SDS-PAGE and western blotting using the anti-α-gal IgM antibody (Enzo).

3.9 Protein Analysis

Selected bands were excised from the gels, and band excisions were placed in water and shipped to MS Bioworks (Ann Arbor, MI, USA) for trypsin digestion and LC-MS/MS analysis of resulting peptides. Gels were washed with 25mM ammonium bicarbonate followed by acetonitrile. They were reduced with 10mM dithiothreitol at 60°C, and were then alkylated using 50mM iodoacetamide at room temperature. Samples were then digested with trypsin (Promega, Madison, WI, USA) at 37° C for 4 hours. The reaction was then quenched using formic acid. Half of each sample was analyzed using nano LC-MS/MS with HPLC system (Waters NanoAcquity, Milford, MA, USA) interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75um analytical column at 350nL/min, and both columns were packed with Luna C18 resin (Phenomenex, Torrance, CA, USA). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS. Data were searched using a local copy of Mascot against the UniProt *Ixodes scapularis*, and NCBI *Amblyomma americanum* databases with monoisotopic mass values, 10ppm peptide mass tolerance, 0.002 Da fragment mass tolerance, and maxed missed cleavages of 2. The Mascot DAT files were parsed into Scaffold (Proteome Software, Portland, OR, USA) for

validation, filtering, and to create a non-redundant list per sample. Data were filtered using 1% protein and peptide false discovery rate (FDR) and requiring at least two unique peptides per protein. Data are available on the ProteomeXchange by using identifier PXD012827.

3.10 Immunolocalization of α-Galactosyl Epitopes

Immunolocalization studies of α-galactose were performed on partially fed salivary glands from *Am. americanum, De. Variabilis,* and *Ix. scapularis*. The tick salivary glands were fixed in 1X PBS containing 4% formaldehyde and stored at 4 °C. The salivary glands were permeabilized using 0.1% Triton X-100 (Sigma-Aldrich, MO, USA) for 30 min and then blocked in 1X PBST containing 5% bovine serum albumin (BSA) for 1 hour at room temperature. Salivary glands were incubated overnight at 4 °C in α-galactose IgM antibody (1:20; Enzo Life Sciences, Farmingdale, NY, USA) in 1X PBST containing 5% BSA, after which they were incubated with an Alexa Fluor 546 goat anti-mouse IgM secondary antibody (1:100) (Life Technologies, Camarillo, CA, USA), and fluorescent dye 633-I phalloidin (1:100) (Abnova, Walnut, CA, USA) in 1× PBS containing 5% BSA for 1 h in the dark. All incubations and washes were maintained on a rocking plate at room temperature unless otherwise indicated. Salivary glands were mounted on glass slides using PROLONG Gold anti-fade reagent with DAPI (4’,6-diamidino-2-phenylindole) (Life Technologies, CA, USA) mounting medium. Tissues prepared in this manner were mounted and viewed under a Zeiss LSM 510 META confocal microscope running ZEN 2009 software (Zeiss, Heidelberg, Germany), using the 10X, 20X, 40X, 63X, and 100X objectives and the 405nm, 545nm, and 633nm wavelength lasers.

3.11 Magnetic Pull-down of α-Galactosyl Containing Proteins

Proteins with terminal α-galactosylations were removed from partially fed *Am. americanum* salivary gland tissue homogenates using DynaBeads α-mouse IgM magnetic beads (Invitrogen, Carlsbad, CA, USA). Briefly, mouse IgM specific magnetic beads were incubated with the α-gal IgM (M86) antibody overnight on a rocker at 4˚C. Antibody/bead complexes were separated from supernatant, and tick salivary gland homogenates were added to the tube and incubated for 20 minutes at 4˚C. Supernatant was removed, and product was washed three times for 5 minutes. Protein and antibodies were released from beads using elution buffer. Products were run in an immunoblot assay to determine successful pull-down, and corresponding bands were excised and sent for LC-MS/MS analysis.

3.12 N-Linked Glycan Profiling

N-linked glycans were released from *Am. americanum, Ix. scapularis,* or *Am. maculatum* unfed salivary glands, partially-fed salivary glands and saliva with an estimated protein concentration of 200 μg, after they were 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 a low w/w percentage acetic acid and the glycopeptides were eluted with a blend of isopropanol in low concentration acetic acid, before being dried by SpeedVac. The dried glycopeptides eluate was treated with a combination of PNGase A and PNGase F 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 permethylated for structural characterization by mass spectrometry

(Anumula & Taylor, 1992). 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 N_2. The permethylated glycans were dissolved with methanol and crystallized with α-dihydroxybenzoic acid (DHBA) matrix. Analysis of glycans present in the samples was performed by MALDI-TOF/TOF-MS using AB SCIEX TOF/TOF 5800 (Applied Biosystems). The permethylated glycans were reconstituted in 100% MeOH and introduced to the mass spectrometer (Thermo Fusion Tribrid Orbitrap) with offline emission. 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 (CID). This data was used to search for a Hex-Hex-HexNAc signature, both with a diagnostic fragment as well as expected neutral losses.

3.13 Indirect basophil activation test

The indirect basophil activation assay experiments were performed by the Commins laboratory at UNC Chapel Hill (NC, USA). For this assay, peripheral blood mononuclear cells (PBMCs) that were acquired from a healthy, non-α-gal allergic donor (a-gal sIgE<0.10) were isolated utilizing a Ficoll–Paque gradient (GE Healthcare, Chicago, IL, USA). Endogenous IgE was stripped from the basophils within the PBMC fraction by incubating the cells with a cold lactic acid buffer (13.4mM lactic acid, 140mM NaCl, 5mM KCl) for 15min. Basophils were the sensitized with plasma from α-gal allergic and non-allergic subjects overnight in RPMI 1640 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 30min with RPMI media, cetuximab (10 μg), rabbit anti-human IgE (1 μg; Bethyl Laboratories Inc., Montgomery, TX, USA), saliva from *Am. americanum* (10 μL), or the partially-fed salivary gland extracts from *Am. americanum* (50 μg), *Am. maculatum* (50 μg), or Ix. scapularis (50 μg). Stimulation reactions were stopped with 20mM 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 Prismversion 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.

3.14 Biotinylated Isolectin B4 capture of α-gal containing proteins

Briefly, Streptavidin HP SpinTrap columns (GE Life Sciences) were loaded with 100 μL of Biotinylated *Griffonia simplicfolia* lectin I (GSL I) B4 (Vector Laboratories, Newark, CA, USA) and 100 μL of binding buffer (TBS, 50 mM Tris, 150 mM NaCl, pH 7.5) for a final concentration of 0.25 mg/mL. The spin column was slowly inverted for one hour at room temperature and centrifuged at 150 RCF. Blocking buffer (TBS, 2mM biotin) was added to the spin columns and allowed to incubate before centrifugation at 150 RCF.

Tick salivary gland proteins were added to the spin column and inverted gently for 10 minutes before centrifugation at 150 RCF. Column was rinsed by a series of 5 washes with wash buffer (TBS, 2M urea, pH 7.5) and centrifuged at 150 RCF each time. Elution was performed using elution buffer (TBS, 0.1M glycine, 2M urea, pH 2.9) and centrifuged at 1,000 RCF.

CHAPTER IV RESULTS*

4.1 Screening of Multiple Tick Species for the presence of α-gal

The species chosen for this study were *Ix. scapularis*, *Am. maculatum, Am. americanum, and De. variabilis* because they are the most prevalent tick species in found in the Southeastern region of the United States, where initially there existed a majority of the AGS-reported cases. In the first experiment, tissues were assessed from the unfed and various fed states of *Am. americanum* using immunoblot analysis. The results indicated that the anti-α-gal antibody had cross-reactivity to partially-fed *Am. americanum* salivary glands and saliva (**Figure 4.1**). Intriguingly, these results showed that unfed *Am. americanum* salivary glands and both unfed and fed midguts lacked any cross-reactivity with anti-α-gal antibodies.

Lack of α-gal cross-reactivity in the immunoblots of unfed lone-star tick tissues prompted us to determine the time-dependent expression of α-gal-containing antigens from one day post-infestation (dpi) to 8 dpi (**Figure 4.2**). Immunoblotting revealed that the expression of α-gal-containing antigens appeared in salivary tissues in a time-dependent manner throughout the feeding process. Saliva from partially-blood-fed *Am. americanum* ticks cross-reacted with anti-α-gal antibodies; however, *Am. maculatum* saliva antigens exhibited no reactivity.

* This chapter contains previously published work (Crispell et al., 2019).

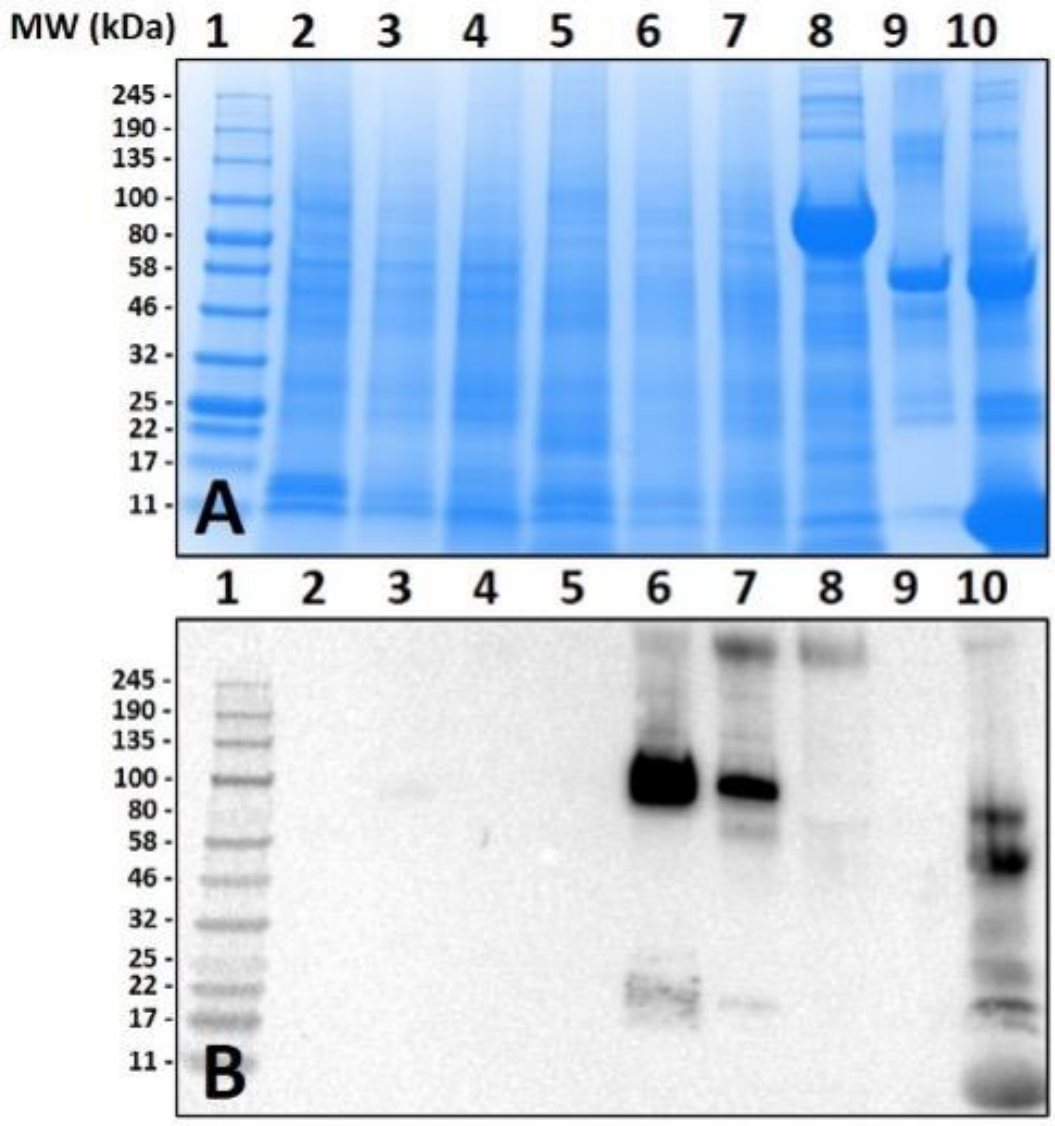


Figure 4.1 *Screening of proteins from Amblyomma americanum for the presence of α-gal.*

Unfed and partially-fed gut tissue and salivary gland tissue homogenates and saliva were run using A) 7.5% SDS-PAGE, B) western blot using anti-gal IgM antibody. Lane 1: broad range (11–245 kDa) pre-stained protein standard, Lane 2: *Am. americanum* unfed midgut tissue, Lane 3: *Am. americanum* 3D partially-fed midguts, Lane 4: *Am. americanum* 11D partially-fed midguts, Lane 5: *Am. americanum* unfed salivary glands, Lane 6: *Am. Americanum* 3D partially-fed salivary glands, Lane 7: *Am. americanum* 11D partially-fed salivary glands, Lane 8: *Am. americanum* 8D saliva (pilocarpine induced), Lane 9: Bovine serum albumin and, Lane 10: Diluted sheep blood.

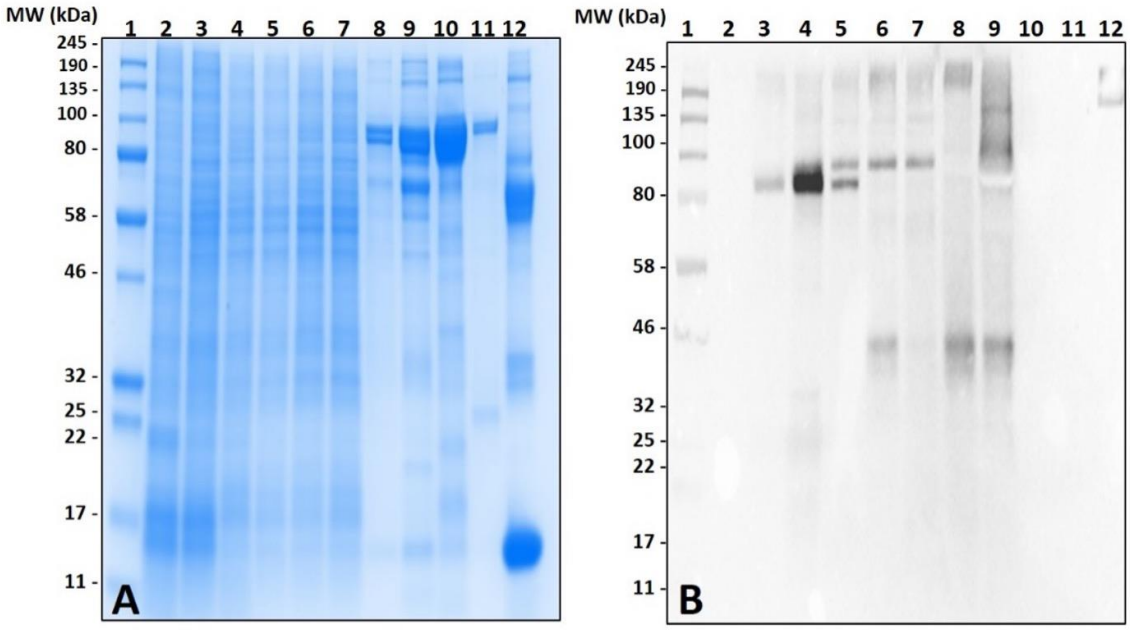


Figure 4.2 *Identification of α-gal in the salivary glands of Amblyomma americanum along the blood meal*

The unfed and partially-fed salivary glands from *Am. americanum* throughout the blood meal were analyzed, along with saliva from *Am. americanum* and *Am. maculatum* drooled with pilocarpine and dopamine. A) SDS-PAGE using Any kDa Mini-PROTEAN TGX gel, B) western blot using anti-gal IgM antibody. Lane 1: A broad range (11–245 kDa) pre-stained protein standard, Lane 2: *Am. americanum* unfed salivary glands, Lane 3: *Am. americanum* 1D partially-fed salivary glands, Lane 4: *Am. americanum* 3D partially-fed salivary glands, Lane 5: *Am. americanum* 5D partially-fed salivary glands, Lane 6: *Am. americanum* 7D partially-fed salivary glands, Lane 7: *Am. americanum* 8D partially-fed salivary glands, Lane 8: *Am. americanum* saliva (dopamine), Lane 9: *Am. americanum* saliva (pilocarpine), Lane 10: *Am. maculatum* saliva (pilocarpine), Lane 11: *Am. maculatum* saliva (dopamine) and, Lane 12: Diluted bovine blood.

Surprisingly, the cross-reactivity of *Ix. scapularis* salivary antigens differed between unfed and partially-fed tissues collected at various time points (**Figure 4.3**). These results depict the immune-reactivity of α-gal antibodies to unfed tissue antigen sizes ranging from 32–50 kDa, and 245 kDa and higher molecular weights. However, the blood meal induces salivary antigens to cross-react with α-gal antibodies consistently in the range of 100–135 kDa (**Figure 4.3B**). Interestingly, α-gal antibodies cross-reacted with unfed

midgut tissue antigens of *Ix. scapularis*, and upon blood feeding this reactivity disappeared (**Figure 4.3D**).

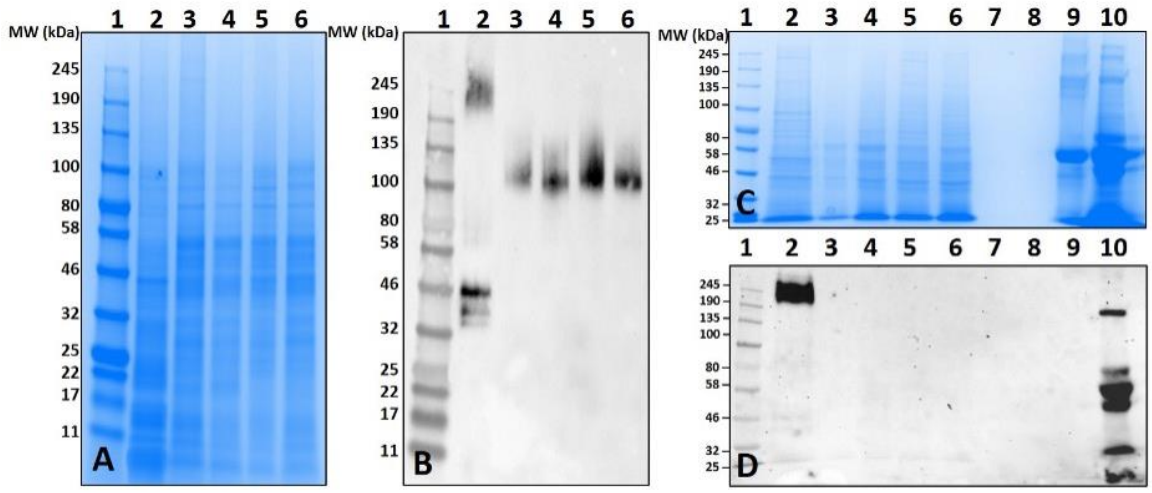


Figure 4.3 *Identification of α-gal in the salivary gland and gut tissues of Ixodes scapularis along the blood meal*

A) SDS-PAGE of *Ix. scapularis* salivary glands using 7.5% Mini-PROTEAN TGX, B) western blot using anti-gal IgM antibody. Lane 1: A broad range (11–245 kDa) pre-stained protein standard, Lane 2: *Ix. scapularis* unfed SG, Lane 3: *Ix. scapularis* 3D partially-fed SG, Lane 4: *Ix. scapularis* 5D partially-fed SG, Lane 5: *Ix. scapularis* 6D partially-fed SG, Lane 6: *Ix. scapularis* 8D partially-fed SG. C) SDS-PAGE of *Ix. scapularis* midgut tissues. Lane 1: A broad range (11–245 kDa) pre-stained standard, Lane 2: *Ix. scapularis* unfed gut tissues, Lane 3: *Ix. scapularis* 3D partially-fed gut tissues, Lane 4: *Ix. scapularis* 5D partially-fed gut tissues, Lane 5: *Ix. scapularis* 6D partially-fed gut tissues, Lane 6: *Ix. scapularis* 8D partially-fed gut tissues, Lane 7-8: blank. Lane 9: Bovine serum albumin and, Lane 10: Diluted sheep blood. D) Western blot using anti-gal IgM antibody.

When investigating *Am. maculatum* saliva, salivary glands and gut tissues during various time-points of the tick blood-meal for the presence of α-gal using the same immunoblotting technique, there were no detectable amounts of signal found under any condition when imaging the blots (**Figure 4.4B**). Similarly, no evidence of α-gal was present in *De. variabilis* unfed or partially-fed salivary glands (**Figure 4.5B**).

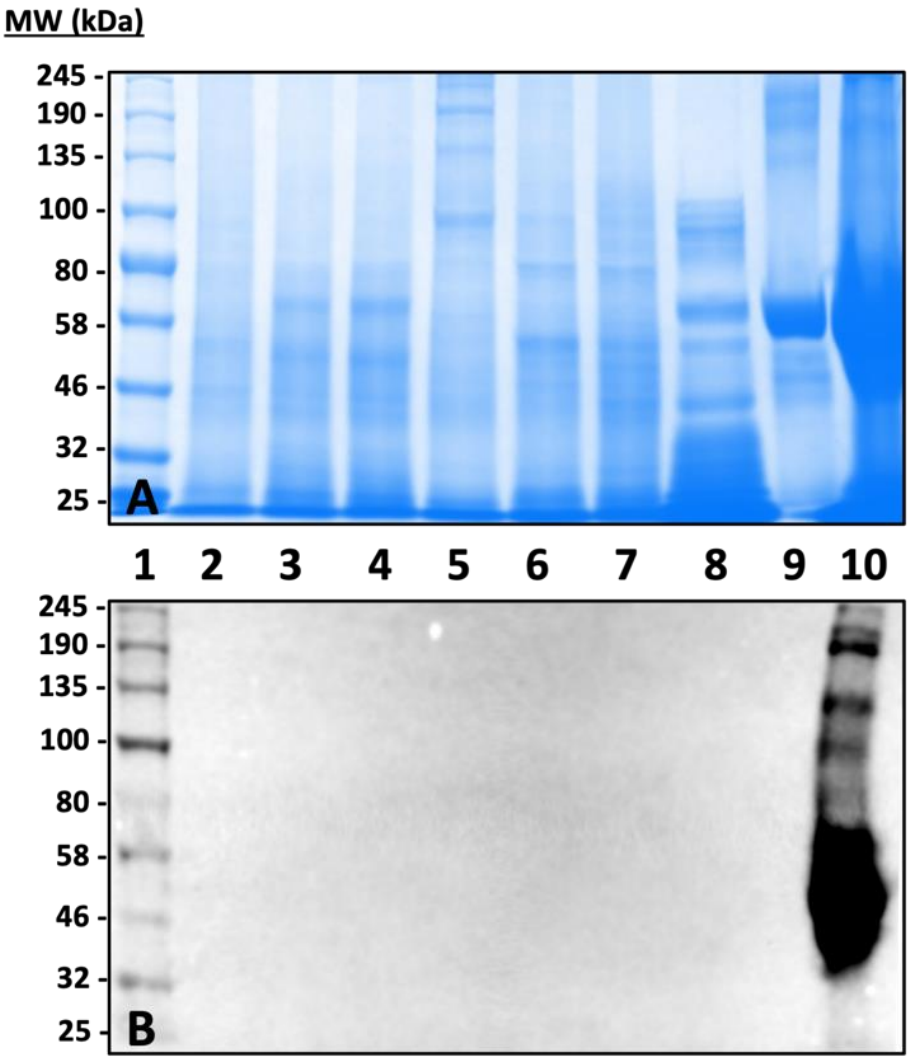


Figure 4.4 *Screening of proteins from Amblyomma maculatum for α-gal*

Unfed and partially-fed gut tissue and salivary gland tissue homogenates and saliva were run using **A)** 12.5% SDS-PAGE, and **B)** western blot using anti-gal IgM antibody. Lane 1: A broad range (11–245 kDa) pre-stained protein standard, Lane 2: *Am. maculatum* unfed midgut tissue, Lane 3: *Am. maculatum* 3D partially-fed midgut tissue, Lane 4: *Am. maculatum* partially-fed 8D midgut tissue, Lane 5: *Am. maculatum* unfed salivary glands, Lane 6: *Am. maculatum* 3D partially-fed salivary glands, Lane 7: *Am. maculatum* 8D partially-fed salivary glands, Lane 8: *Am. maculatum* 8D saliva, Lane 9: Bovine serum albumin and, Lane 10: Diluted sheep blood.

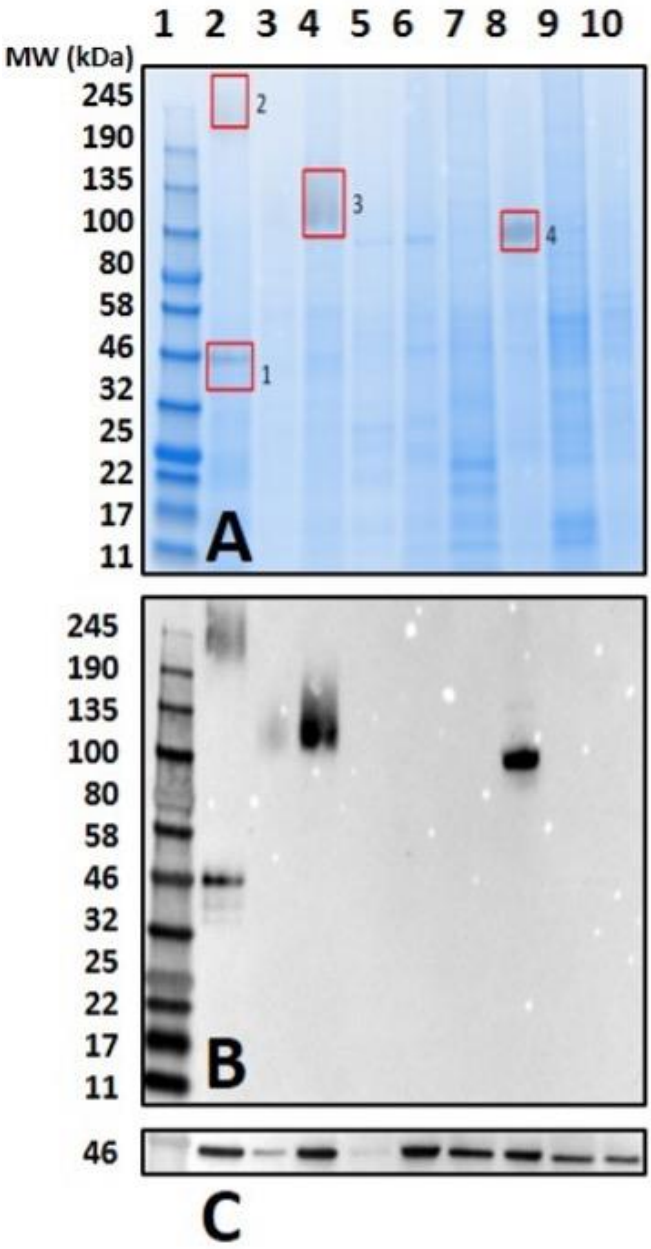


Figure 4.5 *Screening of various tick salivary proteins for the presence of α-gal and the immunoproteomic workflow*

Unfed and partially-fed salivary gland (SG) tissue homogenates from *Ix. scapularis*, *Am. maculatum*, *Am. americanum*, and *De. variabilis* were run using A) an overlay of gel and blot showing excised bands for mass spectrometry analysis. Lane 1: broad range pre-stained protein standard. Lane 2: *Ix. scapularis* unfed SG. Lane 3: *Ix. scapularis* 5D partially-fed SG. Lane 4: *Ix. scapularis* 5D partially-fed SG. Lane 5: *Am. maculatum* unfed SG. Lane 6: *Am. maculatum* 5D partially-fed SG. Lane 7: *Am. americanum* unfed SG. Lane 8: *Am. americanum* 5D partially-fed SG. Lane 9: *De. variabilis* unfed SG. Lane 10: *De. variabilis* 5D partially-fed SG. B) Western blot using anti-gal IgM antibody, C) western blot probed using Beta-actin monoclonal antibody.

4.2 Proteomic analysis of gel excisions

Mass spectrometry (LC/MS-MS) analysis of gel excisions (Red boxes in **Figure 4.5A**) revealed numerous protein peptides of various functions (**Table 4.1** and ProteomeXchange PXD012827). The excision of *Am. americanum* salivary glands contained peptides from proteins such as an abundant hemelipoprotein precursor that contains a VWD domain, glucose-regulated protein grp-94/endoplasmin hsp90 family, and endoplasmic reticulum resident protein glycosyltransferases. Unfed *Ix. scapularis* contained multiple glycoside hydrolases including α-L-fucosidase and α-D-galactosidase, enzymes known to cleave terminal α-L-fucosides and α-D-galactosides. The unfed salivary glands also contained numerous lectins including galectin, hemolectin, and a mannose-binding endoplasmic reticulum-Golgi compartment lectin. In the unfed *Ix. scapularis* salivary glands, there was also observed oligosaccharyl transferases, a sugar transporter protein capable of transporting galactose, heat shock proteins, hemomucin, heme lipoproteins or heme lipoglycoproteins, and ixoderin B. In the partially-fed *Ix. scapularis* salivary glands, it was observed that there were glycoside hydrolases including alpha-mannosidases, alpha-glucosidases, lysosomal glucosidase, and glucosidase II containing a galactose mutarotase domain. Additionally, there were a few glycosyltransferases including oligosaccharyltransferase and α-1,3-glucosyltransferase.

Table 4.1

Select proteins identified from mass spectrometry of gel slices

Sample	Protein identified	Accession	SpC
UF IS LMW (Gel slice 1)	α-L-Fucosidase	ISCW012071 ISCW012070 ISCW003071	2 4 1
	Mannose binding ER-Golgi compartment lectin	ISCW016179	9
	Galectin	ISCW008553	3
	α-D-Galactosidase	ISCW018185 ISCW018187 ISCW018188	17 1 11
	Heme lipoprotein (putative)	ISCW021710	3
	Hsp90 protein (putative)	ISCW014265	15
	Ixoderin B	ISCW013797	2
	Hemomucin	ISCW018609	2
	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase 48 kDa subunit	ISCW017658	13
	Membrane glycoprotein LIG-1 (putative) **Leucine rich and immunoglobulin domains	ISCW002873	1
	Oligosaccharyl transferase	ISCW022325	1
UF IS HMW (Gel slice 2)	Hemolectin **(VWFC, VWFD, [F5/8 type C (Blood coagulation factors V and VIII) Galactose-binding-like superfamily]	ISCW001097	1
	Heme lipoprotein (putative)	ISCW021710	10
	Hemelipoglycoprotein (putative)	ISCW012424 ISCW024299	1 1
	Sugar transporter (putative)	ISCW013894	2
	Oligosaccharyl transferase	ISCW022325	6
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	ISCW024010	3
	Hsp90 protein (putative)	ISCW014265	1
PF IS 100 kDa (Gel slice 3)	Hemelipoglycoprotein (putative)	ISCW012424 ISCW021704 ISCW021709 ISCW024299	14 26 56 8
	Heme lipoprotein (putative)	ISCW021710	112
	α-Mannosidase 2c1	ISCW001703 ISCW016947	1 1
	α-Mannosidase	ISCW011337	1
	Glucosidase II (with galactose mutarotase domain)	ISCW012920	34
	α-Glucosidase	ISCW010663	4
	Lysosomal α-glucosidase	ISCW010662	3
	Hsp90 protein, putative	ISCW014265	23
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	ISCW024010	3
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	ISCW016170	1
	Oligosaccharyl transferase	ISCW022325	11
	Alpha-1,3-glucosyltransferase	ISCW019559	1
AA PF SG (Gel slice 4)	Heme lipoprotein precursor **Contains VWD	ABK40086.2	116
	Endoplasmic reticulum glucose-regulated protein grp94/endoplasmin hsp90 family (putative)	JAG90923.1	14
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit stt3a (putative)	JAG91470.1	7
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit precursor (putative)	JAG91423.1	11
	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 (putative)	JAG92731.1	6
	Oligosaccharyltransferase gamma subunit (putative)	JAG92697.1	1

Samples, protein names, accession numbers, and exclusive spectrum counts (SpC) are listed below (full list of proteins included in spreadsheet).

4.3 Artificial Feeding of Ticks

From these experiments alone, there is has not been enough evidence to implicate ticks in the onset of future hypersensitivity reactions to the α-gal epitope, and more investigation is needed to shed light on the tick/α-gal conundrum. It is understood that non-primate mammals contain an abundance of α-gal, and the tissues used in these experiments come from ticks that are fed on mammals, therefore, it is possible that the detected α-gal from post-attachment ticks could have been sourced from those animals, even though the previously presented data showed that only two species of tick, *Ix. scapularis* and *Am. americanum*, had detectable quantities α-gal. As an attempt to determine if α-gal was sourced from the animal host or synthesized by its own mechanisms, *Am. americanum* females were fed until the in-vitro using an artificial membrane feeding system that contained defibrinated human blood to see if α-gal would be absent in these conditions (**Figure 4.6**).

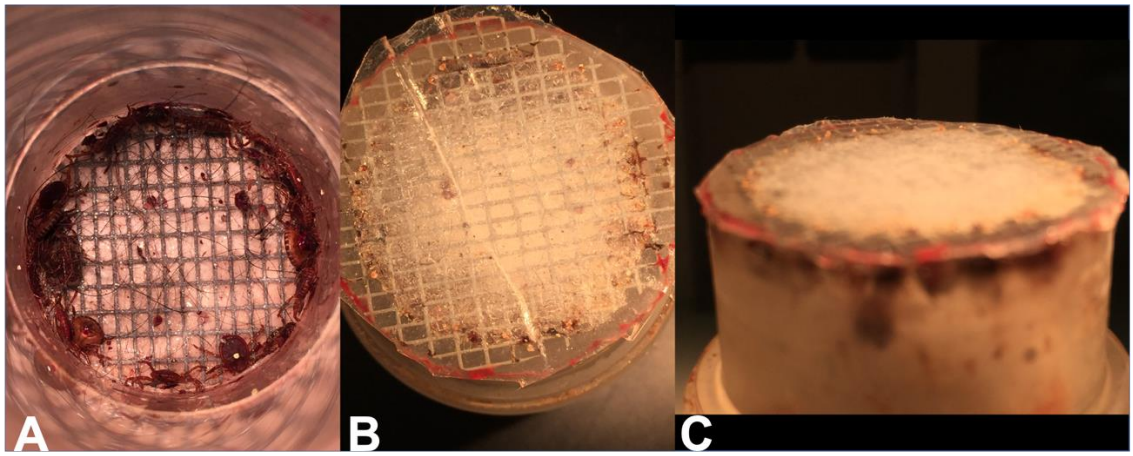


Figure 4.6 *Membrane system for artificial feeding of Amblyomma americanum ticks*

(A) Adult *Am. americanum* ticks attached to the membrane within the feeding cylinder with sheep hair in an attempt to stimulate attachment and feeding. (B) Under side of the membrane during routine cleaning with ticks still attached, (C) side view of underside of membrane showing tick cement cones.

4.4 Western blotting of tick salivary proteins from artificial feeding system and PNGase F treatment

Western blot analysis using the anti-gal antibody (**Figure 4.7**) against salivary glands fed with blood from sheep (lane 3 and 5) was compared with the salivary glands that have been fed human blood (lane 6). There is an observable signal from the anti-gal antibody with antigens contained within the salivary glands of partially-fed ticks that fed on human blood from a human source, and this experiment indicates that α-gal is possibly synthesized within the tick.

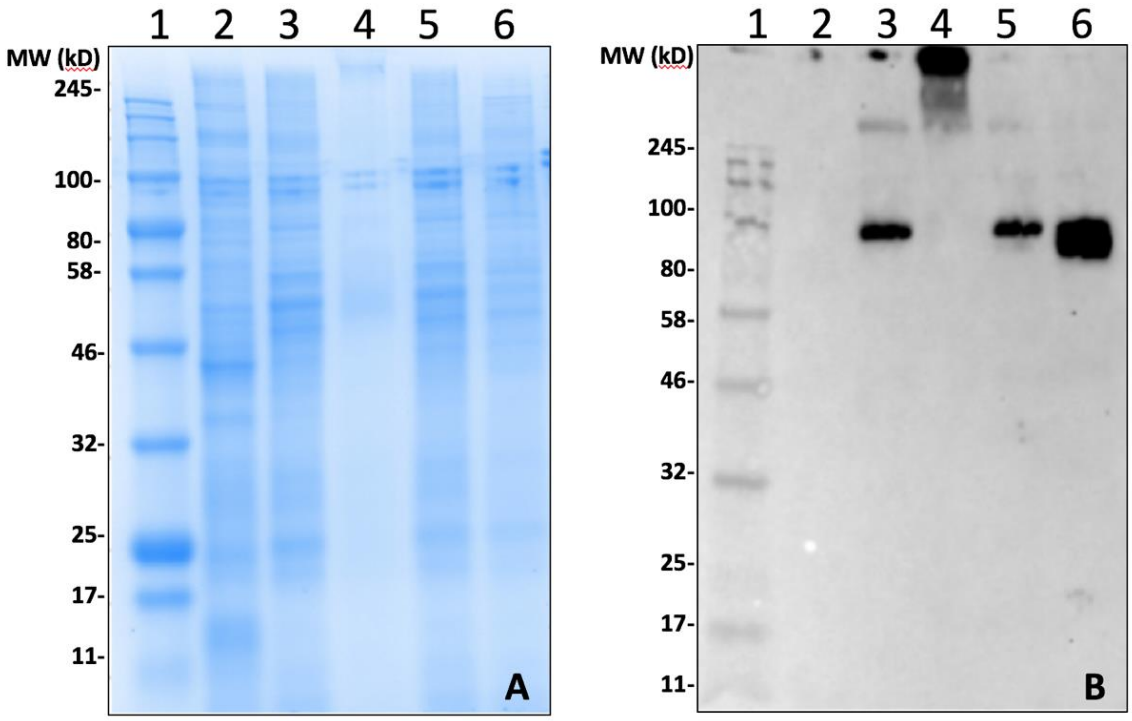


Figure 4.7 *Deglycosylated and human blood-fed Am. americanum salivary gland analysis*

SDS-PAGE (A) and α-gal immunoblot (B). Lane 1 contains a broad range molecular weight ladder (New England Bio-Labs, USA). Lane 2 contains unfed *Amblyomma americanum* salivary glands. Lane 3 contains 7d partially fed *Am. americanum* salivary glands. Lane 4 contains deglycosylated partially fed *Am. americanum* salivary glands. Lane 5 contains 7d partially fed *Am. americanum* salivary glands. Lane 6 contains salivary glands of *Am. americanum* that were fed human blood using an artificial feeding system.

Anti-α-gal antibodies were binding to an α-galactosyl epitope in the salivary glands, but the identity of the protein was still uncertain. To narrow down the nature of the tick α-gal to an O-glycan which might be part of a bacterial lipopolysaccharide or a lipid linked glycan, or an N-glycan of a glycoprotein, the salivary gland protein lysate was incubated in PNGase F to cleave the N-linked glycans chain at asparagine residues. Salivary glands treated with PNGase F (lane 4) were compared with untreated salivary glands (lane 3), and the M86 IgM antibody is binding at the upper-limit of the gel/blot, indicating that there was successful cleavage of the glycan, and that it no longer had the charge necessary to migrate in the polyacrylamide gel. Together, these data suggest that the M86 IgM antibody is binding to N-linked glycans found on tick salivary gland proteins and that the α-gal found on the salivary gland proteins are able to be successfully cleaved using PNGase F.

4.5 Magnetic Pull-down and Proteomics of Tick Salivary Glands

The previously described proteomic experiments using excised bands provided insight into the tick antigens containing α-gal in salivary gland tissues but narrowing this down to unknown proteins based on surface glycosylations presents its own technical challenges and difficulties. IgM-specific magnetic beads were used to capture antigens with α-galactosyl residues using the anti-gal antibody. The quantities of captured antigens were expected to be low, and therefore silver staining was used to visualize antigens (**Figure 4.8A**). I refer to some of the samples as "depleted" because it was my intention to capture and remove all of the α-gal using the M86 antibody bound to magnetic IgM "pull-down" beads. To further verify the specific binding of the captured antigens in the "pull-

down" and "depleted" fractions, the α-gal antibody was used to confirm the cross-reactivity (**Figure 4.8B**). The "depleted" fraction showed the usual cross-reactivity at ~95 kDa, and the "pull-down" fraction showed a band with less intensity at a similar molecular weight. It is likely that the bands at ~80 kDa and ~50–55 kDa are artifacts of the IgM antibodies that were not removed from the α-galactosyl-containing proteins. The magnetic pull-down assay was able to capture the α-gal-containing epitopes from *Am. americanum* salivary gland tissue homogenates and the product was excised, and LC-MS/MS analysis identified the tubulin β-chain of *Am. americanum* (A0A0C9SCB7) (**Table 4.2**). However, the resulting fraction of this experiment provided few peptides for analysis.

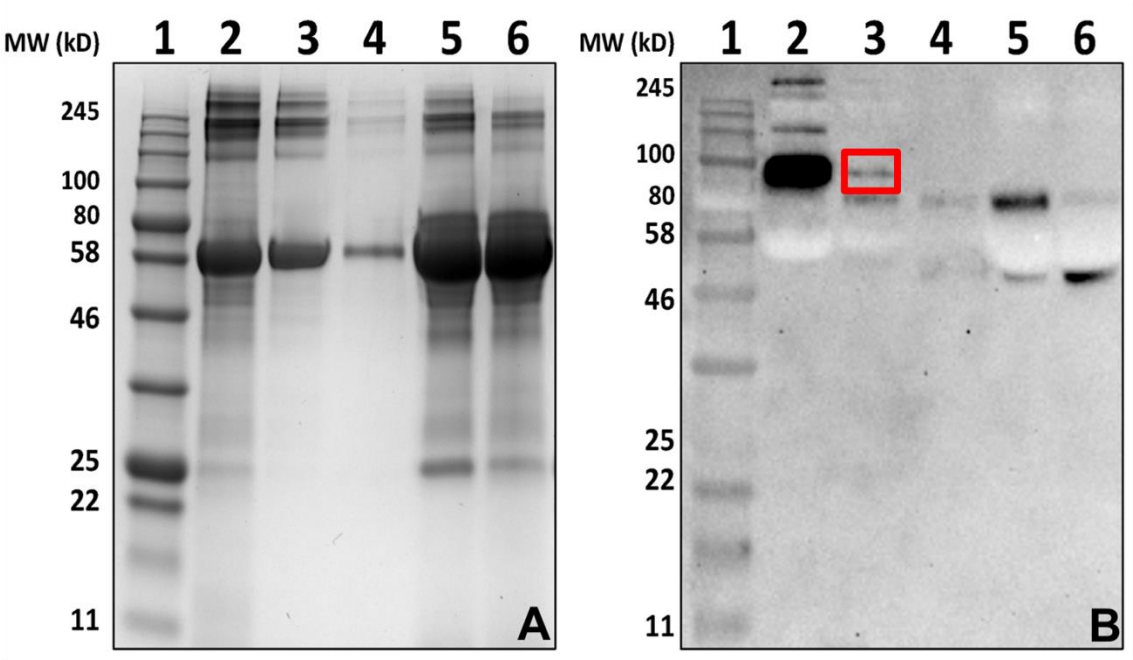


Figure 4.8 *Pull-down of α-gal containing proteins using magnetic beads*

Lane 1 contains broad range molecular weight protein standard, lane 2 contains "depleted" *Am. americanum* salivary glands, lane 3 contains the α-gal pull-down product, lane 4 contains the magnetic beads after incubation with *Am. americanum* salivary glands, lane 5 contains "depleted" α-gal specific IgM, and lane 6 contains α-gal specific IgM. When blotting, α-gal can be detected in lane 1 and 2 at ~95kD, and the IgM heavy chain can be seen at ~80-85kD.

Table 4.2

Protein identification from α-gal magnetic pull-down assay

Protein name	Protein accession numbers	Database sources	Protein molecular weight (Da)	Protein identification probability	Exclusive unique peptide count	Exclusive unique spectrum count	Exclusive spectrum count	Percentage of total spectra	Percentage sequence coverage
Tax_Id=9606 Gene_Symbol=KRT9 Keratin, type I cytoskeletal 9	IPI:CON_00019359.3|SWISS-PROT:P35527	UniProt-Ixodidae-6939_052617.fasta	62,131.00	100.00%	2	3	3	0.07%	3.21%
Tax_Id=9606 Gene_Symbol=KRT14 Keratin, type I cytoskeletal 14	IPI:CON_00384444.5|SWISS-PROT:P02533	UniProt-Ixodidae-6939_052617.fasta	51,622.60	22.90%	0	0	0	0.00%	1.91%
(Bos taurus) Bovine serum albumin precursor	IPI:CON_00708398.1|SWISS-PROT:P02769	UniProt-Ixodidae-6939_052617.fasta	69,294.20	100.00%	12	14	15	0.34%	20.40%
Tax_Id=9606 Gene_Symbol=KRT2 Keratin, type II cytoskeletal 2 epidermal	IPI:CON_00021304.1|SWISS-PROT:P35908	UniProt-Ixodidae-6939_052617.fasta	65,866.40	85.50%	0	0	0	0.00%	0.00%
Trypsin - Sus scrofa (Pig).	IPI:CON_Trypsin|SWISS-PROT:P00761|TRYP_PIG	UniProt-Ixodidae-6939_052617.fasta	24,409.30	100.00%	3	4	4	0.09%	16.50%
Tax_Id=9606 Gene_Symbol=KRT5 Keratin, type II cytoskeletal 5	IPI:CON_00009867.3|SWISS-PROT:P13647	UniProt-Ixodidae-6939_052617.fasta	62,379.60	40.80%	0	0	0	0.00%	0.00%
Tubulin beta chain OS=Ixodes scapularis GN=IscW_ISCW017133 PE=3 SV=1	tr|B7PA92|B7PA92_IXOSC	UniProt-Ixodidae-6939_052617.fasta	49,944.90	99.20%	1	1	1	0.02%	4.04%

The above table shows the proteins that were identified by LC-MS/MS analysis of the gel excision from the magnetic pull-down. Resulting peptides were run against the Ixodidae database.

4.6 Immunolocalization of α-galactosyl epitopes in tick salivary gland tissues

Immunolocalization of α-gal was performed on the unfed and partially fed salivary glands of *Amblyomma americanum* (**Figure 4.9**), *Dermacentor variabilis* (**Figure 4.10**), *Ixodes scapularis* (**Figure 4.11**), and *Am. maculatum* (**Figure 4.12**) to understand the subcellular location of the α-gal epitope on tick salivary proteins. DAPI was utilized to stain nuclei, phallodin to stain F-actin, and M86 antibody to bind to α-gal. These findings indicate that *Am. americanum* and *Ix. scapularis* have detectable α-gal in the acini in proximity to the secretory vesicle. However, there was no visualization of α-gal in the *De. variabilis* or *Am. maculatum* tissues tested, which corresponds with western blotting data previously produced (**Figure 4.5**).

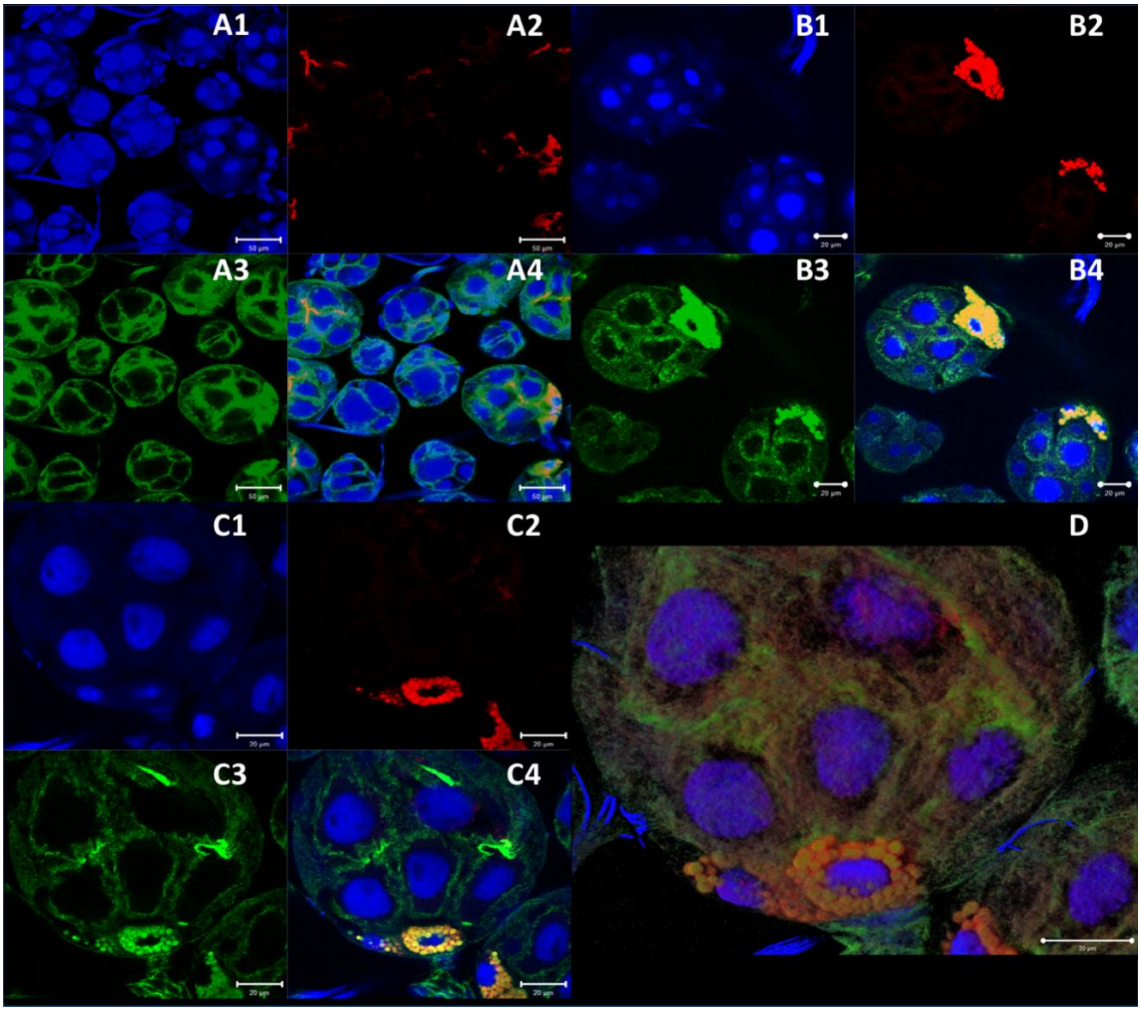


Figure 4.8 *α-gal Immunolocalization of partially fed Am. americanum salivary glands*

40x magnification (A), 63X magnification (B), and 100X magnification (C) using DAPI stained nuclei (1), α-gal IgM (2), F-actin staining with phalloidin (3), and merged images (4). A 3D projection was generated using the Z-Stacks from the 100x magnification with merged DAPI, α-gal, and phallodin (D).

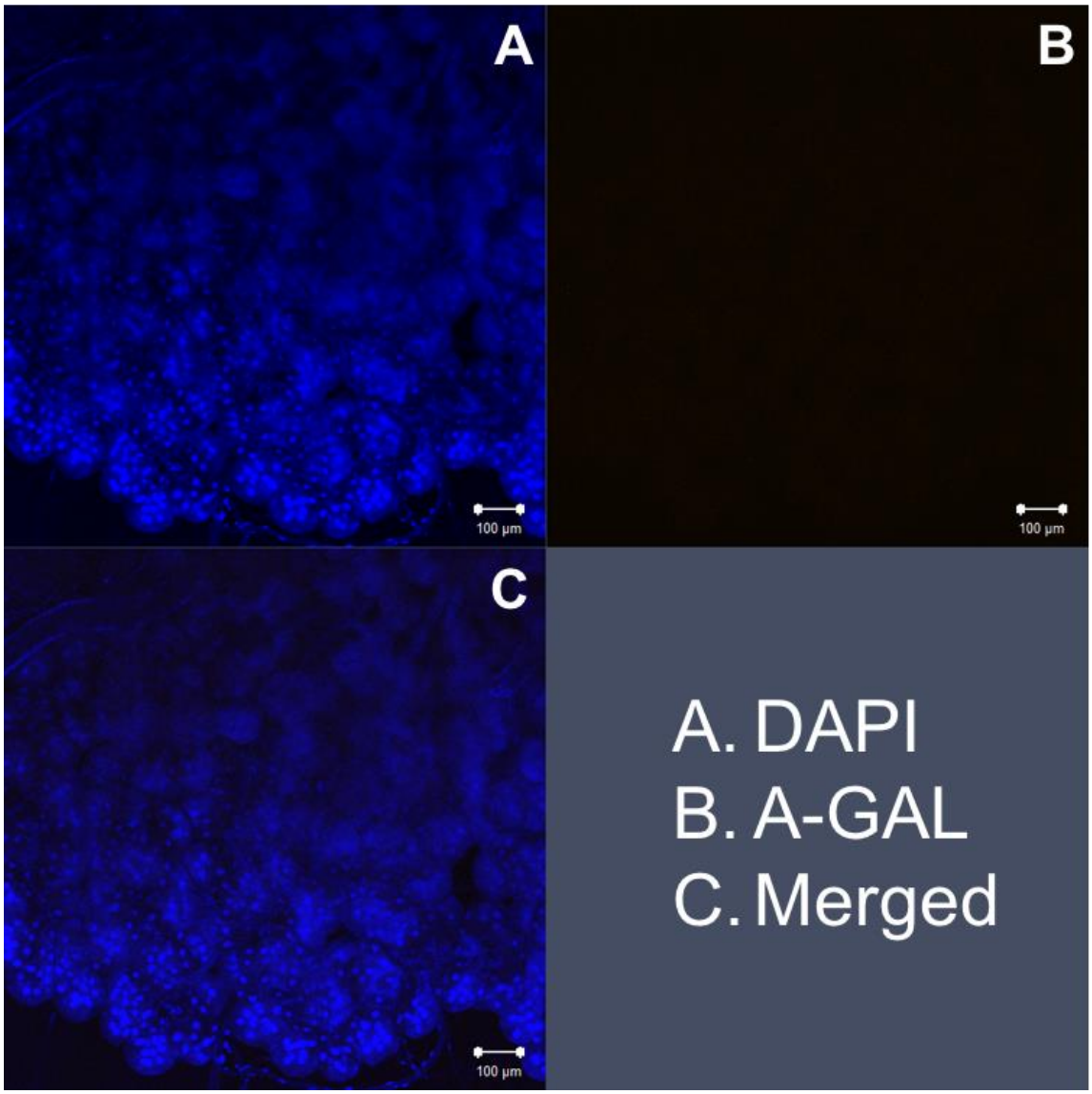


Figure 4.9 *α -galactose Immunolocalization of partially fed De. variabilis salivary glands*

10x magnification of salivary glands (A) DAPI nuclei staining, (B) α-galactose (M86) IgM staining, and (C) merged images.

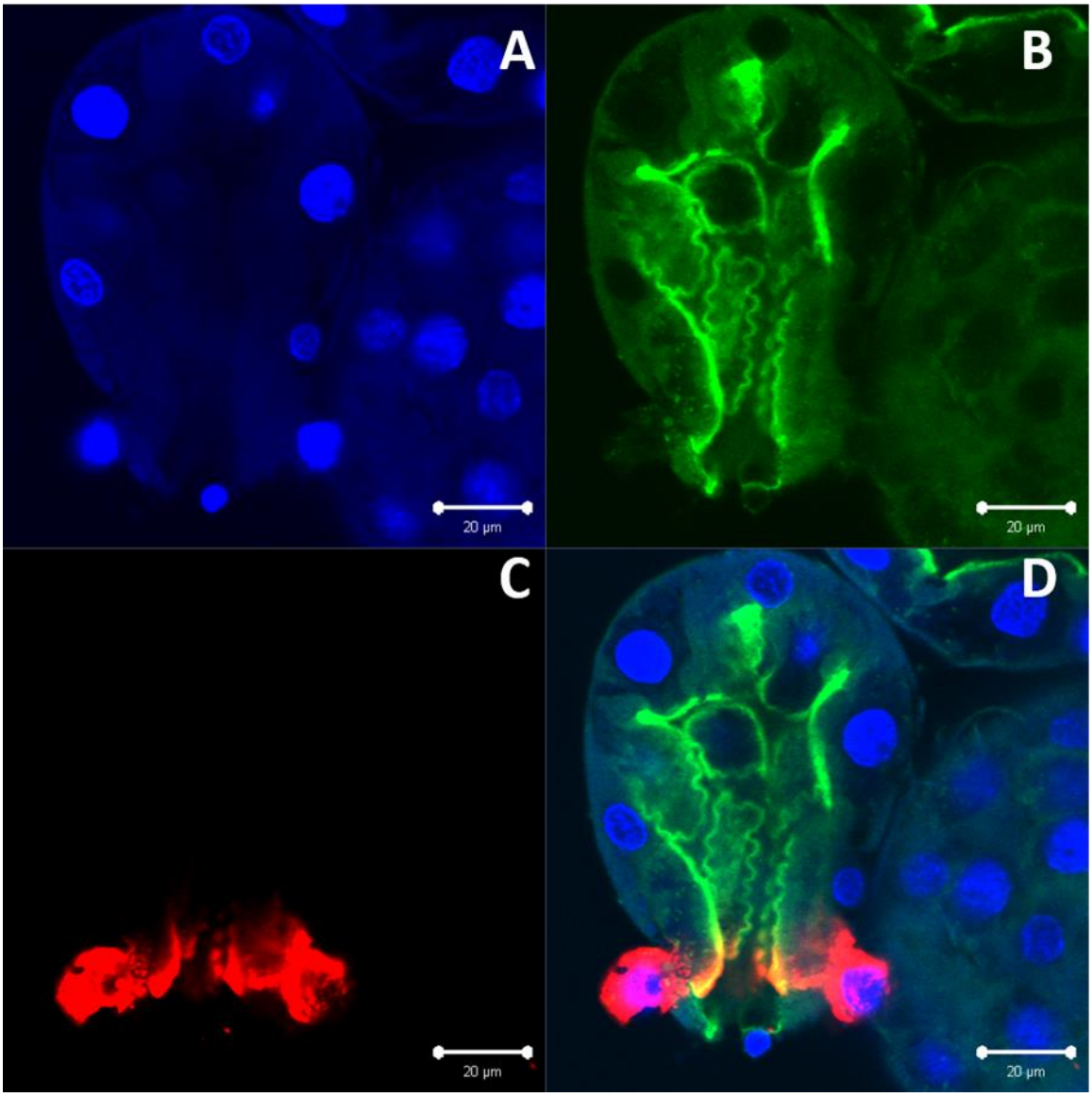


Figure 4.10 *α-galactose Immunolocalization of partially fed Ix. scapularis salivary glands*

100X magnification of salivary glands (A) DAPI nuclei staining, (B) F-actin staining with phalloidin (C) α-galactose (M86) IgM staining, and (D) merged images.

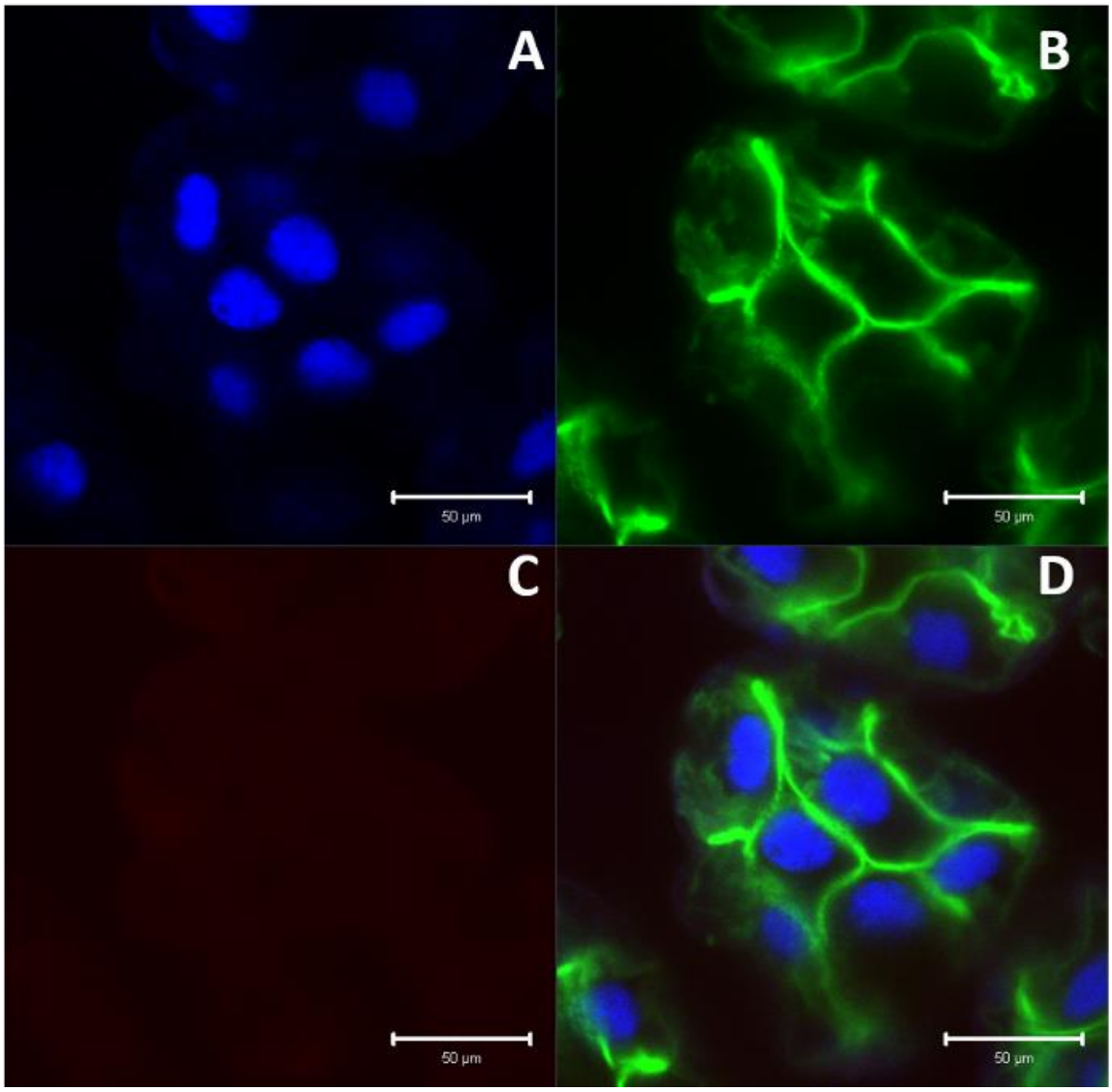


Figure 4.11 *α-galactose Immunolocalization of partially fed Am. maculatum salivary glands*

40X magnification of salivary glands (A) DAPI nuclei staining, (B) F-actin staining with phalloidin (C) α-galactose (M86) IgM staining, and (D) merged images.

4.7 N-linked Glycan Profile of Tick Saliva and Salivary Gland Tissues

N-linked glycan profiling of *Am. maculatum*, *Am. americanum,* and *Ix. scapularis* revealed two critical insights into the glycosylation of tick salivary proteins (**Table 4.3 and 4.4**). First, the unfed salivary glands of both *Am. americanum* and *Am. maculatum*

contained no detectable amounts of α-gal, but glycans in the unfed salivary glands of *Ix. scapularis* contained α-gal. Secondly, the partially-fed salivary glands and saliva from *Am. maculatum* showed no detectable α-gal, which corresponds to the immunoblot and immunolocalization experiments; however, *Am. americanum* and *Ix. scapularis* contained detectable quantities and multiple glycoforms of α-gal (**Table 4.3**). The overall abundance of α-gal glycoforms in *Am. americanum* partially-fed salivary glands tested was greater than 1.12% of the total N-glycans detected, but the saliva consisted of approximately 0.15% of the total N-glycans detected. *Ix. scapularis* unfed salivary gland N-glycans containing α-gal comprised 6.3% of overall N-glycans detected, and more than 1.7% in the partially-fed salivary glands, but only trace amounts, below the quantifiable limit, of α-gal glycans were found within the saliva. The majority of the glycoforms identified were biantennary extended-galactose structures that had core fucosylations, and a few were identified as single-antennary species (hybrid-type) in the non-fucosylated form. This information correlates with the results of immunoblotting experiments and strengthens the theory that some tick species can acquire or synthesize α-gal, while others lack this capacity. Comprehensive details of all identified N-linked glycoforms have been provided (**Appendix B**).

In addition to glycoforms containing α-gal in these samples, multiple pentose-containing species were observed in all three species and in all feeding states, in both salivary glands as well as saliva. While these results do not reflect compositional analysis, MS/MS fragmentation revealed that the pentose was core-mannose attached, similar to xylose-containing structures frequently found in plants. These observed glycoforms were primarily complex-type.

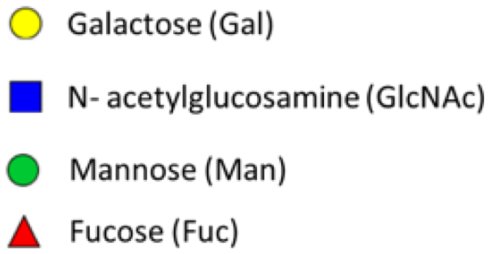
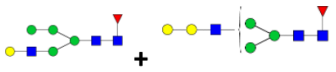
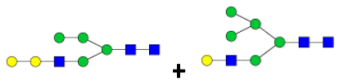
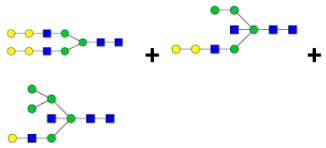
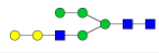
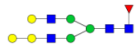
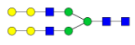
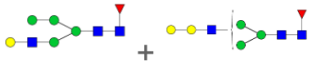
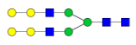


Figure 4.12 – *Key of Glycan Nomenclature used in figures.*

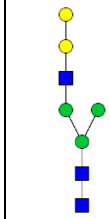
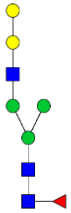
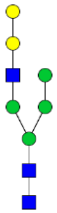
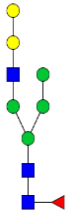
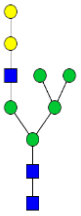
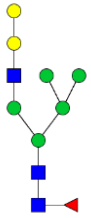
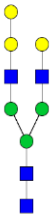
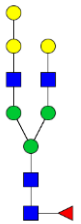
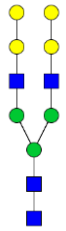
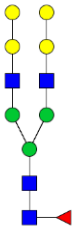
The above color-filled shapes are a guide to the identification of carbohydrate glycoforms found in the tables found within this document. Yellow-filled circles are galactose, blue-filled squares are N-acetylglucosamine, green-filled circles are mannose, and red-filled triangles are fucose.

Table 4.3 *Quantification of α-galactosyl-containing N-linked glycans by NSI-FTMS*

Obs. Mass	Proposed Structures	Graphical Structures	Abundance
***Amblyomma americanum* partially-fed salivary glands**			
1998.999	$(Hex)_2$ $(HexNAc)_1$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		Trace
2029.0142	$(Hex)_3$ $(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$		0.11%
2203.1008	$(Hex)_3$ $(HexNAc)_1$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		0.11%
2274.1356	$(Hex)_3$ $(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$		0.18%
2652.3254	$(Hex)_4$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		0.72%
***Amblyomma americanum* saliva**			
2029.01	$(Hex)_3$ $(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$		Trace
2203.10	$(Hex)_3$ $(HexNAc)_1$$(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		Trace
2233.11	$(Hex)_4$ $(HexNAc)_1$ + $(Man)_3(GlcNAc)_2$		Trace
2448.22	$(Hex)_3$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		Trace
2652.3226	$(Hex)_4$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		0.15%
***Amblyomma maculatum* unfed/partially-fed salivary glands and saliva**			
N/A	None Detected	None Detected	N/A
***Ixodes scapularis* unfed salivary glands**			
2478.246	$(Hex)_4$ $(HexNAc)_2$ + $(Man)_3(GlcNAc)_2$		1.9%
2652.333	$(Hex)_4$ $(HexNAc)_2$ $(Deoxyhexose)_1$ + $(Man)_3(GlcNAc)_2$		4.4%
***Ixodes scapularis* partially-fed salivary glands**			
1999.006	(Hex)2 (HexNAc)1 (Deoxyhexose)1 + (Man)3(GlcNAc)2		1.7%
2478.2444	(Hex)4 (HexNAc)2 + (Man)3(GlcNAc)2		Trace

Glycoforms with α-galactosyl epitope observed in partially-fed *Amblyomma americanum* salivary glands and saliva, and *Ixodes scapularis* unfed and partially-fed salivary glands.

Table 4.4 *Identification of α-galactose containing glycans in Am. americanum saliva.*

										
m/z	**1825**	**1999**	**2029**	**2203**	**2233**	**2407**	**2274**	**2448**	**2478**	**2652**
AMUFSG	NO	NO	NO	NO			NO			
AMPFSG	Unknown	NO	Unknown	Unknown						
AMSALIVA										
AAUFSG	NO	NO	NO	NO						
AAPFSG	NO	YES/MIX	YES/MIX	YES/MIX	YES	YES	YES/MIX		YES	YES
AASALIVA	Unknown	NO	YES	YES	YES			YES		YES

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 ion2

4.8 Basophil activation assay

Because glycan profiling demonstrated the presence of α-gal in salivary samples from *Am. americanum* and *Ix. scapularis* but not *Am. maculatum*, it was important to determine 1) if salivary compounds containing or lacking α-gal moieties could activate basophils primed with α-gal sIgE, and 2) whether activation might reflect species-specific differences in α-gal glycan content. Donor basophils from a healthy, non-allergic control were stripped of IgE and primed overnight with plasma from a subject with α-gal syndrome (α-gal sIgE = 31.3 IU/mL, total IgE = 233 IU/mL). Sensitized cells were exposed to one of the following stimuli for 30 min: RPMI media, crosslinking anti-IgE antibody (positive control), α-gal-containing glycoprotein cetuximab (α-gal positive control), *Am. americanum* saliva, *Am. americanum* partially-fed salivary gland (PF SG) extract, *Ix. scapularis* PF SG extract, or *Am. maculatum* PF SG extract. CD63 expression on lineage-HLA-DR-CD123+CD203c+ basophils was assessed by flow cytometry (**Figure 4.11**). It was found that the frequency of CD63+ basophils was significantly increased following sensitization with α-gal allergic plasma and stimulation with α-gal-containing tick salivary samples from *Am. americanum* (saliva and PF SG extract) and *Ix. scapularis* ($p<0.05$ vs. media). Alternatively, salivary samples from *Am. maculatum* caused small but non-significant increases in CD63+ basophils when the results of all experiments (n=3) were included. Stimulation with PF SG extract from *Ix. scapularis* produced the largest increase in CD63+ basophils, which was consistent with the high level of α-gal content detected via glycan analysis.

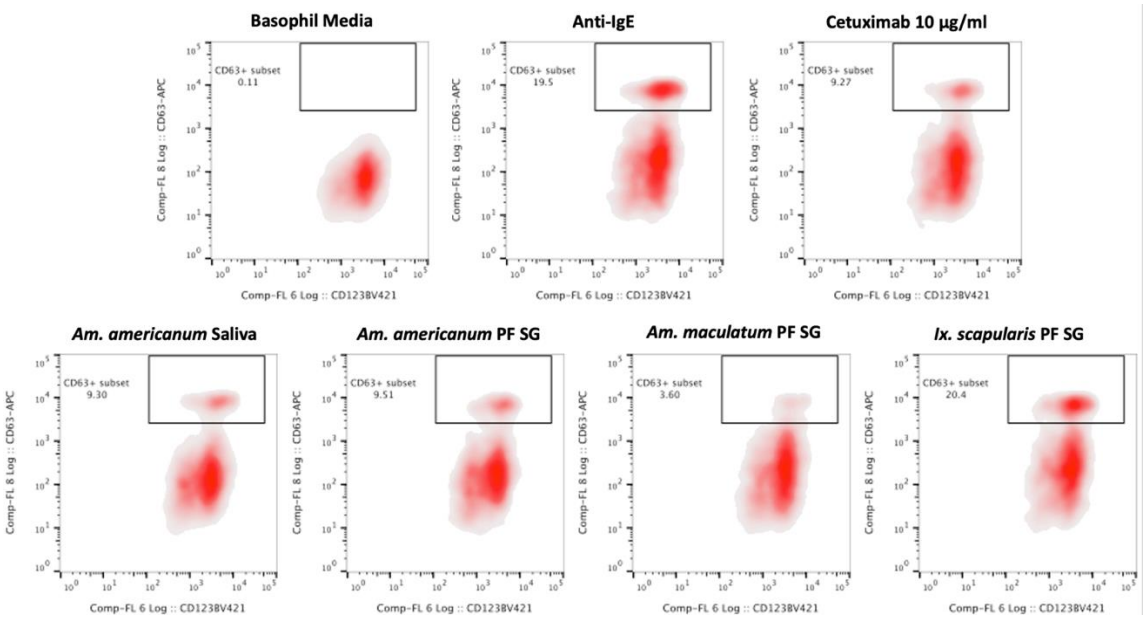


Figure 4.13 *Flow cytometry analysis of human basophil activation by tick salivary proteins*

Donor basophils from a healthy, non-allergic control were stripped of IgE and primed overnight with plasma from a subject with α-gal syndrome (α-gal sIgE = 31.3 IU/mL, total IgE = 233 IU/mL). Sensitized cells were exposed to one of the following stimuli for 30 min: RPMI media, crosslinking anti-IgE antibody (positive control), α-gal-containing glycoprotein cetuximab (α-gal positive control), *Am. americanum* saliva, *Am. americanum* partially-fed salivary gland (PF SG) extract, *Ix. scapularis* PF SG extract, or *Am. maculatum* PF SG extract. CD63 expression on lineage-HLA-DR-CD123+CD203c+ basophils was assessed by flow cytometry.

CHAPTER V - DISCUSSION*

5.1 Main discussion

The lone-star tick has expanded its geographic range from Southwest to the East Coast of the United States. It is a vector for diseases such as spotted fever group rickettsiosis, human monocytic ehrlichiosis, southern-tick-associated rash illness, tularemia, Heartland virus infection, and infection with newly discovered Tacaribe virus (Childs & Paddock, 2003; Goddard & Varela-Stokes, 2009; Paddock & Childs, 2003; Savage et al., 2013; Sayler et al., 2014). In addition to these diseases, this tick species has been associated with delayed anaphylaxis to red meat and is the first example of a blood-feeding ectoparasite causing food allergy in the United States (Commins et al., 2011; Platts-Mills et al., 2015). In fact, a growing body of literature suggests that bites from the lone-star tick (*Am. americanum*) are causing α-gal syndrome (Commins et al., 2011; Commins & Platts-Mills, 2013b; Jackson, 2018; Wuerdeman & Harrison, 2014). It remains unknown whether bites from *Am. americanum* trigger the development of α-gal sIgE in humans due to the presence of α-gal moieties in tick saliva or IgE arises as a class-switched anti-gal response after ecto-parasitic feeding. In this study, I identified the presence of α-gal in *Am. americanum* and the black-legged tick, *Ix. scapularis.* Furthermore, a previous study in Europe used immunohistochemical staining to show the cross-reactivity of the α-galactosyl epitope in the gut tissues of *Ix. ricinus* (Hamsten et al., 2013), and another study using the salivary glands of *Rhipicephalus bursa* and *Hyalomma marginatum* (Mateos-Hernández et al., 2017). This study focused on partially-fed salivary glands because they produce, contain, and secrete saliva that can be injected directly into the host (Karim & Ribeiro,

* This chapter contains previously published work (Crispell et al., 2019).

2015). In theory, if there were no α-gal containing epitopes in tick salivary glands, which are responsible for secreting saliva in to the host during prolonged tick feeding, it is unlikely that α-gal would be present in the tick saliva, and therefore, an increase in α-gal sIgE would most likely reflect a Th2-driven class-switch of the ongoing anti-gal response present in all immunocompetent humans.

However, the immunoblotting results showed that the saliva and salivary glands of *Am. americanum* female ticks express α-gal-containing antigens in a time-dependent manner throughout prolonged blood feeding (**Figure 4.1 and 4.2**). The presence of α-gal-containing antigens in unfed and partially-blood-fed *Ix. scapularis* was evident (**Figure 4.3 and Figure 4.5**). However, the Gulf-Coast tick, *Am. maculatum*, and the American dog tick, *De. variabilis*, lacked the presence of α-gal-containing antigens (**Figure 4.4 and Figure 4.5**). Unlike *Ix. scapularis* unfed midgut tissues, *Am. maculatum* and *Am. americanum* tick species showed no cross-reactivity with α-gal antibodies (**Figure 4.1 and Figure 4.5**). The presence of α-gal cross-reactivity with unfed *Ix. scapularis* was not surprising as it has been reported in sister tick species *Ix. ricinus* using immunohistochemical techniques (Hamsten et al., 2013) and in N-glycan profiling studies (Vancova et al., 2012), and also in a report using flow-cytometry with *Ix. scapularis* (Alberdi et al., 2018). Additionally, the results of basophil activation showed high levels of CD63+ expression following stimulation using *Ix. scapularis* salivary extracts. The initial presence of α-gal-containing antigens in the unfed *Ix. scapularis* midgut and salivary glands is possibly a remnant from a blood meal during the previous life-stage before molting to the adult stage, or the ticks might have cleaved and incorporated the glycans into their own proteins. Interestingly, unfed and partially-blood-fed *Am. americanum* males

showed a lack of cross-reactivity with the M86 α-gal antibodies (my unpublished data), however, a recent study was capable of detecting α-gal in males using N-glycan analysis (Park et al., 2020). A set of more recent experiments show that male *Am. americanum* salivary glands contain α-gal after capillary feeding with bovine blood, but it is no longer detectable after feeding on α-gal knock-out mice (Maldonado-Ruiz et al., 2022). Together, these results lead us to the conclusion that female *Am. americanum* and *Ix. scapularis* ticks express α-gal-containing proteins and might possibly use α-galactose to facilitate successful hematophagy. While it might be possible for the tick to sequester α-gal or the enzymes required to synthesize α-gal from the host during the immature developmental stages (larval or nymphal ticks), adult unfed *Am. americanum* females do not have detectable quantities of α-gal validated by immunoblotting and MALDI-TOF/TOF-MS. However, *Ix. scapularis* unfed females do have detectable quantities of α-gal in the salivary gland tissues, possibly remaining from a previous blood meal. The size of α-gal-containing antigens differs between unfed *Ix. scapularis* salivary glands and partially-blood-fed salivary glands. The switching of α-gal-containing antigens from unfed to fed salivary glands might be a strategy to remain successfully attached to the host for a prolonged period of time. **Figure 5.1** is a reference diagram that shows which methods were used to validate the presence of α-gal in tick tissues and saliva.

	Ix. scapularis	*Am. americanum*	*Am. maculatum*	*De. variabilis*
UF MG	+ IB	- IB/MS	- IB	-
PF MG	- IB	- IB	- IB	-
UF SG	+ IB/MS	- IB/MS	- IB/MS	- IB
PF SG	+ IB/MS/IL	+ IB/MS/IL	- IB/MS/IL	- IB/IL
SALIVA	+ MS	+ IB/MS	- IB/MS	-

IB-Immunoblotting MS-Mass Spectrometry IL-Immunolocalization

Figure 5.1 *Reference diagram of tissues testing positive for α-gal in this study*

The above figure shows which methods validated the presence of α-gal in individual tick tissues at various feeding stages and in the saliva. Samples that contained α-gal are denoted with "+" and red lettering, and tissues and saliva lacking α-gal are denoted with "-" and black lettering.

Incubation of *Am. americanum* salivary gland tissues with PNGase F cleaves internal glycoside bonds of asparagine-linked oligosaccharides. The results indicated that N-linked glycans with terminal α-gal caps were removed from tick salivary proteins (**Figure 4.7**) as α-gal moieties were detected with the M86 antibody above the limit of the protein standard in the polyacrylamide gel that failed to migrate after treatment with PNGase F, as they are uncharged carbohydrate chains. This is important because it reveals that the detected α-gal is part of an N-linked oligosaccharide linked to a glycoprotein within the tick salivary glands, as opposed to an O-linked structure such as a lipopolysaccharide.

These experiments analyzed tissues and saliva that were acquired from ticks fed on mammals that produce α-gal, and thus the host could have served as a potential source of the α-gal carbohydrates detected. To determine if the mammalian host was the source of α-gal carbohydrates, ticks were artificially fed with a membrane feeding system using

human blood as a meal source, which is known to be free of the enzyme N-acetyllactosaminide α-1,3-galactosyltransferase (Galili & Swanson, 1991) and α-gal. However, *Am. americanum* ticks that were artificially fed with human blood still contained α-gal at the same molecular weight as ticks fed sheep blood, as determined using the α-gal M86 IgM antibody (**Figure 4.7**). These results indicate that ticks possibly recycle host glycans or synthesize α-gal using alternative methods. Because there is no evidence that ticks have the necessary galactosyltransferase to produce α-gal, it is possible that the ticks use a fucosidase to cleave fucose residues from human type B blood to produce the α-gal antigens, which could then be cleaved and incorporated into tick salivary proteins or directly secreted back to the host. Another potential source of α-gal could come from bacterial galactosyltransferase enzymes that are used during cell wall biosynthesis (Hamadeh et al., 1996). Combined, these results suggest that ticks do not need to feed on a lower (non-primate) mammal to introduce salivary glycoproteins containing α-gal into humans.

To localize α-gal in tick salivary glands, confocal fluorescence microscopy was utilized to visualize the emission of secondary antibodies against α-gal IgM. Focus was primarily directed towards the partially-fed stage of *Am. americanum* salivary glands, but I also screened *Ix. scapularis*, *Am. maculatum*, and *De. variabilis* for α-gal. The images provided evidence that terminal α-gal residues on salivary glycoproteins are not found in all ticks, as α-gal was present in *Am. americanum* (**Figure 4.9**) and *Ix. scapularis* (**Figure 4.11**), but were absent in *Am. maculatum* (**Figure 4.12**) and *De. variabilis* (**Figure 4.10**). In *Am. americanum* tissues, immunolocalization of α-gal residues was primarily observed with secretory vesicles from ticks that were in the partially-fed state (**Figure 4.9D**). The

presence of terminal α-gal residues near secretory vesicles supports the idea that α-gal can be secreted in the saliva of *Am. americanum*. Together with basophil activation data, these results suggest the potential role of *Am. americanum* saliva antigens as the primary cause of the delayed-type hypersensitivity reaction, although this requires further investigation.

N-glycan profiling of unfed and partially-fed salivary glands and saliva extracted from *Am. americanum* and *Ix. scapularis* revealed the presence of N-linked glycans with terminal α-gal caps; however, they were absent in *Am. maculatum*. The fact that α-gal is present in the saliva, even in trace amounts, supports the idea that ticks play a role in the induction of a hypersensitivity reaction in humans. Humans without α-gal hypersensitivity are known to have as much as 1% of their circulating IgG antibodies that are specific for anti-gal (Galili et al., 1987), which means that the immune system can already recognize this carbohydrate antigen. Because ticks have the ability to remain attached to their host for a prolonged period of time, it is conceivable that small amounts of α-gal and other antigenic molecules being continually secreted into the host could be recognized and initiate an immune response, which resulted in the development of an IgE response directed against α-gal.

α-gal epitopes are commonly expressed on cells and tissues of non-primate mammals, but xylose is found almost exclusively in plants. While I am unsure as to the source, these core-modified glycoforms were found consistently in multiple tissue types and under various conditions, as well as in multiple species. Xylose and core-3-linked fucose may be the most common carbohydrate epitopes recognized by human IgE antibodies (Commins & Platts-Mills, 2009). In the literature, β(1,2)-xylose linked to a core

mannose has been described in the N-glycans of major pollen allergens, as well as a major peanut allergen (Van Ree et al., 2000).

Mass spectrometry of gel excisions (**Table 4.1**) revealed the presence of many proteins and glycoproteins (comprehensive datasets located at ProteomeXchange PXD012827). The protein database for *Ix. scapularis* is well populated and contains many sequences, but the database for *Am. americanum* is scant. The presence of laminin γ-1 was found in both *Ix. scapularis* and *Am. americanum* salivary glands, which has previously been reported to contain an α-gal moiety on the protein, and is suspected of being a common IgE-reactive protein in beef allergy patients in Japan (Takahashi et al., 2014). The alpha chain of type IV collagen was discovered to contain α-gal moieties (Nayak & Spiro, 1991), and it was identified in the unfed *Ix. scapularis* salivary glands. However, a feature of these proteins is that they lack a signal peptide usually associated with secretion and are therefore not likely to be the primary instigators of the human host α-gal sIgE response. An alternative method by which these proteins may be secreted into the saliva could be via exosomes. Purification of exosomes from tick saliva for the identification of α-gal-containing antigens should be carried out in future studies.

There were numerous proteins discovered from both *Am. americanum* and *Ix. scapularis* that could potentially be involved in the α-gal hypersensitivity conundrum. I have presented a narrowed down list of protein candidates in **Table 4.1 and Table 4.2**, and among these discovered many proteins and enzymes involved in carbohydrate metabolism. Multiple glycoside hydrolases in the salivary glands, which could aid the tick in cleavage of its own and host carbohydrates, are attractive candidate molecules. Unfed *Ix. scapularis* contained the enzyme α-D-galactosidase (EC 3.2.1.22), a signal peptide-containing

enzyme responsible for hydrolyzing terminal α-galactosyl residues from glycoproteins and glycolipids that can potentially cleave galactose molecules resulting in free galactose for use in galactosylation elsewhere. Interestingly, I discovered in the lower molecular weight region of *Ix. scapularis* unfed salivary glands, a galactose-binding lectin (galectin) (Uniprot B7Q1V4), and from the higher molecular weight region, a hemolectin. Previously, a group in Japan reported a galectin that can bind galactose containing moieties in *Ornithodoros moubata* ticks (Huang et al., 2007b). It was also found that there was a hemelipoglycoprotein and a heme lipoprotein in *Ix. scapularis*, and heme lipoprotein precursors were also found in *Am. americanum*. A considerable amount of research has also been conducted on hemelipoglycoprotein, which has strong binding specificity towards galactose in the tick *Dermacentor marginatus* (Dupejova et al., 2011). Because lectins are present in the salivary glands, it is possible that they are capable of capturing glycoproteins from the host blood. Conceivably, capture of host glycans by tick lectins and cleavage by glycoside hydrolases in conjunction with tick and bacterial glycosyltransferases could result in the α-gal glycan.

α-1,3-galactosyltransferase enzymes have yet to be discovered in *Am. americanum* or *Ix. scapularis,* but these combined results provide evidence that terminal α-1,3-galactose residues exist in the saliva and salivary glands after the initiation of feeding, both from human and animal hosts, which leads us to believe that there are three possible scenarios that could lead to synthesis:

1) The glycans are captured by lectins and modified with glycoside hydrolases and glycosyltransferases,

2) galactosyltransferase enzymes from bacterial species contained in the normal microbiota or vectored by of *Am. americanum* or *Ix. scapularis* are responsible for the α-gal glycan,
3) some ticks contain an uncharacterized enzyme with a similar or equivalent function to α-1,3-galactosyltransferase, which is yet to be investigated.

Immunoblotting, immunolocalization, and glycan profiling demonstrated that α-gal exists in the salivary proteins of *Am. americanum* and *Ix. scapularis* but not *Am. maculatum.* These data, in tandem with the significant upregulation of CD63+ expression on human basophils indicating activation after inoculation with *Am. americanum* and *Ix. scapularis* salivary antigens, but not *Am. maculatum* (**Figure 4.14**), strengthens the idea that bites from α-gal-containing/producing ticks could be involved with the onset of AGS. This represents a significant step forward in the understanding of the sensitization of humans to carbohydrates by ticks, and the clinical implications of tick bites in the United States and worldwide.

The results described in this study provide new insight into tick physiology and support the possibility of hypersensitivity reactions instigated after parasitism by ticks. This research helps to further the understanding of the process in which *Am. americanum* and *Ix. scapularis* obtain and transmit potentially immunogenic α-gal to the host. My hope is that this mechanism can be used in the future to treat or protect humans from a plethora of medical conditions. This study also highlights the need for allergists and clinicians to consider *Ix. scapularis* and *Am. americanum* bites when diagnosing red meat allergy cases.

CHAPTER VI - CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

Only a few short years ago, many critics would not have taken the involvement of the tick vector with the induction of Alpha-gal syndrome seriously. However, the experimental data that has been provided by this research has given the scientific community new information directly related to the tick's ability to harbor and secrete α-gal containing antigens to a host through its saliva. This work has shown that *Am. americanum*, while not yet shown to harbor the traditional enzymes necessary to synthesize α-gal, still contains α-gal even when fed on human blood that is known to lack α-gal, however, the blood group B antigen closely resembles α-gal with a core fucosylation. The proteomic data generated by these experiments show that *Ix. scapularis* does harbor α-fucosidase enzymes, and it is conceivable that glycans can be modified by tick enzymes during the blood-meal in order to benefit successful attachment and feeding. Since my time working on these projects has come to an end, and my efforts have not been able to definitively narrow down the molecule responsible for AGS onset, there is still further research to be completed to fully understand how ticks sensitize humans against α-gal.

6.2 Future Directions

Much is still unknown about the presence of α-gal in other tick species found throughout North America and around the world. Continued evaluation of new tick species will assist in determining if the α-gal sugars found in this research are found in other geographic areas with reports of AGS. With minimal burden, salivary glands could be

shipped from laboratories worldwide as a collaborative project to create a database of ticks that are capable of presenting α-gal.

Furthermore, consideration should be given to characterizing the tick molecular machinery that is directly involved in synthesis or recycling of host glycans and reintroduction to the host via saliva during the feeding process. Targeted silencing of machinery related to N-glycan synthesis and modification in the tick could further assist understanding how of tick glycan processing occurs and why only specific ticks present α-gal. Specifically, tick α-fucosidase is a promising target for silencing using RNAi in tandem with artificial membrane feeding of human blood. Fucosidases possibly play a role in the tick's ability to cleave fucose residues from the glycans of human blood group B antigens, leaving them with α-gal containing glycans.

Using an α-gal knock-out mouse model would also be useful for both feeding and sensitization experiments using various species of ticks to determine if the tick feeding process is capable presenting α-gal and inducing AGS in the KO mouse model.

I have generated preliminary data that suggests that α-gal containing antigens are capable of being captured using a lectin derived from *Griffonia simplicifolia* (GSL I) (isolectin B4). Briefly, tick salivary gland extracts from *Am. americanum* were incubated in a solution with the biotinylated lectin. A simple dot blot using the M86 antibody shows that α-gal is present in the elution products (**Figure 6.1**). The resulting elution should have LC-MS/MS performed on them to identify the peptides captured. Although a lot of proteomic evidence has been shown in this dissertation and through tangential research, the results have not been narrowed down enough to positively identify which protein or proteins are culprit molecules. Moving forward, isolectin B4 assisted capture is likely a

better method to isolate the α-gal containing antigens than gel excisions of whole tick salivary gland extract.

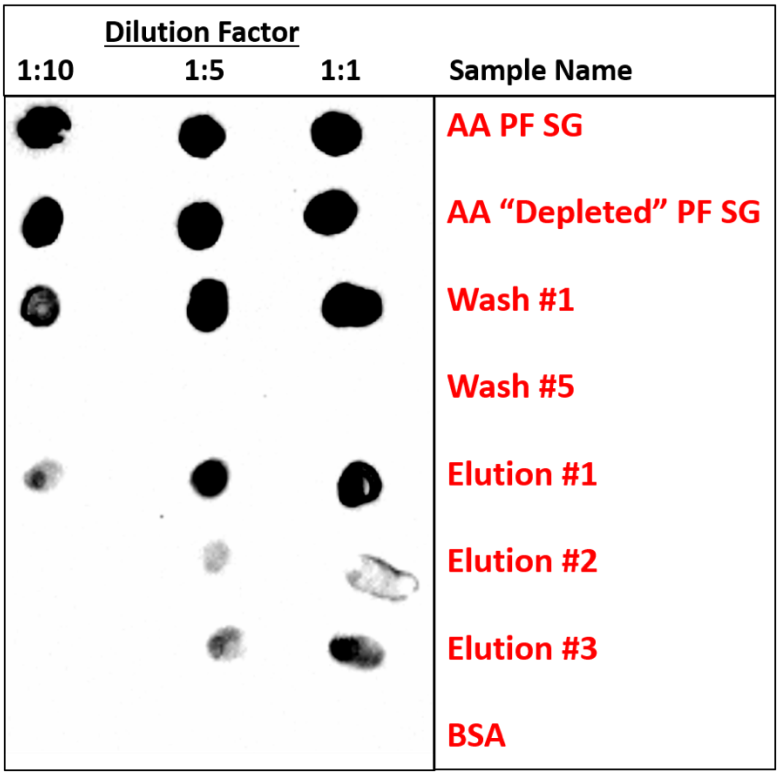


Figure 6.1 *Biotinylated GSL I isolectin B4 capture of α-gal containing antigens*

Am. americanum (AA) partially-fed (PF) salivary gland extracts (SGE) were incubated with biotinylated isolectin B4. AA PF SG contains raw *Am. americanum* (SGE). AA "Depleted" PF SG" contains the SGE after incubation with isolectin B4. Wash #1 is eluant from the first wash on the spin column and Wash #5 is the final eluant from washing. Elution #1 was the first elution from the spin column with elution buffer, and the subsequent elutions were produced by adding more elution buffer the membrane of the spin column and collecting into a fresh tube. Bovine serum albumin (BSA) was used as a negative control. Immunoblotting was performed using the M86 antibody.

Since the time that these experiments were conducted, there has also been the development of an IgG1 antibody (27H8) directed against α-gal (Kreft et al., 2022). Acquisition of this mAb could prove to be a valuable tool in the hands of a group working on α-gal related research using a tick model. The M86 antibody is a pentameric IgM

antibody and is not ideal for immunoprecipitation assays. Use of an IgG antibody could potentially also provide better immunolocalization data.

APPENDIX A – IACUC Approval Letter

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:	**15101501.3 (Replaces 15101501.2)**
PROJECT TITLE:	**Tick Sialome**
PROPOSED PROJECT DATES:	**09/2022-09/2024**
PROJECT TYPE:	**Renewal**
PRINCIPAL INVESTIGATOR(S):	**Shahid Karim**
DEPARTMENT:	**School of Biological, Environmental, and Earth Sciences**
FUNDING AGENCY/SPONSOR:	**USDA NIFA/NIH**
IACUC COMMITTEE ACTION:	**Designated Review Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2024**

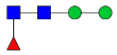
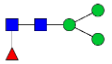
Jake Schaefer, PhD
IACUC Chair

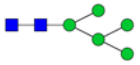
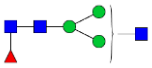
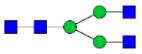
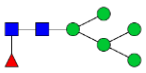
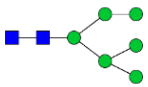
Date: September 13, 2022

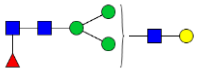
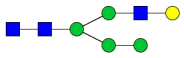
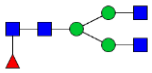
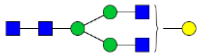
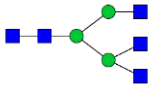
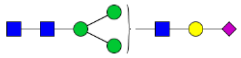
APPENDIX B Glycan Abundance in Ticks

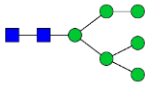
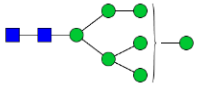
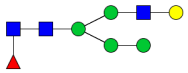
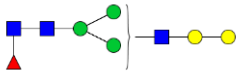
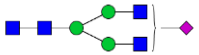
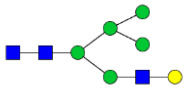
Table B.1

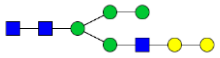
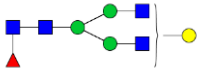
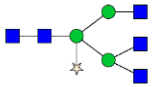
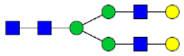
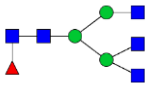
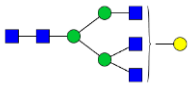
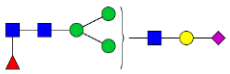
N-glycans identified in tick samples (columns) are marked with percentage of overall abundance (if identified)

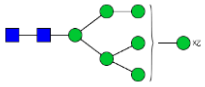
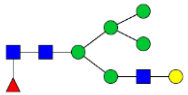
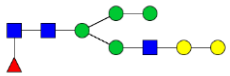
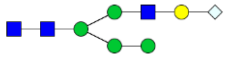
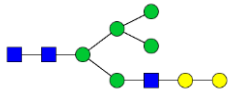
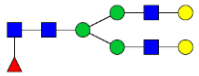
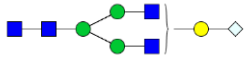
Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G1	885.4331		2.27	1.44	0.19		1.09	0.03		0.00	
G2	967.4867		1.67	1.76		3.42	2.33	0.22	1.80	2.70	3.00
G3	1141.576		0.65		5.02	1.94				3.50	3.60
G4	1171.587		0.82	0.85	2.06	2.69	1.74		0.00	0.00	0.00
G5	1345.678			0.63	2.99	2.08	1.97	0.57	1.50	1.30	0.00
G6	1375.688		0.37	0.70	3.45	2.85	1.68	0.62	0.40	0.70	0.20
G7	1386.702									0.00	2.00

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G8	1416.715			**0.08**		**0.03**					
G9	1549.778		**0.79**	**1.43**	**3.37**	**0.58**	**1.90**	**0.51**		**0.00**	**19.60**
G10	1579.788		**2.12**	**4.15**	**3.57**	**2.56**	**3.61**	**1.88**	**2.10**	**11.50**	**21.70**
G11	1590.801					**0.34**	**2.13**	**0.22**	**4.70**	**4.90**	**3.50**
G12	1620.8148		**0.02**	**0.37**							
G13	1661.831			**0.11**	**0.43**		**0.10**	**0.64**	**0.20**	**0.00**	
G14	1753.8812			**1.07**	**1.39**	**0.05**	**1.69**	**1.73**			
G15	1783.888		**7.61**	**11.16**	**9.00**	**17.28**	**6.47**	**3.13**	**4.40**	**0.00**	**17.60**

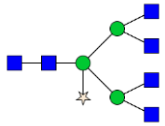
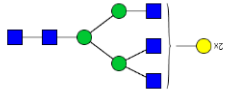
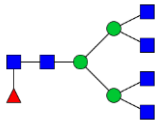
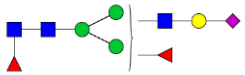
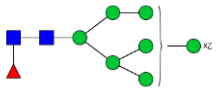
Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G16	1794.904		**0.28**	**1.16**	**0.19**	**0.70**	**1.98**	**0.80**	**4.30**	**14.00**	**0.50**
G17	1821.916								**4.00**	**1.00**	
G18	1824.911		**0.10**	**0.64**	**0.34**	**0.26**					
G19	1835.931		**0.27**	**1.56**	**1.17**	**6.54**	**4.09**	**2.22**	**10.80**	**0.80**	**1.00**
G20	1865.944		**0.09**	**0.34**	**0.03**	**0.24**	**0.50**	**0.83**	**0.10**	**4.10**	
G21	1906.971		**0.60**	**5.06**	**0.05**		**0.04**	**1.29**	**0.50**		
G22	1981.983								**0.00**	**0.10**	

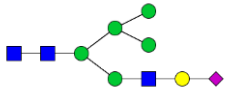
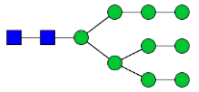
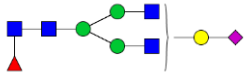
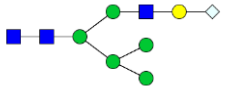
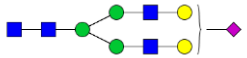
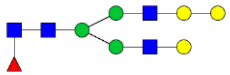
Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G23	1987.988								**3.40**		
G24	1987.9882		**8.88**	**9.93**	**12.79**	**2.70**	**5.77**	**4.51**		**8.60**	**5.30**
G25	1999.005		**0.39**	***Trace***	**0.26**	**1.65**	**1.13**		**4.00**	**1.70**	**0.40**
G26	1999.006			***Trace***						**1.70**	
G27	2012								**1.40**		
G28	2023.016									**4.70**	
G29	2029.013		**0.05**	**0.11**		***Trace***	**0.12**			**0.00**	

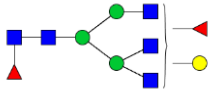
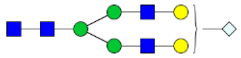
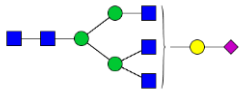
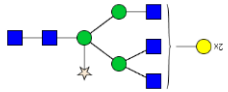
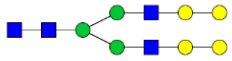
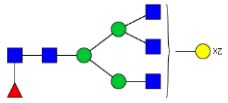
Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G30	2029.013			**0.11**	***Trace***					**0.00**	
G31	2040.0296		**0.29**	**0.81**		**0.79**	**2.46**	**2.95**			
G32	2067.042			**0.62**			**1.14**			**0.00**	**1.10**
G33	2070.048		**0.59**	**0.44**			**0.48**	**1.08**	**0.80**		**0.00**
G34	2081.059		**4.06**	**11.87**	**0.52**	**35.58**	**14.59**	**12.62**	**11.00**	**17.40**	**9.00**
G35	2111.073		**1.52**	**2.36**		**1.64**	**1.91**	**2.35**	**0.10**		
G36	2156.0696				**0.24**						

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G37	2192.088		**8.28**	**7.49**	**13.18**	**2.24**	**3.90**	**6.83**	**3.80**	**9.60**	**5.10**
G38	2203.107		**0.08**	**0.11**		**0.47**	**0.11**			**0.00**	
G39	2203.107			**0.11**	***Trace***					**0.00**	
G40	2216.0934		**0.03**			**0.10**	**0.17**				
G41	2233.117				***Trace***					**0.00**	
G42	2244.13		**1.94**	**1.28**	**0.24**	**1.23**	**2.60**	**2.16**	**12.60**	**0.00**	
G43	2257.126		**0.76**	**0.73**			**1.24**	**0.74**			

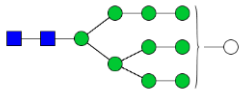
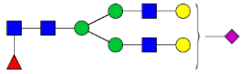
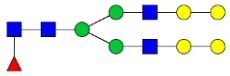
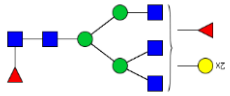
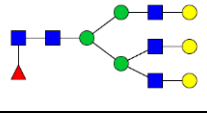
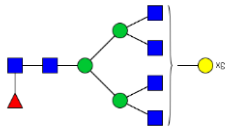
Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G44	2271.143		**0.99**	**0.70**			**1.62**	**1.10**		**0.40**	
G45	2274.14					***Trace***					
G46	2274.1356			**0.18**							
G47	2285.157		**13.18**	**10.97**	**0.42**	**5.61**	**17.17**	**18.78**	**5.60**	**3.60**	**1.40**

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G48	2312.171									**0.10**	
G49	2315.17		**2.36**	**1.14**		**0.33**	**1.62**	**2.09**		**0.00**	
G50	2326.189								**0.10**		**0.00**
G51	2330.1524					**0.47**					
G52	2366.168									**0.00**	

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G53	2390.176									**0.00**	
G54	2396.19		**9.68**	**6.87**	**33.75**	**1.75**	**2.51**	**13.29**	**13.50**	**7.30**	**4.60**
G55	2401.205		**0.22**	**0.16**	**1.33**		**0.02**			**0.00**	
G56	2420.189					**0.49**					
G57	2431.2118		**0.07**					**0.14**			
G58	2448.22			**0.44**	***Trace***						

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G59	2459.241		**0.47**								
G60	2461.215		**1.54**	**0.67**		**0.15**	**0.68**	**1.73**			
G61	2472.2272					**0.16**					
G62	2475.241		**1.64**	**0.60**			**0.89**	**1.16**		**0.10**	
G63	2478.246								**1.90**	***Trace***	
G64	2489.259		**19.72**	**8.34**	**0.61**	**1.68**	**7.57**	**12.88**	**2.20**	***0.10***	**0.10**

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G65	2519.2588		**1.20**	**0.29**				**0.20**			
G66	2530.287		**0.36**	**0.18**					**0.10**	**0.00**	**0.00**
G67	2560.2978		**0.24**								

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G68	2600.29			**0.20**	**3.04**		**0.11**	**0.18**		**0.50**	
G69	2605.293				**0.04**						
G70	2652.333			**0.72**	**0.15**				**4.40**	**0.00**	
G71	2663.339		**0.91**								
G72	2693.364	 	**0.59**						**0.10**	**0.00**	

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G73	2706.352		**0.02**								
G74	2717.3572					**1.38**					
G75	2734.382		**0.75**	**0.07**							**0.20**
G76	2792.3912							**0.51**			

Name	Obs Mass	Proposed Structures	*A. americanum* UF SG	*A. americanum* PF SG	*A. americanum* Saliva	*A. maculatum* UF SG	*A. maculatum* PF SG	*A. maculatum* Saliva	*I. scapularis* UF SG	*I. scapularis* PF SG	*I. scapularis* Saliva
G77	2938.4784		**0.42**	**0.02**							
G78	3142.585									**0.00**	

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