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A STUDY ON ECOLOGICAL DISTRIBUTION OF AND SELECTED GENE EXPRESSION IN AMBLYOMMA AMERICANUM: AN INSIGHT INTO THE ALPHA-GALACTOSE ALLERGY CAUSED BY TICK BITES

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A Thesis Submitted to the Graduate School in Partial Fulfillment of the Requirements for the Degree of Master in Science

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A STUDY ON ECOLOGICAL DISTRIBUTION OF AND SELECTED GENE EXPRESSION IN *AMBLYOMMA AMERICANUM*: AN INSIGHT INTO THE ALPHA-GALACTOSE ALLERGY CAUSED BY TICK BITES

An Abstract of the Thesis by
Leah Cuthill

Ticks transmit a wide variety of pathogens including viruses, bacteria, protozoa, and helminths to vertebrates. Their life cycle depends on blood meals from various hosts as well as on environmental conditions such as the temperature and habitat type. A newly recognized allergic disease (alpha-gal) has been identified and is characterized by a delayed reaction following the consumption of some mammalian meats. The allergy often results in life threatening reactions such as anaphylaxis, hives, and breathing problems 3-6 hours after the consumption of mammalian meat and is often associated with repeated tick bites, specifically by the species, *Amblyomma americanum* (Lone star tick). Not everyone develops the allergy when they are bitten by a lone star tick, and the cause of the reaction is yet to be known. The goal of the present study was to understand the ecological distribution of lone star ticks compared to other tick species as well as optimize and detect expression of selected genes in male and female adult lone star ticks. Ticks were collected during May-September in 2019 from various locations in southeast and northeast Kansas using the flag-drag method. Adults and nymphs were sexed and identified using taxonomic keys, and for a subset the identity was confirmed by PCR. Further study focused on *Amblyomma americanum* and another species *A. maculatum*, which was used as a control group. RNA was extracted from midgut and salivary gland content of dissected ticks using Trizol® based technique. The cDNA was prepared and samples were optimized for and tested by real-time PCR using selected primer sets for housekeeping genes, alpha-gal

synthesis gene families, and others. The data obtained in this study could help in understanding the relative gene expression among males and females of *Amblyomma americanum* and how this difference could account for the development of alpha-gal allergy after tick bites.

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CHAPTER I

INTRODUCTION

A vector-borne disease is defined as an infection transmitted from the bite of an infected arthropod (ECDC 2020). Examples of ectothermic species known to transmit vector-borne diseases to human populations include mosquitos, ticks, triatomine bugs, black flies, and sandflies. Furthermore, pathogens that can be vectored to human or animal populations include bacteria, viruses, helminths, and protozoa (Commins et al. 2011). Weather and climate conditions greatly influence the survival and reproductive rates of vectors and the diseases they carry (Mori et al. 2013). This is because arthropod vectors are ectothermic and typically thrive when habitats are above freezing conditions. Other influences that can change the distribution of vectors may include, but are not limited to habitat destruction, host density, land use, and pesticide application. Importantly, vector-borne diseases are not to be confused with a zoonotic disease which are diseases spread between animals and people (CDC 2017). Zoonotic diseases may be spread by direct or indirect contact with infected animals or originate from food-borne or water-borne sources (CDC 2017). Some infections may also be grouped as a vector-borne and zoonotic disease. Bubonic plague, for example, is caused by the bacterium *Yersinia pestis* and is carried by fleas, which in turn, are harbored by rodent populations (Drancourt 2020).

While the majority of vector-borne diseases are often associated with insects such as mosquitos and fleas, other groups of arthropods pose a great threat to human health and well-being. Ticks, for example, are arachnids closely related to mites and spiders and are regarded as ectoparasites that feed outside of the host (Commins et al. 2011). Ticks are known to transmit the greatest diversity of arthropod-borne pathogens and are also responsible for most cases of vector-borne diseases globally (Eisen et al. 2017). Unlike mosquitos, both male and female ticks can feed on blood meal and thus are capable of spreading vector-borne pathogens. Male mosquitos only feed on nectar whereas female mosquitoes feed on host blood and consequently may transmit pathogens to their hosts. This is a feature common to many other parasitic dipterans. Ticks are completely dependent on vertebrate blood meals for survival in all three of their life cycle stages, consisting of larva, nymph, and adult (Mori et al. 2013). Additionally, females tend to have a greater blood intake than males. Once the tick takes up blood components from its host, blood digestion occurs intracellularly within the cytosol of the midgut cells or within endo-lysosomal vesicles. Hemoglobin, for example, is known to be digested via proteolytic enzymes in the digestive cells of the tick midguts and results in the production of large quantities of heme (Mori et al. 2013). Many parasitic arthropods need to balance vertebrate iron-binding proteins in blood due to the content of serum transferrin or hemoglobin. While the majority of blood-sucking arthropods utilize iron from their hosts, which is especially essential for ovarian development, ticks must exploit iron in a way that prevents toxicity. Transferrin is a known iron transporter in vertebrates and several arthropods, but tick transferrin has only been identified in a few species and is poorly understood compared to other blood-feeding arthropods.

When searching for a blood meal ticks will either rely on ‘questing’, wait under debris until prey is close, or actively hunt prey. Questing is a strategy where ticks will climb a structure, such as a grass blade, and outstretch their front legs in an attempt to grasp a potential host as they brush past (Mori et al. 2013) **(Fig. 1)**. Some species may feature both questing and hunting strategies when searching for a host.

Figure 1. Questing male *Amblyomma americanum* tick (Photo by Leah Cuthill)

The majority of tick populations are limited by geographic range or by host migration events; however, distributions may shift depending on environmental conditions or accidental imports of host species (Mori et al.2013; Werden et al. 2014; Soneshine et al. 2018). Numerous investigations have suggested that increasing global temperatures and humidity have contributed to range expansion of many pathogen-carrying arthropods, including ticks. The extent of freezing temperatures is a factor limiting ticks within a geographic range. A reduction of the duration and extent of freezing temperatures may be an important factor for understanding tick population ranges. Furthermore, various tick species may overlap in their geographic range and feed on the same hosts, thus, increasing the risk of disease transmission to host species. In addition, one of the important hosts of

tick life cycles is white-tailed deer and is known as the primary host for mature ticks. The migration and expansion of deer have a direct impact on the spread of tick-borne diseases **(Fig. 2)**.

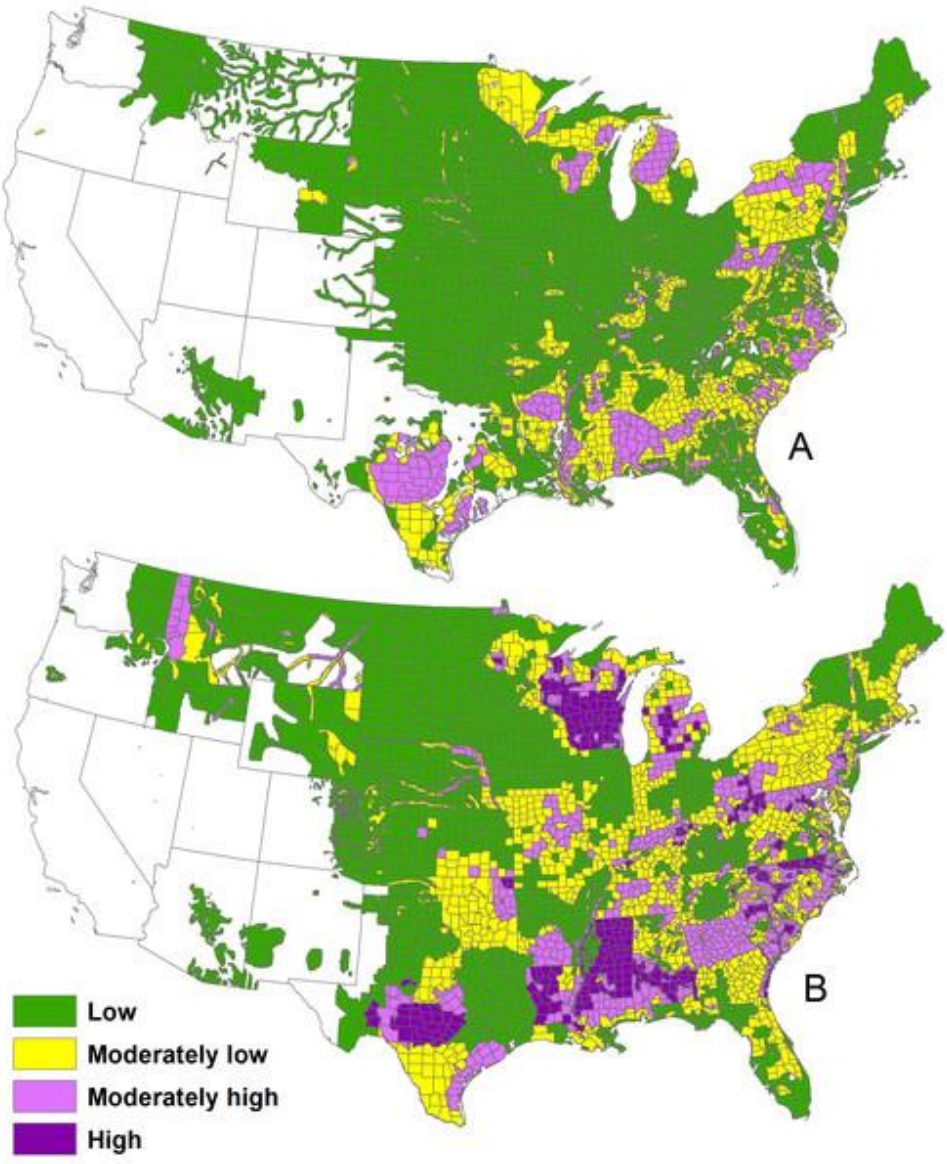


Figure 2. The 1982 white-tailed deer densities (A) and 2001–2005 white-tailed deer densities (B) in the conterminous United States (Hanberry and Hanberry 2020)

The blacklegged tick, or deer tick (*Ixodes* spp.), is the primary vector of Lyme disease, an infectious disease caused by *Borrelia burgdorferi,* as well as other bacterial disease agents. White-tailed deer were extirpated from much of the eastern U.S. by the early 20th century due to unregulated hunting and habitat destruction. Now, 100 years later, here are far more deer in North America than ever before, due to predator removal, etc., to the point where they are a nuisance. Study showed that Midwestern range of *Ixodes scapularis* positively correlate with the emergence of tick-borne diseases (Hamer et al. 2014).

The lone star tick, *A. americanum,* is a highly aggressive tick species known for biting humans in larval, nymphal, and adult stages of its life cycle (CDC 2020). While all three stages are known to feed on humans, the nymph and adult females most frequently bite and transmit pathogens. Their lifecycles begin when the blood-engorged female tick falls from the host and after several days deposits ~5,000 eggs on the soil in a "protected" location, such as in mulch or leaf litter. After dislodging from the host, the female will seek a microclimate, typically an area of high humidity at a soil level that is best suited for survival of the eggs. Females have been shown to search for a favorable microclimate after feeding. Following an incubation period, larvae hatch from eggs and progress through a quiescent (resting) period, then seek a host by questing. The larva then grasps the host and proceeds to move about the host, seeking a preferred feeding site. After acquiring a host, the larva attaches, blood-feeds for 1-3 days, detaches its mouthparts, and then drops from the host to digest its blood meal and molt into a nymph. Nymphs repeat this process; however, after dislodging from this second host they molt into adults. Sizes of ticks in each stage can vary due to genetic and environmental conditions. In laboratory settings, the life

cycle can be shortened to less than 22 weeks under optimal conditions, but is usually 2 years in nature (http://entnemdept.ufl.edu/creatures/urban/medical/lone_star_tick.htm).

It should be noted that populations of lone star ticks are widely distributed in southeastern and eastern United States **(Fig. 3)**. Consequently, this species is increasing range expansion because of reintroduction of deer, changes in habitat and climate and thus presents a serious public health threat to the northeast United States and Canada (Springer et al. 2015). This species also overlaps the range of the American dog tick, *Dermacentor variabilis*, and the Gulf Coast tick, *Amblyomma maculatum.*

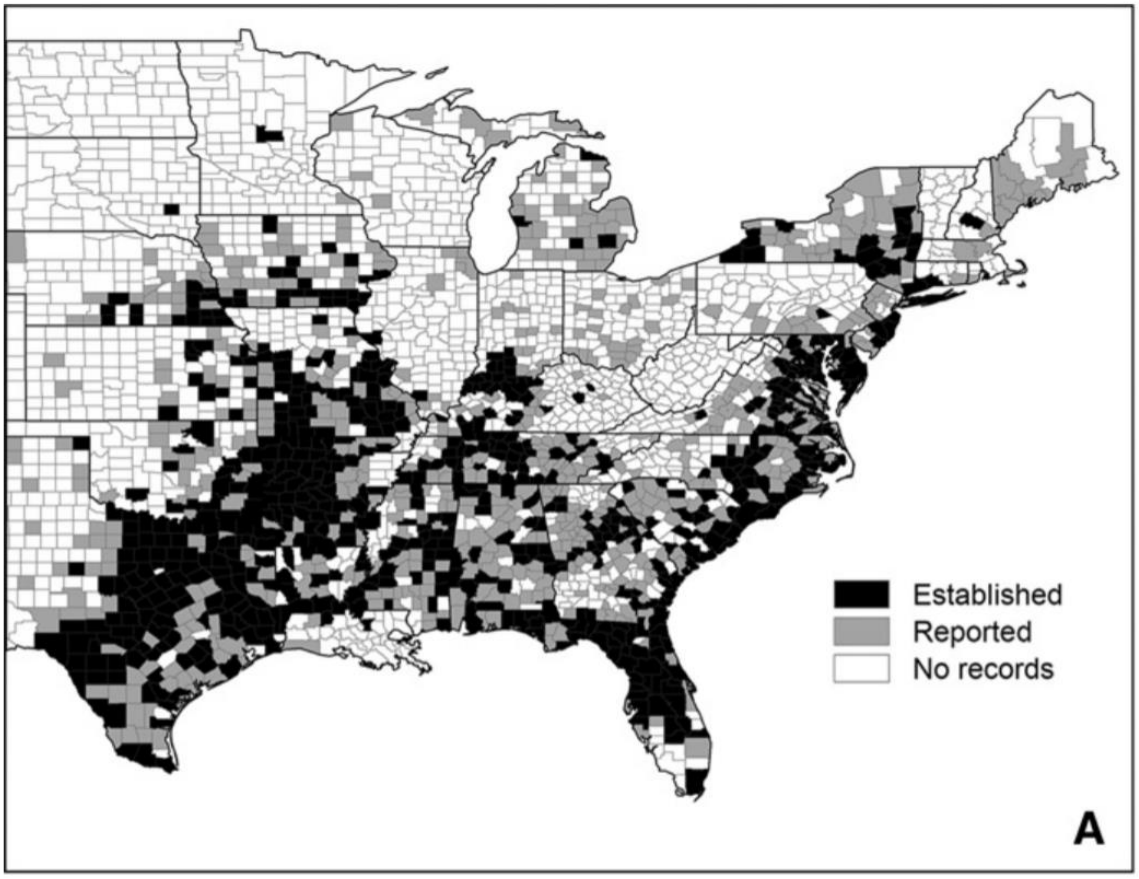


Figure 3. Map depicting spatial distribution of *Amblyomma americanum* based on county-level collection and classification records, cumulative from 1898 through 2012 (established, reported, no records) (Springer et al. 2015)

Adult *A. americanum* females feature a unique white dot or "lone star" on the back of the exoskeleton, while males, larvae, and nymphs lack this feature. Unlike questing species such as *D. variabilis, A. americanum* is regarded as a "hunter tick" which rapidly pursues its prey across many meters when it detects host odor and CO_2 (Mori et al. 2013). However, this species will occasionally both display questing and hunting behavior when searching for hosts. The lone star ticks are known vectors of human elhrlichiosis (*Ehrlichia chaffeensis* and *E. ewingii*), Heartland virus, tularemia (*Francisella tularensis*), and southern tick associated illness (STARI). Additionally, they are suspected to carry disease-causing agents *Rickettsia amblyomii* and *Borrelia lonestari*, a close relative of the Lyme disease pathogen *Borrelia burgdoferi* (Soneshine 2018).

Alpha-gal syndrome (AGS), or the mammalian meat allergy, is an unusual and newly recognized allergen characterized by a delayed reaction following the consumption of mammalian meats (Wolver et al. 2012). Most reports are from southeastern and midwestern U.S. In 2009, there were 24 reported cases of alpha-gal syndrome; however, most recent estimates exceeded 5,000 cases and AGS was identified as the leading cause of anaphylaxis in a southeastern registry of patients (Pattanaik et al., 2018). This allergy often results in life threatening symptoms such as breathing problems, severe hives, and anaphylaxis following mammalian meat consumption **(Fig. 4)**. Other symptoms may include drop in blood pressure, faintness, nausea or vomiting, and severe stomach pain (CDC 2019). Symptoms of alpha-gal allergy usually appear 3 to 6 hours following the consumption of mammalian meats and may vary among different individuals. Many of the common meats reported to cause reactions include beef, lamb, pork, venison, and rabbit.

However, this particular alpha-gal carbohydrate may also be found in some cosmetics, vaccines, gelatin, medications, and dairy products.

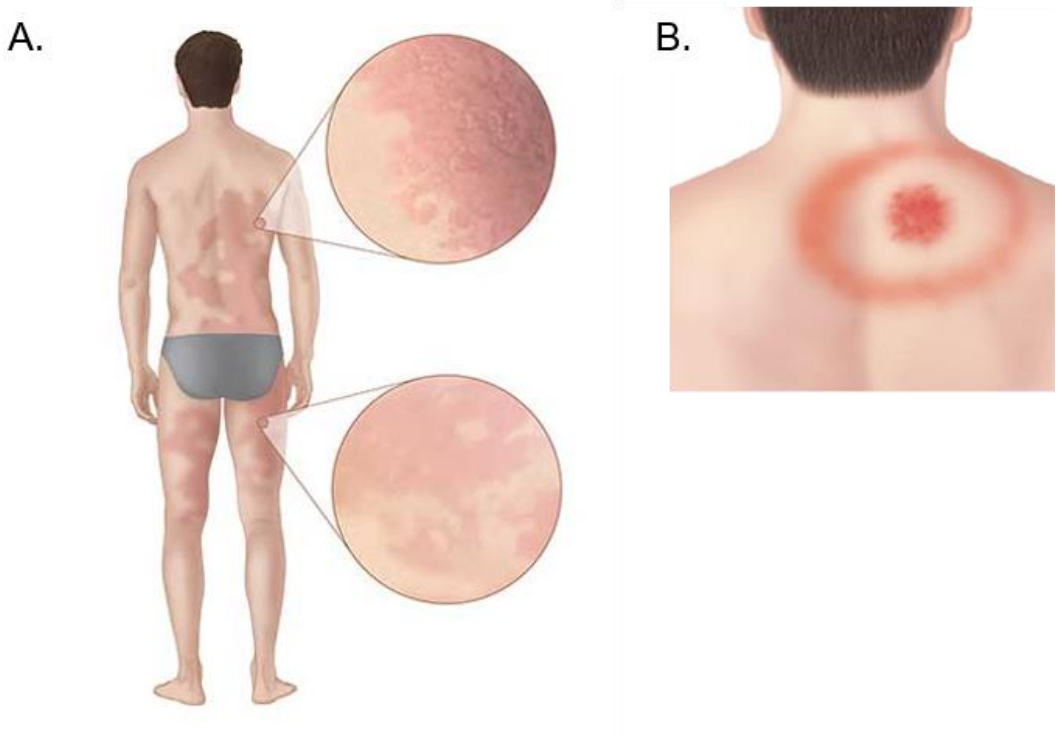


Figure 4. Comparison between alpha-gal rash (A) and Lyme disease rash (B) (CDC 2019).

Salivary glands of ticks possess various secretory proteins with following activities such as anti-inflammatory, anti-hemostatic, immunomodulatory, and anti-complement **(Fig. 5)**. Endogenous protease inhibitors, for example, help mammals regulate many physiological processes, some of which have been recognized in tick saliva. Tick saliva contains one of the largest families of secretory salivary protein, the Kunitz-domain protease inhibitors, which has anti-coagulation activity. Another group of tick salivary protease inhibitor include the trypsin inhibitor-like cysteine rich domain (TIL)-domain inhibitors, which helps ticks defend against parasitic nematodes. Along with these secretory proteins pathogens can also be injected while ticks feed on their vertebrate host.

A recent finding by Commins et al. (2019) suggested for the first time that alpha-gal syndrome (red meat allergy) may develop from ticks' saliva itself. This means that ticks can induce this immune response without requiring the blood meal and this increases the risk of each bite potentially leading to this allergy. Researchers showed that Immunoglobulin E (IgE) antibodies recovered from individuals allergic and not allergic to alpha-gal. When alpha-gal IgE sensitized cells were introduced to *A. americanum* tick salivary gland extract, reactions were the highest compared to that caused by *I. scapularis* whereas there was no reaction with that with *A. maculatum* (Commins et al. 2019).

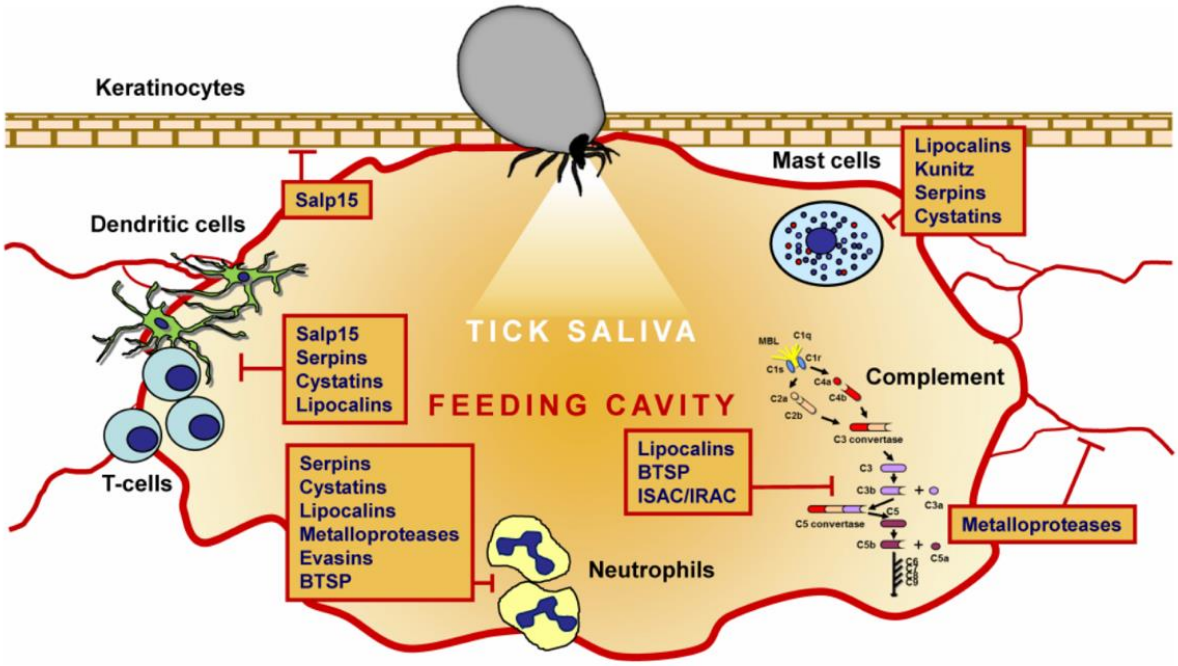


Figure 5. The immunomodulatory and anti-complement activities of the major tick-secreted protein families (Springer and Gagneux 2015)

Alpha-gal syndrome it is the only known carbohydrate allergen to result in this kind of reaction where the etiology is unknown (Mori et al. 2013; van Nunen et al. 2015). Alpha-gal is short for the molecule galactose-alpha-1,3-galactose, and is found naturally in almost

all mammal species **(Fig. 6)**. Humans, apes, and old-world monkeys are unable to synthesize alpha-galactose because they have lost the ability to synthesize the carbohydrate, as anti-alpha galactosyl IgG was produced roughly 28 million years ago (Mori et al.2013; van Nunen et al.2015; Cabezas-Cruz and Espinosa 2018). Consequently, anti-alpha gal IgE antibodies have been associated with tick-induced alpha-gal syndrome and it is suspected that anti-alpha-gal IgG/IgM antibodies might be involved in resistance against leishmaniasis, Chagas disease, and malaria. Presence of alpha-gal in tick saliva is important in the etiology of alpha-gal syndrome. Studies have linked alpha-gal syndrome to the production of IgE antibodies following a bite from certain tick species.

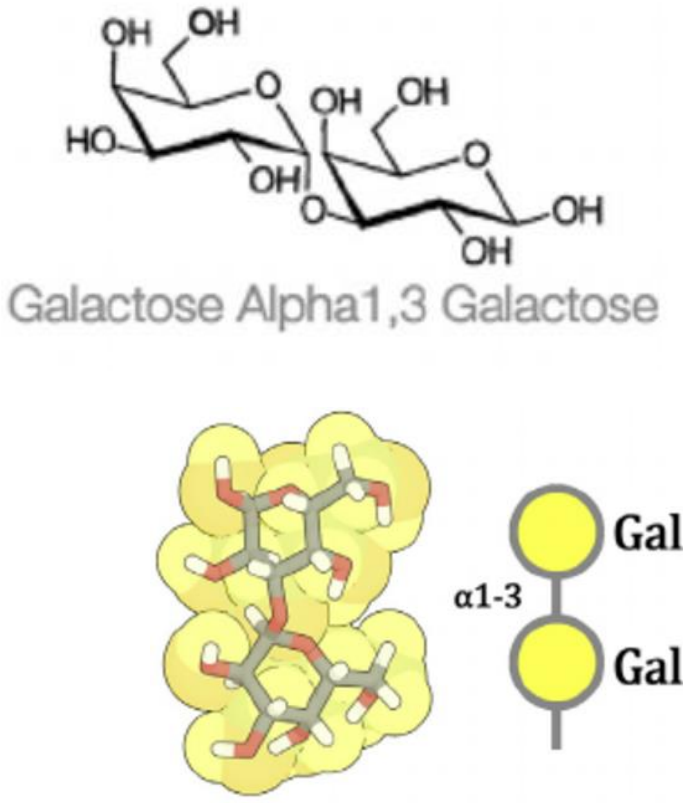


Figure 6. Structure of alpha-gal (Springer and Gagneux 2015)

Both children and adults can develop alpha-gal allergy; however, most cases of alpha-gal allergy appear to be in people >50 years of age and related to bites from *A.*

americanum. In contrast, *Ixodes scapularis*, the main vector of Lyme disease (*Borrelia burgdoferi* infection) in the United States, does not induce IgE to alpha-gal and does not have bite symptoms associated with itching (Steinke et al. 2015). However, if *I. scapularis* is infected with the bacterium *Anaplasma phagocytophilum*, then the genes for alpha-galactose are expressed (Cabezas-Cruiz and Epsinosa 2018). The production of these transferases could be linked to the production of alpha-gal. However, it is currently unknown whether ticks are able to produce endogenous alpha-gal within its system.

Commins (2016) reviewed the literature on various tick species associated with the alpha-gal allergy reported from different countries. **Table 1** below illustrates details of few studies.

Table 1. Association of tick species with alpha-gal allergy

Tick species	Country	Case reports	References
Ixodes holocyclus *I. australiensis*	Australia	Cases reported along eastern seaboard of the Australian continent. Over 800 cases reported. Patients suffering from meat allergy reported exposure to *I. holocyclus.*	van Nunen (2014) Mullins et al. (2012) Cabezas-Cruz et al. (2019)
Ixodes ricinus	Sweden, Germany, Italy, Norway	Sweden experienced meat-induced anaphylaxis near the Stockholm area. Approx. 95 confirmed cases. Over 50 cases reported in Germany. Many reactions followed the consumption of gelatin-containing sweets. 32 patients recalled a tick bite previous to the allergy. Roughly 30 reported in Italy, 1 suspected case from Norway following a *Ixodes ricinus* bite.	Hamsten et al. (2013) Caponetto et al. (2013)
Haemaphysalis longicornis	Korea, Japan	In Japan 2012 a woman developed mammalian meat anaphylaxis and was recently bitten by a tick. Over 80 cases reported. In Korea, male patient experienced delayed pork and beef anaphylaxis. Several more cases have occurred in 2014. Roughly 12 cases reported.	Lee et al. (2013) Sekiya et al. (2012) Takahashi et al. (2014) Cabezas-Cruz et al. (2019)
Amblyomma variegatum	South Africa, Zimbabwe	Roughly 5 cases reported. Two farmers near the coast contracted long-standing mammalian meat allergies as	van Nunen (2014) Cabezas-Cruz et al. (2019)

	adults. A third person from Zimbabwe experienced the allergy as well.

Figure 7. Cross-contamination response of alpha-gal allergy, mild reaction (Photo by Leah Cuthill)

Based on this background the following study questions were framed:

- What is the ecological distribution of *A. americanum* relative to other tick species?
- Which housekeeping gene is suitable for being used as an internal control for gene expression studies in *A. americanum*?
- What are the relative expression levels of genes related to alpha-gal production and other in males and females of *A. americanum*?

CHAPTER II

MATERIALS AND METHODS

Materials required for tick collection and processing:

The material that was needed to collect ticks were 1 m^2 flannel cloth attached to a 0.5 inch-thick wooden flag pole, sterile plastic containers, sharp forceps, and an ice cooler. All collecting personnel were equipped with protective gear such as gloves, all body cover white suit, insect repellents (DEET/Deep Woods), insecticide (Permethrin), and ducktape to seal the gaps between clothing and boot or gloves. Field assistants were also recommended to wear light-colored full-sleeve shirts and long pants. In the laboratory, materials needed were dissecting microscope (stereoscope), liquid nitrogen, bead beater, microcentrifuge tube, DNA isolation kit, sterile plastic pestle, TAE buffer, agar powder, UV Transilluminator, pipettes and pipette tips, vortexer, Ethanol (70% and 100%), 0.5% sodium hypochlorite solution, sterile water, and fine tip brush.

Field collection

Ticks were collected from nine locations in 2019 during late May through September. The method used for collection from wooded and pasture environment was 'Flag and Drag' (Carroll and Schmidtmann 1992). The flag consists of a 1 m^2 wool cloth, tied to a wooden pole and dragged across vegetation by personnel. Ticks questing in tall grass will attach to the flag, which was down every 10 meters for inspection, and carefully

removed from the wool cloth using forceps **(Fig. 8)**. Only adult and nymph ticks were collected; larvae were not collected because of difficulty in identification. Specimens were placed into plastic or metal containers and recorded with GPS coordinates and date, then transported in an ice cooler to the laboratory. Temperature and humidity levels were also recorded on the date and site of collection from https://www.ncdc.noaa.gov/cdo-web/datatools/records.

Figure 8. Flag and drag method for tick collection (Photo by Andra Stefanoni, PSU)

The control group of *A. maculatum* ticks were collected from cattle (designated as K-State samples) with the use of cattle chutes. Juvenile angus cattle were placed within chutes to be weighed, vaccinated, and inspected for ticks. Ticks were removed from the ears of cattle using forceps and placed into plastic bags. Ticks were transported in an ice cooler to the laboratory to be identified with the same process. Furthermore, engorged specimens were separated from the non-engorged ones. Notable habitat features in the area where the K-State samples were collected consisted of pastureland. Specimens of *A.*

americanum collected from the northeastern area were found in deciduous forests, while specimens of the same species collected from the southeastern areas were obtained from a mix of tall grass prairies and deciduous forests **(Fig. 9)**.

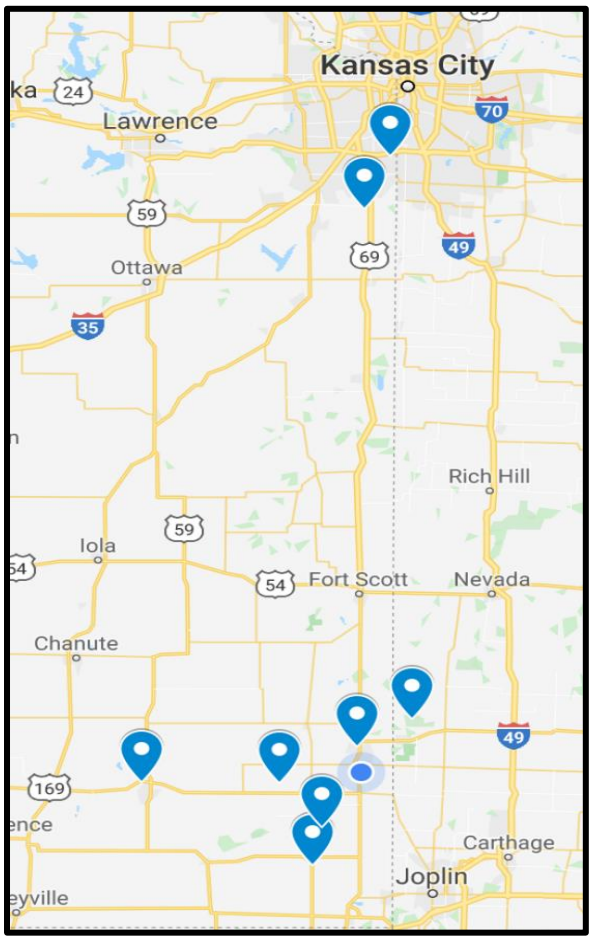


Figure 9. Google map image showing various filed sites for tick collection

Phenotypic identification

Ticks were identified by species using a set of taxonomic keys **(Fig. 10)** (https://tickencounter.org/tick_identification/tick_species). Once in the lab, ticks were placed in a freezer at -20°C for 24 hours before identification began. Ticks removed from -20°C too soon could reanimate and risk escaping during identification. Ticks were identified using a stereoscope and identification key to separate life-stages, species, and sexes **(Fig.**

10). Engorged ticks were identified alongside non-engorged individuals but not processed further. While both nymph and adult ticks were collected and identified using phenotypic identification, only adult samples were used for further analysis. The other species that might be encountered include the black-legged deer tick (*Ixodes scapularis)*, the gulf coast tick (*Amblyomma maculatum)*, and the American dog tick (*Dermacentor variabilis*). Their ranges were listed to determine if they corresponded with the lone star tick's distribution.

Male and female ticks could be differentiated based on the dorsal markings on their exoskeleton. *Dermacentor* and *Amblyomma* male ticks usually displayed multifaceted markings while females displayed simplistic colorations. *Ixodes* typically lack intricate colorations and can be sexed based on the placement of dark coloration on the exoskeleton. Tick species could also be identified based on mouthparts or size. The American dog tick, or *D. variabilis* ticks, for example, have short mouthparts consisting of chelicerae, a hypostome, and palps while *A. maculatum and A. americanum* have longer mouthparts. The sizes of ticks could also be an indicator of species, as *I. scapularis* has one of the smallest adult stages when compared to *D. variabilis, A. americanum, and A. maculatum.* Males and females were separated into plastic petri dishes containing 60 males and 60 females. Each was organized based on species and location. After identification, ticks were again placed into a -20˚C freezer until further molecular analysis. GIS maps were created using ArcGIS software.

TickEncounter Resource Center **Ixodes scapularis (Blacklegged ticks or Deer ticks)**

TickEncounter Resource Center **Dermacentor variabilis (American Dog ticks)**

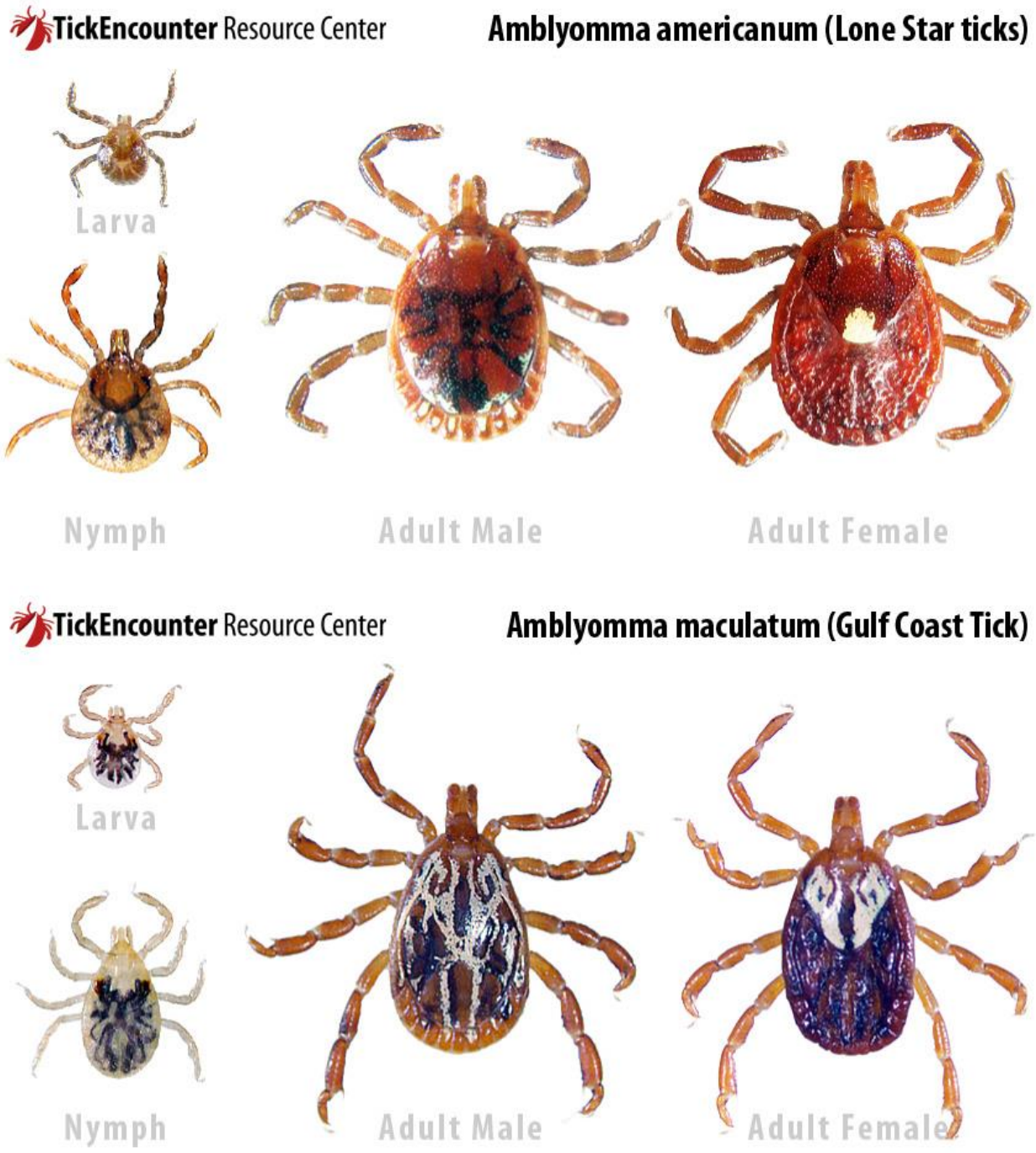


Figure 10. Tick images – larva, nymph, adult male female *of A. americanum, A. maculatum, D. variabilis, I. scapularis*

Tick dissection

Ticks were washed with 95% ethanol for 1 min or until dry. Parafilm was placed onto a glass petri dish and melted using a hot plate. Individual ticks were placed on semi-solid parafilm, using a sterile paintbrush, for 1 min or until wax completely solidified. Ticks could become loose if the parafilm was not properly dried. It was also important to avoid placing ticks on liquid parafilm that could potentially burn the sample. Once the parafilm solidified, a drop of 95% ethanol was placed onto the tick using a plastic dropper. After waiting for 1 min the Ethanol was removed using a sterile brush. A drop of sterile saline buffer was added using another plastic dropper and allowed to sit for 1 min. The dorsal side of the exoskeleton was carefully sliced open using a microscalpel to reveal the digestive and salivary glands. Using a dissection kit, both glands and internal contents were carefully removed **(Fig. 11)**. Two ticks from the same species and same sex and same location were dissected and the internal contents including salivary glands and midgut were pooled in a single microcentrifuge tube containing 500 µl TRIzol®. The specimens were frozen at -20˚C freezer till RNA extraction.

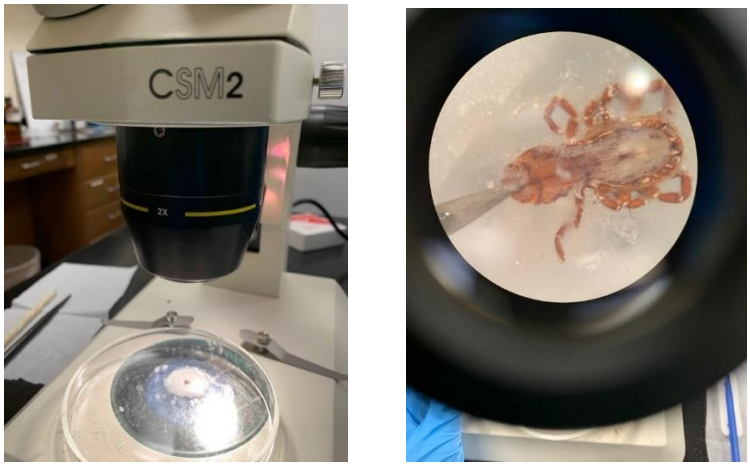


Figure 11. Paraffin embedded tick placed on dissectoscope (left) and dissected *A. maculatum* male tick showing internal contents (right)

Figure 12A: A Female; scutum removed. The complete gut is now visible, as are the anterior salivary glands (arrow) (Edwards 2015).

Figure 12B: Salivary glands (arrow); close-up (Edwards 2015).

RNA extraction

Frozen TRIzol® containing dissected ticks were homogenized with a sterile pestle. To each homogenate 200 µl of chloroform was added and vigorously vortexed for a minute and incubated on ice for 15 min. Following incubation, the samples were centrifuged at 12,000g for 15 min at 4°C to separate phases. Next, the aqueous phase was transferred into a fresh tube and RNA was precipitated by adding 200 µl of isopropanol. The tubes were again incubated for 10 min on ice and centrifuged for 10 min at 12,000g at 4°C. The

supernatant was removed, and the pellet was then washed with 1 ml 70% ethanol by flicking and centrifuged again at 7500g for 10 min at 4°C. The supernatant was discarded gently, the tube was air dried. Once dried, the RNA pellet was dissolved in 20 µl RNase-free H_20.

Total RNA was quantitated using a Nanodrop. First, 3 µl of sterile water was placed onto the optical surface then the lever arm is closed, then lifted and cleaned with a wipe. One µl of water was used to set as blank followed by placing 1µl of RNA sample for measurement. The nucleic acid concentration was measured as ng/µl (for single stranded RNA at 40 µg) while the purity ratios were measured as A260/280 (wavelength). An ideal range of 2.0 A260/A280 was preferred.

Polymerase chain reaction (PCR) amplification of selected genes

First, 10 µl of Promega 2X Master Mix was added into a centrifuge tube followed by 0.5 µl of each of forward and reverse primers (20 pmole) and enough distilled water to bring the volume to 20 µl. One microliter of synthesized cDNA was used as template. The tube was pulse-vortexed 5 times and centrifuged for a few seconds and placed on the thermocycler (BioRad C1000 Touch Cycler). The PCR protocol consisted of 3 different cycles. The first step consisted of denaturation at 95°C for 3 min, followed by another 29 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. The PCR was concluded with a final extension at 72°C for 5 min.

Agarose gel electrophoresis

1% agarose gel was prepared by mixing 0.6 g of agarose powder and 60 ml 1X TAE. To dissolve the powder, the mixture was microwaved for 3 min with 30 sec intervals of cooling. If particles were still visible, the mixture would be heated again until it appeared

clear and homogenous. Once cooled after 5 min, 2.5 µl Red Safe dye (Intron Scientific) was added using a pipette and mixed for a minute. The homogenous mixture was then carefully poured into a Fisher Biotech Electrophoresis Unit and solidified after 30 min. It should be noted that the gel was gradually poured into a corner of the tray to prevent bubbles, which could interfere with the distribution of DNA or RNA bands. Samples were prepared by adding 4 µl of loading buffer (6X) to the 20 µl PCR reaction and almost all of the sample was loaded on the gel. While for RNA, sample was prepared differently as mentioned below. Five microliters of 100 bp DNA ladder was used as a marker. The gel was run at 75 volts for 40 min.

RNA integrity was determined by gel electrophoresis right before cDNA synthesis. One microliter of RNA was run on a native 1.2% agarose gel prepared with 1X TAE (Tris-acetate-EDTA) buffer and stained with RedSafe dye at 75 volts and was visualized under UV-transilluminator. Afterwards, one microliter of RNA diluted in 7 µl of distilled water was heated to 95˚C for 1 min on the thermocycler and immediately put on ice and then run on 1.2% agarose gel as mentioned above.

cDNA synthesis

In order to synthesize first-strand cDNA, three components were combined in a sterile PCR tube. The first volume was 4 µl of SuperScript VILO MasterMix, the second was X µl of RNA up to 1.0 µg, and of DEPC- treated water to have a volume of 20 µl. Each was gently mixed, briefly centrifuged, and incubated at 25°C for 10 min, followed by incubation at 42°C for 120 min on a thermocycler. The reaction was stopped at 85°C after 5 min. Diluted/Undiluted cDNA was obtained in a PCR tube and stored at -20°C. To obtain a negative RT control, 4 µl of SuperScript VILO MasterMix and 16 µl DEPC-treated water

were added into the sterile PCR tube and incubated at 65°C for 10 min in order to denature the reverse transcriptase.

Real-time PCR

Real-time PCR was performed on cDNA samples derived from males and females of *Amblyomma* spp. to determine relative expression of target genes using housekeeping genes as reference genes to normalize cDNA input. During runs to select the appropriate housekeeping gene as reference, two technical replicates were used to conserve reagents and maximize plate efficiency. Biological replicates varied among various experiments.

In a clean biosafety cabinet, PCR reactions prepared by combining 10 µl of 2X Power SYBR™ Green PCR Master Mix (Applied Biosystems), 1 µl of each forward and reverse primers (1:20 diluted from stock 1 µg μl^{-1}), 7 µl of H_2O, and 2 µl of sample template for a final volume of 20 µl. A no template control was included on each plate. Each reaction was prepared in triplicate, pipetted into the 48 well real-time PCR optical plate, and sealed with optical adhesive film. Real-time PCR optical plates were centrifuged at 500 x g for 1 min to collect reaction and remove bubbles introduced from pipetting. Real-time PCR was performed using an Applied Biosystems StepOne Real-Time PCR System. Thermal cycling conditions were 95˚C for 10 min for initial DNA denaturation, followed by 40 cycles of 95˚C for 15 s and an annealing/extension temperature of 60˚C for 1 min. SYBR green fluoresces was measured after each annealing/extension step. A dissociation melting curve was included after the last annealing/extension step. Baseline and threshold Cq settings were adjusted to Cq threshold determined from primer validation.

Real-time PCR Protocol

A total of 19 µl of Master Mix solution, consisting Master Mix, water, and Primer, was transferred into amplification wells followed by 1 µl of template cDNA. The wells were then sealed tightly to prevent sample spillage and re-sealed again to ensure samples were contained. A piece of parafilm was placed over a vortex machine and the plate was vortexed for 5 sec. Afterwards, the microplate was transferred into a centrifuge for 2 min. The microplate was placed into the PCR machine with the door closed completely to ensure the wells were secured. It was also important to wear gloves and avoid using supplies from other stations during the PCR procedure.

Real-time PCR data analysis

The $2^{-\Delta\Delta CT}$ method was used for relative quantification (rao et al. 2013). The threshold cycle (C_T) was the cycle at which the fluorescence level reaches a certain amount (the threshold). This method directly used the C_T information generated from a RT-PCR system to calculate relative gene expression in target and reference samples, using a reference gene as the normalizer. A target sample may be, for instance, a treated sample (tick adult females), while a reference sample is an untreated control (tick adult males). Target and reference samples can also be samples from different tick species. To correct for differences in the amount of DNA/RNA added for each sample and to reduce variation caused by PCR set-up and the cycling process, reference genes or internal control genes were used to normalize the PCRs. Housekeeping genes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, ubiquitin, and histone, were used as reference genes because their expression levels remain relatively stable in response to any

treatment. With respect to the $\Delta\Delta C_T$ of the $2^{-\Delta\Delta CT}$ method, the first ΔC_T was the difference in threshold cycle between the target and reference genes:

$\Delta C_T = C_T$ (a target gene) − C_T (a reference gene).

$\Delta\Delta C_T = \Delta C_T$ (a target sample) − ΔC_T (a reference sample)

The final result of this method was presented as the fold change of target gene expression in a target sample relative to a reference sample, normalized to a reference gene. The relative gene expression was usually set to 1 for reference samples because $\Delta\Delta C_T$ is equal to 0 and therefore 2^0 is equal to 1. The average C_T value of each technical replicate was used to analyze the stability of each candidate reference gene.

Statistical analysis

A t-Test: Two-Sample Assuming Equal Variances was performed with $\alpha = 0.05$ to compare the RNA extraction efficiency among male and female adult ticks. All real-time data were expressed as mean ± SD, which was calculated by Applied Biosystems StepOne Real-Time PCR software.

CHAPTER III

RESULTS AND DISCUSSION

A total of nine locations in Kansas were sampled over a period of four months in 2019. The GIS maps below show the locations sampled along with tick abundance **(Fig. 15A)** as well as the prevalence of *A. americanum* in those locations **(Fig. 15B)**. The majority of sampling was carried out at four locations where tick abundance ranged from 129 – 386 **(Fig. 15A)**. Prevalence of *A. americanum* (lone star tick) ranged from 11 – 206 in those locations. The distance between the locations at northeast and southeast Kansas was ~ 150 miles **(Fig. 15B)**. This suggests how evenly the species *A. americanum* is distributed in the eastern region of the state. An unofficial survey among local residents and local doctors revealed presence of alpha-gal allergy among people in these regions (Kansas City, Pittsburg, Parsons).

The range of *A. americanum* is expanding northeast into areas previously thought to be uninhabited by this species (Dahlgren 2016). While higher temperatures and humidity levels are thought to be the main cause of the population shift, it is now speculated that reductions of woodland habitat from anthropogenic deforestation and agricultural development, in addition to the increase of white-tailed deer, that can correlate with the changes in distribution of *A. americanum* (Springer 2015). Relative abundance of this species is highest in areas of woodland habitats where dense understory vegetation enables

a microclimate habitat. The majority of *A. americanum* were collected in wooded areas, Bucyrus and Nathans Farm, which were reported to have an abundance of white-tailed deer and an abundance of understory vegetation. Tallgrass prairie and pastures featured the least number of specimens collected.

The second most abundant tick found was *D. variabilis,* which range occurs though much of the eastern United States where the climate and environmental conditions favour warm and humid summers (Dergousoff et all. 2013). The majority of specimens collected were obtained from Prairie State Park in areas featuring tallgrass prairie, approximately 24 out of the 48 ticks collected in that location **(Fig. 15A)**. Wild bison (*Bison bison)* were noted to be native to the area in addition to white-tailed deer. The tallgrass prairie habitat also featured the highest *A. maculatum* collection. There were a few *D. variabilis* specimens collected in wooded areas of southeastern and northeastern Kansas. However, the majority found were collected in southeastern areas. Records of *D. variabilis* display its preferred habitats include both boreal forests and prairies, though its geographic range is believed to be shifting west into the habitat of *Dermacentor andersoni*. Another notable adaptation of *D. variabilis* is its adaptation to coexist with other tick species that share the same hosts. Previous studies showing the range of *A. americanum* overlaps considerably with the reported cases of alpha-gal allergy in patients **(Figs. 13A and B)**. Commins et al. (2011) reported that tick bites in the U.S. can induce IgE antibodies to alpha-gal and also generated a map showing distribution of known cases of patients with delayed anaphylaxis to red meat **(Fig. 13B)**. Dots indicated single cases, smaller black stars represented 5-24 cases within a state, and > 25 in state cases were indicated by larger black stars.

Figure 13A. Known distribution of *A. americanum* (CDC 2020)

Figure 13B. Known distribution of alpha-gal cases in patients with the serum containing IgE antibodies to alpha-gal (Commins et al. 2011)

While the majority of *A. maculatum* specimens were found in Southeast Kansas, there was a specimen found in Tomahawk Creak located Northeast. The habitat was wooded with a mix of tall grass, which differed from the specimens of *A. maculatum* that were primary found on cattle in pastures or prairie habitats. Historically, *A. maculatum* was found in many Central and South American countries bordering the Caribbean Sea and Gulf of Mexico (Soneshine 2018). Within the United States, the species was limited to south Atlantic and southeastern states. The range has now expanded norward into Midwestern states such as Oklahoma, Kansas, Arkansas, Tennessee, and even as far west as Arizona **(Fig. 14)**. Notably, the range of *A. maculatum* does not correlate with the range of alpha-gal allergy reports compared to *A. americanum* (CDC 2020).

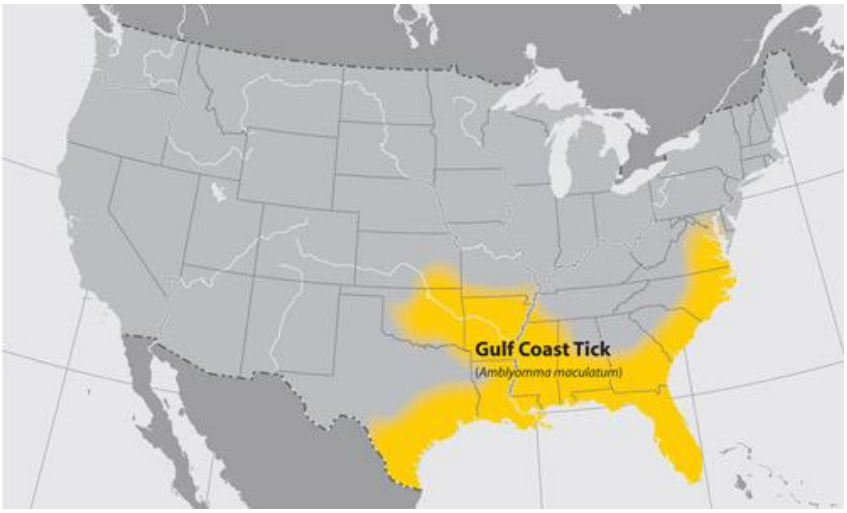
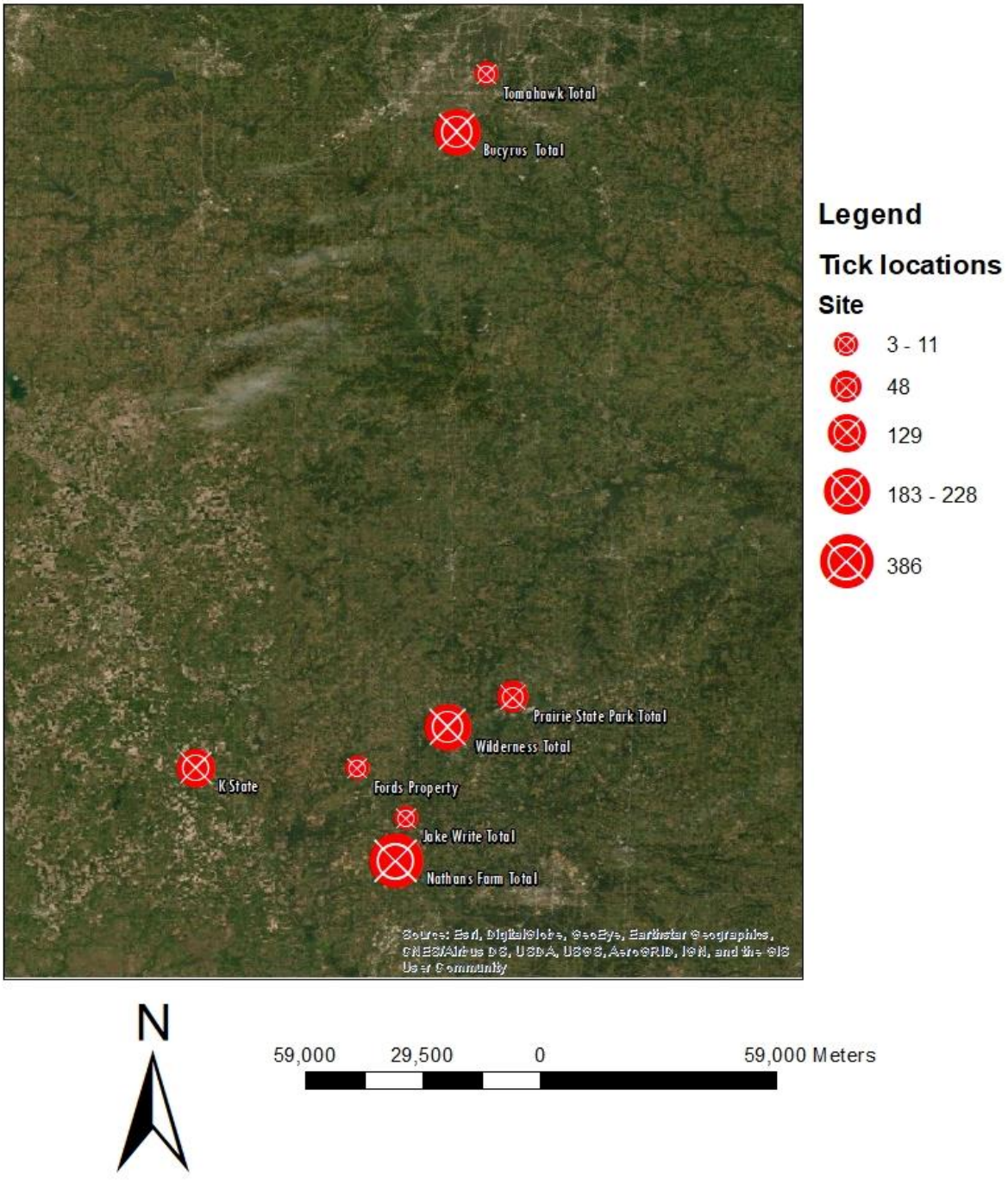


Figure 14. Known distribution of *A. maculatum* (CDC 2020)

N-linked alpha-gal containing proteins in tick salivary glands reveal that *A. americanum* have alpha-gal in their saliva and salivary glands but not *A. maculatum* (Crispell 2019). This study featured data to support that certain tick species may be specific to the development of alpha-gal specific IgE and hypersensitivity reactions.

A.

Tick Abundance and Sites



B.

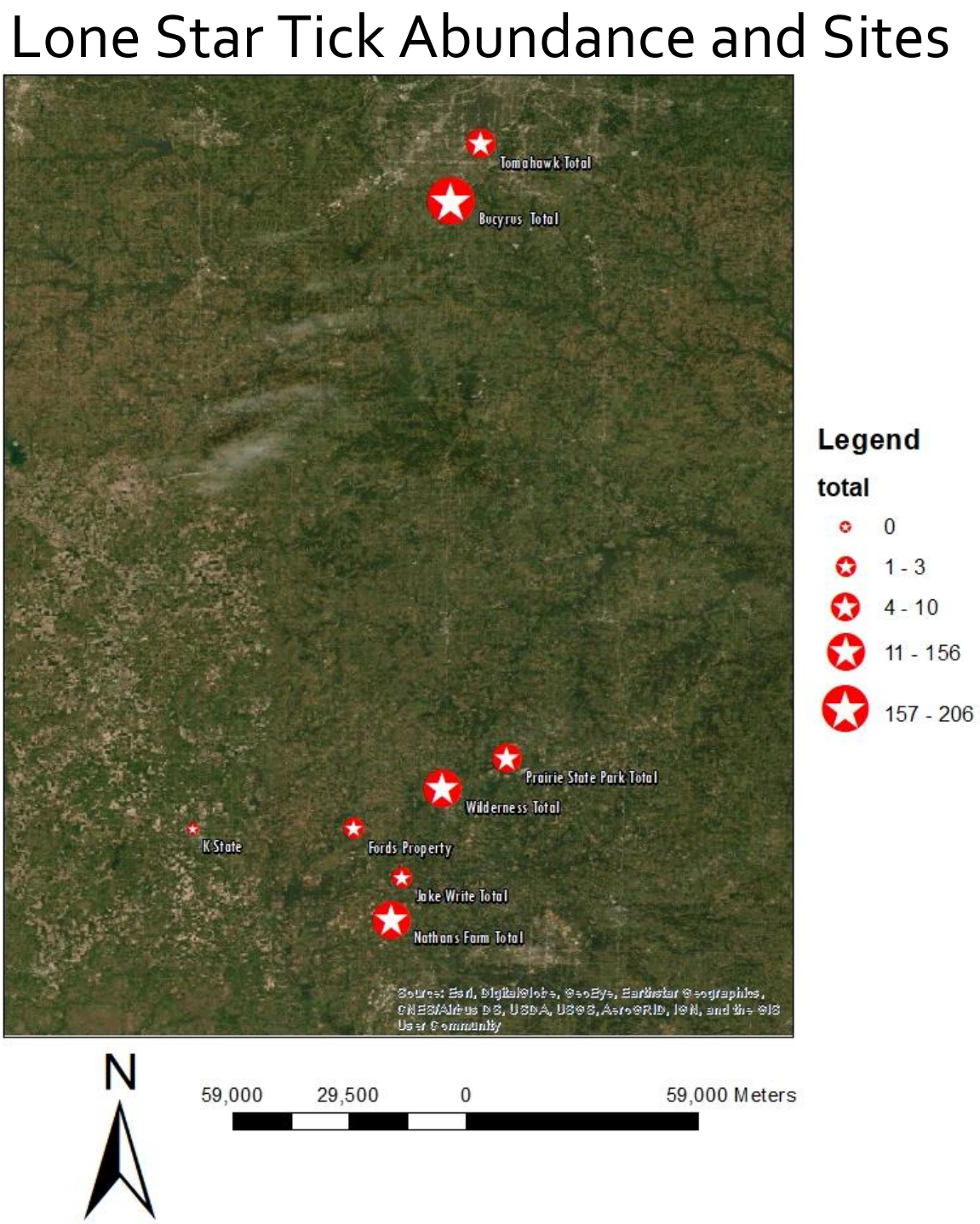


Figure 15. GIS locations, (A) tick abundance and sites, (B) lone star tick abundance and sites. Specimens consisted of adult ticks only.

A total of 998 ticks (869 by flag and drag; 129 by other method) were collected and identified using taxonomic keys. Three prominent species were collected using flag/drag method and identified as: *D. variabilis* (n=62, comprised of females=38 and males=24), *A. americanum* (n=536, comprised of females=309 and males=227), and *A. maculatum* (n=15 comprised of females=10 and males=5) totalling to 613 adults. Majority were females (357, 58.2%) compared to males (256, 41.8%) **(Fig. 16A)**. Several nymphs (256) were also collected and they were all identified as *A. americanum*. Feedlot cows at K-State agricultural extension was sampled using a different approach as described in methods. A total of 129 ticks were collected and identified. The majority were *A. maculatum* (127 out of 129) and the rest two were *D. variabilis* females. In contrast to the flag/drag method, *A. maculatum* were mostly presented by males (69) followed by females (58). Pictures of ticks under dissectoscope were captured and are displayed in **Fig. 16B**.

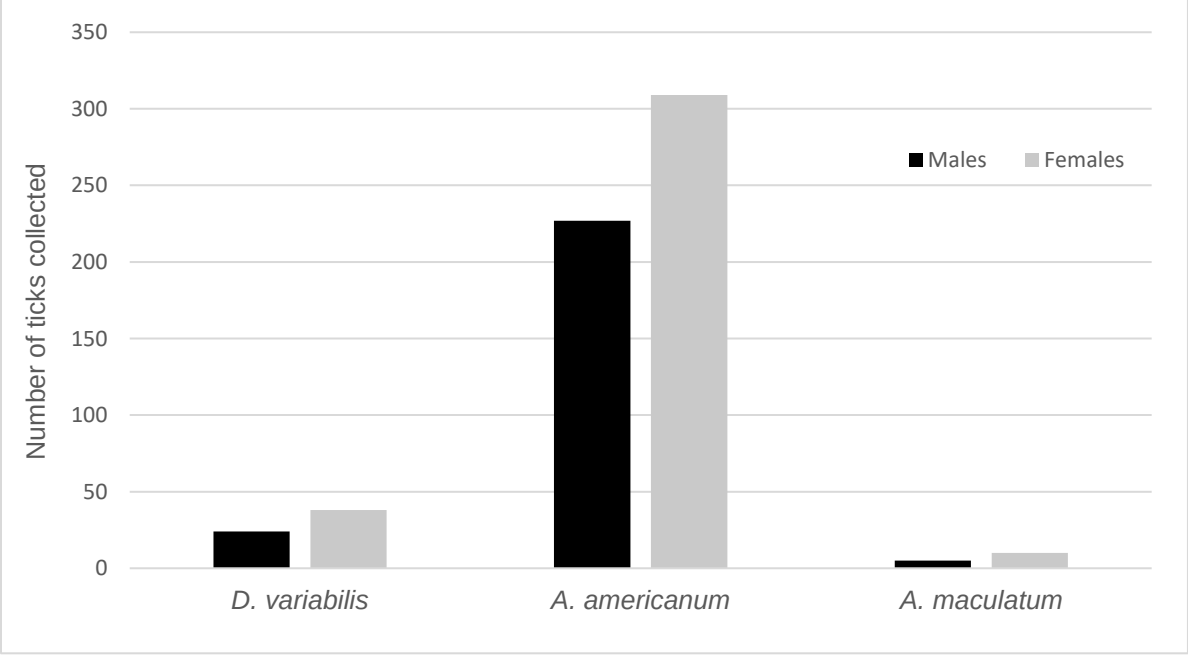


Figure 16A. Distribution of tick species using flag/drag method

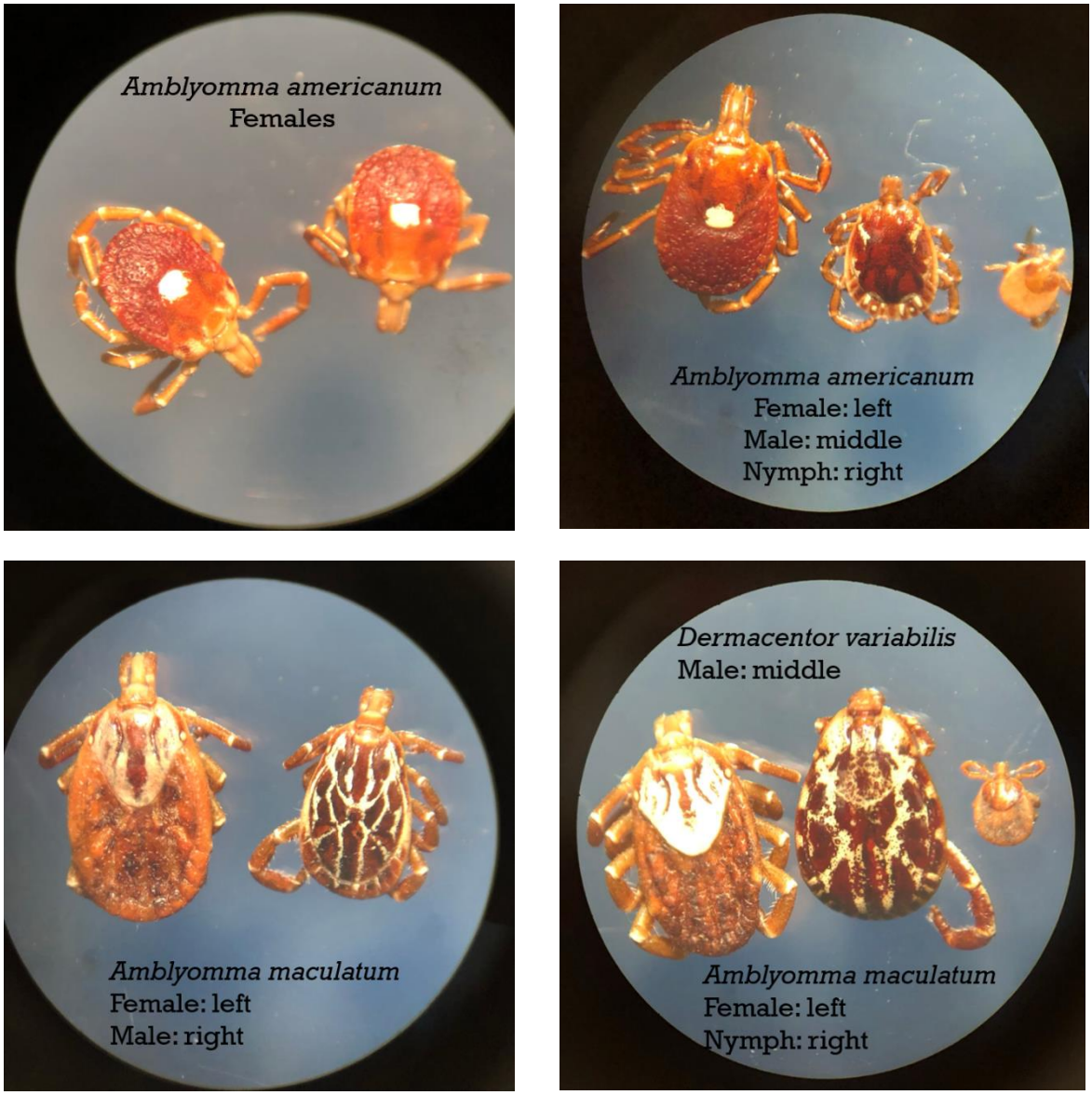


Figure 16B. Identification of ticks under dissectoscope using taxonomic ID keys (Photo by Leah Cuthill)

Further study focused on only adult males and females of *A. americanum* and *A. maculatum*. The identity of a few representative ticks from both species were confirmed by PCR. The primers used for PCR are illustrated in **Table 2**.

Table 2. Details of primers used for tick species identification

Gene name	Forward Primer 5'→3'	Reverse Primer 5'→3'	Length (bp)
Amac_ITS2*	TTGTGCGGGAAACGACCGGGTGT	AACGCTCGTAACGAGATACGCG	193
Aam_ITS2Shnew[#]	AAGCCCGCGCTCCAAGC	GCAGCAGTTCGGCTACACGTA	214

Primers were adopted from *Zemtsova et al. 2014 and [#]Shone et al. 2006.

Table 3. Identification of ticks from their corresponding locations

	GIS coordinates		#times sampled	*Amblyomma americanum*		*Amblyomma maculatum*		
	Latitude	Longitude		male	female	male	female	**Total***
Location A	37.15379	-94.8303	5	74	79	0	0	386
Location B	38.79468	-94.6941	2	83	123	0	0	228
Location C	37.25048	-94.8067	1	1	1	0	0	10
Location D	37.52198	-94.5657	1	2	4	5	9	48
Location E	37.45353	-94.7127	5	62	94	0	0	173
Location F	38.92534	-94.6239	1	4	6	0	1	11
Location G	37.36134	-94.9176	1	1	2	0	0	3
Location H	37.381041	-94.6324	1	0	0	0	0	10**
Location I	37.36372	-95.2830	7	0	0	69	58	129

Locations A-H were sampled using flag/drag method; Location I was sampled by de-ticking using forceps.

The samples that are shaded were used in further molecular analysis.

Less times sampled in locations were not included in molecular study (C, D, F, G, H).

Location H: Only *Dermacentor* species and a few nymphs were collected from this site.

*, total numbers include *Dermacentor* adults and all species nymphs.

**, Only *Dermacantor* were identified in this location.

Among those nine locations (A-I), five were sampled sporadically using flag-drag and resulted in a cumulative 82 ticks **(Table 3)**. Further molecular analysis included equal number of males and females from high *A. americanum* density areas. *Amblyomma maculatum* males and females from location I were as a control, as they belong to the same genus but do not feature genes that produce alpha-gal (Crispell 2019). To increase the yield of RNA, two tick samples from same species, same location, and same sex were pooled together **(Table 4)**.

Table 4. Details of ticks used for molecular analysis

	***Amblyomma americanum* (tested)**		***Amblyomma maculatum* (tested)**		**Total tested**
	male	female	male	female	
Location A	74 (30)	79 (30)	-	-	60
Location B	83 (30)	123 (30)	-	-	60
Location E	62 (30)	94 (30)	-	-	60
Location I	-	-	69 (30)	58 (30)	60
Total	180	180	30	30	240

Genomic DNA was not used for detection of genes related to alpha-gal because the allergy was caused by the injection of tick salivary proteins into the host via tick bites (Springer et al. 2016, Simo et al. 2017). Therefore, it was reasonable to focus on the total RNA in the tick which would get translated into protein eventually. Due to unstable structure of RNA, these were converted into stable double stranded cDNA or complementary DNA. This cDNA was further used to analyse relative gene expression

The following table shows the quantitation of extracted RNA using nanodrop. In most of the cases, two samples were pooled, exceptions are noted **(Table 5)**. Concentration of RNA was noted to be sufficient to synthesize cDNA. Ideal pure RNA should have an A_{260}/A_{280} between 1.8 - 2.0. Particularly, *A. maculatum* female samples yielded poor quality of RNA with a ratio < 2.0.

Table 5. Nanodrop quantitation of extracted RNA from *Amblyomma americanum* and *A. maculatum*

Sample ID.	**Nanogram per microliter**	**A_{260}/A_{280}**
Location A: *A. americanum* females		
NF - F1#	854	2.52
NF - F2	492	2.12
NF - F3	652	2.32
NF - F4	574	2.22
NF - F5	456	2.13
NF - F6	409	2.07
NF - F7	329	2.01
NF - F8	293	2.01
Location A: *A. americanum* males		
NF - M1#	256	2.08
NF - M2	324	2.06
NF - M3	305	2.04
NF - M4	228	2.07
NF - M5	252	2.06
NF - M6	189	1.93
NF - M7	229	2.01
NF - M8	200	1.98

Location I: *A. maculatum* females		
KS - F1#	122	1.87
KS - F2	1613	1.97
KS - F3	1782	1.84
KS - F4	1338	1.77
KS - F5	1573	1.82
KS - F6	1609	1.42
KS - F7	880	1.86
KS - F8	1732	0.92
Location I: *A. maculatum* males		
KS - M1#	768	1.96
KS - M2	971	2.03
KS - M3	267	1.90
KS - M4	1075	1.96
KS - M5	997	1.99
KS - M6	896	1.88
KS - M7	300	1.84
KS - M8	576	1.93

#, denotes RNA extraction from single tick sample

RNA extraction efficiency was remarkably different between the two *Amblyomma* spp.

Fig. 18A and 18B depicts the varied RNA concentration among males and females of *A. americanum* and *A. maculatum.* Statistical analysis showed significant difference between RNA extraction efficiency among males (p=0.0007) and females (p=0.0015) of two species. It was noted during dissection that the body size of *A. maculatum* was larger

than that of *A. americanum* as well as salivary glands were more prominent in *A. maculatum* and isolated efficiently (**Fig 17**).

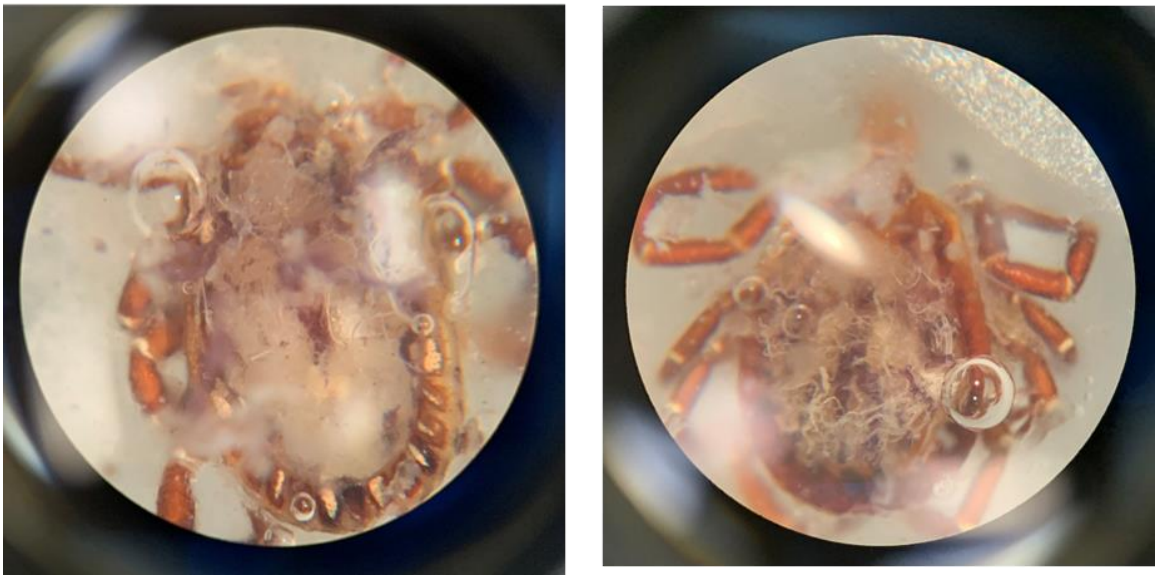
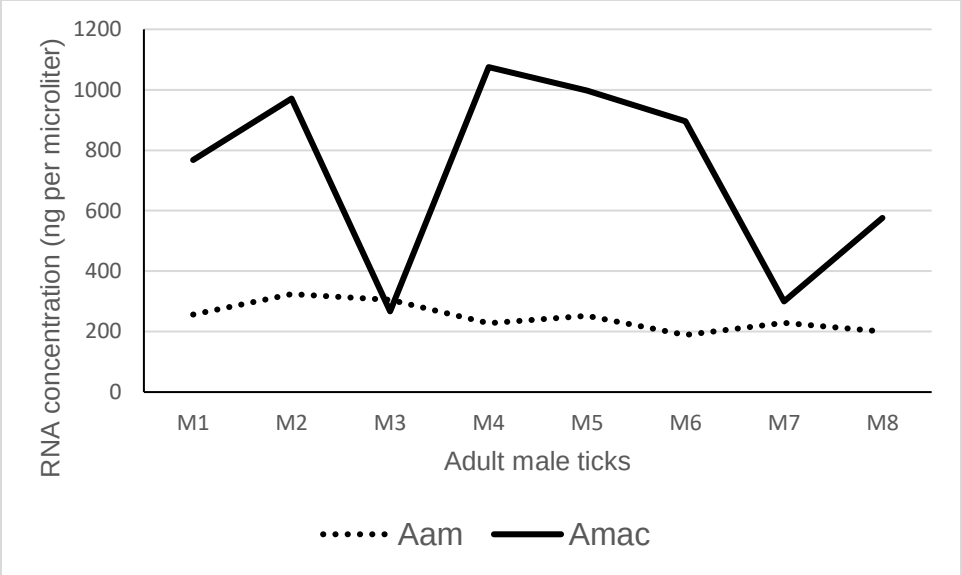


Figure 17. Dorsal view of midgut and salivary glands, *A. maculatum* male (left) (A) and *A. americanum* female (right) (B) (Photo by Leah Cuthill)



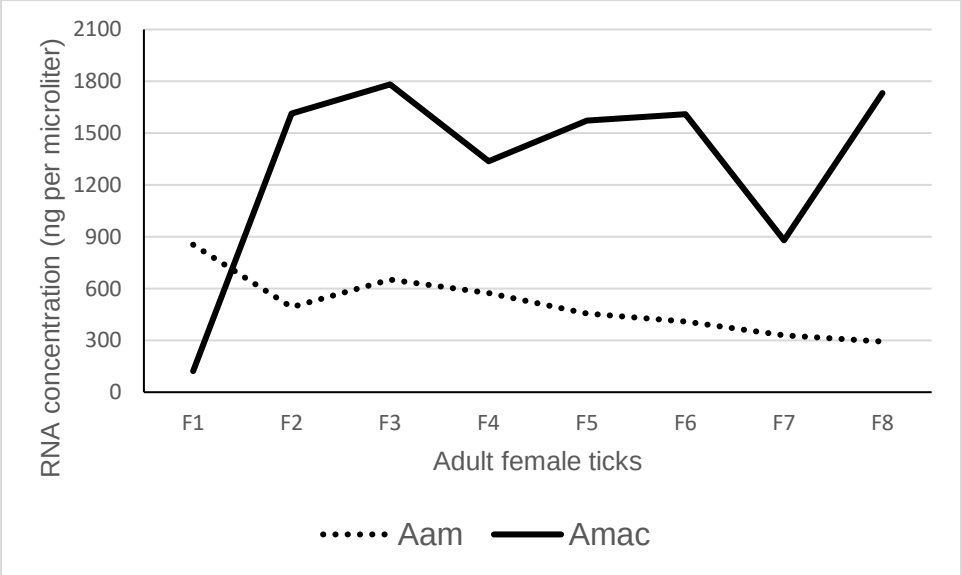


Figure 18. RNA extraction efficiency among males (A) and females (B) of *A. americanum* (Aam) and *A. maculatum* (Amac)

For amplification of housekeeping genes via real-time PCR, the following sets of primers were used **(Table 6)**. Out of these primers, the most suitable primer-set for amplification of housekeeping genes was selected based on initial runs of real-time PCR.

Table 6. Details of primers used for real-time PCR

	Gene name/ function [GenBank acc.no.]	Forward Primer 5'→3'	Reverse Primer 5'→3'	Length (bp)
1.	Aam GAPDH	CTTCTTGGCACCTCCATCAA	GGTGCAGAGTTTGTGGTAGAG	
2.	Aam Histone H3 [759084459]	GAAGCCAGTGAGGCATACTT	GCTCGATATCCTTTGGCATGA	276
3.	Aam Calrecticulin [3924592]	ACTACAAGGGCAAGAACCACCTCA	TCGATCTGCTTGGGTTTCCACT CA	1379
4.	Aam GST /glutathione S-transferase [196476640]	CTTCCCGAATTTGCCCTACTA	CGTCAAGACCGTGCTTCTTT	868
5.	Aam Ubiquitin [759085449]	GCTGTCCGACTACAACATTCA	GGGTGGTGTAGTTCTTCTTCTT	471
6.	Aam β-Actin [759085843]	TCCTATCCTCACCCTGAAGTA	ACGCAGCTCGTTGTACAAG	543
7.	Aam HSP/ heat shock protein 10 [196476687]	CTCTTGTACGCAGGCTTCTTA	TATCATGATGCCGCCCTT	281
8.	Amac_Actin/ Cytoskeletal structural protein [JO842238]	TGGCTCCTTCCACCATGAAGATCA	TAGAAGCACTTGCGGTGCACAATG	169
9.	Amac_EF1/ Component of eukaryotic translational apparatus [JO842576]	CGTGCCAATGCCACCAATCTTGTA	TGGTCCATTGAGCGAAAGTCTGGA	173
10.	Amac_GAPDH/ Oxireductase in glycolysis and gluconeogenesis [JO842341]	CACCCATCACAAACATGGGTGCAT	TTTCAGGAAATGAAGCCTGCCAGC	175
11.	Amac_alpha-tubulin/ Component of microtubules [JO841052]	AACGCAGCTATTGCAGCCATCAAG	GTGGTGTTCGACAACATGCACACA	161

12.	Aam_S6/ serine protease inhibitor (serpin) 6 [internal control]	CTGCTATCAGCGAGAGCACGCA	TCTGCGTGAAATTTCTGTCATTCTGGA
13	Tick_actin [loading control]	GGACAGCTACGTGGGCGACGAGG	CGATTTCACGCTCAGCCGTGGTGG
14.	Amac_Actin	TGGCTCCTTCCACCATGAAGATCA	TAGAAGCACTTGCGGTGCACAATG
15.	Amac_GAPDH	CACCCATCACAAACATGGGTGCAT	TTTCAGGAAATGAAGCCTGCCAGC
16.	b4galt7_979	CGCTGCTCATATCCTTCAGC	TATCACCACCGACTCCGTTC
17.	a4galt-1_908	ATTACGAGCGGCATCGCTTA	AACGTGTCGTCGCAAGGTAA
18.	a4galt-2_262	CTCTCCGGAATCTTGGACTG	CTCTCCGGAATCTTGGACTG
19.	Tick rpS4	GGTGAAGAAGATTGTCAAGCAGAG	TGAAGCCAGCAGGGTAGTTTG
20.	Aam_immunity	AGACGAACTAAACGCAAGGG	GTTTTTGTTTAGGAACGCGG
21.	Aam_ lipocalins	GGCATCAATGGAAGTTGCTT	GTGTGTGAACGGACTGAACG
22.	*Aam*_IGFBP-rP1	GACGGGCCGTGCGGCGAGGA	CCAGTAGGTGGCCGTGTCCTCTGG

Primers 1-7 were adopted from Bullard et al. 2016; Primer 8-11 were adopted from Browning et al. 2012; Primers 12-13 were adopted from Chalaire et al. 2011

Because of larger size of the body, the salivary glands and midgut contents of dissected *A. maculatum* was much more than the other dissected species. This was apparent once higher concentration of RNA was obtained from the same number of pooled contents. The native agarose gel **(Fig. 19)** below confirmed the concentration variation among RNA samples isolated from *A. americanum* and *A. maculatum*. The native gel showed that RNA was not degraded. To view eukaryotic specific band of RNA such as 28S and 18S rRNA, the extracted RNA was denatured by heat and run on the native agarose gel **(Fig. 20)**. RNA smear was observed with some noticeable bands. In ideal condition, RNA could be run on a denaturing agarose gel supplemented with formaldehyde. Since further downstream processing of the RNA in our study was aimed for real-time PCR, larger RNA fragments were not required. Therefore, the integrity of RNA as observed by the native agarose gels was indicative of good quality RNA for PCR purpose.

NF-3(F) NF-8(F) NF-2(M) NF-6(M) KS-3(F) KS-7(F) KS-3(M) KS-4(M)

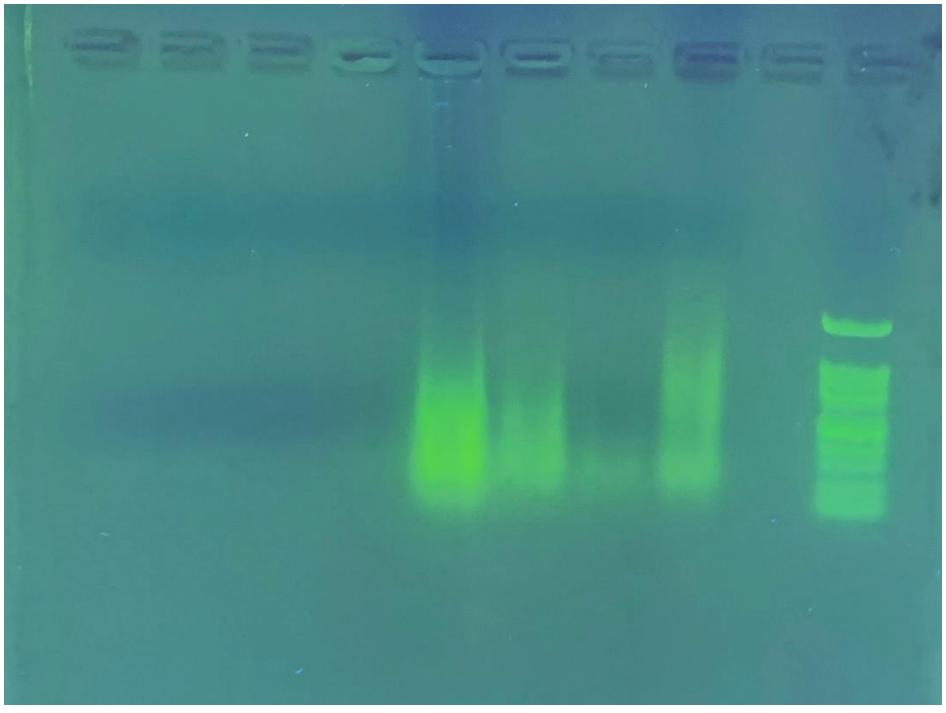


Figure 19. Native agarose gel (1.2%) showing isolated RNA

NF-1(F) NF-4(F) NF-1(M) NF-3(M) KS-2(F) KS-5(F) KS-1(M) KS-5(M)

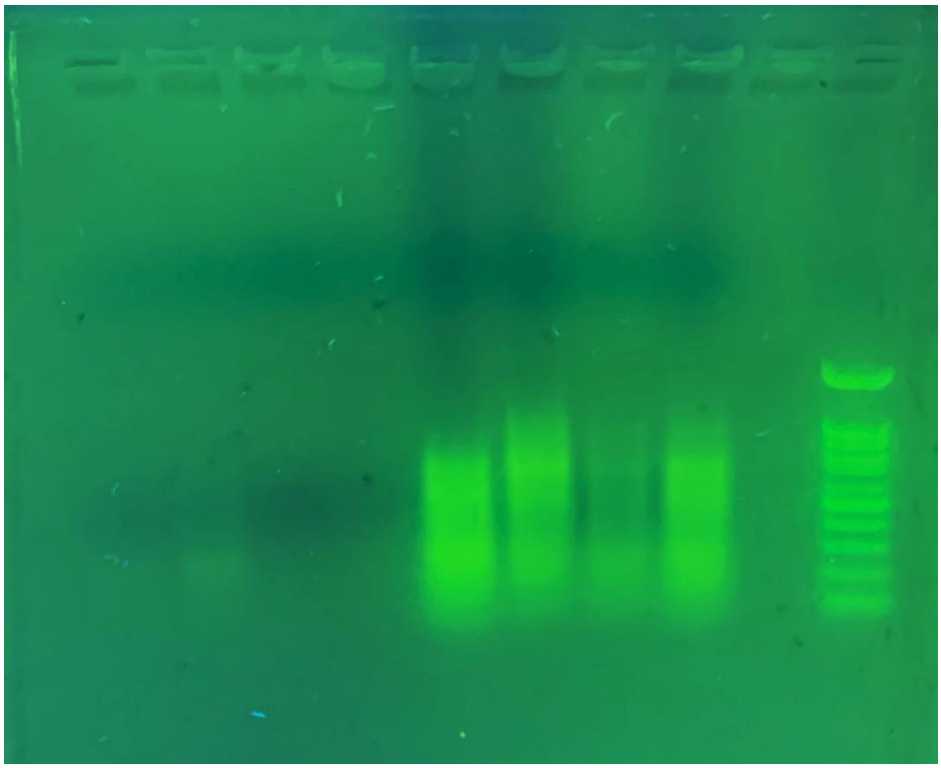
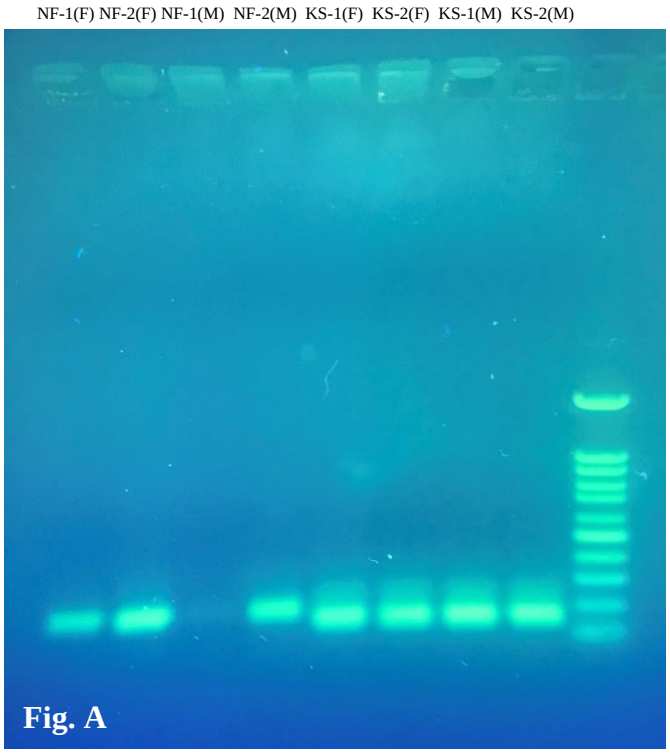
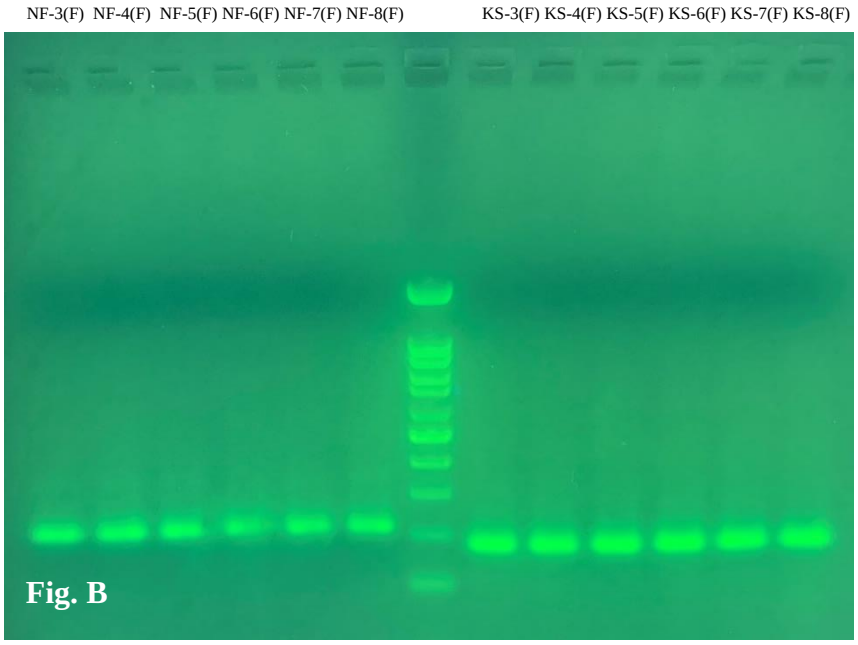


Figure 20. Native agarose gel (1.2%) showing heat-denatured isolated RNA

Once cDNA was synthesized PCR was performed on individual samples to confirm the taxonomic ID of the tick species. Species-specific primer sets were used to detect and differentiate the ITS2 genomic regions of *A. americanum* and *A. maculatum* species. Agarose gels **(Figs. 21A, B, and C)** shows amplification of 214 bp of ITS2 genomic region from *A. americanum* and 193 bp of the same genomic region from *A. maculatum*. Since tick species were confirmed using molecular technique, downstream real-time PCR did not require any species level identification.





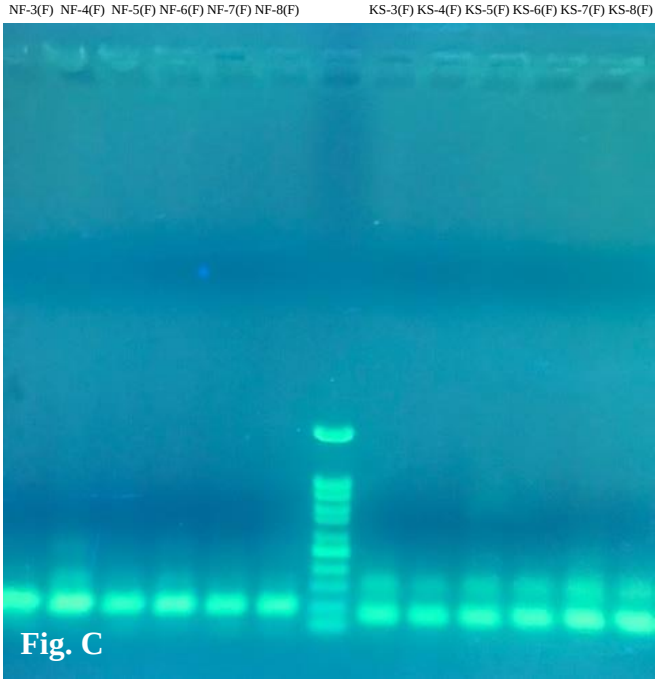


Figure 21A-C. Agarose gels (1%) showing PCR amplification of ITS2 genomic region of A. *americanum* and *A. maculatum*

Expression of housekeeping genes and target genes in A. americanum males and females were studied using real-time PCR. Four sets of housekeeping genes were tested for optimization and use as internal control or reference gene. The primer sets used for the

amplification of GAPDH, actin, ubiquitin, and histone. In this assay, the cDNA template concentration was also optimized. Two cDNA dilutions (1:20 and 1:40) were tested for dissected individual and two pooled adult ticks. **Fig. 22A** showed the mean C_T values plotted for all four housekeeping genes. The negative control was without cDNA template and two technical replicates were used. Negative control for GAPDH showed high C_T values indicating possible primer-dimer formation. The annealing temperature of real-time PCR reaction could be modified to achieve a better result for this primer set. However, all obtained C_T values were >28. Therefore, cDNA template concentration was increased in the next assay.

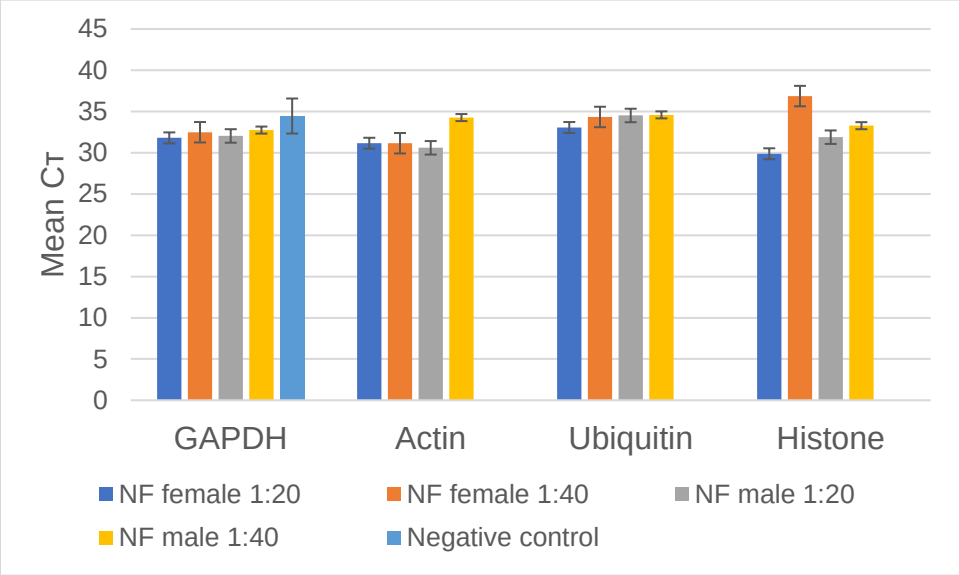


Figure 22A. Expression of housekeeping genes among *A. americanum* males and females (variable tested were cDNA dilutions)

In the next assay to further optimize the housekeeping gene, three biological replicates i.e. 3 males and 3 females were used with two technical replicates and no template negative control. The cDNA template concentration used was 1:10 dilution. Out of three primer sets tested for GAPDH, histone, and actin, the most suitable reference gene

was considered to be actin based on lower and consistent C_T values in both males and females and no amplification in the negative control **(Fig. 22B)**.

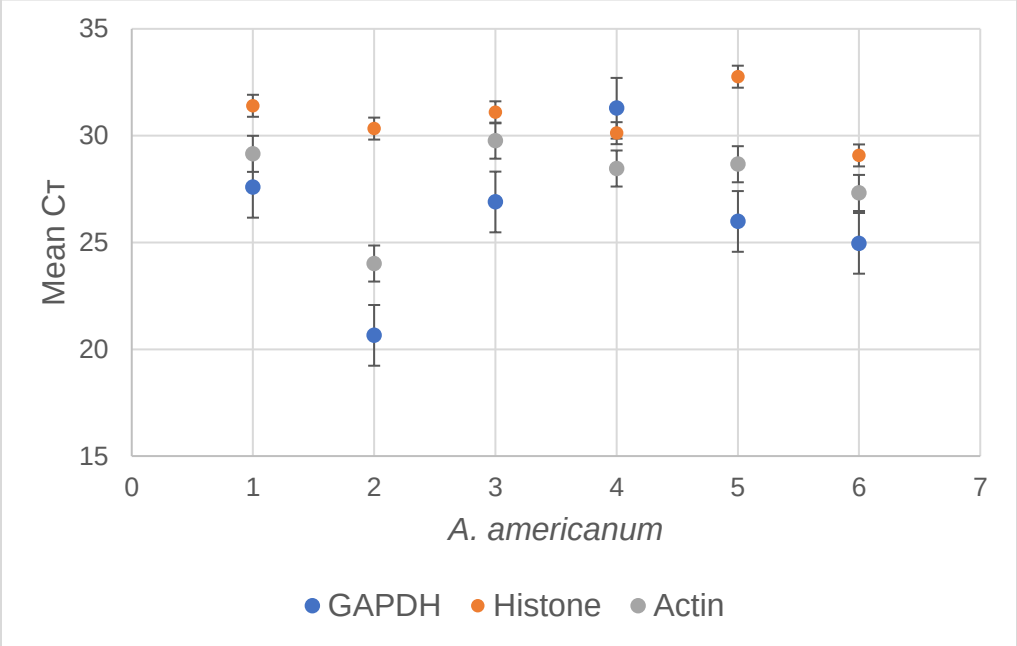
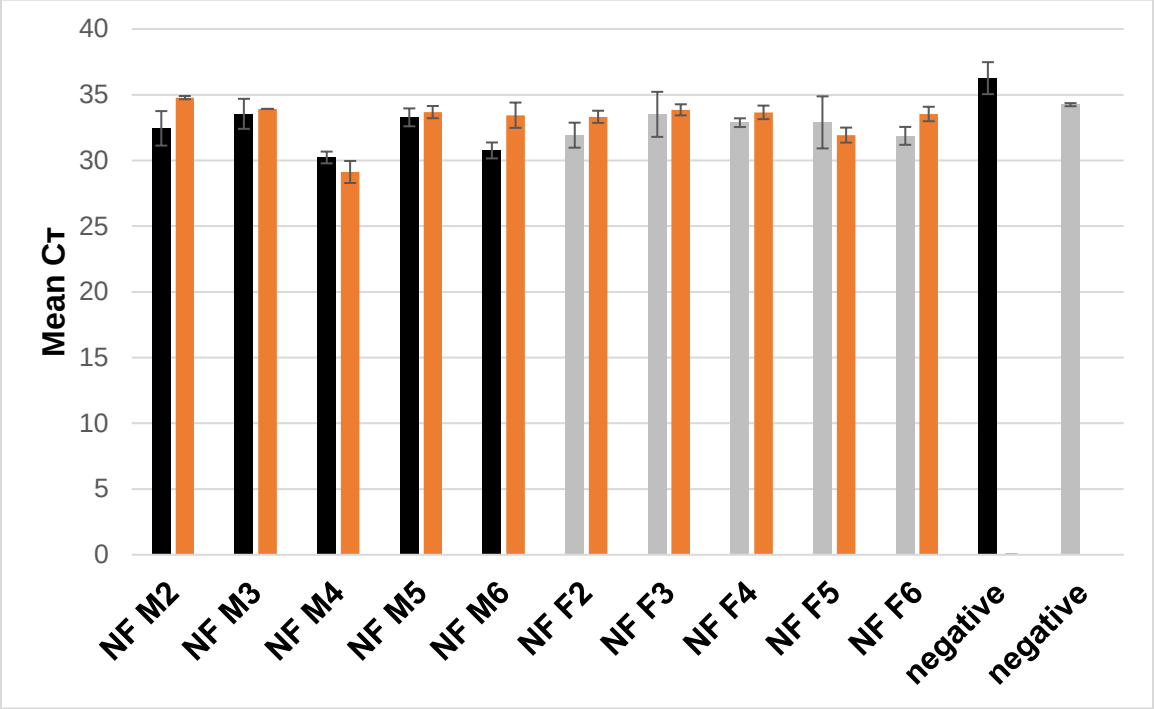


Figure 22B. Expression of housekeeping genes with 1:10 diluted cDNA among *A. americanum* males and females

Further, the expression of a gene encoding salivary gland protein serine protease inhibitor (Serpin 6) was compared among *A. americanum* males and females with respect to the reference gene actin. Serpin 6 is secreted into tick saliva during tick feeding. For this assay, 1:10 diluted cDNA was used as template, five biological replicates (5 males and 5 females) and two technical controls were used. The mean C_T values varied between 28 and 35, which was in the upper range and not desired. Moreover, Serpin 6 negative controls showed C_T values indicating primer-dimer formation. This needed to be addressed by modifying the annealing temperature of the PCR reaction **(Fig. 23A)**. The fold change in expression relative to the internal control gene actin was calculated using $2^{-\Delta\Delta}C_T$ method as described in chapter 2 and was plotted in **Fig. 23B**. The difference between fold gene

expression between males and females was not significant (*P*=0.388672454). This was expected since both males and females bite on host and needs Serpin 6 expressed so that blood does not coagulate at the feeding site. Male ticks feed on blood briefly and do not engorge like the females.



Black bar: Serpin-male, Grey bar: Serpin-female, Orange bar: Actin male and female

Figure 23A. Relative expression of serine protease inhibitor (Serpin 6, a tick salivary protein) against internal control housekeeping gene actin among *A. americanum* males and females

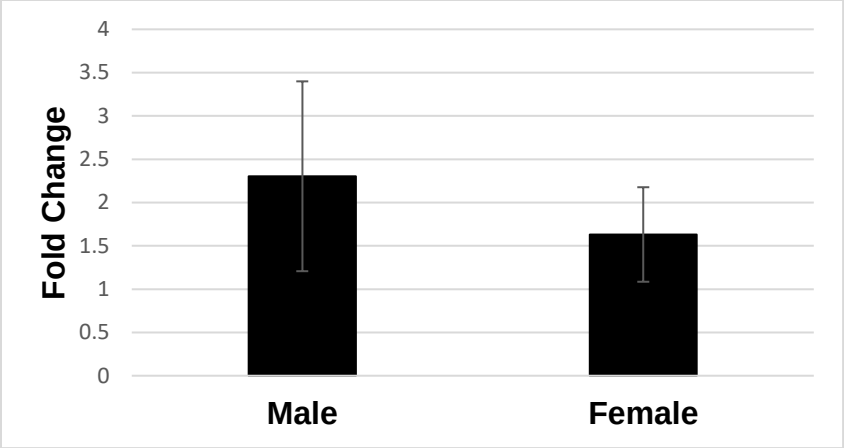


Figure 23B. Relative expression of Serpin 6 gene among *A. americanum* males and females. Bars represent average of 5 biological replicates (5 males and 5 females) ± standard error.

Further assays focused on galactosyltransferase gene family. Galactosyltransferase (GALT) is a type of glycosyltransferase that catalyzes the transfer of galactose via α1-2, α1-3, α1-4, α1-6, β1-3 and β1-4 linkages to diverse acceptor structures. The α-Gal epitope and the anti-α-Gal antibodies have been associated tick-induced allergy. The presence GALTs involved in the α-Gal synthesis pathway in *Ixodes* ticks has been shown (Cabezas-Cruz et al. 2018). In this assay, three primer sets were tested which targeted the followings: α-1,4-galactosyltransferases-1 (α-gal-1), α-1,4-galactosyltransferases-2 (α-gal-2) and β-1,4-galactosyltransferase-7 (β-gal-7). The 1:10 diluted cDNA was tested as template, two technical replicates and two biological replicates (2 males and 2 females) were used. The internal control gene was actin. α-gal-2 primer sets did not show any C_T values in the real-time PCR. α-gal-1 primer set showed C_T values in the negative control indicating primer-dimer formation which needed to be further optimized by modifying annealing temperature of the PCR reaction. β-gal-7 primer sets yielded PCR product with reasonably low C_T

values for both males and females **(Fig. 24)**. This is interesting finding, since these galactosyltransferase genes were not tested in *A. Amblyomma* previously. More assays were needed to establish the difference in expression of alpha-gal genes among males and females.

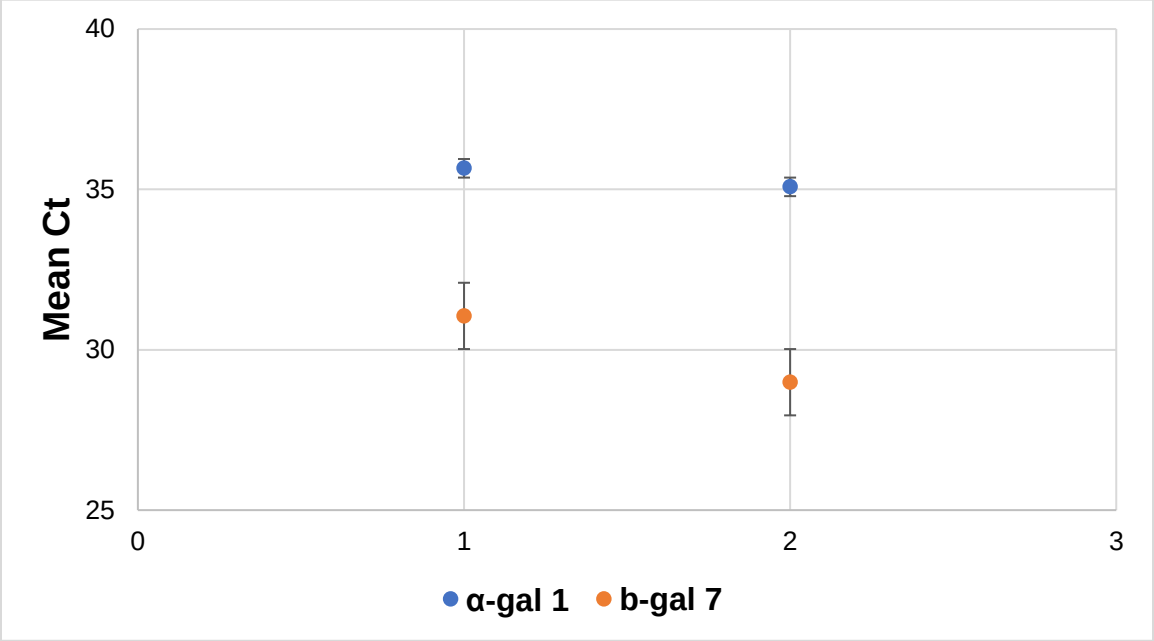


Figure 24. Relative expression of galactosyltransferase genes among *A. americanum* males and females against internal control housekeeping gene actin.

CONCLUSIONS

The study question posed in the beginning of this study have been addressed as follows:

1. What is the ecological distribution of *A. americanum* relative to other tick species?

In terms of ecological distribution of tick-species in northeast and southeast Kansas, our findings were corroborated with previous studies. Our data showed that *Amblyomma americium* was the most abundant species in both locations as collected by flag and drag method. This technique did not draw many *Dermacentor* or *Ixodes* spp. Tick collection from cattle chutes yield only *Amblyomma maculatum* which resides primarily in their ears.

2. Which housekeeping gene is suitable for being used as an internal control for gene expression studies in *A. americanum*? - A set of housekeeping genes were tested by real-time PCR using male and female *A. americanum* and the most appropriate one was found to be actin. The real-time PCR assay showed consistent expression of actin in both male and female adult ticks with suitable C_T values. This was used as internal control in further assays.

3. What are the relative expression levels of genes related to alpha-gal production and other in males and females of *A. americanum*? - Three galatosyltransferase genes involved in synthesis of alpha-gal were tested in *A. americanum* male and female; out of them two genes were expressed in both. A salivary gland protein (Serpin 6) modulating blood coagulation in host while tick feeding was also tested; this did not give any conclusive result. Furthermore, differential expression of genes coding for proteins/enzymes associated with salivary secretion or innate immunity of male and female *A. americanum* and *A. maculatum* ticks are being studied following real-time PCR approach.

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APPENDIX

Appendix A

Step-wise tick dissection protocol:

- Ticks were washed with 95% ethanol for 1 minute or until dry.
- Place melted parafilm onto glass petri dish.
- Observe through stereoscope.
- Wait for wax to cool for 1 minute, add tick and press down until secured.
- After another minute, place a drop of 95% Ethanol onto tick using a plastic dropper.
- Wait for 1 minute, then remove Ethanol using a sterile brush.
- Apply a drop of saline buffer and wait for 1 minute.
- Use a microscalpel to cut the dorsal side of the exoskeleton to reveal the digestive glands and salivary glands.
- Using a dissection kit, both glands and midgut were carefully removed and placed into a labelled Eppendorf containing TRIZOL for further processing

Appendix B

Step-wise protocol for RNA extraction using TRIZOL:

1. Add 1ml TRIzol to the sample and homogenize
2. Add 200 µl chloroform to the homogenate
3. Vortex vigorously
4. Incubate on ice for 15 minutes
5. Centrifuge to get phase separation (12,000g for 15 minutes at 4°C)
6. Transfer the aqueous phase to a fresh tube
7. Precipitate the RNA by mixing with 0.5ml isopropanol
8. Incubate on ice for 10 minutes
9. Centrifuge for 10 minutes at 12,000g at 4°C
10. Remove the supernatant
11. Wash pellet with 1ml 70% ethanol by flicking
12. Centrifuge at 7500g for 10 minutes at 4°C
13. Remove supernatant
14. Air dry
15. Dissolve RNA pellet in appropriate volume of RNase-free H_2O (20 µl)

Appendix C

cDNA synthesis protocol from Invitrogen:

invitrogen PRODUCT INFORMATION SHEET

SuperScript™ VILO™ MasterMix

Catalog Number 11755-050, 11755-250, and 11755-500
Doc. Part No. 100012386 **Pub. No.** MAN0004286 **Rev.** A.0

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

Invitrogen™ SuperScript™ VILO™ MasterMix provides the high-temperature capability of SuperScript™ III Reverse Transcriptase (RT) in an optimized format for generating first-strand cDNA for use in real-time quantitative RT–PCR (qRT–PCR). This formulation can be used with very low and very high amounts of input RNA (up to 2.5 µg total RNA in a 20-µL reaction).

SuperScript™ VILO™ Master Mix includes SuperScript™ III RT, RNaseOUT™ Recombinant Ribonuclease Inhibitor, a proprietary helper protein, random primers, $MgCl_2$, and dNTPs.

SuperScript™ III RT is an engineered version of M–MLV RT with reduced RNase H activity and increased thermal stability. The enzyme can be used to synthesize cDNA at a temperature range of 42–55°C. Because SuperScript™ III RT is not significantly inhibited by ribosomal and transfer RNA, it can be used to synthesize cDNA from total RNA. RNaseOUT™ Recombinant Ribonuclease Inhibitor safeguards against the degradation of target RNA due to ribonuclease contamination.

Synthesize first-strand cDNA

The following protocol has been optimized for generating first-strand cDNA for use in two-step qRT–PCR. The reaction volume may be scaled as needed up to 100 µL. A negative RT control protocol is provided below.

1. For a single reaction, combine the following components in a sterile PCR tube or plate well on ice.

Component	Volume
SuperScript™ VILO™ MasterMix	4 µL
RNA (up to 2.5 µg)	X µL
DEPC-treated water	to 20 µL

2. Gently mix and incubate at 25°C for 10 minutes.
3. Incubate at 42°C for 60 minutes.
4. Terminate the reaction at 85°C at 5 minutes.
5. Use the diluted or undiluted cDNA in qPCR or store at –20°C.

Prepare negative RT control

1. For a volume of RNA = X µL, add the following to a sterile PCR tube or plate well on ice.

Component	Volume
SuperScript™ VILO™ MasterMix	4 µL
DEPC-treated water	16 – X µL

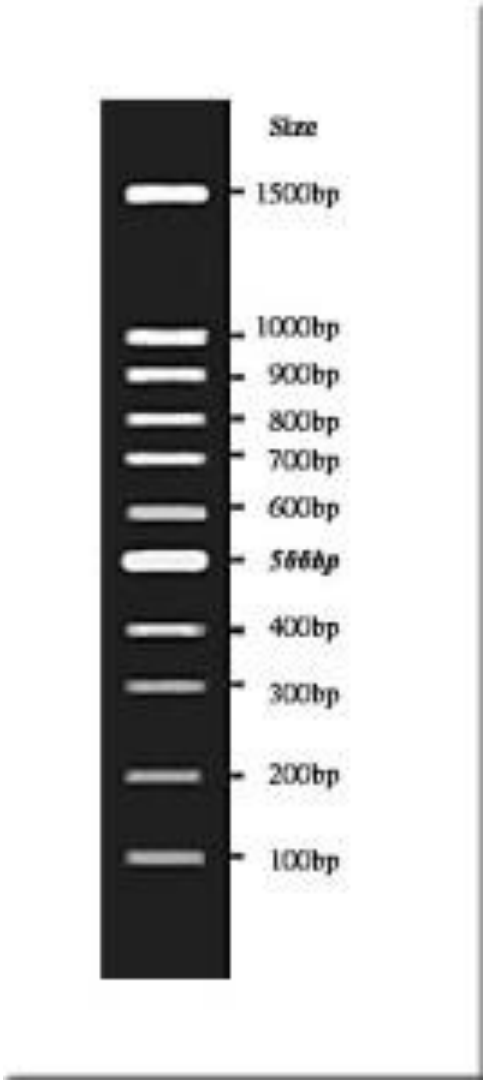
2. Incubate at 65°C for 10 minutes to denature the reverse transcriptase.
3. Add X µL of RNA (up to 2.5 µg) for a total reaction volume of 20 µL.
4. Proceed with steps 2–5 from "Synthesize first-strand cDNA" on page 3.

Appendix D

Information on 100 bp ladder

100BP DNA Ladder | Bullseye 100BP DNA Ladder | 100BP DNA Marker

Bullseye 100bp DNA Ladder



- Ready to use
- Contains 11 DNA bands: 100-1500bp.
- Clearly identifiable 500bp band as reference
- 500ng DNA/6µl/loading
- Easy to load
- Stable at room temperature
- Supplied with 6x sample loading buffer

Bullseye 100bp DNA Ladder consists of 11 DNA fragments ranging in size from 100-1500 base pairs (bp). 6µl will yield at least 30ng DNA in any single band. The intensity of the 500bp band has been increased to serve as a reference for easy identification.

Size: 1200µl
Storage: Store at -20°C.
Concentration: 500ng/6µl
Loading Buffer Composition:

10mM Tris-HCl
1mM EDTA (pH 8.0)
0.02% Bromophenol blue
0.02% Xylene cyanol
5% Glycerol

Usage: Add at least 6µl Bullseye 100bp DNA Ladder directly to wells designated for markers. You may need more than 6µl of ladder, depending on well size and level of intensity needed to visualize the bands.

Appendix E

Step-wise protocol for RT PCR

1. Gloves must be worn when handling anything at this station
2. Do not use any supplies from other stations when doing PCR
3. Do not use Kimwipes below or on top of the microplates during this protocol.
4. Pipet 19 __ of Mater Mix solution (containing Master Mix, Water, and Primer) into appropriate wells.
5. Seal the wells completely. Re-seal the wells once again to ensure the samples were contained within the wells.
6. Place a piece of parafilm over the vortex machine and vortex the plate for 5 seconds.
7. Place the piece of parafilm over the vortex machine and vortex the plate for 5 seconds
8. Set the timer for 2 min. and centrifuge the microplate for the full 2 min.
9. Place the microplate in the PCR machine ensuring the plate is fully in the wells then close the door completely.
10. Run the sample.

A tick study in local wildlife areas is giving students field and lab experience while providing scientists valuable data.

Tick busters

WHILE MOST PEOPLE SPEND their summers avoiding ticks, graduate student Leah Cuthill hopes to find them. She and Assistant Professor Anuradha Ghosh have been collecting them as part of a four-year surveillance study and research project that could shed some light on tick prevalence and tick-borne illnesses in Southeast Kansas.

Ticks carry the pathogen for Lyme disease, Rocky Mountain Spotted Fever, Tularemia, Ehrlichiosis, and host other potentially serious illnesses; Cuthill was inspired to do the project by a potential tick-borne illness she contracted.

"This area is a hub for ticks because of the heat and high humidity," Ghosh said. "As our climate warms, ticks are prevailing in this direction following the migration of their hosts — they're very sensitive to temperatures and humidity. We're studying the distribution of various tick species and whether it's going up or down. We're watching for an invasion, as well."

After collecting ticks in small vials from several locations, they freeze them in the lab in the Biology Department, where they are able to identify the tick species and do a molecular analysis to determine what pathogens the ticks carry.

Courtesy: Spring 2020 PittState Magazine

Other media releases:

https://www.koamnewsnow.com/news/psu-students-and-staff-stay-one-step-ahead-of-local-ticks/1111972320

https://www.fourstateshomepage.com/news/pitt-state-wildlife-research-centers-around-four-state-ticks/

https://www.morningsun.net/news/20190809/local-officials-researchers-aim-to-increase-awareness-of-disease-carrying-pests