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Audrey Long 4/12/2023

Natural *Rickettsia* infection rate in *Amblyomma americanum* and *Dermacentor variabilis* in Georgia, United States.

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By

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B.S.A Human Development and Family Sciences

University of Texas at Austin

2020

Thesis Committee Chair: Gonzalo Vazquez-Prokopec, PhD

An abstract of

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Abstract

Natural *Rickettsia* infection rate in *Amblyomma americanum* and *Dermacentor variabilis* in Georgia, United States.

By Audrey Long

Purpose: *Rickettsia* prevalence in Georgia, United States is poorly understood. *Amblyomma americanum* ticks have the potential to be a vector for human illness due to *Rickettsia* spp. pathogens, and understanding the infection rate compared to *Dermacentor variabilis*, the vector associated with Rocky Mountain Spotted Fever transmission, is important in determining the risk of pathogen transmission to humans in Georgia.

Methods: Host-seeking ticks were collected using flagging methods, then pooled and DNA was extracted. Two molecular methods of detection were used, a real-time PCR and a conventional PCR both designed to test for the *Rickettsia* genus. 64 pools of ticks were tested using both methods, and sanger sequenced to determine the *Rickettsia* species found in positive ticks.

Results: One hundred percent of the 32 pools of *A. americanum* ticks tested positive for *Rickettsia* genus bacteria. Sequencing results from these ticks were one hundred percent *Rickettsia amblyommatis*. 9.38 % (three pools) of *D. variabilis* ticks were positive for rickettsia. Two pools was sequenced as *R. amblyommatis* and the other as *R. montanensis*. No samples were positive for *R. rickettsii*. The sensitivity of both tests (100%) were the same, however the specificity of the real-time PCR was 72.4% compared to the 100% of the conventional PCR.

Conclusions: *A. americanum* may be an important vector of Spotted Fever Group Rickettsiae infection in humans. The conventional PCR was a better method for detecting *Rickettsia* spp. in ticks, due to the occasional false positives in the real-time PCR test.

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Table of Contents

Page

Introduction

A. Introduction and rationale

B. Hypothesis

C. Significance

A. Introduction and Rationale

Ticks in the United States are expanding their geographic ranges as populations and suitable habitats increase, which has resulted in tick-borne illnesses becoming the most prevalent vector borne disease in the United States (Molaei et al., 2019). This rise in cases is mostly attributed to Lyme disease, but there also has been an increase in non-Lyme disease reports since 2004. In 2018, there were approximately 50,000 cases of tickborne illnesses and 8,000 of those were not diagnosed as Lyme disease. Lyme disease, caused by the bacterium *Borrelia burgdorferi*, is transmitted by *Ixodes* species ticks. These are also known as prostriate hard ticks, and while the Centers for Disease Control (CDC) issued a guide to prostriate tick surveillance in 2018, there was a recent expansion in recommendations to include metastriate hard ticks or non-*Ixodes* ticks (CDC Southeastern COE, 2021). Human biting metastriate tick genera in the United States include *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. In Georgia, the most commonly collected metastriate ticks include *Amblyomma americanum* and *Dermacentor variabilis*.

The Lone Star tick, also known as *Amblyomma americanum*, is a tick of the family Ixodidae that inhabits the southeastern United States and is one of the most frequently identified *Amblyomma* species in this region (Higuita et al., 2021). In Georgia, *A. americanum* ticks are found in approximately the same frequency as *Ixodes* species ticks. From 2005- 2021, 2,203 *I. scapularis* ticks were collected for surveillance with the CDC, compared to 2,730 *A. americanum* ticks

(CDC Southeastern COE, 2021). Lone Star ticks are also found in more life stages than other ticks in Georgia. Adults, nymphs, and larva are found in great numbers throughout the tick season which is significant as each of these life stages can transmit disease (O'hara et al., 2008).

The Lone Star tick is an important vector for human pathogens, specifically in the Southeastern United States. Until relatively recently, the Lone Star tick was considered a nuisance species that primarily affected livestock (Hair & Howell, 1970). The Lone Star tick is the vector for Heartland virus, Bourbon virus, *Ehrlichia chaffeensis, E. ewingii,* Southern Tick associated Rash Illness (STARI), and Tularemia (Higuita et al., 2021). There are also reports of *A. americanum* as a competent vector for Rocky Mountain Spotted fever rickettsiae, though there are multiple studies that show the *Rickettsia* spp. found in Lone Star ticks may be non-pathogenic (Goddard & Varela-Stokes, 2009; Higuita et al., 2021). *A. americanum* has been expanding its geographic range to the Midwestern, Northeastern, and Eastern Canadian regions of North America (Molaei et al., 2019). Ticks have also been known to carry more than one pathogen at a time, making them a significant risk to human and animal populations (Madison-Antenucci et al., 2020).

Rocky Mountain Spotted Fever (RMSF) is caused by an intracellular bacterium, *Rickettsia rickettsii*, and is widely spread across the Americas. *Dermacentor* spp. ticks are the primary vector that transmits *R. rickettsii* in the United States, with *D. andersoni* reported as a vector in Western US. In Eastern United States, *Dermacentor variabilis* is the primary vector of RMSF, also known as the American Dog tick (Azad & Beard, 1998). Some *Amblyomma* spp. have been reported in Central and South America as the vector for *R. rickettsii* (Levin et al., 2017). There has been reported detection of *R. rickettsii* in field collected ticks in *Amblyomma americanum* from Kansas and one case of RMSF in North Carolina was found to be linked to a Lone Star tick

(Breitschwerdt et al., 2011; Levin et al., 2017). These studies indicate *A. americanum* is a possible vector for RMSF and other Spotted Fever group Rickettsiae diseases.

Spotted fever group rickettsioses are a group of diseases caused by some species from the genus *Rickettsia,* with RMSF being the most lethal if left untreated (Parola et al., 2005). After the discovery of multiple causes of Rickettsial-like illnesses, the Centers for Disease Control changed the notifiable condition to Spotted Fever Group Rickettsiosis (SFR) in 2010, due to the inability to distinguish between *R. rickettsii* and other Rickettsial diseases on common serologic tests (CDC, 2019). SFR prevalence has risen from 495 in 2000 to 5,207 cases in 2019 in the United States, however, it is unclear how many of these were RMSF and how many were less severe cases of SFR (CDC, 2019). SFR has been reported in all 48 continental states, although 5 eastern states (Arkansas, Missouri, Tennessee, North Carolina, and Virginia) account for approximately 50% of all cases in the US (CDC, 2019). Georgia boarders the states with the highest cases of spotted fever group rickettsioses, and with the changing ecology of tick-borne disease transmission, it is important to understand the current prevalence of *Rickettsia* in Georgia's tick population, and the risk Lone Star ticks could pose in the future.

B. Research Question and Hypothesis

Research Question

Do *A. americanum* ticks, collected in Georgia, have a higher percent positivity of pathogenic *Rickettsia* infection than *D. variabilis*?

Hypothesis

Rickettsia pathogens will be detected from field collected *A. americanum* ticks at a higher percent positivity than *D. variabilis* ticks.

There is a **need** to understand *Rickettsia* infection rate in ticks in Georgia, using molecular methods of identification. The **goal** of this study is to use Polymerase Chain Reaction (PCR) methods to detect *Rickettsia* DNA in host seeking ticks collected in Georgia, then genomic sequencing to determine the species of any positive ticks.

Aims

- To determine the presence or absence of *Rickettsia* pathogens in field collected ticks in Georgia.
- To compare the percent positivity of *Rickettsia* pathogens detected in *Amblyomma americanum* and *Dermacentor variabilis* ticks.
- To assess the efficacy of the two molecular methods employed to detect *Rickettsia* in pooled ticks.

C. Significance

Reported tick-borne disease cases have approximately doubled from 2004 to 2016 in the United States, and issues with surveillance and limitations with diagnostic capabilities suggests the actual prevalence of disease is underestimated (Paules et al., 2018). There have been at least five identified Rickettsia species that have been connected to human disease, *Rickettsia rickettii, R. conorii, R. parkeri, R. candanesis, and R. sibirica* (Parola et al., 2005; Jiang et al, 2012). *R. amblyommii* (now *R. amblyommatis*) has also been connected to human disease and detected in Lone Star ticks collected from outdoor workers skin at roughly 60% in North Carolina (Lee et al., 2014). There are many other *Rickettsia* species with undetermined pathogenicity to humans. The complexity of the interaction between humans, vector, host, and environment, typically results in most cases presenting in rural settings where they may be misdiagnosed (Stuart &

Stuart, 2021). Serology is typically used to diagnose patients with Rickettsial infections, though this is nonspecific between species and often the first few days of infection are negative which is the time in which a patient may present for treatment with a fever (Robinson et al., 2019). Clinical diagnosis is also typically nonspecific, due to the symptoms being similar to other vector borne diseases (Lokida et al., 2020). Rickettsia causes disease world-wide and understanding the ecology of these pathogens in a changing transmission environment is essential in understanding the consequences to human driven land-use change and improving diagnostic capabilities.

Literature Review

A. Background

   a. The Lone Star Tick

   b. The American Dog Tick

B. Previous Research

C. Tick Surveillance

A. Background

This literature review will outline the background and existing research done on the pathogen *Rickettsia* spp., and its vectors, demonstrate the gaps in research, and address current vector surveillance in the United States. *Amblyomma americanum* is expanding its geographic range in the United States and, although this species of tick was historically considered a pest, it has been shown to transmit a wide range of human-pathogenic diseases. This includes pathogens that cause Ehrlichiosis, Tularemia, Southern Tick Associated Rash Illness (STARI), Bourbon virus, Heartland virus, and can cause Alpha-Gal syndrome (AGS) resulting in a red meat allergy (Higuita et al., 2021). The Lone Star tick has been discovered to be a vector of *Rickettsia* pathogens, though the prevalence and geographic distribution is still uncertain.

Ticks are ectoparasites with 850 known species and can be found on every continent. There are three families of ticks, Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttallienlinae. The family of ticks with the greatest importance to humans, and thus the focus of most research, is Ixodidae (O'hara et al., 2008). *Amblyomma Americanum,* also known as the Lone Star tick, is a hard tick (Ixodid) species that is considered one of the most aggressive human-biting ticks in the United States (Childs & Paddock, 2003). *A. americanum* was discovered in the US in 1974 and

was considered a nuisance until the 1990s when it was connected to the transmission of both *Ehrlichia chaffeensis* and *E. ewingii* (Ewing et al., 1995).

a. The Lone Star Tick

Lone Star ticks are non-nidicolous ectoparasites with three active life stages of larva, nymphal, and adult. Each life stage requires a blood meal prior to molting into the next stage. Adult female ticks have a white ornate marking on their scutum, while males have white marks along the entirety of their dorsal body (O'hara et al., 2008). *A. americanum* ticks have long mouthparts to deeply penetrate the skin of their hosts, and during feeding the ticks suppress the inflammatory response to prevent detection. Ticks drop off their host due primarily to photoperiod and feeding times vary depending on the needs of their life stage (O'hara et al., 2008). *A. americanum* ticks are commonly found in forested areas with dense underbrush, particularly leaf litter. Abundance of ticks in woodlands is dependent on the availability of animal hosts, however, large numbers of ticks can be found where hosts are bedded. White-tailed deer are very important hosts as they are sources of a blood meal for each life stage of the tick, but Lone Star ticks are non-specific and are known to bite many mammals and birds at every life stage (Childs & Paddock, 2003). *A. americanum* are distributed in Eastern United States, and their range is expanding to include more Midwestern states and Southeastern Canada (Childs & Paddock, 2003; Higuita et al., 2021).

Historically, New Jersey was considered the northern limit of Lone Star ticks' geographic range, however, currently these ticks have been reported as northern as Canada (Molaei et al., 2019). This expansion may be due in part to rising global temperatures, reforestation, and ecologic changes. Land-use change, like deforestation, have reduced that availability of potential wildlife hosts, but as white-tailed deer and other hosts repopulate and reforestation occurs, ticks can

repopulate these areas (Molaei et al., 2019). Higher temperatures can expand which geographic range that these ticks can survive in, and also increase their active season, which is typically April to September (Molaei et al., 2019; Hair & Howell, 1970).

b. The American Dog Tick

The American Dog tick, *Dermacentor variabilis*, is found in the Eastern-Midwestern United States and Southern Canada. *D. variabilis* is a vector for Rocky Mountain Spotted Fever, Tularemia, and Anaplasmosis (O'hara et al., 2008). Though *D. variabilis* is a human-biting tick, it has also been associated with canine paralysis (O'hara et al., 2008). *D. variabilis* is a three-host tick and has been associated with field and forest habitats. *Dermacentor* species of ticks have been known to exhibit behavioral diapause, a state of low metabolic activity in which the tick is not actively seeking hosts (O'hara et al., 2008; McEnroe, 1985). *Dermacentor variabilis* is thought to be the primary vector for Rocky Mountain Spotted Fever because *Rickettsia rickettsii* has been isolated from ticks collected from RMSF patients in endemic areas, and it is a relatively common tick found in areas with high cases of RMSF (Feng et al., 1980). Even established vectors, such as *D. variabilis*, have a relatively low prevalence of *R. rickettsii* in endemic areas, with other *Rickettsia* spp. detected more frequently (Kakumanu et al., 2018).

B. Previous Research

Zenda et al. (2011) conducted a study that compared entomological inoculation rates (EIRs) of *Rickettsia rickettsii* from field collected ticks in Kansas and Missouri. Ticks were collected using drag-sampling in six sites in Kansas and three sites in Missouri, ticks were homogenized, extracted, and a conventional PCR with three separate targets was performed (*gltA*, *rOmpA*, and *rOmpB*). Samples were then sent to be sequenced. There were a total of 464 adult *A.*

americanums and 406 nymphs, while there were only 169 adult *D. variabilis* collected. 94% of adult *A. americanum* and 93% of nymphs were positive for *R. amblyommii,* with four ticks positive for *R. rickettsii*. Two of those ticks were also infected with *R. amblyommii*, which suggested coinfection may be possible. Only 4.7% of *D. variabilis* ticks collected were positive for *R. amblyommii.* Entomological Infection Rates (EIRs) were calculated for each pathogen and found that it was 28 times more likely to encounter *R. amblyommii* than *R. rickettsii* in these areas (Zenda et al., 2011).

Fritzen et al. (2011) compared common tick-borne pathogens between the American Dog Tick and the Lone Star Tick in Kentucky. 287 ticks were collected from 2007-2008 by taking them off various mammal hosts (including humans) across six counties. 179 of these ticks were *D. variabilis* while the other 108 were *A. americanum* ticks. Ticks were screened for *Rickettsia* spp, *Ehrlichia* spp, and *Borrelia* spp using conventional PCR. 14.3% of ticks were positive for *Rickettsia* spp, 6.3% of ticks were infected with two different *Ehrlichia* species, and one Lone Star tick was positive for *Borrelia lonestari*. No ticks were positive for *Rickettsia rickettsii*. All positive samples were sent for sequencing to confirm the PCR result and species. A Pearson's chi squared test was performed to determine the infection rate between Lone Star ticks (39%) and the American Dog tick (10%) ($P < 0.0002$).

Lee et al. (2014) collected attached ticks from outdoor workers in North Carolina in 2011 and 2012 and tested each tick for *Rickettsia* using PCR, cloning, and nucleotide sequencing. Approximately 90% of ticks collected were *A. americanum* with a total of 874 ticks included in the study. *Rickettsia* species were identified from 60.9% of all tick species, with *A. americanum* as much as 70% prevalence and *D. variabilis* approximately 20%. The most common *Rickettsia*

infection in every tick species was *R. amblyommii* (85%). The study concluded by saying *R. amblyommii* was the most common *Rickettsia* exposure for outdoor workers in North Carolina.

Trout Fryxell et al. (2015) tested ticks collected from canines and white-tailed deer in Arkansas and tested for *Rickettsia* DNA. 1,415 ticks were taken from 156 canines, and 1,569 ticks were from 250 hunted deer. Ticks were then identified by species and life stage, for a total of five species collected: *A. americanum, Ixodes scapularis, D. variabilis, A. maculatum*, and *Rhipicephalus sanguineus*. Conventional PCR was performed with three gene targets, the *gltA*, *rOmpA* and the *rOmpB* genes. Blood was also taken from each specimen and tested as well. None of the blood collected from the deer had *Rickettsia* DNA, and only two of the canines tested positive, while 502 of the ticks tested positive. 37% of the *A. americanum* ticks and 4% of the *Dermacentor variabilis* ticks were positive. Sequencing resulted in primarily *R. amblyommii* (188), two *R. parkeri*, and one was *R. andeanae.* The study concluded by mentioning the lack of *Rickettsia rickettsii* DNA detected and Arkansas being within the five states in which 60% of all RMSF cases are reported and suggested future studies that focused on the range expansion of ticks (Trout Fryxell et al., 2015).

Hecht et al. (2019) tested 883 *Dermacentor variabilis* ticks collected from 12 states in the US for *Rickettsia*. Ticks were collected across the United States and were identified and extracted. Three real-time PCRs were designed for *R. belii* and *R. rickettsii* with a *Rickettsia* genus assay as well. Only one tick was positive for *R. rickettsii* DNA, while 87 were positive for *R. belii.*, 47 having *R. montanensis*, 11 having *R. amblyommatis*, 3 positive for *R. parkeri*, and 2 positive for *R. rhipicephali*. This study confirms the prior research of *R. rickettsii* being relatively low in prevalence in populations of the American Dog tick (Hecht et al., 2019).

Egizi et al. (2020) conducted a study in Monmouth County, New Jersey that tested *A. americanum* nymphs for three different species of SFGR. Ticks were collected using drag sampling for five transects in 19 sites in Monmouth County. The Real-time PCR targets the *17-kDa* gene, the *OmpB* gene specific to *R. amblyommatis* and the *OmpB* gene specific to *R. rickettsii* and *R. parkeri*. Samples positive for the first run were then run on the second duplex to determine any co-infections. 1,858 nymphs were tested and 25% of them were positive for *R. amblyommatis*, with only one tick positive for *R. rickettsii*. These positive samples were then sequenced with over 99% confidence. No co-infections or *R. parkeri* infections were found. Egizi et al. (2020) concluded that Monmouth County had an infection rate of 0.05% of *Rickettsia rickettsii*. This study was also conducted in a region with established Lyme transmission and a robust tick surveillance program.

These studies confirm the low prevalence of *Rickettsia rickettsii* in field collected ticks, in *Dermacentor* species ticks or otherwise. They also support the higher prevalence of infection with *Rickettsia* spp. in *A. americanum* compared to *D. variabilis* across all studies mentioned. While these studies are focused on the east coast of the United States, none are focused specifically on Georgia. My study will focus on addressing this gap in research.

C. Tick Surveillance and Importance to Global Health

Public health surveillance is defined by the continuous, systemic collection, analysis, and interpretation of health-related data essential to the planning, implementation, and evaluation of public health practice (Eisen & Paddock, 2021). Tick Borne Disease (TBD) surveillance can identify where cases have occurred, however ticks need to be attached for a variable length of time to successfully transmit different diseases. In addition, the incubation period can provide a discrepancy between where cases are reported and where they interacted with ticks. Reporting

TBD is also dependent on specific diagnosis, and not all TBDs are nationally notifiable diseases. Tick surveillance, consisting of various methods of tick collection and testing, provides estimates of tick abundance and distribution to help inform local health workers what diseases to consider and to inform emerging pathogen discovery (Eisen & Paddock, 2021). A survey was given to 140 vector borne disease experts across the US to assess their tick surveillance, and while two-thirds of the experts participated in active surveillance, less than half conducted active surveillance (Madder et al., 2020). When asked about the challenges to implementing more active and consistent surveillance, participants mentioned lack of consistent funding and infrastructure, limited trained personnel, and a discrepancy in methods for best practices. TBD and tick surveillance are also an important component of One Health Surveillance. One Health is an approach to public health that focuses on the connection between humans, animals, and the environment and how public health problems can't always be simplified to just one sector. One Health also emphasizes interdisciplinary collaborations and communication between many different sectors, professions, and countries (Filter et al., 2021). A challenge One Health faces in its effective implementation includes the silofication of different sectors, there may be some overlap, but typically only for specific diseases. Tick surveillance also has similar challenges in the US. The United States lacks a national tick surveillance program and in the absence of that most surveillance is done by State Health Departments and university studies to determine the distribution of ticks. These efforts are beneficial but lacks uniformity across the country and may be an underrepresentation of the risk to humans (Eisen & Paddock, 2021). This study aims to assist in these efforts by contributing to the knowledge of tick distribution and abundance, and investigating a pathogen that causes significant human illness.

Methods

A. Introduction

B. Procedures

    a. Tick collection
    b. Tick processing
    c. Population and Sample
    d. DNA extraction
    e. Real-time PCR
    f. Conventional PCR
    g. Genomic Sequencing

C. Plans for Data Analysis
D. Ethical Considerations
E. Limitations and Delimitations

A. Introduction

The purpose of this study is to determine the percent positivity of *Rickettsia* bacteria in field collected ticks in Georgia. This involved the collection of ticks from different field sites from various ecoregions in Georgia (see figure 1). After collection, testing was performed at Rollins School of Public Health Biosafety Level 2 laboratory. Two types of Polymerase Chain Reactions (PCR) were performed, and positive samples were sent to be Sanger sequenced (Psomagen, Inc.). To test the hypothesis, the species of *Rickettsia* was determined to be pathogenic or not based on prior research, and the percent positivity was compared between *Dermacentor variabilis* ticks and *Amblyomma americanum* ticks.

B. Procedures

a. Tick Collection

Site selection

Field site selection was determined based on counties with established *A. americanum* reported in Georgia. Two historical field sites, Kinderhook (KH) and Stalling Road (ST), were used to collect *A. americanum* in enough quantities, while northern and southern Georgia sites were added to determine abundance across Georgia. For this study, Lone Star tick pools were tested from different collection dates to create a sample from the total collected in the 2022 field season (N=32 pools). The *Dermacentor* ticks were collected across all of Georgia, and the entirety of the sample was tested due to the discrepancy of abundance between species (N=32 pools).

KH and ST are part of the Piedmont ecoregion and mainly consist of deciduous forests. See figure 1 for locations of field sites used in this study. Sites selected for this study were used if sampling sites had higher abundances and the field team sampled the same site more than once throughout the field season, to give the results a variability based on the timing of collection.

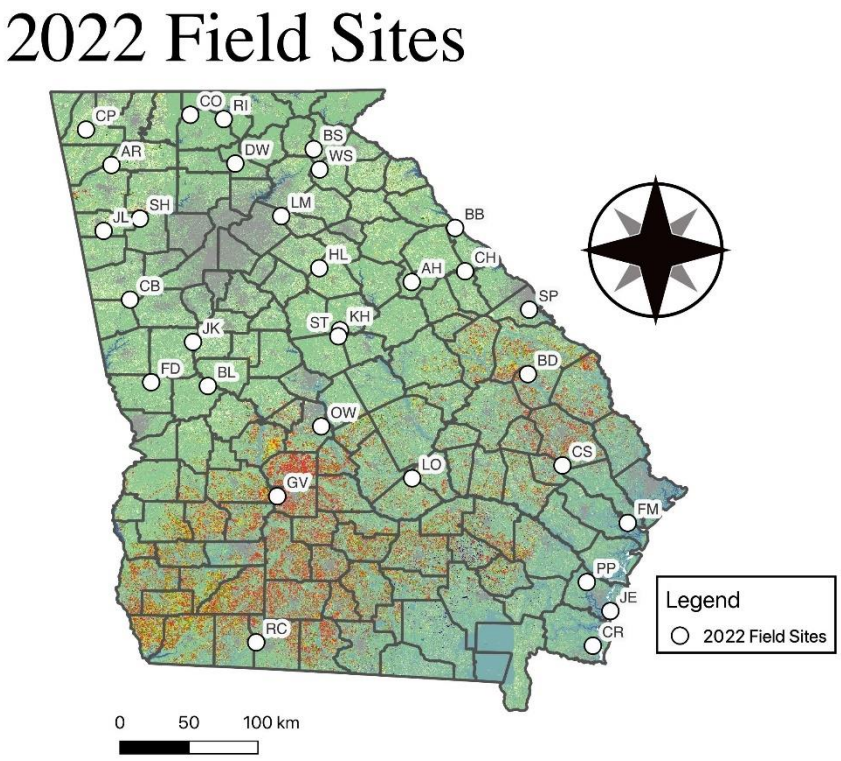

Figure 1. A map of the 2022 field season collection sites in their relation to counties in Georgia. Raster digital data was used to display land-use in Georgia (USDA & NASS, 2023). Refer to appendix A for more information about field sites.

Tick Sampling

Host seeking ticks were collected using the flagging method, in which a white cloth is attached to a wooden rod and dragging the flag over vegetation and leaf litter in a figure eight motion in front of the sampler. At the historical sites (KH and ST), the study team performed free flagging and continuously sampled until one hour had passed. The flag was inspected for ticks frequently, and ticks found were picked off the flag using metal tweezers and kept in plastic vials to keep them until they can be transported. These sites were sampled this way because the presence of ticks in these areas have been established. The State Parks and Wildlife Management Areas sampled in this study were done so by transect sampling. The study team measured out five 15m

by 50m transects for a total of 750m^2 for each sampling site. Selecting these transect areas also involved varying habitat types to determine abundance in different environments. Field collections were performed from April to August of 2022, and if presence of Lone Star ticks was determined, the sites were visited more than once during this time. Sites without Lone Star ticks were not revisited.

b. Tick Processing

Ticks were transported to the laboratory alive and kept in plastic vials in an incubator to maintain adequate humidity and temperature. Species and life stages were identified using a taxonomic key under a dissecting microscope (Martins et al., 2014). Ticks were pooled into 25 nymphs or 5 adult male or females and washed for five minutes to remove any external contaminants with hydrogen peroxide, 10% bleach, and three times with distilled water. Ticks were then crushed with 1 mL of Minimum Essential Medium (Sigma-Aldrich, Inc.) in glass conical tubes, and then stored in an -80 ℃ freezer.

c. Population and Sample

The population of ticks collected per field site were used to represent tick abundance across Georgia's counties. This is only representative of tick species with active questing seasons from March to August in the Southeast United States. The sample size used to test for *Rickettsia* was determined based on the total number of tick pools of *Dermacentor variabilis* ticks collected (N= 32). The total number of tick pools tested and sequenced was 64. This was a sufficient sample size as the percent positivity rate of *Amblyomma* for a PCR designed to detect the *Rickettsia* genus was reported to be over 90% in Kansas (Zenda et al., 2011).

d. DNA Extraction

DNA extractions were performed on each pooled sample by using 140 µL of each sample and following the Qiagen DNeasy Insect Protocol (Qiagen Inc. Rohm and Haas Company, Valencia, CA) which involved a 12-hour incubation period at 56℃. On the last step of the extraction protocol, the researchers used 100 µL of elution buffer to create more concentrated samples after the final step. Extracted samples were then stored in a -20℃ Freezer. Each round of extractions (typically 10-20 samples) included a processing negative control of molecular water to ensure contamination was not a problem during the extraction step.

e. PCR

Positive Control

R. rickettsii is a BSL 3 agent, so no positive control with active bacteria could be used. This study was designed to test the ticks using a variable gene from the *Rickettsia* genus, Citrate Synthase *gltA* gene (GenBank Accession no. NC_010263.3). A gBlock was ordered using a GenBank sequence of *Rickettsia rickettsii* from the Iowa strain (Integrated DNA Technologies, Inc.). See appendix B for full gene sequence used.

Real-time PCR

Real-Time Polymerase Chain Reaction (RT-PCR) was used on every tick sample, along with a positive control (gBlock), a negative control (molecular water), and the processing control from the date of extraction. RT-PCR was performed using primers designed for *Rickettsia rickettsii* citrate synthase gene, gltA (Stenos et al., 2005). Each reaction was run with 12.5 µl of iQ Supermix (Bio-Rad Laboratories, Inc.), 1.0 µl of the forward and reverse primer, 1.0 µl of the probe (Integrated DNA Technologies, Inc.), and 4.5 µl of molecular water, and 5.0 µl of extracted DNA. The samples are run at 50 ℃ for 3 minutes, 95 ℃ for 5 minutes, and 60 cycles

of 95 ℃ for 20 seconds and 60 ℃ for 40 seconds and imaged after each cycle. The primers and probe amplified a 74 base pair fragment of a highly conserved region of the *Rickettsia* genus and run on a BioRad RT-PCR machine, see table 1 for primer sequences (BioRad, CFX96 touch thermal cycler).

Conventional PCR

Conventional PCR was performed on samples that tested positive from the RT-PCR testing. RT-PCR has a higher chance of false positives, so confirming positivity with conventional PCR was done before sending for sequencing. Samples were tested using primers designed for the same citrate synthase gene and run on an Eppendorf thermocycler (Labruna et al., 2004). Samples are run at 95 ℃ for 3 minutes, then cycled 40 times between 95 ℃ (15 seconds), 48 ℃ (30 seconds), 72 ℃ (30 seconds), and then 72 ℃ for 7 minutes. After the PCR cycling is complete, the samples are then imaged using Gel electrophoresis on a 1.5% agarose gel stained with Ethidium Bromide. The gel was run for 45 minutes on 80 Volts. After the gel was imaged, the amplifications were compared to the positive control and the DNA ladder (New England BioLabs, Inc.). If the sample was confirmed positive, the PCR product is cleaned using the Qiagen PCR Purification kit and sent for sanger sequencing (Qiagen Inc. Rohm and Haas Company, Valencia, CA).

Table 1. Polymerase Chain Reaction primers and probe sequences.

Target	PCR	Primer	Sequence 5'-3'	Product Size (bp)	Citation
gltA	Real-time PCR	CS-F	TCGCAAATGTTCACGGTACTTT	74	Stenos et al., 2005
		CS-R	TCGTGCATTTCTTTCCATTGTG		
		CS-Probe	6-FAM-TGCAATAGCAAGAACCGTAGG CTGGATG-BHQ-1		
gltA	Conventional PCR	CS-F 78	GCAAGTATCGGTGAGGATGTAAT	401	Labruna et al., 2004
		CS-R 323	GCTTCCTTAAAATTCAATAAATCAGGAT		

f. Genomic Sequencing

Samples that tested positive for *Rickettsia* (samples with a band size of 401 bp determined by gel electrophoresis) were sent out to be sanger sequenced (Psomagen, Inc.). If a sample tested positive on Real-time PCR (Ct value < 38) but no band was detected using conventional PCR, the sample was also sent for sequencing to determine which method is more accurate. All samples sent for sequencing were at least 12 µl of PCR product. Samples were sent with dry ice to maintain at least 4 ℃ temperature during transportation.

C. Plans for Data Analysis

To compare the efficacy of each PCR method, a McNemar Chi-squared test for paired data was performed to determine whether the results are statistically different than the results of the sequencing.

D. Ethical Considerations

As this project consisted of arachnid collection and testing, it was not considered Human Subjects Research, and Emory Institutional Review Board approval was not required.

E. Limitations and Delimitations

There are some weaknesses in the study design and methodology of this project. One such issue is the disparity between the abundances between tick species which may make any analyses difficult to perform. There was also a difference in abundance between field sites, with most of the ticks being collected from KH or ST. This is due to the established presence of ticks at these sites, from all life stages. However, this makes the risk of Rickettsial disease difficult to determine. It is also not representative of the entire state of Georgia, only the locations that were sampled and tested. Only 64 tick pools were tested, which reduced the sample size of Lone Star ticks significantly and there may be differences in results if every tick was tested and sequenced. This was due to the high positivity rate noticed in Lone Star ticks for all genus *Rickettsia* PCR.

Results

A. Overview of Results

B. Findings

A. Overview of Results

In general, many more *A. americanum* were collected in Georgia compared to *D. variabilis* from March to August. All *D. variabilis* pools were tested, while only 32 pools of *A. americanum* were tested (N=64). No pools of *A. americanum* or *D. variabilis* were positive for *Rickettsia rickettsii*. All (100%) of *A. americanum* pools tested were positive on both PCRs and were sequenced with over 90% confidence as *Rickettsia amblyommatis*. Two pools of *D. variabilis* (6.25%) were positive on PCR and sequenced as *R. amblyommatis*, and one pool was positive for *R. montanensis* (3.125%). All processing controls tested were negative, meaning no contamination occurred during the DNA extraction step.

B. Findings

Table 2. Cumulative abundance of host seeking ticks by species and life stage.

Tick species	Female total	Male total	Nymph Total	Cumulative Tick Total
A. americanum	298	305	2700	3303
Ixodes spp.	24	41	0	65
D. variabilis	29	24	0	53
A. maculatum	9	4	0	13
Total	360	374	2700	3434

All 32 pools of *D. variabilis* ticks (total of 54 adult ticks) were tested for *Rickettsia*. As seen in table 2, no nymphs of other species were found during the 2022 field season. A sample of 32 pools from the *A. americanum* (a total of 359 adult and nymph ticks) ticks were randomly selected. The discrepancy between total tick numbers tested between species was due to the pooling of 25 nymphs in *A. americanum* ticks.

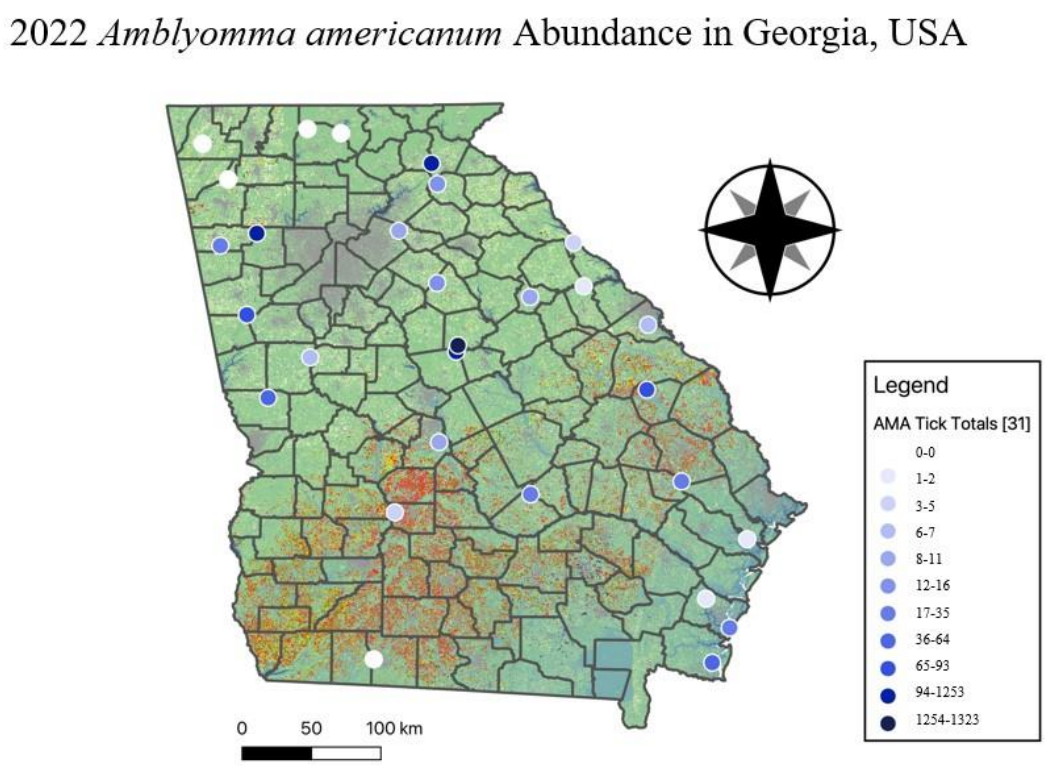

Figure 2. A map of *A. americanum* abundance collected in Georgia during the 2022 field season. The state of Georgia is visualized by county and via raster data representing land-use (USDA & NASS, 2023). Refer to appendix A for more information about field sites.

As seen in figure 2, most host-seeking ticks were collected from two sites (KH and ST). Due to the sample size tested using PCR, the positivity rate was not included in the map as to not misrepresent the geographic risk in Georgia. Eastern Georgia seems to have a lower abundance of *Amblyomma* species ticks, however as seen in the map, tick abundance is variable across the state. Overall, there were a total of 3,303 *A. americanum*, 63 *Ixodes* spp., 53 *D. variabilis*, and 13 *A. maculatum* ticks collected. A subset of the ticks collected (N= 32 pools) were tested to determine the percent positivity of *Rickettsia* in the Lone Star tick.

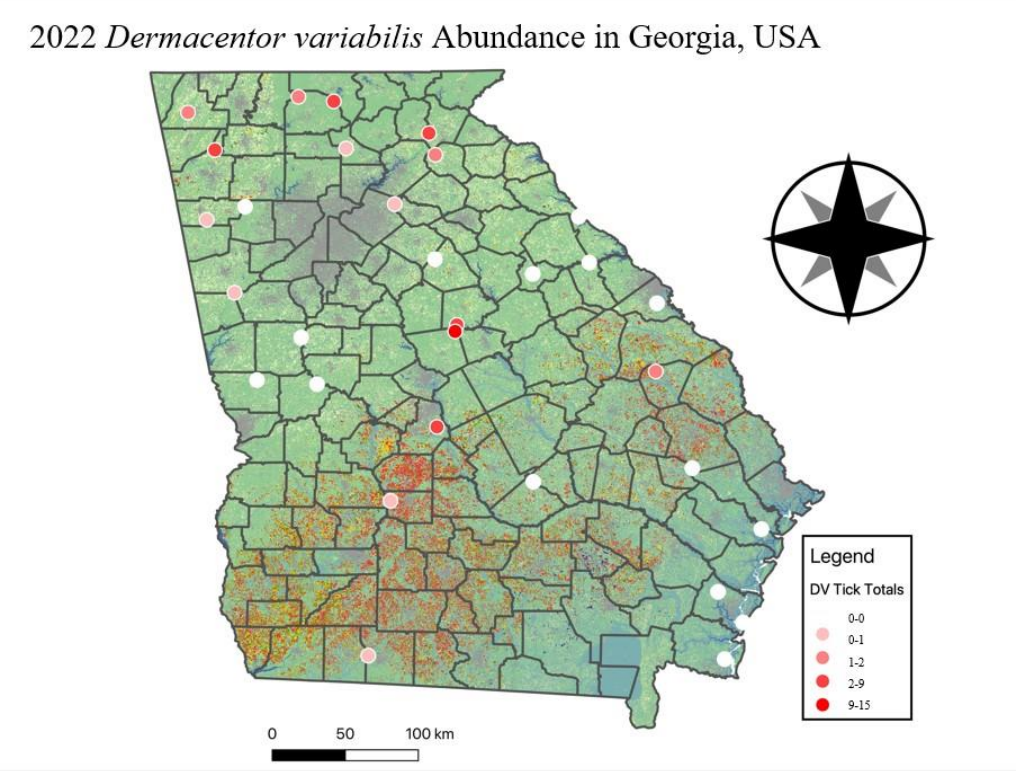

Figure 3. A map of *D. variabilis* abundance collected in Georgia during the 2022 field season. The state of Georgia is visualized by county and via raster data representing land-use (USDA & NASS, 2023). Refer to appendix A for more information about field sites.

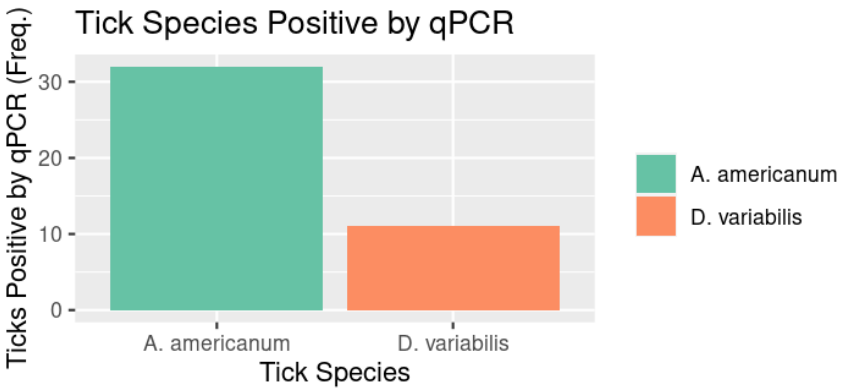

Figure 4. A graph illustrating the positive tick pools by real-time PCR for each tick species tested.

32 pools of *A. amblyomma* ticks were positive, while 11 pools of *D. variabilis* were positive. Positive results were determined by Ct values (<38). As seen in Figure 4, all pooled *A. americanum* samples were positive for *R. amblyommatis*. There were also no coinfections found in these samples. No false positives were detected from the Real-time PCR due to the high prevalence of *Rickettsia* found in the samples.

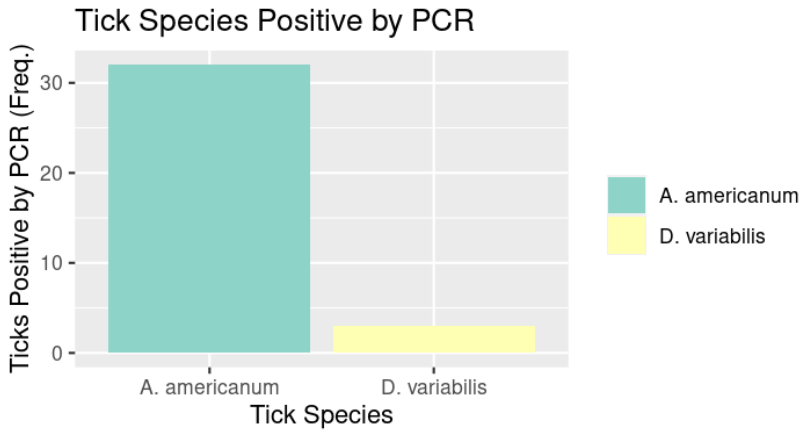

Figure 5. A graph illustrating the positive tick pools by conventional PCR.
32 pools of *A. amblyomma* ticks were positive, while 3 pools of *D. variabilis* were positive. Positive results were determined by Ct values (<38).

Figure 5 shows the relatively low prevalence of *Rickettsia* found in the *D. variabilis* samples. A sample, collected for Grand Bay Wildlife Management Area (GB), and a sample from Stalling Road (ST) were both positive for *Rickettsia amblyommatis*. GB is in Lanier County and is in the Southern Coastal Plains ecoregion of Georgia. ST is in Jones County and is in the Piedmont ecoregion of Georgia. Another sample, of *D. variabilis* from Arrowhead Wildlife Management Area (AR), was positive for *Rickettsia montanensis*. The AR field site is in Floyd County and is found in the Ridge and Valley ecoregion of Georgia.

Table 3. An illustration of the true positives and true negatives based on sequencing results and by testing method. Results of sequencing are used to confirm the reliability of each test.

Type of Test	**Result of Test**	**Results of Sequencing**		**Probabilities**
		Positive by sequencing	Negative by sequencing	
PCR	Positive	35	0	*Sensitivity*= (35/(35+0)= 100%
	Negative	0	29	*Specificity*= (29/29+0)= 100%
qPCR	Positive	35	8	*Sensitivity*= (35/35+0)= 100%
	Negative	0	21	*Specificity*= (21/21+8)= 72.4%

In conclusion, the Real-Time PCR method had 8 false positives, while the conventional PCR had zero (McNemar's $\chi 2 = 6.125$, $df = 1$, $P = 0.013$).

Discussion

A. Discussion
B. Limitations and Suggestions
C. Conclusion

A. Discussion

Historically, *A. americanum* has been suspected as being the vector for RMSF as far back as 1943 (Parker et al., 1943). A total of 114 *A. americanum* nymphs were collected outside a home in Oklahoma where a resident had just recovered from RMSF. These nymphs were placed on guinea pigs and every guinea pig became sick with a spotted fever-like illness. This study concluded that *A. americanum* was the most prominent tick in the area, as the resident's dogs were found to have only one adult *D. variabilis* and *A. maculatum*, and many *A. americanum*. There was also a CDC confirmed case of RMSF in North Carolina caused by the bite of a Lone Star tick. In this case, the tick was confirmed to be *A. americanum* by PCR, the patient was seroconfirmed to have Spotted Fever, and PCR was performed both on the patient's blood and on the tick and the sequencing resulted in *Rickettsia rickettsii* (Breitschwerdt et al., 2011). These studies, the aggressive human biting behavior of these ticks, and the geographic overlap of these vectors and RMSF cases have provided evidence that the Lone Star tick may be an important vector of RMSF (Richardson et al., 2023). However, there are studies that question the role of *A. americanum* in the transmission of RMSF.

In this study, no *R. rickettsii* was detected in the ticks tested. However, *R. amblyommatis* was detected in *A. americanum* ticks at an alarmingly high prevalence. There has been some evidence that *R. amblyommatis* can cause human-illness and its inhibitory role in the diagnosis of other Rickettsia species. Rickettsia is a diverse bacterium that is split into four groups, the Spotted Fever Group (SFG), the *R. bellii* group, the typhus group, and *R. canadensis* group (Richardson

et al., 2023). These pathogens can be transmitted by many arthropod vectors, but the tick-borne human affecting illnesses are found in the Spotted Fever group. SFG Rickettsia need vertebrate reservoirs to maintain their lifecycle, and can be transmitted to other ticks transovarially and transstadially (Richardson et al., 2023). *R. amblyommatis* was originally discovered in 1973 in Tennessee in *A. americanum.* Since it's discovery, *R. amblyommatis* has an over 90% prevalence in Lone Star ticks found in the United States (Lee at al., 2014; Richardson et al., 2023). *R. amblyommatis* was also found to be the cause of a rash in 2006 and has to potential to cause human illness (Billeter et al., 2007). However, *R. amblyommatis* has shown variable pathogenicity in past animal studies (Yen et al., 2021; Clark et al., 2015).

R. amblyommatis has also been found to have a protective effect against other, more lethal, *Rickettsia* infections. Guinea pigs, when inoculated with *R. amblyommatis* did not develop illness, and were then exposed to lethal doses of *R. rickettsii* and the inoculated guinea pigs stayed healthy. The control guinea pigs became ill and died (Blanton et al., 2014). Previous studies have also illustrated the ticks infected with *R. amblyommatis* spent less time questing than uninfected ticks (Richardson et al., 2022).

It is not surprising that *A. americanum* ticks were infected with *R. amblyommatis* at such high rates, when comparing to previous studies and taking into consideration the pooling of ticks. *D. variabilis* is historically infected with *Rickettsia* at a low prevalence and the lack of *R. rickettsii* is altogether not unusual (Lee et al., 2014). However, isolation could not be performed, so these results may not reflect natural infection. One pooled sample of *D. variabilis* ticks tested positive with *R. montanensis,* which was previously thought to be nonpathogenic to humans, but was discovered in 2012 to be the cause of a Spotted Fever-like illness (McQuiston et al., 2012). *R. montanensis* may act like *R. amblyommatis* in the sense that there is evidence that infection with

R. montanensis prevents coinfection with other *Rickettsia* diseases (Baldridge et al., 2010). It is plausible that *R. montanensis* and *R. amblyommatis* may be responsible for some of the SFG cases in the United States (Lippi et al., 2021).

B. Limitations and Suggestions

Some limitations to this study include the primer and probe design. The primer designed for the entire *Rickettsia* genus was useful for a low prevalence sample, such as the *D. variabilis* ticks. However, the non-specific primer and probe design made the detection of different species infecting the A. americanum ticks difficult. Due to time constraints and financial factors, it wouldn't be ideal to sequence every positive *A. americanum* sample to determine whether it is *R. amblyommatis* or a different *Rickettsia* species. In the future, a primer assay that differentiates between *R. amblyommatis* and other *Rickettsia* species would be more useful when testing A. americanum specifically. This would also allow the positive samples with unique *Rickettsia* species to be whole genome sequenced instead of just sanger sequenced. Phylogenic analyses would be useful in beginning the SFG *Rickettsia* surveillance in the future.

Another potential limitation to this study was the lack of a Biosafety Level 3 laboratory. All laboratory work was performed in a BSL 2 lab, and while this was sufficient for detecting *Rickettsia* in field collected ticks, it also limited any further inquiries. *Rickettsia* cannot be cultured or isolated properly unless in a BSL 3 laboratory. Additionally, the real-time PCR was not specific enough to be used for *Rickettsia* screening in the future. As seen in the *D. variabilis* results, there were many false positives in only the real-time PCR that the conventional PCR lacked. This method needs to be further troubleshooted or other primers and probes can be tested for future screening efforts.

A. Conclusion

In conclusion, this study found a high rate of *R. amblyommatis* infection in field collected *A. americanum* ticks. The *D. variabilis* ticks tested had a lower rate of infection and one tick pool was also infected with *R. montanensis*. These findings are consistent with previous research, as Lone Star ticks have a 90% infection rate with *R. amblyommatis.* In future studies, primers that exclude *R. amblyommatis* should be developed and used alongside the primers described in this study to allow for the easier identification of *R. rickettsii* or other species infecting *A. americanum.* Another area for future investigation is the determination of the pathogenicity of *R. amblyommatis* in humans, as most previous research in this area uses animal models to varying success.

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Appendix

A. Table describing field sites in more detail.

Site Name (Site Code)	County	Ecoregion	Habitat Types	Method of Sampling
Kinderhook Road (KH)	Putnam County	Piedmont	Oak-Hickory Forest, Bottomland Forest	Free Flagging
Stalling Road (ST)	Jones County	Piedmont	Oak-Hickory-Pine Forest	Free Flagging
River Creek The Rolf and Alexandra Kauka WMA (RC)	Grady County	Southeastern Plains	Blackbelt Prairies, Longleaf Pine-Scrub Oak Woodlands	Transect
Grand Bay WMA (GB)	Lanier County	Southern Coastal Plain	Bottomland Forest, Forested Depressional Wetlands	Transect
Big Dukes Pond WMA (BD)	Jenkins County	Southeastern Plains	Pond cypress swamp, pond cypress savanna, slash pine-mixed hardwoods, bay swamp and	Transect
Sheffield WMA (SH)	Bartow County	Piedmont	Montane Longleaf Pine-Hardwood Forest	Transect
Arrowhead WMA (AR)	Floyd County	Ridge and Valley	Mesic Pine Hardwood Forests	Transect
Cohutta WMA (CO)	Douglas County	Piedmont	Pine-oak woodlands	Transect
AH Stephens Memorial State Park (AH)	Taliaferro County	Piedmont	Cove forest, hardwood forest	Transect
Joe Kurz WMA (JK)	Meriwether County	Piedmont	Pine forest, Grassland	Transect
Bobby Brown State Park (BB)	Elbert County	Piedmont	Oak-Hickory Forest,	Transect

			Bottomland Forest	
FD Roosevelt State Park (FD)	Harris County	Piedmont	Pine-oak woodlands	Transect
Little Ocmulgee State Park (LO)	Wheeler County	Southeastern Plains	Grassland and early Successional Habitats, Mesic Hardwood Forest	Transect
Grand Bay WMA (GB)	Lanier County	Southern Coastal Plains	Pine and Hardwood Forest	Transect
James H "Sloppy" Floyd State Park (SF)	Chattooga County	Ridge and Valley	Oak Woodlands	Transect
Dawson WMA (DW)	Dawsonville, GA	Blue Ridge	Pine Forest	Transect
Fort Morris State Historic Site (FM)	Liberty County	Southern Coastal Plain	Bottomland Forest, Coastal Shrub Wetlands, Brackish Marsh, Coastal Dune	Transect
Jekyll Island (JE)	Glynn County	Southern Coastal Plain	Bottomland Forest, Coastal Shrub Wetlands, Brackish Marsh, Coastal Dune	Transect
Buck Shoals (BS)	White County	Ridge and Valley	Pine-oak woodlands	Transect
Laura S. Walker State Park (LW)	Ware County	Southern Coastal Plain	Deciduous woodlands	Transect
Johns Mountain WMA (JM)	Floyd County	Ridge and Valley	Hardwood forest	Transect
Spirits Creek WMA (SP)	Richmond County	Piedmont	Hardwood forest	Transect
Clarks Hill WMA (CH)	Lincoln County	Piedmont	Oak-Hickory-Pine Forest	Transect

Paulks Pasture WMA (PP)	Glynn County	Southern Coastal Plain	Bottomland Forest, Coastal Shrub Wetlands, Brackish Marsh, Coastal Dune	Transect
Altamaha WMA (AM)	Mcintosh County	Altamaha WMA	Bottomland Forest, Coastal Shrub Wetlands, Brackish Marsh, Coastal Dune	Transect
Hard Labor Creek State Park (HL)	Morgan County	Piedmont	Bottomland Hardwood Forest	Transect
JL Lester WMA (JL)	Polk County	Piedmont	Deciduous Woodlands	Transect
Chattahoochee Bend State Park (CB)	Coweta County	Piedmont	Deciduous Woodlands	Transect
Rich Mountain WMA (RI)	Gilmer and Fannin counties	Blue Ridge	Pine Forest	Transect
Crockford Pigeon WMA (CP)	Walker County	Blue Ridge	Pine Forest	Transect
Oaky Woods WMA (OW)	Houston and Pulaski Counties	Piedmont	Deciduous Woodlands	Transect
Georgia Veterans Memorial State Park (GV)	Crisp County	Southeastern Plains	Sandhill Pine Woodland, Grassland and early Successional Habitats	Transect
Wilson Shoals WMA (WS)	Banks and Habersham counties	Ridge and Valley	Pine-oak woodlands	Transect
Little Mulberry Park (LM)	Gwinnett County	Piedmont	Deciduous woodlands	Transect

B. gBlock HiFi Gene Fragment ordered using GenBank Accession no. NC_010263.3 FASTA result:

>NC_010263.3:c1213803-1212496 Rickettsia rickettsii str. Iowa, complete sequence

ATGACCAATGAAAATAATAATGATTCAGAATTTGCTGAATTAAAAATCAGAGGAAAAATATTTAAATTAC

CTATACTTAAAGCAAGTATCGGTGAGGATGTAATCGATATAAGTAGGGTATCTGCGGAAGCCGATTGCTT

TACTTACGACCCGGGTTTTATGTCTACTGCTTCTTGTCAGTCTACTATCACCTATATAGACGGTGATAAA

GGAATCTTGCGGCATCGAGGATATGATATTAAAGACTTAGCTGAGAAAAGTGATTTTTTAGAAGTAGCAT

ATTTACTGATTTATGGGGAACTACCAAGTGGCGAGCAGTATAATAATTTCACTAAACAGGTTGCTCATCA

TTCATTAGTGAATGAAAGATTACACTATTTATTTCAGACCTTTTGTAGCTCTTCTCATCCTATGGCTATT

ATGCTTGCGGCTGTCGGTTCTCTTGCGGCATTTTATCCTGATTTATTGAATTTTAAGGAAGCAGATTACG

AACTTACCGCTATTAGAATGATTGCTAAGATACCTACCATCGCTGCAATGTCTTATAAATATTCTATAGG

ACAACCGTTTATTTATCCTGATAATTCGTTAGATTTTACCGAAAATTTTCTGCATATGATGTTTGCAACG

CCTTGTACGAAATATACAGTAAATCCAATAATAAAAAATGCTCTTAATAAGATATTTATCCTACATGCCG

ATCATGAGCAGAATGCTTCTACTTCAACAGTCCGAATTGCCGGCTCATCCGGAGCTAACCCTTTTGCTTG

TATTAGCACGGGTATTGCCTCACTTTGGGGACCTGCTCACGGCGGGGCTAATGAAGCGGTAATAAATATG

CTTAAAGAAATCGGTAGTTCTGAGTATATTCCTAAATATATAGCTAAAGCTAAGGATAAAAATGATCCAT

TTAGATTAATGGGTTTTGGTCATCGTGTATATAAAAACTATGACCCGCGTGCCGTAGTACTTAAAGAAAC

GTGCAAAGAAGTATTAAAGGAACTCGGGCAGCTAGACAACAATCCGCTATTACAAATAGCAATAGAACTT

GAAGCTATCGCTCTTAAAGATGAATATTTTATTGAGAGAAAATTATATCCAAATGTTGATTTTTATTCGG

GTATTATCTATAAAGCTATGGGTATACCGTCGCAAATGTTCACGGTACTTTTTGCAATAGCAAGAACCGT

AGGCTGGATGGCACAATGGAAAGAAATGCACGAAGACCCTGAACAAAAAATCAGCAGACCTAGACAGCTT

TACACCGGTTATGTACATAGAGAGTATAAGGGTATTCGGGAGAGGTAA

C. Tick abundance broken down for each field site, by life stage, tick species, and sex.

Field Site	Tick spp	Female total	Male total	Nymph Total	Tick Total	Site total
	A.americanum	142	127	1054	1323	
	Ixodes. spp	7	9	0	16	
	D. variabilis	3	0	0	3	
KH	*A. maculatum*	1	1	0	2	1344

	A.americanum	105	132	1016	1253	
	Ixodes. spp	11	20	0	31	
	D. variabilis	6	9	0	15	
ST	*A. maculatum*	1	1	0	2	1301
	A.americanum	0	0	111	111	
	Ixodes. spp	0	0	0	0	
	D. variabilis	3	1	0	4	
BS	*A. maculatum*	2	1	0	3	118
	A.americanum	1	0	21	22	
	Ixodes. spp	0	2	0	2	
	D. variabilis	0	0	0	0	
LO	*A. maculatum*	0	0	0	0	24
	A.americanum	7	11	46	64	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
FD	*A. maculatum*	0	0	0	0	64
	A.americanum	1	4	77	82	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	1	0	1	
CB	*A. maculatum*	0	0	0	0	83
	A.americanum	6	2	27	35	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	1	0	0	
JE	*A. maculatum*	0	0	0	0	35
	A.americanum	2	0	20	22	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
CS	*A. maculatum*	0	0	0	0	22
	A.americanum	12	3	27	42	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
CR	*A. maculatum*	0	0	0	0	42
	A.americanum	0	0	7	7	
	Ixodes. spp	0	0	0	0	
	D. variabilis	2	1	0	3	
OW	*A. maculatum*	0	0	0	0	10
	A.americanum	5	5	105	115	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	0	0	0	
SH	*A. maculatum*	0	0	0	0	116
	A.americanum	5	5	83	93	
	Ixodes. spp	0	0	0	0	
BD	*D. variabilis*	0	2	0	2	95

	A. maculatum	0	0	0	0	
	A.americanum	2	2	9	13	
	Ixodes. spp	0	1	0	1	
	D. variabilis	0	0	0	0	
HL	*A. maculatum*	0	0	0	0	14
	A.americanum	2	1	8	11	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	1	0	1	
LM	*A. maculatum*	0	0	0	0	12
	A.americanum	1	2	2	5	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
BB	*A. maculatum*	0	0	0	0	5
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	4	5	0	9	
AR	*A. maculatum*	0	0	0	0	9
	A.americanum	1	2	3	6	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
JK	*A. maculatum*	1	0	0	1	7
	A.americanum	1	0	0	1	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
CH	*A. maculatum*	1	1	0	2	3
	A.americanum	0	2	4	6	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
SP	*A. maculatum*	0	0	0	0	6
	A.americanum	2	1	13	16	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	1	0	2	
WS	*A. maculatum*	0	0	0	0	18
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	1	0	2	
CO	*A. maculatum*	0	0	0	0	2
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	2	1	0	3	
RI	*A. maculatum*	0	0	0	0	3
	A.americanum	0	2	21	23	
JL	*Ixodes. spp*	0	0	0	0	26

	D. variabilis	1	0	0	1	
	A. maculatum	2	0	0	2	
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	2	0	0	2	
CP	*A. maculatum*	0	0	0	0	2
	A.americanum	1	2	41	44	
	Ixodes. spp	0	0	0	0	
	D. variabilis	0	0	0	0	
BL	*A. maculatum*	1	0	0	1	45
	A.americanum	2	0	3	5	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	0	0	1	
GV	*A. maculatum*	0	0	0	0	6
	A.americanum	0	0	8	8	
	Ixodes. spp	1	0	0	1	
	D. variabilis	0	0	0	0	
AH	*A. maculatum*	0	0	0	0	9
	A.americanum	0	2	0	2	
	Ixodes. spp	3	4	0	7	
	D. variabilis	0	0	0	0	
PP	*A. maculatum*	0	0	0	0	9
	A.americanum	0	0	1	1	
	Ixodes. spp	2	5	0	7	
	D. variabilis	0	0	0	0	
FM	*A. maculatum*	0	0	0	0	8
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	0	0	1	
RC	*A. maculatum*	0	0	0	0	1
	A.americanum	0	0	0	0	
	Ixodes. spp	0	0	0	0	
	D. variabilis	1	0	0	1	
DW	*A. maculatum*	0	0	0	0	1
Total						3440

D. *D. variabilis* pooled samples Polymerase Chain Reaction (PCR) and sequencing results.

D Var Vial #	Size	Sex M	Sex F	qPCR	PCR	Sequencing
118	5	3	2	Neg.	Neg.	-
133	2	2	0	Neg.	Neg.	-
139	1	0	1	Pos.	Neg.	-
140	1	0	1	Pos.	Pos.	*R. amblyommatis*
150	2	2	0	Neg.	Neg.	-
167	1	1	0	Pos.	Neg.	-
188	3	2	1	Neg.	Neg.	-
233	1	0	1	Neg.	Neg.	-
236	1	0	1	Neg.	Neg.	-
248	6	4	2	Neg.	Neg.	-
249	1	1	0	Neg.	Neg.	-
250	2	0	2	Pos.	Pos.	*R. montanensis*
272	1	0	1	Neg.	Neg.	-
288	1	0	1	Pos.	Neg.	-
297	3	2	1	Pos.	Pos.	*R. amblyommatis*
301	3	0	3	Neg.	Neg.	-
308	1	1	0	Neg.	Neg.	-
309	1	0	1	Pos.	Neg.	-
310	1	1	0	Pos.	Neg.	-
314	1	1	0	Pos.	Neg.	-
319	1	1	0	Pos.	Neg.	-
328	1	1	0	Neg.	Neg.	-
329	1	0	1	Neg.	Neg.	-
330	1	0	1	Neg.	Neg.	-
331	2	1	1	Neg.	Neg.	-

	332	1	0	1	Neg.	Neg.	-
	333	1	0	1	Neg.	Neg.	-
	337	1	0	1	Neg.	Neg.	-
	339	1	0	1	Neg.	Neg.	-
	341	1	0	1	Neg.	Neg.	-
	350	3	1	2	Pos.	Neg.	-
	363	2	1	1	Neg.	Neg.	-
Total	**32**	**54**	**25**	**29**	**11**	**3**	**3**

E. *A. americanum* pooled samples PCR and sanger sequencing results.

	AMA Vial #	Size	Sex M	Sex F	Nymph	qPCR	PCR	Sequencing
	169	5	5	0	0	Pos.	Pos.	*R. amblyommatis*
	179	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	202	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	227	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	201	4	4	0	0	Pos.	Pos.	*R. amblyommatis*
	210	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	226	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	195	5	5	0	0	Pos.	Pos.	*R. amblyommatis*
	221	26	0	0	26	Pos.	Pos.	*R. amblyommatis*
	212	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	183	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	187	28	0	0	28	Pos.	Pos.	*R. amblyommatis*
	209	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	203	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	196	3	3	0	0	Pos.	Pos.	*R. amblyommatis*

	204	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	98	4	0	4	0	Pos.	Pos.	*R. amblyommatis*
	35	3	1	2	0	Pos.	Pos.	*R. amblyommatis*
	106	3	0	3	0	Pos.	Pos.	*R. amblyommatis*
	15	6	3	3	0	Pos.	Pos.	*R. amblyommatis*
	104	19	0	0	19	Pos.	Pos.	*R. amblyommatis*
	130	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	115	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	123	5	5	0	0	Pos.	Pos.	*R. amblyommatis*
	240	7	0	0	7	Pos.	Pos.	*R. amblyommatis*
	19	7	0	7	0	Pos.	Pos.	*R. amblyommatis*
	20	5	0	5	0	Pos.	Pos.	*R. amblyommatis*
	44	13	0	0	13	Pos.	Pos.	*R. amblyommatis*
	313	2	0	2	0	Pos.	Pos.	*R. amblyommatis*
	39	4	4	0	0	Pos.	Pos.	*R. amblyommatis*
	7	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
	8	25	0	0	25	Pos.	Pos.	*R. amblyommatis*
Total	**32**	**359**	**30**	**61**	**268**	**32**	**32**	**32**