Presence of *Borrelia*, *Rickettsia*, and *Ehrlichia* in Field-collected Ticks on Candlers Mountain, Virginia

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Abstract

Tick survey is an important factor in the determination of tick-borne disease in an area. A tick survey was done on Candlers Mountain in Lynchburg, Virginia, to look for the presence of *Borrelia*, *Rickettsia*, and *Ehrlichia*. With the help of CO_2 traps, 116 ticks were collected, including 75 adult lone star ticks and 3 adult blacklegged ticks. The use of the CO_2 trap was successful as a tick *capturing* method, but a preference was seen in the capture of lone star ticks. The ticks' DNA will then be extracted and analyzed for the presence of pathogens in future work.

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Introduction

Tick-borne disease is found throughout the world and is prevalent in the United States. As good stewards of creation, one way mankind can help diminish disease frequency is by disease prevention. To prevent disease in both animals and humans, one must be aware of possible risks found in our environments. Ticks are major world-wide vectors of disease such as Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis. This study documents efforts made to determine the abundance of ticks in a specific area and if they are vectors of Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis.

There are numerous pathogenic vectors that plague the world with dangerous, even fatal diseases. One common global pathogenic vector is the tick (*1*). On average, 30,000 cases of Lyme disease in the United States are reported to Centers for Disease Control and Prevention per year (CDC) (*2*). Rocky Mountain spotted fever occurs in six cases per million people in the United States (*3*). The incidence of ehrlichiosis in 2010 was determined to be 2.5 cases per million people in the U.S. (*4*). The way ticks transmit pathogens depends on the life stage and species of tick. Ticks go through four life stages: egg, six-legged larva, eight-legged nymph, and adult. The actual feeding stages of the tick occur in the larva, nymph, and adult stages. Ticks find their hosts through a number of different means. They are attracted to body odors, vibration, moisture, heat, and CO_2. Once a tick finds a host, it locates a feeding spot where it inserts its hypostome into the host's skin and anchors itself. The tick will feed over a period of several days before it detaches itself (*5*). While feeding, ticks excrete minute quantities of saliva that contain

numbing chemicals. It is through this saliva that the disease carried by the tick is transmitted to its host.

Knowledge of the presence of disease-causing agents carried by ticks in an area is important to both public awareness and health. Tick collection is required for disease surveillance and depends on a comprehensive understanding of ticks' habitats, life cycles, and diseases they may carry. There are a number of different methods to capture ticks in field research, and once captured, a multitude of tests can be conducted to detect the possible presence of disease (*6, 7*).

Background

The purpose behind this two-year study is to determine whether or not the ticks caught at a specific location in Virginia carry the pathogenic organisms that cause Lyme disease, Rocky Mountain spotted fever, or ehrlichiosis. A pre-determined site is used on top of Candlers Mountain in Lynchburg, Virginia. This mountain is of special interest since it is a popular site that is frequented by many locals and their pets. The mountain contains numerous hiking and biking trails as well as Liberty University's Snowflex Center attraction. It is important to test the area for disease as a courtesy to the public. Due to the high volume of people and their pets that visit Candlers Mountain, it is vital for the public to be aware of any possible disease-carrying agents. After the publication of this work along with local oral presentations, the public can be enlightened on disease presence and take the necessary measure of disease prevention for both themselves and their pets. When people are educated about the dangers their surroundings can present, they can be better prepared and take the proper precautionary measures to prevent disease contraction.

Disease Vectors

Ticks are arthropods that can live from one to three years during which they will progress through several developmental stages: the egg, the six-legged larvae, the eight-legged nymph, and finally an adult (*8*). Ticks are obligate feeders, in that they must obtain a blood meal in order to survive and molt into their next developmental stage. Three pathogenic microorganisms in ticks that can be transmitted to humans are Lyme Disease, Rocky Mountain spotted fever, and ehrlichiosis (13). It is a common misconception that ticks are born with these perilous diseases when in fact it is after they have fed on an infected host's blood that they then will carry the causative agents for disease in their midgut and or salivary glands (*9*). Ticks have piercing mouthparts which assist in their feeding process by penetrating their host's skin and anchoring themselves, making use of the cement-like substance excreted from their salivary glands (*10*). Ticks' saliva contains molecules that prevent a host's natural hemostasis response through inhibitors of platelet aggregation and inhibitors of molecules involved in the coagulation cascade (*11, 12*). Thus, a tick can remain undetected by its host since both the host's pain and itch response are blocked.

Species of Ticks

The Lone Star Tick

The lone star tick (*Amblyomma americanum*) is located throughout south-central, southeastern, and eastern states (*4*). They can be spotted year round but experience a peak in activity between May and July (*13*). They are normally found in wooded areas, typically inhabited by a large number of potential host mammals. Both the larvae and nymph typically feed on smaller mammals such as rabbits, squirrels, and birds as well as

domestic animals and white-tailed deer. They will even feed on humans if the chance arises. The adults are habitually found on white-tailed deer, farm animals, dogs, and humans. The lone star tick is similar in body size to the dog tick but larger than the blacklegged tick. The males have dark drown bodies while the females are closer to a reddish-brown shade (*14*). The female has a silvery or white spot on the posterior end of her scutum (Fig. 5).

Figure 5. An adult female lone star tick caught on duct tape seen through a microscope.

Male lone stars are generally smaller than the females and will exhibit white streaks or spots near the margins of its body (*15*). They have a more circular body shape in contrast with the dog and blacklegged tick (*14*).

The Blacklegged Tick

The blacklegged tick (*Ixodes scapularis*) is also known as the deer tick. They are mostly found on the east coast in areas with an abundance of undergrowth and forest.

Their preferred hosts as adults are the white-tailed deer, while the larvae favor smaller mammals, having a higher predilection for the white-footed mouse (*16*). The adults are active from October to the spring and summer months, which is when they reach their peak in activity (*17*). Although they are the smallest of the three ticks this study examined, they are easily identifiable due to their orange-red body around their scutum and very dark legs (*18*). They are often hard to spot when they feed on a host due to their small stature, as the adults grow to only about 3 mm in length. They do not carry any distinctive markings; however, the male can be identified as distinct from a female since they tend to have very dark brown coloring, while the females display a lighter reddish-brown (*16*) (Fig. 6).

Figure 6. An adult female deer tick caught on duct tape seen through a microscope.

The American Dog Tick

The American Dog Tick (*Dermacentor variabilis)* is mainly found throughout eastern and southern United States in shrub land and grassy fields where there is little tree coverage. They prefer to live in these secondary growth areas since they require a certain

amount of moisture and humidity to prevent desiccation (*17*). In Virginia, the dog tick tends to become active in early April through late August or early September. Their peak activity occurs between the months of April and May and once again in the month of July (*19*). They are common wherever livestock or domestic animals reside (*3*). The female dog tick is distinguished from the male since she has a shorter scutum while the male's covers most of its dorsal surface. Their scutum has a unique off-white coloring while their bodies are typically brown to reddish-brown in appearance. Males are around 3.6 mm long and the females are slightly bigger, around 5 mm in length (*16*). As the female dog tick feeds, it will distend with blood and, once engorged, become balloon-like in appearance with a length of up to 15 mm and 10 mm in width (*19, 20*). The most common ricketsial disease in the U.S. is Rocky Mountain spotted fever, caused by the pathogen *Rickettsi rickettsiican*. It can be transmitted by the Rocky Mountain wood tick (*Dermacentor andersoni*), the brown dog tick (*Rhipicephalus sanguineus*), and the American dog tick (*Dermacentor variabilis*) (*21*). In the 2015-2016 collection, no American dog ticks were captured so no further reference to this species or the diseases they may carry will be made in the research.

Tick Disease

Ehrlichiosis

Human ehrlichiosis has two subtypes in the U.S.: human granulocytic ehrlichiosis (HGE) is caused by *Anaplasma phagocytophilum* (formerly known as *Ehrlichia phagocytophila* or *Ehrlichia equi*) and human monocytic ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis*. HGE is found in the northeastern and upper midwestern states while HME most often occurs in south-central and southeastern states. *Ehrlichia*

chaffeensis can be carried by both the dog tick as well as the Lone Star tick (*Amblyomma americanum*) (*21*).

The symptoms of ehrlichiosis are very similar to those of influenza. After seven days a patient can experience chills, cough, malaise, fever, and myalgia along with the development of a rash. HME and HGE present with similar symptoms(*21*).

Doxycline is the treatment of choice in diagnosed ehrlichiosis. Alternatives include tetracyclin or chloramphenicol. Treatment should continue three days after the fever subsides and a minimum of five to seven days until indication of clinical signs of improvement (*21*).

Lyme Disease

The most common vector borne disease in the U.S. is Lyme disease (*21*). The known strains of Lyme come from the *Borreli*a genus, such as *B. afzelii, B. garinii, and B. burgdorferi*, which are the main species found in the United States *(5).* In the United States, the foremost tick vector for Lyme disease is the blacklegged tick. Natural reservoirs for *B. burgdorferi* are the white-footed mouse and other common small mammals; however, they will also feed on livestock, pets, and humans (*21*).

Not all species of *Ixodes* have the capability to transmit disease transstadially (*5*). When a larvae or nymph feeds on an infected host, such as a white-footed mouse, with Lyme disease, it is then considered infected and can transmit the disease to humans. An infected nymph has to be attached to its next host for up to 36 to 48 hours before the risk of transmission of disease presents itself. For an infected adult, it has to be attached to a host for 48 to 72 hours or longer for the risk of disease transmission to present itself (*21*).

Symptoms of Lyme disease progress generally in three stages. The first stage begins seven to ten days after a person is bitten by an infected tick. In this stage, the patient will usually develop a rash near the site of the tick bite which can expand up to 50 cm in diameter (*21*). The patient can also experience influenza symptoms such as fevers, coughs, and headaches (*22*). In the second stage of the infection, the patient will continue to experience fevers as well as experience adenopathy and central nervous system disorders. As the patient progresses into the third stage, symptoms of chronic arthritis can occur, neurological abnormalities can form, dermatitis can develop and the central nervous system can become impaired (*21*). In chronic cases, Lyme disease has proven to be fatal (*22*).

Yet the risks of infection with *B. burgdorferi* are minimal, especially if the tick is removed before the 36-hour mark. If symptoms do arise, antibiotic treatment is typically curative. Often, doctors will prescribe oral forms of doxycycline or amoxicillin for a period of 14 to 21 days. For those patients with more chronic infections, intravenous antibiotics are usually implemented and will last for at least 30 days (*21*).

Study

Purpose and Hypothesis

The first half of the study aims to determine the most effective way to capture the ticks. Once ticks are captured, they are brought to the lab to be stored. The second half of the study consists of testing the ticks for the disease causing agents of Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis through a DNA analysis.

The head of this research project, Dr. Davis McGuirt, is still a practicing veterinarian in Virginia and has personally dealt with tick-borne disease in his patients.

Dr. McGuirt has diagnosed the presence of canine Lyme disease, anaplasmosis, and ehrlichiosis in his practice and has noted a greater number of ehrlichiosis cases over the years. However, the tests used to detect these diseases are solely antibody tests, which can only show that the canine has been exposed to a pathogen; thus, one cannot tell if the dog is currently infected with the disease. Therefore, the DNA of the ticks must be examined to give an accurate assessment of whether disease-causing agents are there.

Since this research is still in its preliminary stages, the predictions and hypotheses are being tested by just two seasons of data. The hypothesis is that the ticks that will be captured will consist of the blacklegged tick, the American dog tick, and the lone star tick. All three of these ticks are native to Virginia and thus predicted to be found on Candlers Mountain in Lynchburg. After DNA analysis of these ticks, an expected discovery of the presence of *Ehrlichia* is assumed due to the greater number of ehrlichiosis cases in the area.

Phase One: Ongoing Collection

Specific Site Location. The site where the study was conducted was near a popular hiking trail on Candlers Mountain. In McNemee's research, it was determined that ticks often congregate alongside of trails, hence the reason for the choice of location (*23*). The designated area of testing was about 30 feet away from the trail in a dried up stream-bed. This location was covered with leaf litter and surrounded with a relatively dense population of shrubbery and scattered large deciduous trees (Fig. 1).

Figure 1. Photo of the dried up stream-bed where the research site is located on Candlers Mountain, Lynchburg, Virginia.

Finding the Host. Ticks engage in a behavior called questing to attach to their hosts. Ticks will climb up blades of grass, brush, or limbs and reach out their legs and wave back and forth as a potential host approaches. Once the host brushes past the tick, it clings onto the host and quickly climbs aboard. Some ticks will wander and explore the host for an area where the skin is thin while others will attach immediately (*24*).

Collection Methods. Common capture techniques applied in tick collection are the flagging method, by CO_2 traps, and by wildlife traps. The flagging method, also known as a tick sweep, utilizes a sheet of cloth that is dragged over an environmental area of study. Ticks are attracted to vibrations and if there are ticks present in search of a host, they may cling to the cloth as it passes by. There are various types of tick-capturing

flags. A competent design is one that allows the user to stand upright while the flag still remains parallel to the ground. The flag design utilizes the surface area of the flag as much as possible, thus covering the most space in an efficient manner. The material the flag is composed of is of importance as well. A rectangular flannel rubberized-laminate fabric is utilized in studies for its ability to sweep over areas of thick and thorny vegetation without getting snagged (*25*). At one end of the flag is attached a wooden rod with a rope tied on either of the rod's ends which acts as a handle for the collector to hold onto (*10*). As the collector drags the flag, he must consistently check the flag for any attached ticks (*26*). This method is effective in capturing both immature and adult ticks (*6*).

CO_2 traps are another method that has been utilized in collecting ticks. CO_2 is an attractant to ticks as it is a byproduct of cellular respiration and thus an indication of the next bloodmeal candidate. There are a variety of ways to construct effective CO_2 traps but they all have similar key features (*27*). For these traps to work, CO_2 must be produced. The most common way to obtain CO_2 is by the sublimation of dry ice. Dry ice has a very low melting point and will sublimate into CO_2 in normal atmospheric conditions (*28*). The effectiveness of CO_2 as an attractant is based on the time duration the dry ice has been left out for, as well as the trap's distance from surrounding ticks (*26*). As the ticks are attracted to the CO_2 bait and move toward the gas, there must be a means by which the ticks are collected. A simple way to capture the incoming ticks is by tape. Tape will trap the ticks in place and thus obviate the task of capturing the ticks by hand as they come by. A possible area of concern in using CO_2 traps, however, is that they may not attract every tick species uniformly. Results with the aid of CO_2 traps show an

unusual capture rate of lone star ticks compared to deer ticks, even when in areas in which deer ticks are the predominant species (*29*). This suggests a skewed representation of the diversity of species of ticks within an area. Since different species of ticks can be carriers of different pathogens, this could misrepresent the presence, or lack thereof, of certain disease in the area of study. An additional area of concern is the stickiness of the tape itself. If the tape is not effective in holding down the legs of the moving tick, then the ticks may move on past the trap. The tapes used on a CO_2 trap should therefore be experimentally tested with a force transducer and a sample of a tick limb or a simulation of one.

A third method of collection is by wildlife trapping. This method implements possible tick host traps to collect ticks off hosts. Traps are utilized for smaller mammals such as rabbits, squirrels, voles, chipmunks, and most commonly mice. Once the animals are caught, they are put under anesthesia and examined for ticks and then released. Most often the larva and nymph stages are found on these smaller mammals. There are variations in using wildlife as a means to capture ticks. For example, deer killed in hunting season have often been examined for ticks and for presence of disease. Adult ticks are most often found on large mammals, such as the deer. Another variation of tick capture via wildlife is practiced in some veterinary clinics. When veterinarians conduct routine thorough examinations of their patients, they, at times, spot ticks that have gone undetected to their owners. These ticks are then collected for research purposes (*10*). A precaution in utilizing any of these methods is that different tick species have host preferences that may also change as they molt from one stage to another (*6*).

Method and Materials for Capture. A variety of methods were exercised to capture ticks in the attempt to create the most effective means of capture. The initial technique entailed the flagging method. In this trial, a 2 m x 1.2 m sheet of white flannel cloth fabric was used with a wooden rod attached at the end of the flag. A 1.0 m rope was attached to either ends of the rod to create a handle. The flag was dragged across the research site methodically and raised every few minutes to look for any ticks that happened to cling to the passing sheet. This method was discarded early on in the study as it presented a number of problems. The flag frequently got snagged on the underbrush of the testing site, which presented issues in the general sweeping method. Another problem was that ticks would not affix themselves onto the flag. In certain instances, ticks that had grabbed the flag would fall off shortly before the flag was raised up for inspection. This presented the problem of missing potential ticks as well as a misrepresentation of numbers. This lead to the conclusion that the flagging was not an effective mode of capture.

The second method introduced was the use of a CO_2 trap. This trap was composed of a Styrofoam cooler with approximate dimensions of 45 cm long, 30.5 cm wide, and 30.5 cm tall. Two 1.3 cm diameter exhaust holes to allow the carbon dioxide to be released, were punctured towards the base of each wall of the cooler. Inside the container was placed a small amount of dried ice. A wire cage was placed inside the cooler so that the dry ice would not block these exhaust holes. The foam container was placed on top of a plastic platform lined with an adhesive. The tapes that were tested on the plastic lids included Gorilla duct tape, clear packaging tape, and double-sided carpet tape. Two traps

were prepared and set out together (with a rock placed on top of the lids to weigh the traps down) and placed approximately three to seven meters apart (Fig. 2).

Figure 2. The original CO_2 trap used in the 2015 tick collection.

The traps were left over night and checked the next day. If ticks had been captured, they were removed from the tape with tweezers and placed in sealed plastic bags. The bags were dated and the number of ticks was noted on the bag. The bagged ticks were brought back to the lab where each one was identified for its gender, age, and species with a microscope. The ticks were then placed in a -80°F (-26.7°C) cooler to remain preserved for future DNA analysis work.

Force Transducer. Various tapes were experimentally tested in the lab using a force transducer to test the strengths and stickiness of different tapes; this was done to identify the most effective tapes in capture methods and to decrease the chances of captured ticks escaping. In the lab, a simulation of a tick leg was created by using a thin

wire with a hooked end. The simulated tick leg was placed against Pic Window Fly Traps tape, strings of Pic Fly Ribbon tape, yellow lab tape, duct tape, and clear packing tape and the force needed to pull the leg off was measured by the force transducer in grams of pressure. After analysis of the data, it was determined that yellow lab tape was the stickiest and strongest, with the duct tape coming in second and the clear packaging tape being the least effective. Although this was a good measure of a tape's relative strength and stickiness, the accuracy of these data also depended on each tapes' ability to maintain its stickiness and strength when subjected to such environmental variables as rain, humidity, and wind. It was decided to utilize duct tape as opposed to the yellow lab tape. Their reasoning behind this decision was that the lab tape was narrower than the duct tape, making it hard to create a fence around the platform of the CO_2 trap. Additionally, duct tape is known for its resilience even when rained on, since it is an adhesive that can be used outdoors. Even though lab tape was stronger and stickier, it was believed that the duct tape would be the most effective method to employ.

Results and Discussion of Capture. Not every time the CO_2 traps were set out did it result in a tick capture; nonetheless, the results of successful tick captures are as follows: The 2015 tick collection was conducted from March to June of 2015. On March 13, one adult male lone star tick was captured. On March 23, one adult male and one adult female lone star tick were captured, along with one adult female blacklegged tick. On April 5, five adult male lone star ticks, seven adult female lone star ticks, and one adult female blacklegged tick were captured. The next outing was peculiar in that on April 22, while the CO_2 traps were being prepped, it was observed that ticks were immediately coming out of the leaf litter and seen crawling toward the attractant. Three

adult male lone star ticks and one adult female lone star tick were caught. The largest collection occurred May 14, resulting in 15 adult male and 20 adult female lone star ticks, one blacklegged tick, and five unidentified nymphs (Table 1).

Table 1. Total ticks caught in 2015-2016.

Total Ticks Caught

Date of capture	3/13/15	4/5/15	4/22/15	5/14/15	3/?/16	4/1/16	4/29/16	5/25/16	6/14/16	8/30/16-9/1/16
Adult Male Lone Star	1	5	3	15		2		3	4	
Adult Female Lone Star	1	7	1	20	2	2	1	6	2	
Nymph Lone Star								2		9
Larvae Lone Star										22
Adult Male Blacklegged										
Adult Female Blacklegged	1	1		1						
Nymph Blacklegged										
Larvae Blacklegged										
Adult Male Dog										
Adult Female Dog										
Nymph Dog										
Larvae Dog										
Unknown nymph				5						

The 2016 tick collection was conducted from March to September 2016. For an unknown reason the exact date of the first March 2016 outing was not recorded; nevertheless, two adult female lone star ticks were caught. On April 1, two of each adult male and female lone star ticks were captured. The March 29 outing resulted in only one adult female lone star tick. May 25 was the largest capture of adult ticks: three male lone star ticks, six adult female lone star ticks and one lone star nymph. On June 14, the traps apprehended four adult male lone stars and two adult female lone stars. Due to a separate tick study, the final outing lasted over a period of two days, from August 30 to September

1. Over these several days, nine nymph lone star ticks and 22 larvae lone star ticks were captured (Table 2).

Table 2. Comparison of adult ticks captured in 2015-2016.

Total Ticks Caught by Year			
Year of Capture	**2015**	**2016**	**2015-2016**
Total Adult Male Lone Star	24	9	33
Total Adult Female Lone Star	29	13	42
Total Adult Male Blacklegged	0	0	0
Total Adult Female Blacklegged	3	0	3
Total Adult Ticks	**56**	**22**	**78**

From the data collected it can be observed that the traps caught mostly lone star ticks with a few blacklegged tick captures and no American dog tick captures (Fig. 3).

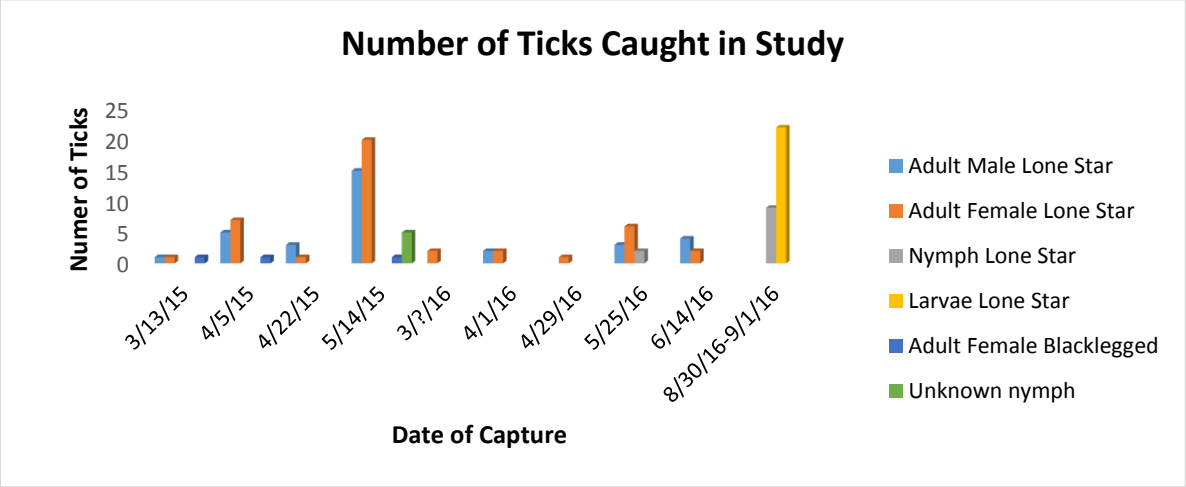

Figure 3. Graph of the number of total ticks and life stages caught in 2015-2016.

There is an interesting peak in the larvae and nymph lone star ticks caught on the last 2016 outing. The similarity in the data occurs in the month of May, when the most adult male and female lone star ticks were captured in both 2015 and 2016.

Figure 4 shows that more adult female lone star ticks were captured over the two years than adult male lone star ticks.

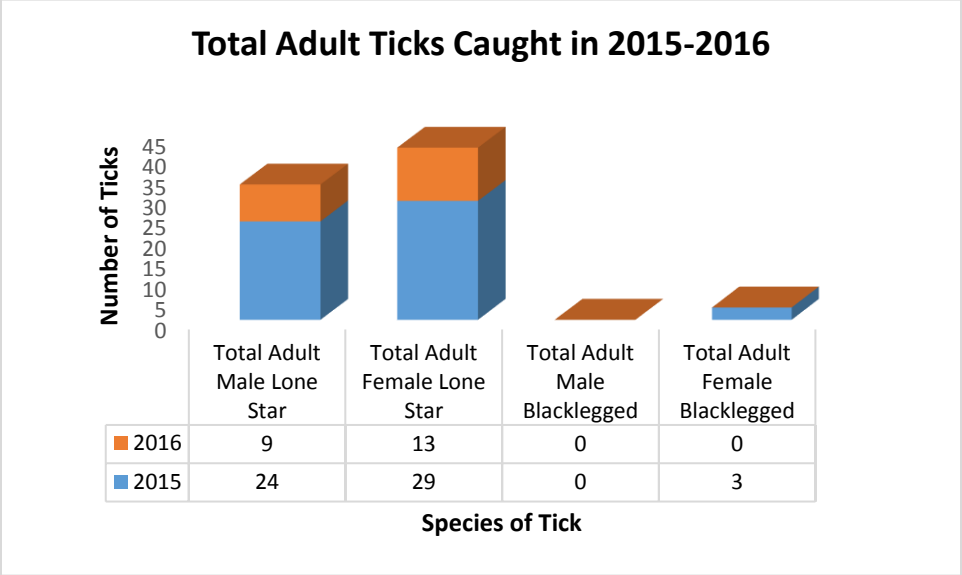

Figure 4. Chart and graph comparison of the total number of adult lone star and blacklegged ticks caught in 2015-2016.

The data also show that in 2015, a total of 56 adult lone star ticks were captured, while in 2016, only 22 were caught. This represents a 60.71% decrease in total lone star tick capture, which is noteworthy since the 2016 capture was conducted over a period of roughly five months, while the 2015 capture was conducted for only three months. The discrepancies in overall data could be due several separate issues. The first and most obvious one is that the months and days of capture in 2015 and 2016 do not coincide with one another. Ticks are seasonal arthropods; thus, they will experience peaks in activity throughout the year as well as a spike in numbers after mating season. Weather is another big contributor to the activity of ticks. Tick activity can be affected by the relative humidity, temperature, soil temperature, precipitation, and sun duration (*30-32*).

Another consideration is the relative aggressiveness of each individual tick species in the study. Lone star ticks are known for being aggressive hunters and will move much faster than other ticks in pursuit of their hosts. The American dog is also

known to be fast moving, while the blacklegged tick is less likely to crawl horizontal distances for any length of time (*6, 26*).

There is always the possibility that ticks may struggle to locate a host as well. If there is a shortage of host candidates, whether because of a fatal disease epidemic or a reduced fecundity in the host population, the chances of a tick locating a host are reduced. Since ticks are obligate blood feeders, in order to mature and molt into each new life stage they must obtain a blood meal. If they are unable to find one, the ticks will die.

The actual trap could have caused differences in data since a smaller sized trap was used in 2016 than in 2015. This change could have reduced the levels of CO_2 being expelled from the traps since smaller portions of dry ice could fit inside these containers. Furthermore, altering the way in which the trap was set with tape along the side as opposed to adhering to the top of the platform may have deterred ticks from crawling up the side of the tape to reach the CO_2; however, this is unlikely due to the aggressive behavior of ticks.

An alternative explanation for the difference in number could be due to the fact that dozens of ticks were removed from the research site. These changes could have impacted the dynamics of the tick population, which could have reflected in our results and been the reason behind why less ticks were caught in 2016.

When the duct tape was used, even after experiencing environmental rainfall and humidity, the tape maintained its resilience. After several days in the field, the duct tape remained sticky and continued to trap ticks attracted to the CO_2. Thus, all further collections were conducted with duct tape on the traps. The field-collection was conducted in the years 2015-2016 and, as an ongoing project, will continue into 2017. As

the study progressed, the tape was replaced as it lost its stickiness and became less effective due to weathering outside. A switch to the use of smaller Styrofoam containers (roughly 30 cm long, 24 cm wide, and 24 cm tall) was made in 2016, in order to make the traps easier and less awkward to carry. After noticing that unwanted debris and passing insects were being caught on the tape, another change was made to the design of the trap. By attaching the tape standing up alongside the perimeter of the plastic lid in lieu of placing it on top of the lid, a fence of sorts was created onto which ticks were still able to crawl up and get stuck but blocked other passing insects and blowing leaves from attaching.

Phase Two: Ongoing DNA Analysis

Preparing Ticks for DNA Analysis. Once the ticks have been captured, analyses did commence. It is necessary to extract both DNA and RNA from ticks to analyze protozoan, bacterial, and viral pathogens (*7*). However, before any testing could commence, the ticks must be sterilized to rid any foreign containments found on the outside of the tick which could affect results; a bath of 70 percent ethanol and water is most often used as a sterilizing agent (*33*). Once the ticks were sterilized, the hard exoskeleton must be removed in order to obtain tissue that can be crushed.

DNA Analysis. Once the softer tissue was exposed, the process of extracting the DNA proceeded. There are a variety of ways in which to extract tick DNA to analyze it for the presence of diseases. The utilization of a Thermo kit is recommended for high DNA yields along with bead beating and cutting the ticks before DNA extraction (*34*). Other methods include the use of proteinase K digestion followed by a commercial kit. This kit successfully extracts about 77% of tick DNA. The mortar crushing method,

followed by proteinase K digestion and then phenol/chloroformextraction, gives a 97% extraction success. While using fine crushing along with bead beaters, a proteinase K digestion and an implemented commercial kit can give a 100% DNA extraction success (*35*).

When the DNA had been extracted, the process of specific DNA sequence isolation began. Every organism has a unique DNA sequence that can be used to distinguish it from other species (*36*). This is also true for pathogens such as those that cause Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis in humans. Since these diseases can be caused by a number of different genera and species, a genus specific primer sequence was utilized. A genus specific primer sequence could identify the possible presence of the desired pathogen (*37*).

After the desired DNA sequence was isolated, the strain was amplified using polymerase chain reaction (PCR) tactics (*38*). A high-quality PCR amplification test will provide the best amplified sequence. To further improve the results of PCR, one can remove any possible PCR inhibitors such as the presence of mammalian blood found in extracted tick DNA (*39*). The removal of reproductive organs can also reduce PCR inhibition (*40*). The amplified DNA was then run on a gel electrophoresis against a positive and negative control for the presence of a certain disease.

Method and Materials of DNA Analysis. The second phase of the experiment is still ongoing but will be introduced and the subsequent steps explained. It is important to take precautionary measures when handling these ticks in the lab since they are potential carriers of harmful pathogens. Safety protocol was used when handling the ticks, such as constantly wearing gloves and proper lab attire at all times. When the ticks were

collected, they were placed in separate plastic bags according to date and species and then placed in -26.7°C cooler. In the lab, this separation should be rigorously maintained and can be further reinforced through the division by gender of each tick. Before experimentation, all ticks were placed in a 70% ethanol solution for five minutes to remove any surface pathogens that could interfere with the results of the experiment. After the ticks were done soaking, they were removed and placed on a paper towel to air dry. Frozen ticks must be allowed to thaw to room temperature while taking care to avoid refreezing afterwards as this can lead to reduced DNA size.

Once the ticks dried, DNA preparation followed. The materials needed to complete the DNA purification include: the DNeasy Blood & Tissue Kit, pipets and pipet tips, scalpel, vortex, microcentrifuge tubes (1.5 ml or 2ml), microcentrifuge with rotor for 1.5 ml and 2 ml tubes, a rocking platform for heating at 56°C and 70°C, ethanol (96-100%), and carrier RNA solution. A DNeasy Blood & Tissue Kit was used to isolate the tick DNA and followed the procedure found in the QIAGEN Supplementary Protocol (*41*). The purified tick DNA was then labeled and could be stored at -20°C if needed. To determine the amount of DNA purified and its relative level of purity, a nanodrop machine with an elution buffer was implemented.

The next step in the process was to prepare the DNA for PCR. To begin the PCR set up, though, it is necessary to obtain the proper primers necessary to test the tick DNA for the pathogens that cause Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis. The DNA sequence for *Borellia burgdorferi*, the disease causing agent of Lyme disease is indicated in Figure 7.

ATGATTATCAATCATAATACATCAGCTATTAATGCTTCAAGAAATAATGGCATTAA
CGCTGCTAATCTTAGTAAAACTCAAGAAAAGCTTTCTAGTGGGTACAGAATTAAT
CGAGCTTCTGATGATGCTGCTGGCATGGGAGTTTCTGGTAAGATTAATGCTCAAA
TAAGAGGTTTGTCACAAGCTTCTAGAAATACTTCAAAGGCTATTAATTTTATTCAG
ACAACAGAAGGGAATTTAAATGAAGTAGAAAAAGTCTTAGTAAGAATGAAGGA
ATTGGCAGTTCAATCAGGTAACGGCACATATTCAGATGCAGACAGAGGTTCTATA
CAAATTGAAATAGAGCAACTTACAGACGAAATTAATAGAATTGCTGATCAAGCTC
AATATAACCAAATGCACATGTTATCAAACAAATCTGCTTCTCAAAATGTAAGAAC
AGCTGAAGAGCTTGGAATGCAGCCTGCAAAAATTAACACACCAGCATCGCTTTCA
GGGTCTCAAGCGTCTTGGACTTTAAGAGTTCATGTTGGAGCAAACCAAGATGAA
GCTATTGCTGTAAATATTTATGCAGCTAATGTTGCAAATCTTTTCTCTGGTGAGGG
AGCTCAAACTGCTCAGGCTGCACCGGTTCAAGAGGGTGTTCAACAGGAAGGAGC
TCAACAGCCAGCACCTGCTACAGCACCTTCTCAAGGCGGAGTTAATTCTCCTGTTA
ATGTTACAACTACAGTTGATGCTAATACATCACTTGCTAAAATTGAAAATGCTATT
AGAATGATAAGTGATCAAAGAGCAAATTTAGGTGCTTTCCAAAATAGACTTGAAT
CTATAAAGGATAGTACTGAGTATGCAATTGAAAATCTAAAAGCATCTTATGCTCA
AATAAAAGATGCTACAATGACAGATGAGGTTGTAGCAGCAACAACTAATAGTAT
TTTAACACAATCTGCAATGGCAATGATTGCGCAGGCTAATCAAGTTCCCCAATAT
GTTTTGTCAT

Figure 7. DNA sequence of *Borellia burgdorferi* (*42*)

The DNA sequence for *Rickettsi rickettsiican*, the disease-causing agent of Rocky Mountain Spotted Fever is as shown in Figure 8.

ATCAAAAAAATTTATAAATCGTAATTCATTTCTAATTTCCTAAAATTAGGGTTATA
CTGACTGAATGAGGTATATACATTATGACTAATGGCAATAATAATAACTTAGAAT
TTGCAGAATTAAAAATTAGAGGTAAACTATTTAAGTTACCTATACTTAAAGCAAG
TATCGGTAAAGATGTAATCGATATAAGTAGGGTATCTGCGGAAGCCGATTACTTT
ACTTATGATCCGGGTTTTATGTCTACTGCTTCTTGTCAATCTACTATCACATATATA
GACGGTGATAAAGGCATATTATGGTATCGAGGATATGATATTAAAGACTTAGCT
GAGAAAAGTGATTTTTTAGAAGTGGCATATTTGATGATTTATGGGGAGCTACCAA
GTAGTGATCAGTATTGTAATTTTACTAAAAAGGTTGCTCATCATTCATTAGTGAAT
GAAAGATTACACTATTTATTTCAAACCTTTTGTAGTTCTTCTCATCCTATGGCTATT
ATGCTTGCAGCTGTTGGTTCTCTTTCAGCATTCTATCCTGATTTATTAAATTTTAAT
GAAACAGACTATGAACTTACCGCTATTAGAATGATTGCTAAGATACCTACTATCG
CTGCAATGTCTTATAAATATTCTATAGGGCAACCGTTTATTTATCCTGATAATTCAT
TAGATTTTACCGAAAATTTTCTACATATGATGTTTGCAACTCCTTGTACTAAATATA
AAGTAAATCCAATAATAAAAAATGCTCTTAATAAGATATTTATCTTACATGCAGAC
CATGAGCAGAATGCTTCTACTTCAACAGTTCGGATTGCTGGCTCATCAGGAGCTA
ATCCTTTTGCATGTATTAGCACTGGTATTGCATCACTTTGGGGGCCTGCTCACGGC
GGGGCTAATGAAGCAGTGATAAATATGCTTAAAGAAATTGGCAGTTCTGAGAAT
ATTCCTAAATATGTAGCTAAAGCTAAAGATAAGAATGATCCATTTAGGTTAATGG
GTTTTGGTCATCGAGTATATAAAAGCTATGACCCGCGTGCCGCAGTACTTAAAGA
AACTTGTAAAGAAGTATTAAATGAATTAGGTCAGTTAGACAATAATCCGCTGTTA
CAAATAGCAATAGAACTTGAAGCTCTCGCTCTTAAAGATGAATATTTTATTGAAA
GAAAATTATATCCAAATGTTGATTTTTATTCAGGCATTATCTATAAAGCTATGGGT
ATACCGTCGCAAATGTTCACTGTACTTTTTGCAATAGCAAGAACCGTAGGTTGGA
TGGCACAATGGAAAGAAATGCACGAAGATCCTGAACAAAAAATCAGTAGACCTA
GACAGCTTTACACTGGTTATGTACATAGAGAGTATAAGTGTATTGTAGAAAGAAA
GTGACGCATTAAAATTGTATGCATTAGCAATTTAGTGATTTAGATGAAGCATTAA
GAAAATCTAATTAATTCATCT

Figure 8. The DNA sequence of *Rickettsi rickettsiican* (*43*).

The DNA sequence for *Ehrlichia chaffeensis*, the disease-causing agent of ehrlichiosis is indicated in Figure 9.

ATGCTAAGGATTCTATTTTTATTAAGCTTAGTAATACTAGTGGCAAGTTTTCCACT
AATAAATAACTGGTTATCTAATAAATCTGGTAAGCCTATAGTAGATAAAGATACA
ATTATTGCAATTATTGAAGAATATATATCAAATTACCCTCAAAAAGTGATAGATCT
GCTCACCAAAGGGCAAGTGCGGGCAGAGAATGAAGAAATGAGTCAAAACATAA
AAAAATACAAATCTGAATTGGAAAATACTTCATATCCTTCAGCTGGAAATAAAGA
TAGTAAAATAGTATTTGTAGAGTTCTTTGATTACTCATGCGGCTATTGCAAAATGA
TGTCTGAAGATATGAAACAAATAGTACAAGACGGTAAAGTGCATGTTATATTCAG
AGATTTTCCAATACTTGGTGAGTCTTCACTCAAAGTTGCCCAAGCAGCACTAGCT
GTACATATGATTAATCCAAATAAGTACATAGACTTCTATTATGCAGCACTACATTA
CAAGCAACAGTTTAATGATGAGTCAATATTAAGTATCATAAAATCAATAGGTATA
ACTGAAGAAGACTTCAAAGTATCATTAGCAAAAAATGCTGATGCTATAGACAAA
ATGATACAATCTACCAGAGAACTAGCACAGAACATTAATATAAGGGGCACTCCTG
CTATCATAGTAGGGGATACATTTATCGGTGGTGCAGCTGATATATCAACTTTAAG
AAGTAAAATAGATATGCAGAAATAA

Figure 9. The DNA sequence of *Ehrlichia chaffeensis (44).*

Each DNA sequence was then plugged into Primer3, an online tool used to design primers for PCR experiments (*45*). When the sequence was entered, adjustments could be made to the general parameters, sequence settings, and internal oligio parameters to get a primers sequence that was genus specific to the pathogen and the needed size to be able to run on the PCR machine. Once the forward and reverse primers were determined, these six primers were ordered from Integrated DNA Technologies, a supplier of custom made nucleic acids used for research (*46*). These primers have to be made into a working stock. Whenever the primers were dealt with, they were placed in ice to keep the DNA from denaturing. The work bench has to be sterilized with 70% ethanol as well. A 50 ml pipette was implemented to place sterile (Dnase and Rnase free) H_2O into a 50 ml tube. With a 1000 μl pipette the following was added to six individual 1.5 ml micro centrifuge

tubes in a 1:10 ratio with the sterile H_2O: 319 µl of *Rickettsia* primer forward, 298 µl *Rickettsia* primer reverse, 322 µl, *Ehrlichia* primer forward, 383 µl *Ehrlichia* primer reverse, 325 µl *Borrelia* primer forward, 369 µl *Borrelia* primer reverse. To prepare the primers for PCR, three PCR tubes were prepared with 12.5 µl Taq Red, 1µl of a forward primer, 1µl of the reverse primer to that same pathogen, 1µl 100mg/µl gDNA, and 25µl of sterile H_2O. The PCR followed the following protocol: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, and then a final extension at 72 °C for 7 min (*34*). A 1.5% agarose with added EtBr for the electrophoresis was prepared and the proper DNA ladder for comparison loaded. The electrophoresis was loaded with the three samples and allowed to run at 80V for 1.5 hours. When completed, the power was turned off, electrodes disconnected, and the gel was carefully removed. A UV light was used to detect the DNA fragments on the gel (*47*).

Discussion of DNA Analysis. The project is still ongoing and this second phase of research is in its preliminary stages. There is currently no lab data to report.

Conclusion

In general, it appears that the use of the CO_2 traps was effective in capturing both adult female blacklegged ticks and lone star larva, nymphs, and female and male adult ticks in the specific research site on Candlers Mountain in Lynchburg, Virginia. Nevertheless, other methods should be implemented in a field collection since there was an observable tendency to exclusively capture lone star ticks than other species of ticks. The duct tape used for the traps worked efficiently and effectively in the study; however, the way the tape is attached to the platform should enjoy more extensive experimentation

since this could change the number of ticks being captured. For a better comparison of data year to year, the traps should be set out on the exact dates as the previous years. When the DNA analysis of these ticks is launched and there is data to be analyzed, a statement of the presence of disease can be made. Once there is an understanding of the state of disease presence on Candlers Mountain, a plan can be made for those who visit the mountain to protect them and their pets from getting infected. If in fact disease is present, locals will be much more likely to take precautionary measures to avoid chance encounters with diseased ticks. There is a possibility that the pathogens that cause Lyme disease, Rocky Mountain spotted fever, and ehrlichiosis are present in Lynchburg, Virginia since their vectors, the blacklegged tick, the lone star tick, and the American dog tick, all inhabit Virginia. This research was presented at Liberty University's Illuminate Research Symposium as well as Virginia Academy of Science at the University of Mary Washington (Fig. 10).

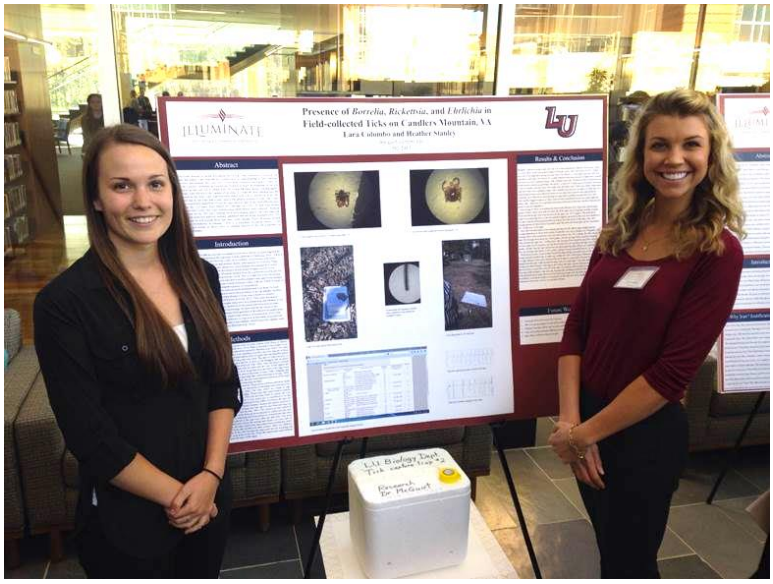

Figure 10. Photo of the co-researchers (Heather Stanley to the left and Lara Colombo to the right) presenting the tick collection data from year 2015 at Liberty University's Illuminate Research Symposium.

During the presentation of this research, many questions arose regarding the trending topic of the meat allergy which can be carried by the lone star tick and which is specifically triggered by the sugar alpha-gal. When an infected lone star bites, its saliva, containing this sugar, is injected into the host. The human body recognizes this as a foreign body and will synthesize antibodies to kill the threat. However, this specific sugar is found in all mammals except for apes and humans. As a result, when the infected person introduces red meats, such as pork and beef, into their body, they have an allergic reaction. The person's IgE antibodies bind to the allergen, causing a massive amount of histamine and other chemicals to be released (*48*). The body at this point has become sensitive to alpha-gal and will overact anytime it is found. Due to the expandability of this project, the decision was made to pursue this new avenue of study into the allergy produced by a tick bite. Significant efforts will be made in researching and testing ticks caught on Candlers Mountain for the presence of the sugar allergen.

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