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Determining the Prevalence and Distribution of Tick-Borne Pathogens in Southeastern Virginia and Exploring the Transmission Dynamics of Rickettsia Parkeri in Amblyomma Maculatum

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DETERMINING THE PREVALENCE AND DISTRIBUTION OF TICK-BORNE PATHOGENS IN SOUTHEASTERN VIRGINIA AND EXPLORING THE TRANSMISSION DYNAMICS OF *RICKETTSIA PARKERI* IN *AMBLYOMMA MACULATUM*

by

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ABSTRACT

DETERMINING THE PREVALENCE AND DISTRIBUTION OF TICK-BORNE PATHOGENS IN SOUTHEASTERN VIRGINIA AND EXPLORING THE TRANSMISSION DYNAMICS OF *RICKETTSIA PARKERI* IN *AMBLYOMMA MACULATUM*

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Old Dominion University, 2015
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Tick-borne pathogens are an increasing threat to human and animal health worldwide. In the United States, cases of Lyme disease, spotted fever rickettsioses, ehrlichiosis and anaplasmosis are on the rise. Factors related to emergence include appearance of new pathogens, recognition of existing pathogens and environmental changes that result in new exposure events. Despite the rise in tick-borne disease incidence within many states, including Virginia, there is a paucity of data related to the prevalence and distribution of ticks and tick-borne pathogens.

The first aim of this dissertation research was to determine the tick-borne pathogen composition within tick populations in southeastern Virginia. Since 2009, the vector ecology laboratory at Old Dominion University has been conducting year-round surveillance of tick populations within Hampton Roads. This research explores the pathogen composition within these tick populations, with a particular focus on emerging pathogens, including *Rickettsia parkeri* and *Ehrlichia chaffeensis*.

The second aim of this research was to determine the transmission dynamics of *R. parkeri* within its vector, *Amblyomma maculatum*. Although some rickettsiae are transovarially transmitted in ticks, little is known about the frequency and efficiency of

this transmission route, and nothing is known regarding the transmission strategy of *R. parkeri* in *A. maculatum*. By understanding the dynamics of pathogen transmission within the tick, a broader knowledge of the disease system can be attained, and mathematical models to explore these dynamics can be parameterized.

The third aim of this research was to explore the potential for *R. parkeri* to spill over from *A. maculatum* populations into populations of *A. americanum*. *Amblyomma americanum* is an aggressive human-biting tick, represents 95% of the ticks encountered in southeastern Virginia, and is the most common tick found attached to humans in the southeastern and mid-Atlantic United States. Because of its common association with humans, *A. americanum* and the pathogens it transmits are important threats to human health in southeastern states. The competence of *A. americanum* as a vector of *R. parkeri* was also investigated.

This dissertation is dedicated to my husband, Dexter, for his enduring support, encouragement and friendship. Also to Mom, Dad and Rachael, all of whom have provided me support, laughter and fortitude.

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INTRODUCTION AND BACKGROUND

Vector-borne diseases are an important threat to human health worldwide. Diseases such as malaria and the reemergence of dengue fever and yellow fever in certain areas have had a major influence on human health, killing over 1.2 million people annually (WHO, 2015). Controlling vector-borne diseases poses a daunting task to scientists and health officials as many of these diseases operate primarily within a sylvatic (non-human) cycle, where humans and domestic animals are merely incidental hosts. The enzootic cycles of vector-borne disease agents are often complex, requiring both invertebrate vectors and vertebrate hosts, each of which has its own suite of ecological specifications required for survival.

Ticks are hematophagous, ectoparasitic arthropods which rely on multiple vertebrate bloodmeals to complete their life cycle and reproduce. In the United States ticks are the most common agents of vector-borne pathogens and worldwide are second only to mosquitoes as arthropod vectors of pathogens (Goodman et al., 2005). Ticks transmit a variety of pathogens including bacteria, viruses and protozoa, and tick-borne diseases are increasingly being recognized as threats to human health worldwide. Lyme disease and tularemia are two severe illnesses caused by tick-transmitted pathogens in both the New and Old Worlds (Gubler, 1998). In the United States, Lyme disease and spotted fever rickettsioses cases have been on the rise; in addition infections such as ehrlichiosis and anaplasmosis, caused by the emerging tick-borne pathogens *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum*, respectively, have also increased (Dahlgren et al., 2011). Factors affecting emergence of these pathogens

include the recognition of these organisms as pathogens and environmental changes that lead to or result in new exposure events (Lederberg et al., 1992). *Ehrlichia chaffeensis* and *Rickettsia parkeri*, the bacterial causative agents of ehrlichiosis and Tidewater spotted fever, respectively, are two emerging tick-borne pathogens associated with human disease in the southeastern United States.

Ticks also have a long history as significant pests of livestock and pose a severe threat to the global cattle industry. The cattle tick, *Rhipicephalus sanguineus*, is estimated to have an annual economic impact of $2 billion on the livestock industry in Brazil (Grisi et al., 2002). While ticks parasitizing livestock impose indirect effects, such as disease caused by multiple bacterial and parasitic agents, the direct effects of tick attachment are also important. The Gulf Coast tick, *Amblyomma maculatum* Koch, which has long been known as a pest of cattle in the United States, inflicts damage to the skin during feeding leading to inflammation, edema and secondary infections that can have permanent health effects (Williams et al., 1978; Sonenshine et al., 1991; Teel et al., 2010).

In Virginia, ticks associated with human disease include of members of the Ixodidae (hard-bodied) family. Most Ixodidae have three life stages in which they actively quest for a host: larva, nymph and adult. Ixodidae associated with human pathogens in Virginia include *Amblyomma americanum* (L.), *Dermacentor variabilis* Say and *Ixodes scapularis* Say. In 2011, populations of other disease-relevant species, *A. maculatum* and *Ixodes affinis* Neumann, were described in Virginia for the first time (Wright et al., 2011; Nadolny et al., 2011). Although *I. affinis* is not known to feed on

humans, it is thought to be important in the enzootic maintenance of *Borrelia burgdorferi* sensu stricto, the agent of Lyme disease (Oliver et al., 1996).

The research presented in this dissertation will primarily focus on bacterial tick-borne pathogens belonging to the order Rickettsiales. Members of Rickettsiales belong to the α-subdivision of the Proteobacteria and include the family rickettsiaceae, in which the genus *Rickettsia* is located. Interestingly, rickettsiae are the closest extant relatives of mitochondria and have been the subject of several studies assessing the history of organelles (Andersson et al., 1998; Emelyanov, 2001; Fitzpatrick et al., 2006). Another family belonging to the Rickettsiales order is Anaplasmataceae, which includes the genera *Anaplasma*, *Ehrlichia*, *Wolbachia* and *Neorickettsia* (Paddock et al., 2003; Dumler et al., 2001). A common theme among the Rickettsiales is the necessity for intracellular replication (Dumler et al., 2001).

The *Rickettsia* genus consists of four groups. The typhus group (TG) includes *Rickettsia prowazekii*, the agent of louse-borne epidemic typhus, and *Rickettsia typhi*, the agent of flea-borne murine typhus. From 1917 to 1923, *R. prowazekii* is thought to have caused nearly 3 million deaths in Russia during the revolutionary war (Zinsser et al., 1963) and a century before that, epidemic typhus in soldiers was likely a major factor contributing to Napoleon's defeat in Russia (Raoult et al., 2006). The spotted fever group (SFG) contains highly pathogenic members, such as *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), which is capable of causing a characteristic spotted rash during human infections. In 2004, Rocky Mountain spotted fever reached its highest incidence in the U.S. (Dumler and Walker, 2005). Although the

incidence of RMSF has increased in recent decades, the case fatality rate has dramatically decreased; a potential reason for this is that other less-pathogenic SFGR are misdiagnosed as RMSF. Both the TG and the SFG rickettsiae are exclusively carried by and transmitted by blood-feeding arthropods (Perlman et al., 2006). Two other rickettsial groups are the ancestral group and transitional group, formed on the basis of present day phylogenetic studies (Gillespie et al., 2007; Sonenshine and Roe, 2013; Stothard et al., 1994; Vitorino et al., 2007). In addition to being carried by hematophagous arthropods such as ticks, fleas and lice, rickettsiae are also known to utilize other hosts, such as beetles, flies and wasps, as well as non-arthropods such as leeches and amoebae (Perlman et al., 2006). Rickettsiae often appear to have a long, symbiotic history with their hosts; some species also appear to cause reproductive manipulation such as male-killing and parthenogenesis (Perlman et al., 2006).

The primary focus of this research was to explore the distribution and transmission dynamics of members of the SFGR, in addition to determining the prevalence and distribution of *Ehrlichia* species present in Virginia tick populations.

Spotted Fever Group Rickettsiae. The SFGR consists of over 20 distinct species and subspecies, some of which cause infections in humans and are distributed worldwide. The prototypical SFGR, *R. rickettsii*, has caused significant mortality over the past century (Sonenshine and Roe, 2013). Since the early 1900s, when Howard Ricketts first suggested the etiology of RMSF to be tick-borne, improvements in molecular techniques for determining phylogeny have resulted in a much broader knowledge of the diversity of the SFGR (McDade and Newhouse, 1986). Approximately 75% of human

diseases caused by the SFGR were discovered after 1984, highlighting the importance of molecular techniques such as PCR in the discovery of these disease-causing agents (Parola et al., 2005).

The SFGR are found on multiple continents and appear to exclusively be transmitted by hard ticks (Ixodidae). Although SFGR have occasionally been detected in soft ticks (Argasidae), there is no evidence that soft ticks are capable of transmitting these pathogenic rickettsiae to humans (Sonenshine and Roe, 2013). In addition to the pathogenic members of the SFGR, multiple SFGR species having no established pathogenicity to humans have been discovered in the Americas; these include *R. amblyommii*, *Candidatus* R. andeanae, *R. peacockii* and *R. rhipicephali*. Many of these organisms of undetermined pathogenicity are found in tick species which frequently bite humans (Sonenshine and Roe, 2013).

An uncommon trait among pathogenic tick-borne bacteria found in the SFGR is the ability to be transmitted transovarially within tick populations, from females to offspring. Certain SFGR are vertically transmitted, including *R. rickettsii* (Burgdorfer and Brinton, 1975), *R. montanensis*, and *R. rhipicephali* (Niebylski et al., 1999). Interestingly, each of these organisms has detrimental effects on tick survival and reproduction in laboratory colonies. The capacity for transovarial transmission likely stems from the long history of *Rickettsia* as arthropod endosymbionts (Perlman et al., 2006) and may lessen the dependence of some SFGR on vertebrate hosts for transmission, thus ensuring their persistence in tick populations.

The lone star tick, *Amblyomma americanum*. Commonly known as the lone star tick, *A. americanum* was first described by Carl Linnaeus in 1758. *Amblyomma americanum* is a three-host, non-nidiculous tick with all three life stages (larva, nymph and adult) known to seek out large vertebrate hosts, including humans. Currently, the distribution of *A. americanum* spans much of the southeastern and mid-Atlantic portion of United States (Fig. 1) and has been expanding northward, with populations reported as far north as New York, Connecticut and Rhode Island (Ginsberg et al., 2002; Ijdo et al., 2000). During the warmer months *A. americanum* can be found in various habitat types, predominantly in the near vicinity of woodland areas. Habitats that are well-suited for white-tailed deer (*Odocoileus virginianus*) are often areas where *A. americanum* are

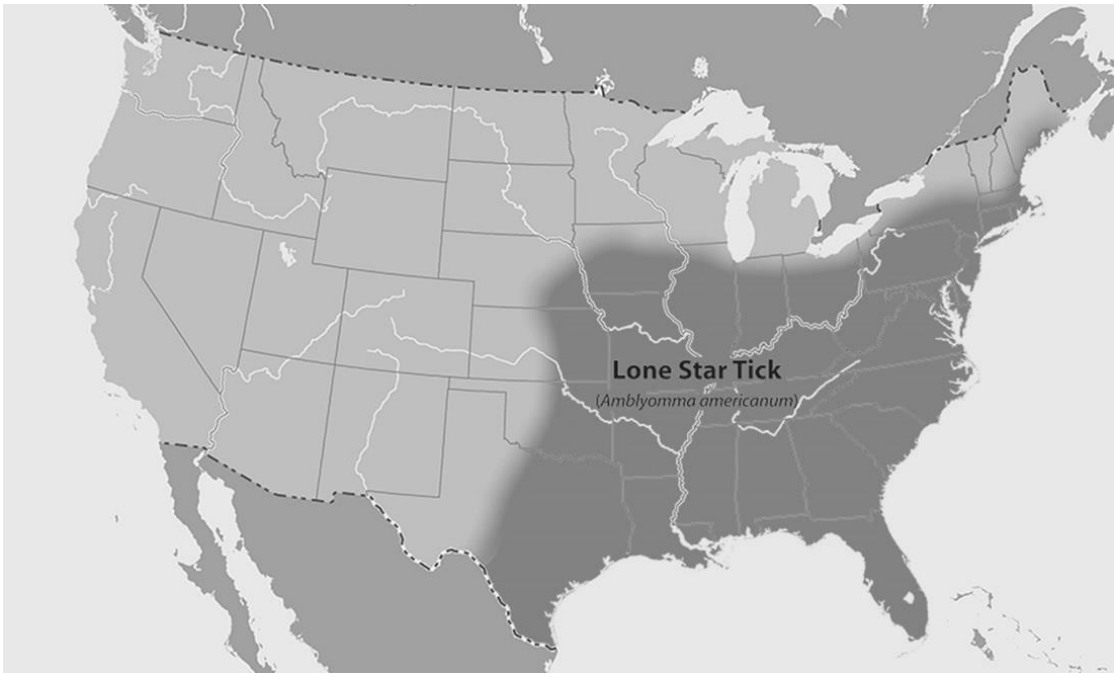

Fig. 1. Distribution of the lone star tick, *Amblyomma americanum*, in the United States. Figure adapted from CDC website (http://www.cdc.gov/ticks/geographic_distribution.html, accessed June 10th, 2015).

encountered (Semtner et al., 1971), likely due to the proclivity of *A. americanum* to utilize white-tailed deer as a host. These habitat types include areas of woody vegetation, particularly along the woods' edge (Bartlett, 1938), and brushy rangeland (Semtner et al., 1971).

In the southeastern states *A. americanum* is the most abundant human-biting tick encountered (Stromdahl and Hickling, 2012; Merten and Durden, 2000; Nadolny et al., 2014), representing approximately 95% of ticks collected on flags in Virginia (Nadolny et al., 2014); and 70-95% of ticks collected from humans residing in New Jersey, Maryland, Virginia, Kentucky, and South Carolina (Stromdahl and Hickling, 2012). Given the high density and aggressive questing behavior of *A. americanum* it is not surprising that this species is commonly found parasitizing humans.

In the past *A. americanum* has primarily been considered a nuisance species; this tick is now recognized as the primary vector of several pathogenic organisms. Pathogens transmitted by *A. americanum* include *Ehrlichia chaffeensis* and *E. ewingii*, which cause infections in humans now referred to generally as "ehrlichiosis" (CDC, 2015a). A third pathogenic *Ehrlichia* species associated with *A. americanum*, Panola Mountain Ehrlichia (PME) has recently been described (Loftis et al., 2008). *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever, and *Francisella tularensis*, the agent of tularemia, have also been found in *A. americanum* although the infection rates for these pathogens in field-collected *A. americanum* are extremely low (Stromdahl et al., 2011; Eisen et al., 2007). The Lyme disease-like human illness known as southern tick-associated rash illness (STARI) has also been associated with the bite of *A. americanum*, although the

exact etiology of this disease is unclear (Masters et al., 2008). Recently, an emerging disease caused by the Heartland virus, a member of the Phlebovirus genus, has caused severe illness in two residents of Missouri (McMullan et al., 2012) and has been detected in populations of *A. americanum* (Savage et al., 2013).

Human monocytic ehrlichiosis, now referred to as "ehrlichiosis", is an infection caused by *E. chaffeensis* or *E. ewingii* (Paddock et al., 2003). *Ehrlichia chaffeensis* and *E. ewingii* are obligate intracellular Gram-negative bacteria transmitted by *Amblyomma americanum*. Ehrlichiosis is reported most commonly from adults over the age of 40, with men affected more than women. Occupational and recreational activities involving rural locations are documented risk factors for contracting the disease (Paddock et al., 2003). Most infections with *E. chaffeensis* are mild and resolve over time, but if left untreated *E. chaffeensis* infections can become severe, leading to serious conditions such as meningoencephalitis, acute renal failure, myocarditis and gastrointestinal hemorrhage. The risk of these complications are greater in adults over the age of 60 and for immunocompromised individuals (Goodman et al., 2005). Newly released 2010 census data indicate that the 65 and older population grew faster in the last decade than previously seen in census history, and at a faster rate than the U.S. population as a whole (U.S. Census Bureau). This suggests that a larger proportion of the U.S. population may be at risk for contracting ehrlichiosis than ever before.

The Gulf Coast tick, *Amblyomma maculatum.* *Amblyomma maculatum*, commonly known as the Gulf Coast tick, is increasingly recognized as an arthropod of not only economic and veterinary interest but also of medical importance. Native to the

Western Hemisphere, populations of *A. maculatum* are found in North, South and Central America. In the United States, *A. maculatum* is reported in southeastern states (Fig. 2), including Alabama, Florida, Georgia, Mississippi, South Carolina, Arkansas, Oklahoma, Kentucky, Tennessee, Texas, and Kansas (Paddock et al., 2004, Merten and Durden, 2000, Goddard and Norment, 1983). Populations of *A. maculatum* in the mid-1900s were described as being within 150 miles of the coast (Bishopp and Hixson, 1936; Cooley and Kohls, 1944; Bishopp and Trembley; 1945). In 1973, inland populations were reported in Oklahoma, likely introduced through the importation of livestock (Semtner

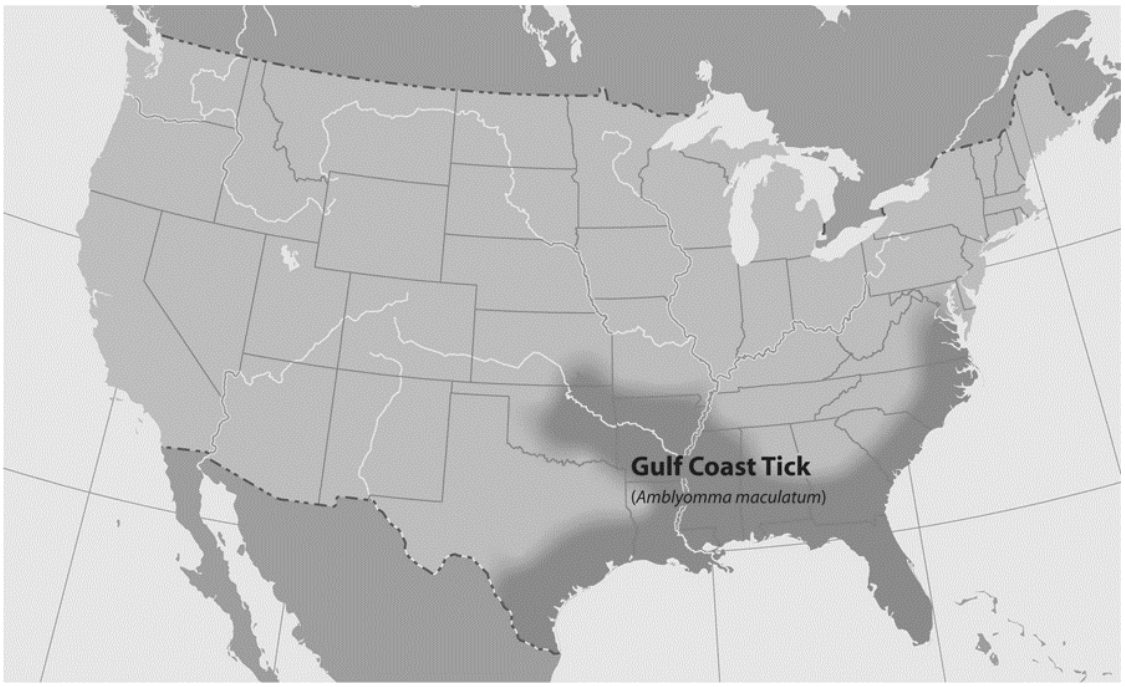

Fig. 2. Distribution of the Gulf Coast tick, *Amblyomma maculatum*, in the United States. Figure adapted from CDC website (http://www.cdc.gov/ticks/geographic_distribution.html, accessed June 10th, 2015).

and Hair, 1973). In the U.S., *A. maculatum* are commonly associated with coastal upland and tall-grass prairie habitats (Bishopp and Hixson, 1936; Hixson, 1940; Semtner and Hair, 1973; Fleetwood, 1985; Teel et al., 1998). Larvae and nymphs feed primarily on birds and rodents, whereas adults commonly seek larger hosts, such as carnivores and ungulates, including white-tailed deer and cattle (Teel et al., 2010).

In the United States, *A. maculatum* serves as the principal vector of *R. parkeri*, the agent of Tidewater spotted fever (also known as *R. parkeri* rickettsiosis or American Boutoneusse fever) (Paddock et al., 2010). Although human infections with *R. parkeri* have been associated only with *A. maculatum* (Paddock et al., 2010), *R. parkeri* has been detected in *A. americanum* collected from Tennessee and Georgia (Cohen et al., 2009). *Rickettsia parkeri* is an obligate intracellular α-proteobacterium belonging to the spotted fever group of rickettsiae (SFGR). Although the organism was first described in 1937 (Parker et al., 1939) it was not known to be pathogenic to humans until 2002, when *R. parkeri* was isolated from a resident of southeastern Virginia (Paddock et al., 2004). Since then over 37 confirmed human cases of infection caused by *R. parkeri* have been described, mostly from the southeastern United States (Paddock & Goddard, 2015). The infection caused by *R. parkeri*, Tidewater spotted fever, is generally less severe than Rocky Mountain spotted fever (RMSF). Symptoms associated with Tidewater spotted fever include fever, headache, an occasional rash and an eschar at the site of tick attachment (Paddock et al., 2003; Whitman et al., 2007; Romer et al., 2011). As of 2010, the Centers for Disease Control and Prevention (CDC) no longer reports RMSF cases as such; instead, a broader category, “Spotted Fever Rickettsiosis”,

has taken its place. This change occurred because of a lack of ability to serologically differentiate between human cases caused by *R. rickettsii*, *R. parkeri* and other potentially pathogenic SFGR (CDC, 2015b).

The American dog tick, *Dermacentor variabilis.* *Dermacentor variabilis*, commonly referred to as the American dog tick, is widely distributed throughout the United States (Fig. 3), extending north into Canada and south into Mexico (Bishopp and Trembley 1945). Larvae and nymphs are commonly found parasitizing small mammals; in Virginia, Sonenshine et al. (1966) reported nearly 98% of *D. variabilis* larvae and nymphs were found on two species, the white-footed mouse (*Peromyscus leucopus*) and the meadow vole (*Microplus pennsylvanicus*). Vertebrate hosts for adult *D. variabilis*

Fig. 3. Distribution of the American dog tick, *Dermacentor variabilis*, in the United States. Figure adapted from CDC website (http://www.cdc.gov/ticks/geographic_distribution.html, accessed June 10, 2015).

include domestic and wild medium-sized mammals, such as dogs, cats, horses, raccoons, Virginia opossums and squirrels (Anderson & Magnarelli, 1980). The most important vegetative types for finding *D. variabilis* adults are low, deciduous grass (Sonenshine et al., 1966). Incidentally, the density of *P. leucopus* and *M. pennsylvanicus* are highest in this class of vegetation (Sonenshine et al., 1966).

Dermacentor variabilis has historically played an important role as the primary vector of *R. rickettsii*, the agent of Rocky Mountain spotted fever. Although this tick has long been associated with *R. rickettsii* in the U.S., present-day surveys indicate a very low prevalence of *R. rickettsii* in *D. variabilis* populations (Moncayo et al., 2010; Stromdahl et al., 2011; Nadolny et al., 2014). The rickettsial endosymbiont, *Rickettsia montanensis*, is more commonly found in *D. variabilis,* albeit in low numbers (<5%) (Carmichael et al., 2010; Stromdahl et al., 2011). Although *R. montanensis* was not believed to be pathogenic, a recent report of febrile illness has been associated with a bite of a *R. montanensis*-infected *D. variabilis* (McQuiston et al., 2012). This tick is also known to be a competent vector of *Francisella tularensis*, the causative agent of tularemia (Reese et al., 2011). Although prevalence of this pathogen in field-collected *D. variabilis* is quite low (<0.1%) (Hopla, 1953; Calhoun and Alford 1955), the efficiency and speed at which *F. tularensis* can be transmitted suggests that *D. variabilis* may be an important source of tularemia infections (Reese et al., 2011).

The blacklegged tick, *Ixodes scapularis.* *Ixodes scapularis*, commonly referred to as the blacklegged tick, is the primary eastern U.S. vector of *Borrelia burgdorferi* sensu

strico, the agent of Lyme disease, and is arguably the most significant arthropod vector within the United States. In the western U.S., the primary vector of *B. burgdorferi* s.s. is *Ixodes pacificus*. The range distribution of *I. scapularis* includes the eastern seaboard, the upper-Midwest and several southeastern states (Fig. 4). *Ixodes scapularis* utilizes a wide variety of vertebrate hosts. Adult *I. scapularis* feed primarily on medium to large mammals, including white-tailed deer (Anderson and Magnarelli 1980; Schulze et al., 1986). Immature *I. scapularis* feed on smaller animals, including mammalian, avian and reptilian hosts. Mice and skinks are particularly important hosts for larvae and nymphs (Keirans et al., 1996).

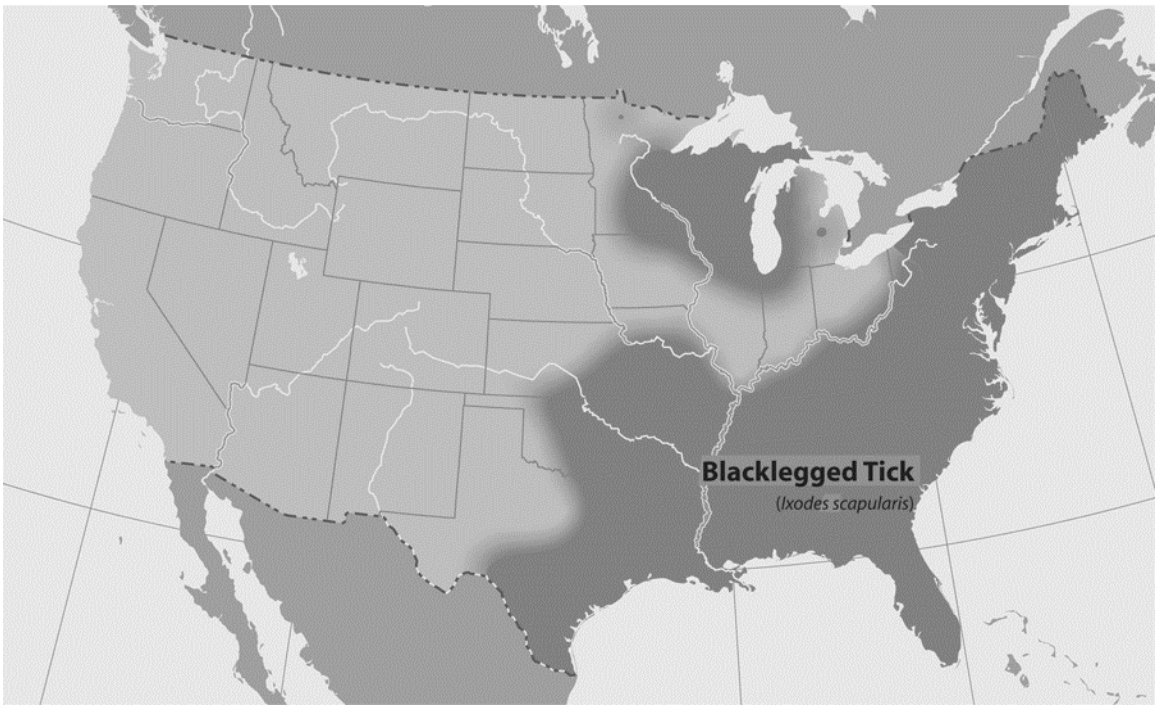

Fig. 4. Distribution of the blacklegged tick, *Ixodes scapularis*, in the United States. Figure adapted from CDC website (http://www.cdc.gov/ticks/geographic_distribution.html, accessed June 10, 2015).

Other pathogens transmitted by *I. scapularis* include *Anaplasma phagocytophilum*, the agent of anaplasmosis, and *Babesia microti*, the agent of babesiosis, although these species are usually only associated with the northern populations of *I. scapularis* in the U.S. (Stromdahl et al., 2011).

Ticks and Tick-borne Diseases of Virginia. Currently the most commonly reported tick-borne disease in Virginia is Lyme disease, followed by spotted fever group rickettsioses (formerly reported as Rocky Mountain spotted fever) and ehrlichiosis/anaplasmosis (reported together). Ten-year trends (2004 to 2013) indicate a general increase in reported cases of tick-borne diseases in Virginia, with the exception of tularemia (Fig. 5).

The most recent surveys of the Virginia tick population were conducted in the 1970s by Sonenshine (1979) and Garrett and Sonenshine (1979), who determined the composition and relative densities of human-biting ticks by flagging vegetation. These surveys revealed the most commonly encountered tick to be the American dog tick, *D. variabilis*, followed by smaller yet well-established populations of *A. americanum* and *I. scapularis* (Sonenshine, 1979; Garrett and Sonenshine, 1979). At the time these surveys were conducted, the only reportable tick-borne disease in Virginia was Rocky Mountain spotted fever; there were 90 cases reported in the state in 1979 (VDH). The RMSF case fatality rate at that time was approximately 5% (CDC); however, since 2000, the RMSF case fatality rate has steadily decreased and is now approaching 0% (CDC).

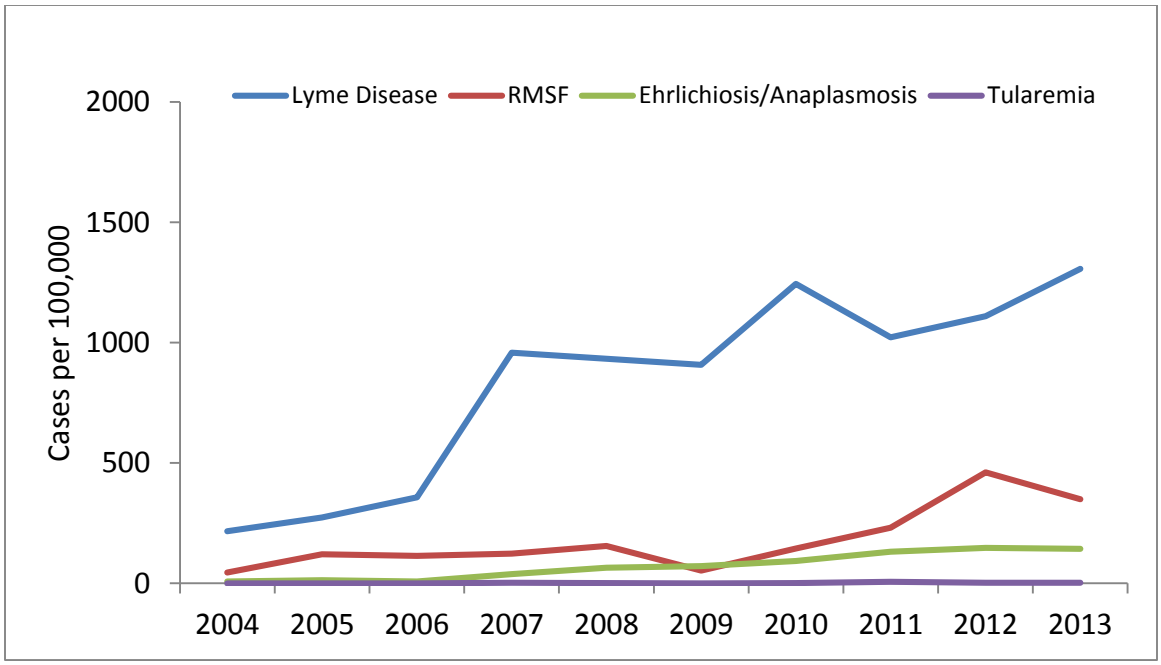

Fig. 5. Tick-borne disease cases per 100,000 people per year from 2004 – 2013 in Virginia. In 2010, the case definition of RMSF changed to "spotted fever group rickettsioses". Figure adapted from the Virginia Department of Health (http://www.vdh.virginia.gov/Epidemiology/Surveillance/SurveillanceData/ReportableDisease/Tables/table2a_trend13.pdf, accessed June 10, 2015).

RESEARCH OBJECTIVES

The purpose of this dissertation research was to determine the composition and prevalence of tick-borne pathogens present in tick populations within southeastern Virginia. Of primary interest are spotted fever group rickettsiae (SFGR) and ehrlichiae present in commonly-encountered, human biting ticks. Furthermore, this research aimed to expand our knowledge of the ecology and evolutionary history of tick-borne diseases by exploring the transmission dynamics of *R. parkeri* within the newly-established, highly-infected *A. maculatum* populations in Virginia.

The final research objective is to explore the potential of *A. americanum* to serve as a secondary vector of *R. parkeri*, potentially increasing the risk of exposure of this pathogen to humans. By pairing pathogen prevalence data derived from field investigations with transmission dynamics derived from laboratory procedures, new insights could be made about the nature of the relationship between rickettsial organisms and ticks. This research provides useful information about the nature of tick-pathogen dynamics, thus increasing our understanding of and potential to control tick-borne disease.

CHAPTER 1

RICKETTSIA PARKERI IN GULF COAST TICKS, SOUTHEASTERN VIRGINIA, USA

INTRODUCTION

Rickettsia parkeri is a gram negative, obligate intracellular bacterium belonging to the spotted fever group rickettsiae (SFGR). First isolated from *Amblyomma maculatum* Koch in 1937 (Parker et al., 1939), *R. parkeri* was not considered to be pathogenic to humans until 2002, when it was isolated from a man residing in the Tidewater region of Virginia presenting with signs and symptoms of a spotted fever-like illness (Paddock et al., 2004). Since then, three cases have been reported from southeastern Virginia (Paddock et al., 2004; Whitman et al., 2007; Wright et al., unpublished), and over 37 human infections caused by *R. parkeri* have been reported from the United States, primarily in southeastern states (Paddock & Goddard, 2015). Infection with *R. parkeri*, referred to either as Tidewater spotted fever or *R. parkeri* rickettsiosis, is clinically very similar to Rocky Mountain spotted fever and cannot reliably be differentiated based on serological assays. Because of the inability to differentiate illnesses caused by SFGR, Rocky Mountain spotted fever and other closely-related infections are now simply reported as “spotted fever rickettsioses”.

The Gulf Coast tick, *A. maculatum*, is distributed throughout many of the southeastern states in the U.S. and is the primary vector of *R. parkeri*. The Gulf Coast

Chapter adapted from Wright C.L., Nadolny R.M., Jiang J., Richards A.L., Sonenshine D.E., Gaff H.D., Hynes W.L., 2011. Emerg. Infect. Dis. 17, 896–898.

tick has been found in multiple states, including Alabama, Arkansas, Delaware, Florida, Georgia, Kansas, Kentucky, Maryland, Mississippi, Missouri, Oklahoma, North Carolina, South Carolina, Tennessee, and Texas (Varela-Stokes et al., 2011; Paddock et al., 2004; Merten and Durden, 2000; Goddard and Norment, 1983, Florin et al. 2014, Brown et al. 2011). The reported prevalence of *R. parkeri* in *A. maculatum* ranges from 5 – 35% in *A. maculatum* populations (Cohen et al., 2009; Sumner et al., 2007; Trout et al., 2010; Varela-Stokes et al., 2011).

Isolated *A. maculatum* have in the past been reported from Virginia on occasion (Sonenshine et al., 1965; Levine et al., 1991) but these collections were attributed to drop off events from migratory birds rather than from established populations. The purpose of this research was to determine whether *A. maculatum* is now established in southeastern Virginia and to find the proportion of ticks infected with *R. parkeri*.

MATERIALS AND METHODS

Multiple locations in southeastern Virginia were sampled on a weekly or bi-weekly basis from 2010 – 2013 (Fig. 6). Questing ticks were sampled from a variety of transects within each field site in order to represent different habitat types, including grassland, closed-canopy forest and forest edge. Ticks were collected by flagging each established transect, ranging in length from 100 – 2000 m, using white denim flags attached to denim rods as described by Ginsberg and Ewing (1989). Ticks were identified to species based on morphological features (Kierans and Litwak, 1989) and confirmed as necessary by molecular identification based on sequencing of the 16S gene (Nadolny et al., 2011). Ticks were stored, without ethanol, at -80 °C until DNA extraction. Each adult tick was bisected longitudinally using a sterile scalpel blade. One half was saved at -80 °C and the other was used for DNA extraction and molecular analysis. Prior to DNA extraction, each tick was subjected to bead-beating using an equal mixture of 1mm and 5mm glass beads for 30 – 60 seconds on a mini bead-beater (BioSpec Products, Inc., Bartlesville, OK). DNA was extracted from individual adult halves using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Inc., Valencia, CA), following the manufacturer's instructions, and stored at -20°C prior to processing.

DNA samples were tested for *R. parkeri* DNA by real-time PCR using a MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Testing for *R. parkeri* DNA was by amplification and detection of a fragment of the *ompB* gene by using Rpa129F and Rpa224R primers and Rpa188 as the probe (p. 108). Samples negative

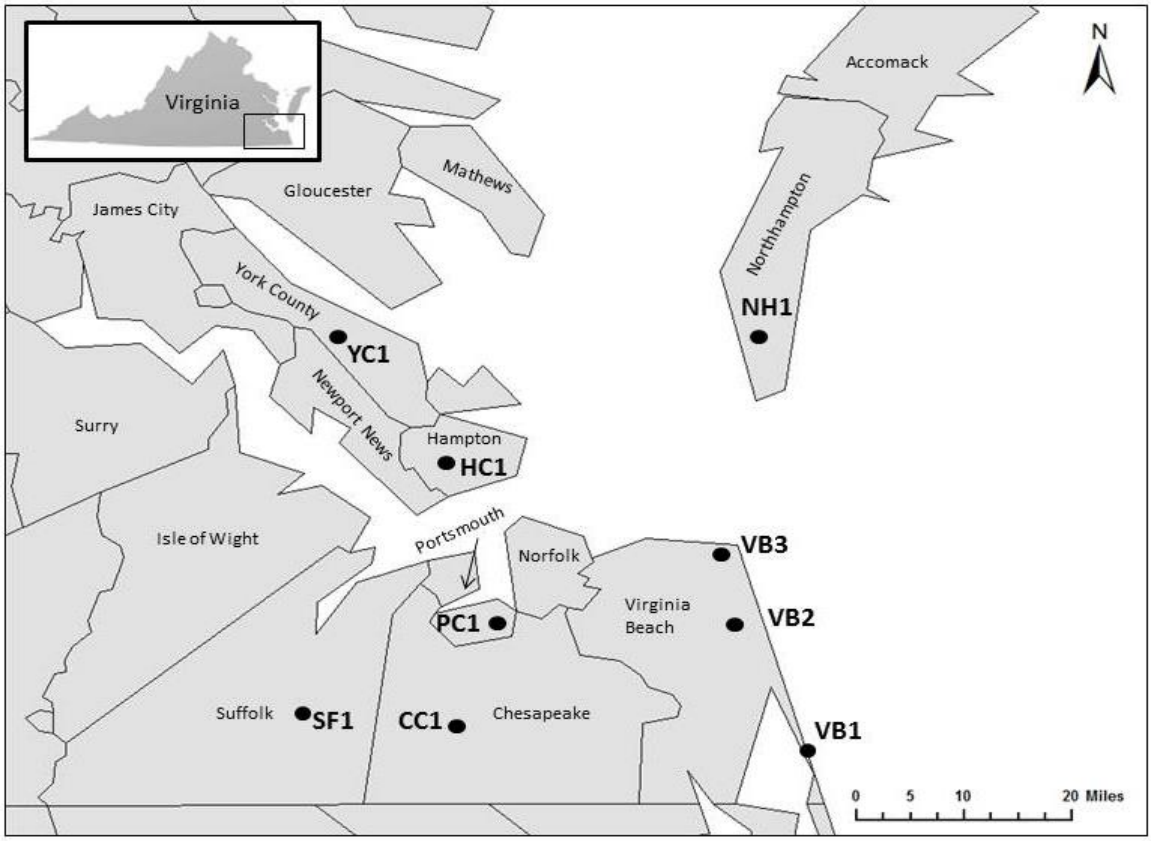

Fig. 6. Locations where *Amblyomma maculatum* were collected between 2010 and 2013.

for *R. parkeri* DNA were tested for *Rickettsia* spp. by amplifying a 111-bp fragment of the 17-kDa antigen gene (p. 108).

Three representative *A. maculatum* samples positive for *R. parkeri* by real-time PCR were confirmed by sequencing of a 540-bp fragment of the *ompA* gene. The fragments were amplified on an iCycler (Bio-Rad) using primers 190-FN1 and 190-RN1 (p. 108). Samples positive for *Rickettsia* spp. but negative for *R. parkeri* had their *ompB* gene amplified and sequenced using primers RompB11F and RompB1902R (p. 108). All PCR products for sequencing were purified using Wizard PCR Preps DNA Purification

System (Promega, Madison, WI, USA), and sequencing reactions were performed using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) as described by the manufacturer and using appropriate primers (p. 108). Sequence similarities were identified by a BLAST search (http://blast.ncbi.nlm.nih.gov).

RESULTS

From 2010 – 2013, a total of 346 adult and 10 nymphal *A. maculatum* were collected on flags from nine locations in southeastern Virginia (Fig. 6). A total of 67 *A. maculatum* were collected in 2010, 97 in 2011, 139 in 2012 and in 2013, 53 were collected (Table 1). *A. maculatum* were found from April to September, with peak collections occurring during June in 2010, 2011 and 2012 and July of 2013 (Fig. 7).

Overall prevalence of *R. parkeri* during 2010-2013 was 49.1% in adults (n=346) and 0% in nymphs (n=10). Prevalence in adults ranged from 30.2% in 2013 to 55.7% in 2011 (Table 1). Prevalence varied by location, ranging from 0% at some locations to 100% (n=1) at others (Table 2). A single *A. maculatum* adult collected from the Portsmouth site in 2010 tested negative for *R. parkeri* but positive for *Rickettsia* spp. by real-time PCR. Sequencing of a fragment of the *ompB* gene revealed this isolate to contain DNA with a 100% match to *Candidatus* Rickettsia andeanae isolate T163 (GenBank accession no. GU395297.1), a rickettsia initially found in Peru (Jiang et al., 2005.)

Table 1
Prevalence of *Rickettsia parkeri* in *Amblyomma maculatum* nymphs and adults collected from southeastern Virginia from 2010-2013.

Year	Life Stage	Number positive	Number tested	Prevalence
2010	Adult	28	65	43.1
	Nymph	0	2	0.0
2011	Adult	54	97	55.7
	Nymph	0	0	0.0
2012	Adult	73	131	55.7
	Nymph	0	8	0.0
2013	Adult	16	53	30.2
	Nymph	0	0	0.0
Total	Adult	171	346	49.4
	Nymph	0	10	0.0

Table 2
Prevalence of *Rickettsia parkeri* in *Amblyomma maculatum* (nymphs and adults) from multiple locations in southeastern Virginia.

Year	Site	County	Number Positive	Number Tested	Prevalence (%)
2010	VB1	Virginia Beach	0	10	0.0
	PC1	Portsmouth	1	3	33.3
	CC1	Chesapeake	27	54	50.0
2011	VB1	Virginia Beach	1	5	33.3
	YC1	James City	1	1	100.0
	VB3	Virginia Beach	0	1	0.0
	HC1	Hampton	1	2	50.0
	VB2	Virginia Beach	0	1	0.0
	CC1	Chesapeake	51	87	58.6
2012	VB1	Virginia Beach	11	36	30.6
	YC1	James City	6	13	46.2
	NH1	Northampton	0	1	0.0
	HC1	Hampton	1	2	50.0
	CC1	Chesapeake	55	87	63.2
2013	VB1	Virginia Beach	8	31	25.8
	SF1	Suffolk	0	6	0.0
	CC1	Chesapeake	8	16	50.0
2010 – 2013	All	All	171	356	48.0

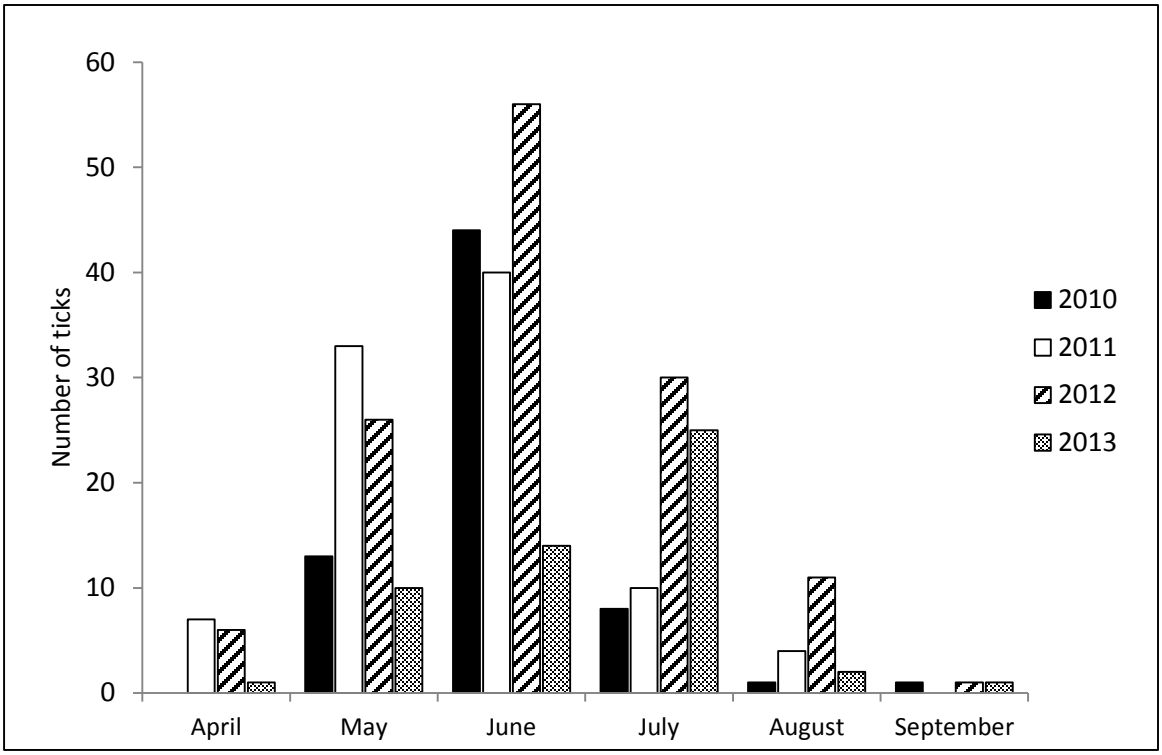

Fig. 7. Phenology of adult *Amblyomma maculatum* collected from southeastern Virginia from 2010 to 2013.

DISCUSSION

Discovery of the numbers and life stages of *A.maculatum* ticks in widely dispersed locations indicates that they are now established in southeastern Virginia. Finding adult *A. maculatum* ticks at the Portsmouth site was unexpected because this is a site devoid of white-tailed deer, a major host for adult ticks (Scrifres et al., 1988; Barker et al., 2004). The *R. parkeri* prevalence of 30.2 – 55.7% in adult *A. maculatum* ticks collected from southeastern Virginia differs from reported prevalences of *R. parkeri* in *A. maculatum* ticks elsewhere in the United States. *R. parkeri* prevalence in *A. maculatum* collected from Florida and Mississippi from 2005 to 2007 was 21.9% and 38.6%, respectively (Paddock et al. 2010), while the average prevalence in *A. maculatum* collected from Florida, Kentucky, Mississippi and South Carolina from 1996 to 2005 was 11.5% (Sumner et al., 2007). For *A. maculatum* collected from Georgia, a prevalence of 5%–11.5% has been reported (Cohen et al., 2009). In Arkansas, only 3 of 207 *A. maculatum* ticks contained *R. parkeri* (Trout et al., 2010). Despite the high percentage of *R. parkeri* in the southeastern Virginia ticks, 141 of 244 positive samples from 2010 – 2013 came from one collection site in Chesapeake, VA. One explanation could be that *R. parkeri* is transovarially transmitted. Currently, there is no evidence that *R. parkeri* is transmitted transovarially by *A. maculatum* ticks, although transovarial transmission of *R. parkeri* has been shown in *A. americanum* (Goddard, 2003) and *Amblyomma triste* ticks (Nieri-Bastos et al., 2013) under laboratory conditions.

A single *A. maculatum* tick was also found to contain *Candidatus* Rickettsia andeanae, which has rarely been reported in the United States (Paddock et al., 2010).

Whether *Candidatus* Rickettsia andeanae is pathogenic to humans is unknown, although it has been suspected to cause infections in persons in Peru (Jiang et al., 2005). Further research is still needed to identify the potential vertebrate reservoir or amplifier host(s) of *R. parkeri*. This information could be useful for controlling the transmission of *R. parkeri* to and from the vector, as well as predicting where *R. parkeri* may be present. Studies relating to transovarial and transstadial transmission of *R. parkeri* in *A. maculatum* ticks would also be useful for predicting the spread of infections. Because *R. parkeri* is known to cause infection in humans, the presence of this pathogen in southeastern Virginia should be a health concern to persons in this region.

CHAPTER 2

TICKS AND SPOTTED FEVER GROUP RICKETTSIAE OF SOUTHEASTERN VIRGINIA

INTRODUCTION

Ticks are ectoparasitic vectors of disease-causing microorganisms with complex life histories that are intimately tied to their hosts and the habitats in which they are found. Each species of human-biting tick is a vector for a different suite of infectious agents. There is great variation in the dominant tick species in different regions of the United States (Merten and Durden, 2000; Stromdahl and Hickling, 2012). Much of the recent work on the ecology of ticks and their associated pathogens has been done in the northeastern United States, where Lyme disease has become a major human health threat in recent decades (Ostfeld et al., 1995; LoGiudice et al., 2003). However, recent increases in rickettsial infections such as those caused by *Rickettsia parkeri* and related organisms such as *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* have drawn the focus of some research to the southeastern United States. The community of human-biting ticks in the southeast is dramatically different than that of the northeast. In the southeast, *Amblyomma americanum*, the lone star tick, is the primary human-biting tick, while in the northeast, the blacklegged tick, *Ixodes scapularis*, and the American dog tick, *Dermacentor variabilis*, are the predominant human-biting ticks (Merten and Durden, 2000; Stromdahl and Hickling, 2012).

Chapter adapted from *Nadolny, R.M., *Wright, C.L., Sonenshine, D.E., Hynes, W.L., Gaff, H.D., 2014. Ticks Tick Borne Dis. 5, 53-57. *Equal contributions

Although differences in the dominant human-biting ticks have been noted, there is a dearth of long-term surveillance on the tick communities in the southeast, the pathogens that questing southeastern ticks carry, and how these numbers are changing over time. The range expansion of tick populations from the southeast into the mid-Atlantic region has had a serious impact on human cases of tick-borne diseases. Perhaps the most dramatic change has been the increase in numbers of *A. americanum* and its associated pathogens during the past several decades (Childs and Paddock, 2003; Paddock and Yabsley, 2007). Increased white-tailed deer (*Odocoileus virginianus*) populations and expanded forested habitat have led to the increase in *A. americanum* ticks and an expansion of their range (Paddock and Yabsley, 2007). Since 2010, 2 new species of tick have established permanent populations in southeastern Virginia. The Gulf Coast tick, *Amblyomma maculatum*, has established several populations in southeastern Virginia and brought with it *R. parkeri*, the agent of Tidewater spotted fever (also known as "*Rickettsia parkeri* rickettsiosis") (Wright et al., 2011). In addition, *Ixodes affinis*, a known sylvatic vector for *Borrelia burgdorferi* s.s., the causative agent of Lyme disease, has also established populations throughout southeastern Virginia (Nadolny et al., 2011). The addition of 2 new species to a tick community has unpredictable impacts on pathogen prevalence and tick species community structure. The spotted fever group rickettsiae (SFGR) consist of multiple species ranging from nonpathogenic organisms such as *Rickettsia amblyommii* to potentially lethal pathogens such as *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever. *Rickettsia amblyommii* is found primarily in *A. americanum*, and reported prevalence is

variable, ranging from 0% to 84% in the United States (Childs and Paddock, 2003; Mixson et al., 2006; Moncayo et al., 2010; Smith et al., 2010). Although it is has not been conclusively established that *R. amblyommii* is pathogenic to humans, there is some evidence suggesting that it is capable of causing human infection (Apperson et al., 2008; Billeter et al., 2007). Another SFGR, *R. montanensis*, is associated with *D. variabilis*. Although generally considered to be nonpathogenic, *R. montanensis* was recently associated with an afebrile rash illness (McQuiston et al., 2012). Reported prevalence of *R. montanensis* in *D. variabilis* is generally low, ranging from 3.2% in *D. variabilis* collected from humans (Stromdahl et al., 2011) and 3.8% in *D*. *variabilis* from Maryland (Ammerman et al., 2004) to 10% in *D. variabilis* from Tennessee (Moncayo et al., 2010). *Rickettsia parkeri* is a pathogenic SFGR primarily transmitted by *A*. *maculatum*. Reported prevalence of *R. parkeri* in *A. maculatum* ranges from 1.4% to 43.1% (Paddock et al., 2010; Sumner et al., 2007; Cohen et al., 2009; Jiang et al., 2012; Trout et al., 2010; Wright et al., 2011; Fornadel et al., 2011; Varela-Stokes et al., 2011; Ferrari et al., 2012). Also found in *A. maculatum* is *Candidatus* Rickettsia andeanae, a SFGR of unknown pathogenicity. Reported prevalence of *Candidatus* R. andeanae in ticks varies from 0.6% to 10% (Fornadel et al., 2011; Jiang et al., 2012; Varela-Stokes et al., 2011; Wright et al., 2011; Ferrari et al., 2012; Sumner et al., 2007; Paddock et al., 2010). We have been conducting active tick surveillance through-out southeastern Virginia since 2009, with intensive year-round surveillance since 2010. The purpose of our surveillance has been to describe the tick and tick-borne disease-causing pathogen populations in southeastern Virginia, determine which species are the most abundant, and document

changes in species composition overtime. Ticks collected from the region have been tested for several members of the SFGR. All of this information will be used to develop and parameterize predictive mathematical models assessing the risk of human disease in this region. Here, we describe the results of our surveillance and the rates of SFGR infection found in the ticks of southeastern Virginia.

MATERIALS AND METHODS

Questing ticks were collected from 13 sites in 8 counties and cities throughout southeastern Virginia, comprising a mix of habitats and degrees of human disturbance (Fig. 8). Sites were selected across the region to provide a variety of landscapes including 3 urban parks, 4 government installations, 4 state parks/preserves, a National Wildlife Refuge, and a Nature Conservancy site. The majority of the sites were chosen to parallel studies conducted in the region by Sonenshine and others from the 1960s through the 1980s (Sonenshine et al., 1966, 1995; Sonenshine, 1979). Additional sites were added to so that there was a sampling site in every county and city in the region. Each site was composed of one or more transects ranging from100 m to 800 m long, with one 2000 m long transect through the Nature Conservancy site in a late secondary successional tract. Each individual transect is confined to one particular habitat for a total of approximately 1500 m of grass-dominated, 1800 m of edge, and 3000 m of wooded habitat. The number of transects at each site was determined by the variety of habitats present with the goal of having one grass-dominated habitat, one closed canopy wooded habitat, and one edge habitat. The same transects have been flagged each time the site was visited, resulting in a consistent area being sampled during each collection trip. Sampling was conducted at 10 sites comprising 24 transects in 2010, 13 sites comprising 33 transects in 2011, and 12 sites comprising 29 transects in 2012. Each site was sampled monthly during the winter months when tick density was low, but was sampled weekly or bi-weekly when tick densities are high from May through October (Table 3). Questing ticks were collected using flags constructed from 1 m^2 white denim

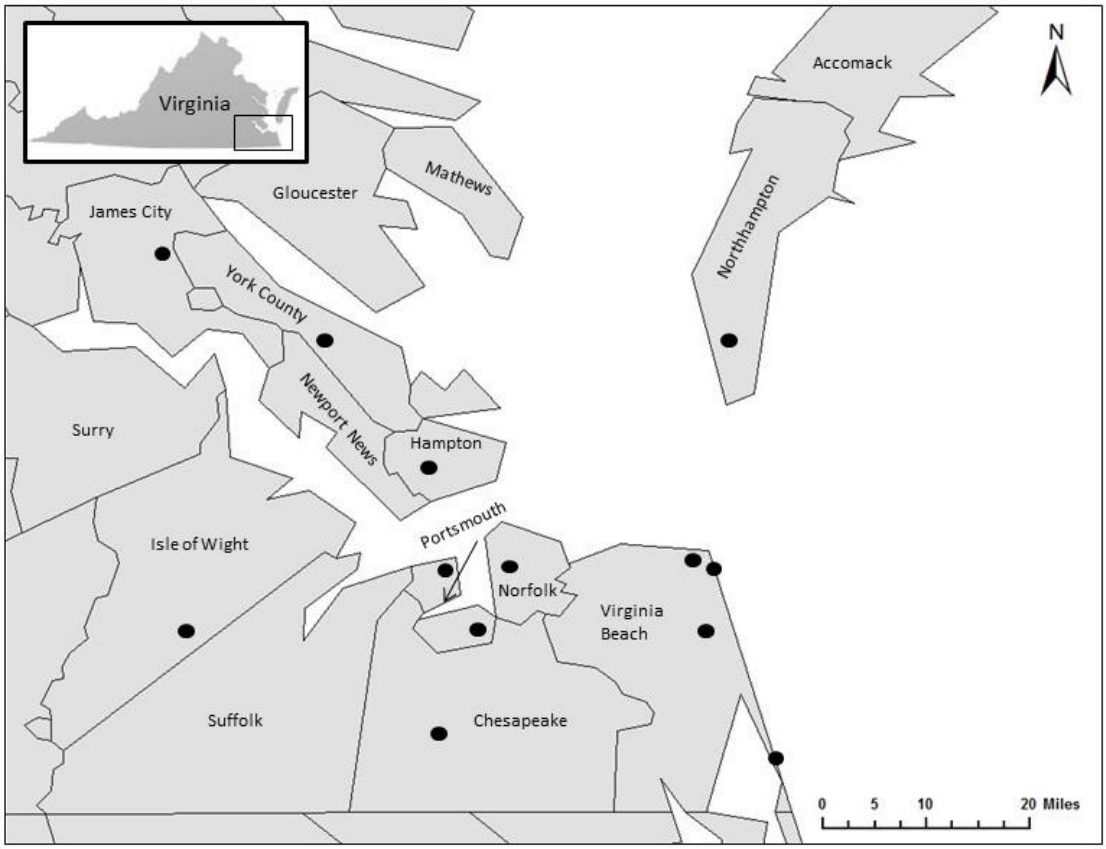

Fig. 8. Map of collection sites throughout southeastern Virginia.

squares attached to dowel rods and swept through low vegetation and along the ground as described previously (Ginsberg and Ewing, 1989). Flags were inspected for ticks every few meters; careful training was provided to ensure consistency of collection. Adult and nymphal ticks were collected from the flag with forceps, while larvae were collected immediately with masking tape before being placed in vials and brought back to the lab for morphological identification. Flags were carefully checked between transects and washed between sites to ensure collection of all ticks and prevent contamination of the next sample. Each tick was morphologically identified to species (Keirans and Clifford, 1978; Keirans and Litwak, 1989; Oliver et al., 1987) and then frozen at −20°C until processed for pathogen testing. *Amblyomma americanum* and *D. variabilis* adults were pooled in groups of up to 10 for DNA extraction and pathogen testing; *A. americanum* nymphs were grouped in pools of up to 25 for testing. Pools were restricted to ticks collected from the same transect on the same day. All other ticks were individually extracted and tested. Every adult tick was cut in half; one half used for analysis, the other half stored at −80°C for future analyses or validations. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol and stored at −20°C until analyzed. DNA samples were tested for rickettsial DNA by real-time PCR using a MiniOpticon Real-Time PCR System (BioRad Laboratories, Hercules, CA). Samples were tested for *Rickettsia* spp., *R. amblyommii*, *R. montanensis*, *R. parkeri*, or *Candidatus* R. andeanae DNA using quantitative PCR (qPCR) (p. 108). Reactions were carried out using EconoTaq PLUS 2X Master Mix (Lucigen Corp., Middleton, WI), 0.5–0.7 µM of each primer, 0.4–0.5 µM probe, 5–8 mM MgCl2, and 2–5

µL DNA template in a final reaction volume of 20 µL. The PCR protocol for all reactions consisted of 50°C for 2 min, 95°C for 2 min, and then 45 cycles of 95°C for 15 s and 60°C for 30 s. A select number of positive samples were periodically sampled and *Rickettsia* species confirmed by DNA sequencing.

Table 3
The number of sites sampled and total transect sampling events during 2010 – 2012. Also reported are the total number of ticks collected and the average number of ticks collected per sampling event each year.

	2010	**2011**	**2012**
Sites sampled	10	13	12
Transect sampling events	840	968	1921
Total ticks collected	13263	17055	34216
Average ticks/sampling event	15.8	17.6	17.8

Because *A. americanum* and *D. variabilis* tick DNA was extracted in pools, a maximum likelihood estimation (MLE) was used to approximate true infection rate of *R. amblyommii* and *R. montanensis*, respectively. The software used to perform MLE was developed by Brad Biggerstaff and acquired from the Centers for Disease Control and Prevention website (CDC, 2013a).

Table 4
Number of questing ticks collected each year (excluding *Amblyomma americanum* larvae).

Tick Species	2010	2011	2012	Total	Percentage of Total
I. brunneus	0	0	3	3	0.02%
I. scapularis	104	208	231	513	2.7%
D. variabilis	280	326	416	1022	5.5%
A. maculatum	74	116	145	335	1.8%
I. affinis	81	152	177	410	2.2%
A. americanum	3250	5450	7731	16431	87.8%
H. leporispalustris	2	4	5	11	0.06%
Total	3789	6238	8698	18725	100.0%

RESULTS

From 2010 to 2012, 66,590 questing ticks (37,450 ticks, excluding *A. americanum* larvae) were collected from southeastern Virginia. *Amblyomma americanum* comprised 96.5% of all ticks collected. The majority of the *A*. *americanum* were larvae collected during the late summer, comprising 76.5% of the *A*. *americanum* collected. Excluding larvae, *A. americanum* adults and nymphs still accounted for more than 87% of ticks collected (Table 4). Four other species of ticks were commonly collected using flags, including *D. variabilis, I. scapularis*, and *A. maculatum*, as well as *I. affinis* (Table 4). These trends were similar for grass, edge, and wooded sampling sites. The one site undergoing late secondary succession had higher percentages of *D. variabilis* and *A. maculatum*, but was still dominated by *A. americanum*.

There was an increase in the total number of ticks collected each year (Table 4), which can be explained by the addition of transects in 2011 and an increase in transect sampling events each year. Sites that were sampled monthly or bi-weekly in 2010 and 2011 were increased to weekly sampling in 2012. However, even with the increase in collections in 2012, the average number of ticks collected per sampling trip remained roughly the same between 2011 and 2012.

Rickettsia amblyommii was detected in over 72.5% of *A. americanum* pools tested (Table 5). Maximum likelihood estimates showed *R*. *amblyommii* present at an average prevalence (adults and nymphs) of 26.9% in 2010 and 54.9% in 2011. *Rickettsia montanensis* was found in 5.6–6.9% of *D. variabilis* pools tested. Maximum likelihood estimates showed *R. montanensis* prevalence to be 1.5% in 2010 and 2.0% in 2011. All

D. *variabilis* pools were initially tested for *Rickettsia* spp. DNA with positive samples being tested for *R. montanensis*. All samples positive for *Rickettsia* spp. were positive for *R. montanensis*. No evidence of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever, was found. *Rickettsia parkeri* prevalence in *A. maculatum* ranged from 43.1–55.7%. In 2010, one *A*. *maculatum* adult was found to be positive for *Candidatus* Rickettsia andeanae (Table 5).

Table 5
Prevalence of SFGR in ticks collected from southeastern Virginia, 2010-2012.

Year	Organism	Tick Species	Life Stage	# Positive/ # Tested	# Individuals	Positive Pools (%)	MLE Prevalence (%)
2010	*R. montanensis*	*D. variabilis*	Adult	4/71	268	5.6	1.5
	R. amblyommii	*A. americanum*	All	265/326	2509	81.3	26.9
			Adult	163/195	1191	83.6	33.4
			Nymph	102/131	1318	77.9	19.6
	R. parkeri	*A. maculatum*	All	28/67	67	41.8	N/A
			Adult	28/65	65	43.1	N/A
			Nymph	0/2	2	0.0	N/A
	Candidatus R. andeanae	*A. maculatum*	All	1/67	67	1.5	N/A
			Adult	1/65	65	1.5	N/A
			Nymph	0/2	2	0.0	N/A

Table 5. Continued.

Year	Organism	Tick Species	Life Stage	# Positive/ # Tested	# Individuals	Positive Pools (%)	MLE Prevalence (%)
2011	*R. montanensis*	*D. variabilis*	Adult	5/72	254	6.9	2.0
	R. amblyommii	*A. americanum*	All	65/82	252	79.3	54.9
			Adult	29/40	127	72.5	44.3
			Nymph	36/42	125	85.7	67.3
	R. parkeri	*A. maculatum*	All	54/97	97	55.7	N/A
			Adult	54/97	97	55.7	N/A
			Nymph	0/0	0	-	-
	Candidatus R. andeanae	*A. maculatum*	All	0/95	95	0.0	N/A
			Adult	0/95	95	0.0	N/A
			Nymph	0/0	0	-	-
2012	*R. parkeri*	*A. maculatum*	All	72/139	139	51.8	N/A
			Adult	72/131	131	55.0	N/A
			Nymph	0/8	8	0.0	N/A
	Candidatus R. andeanae	*A. maculatum*	All	0/139	139	0.0	N/A
			Adult	0	131	0.0	N/A
			Nymph	0	8	0.0	N/A

DISCUSSION

We conclude that the tick community in southeastern Virginia is overwhelmingly dominated by *A. americanum*, with smaller but well-established populations of *D. variabilis*, *I. scapularis*, *A. maculatum*, and *I. affinis*. While populations of *A. americanum*, *D. variabilis*, and *I. scapularis* were expected in southeastern Virginia, populations of *I. affinis* and *A. maculatum* were unexpected when this surveillance project began. These five tick species are ones a large host, e.g., white-tailed deer, are likely to encounter. *Ixodes brunneus* and *Haemaphysalis leporispalustris* were collected only incidentally by flagging, but it is likely that host-targeted collections of birds would yield substantial numbers of these ticks. The sheer numbers of *A. americanum* make it difficult to encounter anything other than this species during the times of year and in the habitats where they are abundant. This "lone star effect" swamps out the other species, especially during larval season and makes it impossible to assume that anything that might be true for tick population ecology in the northeast is applicable to tick communities in the southeast. The results of our survey may be a more accurate representation of tick communities in other areas of the southeast, particularly along the coastal plain.

Rickettsia amblyommii and *R. parkeri* are the dominant SFGR found in *Amblyomma* spp. ticks in southeastern Virginia. Although it has not been established that *R. amblyommii* is pathogenic to humans, *R. parkeri* is a known pathogen, and its high prevalence in *A. maculatum* in southeastern Virginia (43–55%) indicates that this tick may be an important disease vector in the region. Other reports of *R. parkeri*

prevalence in southern states have generally been lower, ranging from 1.5% (Trout et al., 2010) to 33% (Varela-Stokes et al., 2011). *Rickettsia parkeri* prevalence in northern Virginia (Fornadel et al., 2011) was found to be comparable (41.4%) to rates reported in this study, indicating that *R. parkeri* prevalence in Virginia is higher than in other states to the south and west.

Interestingly, no *R. rickettsii* was found in *D. variabilis* collected in 2010 and 2011. The absence of *R. rickettsii* and low prevalence of *R. montanensis* have also been observed in ticks collected from Tennessee in 2007 and 2008 (Moncayo et al., 2010) and from *D. variabilis* collected off of humans from 1997 to 2009 (Stromdahl et al., 2011). Despite the lack of *R. rickettsii* detected in this and other studies, reported cases of spotted fever group rickettsiosis continue to rise in Virginia. From 2002 to 2010 there was a 3-fold increase in the number of RMSF cases in Virginia (VDH, 2013); in 2011, RMSF cases were no longer reported as such by the CDC and were instead reported as a "spotted fever group rickettsiosis" (VDH, 2013). From 2010 to 2011, there was a 1.5-fold increase in the number of SFGR cases reported in Virginia with nearly a 3-fold increase in the eastern region of Virginia (VDH, 2013). Furthermore, the case fatality rate of Rocky Mountain spotted fever nationwide has decreased from 28% in 1944 to <1% beginning in 2001 (CDC, 2013b). Our study reports no *R. rickettsii* in 522 questing *D. variabilis* tested from 2010 and 2011, which supports data from the Army Public Health Command's Human Tick Test Kit Program. They reported no ticks positive for *R. rickettsii* out of 106 ticks biting military personnel at Ft. Eustis, Virginia, from 1997 through 2012 (Stromdahl, pers. communication). Given the lack of *R. rickettsii* found in ticks in Virginia

and other states, we suggest that this organism is no longer the primary cause of human infection diagnosed as spotted fever group rickettsiosis. Given the high prevalence of other SFGR, including *R. parkeri*, in southeastern Virginia, it is likely that these organisms have contributed to human cases reported as RMSF. It has also been suggested that *Ehrlichia chaffeensis*, a closely related species transmitted by *A. americanum* which causes acute symptoms similar to RMSF, may contribute to this misdiagnosis (Stromdahl et al., 2011).

In conclusion, *A*. *americanum* is by far the most commonly encountered human-biting tick in southeastern Virginia, harboring a high prevalence of *R. amblyommii*. The composition of the tick community is undergoing changes as two invading species, *A. maculatum* and *I. affinis*, now have established populations in this region. The SFGR populations have also undergone changes with the absence of *R. rickettsii* and the influx of *R. parkeri*-infected *A. maculatum*, posing a new health threat to humans. It remains to be seen how the dynamic ecology of human-biting ticks and their associated SFGR will impact human health in the mid-Atlantic region in coming decades. Information about tick and SFGR ecology collected in this study will help parameterize models that will be used to predict the risk of tick-borne infections.

CHAPTER 3

PREVALENCE OF *EHRLICHIA CHAFFEENSIS* AND *EHRLICHIA EWINGII* IN *AMBLYOMMA AMERICANUM* AND *DERMACENTOR VARIABILIS* COLLECTED FROM SOUTHEASTERN VIRGINIA, 2010-2011

INTRODUCTION

The lone star tick*, Amblyomma americanum* (L.) (Acari: Ixodidae), is found throughout the southeastern United States with populations extending west to central Texas and north to Iowa (Childs and Paddock, 2003). The eastern range of *A. americanum* extends through the mid-Atlantic region, with populations intermittently reported in New England states including Maine (Kierans and Lacombe, 1998), Connecticut and Rhode Island (Ijdo et al., 2000). *Amblyomma americanum* is the most commonly reported tick species collected from humans in the southeastern and mid-Atlantic U.S., representing over 60% of ticks collected from humans from New Jersey, Maryland, Virginia, Kentucky and South Carolina from 2004-2010 (Stromdahl and Hickling, 2012). In southeastern Virginia, *A. americanum* is the most commonly encountered human-biting tick, constituting over 95% of questing ticks collected from 2010-2012 (Nadolny et al., 2014). Because of the abundance of this tick in the southeastern U.S. and its propensity to feed on humans, pathogens transmitted by *A. americanum* pose an important threat to human health.

Chapter adapted from Wright, C.L., Gaff, H.D., Hynes, W.L. 2014. Ticks Tick Borne Dis. 5, 978-982.

Ehrlichia chaffeensis and *Ehrlichia ewingii* are the causative agents of human ehrlichiosis and are transmitted to humans and animals by infected *A. americanum* (Anziania et al., 1990 and Ewing et al., 1995). These *Ehrlichia* spp. have also been found in the American dog tick, *Dermacentor variabilis* (Say) (Murphy et al., 1998; Steiert and Gilfoy, 2002), although it is unclear whether *D. variabilis* is capable of transmitting these pathogens. Here we describe the prevalence of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in ticks collected from southeastern Virginia.

MATERIALS AND METHODS

Questing adult and nymphal *A. americanum* and adult *D. variabilis* were collected on flags from April through September of 2010 and 2011 from multiple locations representing 11 independent cities and counties in southeastern Virginia (Fig. 9). Nine sites were sampled on a weekly basis in 2010 and 12 sites were sampled on a weekly basis in 2011 (Nadolny et al., 2014). Within each site, the area of each transect was recorded so that density of host-seeking ticks encountered during each sampling event could be determined. Ticks were identified to species morphologically (Keirans and Litwak, 1989; Keirans and Durden, 1998) and individuals were pooled prior to DNA extraction. Adult *A. americanum* and *D. variabilis* collected at the same location in the same week were morphologically identified and then pooled into groups of up to 10. *Amblyomma americanum* nymphs were pooled into groups of up to 25. Prior to extraction all adult ticks were cut in half, one half was used for DNA extraction and the other stored at -80°C for future use. All ticks were homogenized by bead-beating with 1 mm glass beads. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc. Valencia, CA) following the manufacturer's protocol and stored at -20°C.

Samples were tested separately for *E. chaffeensis* and *E. ewingii* DNA using real-time quantitative PCR (qPCR) assays specific to each species. Both species were detected using TaqMan qPCR assays targeting the 16S rRNA gene (p. 108). The *E. chaffeensis* assay used 200nM of forward primer, 800nM of reverse primer, 100nM probe and 3µM $MgCl_2$. The *E. ewingii* assay used 800nM of forward and reverse primers, 100nM probe and 5µM $MgCl_2$. Thermocycling conditions for both assays consisted of

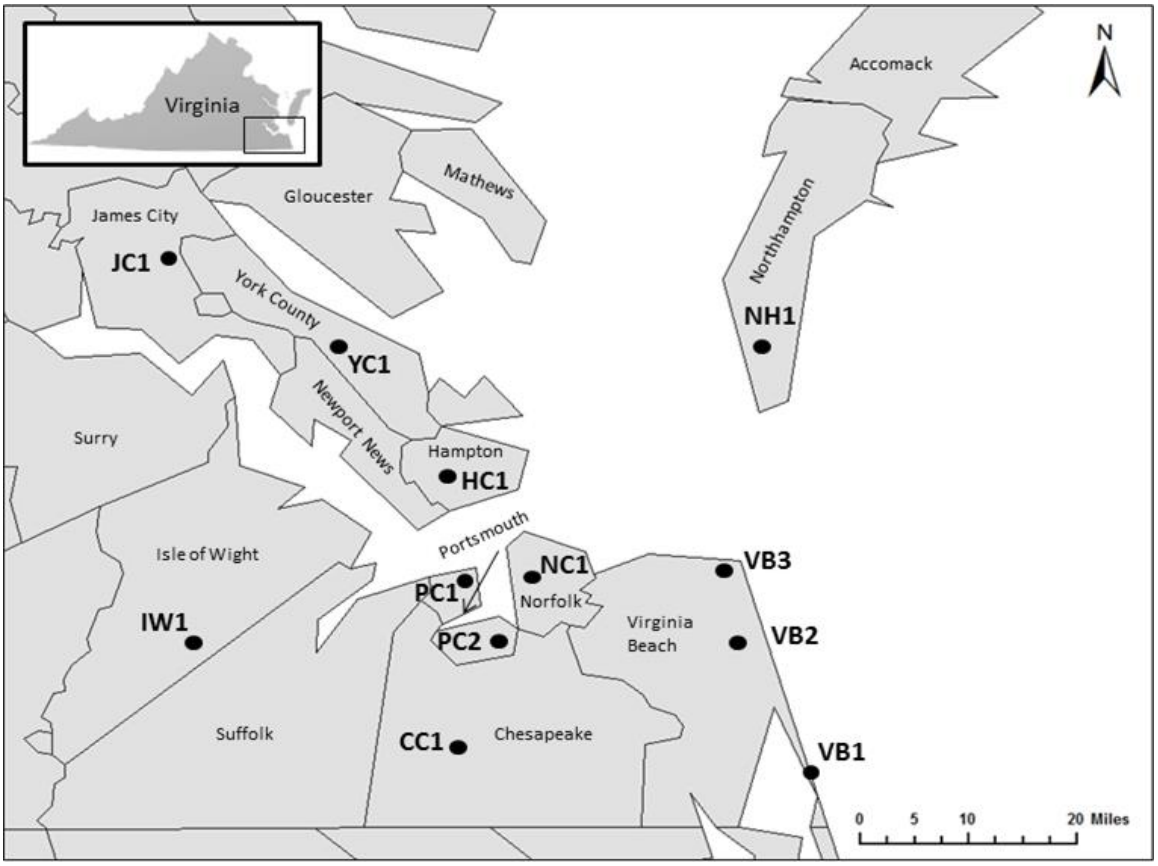

Fig. 9. Map of southeastern Virginia showing the location of the sites where ticks were collected in 2010 and 2011.

95°C for 10 min followed by 40 cycles of 95°C for 15s and 57°C for 60s.

A subset of qPCR-positive samples were confirmed by using a nested PCR assay to sequence either the *groEL* gene of *E. chaffeensis* (Tabara et al., 2007) or the *p28* gene of *E. ewingii* (Gusa et al., 2001). A total of 38 *E. chaffeensis* positive samples and 6 *E. ewingii* positive samples were sequence-confirmed. Sequences were analyzed by performing a BLAST search on GenBank (Altschul et al., 1990).

Because *A. americanum* samples were pooled prior to extraction, a maximum likelihood estimation (MLE) was used to approximate the true prevalence of *E. chaffeensis* and *E. ewingii* in the tick population. The software used to perform MLE (Biggerstaff, 2008) was acquired from the Centers for Disease Control and Prevention website (CDC).

RESULTS

A total of 605 *D. variabilis* adults and 8700 *A. americanum* adults and nymphs were collected during 2010 and 2011. The highest numbers of both species were collected in May and June in both years (Fig. 10). Although both *E. chaffeensis* and *E. ewingii* were detected in *A. americanum*, no evidence of either pathogen was found in the *D. variabilis* tested. Sequence confirmation of 44 positive samples showed either ≥99% match to *E. chaffeensis* or 100% match to *E. ewingii* in a BLAST search. A total of 967 and 981 *A. americanum* pools were tested for *E. chaffeensis* and *E. ewingii*, respectively. Because testing for each pathogen was performed at different times, not every sample was available for testing in both assays. Overall prevalence based on the MLE of *E. chaffeensis* in *A. americanum* adults and nymphs was 0.9% in 2010 and 0.6% in 2011; *E. ewingii* prevalence was 1.5% and 1.3% in 2010 and 2011, respectively (Table 6). A higher prevalence of both *Ehrlichia* spp. was found in adults than in nymphs, with adults having an approximate ten-fold greater prevalence of both pathogens (Table 6). In adults, prevalence of *E. chaffeensis* varied by location (Table 7), ranging from 0 to 4.3% (mean = 1.6 ± 1.4) in 2010 and 0 to 5.1% (mean = 1.1 ± 1.6) in 2011. Prevalence of *E. ewingii* in adults also varied by location, ranging from 0 to 8.2% (mean = 3.1 ± 2.6) in 2010 and 0 to 7.7% (mean = 2.8 ± 2.8) in 2011. The higher prevalence of *E. ewingii* relative to that of *E. chaffeensis* in adult *A. americanum* was mainly driven by one site in Virginia Beach, which had the highest prevalence of all sites (8.2% in 2010 and 7.7% in 2011). Although greater numbers of both *A. americanum* and *D. variabilis* were collected during May and June, there were no apparent spatial or temporal trends in

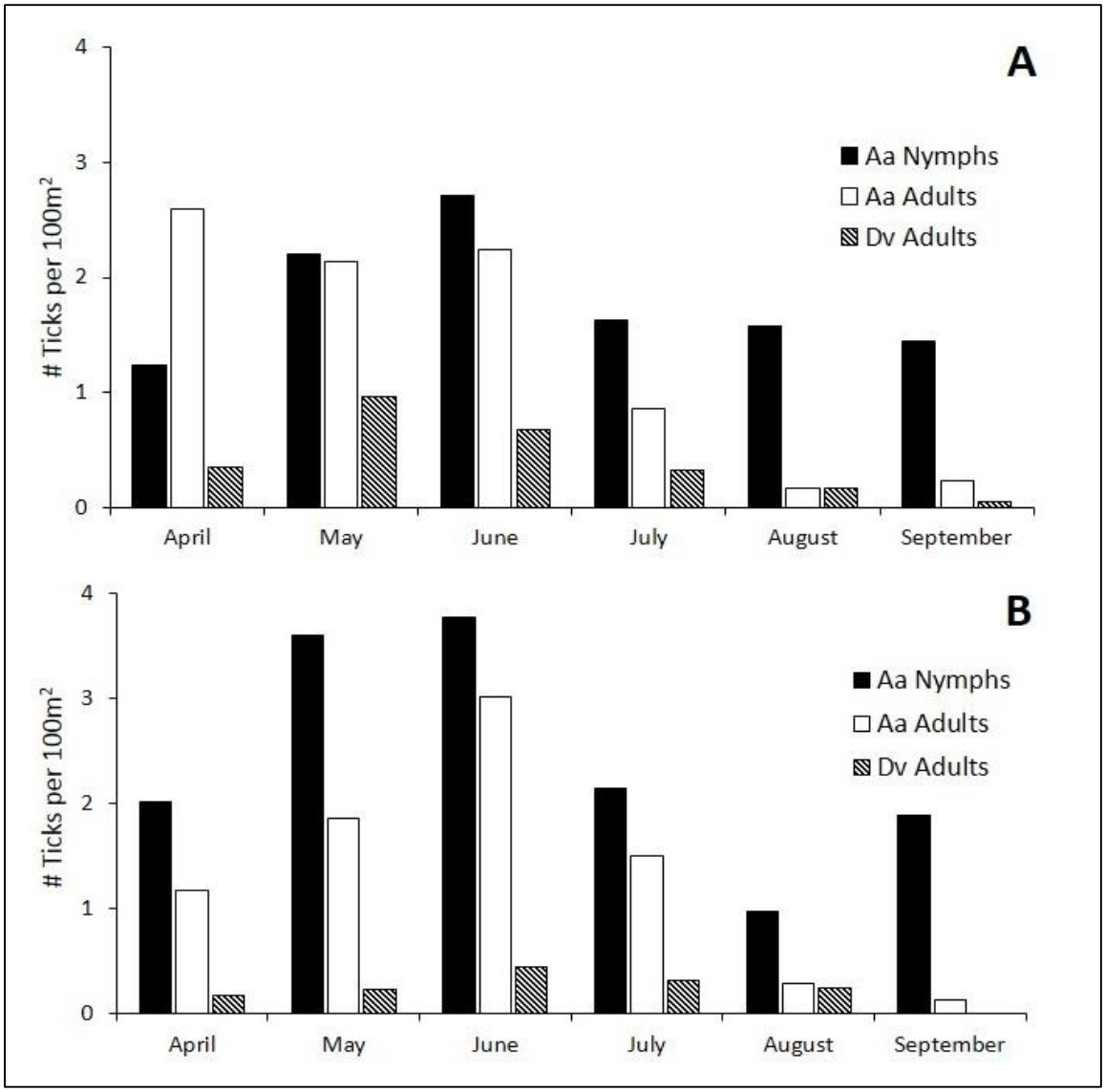

Fig. 10. Phenology of *Amblyomma americanum* nymphs and adults and *Dermacentor variabilis* adults collected during 2010 (A) and 2011 (B) from southeastern Virginia.

Table 6

Pooled and maximum likelihood estimated (MLE) prevalence of *Ehrlichia* spp. in questing adult and nymphal *Amblyomma americanum* and adult *Dermacentor variabilis* collected on flags from multiple sites within southeastern Virginia. To assess true pathogen prevalence from pooled DNA samples, a MLE calculation was used (Biggerstaff, 2008).

Tick	Year	Organism	Life Stage	Number Pools Positive	Number Pools Tested	# of Ticks	Pools Positive (%)	MLE (%)
A. americanum	2010	*E. chaffeensis*	All	28	417	3134	6.7	0.9
			Adult	25	221	1363	11.3	2.0
			Nymph	3	196	1771	1.5	0.2
		E. ewingii	All	45	426	3095	10.5	1.5
			Adult	42	231	1343	18.2	3.4
			Nymph	3	195	1752	1.5	0.2
	2011	*E. chaffeensis*	All	26	550	4814	4.7	0.6
			Adult	20	254	1483	7.8	1.4
			Nymph	6	296	3331	2.1	0.2
		E. ewingii	All	59	555	4813	10.6	1.3
			Adult	48	262	1532	18.3	3.5
			Nymph	11	293	3281	3.2	0.3
D. variabilis	2010	*E. chaffeensis*	Adult	0	69	259	0	-
		E. ewingii	Adult	0	69	259	0	-
	2011	*E. chaffeensis*	Adult	0	76	228	0	-
		E. ewingii	Adult	0	76	228	0	-

Table 7

Maximum likelihood estimated (MLE) prevalence of *Ehrlichia chaffeensis* (left) and *Ehrlichia ewingii* (right) in adult and nymphal *Amblyomma americanum* collected from various locations (Fig. 9) within southeastern Virginia in 2010 and 2011. The total number of individuals represented within pooled DNA samples is indicated.

Year	Site	*Ehrlichia chaffeensis*				*Ehrlichia ewingii*			
		MLE Prevalence (%)		# Ticks Represented		MLE Prevalence (%)		# Ticks Represented	
		Adults	Nymphs	Adults	Nymphs	Adults	Nymphs	Adults	Nymphs
2010	All	1.95	0.17	1363	1771	3.44	0.17	1343	1752
	CC1	4.33	0.85	50	121	3.50	0	59	99
	IW1	2.43	0	40	52	2.40	0	41	53
	JC1	1.09	0	92	155	5.80	0	82	153
	NH1	0	0	15	3	0	0	15	3
	PC1	2.23	0.27	630	746	2.10	0.13	624	733
	PC2	0	0	21	0	0	0	21	0
	VB1	1.04	0	93	183	3.50	0	91	185
	VB2	1.30	0	239	302	8.20	0.65	233	317
	YC1	2.30	0	183	208	2.40	0	177	208
2011	All	1.41	0.18	1483	3331	3.47	0.34	1532	3281
	CC1	0	0	203	495	2.09	0.41	203	495
	HC1	0	0	15	33	2.22	0	95	41
	IW1	5.08	0.65	41	152	5.58	0	38	48
	JC1	0	0	22	31	0	0	22	31
	NC1	0	0	4	2	0	0	4	2
	NH1	2.59	0	209	119	4.40	0	209	119
	PC1	1.31	0.06	393	1760	1.13	0.18	366	1707
	PC2	0	0	4	1	0	0	4	0
	VB1	2.05	0	101	83	7.06	1.31	97	77
	VB2	1.94	0.95	216	451	7.72	0.91	217	451
	VB3	0	0	1	3	0	0	1	3
	YC1	0.74	0	274	201	3.18	0	276	202

prevalence of either *Ehrlichia* spp. (Table 7).

To validate the accuracy of the maximum likelihood estimation, leftover individual adult *A. americanum* halves from pools which tested positive for *E. chaffeensis* were extracted and tested by qPCR for *E. chaffeensis*. A MLE was then performed to determine *E. chaffeensis* prevalence within these individually extracted samples. Pooled samples had an overall *E. chaffeensis* prevalence of 1.95% in 2010, whereas when MLE analysis of individually extracted samples indicated a prevalence of 2.01%. Since these prevalence values are similar, this experiment validates the accuracy of the MLE calculation, which has been used extensively to estimate the prevalence of vector-borne disease agents in studies with pooled samples.

DISCUSSION

We describe the collection of both *A. americanum* and *D. variabilis* in southeastern Virginia, as well as the presence of both *E. chaffeensis* and *E. ewingii* in questing *A. americanum* nymphs and adults. Although *D. variabilis* has occasionally been shown to harbor these pathogens, we found no evidence of either pathogen in any *D. variabilis* collected in this study. The prevalence of *E. chaffeensis* (1.4 – 2.0%) and *E. ewingii* (3.4 – 3.5%) in adult *A. americanum* is comparable to the prevalence of *E. chaffeensis* (2.2%) and *E. ewingii* (2.2%) determined in another study assessing the rate of *Ehrlichia* spp. infection in *A. americanum* adults collected in 2012 (Gaines et al., 2014). The study, which assessed the prevalence of *Ehrlichia* spp. in *A. americanum* adults collected throughout the state of Virginia, found that *E. chaffeensis* prevalence ranged from 0 – 24.5% and *E. ewingii* prevalence ranged from 0 – 14.3% (Gaines et al., 2014). The lower infection prevalence in *A. americanum* nymphs is consistent with other studies assessing the prevalence of *Ehrlichia* spp. in questing ticks. *Amblyomma americanum* nymphs collected in Maryland were determined to have an *E. chaffeensis* minimum infection rate (MIR) of just 0.8%, while adults showed a MIR prevalence of 3.5% (Stromdahl et al., 2000). Given that the white-tailed deer (*Odocoileus virginianus*) is a known reservoir of *Ehrlichia* spp. (Ewing et al., 1995; Lockhart et al., 1997) it is not surprising that questing *A. americanum* adults, which have taken two bloodmeals in their lifetime, would have a greater prevalence than questing nymphs, which would have taken just one bloodmeal.

Other studies have noted the great abundance of *A. americanum* present in the southeastern and south-central United States in relation to other sympatric tick species (Stromdahl and Hickling, 2012; and Nadolny et al., 2014). All three *A. americanum* life stages (larva, nymph and adult) are known to aggressively parasitize humans and multiple concurrent tick bites of this species are often reported. Stromdahl and Hickling (2012) observed that approximately 15% of persons submitting *A. americanum* to the DOD for testing submitted multiple specimens. Because of the high proportion of *A. americanum* in this area and the propensity of this species to seek out human hosts in both the nymphal and adult stages, pathogens present even in low numbers within these populations warrant attention as concerns to public health. Furthermore, this study found no uniformity in geographic distribution of either *Ehrlichia* species in *A. americanum,* indicating a potential for disease "hotspots" in areas where these pathogens are more abundant.

CHAPTER 4

EXPERIMENTAL VERTICAL TRANSMISSION OF *RICKETTSIA PARKERI* IN THE GULF COAST TICK, *AMBLYOMMA MACULATUM*

INTRODUCTION

Rickettsia parkeri is one of the spotted fever group rickettsiae (SFGR), a collection of obligate intracellular bacteria transmitted to humans and other animals by invertebrate vectors. First discovered in 1937 in the Gulf Coast tick, *Amblyomma maculatum* Koch (Parker et al., 1939)*, R. parkeri* was recognized as being pathogenic to humans in 2002 when it was isolated from a man residing in Virginia (Paddock et al., 2004). Since then there have been over 37 documented cases of *R. parkeri* infection, referred to either as "Tidewater spotted fever" or "*R. parkeri* rickettsiosis," in the United States (Paddock and Goddard, 2015). Infection with *R. parkeri* can be difficult to differentiate from infections caused by other SFGR, including *R. rickettsii*, the agent of Rocky Mountain spotted fever (RMSF); because of this, *R. parkeri* infections may have been misreported as cases of RMSF. Many SFGR infections manifest similarly, often characterized by a macropapular rash and flu-like symptoms. Serological testing fails to discriminate between infections resulting from SFGR species and such infections are now reported as "Spotted Fever Rickettsiosis". This new category encompasses infections caused by other members of the SFGR, including *R. parkeri*.

In the U.S. the primary vector of *R. parkeri* is *A. maculatum*, although

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Dermacentor variabilis Say and *Amblyomma americanum* (L.) have been shown to harbor *R. parkeri* (Cohen et al., 2009; Henning et al., 2014). In South America, *R. parkeri* has been detected in *Amblyomma triste* Koch (Silveira et al., 2007; Venzal et al., 2004; Nava et al., 2008) and *Amblyomma tigrinum* Koch (Tomassone et al., 2010; Romer et al., 2014). The prevalence of *R. parkeri* within the U.S. in *A. maculatum* populations ranges from 5 to 56% (Cohen et al., 2009; Sumner et al., 2007; Trout et al., 2010; Paddock et al., 2010; Fornadel et al., 2011; Varela-Stokes et al., 2011; Wright et al., 2011; Ferrari et al., 2012; Jiang et al., 2012; Nadolny et al., 2014). Historically the geographic range of *A. maculatum* in the U.S. has been the southeastern U.S., but recently this tick has been expanding northward. Tick surveys conducted in Virginia in the 1970s produced the occasional *A. maculatum* (Sonenshine, 1979); however, no established populations were encountered and their sporadic presence was suggested to result from migratory birds. Now, multiple established populations of *A. maculatum* are reported in southeastern (Wright et al., 2011) and northern (Fornadel et al., 2011) Virginia. Interestingly, these northernmost populations of *A. maculatum* have a higher prevalence of *R. parkeri* (41.4 to 55.7%) than populations in the southern U.S. (5 to 33%). The reason for the geographic variation in *R. parkeri* prevalence is unclear.

While *R. parkeri* is a pathogenic SFGR transmitted primarily by *A. maculatum* in the U.S., little is known about the enzootic cycle of *R. parkeri* and its interaction with *A. maculatum*, including how the pathogen is maintained within *A. maculatum* populations. Pathogen transmission in ticks is complex and not fully understood; in general ticks acquire and transmit pathogens vertically and/or horizontally. Vertical

transmission occurs when a pathogen is transmitted from an infected female to her offspring. Horizontal transmission occurs when a pathogen is transmitted to and from a vertebrate host. This study investigates the vertical transmission of *R. parkeri* within a field-derived, naturally-infected *A. maculatum* colony and determines whether this pathogen imposes any reproductive fitness costs on the tick.

MATERIALS AND METHODS

Tick rearing. Two separate colonies of *A. maculatum* were derived from ticks collected from a Virginia field site and propagated in the tick rearing facilities at Old Dominion University. Questing adult *A. maculatum* (n=18) were collected from Back Bay National Wildlife Refuge in Virginia Beach in July of 2013 by flagging. Questing adult ticks were identified to species level based on morphological characteristics (Keirans & Litwak 1989). Male and female ticks were placed on a pathogen-free New Zealand white rabbit and allowed to feed to repletion. Replete females (n=13) were placed into individual vials and stored in an incubator kept at 26°C, with 94% relative humidity with 14 hours of light and 10 hours of dark. When females had completed oviposition, which lasted approximately 21 days, DNA from individual females and from a sample of each egg mass was extracted and tested for *R. parkeri* (methods described below). To ensure the absence of other SFGR in the *R. parkeri*-negative egg masses, DNA from these samples was subjected to a sensitive *Rickettsia* genus level real-time PCR assay (described below). Upon testing negative, these individual eggs were propagated as the *R. parkeri*-free colony, and the positive egg masses were propagated as the *R. parkeri*-infected colony. Offspring from each female (eggs, larvae and nymphs) were propagated in separate vials and on separate animals and adults from each colony were pooled together and fed on single animals to propagate the new generations. To ensure the absence of *R. parkeri* in the *R. parkeri*-free colony, individuals from F_1-F_3 were randomly sampled and subjected to the *R. parkeri* real-time PCR assay.

Each colony was propagated on separate animals for three generations. Larval and nymphal *A. maculatum* were fed on pathogen-free mice while adults were fed on either New Zealand white rabbits or Hartley guinea pigs. Tick feeding protocols were performed according to the approved Institutional Animal Care and Use Committee (IACUC) protocols 10-018, 10-032 and 12-003.

DNA extraction. The infection status of *A. maculatum* was determined using real-time PCR on DNA extracted from *A. maculatum* eggs, larvae, nymphs and adults. Skin tissue from the area where ticks were attached was sampled from rabbits and guinea pigs after feeding *R. parkeri*-infected F_1 and F_2 adults. Quantitative real-time PCR (qPCR) was used because it allows for sensitive and specific amplification of *R. parkeri* DNA (Jiang et al., 2012). To extract DNA from eggs and larvae, individual eggs and larvae were placed in 0.2 mL PCR tubes and manually crushed with a sterile toothpick; 10 µL of ddH_2O was added to each tube followed by incubation at 95°C for 10 minutes. DNA samples were placed on ice and used immediately for real-time PCR. DNA from whole nymphs, adults and skin tissue was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Prior to extraction, the ticks or skin tissue were placed in a 2 mL microcentrifuge tube containing an equal mixture of 1 mm and 2.5 mm glass beads and homogenized in a bead-beater (BioSpec Products, Inc., Bartlesville, OK) for 30 - 45 seconds. DNA was extracted following the manufacturer's protocol with the DNA eluted in 50-100 µL of buffer AE; the eluted DNA was stored at -20°C until processed.

Pathogen detection. To determine whether ticks were infected with *R. parkeri*, a real-time PCR assay specific to the *ompB* gene of *R. parkeri* was used (Jiang et al., 2012).

Although no quantification was performed, real-time PCR was used because of the sensitivity allowing for detection of low copy number targets. DNA from F_1 egg masses negative for *R. parkeri* was tested for the presence of other *Rickettsia* spp. using the 17-kD antigen gene in a real-time PCR assay specific to SFG *Rickettsia* (Jiang et al., 2012). All PCR reactions were performed using a Mini-Opticon Real-Time PCR System (BioRad Inc., Hercules, CA). Reactions were carried out in 20 µL volumes using 2X EconoTaq PLUS Master Mix (Lucigen Inc., Middleton, WI) and 3 µL of DNA template. The *R. parkeri* assay used 0.7 µM each of Rpa129F and Rpa224R and 0.4 µM of Rpa188probe (p. 108) with a final $MgCl_2$ concentration of 8 mM. The *Rickettsia* genus assay used 0.5 µM each of R17K128F2 and R17K238R and 0.4 µM of R17Probe (p. 108) with a final $MgCl_2$ concentration of 5 mM. The PCR protocol for both assays consisted of a 2 minute denaturation at 95°C followed by 45 cycles of 95°C for 15s and 60°C for 30s.

To confirm the presence of *R. parkeri* in colony-reared *A. maculatum*, DNA was extracted from a subset of F_2 adults from the infected colony and a nested PCR reaction targeting the *ompA* gene was performed (Fournier et al., 1998); the resulting amplicon was sequenced. Outer primers 190-3588F and 190-5238R were used to amplify a 1631 bp fragment, and nested primers RhoA4336F and 190-5044R (p. 108) were used to amplify a 709 bp fragment. Reactions were carried out in 20 µL volumes using 2X EconoTaq PLUS Master Mix (Lucigen Inc., Middleton, WI), 0.5 µM of each primer, and 3 µL of DNA template (outer reaction) and 1 µL PCR product (inner reaction). Thermocycling conditions consisted of 95°C for 3 minutes followed by 35 cycles (outer) and 20 cycles (inner) of 95°C for 30 seconds, 45°C for 60 seconds and 72°C for 2 minutes,

followed by a final extension at 72°C for 7 minutes. PCR products were visualized on a 1.8% agarose gel, purified and a sequencing reaction performed following the BigDye Terminator v3.1 Cycle Sequencing Kit specifications (Life Technologies, Grand Island, NY, USA). Purified products from sequencing reactions were run on an ABI 3130xL Genetic Analyzer (Life Technologies, Grand Island, NY, USA), and a BLAST search was performed on *ompA* sequences.

Fitness estimates. Fitness costs resulting from *R. parkeri* infection were calculated by comparing *R. parkeri*-free and *R. parkeri*-infected *A. maculatum* colonies. Fitness measures included the egg production index (EPI), where the weight of the egg mass was determined approximately 21 days after the first day of oviposition; estimated reproduction (ER), where the approximate number of larvae resulting from 1 g of eggs (~19,610 eggs) was determined by weighing groups of 20 eggs calculating the average weight per egg; and the percent reduction of ER (PRER) (Bennett 1974, Drummond et al., 1973, Paião et al., 2001). These values were determined using the following equations:

$$\textbf{EPI} = \frac{\text{egg weight (g)}}{\text{initial female weight (g)}} \times 100$$

$$\textbf{ER} = \frac{\text{egg weight (g)}}{\text{initial female weight (g)}} \times \%\ \text{hatching} \times 19{,}610$$

$$\textbf{PRER} = \frac{\text{ER (clean)} - \text{ER (infected)}}{\text{ER (clean)}} \times 100$$

Climbing experiments. Differences in questing behavior of *R. parkeri*-free and *R. parkeri*-infected *A. maculatum* adults were determined by measuring the height to which questing adults would climb. A total of 76 F_1 adults (38 male, 38 female) and 62 F_2 adults (33 male, 29 female) were observed. All climbing experiments were performed in a walk-in incubator kept at temperature of 19.4-21.7°C and relative humidity of 83-91%. Adult ticks were placed at the base of a 100 cm wooden rod measuring approximately 1 cm in diameter. Ticks were allowed to climb for 30 minutes and the highest height (cm) to which each tick climbed was recorded.

Statistical analyses. To determine any significant differences between *R. parkeri*-free and *R. parkeri*-infected *A. maculatum* fitness results, including climbing experiments, Mann-Whitney U tests were performed. To determine significant differences in molting success between *R. parkeri*-free and *R. parkeri*-infected *A. maculatum* Fisher's exact tests were used. Values were considered significant at the $\alpha=0.05$ level. All statistical analyses were performed using SPSS software (IBM, Inc.).

RESULTS

Colony establishment and tick fitness. DNA from all 13 replete F_0 females and from 7 of 13 F_1 egg mass samples tested positive for *R. parkeri*. Although unlikely that all 13 females were initially infected *R. parkeri*, it is possible that *R. parkeri*-negative individuals became infected due to feeding in close proximity to infected ticks. To ensure the absence of *R. parkeri* in the *R. parkeri*-free clutches, samples from each successive life stage (larvae, nymphs, adults) resulting from each of the six *R. parkeri*-negative egg clutches were tested for *R. parkeri*. Of the six negative egg clutches, two pools of hatched larvae were positive for low levels of *R. parkeri* DNA. These two clutches were discarded so that only individuals from lines showing consistently-negative PCR results, from eggs, larvae, nymphs and adults, were used to propagate the uninfected colony. Pools of larvae and nymphs that were *R. parkeri*-negative were also shown to be *Rickettsia* spp. negative based on the genus-level real-time PCR. *Amblyomma maculatum* colonies were reared in the laboratory for three generations, through the F_3 adult stage. *R. parkeri* was detected in the infected colony in each generation and life stage by real-time PCR. The presence of *R. parkeri* was additionally confirmed by sequencing and performing a BLAST search on a 599 bp fragment of the *ompA* gene of two *R. parkeri*-infected F_2 adult *A. maculatum* DNA extracts. The *ompA* sequences were 100% identical with GenBank accession numbers KJ741849, CP003341 (*R. parkeri* strain Portsmouth) and U83449 (*R. parkeri* Maculatum 20). *R. parkeri ompA* sequences were deposited into GenBank under accession numbers KP235202 and

KP235203. The F_1 egg masses negative for *R. parkeri* also tested negative for *Rickettsia* spp., indicating that no other SFGR was present in the *R. parkeri*-free colony.

Within the three generations of *R. parkeri*-infected *A. maculatum*, no significant difference in reproductive fitness was observed between *R. parkeri*-infected and *R. parkeri*-free *A. maculatum* colonies. Although the overall engorged female weight, egg mass weight, and egg production index was generally higher in the *R. parkeri*-free colony than in the *R. parkeri*-infected colony, these differences were not statistically significant (Table 8). Additionally, there were no significant differences in the egg hatchrates or estimated reproduction between the colonies. The overall percent reduction in estimated production (PRER) was -4.73%, indicating the estimated reproduction of the *R. parkeri*-infected colony was slightly higher than that of the uninfected colony (Table 9).

To determine whether *R. parkeri* affects molting success of *A. maculatum*, the molt success of fed larvae and of fed nymphs of both the *R. parkeri*-infected and *R. parkeri*-free colonies was determined (Table 10). Although there was no data for F_1 larval molt success, a highly significant difference was observed between *R. parkeri*-free and *R. parkeri*-infected F_2 larval molting ($p<0.001$), with significantly more *R. parkeri*-free larvae failing to molt. A significant difference ($p=0.035$) was also observed between F_1 nymphs, with fewer *R. parkeri*-free nymphs successfully molting. No other significant differences were observed in molt success.

Table 8

Egg production index, engorged female weight, and egg mass of *Rickettsia parkeri*-free and *Rickettsia parkeri*-infected *Amblyomma maculatum.* Infected lines represent offspring from a single egg clutch that consistently tested positive for *Rickettsia parkeri*. *Rickettsia parkeri*-free lines represent offspring from a single egg clutch that consistently tested negative for *Rickettsia parkeri*. F_1 and F_2 generations were propagated from adults from cohorts that consistently tested positive (infected line) or negative (*Rickettsia parkeri*-free) for *Rickettsia parkeri* by qPCR. Values represent mean ± standard error.

Generation	Engorged female weight (mg)		Egg mass weight (mg)		Egg Production Index	
F_0	*Infected (n=7)*	*Rp-free (n=4)*	*Infected (n=7)*	*Rp-free (n=4)*	*Infected (n=7)*	*Rp-free (n=4)*
	948.5 ± 232.0	1072.9 ± 138.4	585.9 ± 155.2	641.1 ± 96.6	61.5 ± 4.3	59.9 ± 6.0
F_1	*Infected (n=6)*	*Rp-free (n=6)*	*Infected (n=6)*	*Rp-free (n=6)*	*Infected n=6*	*Rp-free n=6*
	867.3 ± 147.9	996.2 ± 129.2	531.2 ± 101.5	603.8 ± 88.3	61.1 ± 3.2	61.0 ± 7.8
F_2	*Infected (n=5)*	*Rp-free (n=4)*	*Infected (n=5)*	*Rp-free (n=4)*	*Infected n=5*	*Rp-free n=4*
	982.4 ± 104.6	1082.4 ± 127.0	545.2 ± 54.1	672.1 ± 117.5	55.8 ± 6.0	61.8 ± 4.3
Total (F_0 – F_2)	*Infected (n=18)*	*Rp-free (n=14)*	*Infected (n=18)*	*Rp-free (n=14)*	*Infected (n=18)*	*Rp-free (n=14)*
	930.9 ± 174.2	1010.4 ± 153.8	556.4 ± 113.3	617.2 ± 105.5	59.5 ± 4.5	60.9 ± 6.0

Table 9

Larval hatchrates, estimated reproduction (ER), and percent reduction in estimated reproduction (PRER) of *Rickettsia parkeri*-free and *Rickettsia parkeri*-infected *Amblyomma maculatum*. Values presented in tables 1 and 2 are derived from the same females and egg clutches. Values represent mean ± standard error.

Generation	Hatchrate		Estimated Reproduction		PRER
F_0	*Infected (n=7)*	*Rp-free (n=4)*	*Infected (n=7)*	*Rp-free (n=4)*	
	0.90 ± 0.18	0.76 ± 0.11	10,857.9 ± 2431.2	9011.4	-20.5%
F_1	*Infected n=5*	*Rp-free n=6*	*Infected n=5*	*Rp-free n=6*	
	0.98 ± 0.023	0.88 ± 0.12	11,897.6 ± 522.0	10,563.4 ± 2259.5	-12.6%
F_2	*Infected n=5*	*Rp-free n=4*	*Infected n=5*	*Rp-free n=4*	
	0.79 ± 0.093	0.88 ± 0.12	8,673.8 ± 1748.0	10,700.1 ± 2051.8	18.9%
Total (F_0 – F_2)	*Infected n=17*	*Rp-free n=14*	*Infected n=17*	*Rp-free n=14*	
	0.89 ± 0.14	0.84 ± 0.12	10,521.3 ± 2181.0	10,136.9 ± 1984.8	-4.73%

Table 10
Molt success of infected and clean-line *Amblyomma maculatum*. Molt success was determined by counting individuals that successfully molted and those that failed to molt (died), post-bloodmeal. Molt success rates were determined by sampling all individuals of each colony.

Generation	Molt Success (Larva to Nymph)		Molt Success (Nymph to Adult)	
	Infected	Clean	Infected	Clean
F_1	No data	No data	41/41 (100%)	8/10 (80%)*
F_2	181/212 (85%)	276/381 (72%)*	23/26 (88%)	54/58 (93%)
F_3	78/94 (83%)	35/38 (92%)	13/15 (87%)	9/10 (90%)
Total	259/306 (85%)	311/419 (74%)*	77/82 (94%)	76/83 (92%)

*Molting success between infected and clean groups were significantly different (p<0.05).

No differences in the questing height of *R. parkeri*-infected and uninfected *A. maculatum* adults, sampled from both the F_1 and F_2 generations, were observed. The uninfected adults climbed an average of 65.9 cm (± 3.5 cm, standard error) and the infected adults climbed an average of 63.0 cm (± 3.8 cm, standard error). This difference in questing heights was not statistically significant.

***R. parkeri* transmission**. *Amblyomma maculatum* transovarially (female to offspring) and transstadially (between life stages) transmitted *R. parkeri.* Efficiency of transovarial transmission was determined by calculating the percentage of positive eggs of the total number of eggs tested. The overall efficiency of transovarial transmission was 83.7%, ranging from 66.7% in the F_2 generation to 100% in the F_3 generation (Table 11). Transstadially, the larva-to-nymph and nymph-to-adult transmission rates of *R. parkeri* were both 100% for all three generations (Table 11).

Skin tissue DNA from the rabbit and the guinea pig on which F_1 and F_2 *R. parkeri*-infected adults fed, respectively, tested positive for *R. parkeri*.

Table 11

Efficiency of transovarial and transstadial transmission of *Rickettsia parkeri* in *Amblyomma maculatum.*

Generation	Life Stage	# positive / # tested (% infected)	# hatched or molted / total #
F_1	Eggs	49/56 (87.5%)	627/700 (90%)
	Larvae	60/70 (85.7%)	No data
	Nymphs	17/17 (100%)	41/41 (100%)
	Adults	15/15 (100%)	-
F_2	Eggs	40/60 (66.7%)	490/500 (98%)
	Larvae	43/60 (71.6%)	181/212 (85%)
	Nymphs	43/50 (86%)	23/26 (88%)
	Adults	4/7 (57.1%)	-
F_3	Eggs	50/50 (100%)	393/500 (79%)
	Larvae	49/50 (98%)	78/94 (83%)
	Nymphs	16/16 (100%)	13/15 (87%)
	Adults	11/11 (100%)	-

DISCUSSION

Despite knowing that *A. maculatum* is the primary vector for *R. parkeri* in the U.S., no studies have assessed the vertical transmission dynamics for this pathogen in its host vector. The current study addresses questions related to how *R. parkeri* is maintained within a naturally-infected, laboratory-reared Gulf Coast tick colony, and whether *R. parkeri* imposes any reproductive fitness costs on this tick. To minimize fitness effects resulting from potential colony inbreeding, *A. maculatum* colonies were only propagated and observed for three generations.

Although no studies specifically assessing vertical transmission of *R. parkeri* in *A. maculatum* have been reported, other studies investigated various routes of *R. parkeri* transmission in a number of tick species. Goddard (2003) determined that the lone star tick, *Amblyomma americanum*, can vertically maintain *R. parkeri* infection for two generations after infection by inoculation. Horta et al. (2010) investigated horizontal transmission of *R. parkeri* between big-eared opossums (*Didelphis aurita*) and *Amblyomma* spp. and found that one *A. cajennense* (2%) became infected with *R. parkeri* after taking a bloodmeal. Moraru et al. (2013) determined that *A. maculatum* nymphs feeding on either northern bobwhite quail (*Colinus virginianus*) or hispid cotton rats (*Sigmodon hispidus*) infected with *R. parkeri* failed to acquire the pathogen.

Nieri-Bastos et al. (2013) studied *R. parkeri* transmission in the related tick species, *Amblyomma triste* Koch, a vector for the pathogen in South America. Unlike the results obtained with *A. maculatum* in this study, *R. parkeri* significantly reduced the molting success of engorged *A. triste* nymphs. The opposite was seen in *R. parkeri*-

infected *A. maculatum*, with these ticks showing a significantly increased molt success with F_2 larvae and F_1 nymphs. No significant differences in reproductive fitness, as assessed by measuring egg production, were observed in either *A. maculatum* or *A. triste*; however, in the F_1 and F_2 generations of *A. triste*, the egg hatch rates of the infected group was significantly lower than the control group (Nieri-Bastos et al., 2013). Such a decrease was not observed with *A. maculatum*. Furthermore, no differences in questing height between infected and non-infected ticks were observed. These data suggest that *R. parkeri* may be better adapted to an association with *A. maculatum* than to one with *A. triste*. Taken together, the results from prior investigations and the present study indicate that *R. parkeri* is efficiently maintained by vertical transmission in ticks and less-efficiently so by horizontal transmission between ticks and vertebrate animals.

Another route of pathogen transmission to ticks is transmission by co-feeding, which occurs when a tick ingests a microorganism obtained during a bloodmeal due to a recent or proximal feeding event by an infected tick. Co-feeding transmission differs from horizontal transmission in that it occurs in the absence of a systemic infection in the animal (Randolph et al., 1996). Efficient transmission by co-feeding was observed with *Rickettsia conorii*, with 92-100% of uninfected *Rh. sanguineus* nymphs acquiring the pathogen by co-feeding with infected adults (Zemstova et al., 2010). Although co-feeding transmission was not investigated in this study, it is interesting to note that all 13 field-caught females that were fed on a rabbit tested PCR-positive for *R. parkeri* after simultaneously taking a bloodmeal, despite only 7 of the 13 resulting egg masses testing

positive for the pathogen. *R. parkeri* prevalence in southeastern Virginia ranges from 43.1 to 56% (Wright et al., 2011; Nadolny et al., 2014) making it unlikely that all 13 females collected by flagging were initially positive for *R. parkeri*. Thus, it is possible that uninfected female ticks acquired *R. parkeri* by co-feeding with infected ticks on a rabbit but were not transmitted transovarially as the rickettsiae were unable to establish infection within the ovaries prior to oviposition.

Given the high prevalence of *R. parkeri* in certain *A. maculatum* populations and the efficiency by which female ticks transovarially transmit this microorganism, it is not surprising there were no fitness costs to the infected ticks. Given the ability of *A. maculatum* to efficiently maintain *R. parkeri* without detriment to reproduction or questing behavior, these results indicate the tick is a suitable reservoir host in this enzootic cycle and suggest that vertebrate animals may not be absolutely necessary for *R. parkeri* to be maintained in *A. maculatum* populations. The propensity of *Rickettsia* spp. to rely on vertical transmission stands in stark contrast with other members of the Rickettsiales order, such as *Ehrlichia* and *Anaplasma*, which rely heavily on vertebrate reservoir hosts and horizontal transmission to survive (Munderloh and Kurtti, 1995; Long et al., 2003).

Perlman et al. (2006) suggested that given the long history of *Rickettsia* spp. as a vertically-transmitted invertebrate endosymbiont, this genus would eventually evolve horizontal transmission strategies. Although this study did not specifically address the efficiency of horizontal *R. parkeri* transmission, this idea was substantiated in part in that *R. parkeri* was found to be efficiently maintained by transovarial transmission. The

lack of fitness cost to the invertebrate host and gaining of horizontal transmission are factors that could have led to *R. parkeri* emerging as a pathogen of vertebrate animals and humans.

Not all *Rickettsia* species have symbiotic relationships with invertebrates. *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever has been shown to result in mortality of *Dermacentor andersoni,* one of the vectors for this pathogen (Niebylski et al., 1999). The detrimental effect of this SFGR on its vector may explain the low prevalence of this organism in field-collected ticks (Nadolny et al., 2014, Moncayo et al., 2010; Stromdahl et al., 2011). In contrast, *R. parkeri* has a relatively high prevalence (20 - 56%) in U.S. *A. maculatum* populations which may in part be explained by the absence of any fitness costs and potential for increased molt success. The lack of detriment to its vector and increased molt success could be an important facilitator of higher rates of infection in *A. maculatum* populations, which in turn could have negative consequences for public health.

CHAPTER 5

RICKETTSIA PARKERI TRANSMISSION TO *AMBLYOMMA AMERICANUM* BY CO-FEEDING WITH *AMBLYOMMA MACULATUM* AND POTENTIAL FOR SPILLOVER

INTRODUCTION

Commonly known as the lone star tick, *Amblyomma americanum* (L.), was first described by Carl Linnaeus in 1758. *A. americanum* is a three-host, non-nidiculous tick with all three life stages (larva, nymph and adult) known to seek out large vertebrate hosts, including humans. Currently the distribution of *A. americanum* spans much of the southeastern and mid-Atlantic portion of United States. The range of this tick has been expanding, with populations reported as far north as New York, Connecticut and Rhode Island (Ginsberg et al., 2002; Ijdo et al., 2000) and, more recently westward in Nebraska (Cortinas and Spomer, 2013). In the southeastern states, *A. americanum* is the most abundant human-biting tick encountered (Stromdahl and Hickling, 2012; Merten and Durden, 2000; Nadolny et al., 2014), representing approximately 95% of questing ticks collected in Virginia (Nadolny et al., 2014) and 70-95% of ticks collected from humans residing in New Jersey, Maryland, Virginia, Kentucky, and South Carolina (Stromdahl and Hickling, 2012).

Rickettsia parkeri is a member of the spotted fever group rickettsiae (SFGR) and causes an infection in humans referred to as "Tidewater spotted fever" (Wright et al., 2011), "*R. parkeri* rickettsiosis" (Paddock et al., 2004; Paddock et al., 2008) or

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"American Boutonneuse fever" (Goddard, J. 2004). Although *R. parkeri* is primarily transmitted in the U.S. by the Gulf Coast tick, *Amblyomma maculatum* Koch, *R. parkeri* has been found at a very low prevalence in *A. americanum* collected from the southeastern U.S. From 2005-2007, only 1 of 446 *A. americanum* from Tennessee and 1 of 418 *A. americanum* from Georgia tested positive for *R. parkeri* (Cohen et al., 2009). In 2012, *R. parkeri* was detected in 1% of questing *A. americanum* adults (n=206) and 0.4% of questing *A. americanum* nymphs (n=1381) in Virginia (Gaines et al., 2014). Although it is currently unknown whether *A. americanum* is a competent vector of *R. parkeri*, the high proportion and ubiquity of *A. americanum* populations relative to *A. maculatum* populations suggests that *A. americanum* may play a role in *R. parkeri* maintenance and transmission.

R. parkeri is transovarially maintained in *A. maculatum* (Wright et al., 2015). The role vertebrate hosts play in the maintenance and transmission of *R. parkeri* in tick populations is unclear. It is likely that transmission to *A. americanum* is through a shared vertebrate host. White-tailed deer (*Odocoileus virginianus*) and coyotes (*Canis latran*s) are preferred hosts of adult *A. americanum* (Childs and Paddock, 2003) and adult *A. maculatum* are often found parasitizing large mammals, such as carnivores and ungulates, including white-tailed deer (Teel et al., 2010). *A. americanum* larvae and nymphs will feed on small animals, such as ground-dwelling birds and rodents, but will also actively seek out medium and large-sized hosts, such as wild turkey (*Meleagris gallopavo*), red foxes (*Vulpes vulpes*) and white-tailed deer (Teel et al., 2010). *A. maculatum* larvae and nymphs are rarely found on medium and large animals and

preferentially feed on smaller hosts such as passerine birds and rodents (Teel et al., 2010).

Rickettsia amblyommii is another SFGR member, and although there is only limited evidence of it being pathogenic to humans (Apperson et al., 2008; Billeter et al., 2007), it is found in up to 84% of tested *A. americanum* populations (Childs and Paddock, 2003; Mixson et al., 2006; Moncayo et al., 2010; Nadolny et al., 2014; Sayler et al., 2014; Smith et al., 2010). In southeastern Virginia, the prevalence of *R. amblyommii* in *A. americanum* adults collected in 2010 and 2011 was 33.4% and 44.3%, respectively (Nadolny et al., 2014). In *A. americanum* adults collected throughout Virginia during 2012, the prevalence of *R. amblyommii* ranged from 64 to 100% (Gaines et al., 2014).

The first objective of this investigation was to determine whether *R. parkeri* is spilling over into *A. americanum* populations within eastern Virginia. Given the recent range expansion of *A. maculatum* into Virginia and the high prevalence of *R. parkeri* in some of these Gulf Coast tick populations (41-56%) (Wright et al., 2011; Fornadel et al., 2011), it is possible that *R. parkeri* is spilling over into *A. americanum*, facilitated either by rickettsemic vertebrate bloodmeals or by co-feeding alongside *R. parkeri*-infected ticks. The second objective was to determine whether *A. americanum* could acquire *R. parkeri* by co-feeding on guinea pigs with infected *A. maculatum*. Furthermore, this study assessed the extent to which *R. amblyommii*-infected *A. americanum* nymphs were able to acquire and transstadially maintain *R. parkeri*.

MATERIALS AND METHODS

Tick collection. Between June 9th and June 22nd of 2014, transects at sites within 31 counties and independent cities in eastern Virginia were sampled for ticks by flagging vegetation; flagging was carried out as previously described (Ginsberg and Ewing, 1989). Ticks were sampled from a variety of habitat types. Adult *A. americanum* were identified to species level morphologically (Keirans and Litwak, 1989) and stored at -20°C prior to DNA extraction.

DNA extraction and pathogen detection. DNA was extracted from individual *A. americanum* adults using the DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA). Prior to extraction, adult ticks were cut in half longitudinally and subjected to bead-beating for 30-60 seconds at maximum speed on a BioSpec Mini Bead-beater (BioSpec, Inc., Bartlesville, OK) using equal volumes of 5 mm and 1 mm glass beads. DNA extraction was carried out on the tissue homogenates following the manufacturer's protocol; DNA was eluted in 100 µL elution buffer.

All DNA extracts from adult ticks were subjected to a real-time PCR assay targeting the *ompB* gene of *R. parkeri* using Rpa129F, Rpa224R and FAM/BHQ labeled Rpa188probe (p. 108). Extracts testing positive for *R. parkeri* were also tested for *R. amblyommii*. To test for *R. amblyommii*, DNA extracts were subjected to real-time PCR assays targeting the *ompB* gene of *R. amblyommii* using Ra477F, Ra618R and FAM/BHQ labeled Ra532probe (p. 108). Reactions were carried out in 20 µL volumes using 2X EconoTaq PLUS Master Mix (Lucigen Inc., Middleton, WI) and 3 µL of DNA template. The PCR protocol for both assays consisted of a 2 minute denaturation at 95°C followed by

45 cycles of 95°C for 15s and 60°C for 30s. PCR reactions were performed on a Mini-Opticon Real-Time PCR System (BioRad Inc., Hercules, CA).

Tick rearing for transmission experiments. *Rickettsia*-free *A. americanum* nymphs were purchased from the tick rearing facility at Oklahoma State University (OSU) (Stillwater, OK). An engorged *A. americanum* female was collected from a wild boar in Virginia Beach, Virginia, during the spring of 2014. Ticks were housed in an incubator kept at 26°C with 94% relative humidity with 14 hours of light and 10 hours of dark. To ensure absence of *R. amblyommii* in the OSU ticks, 8 nymphs were randomly selected and tested for *R. amblyommii* using real-time PCR, as described above. All OSU nymphs tested negative. A sample of the egg mass laid by the boar-derived adult *A. americanum* was also tested for *R. amblyommii* DNA; this egg mass sample tested positive. Upon hatching, larval ticks from the *R. amblyommii*-positive egg mass were fed on mice.

Pathogen acquisition experiments. To expose *A. americanum* nymphs to *R. parkeri*, nymphs were fed on guinea pigs alongside F_2 females from a field-derived, *R. parkeri*-infected *A. maculatum* colony (Wright et al., 2015). This colony was previously found to be free of other known SFGR by qPCR testing. Two guinea pigs were used, one each for feeding *R. amblyommii*-free (n=11) and *R. amblyommii*-infected (n=11) *A. americanum* nymphs. Five female adult *A. maculatum* were placed on each guinea pig 24-48 hrs prior to infesting with *A. americanum* nymphs to ensure attachment prior to nymphal feeding. Guinea pigs were inspected daily to ensure tick attachment. After *A. americanum* nymphs had fed to repletion, approximately 5 days, replete nymphs were

returned to the incubator and allowed to molt into adults. After adult *A. maculatum* had fed to repletion (approximately 14 days), guinea pigs were euthanized and a 2 cm diameter piece of skin was removed from the area of tick attachment for *R. parkeri* testing by qPCR.

After molting into adults, the salivary glands from each *A. americanum* were dissected from individual ticks to determine whether *R. parkeri* was present. Briefly, adults were surface sterilized with 70% ethanol and allowed to air dry. Salivary glands were aseptically dissected from each tick, washed twice in sterile PBS and individually stored in PBS prior to homogenizing with 1 mm glass beads in a bead-beater on low speed for 10 seconds. The salivary gland homogenate was immediately introduced into confluent flasks of Vero cells. The remaining tick tissue was subjected to DNA extraction using the DNeasy Blood and Tissue kit (Qiagen, Inc. Valencia, CA) and tested for *R. parkeri* and *R. amblyommii* using the real-time PCR assays described above.

Cell culture. Vero cells were propagated in 25 cm^2 flasks according to the protocol developed by Ammerman et al. (2008). Flasks containing confluent layers of Vero cells were inoculated with individual salivary gland homogenates dissected from each *A. americanum* adult (n=22). Cultures containing salivary gland homogenates from adults whose tissue tested negative for *R. parkeri* by qPCR were discarded. Vero cells and spent media were monitored for *R. parkeri* infection by real-time PCR for up to five days after inoculation with salivary gland homogenates. Briefly, Vero cells were centrifuged and DNA from Vero cells and spent media was extracted by incubating at 95°C for 10 minutes.

Statistical analysis. To determine whether significant differences exist between presence of *R. parkeri* in lab-reared *Rickettsia*-free and *R. amblyommii*-infected *A. americanum*, a Fisher's exact test was performed in SPSS (IBM, Inc.).

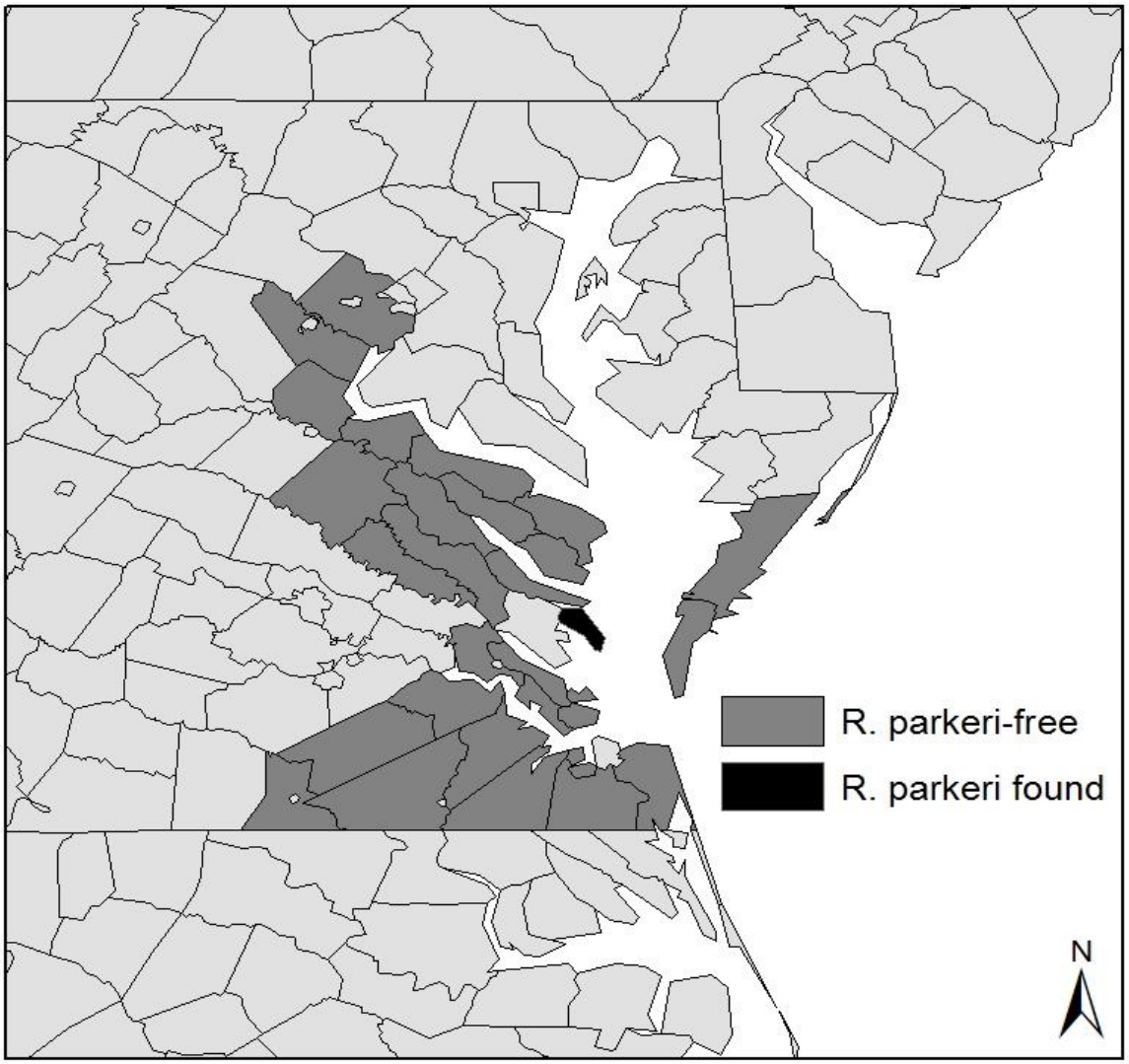

Fig. 11. Virginia cities and counties in which *Amblyomma americanum* were sampled and where *Rickettsia parkeri* was detected in questing adults.

RESULTS

A total of 449 adult *A. americanum* were collected from sites representing 29 counties and independent cities in eastern Virginia during June of 2014 (Table 12). Although sites within Norfolk City and Gloucester County were sampled, no *A. americanum* were found. Of the 449 adult *A. americanum* collected, up to 10 males and up to 10 females from each site or transect within a site, comprising 317 adults (170 female, 147 male) were tested for *R. parkeri* DNA. A single female collected from Mathews County, Virginia (Fig. 11), tested positive for *R. parkeri* (Table 12). This individual tested negative for *R. amblyommii*.

A total of 11 each of *R. amblyommii*-free and *R. amblyommii*-infected *A. americanum* nymphs were co-fed alongside *R. parkeri*-infected adult *A. maculatum* on guinea pigs for 5-7 days. Skin tissue collected from guinea pigs from each feeding event tested positive for *R. parkeri* DNA by real-time PCR, indicating that *A. maculatum* successfully transmitted *R. parkeri* while feeding. Upon molting into adults, 8 of 11 (73%) *R. amblyommii*-free and 2 of 11 (18%) R*. amblyommii*-infected *A. americanum* adults tested positive for *R. parkeri* DNA (p=0.03). All adults from the *R. amblyommii*-positive and *R. amblyommii*-negative *A. americanum* colonies were PCR-positive and PCR-negative for *R. amblyommii*, respectively (Table 13).

To determine whether *R. parkeri* was present in salivary glands and whether the rickettsiae were viable, salivary gland homogenates from adults testing positive for *R. parkeri* by real-time PCR were inoculated into confluent flasks of Vero cells. *R. parkeri* DNA was detected in cell culture from 1/2 (50%) and 4/8 (50%) salivary gland

homogenates from *R. amblyommii*-positive and R. *amblyommii*-free *A. americanum*, respectively, five days post-inoculation (Table 13).

Table 12
Virginia cities/counties in which adult *A. americanum* were collected and number of ticks collected, tested, and determined to contain *R. parkeri* DNA, from each county.

County	**Number adult *A. americanum* Collected**	**Number adult *A. americanum* tested**	**Number adult *A. americanum R. parkeri* positive**
Accomack County	43	21	0
Caroline County	26	26	0
City of Chesapeake	43	19	0
Essex County	39	20	0
Fairfax County	14	13	0
Greensville County	6	6	0
City of Hampton	8	8	0
Isle of Wight County	1	1	0
James City County	29	29	0
King and Queen County	5	5	0
King George County	3	3	0
King William County	1	1	0
Lancaster County	24	10	0
Mathews County	4	4	1
Middlesex County	20	19	0
City of Newport News	11	11	0
Northampton County	15	15	0
Northumberland County	1	1	0
City of Portsmouth	31	16	0
Prince William County	4	4	0
Richmond County	10	10	0
Southampton County	1	1	0
Stafford County	5	5	0
City of Suffolk	9	9	0
Surry County	6	6	0
Sussex County	11	11	0
City of Virginia Beach	12	12	0
Westmoreland County	11	11	0
York County	56	20	0
Total	449	317	1

Table 13

Number of individual adults and number of Vero cell cultures, derived from salivary gland homogenates, testing positive for *Rickettsia parkeri* DNA.

	Number *R. parkeri*-positive adults / Number tested	**Number *R. amblyommii*-positive adults / Number tested**	**Number *R. parkeri*-positive cultures / Number tested**
R. amblyommii-positive *A. americanum*	2/11 (18%)*	11/11 (100%)	1/2 (50%)
R. amblyommii-free *A. americanum*	8/11 (73%)*	0/11 (0%)	4/8 (50%)

*Fisher's Exact Test (p=0.03)

DISCUSSION

The first documented *R. parkeri* infection in a human was in 2002 and since then this pathogen has caused at least 37 reported infections (Paddock and Goddard, 2015), although the true number of cases is likely higher due in part to non-reporting and the cross-reactivity of serological tests (Vaughn et al., 2014). Because of the range expansion of *A. maculatum* into states including Virginia (Wright et al., 2011), Delaware and Maryland (Florin et al., 2014), and the high proportion (42 - 56%) of *A. maculatum* from Virginia infected with *R. parkeri,* this disease agent may now pose an increasing threat to human health.

Although the prevalence of *R. parkeri* is high in *A. maculatum* populations, for each questing *A. maculatum* encountered in southeastern Virginia approximately 50 *A. americanum* are collected (Nadolny et al., 2014). Because *A. americanum* are so widespread in this region it is important to identify pathogens present in this species. Even a low prevalence of pathogens in *A. americanum*, such as *E. chaffeensis*, which ranges in prevalence from 0 to 8.2% in southeastern Virginia (Wright et al., 2014), may be of public health importance due to the high density and ubiquity of the lone star tick. The purpose of this study was to determine whether *A. americanum* are infected with *R. parkeri* in coastal Virginia and also to determine whether *R. parkeri* can be acquired while co-feeding with infected ticks and maintained transstadially. Because *R. amblyommii* is commonly found in *A. americanum*, the potential for this related SFGR to inhibit *R. parkeri* from establishing infection within the lone star tick was also investigated.

Field collections of *A. americanum* from eastern Virginia revealed a single *R. parkeri*-positive adult, indicating that *R. parkeri* is found in *A. americanum* from Virginia, albeit in low (0.3%) frequencies. In 2014, Gaines et al., also demonstrated low numbers (≤1%) of Virginia *A. americanum* harboring *R. parkeri*. In 2009, Cohen et al. reported *R. parkeri* prevalence of <1% of *A. americanum* collected from Tennessee and Georgia.

Although it is apparent from these studies that *R. parkeri* is present at a very low prevalence in *A. americanum* populations, it is unclear whether *A. americanum* is capable of transmitting this organism to humans and other hosts. In 2003, Goddard experimentally infected *A. americanum* with *R. parkeri* by direct inoculation Hemolymph tests and fluorescence antibody tests indicated the presence of *R. parkeri* in *A. americanum* and the ticks were shown to maintain viable *R. parkeri* both transstadially and transovarially for two generations (Goddard et al., 2003).

The present study determined that *A. americanum* can acquire *R. parkeri* by co-feeding alongside infected *A. maculatum* on guinea pigs. Guinea pigs are susceptible to infection with *R. parkeri* (Parker et al., 1939; Goddard et al., 2003), showing clinical signs of infection including fever and scrotal swelling in males. The extent to which *R. parkeri* transmission by co-feeding occurs in other animals is uncertain. It is possible that *R. parkeri* spillover is facilitated in the natural environment by cotton rats (*Sigmodon hispidus*) which have been shown to have persistent infection with *R. parkeri* for up to 7 days after subcutaneous inoculation (Moraru et al., 2013). Furthermore, the presence of *R. parkeri* from cell culture five days post inoculation suggests that at least 50% of the salivary gland homogenates from PCR-positive adults contained potentially viable *R.*

parkeri. This finding suggests that *A. americanum* may be capable of *R. parkeri* transmission and thus may be a competent vector of this pathogen.

Interestingly, *A. americanum* nymphs infected with *R. amblyommii* prior to feeding alongside *R. parkeri*-infected *A. maculatum* were significantly less likely to acquire the pathogen, as detected in adults by PCR post-molting. This finding is consistent with the phenomenon of rickettsial exclusion observed by Macaluso et al. (2002), where the presence of either *Rickettsia montanensis* or *Rickettsia rhipicephali* excluded the transovarial transmission of the reciprocal organism in laboratory-reared *Dermacentor variabilis*. Although this study only investigated transstadial transmission, and not transovarial transmission, the same phenomenon may still be occurring.

In summary, this investigation confirmed the presence of *R. parkeri* in a low proportion of Virginia *A. americanum* populations. Furthermore, this study determined that *A. americanum* can acquire *R. parkeri* by feeding alongside *R. parkeri*-infected *A. maculatum*, and it was also confirmed that *A. americanum* can transstadially transmit *R. parkeri* from nymphs to adults. This experiment also indicated a decreased capacity for *R. amblyommii*-infected *A. americanum* to successfully acquire and/or maintain *R. parkeri*, confirming previous studies of rickettsial exclusion. Although *R. parkeri* was detected in salivary glands of *A. americanum* adults, suggesting that *A. americanum* is a competent vector of *R. parkeri*, future studies should further investigate the capacity for lone star ticks to horizontally transmit this pathogen to vertebrate hosts.

GENERAL DISCUSSION

Tick-borne pathogens are an increasing threat to human and animal health worldwide. Ticks are the most common agents of vector-borne pathogens in the United States and worldwide they are second only to mosquitoes as arthropod pathogen vectors (Goodman et al., 2005). In the U.S., cases of Lyme disease and spotted fever rickettsioses have been on the rise, in addition to infections such as ehrlichiosis and anaplasmosis caused by emerging tick-borne pathogens. Factors related to emergence include appearance of new pathogens, recognition of existing pathogens and environmental changes that result in new exposure events. Despite the rise in tick-borne disease incidence within many states, including Virginia, there is a paucity of data related to the prevalence and distribution of ticks and tick-borne pathogens.

New and emerging tick-borne pathogens are increasingly posing threats to human and animal health. Since the onset of this dissertation research in 2010, at least four tick-borne pathogens have emerged or been associated with human disease for the first time in the United States. These emerging disease agents include Heartland virus, *Borrelia miyamotoi*, *Rickettsia montanensis* and *Rickettsia phillipi* (previously known as *Rickettsia* 364D) (McMullan et al., 2012; Krause et al., 2013; McQuiston et al., 2012; Shapiro et al., 2010). In 2014, a novel *Thogotovirus* was isolated from the blood of a patient, who later died; due to the homology of this novel “Bourbon virus” to other tick-transmitted *Thogotovirus* members, it is likely this organism is also tick-transmitted (Kosoy et al., 2015). The emergence of new tick-borne pathogens is ongoing, posing significant challenges for protecting human and animal health. While molecular tools

are quickly evolving and becoming more usable for rapid detection of these new agents, gaining increased knowledge of the ecology and epidemiology of these organisms and their vectors is often a slow and laborious task.

In addition to the threat posed to human health by emerging pathogens, tick range expansions, likely driven by environmental and anthropogenic factors, are also occurring in the U.S. The results from this and other research investigations indicate that the Gulf Coast tick is expanding its range northward, carrying high infection rates (>40%) of *R. parkeri*. Since 2002, *R. parkeri* has caused infections in at least four residents of the Tidewater region of Virginia, indicating that this organism is contributing to disease now reported broadly as spotted fever rickettsiosis. Given the difficulty in serologically differentiating between infections caused by *R. parkeri* and other SFGR, the true incidence of Tidewater spotted fever in this region remains largely unknown. The lone star tick is another tick species that has been expanding its range in recent decades. Populations of *A. americanum*, which were once limited primarily to the southeastern U.S., are now reported from New York, Connecticut, Rhode Island (Ginsberg et al., 2002; Ijdo et al., 2000) and Michigan (Springer et al., 2014), in addition to more recently-reported established populations in Nebraska (Cortinas et al., 2013). Pathogens carried and transmitted by *A. americanum* include *Ehrlichia* spp., *Borrelia lonestari* and more recently, the Heartland virus. Ticks with expanding ranges present important problems to human health in that when new pathogens are introduced into an area, physicians and public health officials are often unaware of the introduction and unprepared for diagnosing and treating new illnesses.

This research focused on ticks and tick-borne pathogens present and recently introduced into Virginia. Given the high density of *A. americanum* in the southern U.S., it was not surprising that 95% of the ticks encountered during this study were *A. americanum*. All three life stages of this tick actively seek out large hosts, including humans, and are most active during the summer months when many people are outside. In the southeastern U.S., *A. americanum* is the most commonly found tick parasitizing humans, representing >80% of ticks attached to humans in Virginia, South Carolina and Kentucky (Stromdahl and Hickling; 2012). Because of the high density and ubiquity of the lone star tick in the southeastern U.S., even low abundance pathogens may pose an important problem for human health. This research showed that *E. chaffeensis* and *E. ewingii* are detected in up to 8.2% of *A. americanum* populations. Interestingly, prevalence of these pathogens was not constant throughout the region, with some locations having no detectable *Ehrlichia* while other areas have a prevalence of up to 8.2%. This finding highlights the focality of certain tick-borne pathogens and implies that the risk of exposure to a tick-borne pathogen may not be constant throughout a region, even though tick density can remain relatively stable. Gaines et al., (2014) also demonstrated a focal trend in distribution of *E. chaffeensis* and *E. ewingii* in *A. americanum* populations throughout Virginia; of seven regions sampled, four regions had 0% *E. chaffeensis* prevalence, while the prevalence in the three other regions ranged from 2.2% to 24.5%. A possible explanation for this variation in prevalence is that *E. chaffeensis* is largely maintained by white-tailed deer (*Odocoileus virginianus*) populations, so distribution of deer populations and rate of *Ehrlichia* infection in deer

may affect tick infection prevalence. Other potential reservoir hosts for *E. chaffeensis* include goats, coyotes, domestic dogs, red foxes, raccoons and opossums (Paddock and Childs, 2003), although it is unclear the extent to which these hosts play a role in the enzootic maintenance of *Ehrlichia* species.

An additional aim of this dissertation research was to identify and determine the prevalence of various spotted fever group rickettsiae in ticks collected from southeastern Virginia. The most notable member of the SFGR group is perhaps *R. rickettsii*, the agent of Rocky Mountain spotted fever (RMSF). Historically RMSF was a debilitating disease, with a case fatality rate of over 25% (CDC, 2013b). Although the incidence of RMSF has increased in recent decades, the case fatality rate has dropped to nearly 0% (CDC, 2013b), suggesting that other SFGR may be contributing to cases misdiagnosed as RMSF. Vaughn et al. (2014) demonstrated that 7 of 8 serum samples collected from patients diagnosed with RMSF in North Carolina cross-reacted with antibodies specific to *R. amblyommii* and *R. parkeri*. The research presented in this dissertation identified several SFGR from Virginia tick populations, including *R. amblyommii*, *R. montanensis* and *Candidatus* R. andeanae. Of these, *R. montanensis*, which is found in 5.6 – 6.9% of questing *D. variabilis* is southeastern Virginia, was recently associated with human infection (McQuiston et al., 2012). In lone star tick populations within the U.S., *R. amblyommii* prevalence is variable and can be fairly high (up to 84%); in Virginia, *R. amblyommii* prevalence was 26.9% and 54.9% in 2010 and 2011, respectively. Although prevalence of *R. amblyommii* is high, there is limited evidence supporting *R. amblyommii* as a human pathogen (Apperson et al., 2008;

Billeter et al., 2007). *Candidatus* R. andeanae was first detected in ticks from Peru (Jiang et al. 2005) and it is currently unclear whether this organism is pathogenic to humans. *Candidatus* R. andeanae has been found on occasion in *A. maculatum* within the United States, typically at a low prevalence (<5%) (Wright et al. 2011; Jiang et al. 2012; Fornadel et al. 2011; Varela-Stokes et al. 2011; Sumner et al. 2007). Conversely, *A. maculatum* collected from Oklahoma and Kansas from 2011 to 2014 were reported to have a very high prevalence (47 – 73%) of *Candidatus* R. andeanae but no *R. parkeri* was detected within these populations (Paddock et al. 2015). This finding suggests a possible exclusory role between *Candidatus* R. andeanae and *R. parkeri*. In southeastern Virginia, 1 of 301 *A. maculatum* tested was positive for *Candidatus* R. andeanae, indicating a very low prevalence of this organism in Virginia *A. maculatum* populations. Due to the variety and abundance of SFGR in Virginia, and also the ability of these SFGR to cross-react with *R. rickettsii* antigens, it is plausible that these organisms have contributed to the increased reported incidence of RMSF in the U.S.

Another research objective was to investigate the ecological dynamics of *R. parkeri* and *A. maculatum* by determining whether *R. parkeri* can be vertically maintained within tick populations. Other studies indicate that transovarial transmission is common with *Rickettsia* spp. (Goddard, J. 2003; Nieri-Bastos et al., 2013). *Rickettsia rickettsii*, while transovarially transmitted, imparts reproductive fitness costs on *D. andersoni* over several generations (Niebylski et al., 1999). This current research determined that *R. parkeri* is efficiently maintained vertically in *A. maculatum*, being transmitted both transovarially and transstadially. Furthermore, presence of *R. parkeri*

did not appear to confer any costs to reproductive fitness on the tick. Interestingly, *R. parkeri*-infected F_1 nymphs and F_2 larvae were significantly more likely than *R. parkeri*-free ticks to successfully molt, suggesting a potential advantage to *R. parkeri* infection in this tick. The ability of *R. parkeri* to be transovarially transmitted contrasts dramatically with other pathogens such as *E. chaffeensis*, which are not vertically maintained in *A. americanum* and thus rely on the presence of a competent vertebrate reservoir host. These differences in transmission dynamics between *R. parkeri* and *E. chaffeensis* may in part explain the different trends in prevalence seen in field-collected *A. maculatum* and *A. americanum*. While *R. parkeri* prevalence tends to be high and relatively consistent within and among *A. maculatum* populations, ranging from 30.2 – 55.7% in Virginia (Nadolny et al., 2014; Fornadel et al., 2011), *E. chaffeensis* is not always present in *A. americanum* populations and varies in prevalence (up to 25%) (Gaines et al., 2014).

The last objective of this research was to determine whether *A. americanum* could be a suitable vector for *R. parkeri*. This and other studies have established that *R. parkeri* is present in low numbers in lone star tick populations in Virginia, Tennessee and Georgia, ranging in prevalence from 0.2 – 1% (Cohen et al., 2009; Gaines et al., 2014). It should be noted that although *R. parkeri* prevalence is quite low in this tick, *A. americanum* is by far the most abundant human-biting tick species encountered in these states (Stromdahl and Hickling, 2012; Cohen et al., 2009) and pathogens present even in low prevalence may still pose a substantial risk to human health. Goddard (2003) determined that *A. americanum* can acquire *R. parkeri* from an infected guinea pig and maintain the pathogen transovarially, although it was unclear whether *A. americanum*

could transmit the pathogen during a bloodmeal. The present research indicates that nymphal *A. americanum* can acquire *R. parkeri* by co-feeding alongside *R. parkeri*-infected *A. maculatum* on a guinea pig, and that viable *R. parkeri* can be detected in salivary glands of *A. americanum* adults after molting from nymphs. Although it was not conclusively established that *A. americanum* can transmit *R. parkeri* to a vertebrate host, the presence of this pathogen in the salivary glands suggests that *A. americanum* is potentially a competent vector of *R. parkeri*. Interestingly, *A. americanum* nymphs that were previously infected with *R. amblyommii* were significantly less likely to acquire and maintain *R. parkeri*, suggesting that the presence of one *Rickettsia* species may exclude another from colonizing the tick. This phenomenon was also observed by Macaluso et al. (2003) who found that the presence of either *R. rhipicephali* or *R. montanensis* excluded colonization by the reciprocal organism within *D. variabilis*. It is possible that the high prevalence of *R. amblyommii* in *A. americanum* populations may inhibit *R. parkeri* colonization and may effectively prevent *A. americanum* from serving as a vector of *R. parkeri*. Whether *A. americanum* can transmit *R. parkeri* and whether *R. amblyommii* can inhibit *R. parkeri* acquisition and/or transmission are both questions that should be investigated in the future.

CONCLUSIONS

The first aim of this dissertation research was to determine the tick-borne pathogen composition within tick populations in southeastern Virginia, with a particular focus on two emerging pathogens, *R. parkeri* and *Ehrlichia* spp. The results of this research indicate that *R. parkeri* is highly prevalent in Virginia *A. maculatum* populations, with a prevalence of 43 - 56%. *Ehrlichia chaffeensis* and *E. ewingii* were both detected in *A*. *americanum* populations in Virginia. The prevalence of *E. chaffeensis* varied by location, ranging from 0 to 5.08% among *A. americanum* populations, while the prevalence of *E. ewingii* was slightly higher, ranging from 0 to 8.20% among *A. americanum* populations. Other SFGR were also detected in Virginia tick populations, and these include *R. amblyommii*, *R. montanensis* and *Candidatus* R. andeanae.

The second aim was to determine the transmission dynamics of *R. parkeri* within its vector, *A. maculatum*. Although some rickettsiae are transovarially transmitted in ticks, little was previously known about the frequency and efficiency of this transmission route, and nothing was known regarding the transmission strategy of *R. parkeri* in *A. maculatum*. Furthermore, nothing was known regarding whether *R. parkeri* infection results in decreased fitness of the tick. The results from the transmission experiments indicate that *R. parkeri* is efficiently maintained both transovarially and transstadially in *A. maculatum*. No observable declines in tick fitness were observed in the *R. parkeri*-infected colony, and significantly more *R. parkeri*-infected nymphs and larvae succeeded in molting as compared to *R. parkeri*-free ticks. The results of these experiments indicate that *R. parkeri* is maintained in *A. maculatum* populations efficiently by

transovarial and transstadial transmission without any noticeable effects on tick reproduction or survival.

The third aim was to explore the potential for *R. parkeri* to spill over from *A. maculatum* populations into populations of *A. americanum*. *Amblyomma americanum* is an aggressive human-biting tick. It represents 95% of the ticks encountered in southeastern Virginia and is the most common tick found attached to humans in the southeastern and mid-Atlantic United States. Because of its common association with humans, *A. americanum* and the pathogens it transmits are an important threat to human health in southeastern states. The competence of *A. americanum* as a vector of *R. parkeri* was investigated in this study. Of 317 adult *A. americanum* collected from eastern Virginia, one female tested positive for *R. parkeri*. Laboratory studies using guinea pigs indicated that 8 of 11 *A. americanum* nymphs co-feeding alongside *R. parkeri*-infected *A. maculatum* adults acquired *R. parkeri*; and the pathogen was detected in salivary glands of adults after molting. However, *A. americanum* nymphs infected with *R. amblyommii* prior to co-feeding were less likely to acquire *R. parkeri*, with only 2 of 11 nymphs acquiring and transstadially transmitting the pathogen. The results of this study indicate that *R. parkeri* is present at low levels in Virginia *A. americanum* populations, and that *A. americanum* can acquire and transstadially transmit *R. parkeri* by feeding alongside an infected Gulf Coast tick. Because viable *R. parkeri* were detected in the salivary glands of adult *A. americanum*, it is plausible that this tick may serve as a secondary vector of *R. parkeri*.

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PRIMERS AND PROBES USED TO AMPLIFY RICKETTSIAE AND EHRLICHIAE

Specificity	Primer and Probe Sequence (5'-3')	Target Gene	Product Size	Reference
Rickettsia genus	R17K128F2**:** GGGCGGTATGAAYAAACAAG R17K238R: CCTACACCTACTCCVACAAG R17K202TaqP: 6FAM-CCGAATTGAGAACCAAGTA ATGC-BHQ1	*17kD-antigen*	111	Jiang et al., 2012
Rickettsia amblyommii	Ra477F: GGTGCTGCGGCTTCTACATTAG Ra618R: CTGAAACTTGAATAAATCCATTAGTAACAT Ra532Probe: 6FAM-CGCGATCTCCTCTTACACTTGG ACAGAATGCTTATCGCG-BHQ1	*ompB*	142	Jiang et al., 2010
Candidatus Rickettsia andeanae	Rand957F: CGCTGGACAAGTTTATGCTCAAG Rand1062R: GGCAGTAGTACCGTCTGTACCAAC Rand1003FAM: 6FAM-CGCGATGTAGGCGGACAGGT AACTTTTGATCGCG-BHQ1	*ompB*	109	Jiang et al., 2012
Rickettsia montanensis	RMF2832: GCGGTGGTGTTCCTAATAC RMR2937: CCTAAGTTGTTATAGTCTGTAGTG RMB2875: 6FAM-CGGGGCAAAGATGCTAGCG CTTCACAGTTACCCCG-BHQ1	*ompB*	106	Smith et al., 2010
Rickettsia parkeri	Rpa129F: CAAATGTTGCAGTTCCTCTAAATG Rpa224R: AAAACAAACCGTTAAAACTACCG Rpa188probe: 6FAM-CGCGAAATTAATACCCTTATG AGCAGCAGTCGCG-BHQ1	*ompB*	96	Jiang et al., 2012
Rickettsia spp.	190-FN1: AAGCAATACAACAAGGTC 190-RN1: TGACAGTTATTATACCTC	*ompA*	540	Paddock et al., 2004.
Rickettsia spp.	RompB11F: ACCATAGTAGCMAGTTTTGCAG RompB1902R: CCGTCATTTCCAATAACTAACTC	*ompB*	1895	Jiang et al., 2005.
Rickettsia spp.	Outer 190-3588F: AACAGTGAATGTAGGAGCAG 190-5238R: ACTATTAAAGGCTAGGCTATT Inner RhoA4336F: AGTTCAGGAAACGACCGTA 190-5044R: AACTTGTAGCACCTGCCGT	*ompA*	1631	Fournier et al. 1998; Pagac et al. 2014
Ehrlichia chaffeensis	Ech16S-17: GCGGCAAGCCTAACACATG Ech16S-97: CCCGTCTGCCACTAACAATTATT Ech16S-38: 6FAM- AGTCGAACGGACAATTGCTTATA ACCTTTTGGT-BHQ1	*16S*	81	Loftis et al. 2003
Ehrlichia ewingii	Ech16S-17: GCGGCAAGCCTAACACATG Ece16S-99: CAC CCG TCT GCC ACT AAC AAY TAT Eew16S-40: 6-FAM-TCGAACGAACAATTCCTAAAT AGTCTCTGACTATT-BHQ1	*16S*	82	Killmaster et al. 2014

COPIES OF IACUC APPROVAL LETTERS

OLD DOMINION UNIVERSITY

OFFICE OF THE VICE PRESIDENT FOR RESEARCH

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To: Dr. Daniel Sonenshine

From: Dr. Barbara Hargrave, IACUC Vice-Chair

CC: Kersten Wheeler, Office of Research

Re: IACUC protocol #12-003

Date: January 23, 2013

Dear Dr. Sonenshine,

This letter is to inform you that the annual renewal for your protocol, "Rickettsia parkeri: transmission dynamics and dispersal of emerging tick-borne diseases" (IACUC protocol #12-003), has been approved by the Old Dominion University IACUC through the Annual Review process. You are authorized to continue your project.

Your approval expires on March 14th, 2014, at which time you will need to submit another Annual Review form to seek another year of approval. After the third year you must submit an updated protocol form and seek re-approval from the IACUC if you wish to continue your project beyond that date.

Regards,

Barbara Hargrave

Dr. Barbara Hargrave
Vice-Chair, Institutional Animal Care and Use Committee

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To: Dr. Daniel Sonenshine, Department of Biological Sciences

From: Dr. Stephen Beebe, IACUC Chair

CC: Kersten Wheeler, Office of Research

Re: IACUC protocol #10-018 de novo

Date: April 2, 2013

Dear Dr. Sonenshine,
This letter is to inform you that your protocol, NSF IOS project "Endocrinology of Tick Reproduction; a new perspective" (IACUC protocol #10-018 de novo), has been fully approved by the Old Dominion University IACUC through the de novo review process. You are free to begin work on the project immediately. The decision date for this project is April 2, 2013.

Please remember that even minor changes to your protocol including personnel and procedural changes must be submitted to the IACUC using our amendment forms found online at http://www.odu.edu/ao/research/compliance/animals.shtml. The amendment(s) must be submitted to Kersten Wheeler (kwheeler@odu.edu) in the Office of Research in advance of implementing the change. Once these amendments are received and approved, you will be free to implement the changes. Any unapproved deviations in the protocol will be considered non-compliance and could result in a suspension and/or investigation.

In addition, this protocol will need to be renewed annually through the annual review process if you wish to continue your project past April 2, 2014.

Feel free to contact the Office of Research (683-5451) to discuss any questions or concerns during your project.

Regards,

Dr. Stephen Beebe
Chair, Institutional Animal Care and Use Committee

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To: Dr. Daniel Sonenshine, Department of Biological Sciences

From: Dr. Stephen Beebe, IACUC Chair

CC: Kersten Wheeler, Office of Research

Re: IACUC protocol #10-032

Date: July 12, 2012

Dear Dr. Sonenshine,

This letter is to inform you that your protocol, "Endocrinology of tick reproduction: A new perspective (Tick Rearing on Rabbits)" (IACUC protocol #10-032), has been fully approved by the Old Dominion University IACUC. The decision date for this project is July 12, 2012.

Please remember that even minor changes to your protocol including personnel and procedural changes must be submitted to the IACUC using our amendment forms found online at http://www.odu.edu/ao/research/compliance/animals.shtml. The amendment(s) must be submitted to Kersten Wheeler (kwheeler@odu.edu) in the Office of Research in advance of implementing the change. Once these amendments are received and approved, you will be free to implement the changes. Any unapproved deviations in the protocol will be considered non-compliance and could result in a suspension and/or investigation.

In addition, this protocol will need to be renewed annually through the annual review process if you wish to continue your project past July 12, 2013.

Feel free to contact the Office of Research (683-5451) to discuss any questions or concerns during your project.

Regards,

Dr. Stephen J. Beebe
Chair, Institutional Animal Care and Use Committee

PERMISSIONS FOR REPRINTING PUBLISHED MATERIALS

Chapter Number	Journal	License Number	Date Acquired	Reference
1	Emerging Infectious Diseases	Public Domain	N/A	Wright et al., 2011
2	Ticks and Tick-Borne Diseases	3506721379187	Feb. 2014	Nadolny et al., 2014
3	Ticks and Tick-Borne Diseases	3506721245521	Nov. 2014	Wright et al., 2014
4	Ticks and Tick-Borne Diseases	3651671262087	Jun. 2015	Wright et al., 2015
5	Journal of Medical Entomology	3673911208604	Jul. 2015	Wright et al., 2015

VITA

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EDUCATION

Bachelor of Science (Biology) Cum Laude, Old Dominion University, Norfolk, VA. 2010.

EXPERIENCE

ORISE Fellow	DHS-S&T	Washington, DC	05/2015-present
Graduate Research Assistant	Old Dominion University	Norfolk, VA	05/2010-05/2015
Research Intern	USDA-ARS	Pullman, WA	08/2014-11/2014

PEER-REVIEWED PUBLICATIONS

Dissertation-related

- Wright CL, Nadolny RM, Jiang J, Richards AL, Sonenshine DE, Gaff HD, Hynes WL. *Rickettsia parkeri* in Gulf Coast ticks, Southeastern Virginia, USA. 2011. Emerg. Infect. Dis. 17, 896-898.
- Nadolny RM*, Wright CL*, Sonenshine DE, Hynes WL, Gaff HD. 2013. Ticks and Spotted Fever Group Rickettsiae of Southeastern Virginia. Ticks Tick-borne Dis. 5, 53-57. *Equal contributions by both
- Wright CL, Gaff HD, Hynes WL. 2014. Prevalence of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in ticks collected from southeastern Virginia, 2010-2011. Ticks Tick-borne Dis. 5, 978-982.
- Wright CL, Gaff HD, Sonenshine DE, Hynes WL. 2015. Experimental Vertical Transmission of *Rickettsia parkeri* in the Gulf Coast tick, *Amblyomma maculatum*. Ticks Tick-borne Dis. In press.
- Wright CL, Sonenshine DE, Gaff HD, Hynes WL. *Rickettsia parkeri* transmission to *Amblyomma americanum* by co-feeding with *Amblyomma maculatum* and potential for spillover. Journal of Medical Entomology. In press.

Other

- Nadolny RM, Wright CL, Hynes WL, Sonenshine DE, Gaff HD. 2011. J. Vector. Ecol. 36, 464-467.
- Luce-Fedrow A, Wright C, Gaff HD, Sonenshine DE, Hynes WL, Richards AL. 2012. FEMS Immunol. Med. Microbiol. 64, 74-81.
- Wright CL, Hynes WL, White BT, Marshall MN, Gaff HD, Gauthier DT. 2013. Ticks Tick-borne Dis. 5, 48-52.
- Gaines D, Operario D, Stroup S, Stromdahl E, Wright C, Gaff H, Broyhill J, Smith J, Norris D, Lucas A, Houpt E. 2014. Vector Borne Zoonotic Dis. 14, 307-16.
- Florin DA, Brinkerhoff RJ, Gaff H, Jiang J, Robbins RG, Eickmeyer W, Butler J, Nielsen D, Wright C, White A, Gimpel ME, Richards AL. 2014. Syst. Appl. Acarol. 19, 257-262.