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Co-infection dynamics of the tick-borne bacteria, *Rickettsia parkeri* and "*Candidatus* Rickettsia andeanae"

By

Jung Keun Lee

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine

Mississippi State, Mississippi

December 2016

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Co-infection dynamics of the tick-borne bacteria, *Rickettsia parkeri* and "*Candidatus* Rickettsia andeanae"

By

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Title of Study: Co-infection dynamics of the tick-borne bacteria, *Rickettsia parkeri* and "*Candidatus* Rickettsia andeanae"

Pages in Study: 120

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Amblyomma maculatum Koch (Acari: Ixodidae) is the primary vector for *Rickettsia parkeri* which is a causative agent of American Boutonneuse fever. Infection rates of *R. parkeri* in *A. maculatum* populations from the United States are approximately 0-56%. The first human clinical case of *Rickettsia parkeri* infection was reported in 2014 and the patient presented with a febrile illness similar to Rocky Mountain spotted fever but showing milder clinical symptoms. Through 2016, approximately 40 human clinical cases of *R. parkeri* infection have been reported within southern United States. *Amblyomma maculatum* may also be infected with a rickettsia of unknown pathogenicity "*Candidatus* Rickettsia andeanae" which has 0-6.3% infection rates in much of the southeastern United States. However, the interaction between *R. parkeri* and "*Ca*. R. andeanae" is largely unknown. In studies presented here, we found infection rates of *R. parkeri* and "*Ca*. R. andeanae" in questing *A. maculatum* from Mississippi between 2013 and 2015 to be within the reported ranges for the Southeast, though higher than those previously reported for this area; levels of *R. parkeri* were also higher than "*Ca*. R. andeanae" in individual infected ticks. Using animal tick-feeding trials, we found tick

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tissue levels of both rickettsiae to increase over time, while transovarial transmission was most successful for "*Ca*. R. andeanae" when present alone in ticks or co-infecting with *R. parkeri*. Finally, adult ticks transmitted *R. parkeri* to naïve co-feeding ticks, and tick acquisition and survival was more efficient when co-feeding ticks were in close proximity and naïve ticks uninfected with "*Ca*. R. andeanae."

In summary, this dissertation research filled specific gaps in our knowledge of *R. parkeri*-"*Ca*. R. andeanae" interactions in individual *A. maculatum* ticks and tick populations. Through this research, we have contributed to a better understanding of the human pathogen, *R. parkeri*, and sympatric species, "*Ca*. R. andeanae." We anticipate that sharing these data with the research community will lead to a better understanding of the complexities of spotted fever rickettsiosis in the United States.

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DEDICATION

This dissertation is dedicated to my wife, Ji A Lee, and my son, Samuel Damin Lee, who are always with constant support, prayer, and joy.

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CHAPTER I

INTRODUCTION

The first human clinical case of *Rickettsia parkeri* infection was initially thought to be caused by another agent of spotted fever rickettsiosis when it was reported twelve years ago in United States (Paddock et al. 2004). The patient presented with a febrile illness similar to Rocky Mountain spotted fever (RMSF) but showing milder clinical symptoms. Unlike RMSF, which is associated with a red to purple petechial rash on skin, *R. parkeri* infection is characterized by an eschar at the tick-bite site and any associated rash is usually only a maculopapular or papulovesicular eruption on the truck and extremities (Paddock et al. 2004; Paddock et al. 2008). *Rickettsia parkeri* has been detected in up to 56 % of *Amblyomma maculatum* (Gulf Coast tick), the primary tick vector, from the United States (Sumner et al. 2007; Paddock et al. 2010; Varela-Stokes et al. 2011; Wright et al. 2011; Ferrari et al. 2012; Nadolny et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). Through 2016, approximately 40 human clinical cases of *R. parkeri* infection have not only been reported within the southeastern United States range of *Amblyomma maculatum* (Paddock et al. 2004; Raoult and Paddock 2005; Finley et al. 2006; Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Myers et al. 2013; Ekenna et al. 2014; Kaskas et al. 2014; Paddock and Goddard 2015; Drexler et al. 2016; Straily et al. 2016) but have also been reported in the southwestern United States where the vector is *Amblyomma triste*, not *Amblyomma maculatum* (Herrick et al. 2016).

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In contrast, the prevalence of *R. rickettsii*, the agent of RMSF, in *Dermacentor* tick species is usually under 1% despite increasingly reported disease cases. The incidence of RMSF cases reported to the Centers for Disease Control and Prevention (CDC) increased over the last two decades from 345 cases in 1993 to 1815 cases in 2009, when the case definition was modified; cases are now reported as "spotted fever rickettsiosis" to include infection with similar agents (CDC 2010; CDC 2013).

In addition to *R. parkeri,* a newly recognized unnamed species of unknown pathogenicity, "*Candidatus* Rickettsia andeanae" was identified recently in *A. maculatum* (Blair et al. 2004). Through previous research in our laboratory, this organism was found in *A. maculatum* co-infected with *R. parkeri* at a higher co-infection rate than expected by chance alone (Ferrari et al. 2012). This is an unusual phenomenon compared to co-infections of sympatric *Rickettsia* spp. observed in *Dermacentor* species that are rare due to transovarial interference (Burgdorfer and Brinton 1975). The overall objective of this dissertation was to provide a better understanding in our knowledge of *R. parkeri*-"*Ca*. R. andeanae" interactions in individual *A. maculatum* ticks and in tick populations.

Specific aims of this research were to:

1. Estimate the prevalence and DNA levels of *Rickettsia parkeri* and "*Ca.* R. andeanae" in questing wild-caught Gulf Coast ticks, *Amblyomma maculatum*, from Oktibbeha County, Mississippi between 2013 and 2015.

2. Establish tissue tropism, distribution and ovarian transmission of *R. parkeri* and "*Ca.* R. andeanae" in singly- and co-infected Gulf Coast ticks and evaluate rickettsial transmission and distribution to vertebrates (rabbit model) following exposure to singly- and co-infected *R. parkeri* and "*Ca*. R. andeanae."

3. Evaluate acquisition of *R. parkeri* in naïve Gulf Coast ticks during co-feeding with *R. parkeri* infected Gulf Coast ticks in calves depending on "*Ca*. R. andeanae" infection status.

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CHAPTER II

LITERATURE REVIEW

2.1 Life history of the Gulf coast tick, *Amblyomma maculatum*

The Gulf Coast tick (GCT), *Amblyomma maculatum* Koch (1844), was first described by Koch in 1844 from "Carolina" with no specific hosts mentioned (Koch 1844). *Amblyomma maculatum* is a hard tick in the Phylum Arthropoda, Class Arachnida, Subclass Acari, Order Parasitiformes, and Suborder Ixodida. Three families belong to Ixodida: the Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. The Ixodida are characterized by eight legs in nymphal and adult stages, a lack of antennae and no division of body parts into head, thorax and abdomen. Hard ticks (Family Ixodidae) have morphological differences from soft ticks (Family Argasidae) that include a long prominent capitulum (mouth parts) located anteriorly and presence of a hard shell (scutum) covering the dorsal surface partially or entirely. Soft ticks have a soft exoskeleton with no scutum and the capitulum is located under the body. Nuttallielidae contains only a single species, *Nuttalliella namaqua*, in southern Africa and has combined characteristic of the hard and soft ticks.

Amblyomma maculatum is a three-host tick and each stage feeds to repletion only once, leaving the host after each feeding (Sonenshine 1991). *Amblyomma maculatum* ticks parasitize a wide range of vertebrate animals including small mammals, ground-dwelling birds, cattle, horses, deer, goats, sheep, and humans, and have been collected

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from 71 species of birds and mammals in the United States (Bishopp and Hixson 1936; Hixson 1940; Teel et al. 2010). Among hosts used by immature stages, the most common fed on by larvae are birds (60%) and rodents (31%); similarly, nymphal stages also use birds (52%) and rodents (20%) (Teel et al. 2010). In contrast, adult stages of GCTs prefer to attack large wild or domestic animals, and humans (Hooker et al. 1912; Hixson 1940). GCTs attach primarily to the head and neck of mammals and birds after initially using a questing strategy to find their hosts (Hixson 1940; Bishopp and Trembley 1945). The life cycle takes from 6 months to 6 years (usually 2-3 years) depending on environmental conditions, temperature, relative humidity and photoperiod. GCTs spend over 90% of their time off the host, in open environments such as meadows and forest, particularly in coastal upland and tall grass prairies, such as the Gulf Coast Prairies region (Bishopp and Hixson 1936; Hixson 1940; Sonenshine 1991). Newly hatched larval stages aggregate on the lower surfaces of vegetation in early summer and respond to host stimuli such as breath or vibration from ground-dwelling birds and small rodents (Hixson 1940; Stacey 1971). The molting time of engorged larvae is influenced by temperature and photoperiod duration (Bishopp and Hixson 1936; Hixson 1940). In one field study, nymphal GCTs actively sought rodent hosts from May to October in north-central Oklahoma (Barker et al. 2004). The molting time for engorged nymphs is from 21 to 28 days and is mostly affected by temperature (Bishopp and Hixson 1936). Molted adult GCTs are found between March and November, peaking in July through early August in Mississippi (Goddard and Paddock 2005). Male GCTs may remain on the host for longer periods, up to 3.5 weeks after the females drop, and may mate with several additional females during that time. Female GCTs were more prevalent in cattle co-infested with male GCTs

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(Sleeba et al. 2010). Male GCTs frequently cluster in groups of six to nine. Female GCTs began to oviposit between 0 and 58 days after dropping from hosts, depending on daily temperatures (Hixson 1940). Engorged female GCTs convert 52-69% of their body weight to eggs and lay average 8,141 eggs per female; individual egg weight is range from 46.2 to 48.4 μg (Bishopp and Hixson 1936; Drummond and Whetstone 1970; Stacey 1971). These GCT eggs hatch within 14 and 45 days after oviposition depending on microclimatic factors such as average daily temperature, habitat type and seasonal phenology of the vegetation community (Teel et al. 2010).

The distribution of *A. maculatum* was initially described in the 1900s to include the southern United States along the border of Gulf of Mexico and the Atlantic Coast of the U.S. including Georgia, North Carolina, South Carolina and Virginia (Bishopp and Hixson 1936; Cooley and Kohls 1944; Bishopp and Trembley 1945). The greatest prevalence of GCTs coincided within the Mississippi and Atlantic migratory bird flyways (Smith et al. 1996). Later, in 1973, inland populations of GCTs were reported in Oklahoma, likely secondary to the introduction of cattle into Oklahoma from the Gulf Coast region of the southeastern United States as early as 1958 (Semtner and Hair 1973). GCTs are also found in central states such as Kansas, Arkansas, Kentucky and Tennessee (Goddard and Norment 1983; Trout et al. 2010). Outside of the United States, *Amblyomma maculatum* is distributed in parts of Central and South America, along the border of Gulf of Mexico such as Jamaica, Belize, the West Indies, Colombia and Peru (Robinson et al. 1926; Bishopp and Trembley 1945; Blair et al. 2004)

Amblyomma maculatum plays an important role in the transmission of medical and veterinary pathogens. One of them, *Rickettsia parkeri*, is an important emerging

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human pathogen with increasing public health importance in the southeastern U.S. that uses *A. maculatum* as a vector (Raoult and Paddock 2005). *Amblyomma maculatum* is also a vector for American canine hepatozoonosis, a disease caused by *Hepatozoon americanum* in the U.S. (Ewing et al. 2002; Ewing and Panciera 2003). *Amblyomma maculatum* successfully transmits the causative agent of heartwater disease, *Ehrlichia ruminantium*, under experimental conditions, but it has not been implicated in natural transmission of *E. ruminantium* (Mahan et al. 2000). Heartwater disease is endemic in wild and domestic ruminants in sub-Saharan Africa and the Caribbean and *A. maculatum* may be a potential vector in the U.S. if the pathogen is introduced here. *Amblyomma maculatum* can also cause an ascending paralysis in dogs in the eastern U.S. (Gothe et al. 1979). Finally, blood-feeding by adult *A. maculatum* can induce extensive inflammation, edema and abscesses in their hosts. GCTs generally prefer feeding on or in ears of cattle that result in thickening of ears, edema, curling and reducing market value. This ear condition is called 'gotch ear' and has also been described in other livestock such as goats (Edwards et al. 2011). Gotch ear may cause a loss of up to a third portion of the ear in young calves (Bishopp and Hixson 1936; Edwards 2011). Lesions at the *Amblyomma maculatum* biting site on domestic and wild ruminant also contributed to attracting infestations of the primary screwworm, *Cochliomyia hominivorax*, resulting in loss of production before eradication of the fly from North America (Bishopp and Hixson 1936; Hixson 1940).

2.2 Spotted fever group rickettsiae

The genus *Rickettsia* is comprised of obligate intracellular bacteria in the Family Rickettsiaceae which is classified under the Phylum Proteobacteria, Class

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Alphaproteobacteria and Order Rickettsiales. All Proteobacteria are gram-negative with a lipopolysaccharide outer membrane. The Order Rickettsiales consists of Families Rickettsiaceae, Bartonellaceae and Anaplasmataceae (Walker 1988). For the purposes of this dissertation, rickettsiae and rickettsial bacteria refer to *Rickettsia* species only. Human pathogenic *Rickettsia* species are subdivided into the typhus group and spotted fever group. The typhus group includes *R. typhi* and *R. prowazekii,* agents of murine and epidemic typhus, respectively. The spotted fever group rickettsiae (SFGR) is a larger group of over 30 species that includes *R. rickettsii* and *R. conorii,* the agents for Rocky Mountain spotted fever (RMSF) and Mediterranean spotted fever, respectively. Human diseases caused by SFGR in the United States include RMSF (*R. rickettsii*), mite-borne rickettsialpox (*R. akari*) and cat-flea typhus (*R. felis*) (Raoult et al. 1996; Raoult and Roux 1997). Rickettsiae are mainly transmitted to vertebrates via salivary secretion or feces from arthropod vectors, but may also be transmitted by inhalation and blood transfusion. The hard ticks play a critical role as vectors, reservoirs and amplifiers for SFGR. Rickettsiae can multiply in almost all organs of their invertebrate hosts and can be transovarially transmitted to progeny. Once an egg is infected, rickettsial bacteria could be completely transmitted to all subsequent life cycle stages, transstadially (Parola et al. 2005). In addition, naïve ticks at any stage can be infected while feeding on infected hosts. Sexual transmission of rickettsiae from male to female ticks has also been described in *Ixodes ricinus* and *Dermacentor andersoni* ticks (Philip and Parker 1933; Hayes et al. 1980).

Rickettsiae include species that are not only historically important as human pathogens but also recently identified as emerging threats. Epidemic typhus was

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suspected to be responsible for the Athens plague by Thucydides during 5th century B.C. (Walker 1988). Approximately a century ago, the causative agent of RMSF, *Rickettsia rickettsii*, was identified and determined to be transmitted by *Dermacentor* ticks, causing symptoms including fever, rash, headache, nausea, vomiting and muscle pain in infected people (Ricketts 1909; Burgdorfer 1975). The fatality rate was 40% overall in 1940s but decreased to less than 1% in 2007 (Hattwick et al. 1973; Dahlgren et al. 2012). However, the incidence of RMSF cases reported to the Centers for Disease Control and Prevention (CDC) increased in the last two decades from 345 cases in 1993 to 1815 cases in 2009 (CDC 2013). The CDC recently reported that SFGR other than *R. rickettsii* may contribute to the reported RMSF cases, due to cross-reactivity between SFGR and RMSF pathogens on serological tests. This might explain the increased numbers of RMSF cases. Consequently, CDC adjusted the category for reporting RMSF cases to the broader "spotted fever rickettsiosis" in 2010 (CDC 2010).

2.3 *Rickettsia parkeri*

Rickettsia parkeri was first recognized as another causative agent for spotted fever rickettsiosis approximately twelve years ago. In this first case, a 40-year-old previous healthy man from Virginia, U.S. experienced a febrile illness similar to RMSF but with milder clinical symptoms (Paddock et al. 2004). However, unlike RMSF, which causes a red to purple petechial rash on skin in over 90% of infected people, *R. parkeri* infection is characterized by an eschar (dermal and epidermal necrosis) at the tick-bite site and any associated rash is usually only maculopapular or papulovesicular eruption on the truck and extremities (Paddock et al. 2008; Parola et al. 2009). Human cases of spotted fever rickettsiosis caused by *R. parkeri* were subsequently reported in other states

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including Mississippi and Virginia (Paddock et al. 2004; Raoult and Paddock 2005; Finley et al. 2006; Whitman et al. 2007; Paddock et al. 2008; Cragun et al. 2010; Myers et al. 2013; Ekenna et al. 2014; Kaskas et al. 2014; Paddock and Goddard 2015; Drexler et al. 2016; Straily et al. 2016). In one study, serum analysis from 15 U.S. patients who had an earlier diagnosis of RMSF revealed four samples that reacted to a 120-kDa protein of *R. parkeri* suggestive of infection with *R. parkeri* (Raoult and Paddock 2005). Approximately 77 years prior to its association with human disease, *R. parkeri* was first isolated from *A. maculatum* by R.R. Parker from Liberty County, Texas (Parker et al. 1939). In early studies, *R. parkeri* inoculation into guinea pigs led to development of a mild febrile disease that resembled other spotted fever rickettsioses such as RMSF and Mediterranean spotted (Boutonneuse) fever (Parker et al. 1939; Parker 1940). Currently, *Rickettsia parkeri* is regarded as the only other tick-borne SFGR species that has been associated with human disease in the southeastern United States (Raoult and Paddock 2005; Paddock et al. 2008) but it has also been reported southwestern United States where the vector is *Amblyomma triste*, not *Amblyomma maculatum* (Herrick et al. 2016). The prevalence of *R. parkeri* in *A. maculatum* has been reported up to 56 % in the southern U.S. including Florida, Georgia, Mississippi, Oklahoma, South and North Carolina (Sumner et al. 2007; Paddock et al. 2010; Varela-Stokes et al. 2011; Nadolny et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). This range is significantly higher than the infection rate for *R. rickettsii*. The prevalence of *R. rickettsii* in its primary vectors, *Dermacentor* ticks, is rarely over 1%, possibly due to the lethal effect of *R. rickettsia* in infected *Dermacentor* ticks (Philip and Casper 1981; Niebylski et al. 1999). *Rickettsii parkeri* has been detected in 43-56 % of sampled *A. maculatum* in

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Virginia, U.S. (Wright et al. 2011; Nadolny et al. 2014). Interestingly, *R. parkeri* was possibly excluded in *A. maculatum* populations from Kansas and Oklahoma due to infection with "*Candidatus* R. andeanae" (Paddock et al. 2015). Therefore, there are possible interaction between *R. parkeri* and "*Ca*. R. andeanae." *Amblyomma americanum* ticks were determined to be a possible vector of *R. parkeri* in laboratory studies as well as through surveys of field-collected *A. americanum* in Tennessee and Georgia (Goddard 2003; Cohen et al. 2009). In addition, the acquisition of *R. parkeri* in naïve *A. americanum* after co-feeding with *R. parkeri* infected *A. maculatum* has been studied (Wright et al. 2015). *Rickettsia parkeri* was also detected in *Amblyomma triste* ticks in Uruguay, Brazil and Argentina (Pacheco et al. 2006; Silveira et al. 2007; Nava et al. 2008).

2.4 "*Candidatus* Rickettsia andeanae"

Newly recognized but unnamed "*Candidatus* Rickettsia andeanae" was first identified in *A. maculatum* and *Ixodes boliviensis,* both collected from horses in northwestern Peru in 2004, where there was an outbreak of febrile disease in humans (Blair et al. 2004). This novel rickettsia was identified by testing for SFGR from flea pools and individual ticks in the affected area. Sequence analysis of "*Ca.* R. andeanae" using five rickettsial genes, 17-kDa gene, *gltA, ompB, ompA*, and *sca4,* demonstrated sequence identity closest (over 97%) with known SFGR; phylogenetic analyses of those genes also revealed that "*Ca.* R. andeanae" was included within the SFGR but was unique from known SFGR (Blair et al. 2004; Jiang et al. 2005). Within twelve years, "*Ca.* R. andeanae" was reported in the southeastern United States from *A. maculatum* collected in states including Florida, Georgia, Kentucky, Mississippi, Tennessee, Virginia and

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South Carolina with an overall infection rate of approximately 0.6-6.3% in tested samples (Sumner et al. 2007; Paddock et al. 2010; Fornadel et al. 2011; Varela-Stokes et al. 2011; Ferrari et al. 2012; Jiang et al. 2012; Nadolny et al. 2014; Paddock and Goddard 2015; Mays et al. 2016;).

Despite its recent detection, "*Ca.* R. andeanae" is incompletely characterized and any association with human disease is still unknown. In one study, the detection of *R. parkeri* and "*Ca.* R. andeanae" co-infecting *A. maculatum* was significantly higher than expected by random chance, at about 1.7% (12/698 of *A. maculatum*) (Ferrari et al. 2012). In recent paper, a high prevalence of "*Ca*. R. andeanae" was also recently reported in *A. maculatum* from Kansas and Oklahoma, whereas *R. parkeri* was absent (Paddock et al. 2015). Interactions between sympatric rickettsiae in *A. maculatum* have not yet been studied and are unknown. The biologic role of *A. maculatum* as well as an interaction between *R. parkeri* and "*Ca.* R. andeanae" needs to be determined to better understand the SFGR in southeastern United States.

2.5 Co-infection of rickettsiae in ticks

Rickettsiae are highly specialized obligate intracellular pathogens that are maintained in both the vertebrate host and the arthropod vector. The relationships among vertebrate hosts, invertebrate hosts and the rickettsial pathogen play significant role in horizontal and vertical transmission. Efficient maintenance, replication, transstadial and transovarial transmission of pathogens have developed through coevolution of rickettsiae in their vertebrate and tick hosts (Sonenshine 1993; Azad and Beard 1998). The phenomenon of transovarial interference was first reported between *R. rickettsii* and a sympatric non-pathogenic *Rickettsia* sp. (Burgdorfer and Brinton 1975). Co-infections

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with sympatric *Rickettsia* spp. in *Dermacentor* tick species are rare. This reflects the phenomenon where initial infection of ticks by one *Rickettsia* sp. prevents the acquisition and transmission of a second *Rickettsia* sp. that has been described using laboratory studies with *Dermacentor variabilis* and *D. andersoni* ticks (Burgdorfer et al. 1981; Macaluso et al. 2002). Therefore, horizontal transmission is very important for *R. rickettsii* and helps to explain the low frequency in nature of less than 1% (Burgdorfer 1988; Hackstadt 1996). The prevalence of *R. rickettsii*, the causative agent of RMSF, in *Dermacentor* ticks is rarely over 1% despite the increase in RMSF cases reported to the CDC from 345 cases in 1993 to 1,815 cases in 2009 (Walker et al. 2008; Stromdahl et al. 2010; CDC 2013).

Transovarial interference does not apply to all rickettsial bacteria although ticks naturally co-infected with multiple *Rickettsia* spp. are rarely reported. A single *D. variabilis* tick was detected with *R. belli, R. montanensis* and *R. rickettsii* (Carmichael and Fuerst 2006). Evidence of *R. parkeri* and the novel SFGR co-infecting individual *A. maculatum* has been reported in surveys from the southeastern United States (Sumner et al. 2007; Paddock et al. 2010; Varela-Stokes et al. 2011; Ferrari et al. 2012; Lee et al. 2016 *in press*). In one survey, the infection rate of *A. maculatum* co-infected with *R. parkeri* and "*Ca.* R. andeanae*"* was significantly higher than expected by random chance, at about 1.7% (12/698 of *A. maculatum*) (Ferrari et al. 2012). In a recent published study presented as part of this dissertation, we detected *R. parkeri* DNA levels higher than "*Ca*. R. andeanae" level in singly infected ticks, while levels for these two rickettsiae were similar in co-infected ticks (Lee et al. 2016, *In press*). Therefore, there is a possible interaction in these two rickettsiae in GCTs. However, possible interactions between

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sympatric rickettsiae in tick-rickettsial systems such as *R. parkeri* and "*Ca.* R. andeanae" in *A. maculatum* have not been reported and may differ from the *Dermacentor*-rickettsial system. The vector competence of *A. maculatum* as well as any interactions between *R. parkeri* and "*Ca.* R. andeanae," needs to be determined to better understand SFGR maintenance and transmission in the southeastern United States.

2.6 Artificial infection of ticks via capillary feeding

Experimental transmission of tick-borne organisms to laboratory animals using wild-caught infected ticks is rarely performed in the laboratory because most pathogenic organisms, such as *Borrelia burgdorferi*, *R. parkeri* and *R. rickettsii* have been cultured in the laboratory (Broadwater et al. 2002). It has been reported that laboratory mice infected by tick bite have a different immune response than mice infected by syringe inoculation in Lyme disease (Gern et al. 1993). Therefore, animal infections using pathogen-infected ticks are recommended in order to reproduce natural infection. Artificial feeding methods for hematophagous arthropods including ticks, mosquitoes, fleas and blood-sucking flies have been studied. The use of artificially-fed arthropods for transmission of organisms to vertebrate hosts is directly applicable to studying relationships among hosts, arthropods, and arthropod-borne organisms (Rutledge et al. 1964; Moloo 1971; Wade and Georgi 1988; Waladde et al. 1995; Young et al. 1996). For the study of tick-borne disease, artificial feeding methods have been used with fast-feeding argasid (soft) ticks but these methods are more complicated for slow-feeding ixodid (hard) ticks. Ixodid ticks attach to the feeding site for up to 2 weeks, during which time they create a feeding pool and secrete cement to bond the mouthpart on the skin (Sonenshine 1991; Young et al. 1996). Several techniques for artificial infection have

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been tested for hard ticks such as *Dermacentor, Amblyomma, Ixodes*, and *Rhipicephalus.* These techniques include capillary feeding (Chabaud 1950; Burgdorfer 1957; Purnell and Joyner 1967), feeding chambers using natural or artificial membranes (Howarth and Hokama 1983; Waladde et al. 1995; Young et al. 1996; Barre et al. 1998), enema infusion and hemocoelic inoculation (Turell et al. 1997). Additional tactile and olfactory stimuli for artificial feeding may often be provided to enhance attachment and feeing behavior of ticks including high concentrations of carbon dioxide (5-10%) in the chamber, 90-95% relative humidity, and maintaining blood temperature around 37 ˚C (Voigt et al. 1993). However, feeding chambers using natural or artificial membranes are expensive to prepare and maintain without contamination and decomposition (Chabaud 1950). Artificial feeding through enema infusion into ticks may create injuries and kill the ticks (Turell et al. 1997).

Artificial infection of ticks via capillary feeding was originally developed for infection of ixodid ticks. This method has been used for infection in a variety of ixodid tick species including *A. maculatum, A. americanum, Ixodes ricinus, nymphal I. scapularis* and *Dermacentor variabilis* (Chabaud 1950; Monin et al. 1989; Jasinskas et al. 2000; Rechav et al. 2000; Broadwater et al. 2002; Kocan et al. 2005). *Rickettsia parkeri* or "*Ca*. R. andeanae"-infected *A. maculatum* could be produced in the laboratory by feeding larval ticks on infected mice or injecting pathogenic organism into the tick and then allowing ticks to molt into nymphal or adult stages. However, rearing large numbers of infected *A. maculatum* ticks and laboratory animals may be a labor intensive, time consuming and expensive process. Isolation of *Rickettsia parkeri* and "*Ca*. R. Andeanae" in vitro using Vero E6 cells has been performed (Paddock et al. 2010). These rickettsiae

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could be introduced into ticks artificially, without feeding ticks on infected vertebrate hosts, and then infected ticks subsequently used for transmission to naïve vertebrate hosts.

2.7 Co-feeding in vector-borne system

Experimental infection of vertebrates with *R. rickettsii* produces variable degrees of clinical signs and rickettsemia, the presence of detectable circulating rickettsiae in the blood, for 4-14 days depending on inoculation doses in dogs and guinea pigs (Moe et al. 1976; Keenan et al. 1977; Norment and Burgdorfer 1984). Tick acquisition of circulating rickettsiae during periods of rickettsemia is one of the most important routes of *R. rickettsii* transmission to naïve ticks due to the lethal effects of *R. rickettsii* on its vector, *D. andersoni* (Niebylski et al. 1999). In contrast, only transient rickettsemia has been detected in opossums and cattle experimentally infected with *R. parkeri* (Horta et al. 2009; Edwards 2010). Infections with *R. felis* and *R. belli* produced no detectable rickettsemia in opossums (Horta et al. 2009). Rickettsemia in capybaras experimentally infected by *R. rickettsii* was sufficient to infect up to 35% of feeding *A. cajennense* ticks (Souza et al. 2009). Therefore, levels of rickettsemia may be related to differences of rickettsial strain, species or vertebrate hosts. Further, the acquisition of a *Rickettsia* sp. is not likely to be efficient for horizontal transmission from hosts to feeding ticks without presence of rickettsemia in the vertebrate host.

Co-feeding plays critical role for horizontal transmission of some pathogens between infected and uninfected (naïve) arthropods feeding on the same host when that host is not systemically infected by the pathogen. Horizontal transmission by co-feeding has been studied in *R. conorii* (Mediterranean spotted fever or Boutonneuse fever in

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human), *R. parkeri*, *R. massiliae*, certain arboviruses, and *Borrelia burgdorferi* (Lyme disease) (Jones et al. 1987; Labuda et al. 1993; Labuda et al. 1993; Levin and Fish 2000; Matsumoto et al. 2005; Zemtsova et al. 2010; Wright et al. 2015). Naïve *A. maculatum* ticks that acquire pathogens by co-feeding, especially in the nymphal stage, would subsequently increase chances a human exposure because adult *A. maculatum* ticks are known to bite on humans (Goddard 2002). This route of transmission can ensure continuous circulation of pathogens between vectors even in the absence of naïve reservoir hosts and newly acquired rickettsial pathogen may increase genetic diversity. Co-feeding as a strategy to transmit *R. parkeri* from infected adult stages to naïve nymphal stages of *A. maculatum,* as well as the influence of "*Ca*. R. andeanae" in recipient nymphs was evaluated in this dissertation.

2.8 Visualizing rickettsiae in the tick vector and vertebrate host

2.8.1 Ultrastructure of spotted fever group rickettsiae

The morphologically fine ultrastructure of SFGR is similar among species and difficult to differentiate based on ultrastructural examination. There are some characteristic structures on SFGR, such as an electron-lucent halo zone ("slime layer") and the adjacent trilaminar cell wall (TCW) on transmission electron microscopy (TEM) (Hayes and Burgdorfer 1979). The possession of a "halo zone" layer is one of the prominent characteristics in both non-pathogenic SFGR, like *R. rhipicephali,* and pathogenic SFGR, such as *R. rickettsii* and *R. parkeri*. The "halo zone" layer is not a shrink artifact and is suggested to be polysaccharide layer in nature based on TEM (Silverman et al. 1978; Hayes and Burgdorfer 1979). The "halo zone" is absent from *Wolbachia* endosymbionts found in arthropods, including ticks and *Wolbachia* species

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and *Francisella* species can be identified by the presence of multi-membrane cell walls (Niebylski et al. 1997). The TCW is composed of an osmophilic inner mucoprotein layer, a less osmophilic lipopolysaccharide middle layer and an osmophilic lipoprotein outer layer. Morphologically, SFGR are rod to elliptical measuring approximately 1-2 µm long and 0.4µm wide (Hayes and Burgdorfer 1979).

The distribution of the endosymbiont, *Rickettsia peacockii*, appears to be concentrated in female ovaries, where it interferes with transovarial transmission of a secondary *Rickettsia* sp., although it may also be found in specific portions of Malpighian tubules and midgut (Burgdorfer et al. 1981). An endosymbiont of questionable pathogenicity, *R. rhipicephali*, has been identified in salivary glands of *Rhipicephalus sanguineus* and causes moderate degree of cytological damage, especially during and after tick engorgement (Hayes and Burgdorfer 1979). However, the relationship between endosymbiotic rickettsiae and pathogenic rickettsiae in ticks is unknown. The characterization and comparison between nonpathogenic (or endosymbiotic) SFGR and pathogenic SFGR at the ultrastructural level helped to understand the transmission and pathogenicity in *A. maculatum* in this dissertation.

2.8.2 Fluorescence in situ hybridization (FISH) in rickettsial research

Morphological examination of SFGR using transmission electron microscopy (TEM) can be used to identify genera such as *Wolbachia* in tick tissue but cannot distinguish species of SFGR. Monoclonal antibodies used with immunogold labeling in TEM can cross-react to similar antigens among *Rickettsia* species. Fluorescence *in situ* hybridization (FISH) is a highly sensitive cytogenic technique to detect and localize the presence or absence of specific DNA sequence related to rickettsial species in tick tissue,

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including co-infected ticks (Langer-Safer et al. 1982). FISH probes labeled with Cy3 and Cy5 which are the most popular cyanine dyes can be combined for two-color detection. Cy3 and Cy5 dyes emit fluorescence at yellow-green at ~ 550/570nm and red at ~650/670nm, respectively (Gruber et al. 2000). FISH probes labeled with Cy3 and Cy5 successfully identified and localized *Rickettsia* sp. and "*Candidatus* Portiera aleyrodidarum" in the whitefly, *Bemisia tabaci* (Gottlieb et al. 2006). A *Coxiella*-type symbiont was localized in whole specimens of *A. americanum* using the FISH technique (Klyachko et al. 2007). FISH has also been used to visualize *Borrelia burgdorferi* sensu lato, the agent of Lyme disease, in gerbil skin biopsies and whole-body sections of *Ixodes ricinus* ticks (Hammer et al. 2001).

The designs of FISH probes are often based on the 16S ribosomal RNA gene which is the most ideal target for differentiate the bacteria due to high copy number and highly conserved region and availability of known sequences (Olsen et al. 1986). In some cases, it is hard to find suitable regions for FISH probe on the 16S rRNA gene to detect the organisms of interest, despite its length of approximately 1,500 nucleotides. The 23S rRNA gene has been studied as an ideal alternative for FISH probes in other bacteria such as *Escherichia coli* and *Salmonella* species because 23S rRNA genes are also high in copy number in all living cells and have a highly conserved structure (Woese 1987; Fuchs et al. 2001; Fang et al. 2003).

2.8.3 *Rickettsia parkeri* GFPuv strain

The novel green fluorescent protein (GFP) was first isolated from the bioluminescent jellyfish, *Aequorea victoria,* and is composed of 238 amino acid residues exhibiting bright green fluorescence under ultraviolet range (509nm) (Chalfie 1995). The

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widespread usage of GFP in cell and molecular biology led to development of GFP derivatives such as GFPuv, which has higher fluorescent than wild type GFP, and has been inserted into diverse *Rickettsia* spp. including *R. parkeri* (Shaner et al. 2005; Burkhardt et al. 2011). *Rickettsia parkeri* expressing GFPuv and antibodies to GFPuv can be used to detect and identify in vertebrate hosts and tick hosts and was used in these studies.

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2.9 References

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CHAPTER III

RICKETTSIA PARKERI AND "*CANDIDATUS* RICKETTSIA ANDEANAE" IN QUESTING *AMBLYOMMA MACULATUM* (GULF COAST TICK) FROM MISSISSIPPI

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3.1 Abstract

Amblyomma maculatum Koch (Acari: Ixodidae), the primary vector for *Rickettsia parkeri,* may also be infected with a rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae." Infection rates with these rickettsiae vary geographically, and co-infected ticks have been reported. In this study, infection rates of *R. parkeri* and "*Ca*. R.

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andeanae" were evaluated, and rickettsial DNA levels quantified, in a total of 335 questing adult *A. maculatum* collected in 2013 (n=95), 2014 (n=139), and 2015 (n=101) from Oktibbeha County, Mississippi. Overall infection rates of *R. parkeri* and "*Ca.* R. andeanae" were 28.7% and 9.3%, respectively, with three additional *A. maculatum* (0.9%) co-infected. While *R. parkeri*- infected ticks were detected all three years (34.7% in 2013; 13.7% in 2014; 43.6% in 2015), "*Ca.* R. andeanae" was not detected in 2013, and was detected at rates of 10.8% in 2014, and 15.8% in 2015. Interestingly, rickettsial DNA levels in singly-infected ticks were significantly lower in "*Ca.* R. andeanae"-infected ticks compared to *R. parkeri*-infected ticks (P<0.0001). Thus, both infection rates and rickettsial DNA levels were higher for *R. parkeri* than *"Ca.* R. andeanae." Infection rates of *R. parkeri* were also higher, and *"Ca.* R. andeanae" lower, here compared to *A. maculatum* reported previously in Kansas and Oklahoma. As we continue to monitor infection rates and levels, we anticipate that understanding temporal changes will improve our awareness of human risk for spotted fever rickettsioses. Further, these data may lead to additional studies to evaluate potential interactions among sympatric *Rickettsia* species in *A. maculatum* at the population level.

3.2 Introduction

The Gulf Coast tick, *Amblyomma maculatum* Koch (1844), is currently considered native throughout the Western Hemisphere, with North American populations mainly established along the Gulf and Atlantic Coasts of the United States (Teel et al. 2010). Of medical importance, *A. maculatum* is the major tick vector for *Rickettsia parkeri*, an agent of spotted fever group rickettsiosis, of which there are now at least 37 identified human cases in the US (Paddock and Goddard 2015). Reported infection rates

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of *R. parkeri* in *A. maculatum* vary in the southern states and may reach up to 56% in some areas (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Florin et al. 2013, Budachetri et al. 2014, Nadolny et al. 2014, Pagac et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). In addition to *R. parkeri*, *A. maculatum* may be infected with a spotted fever group rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae," first identified in *A. maculatum* and *Ixodes boliviensis* from Peru (Blair et al. 2004). In the southeastern US, "*Ca.* R. andeanae"-infected questing *A. maculatum* were reported at infection rates ranging from 1-6.3% (Sumner et al. 2007, 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, Leydet and Liang 2013, Budachetri et al. 2014, Paddock and Goddard 2015, Mays et al. 2016). A high prevalence of "*Ca*. R. andeanae" was also recently reported in *A. maculatum* from Kansas and Oklahoma, whereas *R. parkeri* was absent (Paddock et al. 2015). Co-infections of *A. maculatum* with *R. parkeri* and "*Ca*. R. andeanae" are not common, but have been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012, Leydet and Liang 2013, Budachetri et al. 2014), with one report documenting co-infections at a rate higher than expected by random chance (Ferrari et al. 2012).

Among the reports of rickettsiae in *A. maculatum*, rickettsial levels within infected ticks are not well-documented. In the present study, we evaluated infection rates of *R. parkeri* and "*Ca*. R. andeanae" and quantified rickettsial DNA levels in questing adult *A. maculatum* from central Mississippi, US, over a 3-year period. By continuing to monitor infection rates and additionally document rickettsial levels of selected pathogenic and (presumably) non-pathogenic tick-associated rickettsiae in this region over time, we

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provide insight into natural maintenance of these organisms and potential changes in risk for pathogen exposure.

3.3 Materials and Methods

3.3.1 *Amblyomma maculatum* collections and assays

Adult questing *A. maculatum* were collected by flagging/dragging in May-September of 2013-2015 from four sites within Oktibbeha County, Mississippi (Figure 1). The 2013 *A. maculatum* samples were previously from another study (Lee et al. 2014). We identified *A. maculatum* in the laboratory based on a standard taxonomic key (Keirans and Litwak 1989) and kept them in a humidity chamber (saturated potassium nitrate, ~93% humidity) until processing. To reduce external contaminants, we washed *A. maculatum* by vortexing 3 min in each of the following: 0.17% sodium hypochlorite, 0.5% benzalkonium chloride, 70% ethyl alcohol and sterile phosphate buffered saline (pH 7.4). Ticks were bisected sagittally and genomic DNA extracted from individual halves using a DNeasy Blood and Tissue Kit (Qiagen, Limburg, Netherlands). The other tick halves were archived (-80°C) and extracted DNA samples stored (-20˚C) until testing.

For quality control, DNA extracts were tested in a PCR assay to amplify a fragment of the tick mitochondrial 16S rRNA gene (Black and Piesman 1994). All tick extracts were positive by this assay, and subsequently screened for *R. parkeri* and "*Ca*. R. andeanae" DNA using a TaqMan® multiplex quantitative (Q)PCR assay with *Rickettsia*-wide primers and species-specific probes. Primers and probes are listed in Table 1, with the exception that concentrations of both QrompB primers in initial screening were at 300 nM. Non-template and positive controls were included in each assay, and reactions tested

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on a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). Extracts that were initially positive by QPCR were re-assayed to quantify rickettsiae by modifying the TaqMan® multiplex QPCR to include TaqMan primers and probe for the *A. maculatum* macrophage migration inhibitory factor (MIF) gene (sequences kindly provided by E. Harris and K. Macaluso, Louisiana State University). In each multiplex QPCR assay, 3µL sample DNA was mixed with Brilliant Multiplex Master Mix 2X (Agilent Technologies, Santa Clara, CA, USA), ROX reference dye (30 nM), and probes and primers (Table 1) in a 25µL reaction volume. We performed QPCR on a Stratagene Mx3005P with a two-step cycling profile consisting of 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. All positive extracts were tested in duplicate and all assays included ten-fold dilutions (10^7 to 10^2) of plasmid template mixture combining plasmids constructed using *R. parkeri* GFPuv Oktibbeha strain, "*Ca*. R andeanae" and *A. maculatum*. Non-template (water) controls were included in each run for quality control. Only data from multiplex QPCR assays with efficiencies between 90% and 110% for all three targets, and R squared values above or equal to 0.985 were used for evaluating rickettsial levels. We calculated levels in each extract using the ratio of *rompB* copy number to tick MIF copy number, for each rickettsial species. An annual and overall index of co-infection was calculated (Ginsberg 2008) using numbers of *A. maculatum* determined positive for *R. parkeri,* "*Ca.* R. andeanae," or both, by QPCR.

We confirmed all 2015 extracts that were positive by *rompB* multiplex QPCR for one *Rickettsia* sp. (n = 60) or both (n = 1) by sequencing *rompA* gene amplicons from species-specific PCR assays (Paddock et al. 2010, Varela-Stokes et al. 2011). PCR amplicons were purified (DNA Clean and Concentrator, Zymo Research, CA, USA),

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bidirectionally sequenced (Eurofins MWG Operon, Huntsville, AL, USA), and sequences aligned (ClustalX2) (Larkin et al. 2007). Consensus sequences were identified using BLAST (Basic Local Alignment Search Tool) analysis in the National Center for Biotechnology Information (NCBI) database.

3.3.2 Statistical Analyses

The occurrence of *R. parkeri* and "Ca. *R. andeanae"* infection in *A. maculatum* was assessed in separate logistic regression using PROC LOGISTIC in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). Gender, year, and gender by year interaction were initially included as explanatory variables. The gender by year interaction was not significant for either outcome and was removed, with the models refit. Penalized maximum likelihood estimation was used for the "Ca. *R. andeanae"* models due to quasi-complete separation of data points because no "Ca. *R. andeanae"* positive ticks were collected in 2013. The effect of year and *Rickettsia* species on rickettsial levels was assessed by ANOVA using PROC MIXED in SAS for Windows 9.4. The two main effects and their interaction were initially included as explanatory variables in the model. The year by *Rickettsia* species interaction was not significant and was removed, with the model refit. Pair-wise comparisons of years were made with Tukey correction of p-values. Due to low sample size for co-infected ticks, a Wilocoxon Signed Rank test using PROC UNIVARIATE in SAS for Windows 9.4 was used to compare "Ca. *R. andeanae"* to *R. parkeri* levels. An alpha level of 0.05 was used to determine statistical significance for all analyses.

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3.4 Results and Discussion

A total of 335 *A. maculatum* were collected between 2013 and 2015; no significant sex bias was observed in our population. Infection rates of *R. parkeri* and "*Ca*. R. andeanae" in *A. maculatum* varied annually, with overall rates of single infections at 28.7% and 9.3%, respectively (Table 2). The odds of detecting *R. parkeri* in 2014 were significantly lower compared to 2013 (OR 0.3; CI 0.156-0.567) and 2015 (OR 0.2; CI 0.110-0.383). In 2013, 33/95 (34/7%) of *A. maculatum* were positive for *R. parkeri* but "*Ca.* R. andeanae" was not detected. Not surprisingly, the odds of detecting *"Ca.* R. andeanae" in 2014 were significantly higher than in 2013 (OR 24.1; CI 1.435-404.8) and for 2015 compared to 2013 (OR 36.9; CI 2.196-618.649). There was no significant difference in infection rates based on *A. maculatum* gender (*R. parkeri* infection *P*= 0.9474; *"Ca.* R. andeanae" infection *P*= 0.6572). The overall co-infection rate was 0.9% (3/335 co-infected *A. maculatum*). The index of co-infection (IC) was calculated (Ginsberg 2008), with the IC between 0 and -11.9 among study years and an overall IC of -5.42, indicating fewer co-infected ticks than expected by chance alone. Rickettsial levels (calculated as the ratio of rickettsial *ompB* copy number to tick MIF copy number) in positive ticks, varied in singly infected ticks. The level of *"Ca.* R. andeanae" (6.76; SE 1.066) was significantly lower than *R. parkeri* (14.26; SE 0.584) (*P* < 0.0001). Rickettsial levels were also significantly different between 2014 and 2013 (Adj P<0.0001), and between 2015 and 2013 (Adj P<0.0001), likely because no *"Ca.* R. andeanae"-infected tick was detected in 2013. However, there was no significant difference in rickettsial levels between 2014 and 2015 (Adj P=0.9081). Finally, there was

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no significant difference in rickettsial levels between *R. parkeri* and *"Ca.* R. andeanae" in co-infected ticks (*P* = 1.0); however, there were only three co-infected ticks to evaluate.

For 2015 extracts positive only for *R. parkeri* by QPCR, 39/44 had consensus *rompA* sequences 100% identical to available *R. parkeri* (e.g. KF782320.1 and KC003476.1). Rickettsial *ompA* amplicons from all extracts positive for "*Ca.* R. andeanae" by initial QPCR (16/16) were also 100% identical to "*Ca.* R. andeanae" (e.g. KF179352.1 and KF030932.1). Of the five *R. parkeri* samples where a consensus sequence could not be resolved, one extract had one unambiguous sequence which demonstrated 99% identity with *R. parkeri* (e.g. KF782320.1 and KC003476.1). Two samples could not be confirmed by sequencing. For the remaining two samples, consensus sequences were 100% identical to multiple rickettsiae including *R. parkeri* (e.g. KP861344.1), an endosymbiont of *A. maculatum* (KP172268.1), and uncultured *Rickettsia* (e.g. JQ914775.1). We considered all 44 extracts positive for *R. parkeri* based on the two QPCR and specific *rompA* PCR assay results prior to sequencing. For *R. parkeri* and *"Ca.* R. andeanae"-specific *rompA* amplicons in the 2015 co-infected tick, a consensus sequence for the *"Ca.* R. andeanae" *rompA* amplicon was 100% identical to *"Ca.* R. andeanae", whereas one unambiguous sequence direction for the *R. parkeri* *rompA* amplicon was 99% identical to *R. parkeri*.

Thus, the three-year infection rate for *R. parkeri* (28.7%) was within the range previously reported for southeastern *A. maculatum* (Sumner et al. 2007, Paddock et al. 2010, Fornadel et al. 2011, Varela-Stokes et al. 2011, Wright et al. 2011, Ferrari et al. 2012, Florin et al. 2013, Budachetri et al. 2014, Nadolny et al. 2014, Pagac et al. 2014, Paddock and Goddard 2015, May et al. 2016). However, it was higher than that

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previously reported for similar sites sampled in Mississippi; 19.1% of *A. maculatum* were singly infected with *R. parkeri* in the location labeled “North” reported by Ferrari et al. (2012). While infection rates for “*Ca.* R. andeanae” varied among study years, the overall infection rate of “*Ca.* R. andeanae” here (9.3%), was higher than the previously reported infection rates in the Southeast, and higher compared to the three-year rate (0%) reported for the “North” location by Ferrari et al. (Paddock et al. 2010, Varela-Stokes et al. 2011, Ferrari et al. 2012, Nadolny et al. 2014). Thus, both “*Ca.* R. andeanae” and *R. parkeri* rates were increased in the current study. This may reflect a combination of temporal changes in the natural maintenance of both rickettsiae due to abiotic and biotic factors, and random fluctuation in rates. Still, “*Ca.* R. andeanae” infection in our sampled *A. maculatum* was lower, and *R. parkeri* higher, to rates recently reported from populations in Kansas and Oklahoma (Paddock et al. 2015). While uncommon, *A. maculatum* co-infected with *R. parkeri* and “*Ca.* R. andeanae” have been reported (Varela-Stokes et al. 2011, Ferrari et al. 2012). In the current study, we detected 0.9% co-infected questing *A. maculatum* (3/335). The overall index of co-infection (IC) was -5.42, demonstrating that the co-infection rate was lower than expected by chance alone, in contrast to a previous study from Mississippi (Ferrari et al. 2012). In our study, infection rates of both *R. parkeri* and “*Ca.* R. andeanae” varied, although only two of the four sites within the county were sampled consistently over the three year period; some sites could not be resampled due to human alterations (e.g. construction). The most notable annual fluctuations were with infection rates of “*Ca.* R. andeanae,” which did not appear to be negatively correlated to *R. parkeri* infection rates.

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Geographical differences in infection rates, particularly the absence of *R. parkeri* and overwhelming presence of *Ca.* R. andeanae" in *A. maculatum* from Kansas and Oklahoma suggest that rickettsial exclusion by transovarial interference may be occurring on a broader population scale (Paddock et al. 2015). Infrequent evidence of "*Ca.* R. andeanae" in *A. maculatum* has been reported where *R. parkeri* is frequently found in this tick vector (Florin et al. 2013, Nadolny et al. 2014, Pagac et al. 2014). We found that the mean rickettsial level for *A. maculatum* singly-infected with *R. parkeri* was significantly higher than for *A. maculatum* singly infected with "*Ca.* R. andeanae." This finding suggests that *R. parkeri* are maintained at a higher bacterial load than "*Ca*. R. andeanae" in questing *A. maculatum*. In contrast, the mean rickettsial levels for *R. parkeri* and "Ca. R. andeanae" in co-infected ticks were similar to each other and both low, compared to singly infected ticks. Using a non-parametric test for statistical analysis of these three ticks, we found no difference in rickettsial levels between the two rickettsial species. Considering the presence of "*Ca.* R. andeanae" and observed exclusion of *R. parkeri* in *A. maculatum* populations from Kansas and Oklahoma (Paddock et al. 2015), determining whether rickettsial levels in these populations vary from those detected here would contribute to a better understanding of geographical differences in *A. maculatum*-rickettsial maintenance. Of note, after completion of this study we were made aware that ticks from a laboratory-reared colony (Oklahoma State University Tick Rearing Facility; OSU) were released on part of Site 2 for an unrelated study by another group. Over the three years, 61 adult *A. maculatum* were collected from Site 2. No tick from this site was positive for *"Ca.* R. andeanae," while 31.1% overall were positive for *R parkeri.* Given that we detected *"Ca.* R. andeanae" but not *R. parkeri* infection in PCR tests of OSU

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colony ticks in the past, we do not suspect that this release significantly impacted our study. The *A. maculatum* collected were more likely from the endemic population.

In summary, the current study demonstrated disparate mean rickettsial levels and infection rates for *R. parkeri* and *"Ca.* R. andeanae" in *A. maculatum* from Oktibbeha Co., Mississippi. This is significant considering *A. maculatum* is a known and primary vector for this human pathogen, and is known to occasionally bite humans (Goddard 2002). Human cases have increased since the first case described in 2004, in part due to increased awareness and reporting (Goddard 2004, Paddock et al. 2004, Goddard and Varela-Stokes 2009, Paddock and Goddard 2015). Currently, the pathogenic potential for "*Ca.* R. andeanae" in *A. maculatum* and human or other vertebrate hosts is unknown. Understanding the relationship between *R. parkeri* and "*Ca.* R. andeanae" in the ticks, and monitoring infection rates and levels, will provide practical information for evaluating changes in human risk for *R. parkeri* rickettsiosis and the role of *"Ca.* R. andeanae" in affecting risk of spotted fever rickettsioses.

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Table 3.1 Primers and probes, with final concentrations, used in TaqMan® Multiplex QPCR assay for quantification of rickettsial levels.

Primer/Probe Name	**Sequence (5'→ 3')**	**Final Concentration**
QrompB_F	AAGTGGTACTTCAACATGGG	400 nM
QrompB_R	GCACCACCTTGGATTAAAG	400 nM
CaRa_probe_FAM	ATCGCGGAAGGTGCTCAAGTTAATG	50 nM
Rp_probe_HEX	ATTTTGGAAGGTGCGCAAGTTAATGC	400 nM
Amac MIF.18F	CCAGGGCCTTCTCGATGT	300 nM
Amac MIF.99R	CCATGCATTGCAAACC	300 nM
Amac MIF.63_Cy5	TGTTCTCCTTTGGACTCAGGCAGC	200 nM

Rickettsial ompB sequences used for design of primers and probes were GenBank accession numbers GU131157 and AF123717.

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Table 3.2 Number of *A. macluatum* [male:female] positive for rickettsial DNA [male:female] out of total *A. maculatum* collected per site in Oktibbeha County, Mississippi over the three year collection period.

	2013			2014			2015		
Site No.	***R. parkeri***	**"*Ca.* R. andeanae"**	**Co-infected**	***R. parkeri***	**"*Ca.* R. andeanae"**	**Co-infected**	***R. parkeri***	**"*Ca.* R. andeanae"**	**Co-infected**
1	16 [6:10]/ 45 [18:27]	0 [0:0]/ 45 [18:27]	0 [0:0]/ 45 [18:27]	15 [9:6]/ 66 [35:31]	12 [4:8]/ 66 [35:31]	2 [1:1]/ 66 [35:31]	13 [7:6]/ 42[22:20]	5 [4:1]/ 42[22:20]	0 [0:0]/ 42[22:20]
2	17 [8:9]/ 50 [23:27]	0 [0:0]/ 50 [23:27]	0 [0:0]/ 50 [23:27]	2 [0:2]/ 10 [1:9]	0 [0:0]/ 10 [1:9]	0 [0:0]/ 10 [1:9]	0 [0:0]/ 1[0:1]	0 [0:0]/ 1[0:1]	0 [0:0]/ 1[0:1]
3	NS [a]	NS	NS	NS	NS	NS	31[13:18]/ 58[24:34]	11[4:7]/ 58[24:34]	1[0:1]/ 58[24:34]
4	NS	NS	NS	2 [1:1]/ 63 [37:26]	3 [2:1]/ 63 [37:26]	0 [0:0]/ 63 [37:26]	NS	NS	NS
Total	33 [14:19]/ 95 [41:54]	0 [0:0]/ 95 [41:54]	0 [0:0]/ 95 [41:54]	19 [10:9]/ 139 [73:66]	15 [6:9]/ 139 [73:66]	2 [1:1]/ 139 [73:66]	44 [20:24]/ 101[46:55]	16 [8:8]/ 101[46:55]	1 [0:1]/ 101[46:55]

[a] Not sampled

Tick extracts initially positive by rompB QPCR were confirmed by rompA amplicon sequencing (2015 samples) and final QPCR analysis for rickettsial levels (2013 - 2015) .

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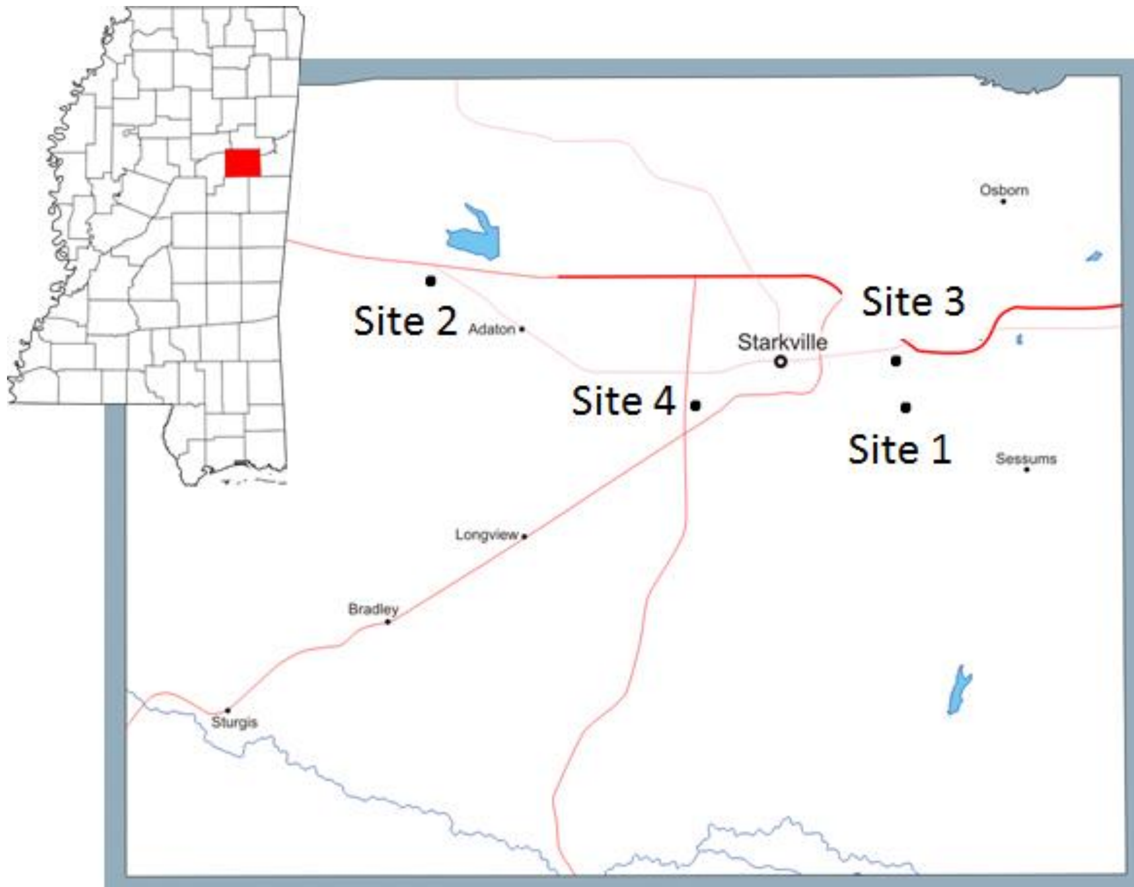

Figure 3.1 Geographical location of *A. maculatum* collection site in Oktibbeha County, MS.

Site points were based on geographical coordinates.

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CHAPTER IV

RICKETTSIA PARKERI AND "*CANDIDATUS* RICKETTSIA ANDEANAE" TROPISM IN TISSUES OF EXPERIMENTALLY INFECTED *AMBLYOMMA MACULATUM* (GULF COAST TICK)

4.1 Abstract

Spotted fever rickettsiosis caused by *Rickettsia parkeri* has been recognized in the United States since 2004. The primary vector for *R. parkeri, Amblyomma maculatum,* may contain another spotted fever rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae". We investigated tissue tropism of *R. parkeri* and "*Ca.* R. andeanae" in singly and co-infected *A. maculatum* prior to, during, and after blood feeding, using multiplex QPCR to quantify rickettsial DNA levels and microscopy to visualize organism in selected tissues. Transovarial transmission of rickettsiae in ticks and transmission of rickettsiae to infested rabbits were also evaluated. Three animal trials were performed using adult capillary-fed *A. maculatum* and 12 rabbits in four experimental groups: 1) control; 2) *R. parkeri* GFPuv; 3) "*Ca*. R. andeanae"; 4) *R. parkeri* GFPuv and "*Ca*. R. andeanae" combined. Salivary glands, midguts and ovaries (females), tick tissues selected for their importance in vertical and horizontal transmission of rickettsiae, were collected and tested on Days 0 (7 days post-capillary feeding), 6 and 12 after placement on rabbits. Rickettsial levels (calculated as a ratio of rickettsial to tick DNA) increased over the three time points and *R. parkeri* levels showed a greater increase in the co-

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infected group than the *R. parkeri*-singly infected group. Tropism for *R. parkeri* for midgut tissues was 2.69 times higher than for salivary gland tissues. No difference in tissue tropism based on the selected tissues was detected for "*Ca*. R. andeanae." Rickettsial transovarial transmission was successful for "*Ca*. R. andeanae", however, it was rarely detected for *R. parkeri*. Seroconversion of rabbits in all rickettsia-exposed groups, and additional microscopic detection of *"Ca.* R. andeanae" in salivary glands support transmission of this sympatric species to a vertebrate host. Results from this study demonstrate that *"Ca.* R. andeanae" is capable of transmission to a vertebrate host by *A. maculatum* and show that "*Ca.* R. andeanae" may be more efficiently transmitted transovarially than *R. parkeri*. Further, similar levels of *"Ca.* R. andeanae" coincident with increased levels of *R. parkeri* in co-infected compared to singly-infected ticks at the end of the tick feeding period suggest a possible synergistic effect. Rickettsial interactions in ticks may impact infection rates of either rickettsia differently depending on how ticks are exposed. Evaluating rickettsial dynamics under additional transmission settings will allow us to better understand natural maintenance of a rickettsial pathogen in the presence of an uncharacterized rickettsia.

4.2 Introduction

The first described case of American Boutonneuse fever, a spotted fever rickettsiosis caused by *Rickettsia parkeri*, was initially believed to be due to an infection with another spotted fever group *Rickettsia* (SFGR) because *R. parkeri* had not, until that time, been considered pathogenic (Paddock et al., 2004). Clinical signs for that index case and subsequent cases of *R. parkeri* rickettsiosis are characterized by an eschar at the tick-bite site, headache, fever, and maculopapular eruption on the trunk and extremities.

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Signs are similar to other spotted fevers including Mediterranean spotted fever caused by *R. conorii,* and Rocky Mountain spotted fever (RMSF), caused by *R. rickettsii,* though *R. parkeri* rickettsiosis is considerably milder than RMSF (Goddard 2004; Paddock et al. 2008; Parola et al. 2009). During the past twelve years, more than 37 human cases of SFGR caused by *R. parkeri* have been reported within in the nine southeastern United States (Paddock et al. 2004; Paddock et al. 2008; Cragun et al. 2010; Myers et al. 2013; Ekenna et al. 2014; Kaskas et al. 2014; Paddock and Goddard 2015). The prevalence of *R. parkeri* in its primary vector, *Amblyomma maculatum,* typically ranges from 10-56 % in the southern US where this tick is endemic (Sumner et al. 2007; Paddock et al. 2010; Varela-Stokes et al. 2011; Ferrari et al. 2012; Nadolny et al. 2014; Mays et al. 2016). The prevalence of *R. parkeri* is significantly higher than that of *R. rickettsii* in *Dermacentor* ticks, which is rarely over 1%, possibly due to the lethal effect of *R. rickettsia* in infected *Dermacentor* ticks (Philip and Casper 1981; Niebylski et al. 1999). However, the interaction of *R. parkeri* with other rickettsial agents in the *A. maculatum* is unknown.

A novel rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae," is now also recognized in *A. maculatum* in the southeastern US at low infection rates (Sumner et al. 2007; Ferrari et al. 2012; Mays et al. 2016). *"Candidatus* R. andeanae" was first identified in *A. maculatum* and *Ixodes boliviensis* in, where an outbreak of febrile disease in humans had occurred (Blair et al. 2004). In addition to being detected in the above tick species, *Rhipicephalus sanguineus* in Peru and *Amblyomma parvum* in Brazil were subsequently reported to harbor *"Ca.* R. andeanae" (Flores-Mendoza et al. 2013; Nieri-Bastos et al. 2014). Unlike the low infection rates of "*Ca.* R. andeanae" within the southeastern range of *A. maculatum,* a high frequency of "*Ca*. R. andeanae"-

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infected *A. maculatum* ticks was detected from populations in Kansas and Oklahoma, at 47% and 73% respectively; *R. parkeri* was not detected from these two states (Paddock et al. 2015).

Co-infections of *A. maculatum* with *R. parkeri* and "*Ca.* R. andeanae" have been reported (Varela-Stokes et al. 2011; Ferrari et al. 2012; Flores-Mendoza et al. 2013). Further analyses of these co-infections in one report described rates higher than what would be expected by random chance (Ferrari et al. 2012), however, these high co-infection rates were not maintained in more recent sampling of *A. maculatum* from Mississippi (Lee et al., 2016 *in press*). Further, rickettsial infection rates in *A. maculatum* from populations in Kansas and Oklahoma show a significantly different trend, with exclusion of *R. parkeri* in the presence of a high prevalence of "*Ca*. R. andeanae" in these ticks (Paddock et al. 2015). This suggests the dynamics among these sympatric rickettsiae at the population level may be more complex than either a simple synergism whereby presence of both species allows for increased occurrence of either, or simple exclusion whereby presence of one prevents the occurrence of the other species. Interactions among these rickettsiae within the individual tick and during host interactions are also not entirely clear.

Animal studies using guinea pigs, mice and rhesus macaque for evaluating *R. parkeri* infection, demonstrate a febrile illness with maculopapular eruptions, vasculitis and eschars, especially evident in non-human primates, where signs are similar to symptoms described in infected humans (Parker et al. 1939; Parker 1940; Grasperge et al. 2012; Banajee et al. 2015). While some insight has been gained into the dynamics of *R. parkeri* infection, rickettsial infection dynamics in the presence of both *R. parkeri* and

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"*Ca*. R. andeanae" are poorly understood. In this study, we tested the hypothesis that rickettsial infection dynamics in *A. maculatum* on rabbit hosts would be similar between groups exposed to either singly infected -*R. parkeri* or -*"Ca.* R. andeanae" but co-infection dynamics would differ in simultaneously exposed ticks. In addition, we hypothesized that unlike *"Ca.* R. andeanae," *R. parkeri* would demonstrate tropism to salivary gland tissues, as *R. parkeri* is known to be transmitted during blood feeding. Finally, considering approximately 50-75% of *A. maculatum* in a population of *A. maculatum* from Kansas and Oklahoma have evidence of "*Ca*. R. andeanae" while *R. parkeri* infection rates are generally lower, we hypothesized that transovarial transmission would be more common for "*Ca.* R. andeanae" than for *R. parkeri*. To address these hypotheses, we used an *A. maculatum*-rickettsia-rabbit model to quantify rickettsiae in selected tick tissues and additionally assessed transmission of rickettsiae to rabbits during tick feeding. Here, we present evidence that both rickettsial species are transmitted to rabbits, while rickettsial levels and vertical transmission differs between rickettsial species.

4.3 Materials and Methods

4.3.1 *Amblyomma maculatum* (Gulf Coast Tick)

Laboratory-reared *A. maculatum* (Gulf Coast tick, GCT) were purchased from Texas A&M University (TAMU), College Station, Texas and Oklahoma State University (OSU), Stillwater, Oklahoma. Both sources of *A. maculatum* colonies were used because previous work in our laboratory demonstrated evidence of "*Ca*. R. andeanae" in OSU colonies, and absence of rickettsiae in TAMU populations; however, this was not always consistent. To assess natural infection of rickettsiae in purchased ticks, a portion of ticks

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from each source were individually tested by PCR for *R. parkeri* and “*Ca*. R. andeanae” using previously published protocols targeting the outer membrane protein A gene (ompA) (Paddock et al. 2010; Varela-Stokes et al. 2011). All *A. maculatum* were maintained in a humidity chamber with saturated KNO3 (approximately 93% relative humidity) until they were used in experimental studies.

4.3.2 *Rickettsia* preparation

A strain of “*Ca.* R. andeanae”, originally isolated in our laboratory from naturally infected *A. maculatum,* was propagated in either Vero cells and *Ixodes scapularis* embryonic cells (ISE6, provided by U.G. Munderloh, University of Minnesota, USA) as described (Ferrari et al. 2013), to generate sufficient rickettsiae for capillary feeding. The Oktibbeha strain of *R. parkeri* transformed with plasmid pRAM18dRGA/Rif/GFPuv (provided by U.G. Munderloh, University of Minnesota, USA) (Burkhardt et al. 2011) was cultured in Vero cells. Infected and uninfected Vero cell cultures were maintained at 37°C, 5% CO_2 in Eagle’s Minimal Essential Medium with 10% fetal bovine serum. For preparation of material to be used in capillary feeding ticks, cells from infected and uninfected flasks were harvested with a cell scraper and the cell suspension passaged three times through 21 gauge (G) needle and then another 3 times through 30 G to release rickettsiae. The cell suspension was spun down at 50 *g* for 5 minutes to remove large clumps of cells and then rickettsial organisms were collected at 10,000 *g* for 10 minutes. An aliquot from this material was saved for DNA extraction (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia CA) and quantitative (Q)PCR using a TaqMan® protocol to specifically quantify *R. parkeri*- and “*Ca*. R. andeanae”(described below) prior to capillary feeding. The concentrations of *R. parkeri* and “*Ca*. R. andeanae” harvested at

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the time of capillary feeding were ~1X10^8 and ~1X10^3 DNA copy numbers/mL, respectively.

4.3.3 Vertebrate hosts

A total of 36 juvenile female New Zealand White rabbits were obtained from Charles River Laboratories, Massachusetts. All rabbits were 9-12 week-old, in good body condition, and purchased in three separate groups of twelve for the three replicate trials. All rabbits were acclimated for one week prior to use in transmission studies and kept within a climate-controlled laboratory animal housing room at the College of Veterinary Medicine, Mississippi State University. During the course of the study, rabbits were kept in individual cages and monitored at least twice a day during tick feeding and once a day after removal of ticks. These studies were approved by the Institutional Animal Care and use Committee (IACUC) at Mississippi State University.

4.3.4 Tick feeding trials

For each of the three replicate trials, twelve rabbits were distributed into four experimental groups, with three rabbits per group, and all rabbits infested with *A. maculatum.* Groups were defined according to what was used for capillary-feeding *A. maculatum*: (1) "*Ca*. R. andeanae" culture, (2) *R. parkeri* culture, (3) mixture of "*Ca*. R. andeanae" and *R. parkeri*, and (4) uninfected Vero cell culture (control). The study design is shown in Figure 1. For groups capillary-fed either *Rickettsia* sp. alone, a live cultured rickettsial suspension containing a known concentration of *R. parkeri* GFPuv or "*Ca*. R. andeanae" was fed to *A. maculatum* using 10µL glass capillary tubes for approximately 2 hours in a 32˚C incubator. For ticks capillary-fed both rickettsiae, we

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mixed *R. parkeri* and "*Ca*. R. andeanae" at the concentration comparable to those used for groups fed either species alone. Because "*Ca*. R. andeanae" levels were generally lower than *R. parkeri* levels for capillary feeding, we additionally used OSU GCTs for "*Ca*. R. andeanae" singly- and co-infected groups. We had previously determined that OSU GCTs were naturally infected with "*Ca*. R. andeanae". The control group was GCTs capillary-fed with uninfected Vero cells which were processed in the same manner as infected cells, tested for rickettsiae by QPCR, and resuspended in the cell culture media. After capillary feeding, GCTs were kept in a humidity chamber for one week. On the day of tick placement (DPT) 0 (one week after capillary feeding), 30 GCTs (male:female=1:1) were removed from each of the four groups and dissected to collect tissues for QPCR and microscopy, as described below. Another 36 GCTs (18:18=male and female) were placed on each of three individual rabbits for each group, with 12 total rabbits used in a single trial, and three trials sequentially. For tick placement, the dorsum of each rabbit was shaved for placement of a chamber measuring ~60cm^2, constructed using a design based on Embers et al. (2013). Rabbits were fitted with Elizabethan collars during tick feeding to discourage chewing of the tick chamber.

On DPT 6, 9 male and 9 female GCTs were removed from each rabbit and all GCTs were dissected for collection of tissues as on DPT 0. On DPT 12, the remaining GCTs were removed from each rabbit's chamber and processed as above. Rabbits were anesthetized during tick placement (DPT 0) and removal (DPT 6 and 12), and monitored twice a day during tick feeding and daily afterwards until completion of the study. For each rabbit, 2-3 of the replete female GCTs collected on DPT 12 were kept in a humidity chamber for oviposition. After oviposition, egg masses were divided, with a subset of

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eggs (approximately 300-500) processed for DNA extraction and rickettsial QPCR. The remainder of the egg mass was kept in the humidity chamber to hatch for subsequent QPCR testing of larvae. A subset of larval mass (approximately 100-300) processed as same as egg mass for rickettsial QPCR. Additionally, on DPT 12, rabbit skin biopsies were taken from the tick biting sites for cell culture and QPCR assay.

Blood was collected in heparin tube from each rabbit on DPT 0, 6, 12, and twice a week thereafter until DPT 33 (Figure 1, arrowhead). Blood samples were divided, with a sample of plasma saved for serological assays (indirect immunofluorescent antibody test; IFA) and whole blood saved for DNA extraction and subsequent QPCR. On DPT 33, rabbits were euthanized, and necropsy performed to collect selected organs, including skin, popliteal lymph node, axillary lymph node, mesenteric lymph node, spleen, lung, heart, liver, and kidney. Samples from these organs were saved at -20˚C for future PCR testing or placed in 10% formalin for histological examination. For evaluation of rickettsiae in tick tissues, we dissected GCTs collected on DPT 0, 6, and 12, and collected salivary glands, midgut and ovaries (in females). Tissues from three individual ticks were first pooled in one tube with PBS and then redistributed into three separate tubes for (1) QPCR assay; (2) microscopy by combined fluorescence *in situ* hybridization and immunohistochemistry (FISH/IHC); (3) and transmission electron microscopy (TEM). Tubes containing tick tissue for DNA extraction and subsequent QPCR were in PBS and were stored in -20˚C until DNA extraction. Tubes for FISH/IHC contained 10% formalin; and tubes for TEM contained Karnovsky's fixative, as described below, and were stored at 4˚C until processing. Additionally, legs were removed from individual

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ticks and pooled for each set of three as above, but used only for DNA extraction and QPCR to evaluate rickettsial infection in hemolymph.

4.3.5 DNA extraction and rickettsial DNA quantification by PCR

Genomic DNA was extracted from tubes of pooled like tissues (salivary glands, midgut, female ovaries, as well as legs) using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia CA) and stored at -20˚C until QPCR testing. Water samples were included with each set of tissues extracted, and were also tested by QPCR to evaluate environmental contamination with rickettsial DNA or QPCR products.

We quantified rickettsial levels relative to tick DNA (ratio) in GCT tissues using a TaqMan® multiplex QPCR assay. To quantify rickettsial DNA, we used Integrated DNA Technologies (IDT), Inc. (Coralville, IA) to design a primer set to amplify a 200-bp fragment of the rickettsial outer membrane protein B (*rompB*) gene from both *R. parkeri* and *"Ca.* R. andeanae", and probes specific to each rickettsia. For amplifying tick DNA, we used a previously designed set of primers and probe based on the *A. maculatum* macrophage migration inhibitory factor (MIF) gene (kindly provided by E. Harris and K. Macaluso, Louisiana State University).

To determine rickettsial DNA level, multiplex QPCR assay was performed in 25 µL reaction with 3µL of DNA extract and in 22 µL Brilliant Multiplex Master Mix 2X (Agilent Technologies, Santa Clara, CA, USA) with probes and primers described in Table 1. This master mix also included reference dye (ROX), used at a 30 nM final concentration according to manufacturer recommendations for the instrument. The cycling profile consisted of 95˚C for 10 min followed by two-step cycling with 40 cycles of 95˚C for 15 s and 60˚C for 1 min, performed on a Stratagene Mx3005P (Agilent

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Technologies, Santa Clara, CA, USA). All samples were analyzed in duplicate. To perform rickettsial DNA quantification, ten-fold dilutions from 10^7 to 10^2 of a plasmid template mixture combining plasmids constructed for *R. parkeri* GFPuv Oktibbeha strain, "*Ca*. R andeanae" and *A. maculatum* MIF were included in each QPCR assay. Non-template (water) controls were also included in each run for quality control. In addition, QPCR data was only accepted when assay efficiencies were between 90% and 110% for all three targets and R squared values above or equal to 0.985; these data were then used for evaluating rickettsial levels in ticks. Thus, rickettsial DNA levels in each tick extract were normalized to adjust for differences in genomic DNA content from individual extracts by calculating the ratio of *rompB* copy number to tick MIF copy number for each rickettsial species.

To test for rickettsial DNA in rabbit skin biopsies collected on DPT 12, approximately 5-15 mg of tissue was used for DNA extraction (Qiagen Inc., Valencia CA). For tissues harvested at necropsy, all tissues used in extraction with the exception of spleen were between 25-30 mg; spleen mass was 8-10 mg. Rickettsial DNA level to rabbit genomic DNA (12S) were quantified for axillary lymph node, biopsy skin at Day 12, necropsy skin at Day 33and spleen using QPCR as the same as tick tissue PCR except rabbit 12S specific primers and probe (Table 1). Of nine whole blood samples from rabbits collected from individual rabbits on sample days throughout the study (Figure 1), samples from DPT 0, 6 and 12 were assessed by QPCR for rickettsial DNA. The rickettsial QPCR assay using rabbit samples was similar to that for tick samples, with the replacement of a rabbit gene target (12S rRNA), and corresponding primers, probe and plasmid controls to determine relative quantities (Table 1).

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4.3.6 Fluorescence in situ hybridization and immunohistochemistry

Tick tissues were fixed in 10% neutral buffered formalin for 20-24 hours, then routinely processed, embedded in paraffin, and 5µm sections placed onto charged slides. To distinguish *R. parkeri* GFPuv and "*Ca*. R. andeanae" under microscopy, we optimized a protocol combining fluorescent in situ hybridization using a RNA probe (riboprobe) targeting "*Ca*. R. andeanae" and fluorescent immunohistochemistry using an antibody to GFPuv. To generate the riboprobe, we used primers designed in the lab for amplifying "*Ca*. R. andeanae" region of 23S-5S intergenic spacer region including T7 promoter site, and based on sequences generated using previously published primers (Stothard et al. 1994). Our in situ 23S primers for amplifying the 300 bp riboprobe were as follows, Rick 23S_F 5'-CCATTAGAGCCGTGGAAGAC-3' and Rick23S_R_T7 5'-TAATACGACTCACTATAGGGCCACCAAGCTAGCAATACAA-3'. To prepare the riboprobe, the "*Ca*. R. andeanae" 23S amplicon was labeled with digoxigenin-UTP by in vitro transcription with T7 RNA polymerase (Roche Diagnostics Corporation, Indianapolis, IN). Sections were first deparaffinized and then steamed at 100ºC for 20 minutes in 10mM sodium citrate buffer (pH 6) to begin antigen retrieval. Sections were agitated in 0.3% Triton™ X-100 (4-1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol (Sigma, St. Louis, MO) at room temperature (RT) for 10 min and digested with 5µg/mL of proteinase K at 37ºC for 20 min. Post-fixation in 4% formaldehyde at 4ºC for 10 min was followed by acetylation (0.25% acetic anhydride) using 0.1M triethanolamine at RT for 10 min, and subsequent denaturation in 70% formamide at 70ºC for 10 min. The sections were hybridized with a digoxigenin-labeled 23S ribosomal RNA probe specific for "*Ca*. R. andeanae" at 43ºC for overnight. The next day, the sections were blocked at

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RT for 20 minutes with bovine serum albumin (BSA) diluted to 5% (Blocker™ BSA (10%) in PBS, Thermo Scientific, Rockford, IL). Sections were then incubated with sheep anti-digoxigenin primary antibody (1:800, Roche Diagnostics, Indianapolis, IN) and mouse anti-GFPuv primary antibody (1:500, R&D systems, Minneapolis, MN) in PBS with 5% BSA at RT for 1 hour. For secondary antibody, slides were incubated with cyanine (Cy)3 conjugated donkey anti-sheep (1:500, EMD Millipore Corporation, Temecula, CA) in PBS with 5% BSA at RT for 60 minutes and then subsequently incubated with Cy2 conjugated goat anti-mouse antibody (1:250, Abcam Inc., Cambridge, MA) in PBS with 5% BSA at RT for 60 minutes. The sections were covered with mounting media (KPL Inc., Gaithersburg, MD) and observed using Olympus BX60 fluorescence microscope (Olympus, Pennsylvania, US) and then ZEISS Axiovert 200 M Inverted confocal laser scanning microscope for selected slides (Zeiss Light Microscopy, Gottingen, Germany). Uninfected Vero cells and Vero cells infected with *R. parkeri* and "*Ca*. R. andeanae" were used as negative and positive controls, respectively. Cell cultures of rickettsiae were first pelleted and resuspended in molten agarose, then plugs placed in separate histological cassettes, paraffin embedded, and processed alongside tick tissue slides.

4.3.7 Transmission electron microscopy (TEM)

Tick tissue samples (salivary gland, midgut, and female ovaries) from each group of GCTs were fixed in Karnovsky's fixative (4% glutaraldehyde and 4% paraformaldehyde) containing 1% DMSO in 0.1M cacodylate buffer at 7.2. Tick tissues were rinsed 4 times with 0.1M cacodylate buffer at pH 7.2 for 15 minutes on ice and then were post-fixed in 2% osmium tetroxide in 0.1M cacodylate buffer at pH 7.2 for 2 hours.

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After washing osmium tetroxide with distilled water, tick tissue were *en bloc* stained with 2% aqueous uranyl acetate overnight and then dehydrated with a serially graded ethanol series. Tick tissues were embedded in Spurr's resin (Electron Microscopy Science, Hatfield, PA) and polymerized overnight at 68-70˚C. Sections were cut from the block at approximately 60-90 nm thickness using a Reichert-Jung Ultracut E Ultramicrotome (Vienna, Austria). Tissue sections were stained with alcoholic uranyl acetate and then lead citrate. Sections were observed on a JEOL 1230 Transmission Electron Microscope (JEOL USA, Peabody, MA) at 120 kV. Cultured *R. parkeri* and "*Ca*. R. andeanae" were suspended in 1% of agarose as positive controls and processed as tissues. Tick tissues confirmed negative for rickettsiae by QPCR were used for negative tissue controls.

4.3.8 Cell culture for rickettsial isolation from skin

For rickettsial isolation from tick biting sites of rabbit skin, a full thickness 6mm in diameter skin (biopsy punch, Miltex Inc., York, PA, USA) was collected from each rabbit on DPT 12. Biopsy sites were within the feeding chamber, adjacent to tick biting site but on intact (undamaged) skin. Samples were cleaned with 70% ethanol, rinsed with autoclaved distilled water and then dried briefly before dividing into portions for DNA extraction, histology, and cell culture; cell culture portions were frozen at -80ºC until processing. A small amount of skin section (50 mg) was triturated into small pieces in fresh Minimum Essential Medium Eagle (MEM) at pH 7.3 (Sigma-Aldrich, St. Louis, MO), 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), 100U/mL of Penicillin, 100 µg/mL of Streptomycin (Pen Strep, Gibco by Life Technologies, St. Louise, MO), and 2.5 µg/mL of amphotericin (Sigma-Aldrich, St. Louis, MO), and then inoculated into a 80-90% confluent flask of Vero cells and maintained for 2 months.

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Small areas of cultured Vero cells were harvested using a cell scraper (BD Biosciences, Bedford, MA) for DNA extraction and rickettsial PCR using species-specific primers targeting the rickettsial outer membrane protein A (*rompA*) gene for *R. parkeri* and "*Ca*. R. andeanae" (Paddock et al. 2010; Varela-Stokes et al. 2011). Amplicons were visualized in 2% agarose gels stained with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, CA, 92008), and examined under ultraviolet light. Cytospins were also occasionally performed, with slides stained by acridine orange (BD, Maryland, US) or Diff-Quick (Dade Behring, Delaware, US) to examine for rickettsiae.

4.3.9 Histopathology

A section from skin biopsy samples as well as tissue samples collected on DPT 33 (skin within the tick chamber, popliteal lymph node, axillary lymph node, mesenteric lymph node, spleen, lung, heart, liver, and kidney) were fixed in 10% neutral buffered formalin, processed routinely, sectioned at 5µm, and stained with hematoxylin and eosin for histopathologic examination.

4.3.10 Indirect fluorescent antibody assay (IFA)

Plasma was separated from a portion of heparinized blood collected on the nine sample days, and was stored at -80ºC until serologic testing by indirect fluorescence antibody assay (IFA). For antigen slides, 12-well-slides were prepared using *R. parkeri* (Portsmouth) grown in Vero cells, and stored in -80˚C until use. Rabbit plasma samples were screened at 1:32 and 1:64 dilutions as previously described (Moraru et al. 2013). For detection, we used FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG (KPL Inc., Gaithersburg, MD). Slides were counterstained with Erichrome™ Black T

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(Fisher Scientific, Waltham, MA, USA), were mounted with Vectashield and DAPI (Vector Laboratories, Inc., Burlingame, CA, USA), then were observed under ultraviolet light. For quality control, positive and negative sera from cattle were applied. Samples were considered seropositive if they demonstrated reactivity at the 1:64 dilution as well as 1:32 dilution; these samples were then serially diluted two-fold to determine end-point titers.

4.3.11 Statistical Analyses

Because of the large number of samples negative for rickettsiae by QPCR, and therefore having ratios of rickettsial to tick DNA ("rickettsial levels") equal to "0," data were not normally distributed. Thus, to determine the effect of experimental group, sample day, tissues, and sex on rickettsial levels we used a mixed model logistic regression using PROC GLIMMIX in SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC, USA). Separate models were fit for "*Ca*. R. andeanae" DNA ratio and *R. parkeri* DNA ratio. The explanatory variables were selected for a multivariable model by manual forward selection starting with experimental groups. The interaction terms of the main effects were tested and removed if not significant. The random effects included in the models were day (rabbit trial), rabbit (trial), and trial. When analyzing serological responses, the data was also not normally distributed due to the large numbers of seronegative samples. Thus, the effects of experimental groups and sample day on IFA titers were also assessed by mixed model logistic regression. Rabbit (trial) and trial were included as random effects. The residual option was used in a random statement with a first order autoregressive covariance structure to account for the repeated measures of

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rabbits. Results were reported as odds ratios. An alpha level of 0.05 was used to determine statistical significance.

4.4 Results

4.4.1 Tick feeding trials

During the three tick feeding trials, no rabbits exhibited clinical signs related to rickettsial infection. While initially screening GCTs from the TAMU source, which had been previously negative for both rickettsial species, we detected "*Ca.* R. andeanae" at low infection rates approximately 5-10%. Subsets GCTs from OSU and TAMU infected with "*Ca*. R. andeanae" were preferentially selected for "*Ca.* R. andeanae"-singly infected or co-infected groups.

4.4.2 *Rickettsia parkeri* level

In salivary gland extracts, the average ratio (AR) of *R. parkeri* in GCTs capillary-fed *R. parkeri* alone (*R. parkeri*-singly infected group) was low 0.003 ± standard error (SE) 0.002 at DPT 0 (7 days after capillary infection) and increased to 0.351 ± SE 0.218 at DPT 12 (Table 2). GCTs capillary-fed both rickettsial species did not have detectable levels of *R. parkeri* on DPT 0, but the AR of *R. parkeri* increased to 83.320 ± SE 43.761 on DPT 12. In midgut tissue extracts, the AR of *R. parkeri* in GCTs capillary-fed *R. parkeri* alone was 0.005 ± SE 0.002 on DPT 0 and increased slightly to 1.076±SE0.630 on DPT 12. The AR of *R. parkeri* in GCTs capillary-fed both rickettsial species on DPT 0 was 0.125±SE 0.053 and demonstrated an increase to 35.621±SE13.490 by DPT 12, which was 3311 times higher than in GCTs capillary-fed *R. parkeri* alone. We used DNA extracts from legs to estimate rickettsial levels in circulating hemolymph from all

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experimental groups. The AR for *R. parkeri* in GCTs from this capillary-fed group remained comparatively low at 0.002±0.001 and 0.092±0.080 on DPT 0 and 12, respectively. However, the AR of *R. parkeri* in the group fed both rickettsial species increased from 0.001±0.001 on Day 0 to 69.582±42.487 on DPT 12 (Table 2).

While the AR of *R parkeri* showed an increasing trend from Day 0 to Day 12, statistical analysis using logistic regression showed that when comparing presence or absence of *R. parkeri* between Day 0, 6, and 12, differences were not significant. Overall, the odds of the *R. parkeri* in the midgut tissue in all groups was 2.691 times greater ($P<0.0001$, CL=1.885 and 3.903) than for *R. parkeri* detection in the salivary gland. In addition, the very low AR of *R. parkeri* in the control group and in the "*Ca*. R. andeanae"-singly infected group was also detected on QPCR (Table 2). While low levels of *R. parkeri* were detected unexpectedly by QPCR in groups that were not capillary-fed *R. parkeri,* when these samples were tested by conventional PCR targeting the *rompA* gene, most (approximately 95%) were negative. Thus, we suspect that occasional contamination occurred in the QPCR assay, albeit at low levels considering the ARs in those groups.

4.4.3 *"Candidatus* Rickettsia andeanae" level

In salivary gland extracts, the AR of "*Ca*. R. andeanae" in GCTs capillary-fed "*Ca*. R. andeanae" alone- was 0.903±0.193 on DPT 0 and increased to 23.636 ±7.393 by DPT 12. The AR of "*Ca*. R. andeanae" in GCTs capillary fed both rickettsial species was 0.582 ± 0.180 on DPT 0 and increased similarly, to 24.102 ± SE 11.928 by DPT 12. (Table 3). In midgut tissue extracts, the ARs of "*Ca*. R. andeanae" in the *"Ca.* R. andeanae" capillary-fed group and the group with GCTs fed both rickettsial species were

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low on DPT 0 and also increased similarly by DPT 12 (Table 3). In the "*Ca.* R. andeanae" fed group, ARs for this rickettsia were similar on DPT 0 and 12 in leg extracts but demonstrated a decrease from 14.247±4.213 on DPT 0 to 4.709±0.999 on DPT 12 in the group capillary-fed both rickettsial species. The low ARs of "*Ca.* R. andeanae" in the control group and the *R. parkeri* group detected on QPCR were likely due to low natural infection of GCTs colonies from OSU and TAMU (Paddock et al. 2010).

Using logistic regression based on positive and negative tissue frequency, there was no significant association among the salivary gland, midgut and leg. Overall, there was a significant association for frequency of detection (presence and absence) on experimental groups ($p<0.0001$) and Days ($p=0.0001$). The odds of detecting "*Ca*. R. andeanae" in the GCT group capillary-fed "*Ca*. R. andeanae" was 1.915 times greater than in the group fed both rickettsial species ($P=0.0435$, CL=1.019 and 3.597).

4.4.4 Transovarial and transstadial transmission

The AR of *R. parkeri* in extracts of female GCT ovaries remained low during the three sample days for the group fed *R. parkeri* alone; transovarial transmission was not detected (Table 4A). While the AR of *R. parkeri* was increased from 0.002±0.001 at DPT 0 to 5.950±2.996 on DPT 12 in co-infected group, transovarial transmission was also not detected. One larval pool of replete female from each set of those tested in the "*Ca*. R. andeanae"-fed and co-infected group was positive for *R. parkeri*. The positive for *R. parkeri* on "*Ca*. R. andeanae"-singly infected group was also identified testing *rompA* gene. The odds of detecting *R. parkeri* in ovary extracts from the co-infected group was 2.868 times greater than in the *R. parkeri* capillary-fed group but was not statistically significant ($P=0.1501$, CL 0.680 and 12.094).

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The AR of "*Ca*. R. andeanae" in ovary extracts from both GCTs capillary-fed "*Ca*. R. andeanae" only and in the co-infected group increased from DPT 0 to 12, but was similar between both experimental groups. Transovarial transmission was detected in approximately 69% and 59% of egg pools from the *"Ca.* R. andeanae"-fed and co-infected group, respectively (Table 4B). In the uninfected Vero control and *R. parkeri* capillary-fed groups, we also detected relatively a low AR of "*Ca*. R. andeanae" transovarial transmission was also consistently detected at low levels in egg and larval pools from these groups, confirming presence of natural infection in the tick population used in these studies.

4.4.5 Fluorescence in situ hybridization and immunohistochemistry

We selected GCT tissues based on QPCR results in order to confirm our ability to differentiate rickettsiae using the combined FISH/IHC technique, as well as confirm the presence of rickettsiae in tissues reported with high rickettsial ratios. The AR range for selected tissues was approximately 10-300 AR range. Using the combined protocol, we were able to detect fluorescent signal consistent with *R. parkeri* and "*Ca.* R. andeanae" (Figure 2a) in slides containing sections from Vero cell cultures infected with either rickettsial species; we also did not see representative signal in uninfected Vero cells (control). We detected rod-shaped organisms approximately 1µm, consistent with *R. parkeri* in salivary gland tissue in co-infected group on DPT 12 (Figure 2b). In addition, signal consistent with "*Ca*. R. andeanae" was detected in the developing eggs in co-infected group on Day 12 (Figure 2c). Similarly, *R. parkeri* signals were detected in the tissues of salivary and midgut in *R. parkeri*-group and co-infected group. Similar rod shaped organisms of "*Ca*. R. andeanae" were confirmed in tissues including salivary

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gland, midgut and ovary from “*Ca*. R. andeanae” group and co-infected groups. However, two different organisms, both *R. parkeri* and “*Ca*. R andeanae” were not observed in one tick tissue in the co-infected experimental group.

4.4.6 Transmission electron microscopy (TEM)

Tissue samples including salivary gland, midgut and ovary were also selected for transmission electron microscopy (TEM) as same manner as FISH/IHC visualization. Transmission electron microscopy (TEM) would not differentiate rickettsial species levels but could reveal approximately 1µm x 0.5µm rod-shaped electron-dense bacteria with halo zone and trilaminar cell wall consistent with *Rickettsia* spp (Silverman et al. 1978; Hayes and Burgdorfer 1979) Tissues including salivary gland, midgut, and ovary from both singly-infected and co-infected groups contained evidence of rickettsial organisms by TEM. A single rickettsial organism was observed in developing egg in ovarian tissues in “*Ca*. R. andeanae”-infected group on Day 12 (Figure 2d). Clusters of rickettsial bacteria were observed in midgut tissues in co-infected group on Day 12 (Figure 2e) and salivary gland in co-infected group on Day 12 (Figure 2f).

4.4.7 Rickettsial transmission to rabbits

Antibody titers against rickettsiae were analyzed by indirect fluorescent antibody assay (IFA) with *R. parkeri* Portsmouth stain. All rabbits from the three trials were seronegative on DPT 0. Some rabbits from treatment groups showed seroconversion (titer 64) on DPT 6, and most were seropositive, by DPT 12, with end-point titers that decreased by the end of the study (Figure 3). The geometric mean titers of rabbits in the three trials for the control group, “*Ca*. R. andeanae” group, *R. parkeri* group, and co-

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infected group on DPT 12 were 117, 59, 128 and 30, respectively, demonstrating exposure to *rickettsia*-infected GCTs in control ticks as well. Overall statistical analysis using logistic regression, the odds of being positive on DPT 12 were 12.5 times greater than DPT 6 (P<0.001, CL 3.888 and 40.140). The odds of being positive on DPT 18 were 6.6 times greater than DPT 6 (P=0.0011, CL 2.155 and 20.508). However, the odds of being positive among experimental groups were not statistically significant by logistic regression (P=0.8648).

On histopathological examination, small aggregates of lymphocytes, plasma cells and few macrophages were present in the deep dermis with slightly reactive blood vessels. In axillary lymph node, small numbers lymphocytes, plasma cell and cellular debris were present in the subcapsular and medullary spaces. Rickettsial isolation from rabbit skin biopsies within the tick feeding chamber was attempted, with cultures maintained for 2 months. Rickettsial organisms were neither detected visually nor by conventional PCR targeting *ompA* gene for any samples in the three trials.

4.5 Discussion

Using laboratory-reared *A. maculatum* that were capillary-fed uninfected cell culture, cultured *R. parkeri* or *"Ca.* R. andeanae", or a mixture of both sympatric rickettsiae, we identified several trends in rickettsial levels during rabbit feeding, transmission of both rickettsiae, and a naturally infected laboratory colony. Both *R. parkeri* and "*Ca*. R. andeanae" DNA levels showed an increasing trend in DNA levels (based on ratios of rickettsial to tick DNA) from DPT 0, when ticks were subsampled a week after capillary feeding, to DPT 6 and 12, when they were allowed to feed on rabbit hosts. This trend was observed in salivary gland and midgut tissues for, although DNA

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extracts from legs removed from ticks, and representative of hemolymph samples, did not show this trend for *"Ca.* R. andeanae" in either the ticks exposed to this culture alone or in a mixture with *R. parkeri.* These trends suggest replication of the bacteria from artificial exposure to feeding on a vertebrate host. While *R. parkeri* levels in ticks capillary-fed this culture were generally low, levels in co-infected ticks exposed to both rickettsial species were higher, and this was consistent among the three tissues tested. We observed variation in levels both within rabbits from an experimental group in a single trial, as well as among the three trials that were performed, but these trends were fairly consistent. In addition, we detected a low level of rickettsiae by our QPCR assay, revealing a natural infection with *"Ca.* R. andeanae" in a population of ticks that we had determined to be rickettsia-negative and selected prior these studies. We observed a mortality rate of approximately 30% in *A. maculatum* that were capillary-fed *R. parkeri*, but this was not as dramatic in ticks capillary fed the mixture of rickettsiae. The ticks selected for capillary feeding in this co-infected group were from OSU which was naturally infected and might cause less lethal effect. The size of OSU ticks was generally larger than TAMU ticks which might cause less lethal effect to this group. We also observed a mortality of approximately 10% for *A. maculatum* in the *R. parkeri* group during the feeding period on rabbits; this was not observed in other groups (data not shown). The mild lethal effect on *R. parkeri* infected ticks was from TAMU which was smaller size than OSU. While there may be speculation that *R. parkeri* has some degree of lethal effect in *A. maculatum,* we used a modified strain (*R. parkeri* Oktibbeha GFPuv) and detected low levels of rickettsiae in this treatment group; dead ticks were not tested. Therefore, lightly infected GCTs might be survived due to less lethal effect. Further, the

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source of ticks for these groups was both TAMU and OSU, and these populations may have different fitness as we observed some differences in tick size. Some degree of *R. parkeri* lethal effect was reported in *A. triste* but lethal effects from *R. parkeri* may differ between *Amblyomma* species (Nieri-Bastos et al. 2013).

While we detected a higher rickettsial level of *R. parkeri* on DPT 12 in tissues from the co-infected group, levels of "*Ca*. R. andeanae" in co-infected group were lower, and similar to levels in group capillary-fed only "*Ca*. R. andeanae". Notably, ticks in either the *"Ca.* R. andeanae" singly or co-infected groups were selected from the OSU source due to low levels of this rickettsiae in culture for capillary feeding. Thus the levels we detected by QPCR may be more representative of natural infection than *R. parkeri* levels. However, this would not account for the higher levels of *R. parkeri* in the co-infected group compared to the singly-infected group, and may suggest an interaction that should be explored further. Still, we found that the odds of detecting *R. parkeri* in the *R. parkeri*-singly infected group and coinfected group were not statistically significantly different. Interestingly, the odds of detecting "*Ca*. R. andeanae" in the "*Ca*. R. andeanae"-singly infected group was 1.92 times greater than in the co-infected group, suggesting that further investigation on the potential for suppressed replication by *R. parkeri,* including other *R. parkeri* strains, are warranted. Nonetheless, the "*Ca*. R. andeanae" levels were maintained in both groups and we observed evidence that this organism was transmitted to rabbits, as at least some rabbits in all groups seroconverted to rickettsiae based on antibodies reactive to *R. parkeri* antigen.

In ovary tissues, we observed transovarial and transstadial transmission of "*Ca*. R. andeanae" in groups capillary fed this organism alone or mixed with *R. parkeri*. "In

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contrast, no transovarial and rare transstadial transmission was detected in *R. parkeri*. This phenomenon might support harmful or lethal effect to *A. maculatum* which was similarly observed on incubation (Day -7) after capillary feeding and during feeding. Alternatively, it may be due to the modified strain of *R. parkeri* we used. Unlike, studies evaluating transovarial interference, where preacquisition of one rickettsia was performed before introduction of a second rickettsial species, we introduced both rickettsiae simultaneously (Burgdorfer and Brinton 1975; Macaluso et al. 2002). While both rickettsiae could be detected in tissues of exposed ticks, only one was observed to be passed to offspring in this group. In this group, we also suspect that some ticks may have been naturally infected considering the low presence of *"Ca.* R. andeanae" in the source. Thus, there is a possibility that the introduction of *R. parkeri* was in the presence of the sympatric rickettsia. Transovarial and transstadial transmission of *R. parkeri* has been reported in *A. americanum* and *A. triste* (Goddard 2003; Nieri-Bastos et al. 2013). Therefore, transovarial and transstadial transmission of *R. parkeri* might react differently on *Amblyomma* species or possible play a role of "*Ca*. R. andeanae." Again, we used modified *R. parkeri* GFPuv which might react differently.

Organisms consistent with rickettsiae, in treatment groups for *"Ca.* R. andeanae" and *R. parkeri* were visualized by the FISH/IHC assay we developed, providing an additional evidence of rickettsial presence in tick tissues. Numerous organisms corresponding to *R. parkeri* were detected in the salivary gland and midgut, however this was not observed within the developing eggs in ovary which were also consistent with rare transovarial transmission of *R. parkeri* that we observed here. We rarely detected "*Ca*. R. andeanae" in the salivary gland and midgut, but "*Ca*. R. andeanae" were

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frequently observed within the developing eggs in ovary. Rickettsial AR was relatively lower in ovary compared to salivary and mildgut which might make difficult to visualize fewer rickettsial organism in the ovarian tissue. Unlike the FISH/IHC protocol where we were able to label rickettsiae with two probes, the ultrastructure of SFGR is similar among species. TEM was included to confirm only the presence of organisms that had characteristics expected for SFGR, including electron dense rod to elliptical 1μm long bacteria, an electron-lucent halo zone ("slime layer") and adjacent trilaminar cell wall (Silverman et al. 1978; Hayes and Burgdorfer 1979). Clusters of rickettsia-like organisms were visualized in the developing eggs, midguts, and salivary glands which were useful technique to differentiate from other genus possibly present in the ticks (Figure 2) (Niebylski et al. 1997). While other prokaryotes may have been present in *A. maculatum* and detectable using TEM, we limited our search to organisms likely to be SFGR.

We were unable to cultivate rickettsiae from the samples of skin biopsied from the tick feeding sites on DPT 12 based on lack of detectable rickettsial DNA by PCR and lack of organism presence in culture cytospins. Rickettsial DNA on blood sample on Day 0 and 6 and the rabbit tissues on Day 33 including axillary lymph node, skin and spleen by QPCR were not detected. Despite the absence of detectable rickettsiae in sampled tissues, exposure to rickettsiae was documented in all treatment groups. Therefore, rickettsial transmission might occur early and neutralize in a short period time. However, antibody titer maintained at least 33 days and could be used for rickettsial exposure test without rickettsemia. As this included the control groups which received ticks from the TAMU source, and low levels of *"Ca.* R. andeanae" were detected in this group, it became apparent that transmission of *"Ca.* R. andeanae" could occur regardless of

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whether ticks were capillary fed cultured organism. Further, detection of "*Ca*. R. andeanae" in the salivary gland of "*Ca*. R. andeanae"-singly infected group and seroconversion in that group further supported this observation.

Histopathological examination of rabbit tissue was unremarkable. Mild lymphoplasmacytic dermatitis was likely due to a low degree of inflammation on tick feeding in that area. No vasculitis was observed which was typical for rickettsial infection (Grasperge et al. 2012; Banajee et al. 2015). Rickettisal DNA from the rabbit tissue was not detected in any experimental group.

In summary, this study examined differential levels of two sympatric rickettsiae within selected tissues of their tick host, *A. maculatum.* Under conditions GCTs were exposed to one or both rickettsiae and then allowed to feed. Specific tissue tropism was not detected for "*Ca*. R. andeanae", with levels similar in tissues examined, whether ticks were singly infected with this SFGR or co-infected with *R. parkeri.* Interestingly, the odds of the *R. parkeri* in the midgut tissue in all groups was 2.691 times greater ($P<0.0001$, CL=1.885 and 3.903) than salivary gland where probably provide favorable condition and nutrients for rickettsia after blood meal. Although high AR was detected on co-infected group for *R. parkeri*, transovarial and transstadial transmission of *R. parkeri* was rarely detected on *R. parkeri*-singly and co-infected groups. While the pathogenicity of "*Ca*. R. andeanae" in humans or other hosts is unknown, we demonstrated transmission of "*Ca*. R. andeanae" in rabbits; no rabbit developed detectable clinical signs, suggesting low pathogenicity in this host. Detection of antibodies in all rabbits provided evidence of exposure. However, the development of a serological assay to distinguish exposure between these SFGR and other tick-borne SFGR is necessary to

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definitively evaluate exposure. This, and evaluation of rickettsial presence in the vertebrate at early time points in feeding, would allow more thorough examination of possible synergistic effects between these bacteria when present in the same tick.

4.6 Acknowledgments

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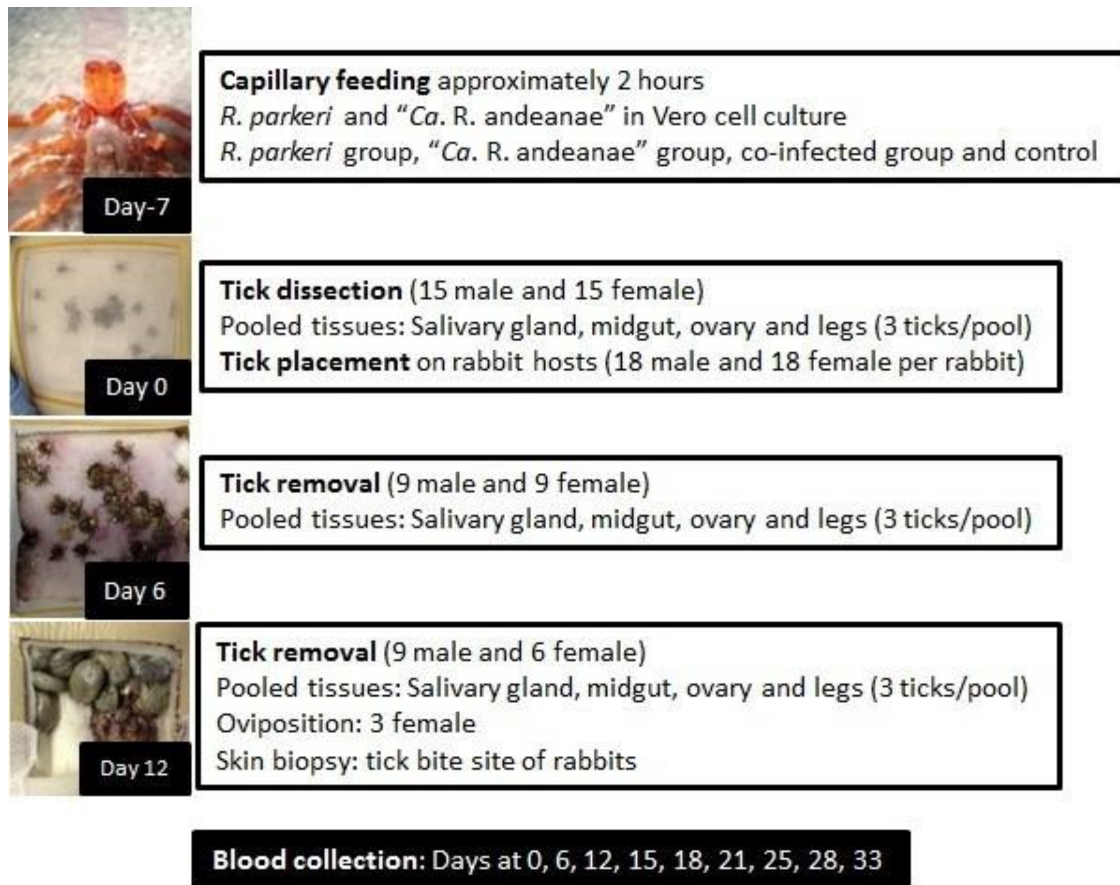

Figure 4.1 Overall study design per trial including tick placement, blood collection and sample collection for all groups (Singly, coinfected and control groups) were capillary fed on Day -7.

Infected and uninfected GCTs were placed on Day 0. GCTs were removed on Day 6 and Day 12. On Day 33, necropsy was performed. Blood samples were collected on days 0, 6, 12, 15, 18, 21, 25, 28, and 33.

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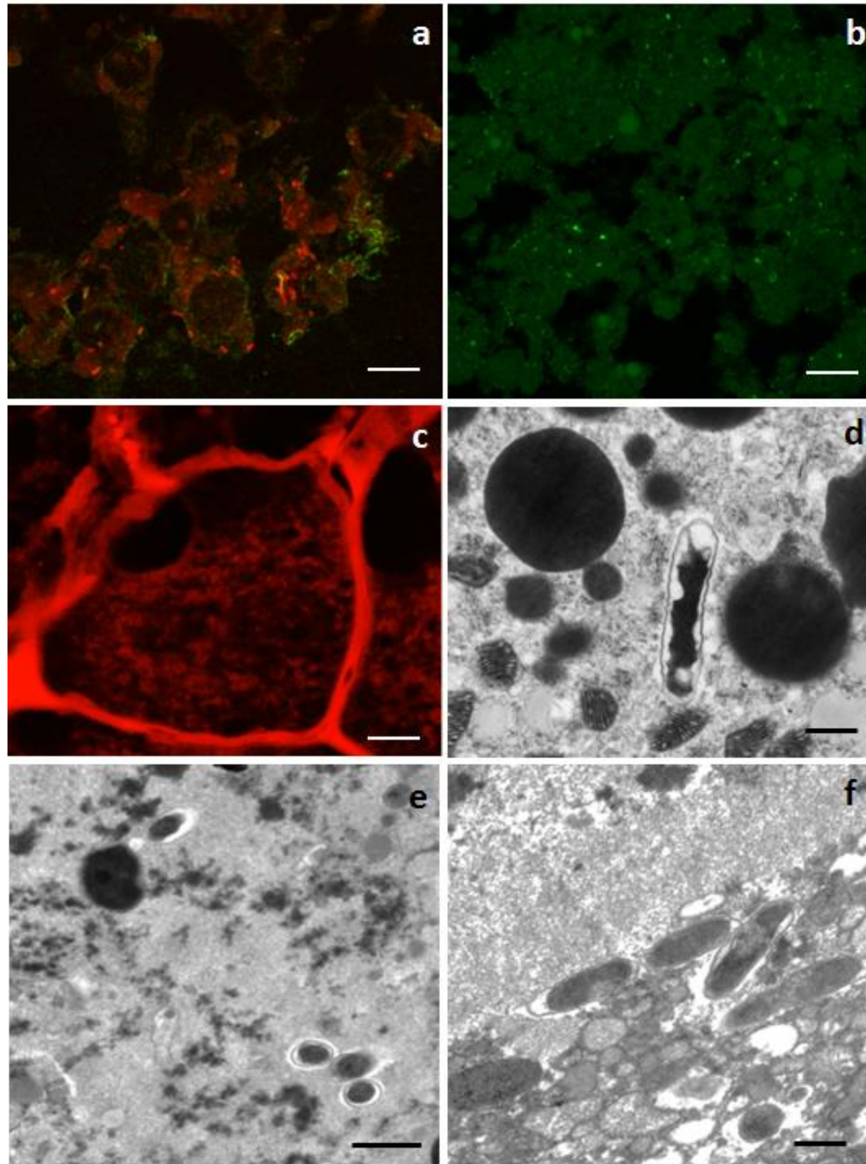

Figure 4.2 Immunohistochemistry and fluorescence in situ hybridization (a-c) and transmission electron microscopy (d-f) revealed rickettsial organisms in the experimentally infected tick tissue.

(a) *R. parkeri* (green, IHC) and "*Ca*. R. andeanae" (orange, FISH) were detected in Vero cells (FISH and IHC, scale bar=10µm). (b) *R. parkeri* were detected in salivary gland in co-infected group on Day 12 (IHC, scale bar=10µm). (c) "*Ca*. R. andeanae" were detected in developing eggs in ovary on co-infected group on Day 12 (FISH, scale bar=10µm). (d-f) Rickettsial organisms were revealed in developing eggs in ovary of "Ca. R. andeanae" group on Day 12 (d. scale bar= 600nm), midgut of co-infected group on Day 12 (e. scale bar=1µm) and salivary gland of co-infected group on Day 12 (f. scale bar=600nm).

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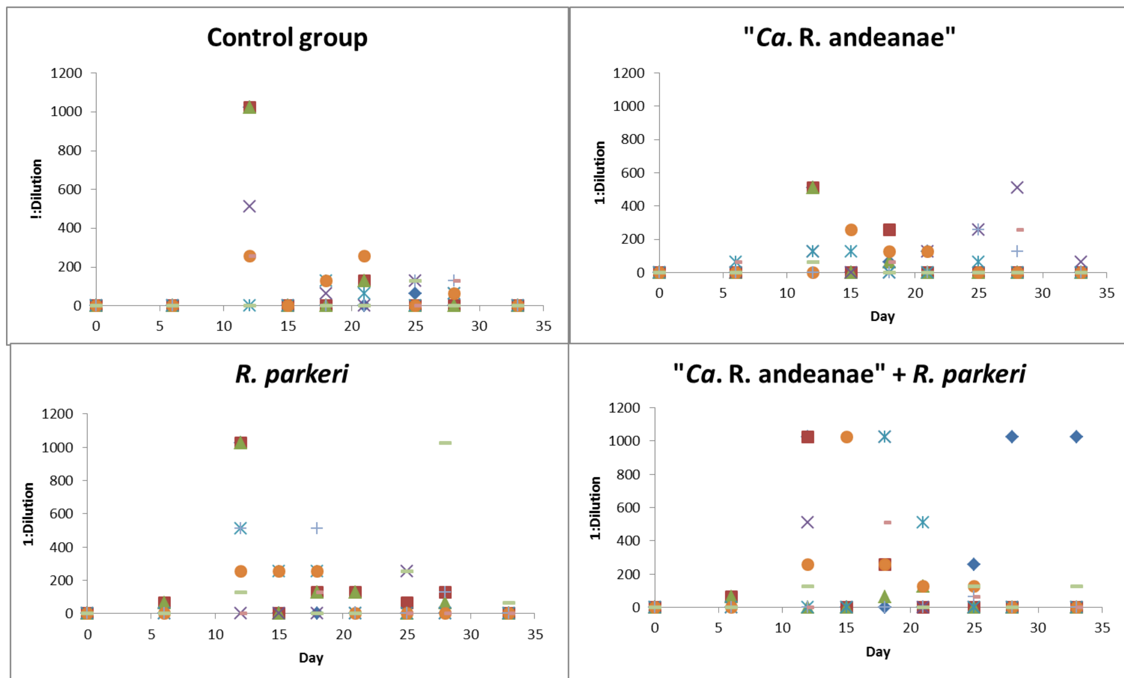

Figure 4.3 Individual antibody titers for each of nine rabbits in experimental groups (three rabbits for each of three trials), as determined by indirect fluorescent antibody assays.

The highest antibody titers measured were ≥1024. Fluorescent antibody assay of rabbit host. Rabbit plasma was serially diluted.

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Table 4.1 Primers and probes, with final concentrations, used in TaqMan® Multiplex QPCR assay for quantification of rickettsial levels.

Primer/Probe Name	**Sequence (5'→ 3')**	**Final Concentration**
QrompB_F	AAGTGGTACTTCAACATGGG	400 nM
QrompB_R	GCACCACCTTGGATTAAAG	400 nM
CaRa_probe_FAM	ATCGCGGAAGGTGCTCAAGTTAATG	50 nM
Rp_probe_HEX	ATTTTGGAAGGTGCGCAAGTTAATGC	400 nM
Amac MIF.18F	CCAGGGCCTTCTCGATGT	300 nM
Amac MIF.99R	CCATGCATTGCAAACC	300 nM
Amac MIF.63_Cy5	TGTTCTCCTTTGGACTCAGGCAGC	200 nM
Rab 12S-F	CTCTAAGAGCCAAAGGAGGATTT	300 nM
Rab 12S-R	TGTCACTTGAGGAGGGTGA	300 nM
Rab 12S Cy5	ATTGAACAAGGCCATGAAGCACGC	200 nM

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Table 4.2 Ratio of R. parkeri copy number to tick MIF copy number in salivary gland, midgut and leg (hemolymph) on experimentally infected rabbit host on Day 0, Day 6, and Day 12.

		Day 0	Day 6	Day 12
Control	Salivary gland	0.003±0.002* (30)**	0.078±0.047 (50)	0.400±0.400 (41)
(media only)	Midgut	0.014±0.004 (30)	0.027±0.014 (50)	0.054±0.053 (41)
	Leg (hemolymph)	0.003±0.003 (30)	0 (50)	0.001±0.000 (41)
"*Ca*. R. andeanae"	Salivary gland	0 (30)	0 (53)	0 (47)
(singly infected)	Midgut	0.001±0.001 (30)	0.012±0.008 (53)	0.006±0.004 (47)
	Leg (hemolymph)	0 (30)	0 (53)	0.001±0.001 (47)
R. parkeri	Salivary gland	0.003±0.002 (30)	0.146±0.067 (46)	0.351±0.218 (39)
(singly infected)	Midgut	0.005±0.002 (30)	0.14±0.066 (46)	1.076±0.630 (39)
	Leg (hemolymph)	0.002±0.001 (30)	0.001±0.001 (46)	0.092±0.080 (39)
"*Ca*. R. andeanae"	Salivary gland	0 (30)	1.084±0.509 (50)	83.320±43.761 (43)
and *R. parkeri*	Midgut	0.125±0.053 (30)	3.699±1.820 (50)	34.793±13.199 (43)
(co-infection)	Leg (hemolymph)	0.001±0.001 (30)	1.543±1.083 (50)	69.582±42.487 (43)

* Average ± Standard error
** Numbers of specimen

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Table 4.3 Ratio of “*Ca*. R. andeanae” copy number to tick MIF copy number in salivary gland, midgut and leg (hemolymph) on experimentally infected rabbit host on Day 0, Day 6, and Day 12.

		Day 0	Day 6	Day 12
Control	Salivary gland	0.007±0.003* (30)**	0.153±0.074 (50)	0.099±0.076 (41)
(media only)	Midgut	0.012±0.009 (30)	0.068±0.039 (50)	0.155±0.125 (41)
	Leg (hemolymph)	0.024±0.011 (30)	0.004±0.002 (50)	0.004±0.003 (41)
"*Ca*. R. andeanae"	Salivary gland	0.903±0.193 (30)	20.544±7.051 (53)	23.636±7.393 (47)
(singly infected)	Midgut	2.403±0.605 (30)	17.310±6.193 (53)	22.851±9.147 (47)
	Leg (hemolymph)	7.728±1.491 (30)	3.338±0.428 (53)	7.203±1.532 (47)
R. parkeri	Salivary gland	0.001±0.000 (30)	0.927±0.743 (46)	0.102±0.071 (39)
(singly infected)	Midgut	0.061±0.028 (30)	0.010±0.009 (46)	1.639±1.103 (39)
	Leg (hemolymph)	0.078±0.042 (30)	0.010±0.005 (46)	0.041±0.040 (39)
"*Ca*. R. andeanae"	Salivary gland	0.582±0.180 (30)	4.643±1.473 (50)	24.102±11.928 (43)
and *R. parkeri*	Midgut	2.863±0.966 (30)	11.180±6.753 (50)	10.385±3.894 (43)
(co-infection)	Leg (hemolymph)	14.247±4.213 (30)	4.899±1.459 (50)	4.709±0.999 (43)

* Average ± Standard error
** Numbers of specimen

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Table 4.4 Ratio of R. parkeri (A) and “Ca. R. andeanae” (B) copy number to tick MIF copy number in ovary extracts on DPT 0, 6, and 12.

A: *R parkeri*

	Day 0	Day 6	Day 12	Eggs	Larvae
Control	0 (15)**	0.001±0.001* (25)	0 (17)	0/26***	0/20
"*Ca*. R. andeanae"	0 (15)	0 (27)	0 (23)	0/26	1/22
R. parkeri	0.009±0.001 (15)	0.114±0.079 (20)	0.173±0.164 (14)	0/16	0/10
Coinfected group	0.002±0.001 (15)	0.217±0.130 (24)	5.950±2.996 (21)	0/22	1/15

B: “*Ca*. R. andeanae”

	Day 0	Day 6	Day 12	Eggs	Larvae
Control	0.012±0.012* (15)**	0.080±0.064 (25)	0.048±0.048 (17)	5/26***	2/20
"*Ca*. R. andeanae"	1.563±0.504 (15)	1.886±0.694 (27)	3.996±1.158 (23)	18/26	14/22
R. parkeri	0.032±0.016 (15)	0.186±0.115 (20)	0.531±0.445 (14)	2/16	2/10
Co-infected group	0.333±0.139 (15)	2.711±2.031 (24)	2.536±0.913 (21)	13/22	9/15

* Average ± Standard error
** Numbers of specimen
*** Numbers of positive masses/Numbers of egg or larvae masses tested

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CHAPTER V

TRANSMISSION OF RICKETTSIAE ASSOCIATED WITH *AMBLYOMMA MACULATUM* (GULF COAST TICK) DURING CO-FEEDING ON CATTLE

5.1 Abstract

Amblyomma maculatum is the primary vector for the human pathogen, *Rickettsia parkeri,* a spotted fever group rickettsia that can be detected in nearly half of some sampled *A. maculatum* populations but may be rare or absent in other *A. maculatum* populations. Infection rates of *R. parkeri* in adult *A. maculatum* appear to correlate inversely with those of "*Candidatus* Rickettsia andeanae," a second spotted fever group rickettsia of unknown pathogenicity. Rickettsial co-infections in *A. maculatum* are documented but the likelihood of co-infections reportedly low. Simultaneous infection of ticks with two rickettsial species is unlikely, and infection with these endothelial cell-associated bacteria via a blood meal is also likely rare. However, ticks are often found co-feeding on hosts, allowing for an opportunity for microbe exchange. In this study, we sought to determine the ability of initially pathogen-free adult *A. maculatum* to transmit *R. parkeri* to *"Ca.* R. andeanae"- infected and –free nymphs via co-feeding on beef cattle, a natural host for adult stages. We performed two co-feeding trials using six beef calves each, with both trials infesting calves for four days with adult *A. maculatum* capillary-fed *R. parkeri* GFPuv, and subsequently adding *A. maculatum* nymphs infected or uninfected

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with *"Ca.* R. andeanae". Trials differed by whether an attempt was made to minimize adjacent feeding within the aural feeding chambers. *R. parkeri* DNA was detected by PCR in approximately 26.8% of "*Ca*. R. andeanae"-free adults after co-feeding as nymphs; however, no *R. parkeri* was detected in "*Ca*. R. andeanae"-infected adults after co-feeding as nymphs. The efficiency of *R. parkeri*-transmission was greater in nymphs allowed to co-feed in close proximity to *R. parkeri*-infected adult *A. maculatum.* Of the twelve calves, rickettsial DNA was not detected in the blood on PCR on early infection (Post infection Days 0, 4, 8) and mild to moderate seroconversion was detected; Results here confirm co-feeding as a method of intra-species horizontal transmission of *R. parkeri* in the absence of host rickettsemia in the vertebrate host and suggest exclusion of *R. parkeri* from "*Ca*. R. andeanae" in *A. maculatum* during co-feeding.

5.2 Introduction

Infection rates of the spotted fever group rickettsial pathogen, *Rickettsia parkeri,* in its principal vector, *Amblyomma maculatum* (Gulf Coast tick)*,* vary geographically. In the southern range of *A. maculatum, R. parkeri* is typically found at rates up to 10-56 % (Sumner al. 2007; Paddock et al. 2010; Varela-Stokes et al. 2011; Ferrari et al. 2012; Nadolny et al. 2014). Interestingly, *R. parkeri* is not detected in Kansas and Oklahoma (Paddock et al. 2015). In addition, *A. maculatum* may be infected with a spotted fever group rickettsia of unknown pathogenicity, "*Candidatus* Rickettsia andeanae". Populations of *A. maculatum* within their southern US range typically have low infection rates with "*Ca.* R. andeanae," in approximately 1-2% of sampled adult ticks (Sumner et al. 2007; Paddock et al. 2010; Fornadel et al. 2011; Varela-Stokes et al. 2011; Ferrari et al. 2012; Jiang et al. 2012; Nadolny et al. 2014). The prevalence of "*Ca*. R. andeanae"

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reported from Kansas and Oklahoma was 47% and 73%, respectively (Paddock et al. 2015). Co-infection of *A. maculatum* with *R. parkeri* and "*Ca.* R. andeanae" appears to be rare but has also been reported (Varela-Stokes et al. 2011; Ferrari et al. 2012; Leydet and Liang 2013; Budachetri et al. 2014). In one report, *A. maculatum* co-infected with *R. parkeri* and "*Ca.* R. andeanae*"* were detected at a higher rate than expected by random chance (Ferrari et al. 2012).

Rickettsiae are obligate intracellular organism and are identified in vascular endothelial cells in cytoplasm and nucleus (Moulder 1985; Raoult and Roux 1997). The acquisition of rickettsiae from rickettsemic vertebrate hosts may be inefficient for naïve feeding ticks if the presence of circulating rickettsiae is transient or is rarely detected in animals (Horta et al. 2009; Horta et al. 2010; Edwards 2011; Grasperge et al. 2012; Moraru et al. 2013). In such cases, co-feeding is a potential way for pathogen or non-pathogen exchange between infected and naïve arthropod vectors while feeding simultaneously on a host without systemic infection (Jones et al. 1987). The efficiency of arthropod co-feeding for pathogen transmission may vary depending on their pathogen and host interaction. The efficiency of co-feeding in transmission of *Borrelia burgdorferi* s.s. from infected nymphs to larvae was only 0-5% (Piesman and Happ 2001). In contrast, transmission of *R. conorii* via co-feeding was very efficient, as between 92% and 100% of naïve *Rhipicephalus sanguineus* acquired *R. conorii* which co-feeding with infected ticks upon a seronegative dog; interestingly, transmission of *R. conorii* dramatically decreased to 8-28.5% when the canine host was seropositive (Zemtsova et al. 2010).

So far, *R. parkeri* co-feeding transmission and possible exclusion from *R. amblyommii* is briefly studied using *A. maculatum* and *A. americanum* but co-feeding

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study between *R. parkeri* and "*Ca*. R. andeanae" has not been studied and unknown (Wright et al. 2015). This study evaluated the acquisition of *R. parkeri* by naïve *A. maculatum* nymphs during co-feeding with *R. parkeri*-infected adult *A. maculatum,* using a beef calf model in the absence of host rickettsemia. In addition, the effect of "*Ca*. R. andeanae" infection status on *R. parkeri* acquisition during co-feeding was evaluated. We hypothesized that *R. parkeri* acquisition by naïve *A. maculatum* during co-feeding with *R. parkeri*-infected *A. maculatum* would be successful for nymphs regardless of *"Ca.* R. andeanae" infection status, but differed in "*Ca*. R. andeanae"-infected *A. maculatum* compared to "*Ca*. R. andeanae"-free *A. maculatum*. This study not only confirmed horizontal transmission of *R. parkeri* by co-feeding but also evaluated the influence of "*Ca*. R. andeanae" on acquisition of *R. parkeri* without detectable rickettsemia in a vertebrate hosts.

5.3 Materials and Methods

5.3.1 Calves

A total of twelve beef calves ranging in breeds commonly found in Mississippi (Angus, Holstein and Brangus) were obtained through a source identified by the College of Veterinary Medicine (CVM), Mississippi State University (MSU). These calves were mostly male and some female. All calves were approximately 3~4 month-old, in good body condition, and had been raised outdoors in an enclosed pen prior to being relocated to MSU CVM. All calves were examined to confirm absence of attached ticks and acclimated for one week in individual rooms of a six-room (approximately 100 ft^2) climate-controlled large animal facility at a biosafety level 2, where they remained for the

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duration of the studies. Calf studies were approved by the Institutional Animal Care and use Committee (IACUC) at Mississippi State University.

5.3.2 *Amblyomma maculatum* (Gulf Coast Tick)

Adult male and female adult laboratory-reared pathogen-free *A. maculatum* were obtained from BEI Resources (National Institute of Allergy and Infectious Disease, National Institutes of Health). To generate sufficient *A. maculatum* for this study, we reared pathogen-free *A. maculatum* adults on specific pathogen-free female New Zealand White rabbits for feeding larval offspring to nymphs, and a subset of nymphs to adult stages. Approximately 500 adults and 1,600 nymphs *A. maculatum* were reared and kept alive in humidity chamber with saturated potassium nitrate until used for studies.

5.3.3 *Rickettsia* preparation

For rickettsial propagation, we used Vero cells maintained at 37ºC, 5% CO_2 in Minimum Essential Medium Eagle (MEM) at pH 7.3 (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) or ISE6 cells (*Ixodes scapularis* embryonic cells, provided by U.G. Munderloh, University of Minnesota) maintained at 33ºC in L15B300 medium with heat inactivated 20% FBS and 10% tryptose phosphate broth. "*Candidatus* R. andeanae" originally isolated from naturally infected *A. maculatum* (Ferrari et al. 2013). *Rickettsia parkeri* (Oktibbeha) transformed with plasmid pRAM18dRGA/Rif/GFPuv (also provided by U.G. Munderloh, University of Minnesota) were separately cultured in Vero cells (Paddock et al. 2010; Burkhardt et al. 2011). *Rickettsia parkeri* GFPuv strain was used for better visualization in the tick and animal host for targeting GFPuv protein. We removed cells from infected

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flasks using a cell scraper and passed the cell suspension three times through a 21 gauge (G) needle and then another three times through 30 G needle to release rickettsial organisms. The cell suspension was spun down at 50 *g* for 5 minutes to remove large clumps of cells and then rickettsial organisms were collected at 10,000 *g* for 10 minutes. We performed DNA extraction on a subsample from both rickettsial preparations by a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia CA) and subsequently performed QPCR for both *R. parkeri*- and "*Ca*. R. andeanae" to evaluate rickettsial quantities (copy numbers) in the inoculum. Rickettsial concentrations were ~$1X10^8$ and ~$1X10^3$ DNA copy numbers/mL in fresh media for *R. parkeri* and "*Ca*. R. andeanae", respectively.

5.3.4 Co-feeding and tick placement

For each of the two trials, six calves were distributed into two experimental groups, one group (n = 3 calves) received "*Ca*. R. andeanae"-infected *A. maculatum* nymphs (capillary-fed day post-tick -6; DPT -6) and the other group (n = 3 calves) received "*Ca*. R. andeanae"-free *A. maculatum* nymphs. All calves received adult *A. maculatum* that had been capillary-fed *R. parkeri* GFPuv seven days prior to infestation (DPT -7). The study design is shown in Figure 1. To infect adult *A. maculatum* with R. *parkeri* GFPuv, each tick was fitted with a 10µL glass capillary tube filled with live cultured *R. parkeri* GFPuv suspension at approximately ~$1X10^8$ copy numbers/mL over its mouthparts while secured to a glass slide using carpet tape (Kocan et al. 2005). Ticks were fed for approximately 2 hours in a 32 ˚C incubator. Nymphal *A. maculatum* in the *"Ca.* R. andeanae" group were similarly capillary-fed, approximately $1X10^3$ DNA copy numbers/mL of "*Ca*. R. andeanae" using 5µL glass capillary(Broadwater et al. 2002; Ferrari et al. 2013). A source of naturally infected nymphs (kindly provided by K.

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Macaluso, Louisiana State University) was used in place of capillary-fed nymphs for one calf in the first trial. Capillary-fed adult and nymphal *A. maculatum* were maintained in vials in a humidity chamber with saturated potassium nitrate (KNO_3) solution (approximately 93% humidity) at room temperature for 7 days and 6 days for adults and nymphs respectively.

All calves were housed individually, in separate rooms of one building throughout both trials. Calves were sedated with xylazine for tick placement and removal; calves were reversed with yohimbine after the procedure. On DPT 0 (seven days after *R. parkeri* capillary feeding), all six calves in a tiral were infested with 30-40 adult *A. maculatum* (20:20=male and female, ranging 12 to 25 per sex) on one ear. To place ticks, the ear was first shaved and a sock was fitted over the ear using OSTO-BOND skin adhesive to adhere the sock to the ear base (OSTO-BOND skin bonding latex adhesive, M.O.C. QC, Canada). Adult *A. maculatum* were added though an opening at the opposite end of sock attachment and were allowed to infest all available space in the ear. The numbers of ticks were determined for proper feeding spaces depending on size of the ear and engorged spaces. A similar ratio of male and female adult was used for pheromone secretion of male to enhance female tick feeding and mating. On Day 4 (6 days after capillary feeding nymphs), 65-100 nymphal *A. maculatum* were added to sock chambers with adult stages. In addition, 10-15 adult *A. maculatum* that had been previously marked with paint were simultaneously added as recipient adults to evaluate *R. parkeri* acquisition by co-feeding. For calves in the group with *"Ca.* R. andeanae"-infected nymphal *A. maculatum,* we used adult recipient *A. maculatum* from Oklahoma State University (OSU) (Tick Rearing Facility, Department of Entomology and Plant Pathology, OSU, Stillwater, Oklahoma)

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because "*Ca*. R. andeanae" were naturally infected in these tick colonies. For the "*Ca*. R. andeanae"-free group, we used pathogen-free adult recipient *A. maculatum* (BEI Resources, NIAID, NIH).

The second calf trial differed from the first trial in that adult *R. parkeri*-infected (donor) *A. maculatum* were enclosed within the inside-chamber for the spatial distance to the nymphs at least 1cm, rather than allowed to move freely with acquisition nymphs and adults as in Trial 1. On DPT 12 (8 days from nymph *A. maculatum* placement), all nymphal *A. maculatum* and fully engorged adult donor *A. maculatum* (donor) were removed from the ear sock chamber. A portion of these engorged nymphs were frozen (-20˚C) for later processing by DNA extraction; a portion was kept in humidity chambers for molting to the adult stage. Engorged donor adult ticks were kept in the humidity chamber for oviposition and male ticks were frozen (-20˚C). On DPT 15, the remaining adult *A. maculatum* (recipient) were removed and kept in the humidity chamber for oviposition and male ticks were frozen (-20˚C) for further rickettsial detection. After oviposition by *R. parkeri*-infected adult *A. maculatum* (donor) and adult *A. maculatum* (recipient) females, portions of egg masses (approximately 100-500 eggs) were frozen (-20˚C) and allowed to hatch to larvae in the humidity chamber. A portion of these larval clutches (~100-300 larvae) were frozen at (-20˚C) for later rickettsial DNA assays to assess transovarial transmission by single QPCR or conventional PCR.

On DPT 33 of study, calves were euthanized and tissues were collected during necropsy. Calf tissues including ear skin, retropharyngeal lymph node, axillary lymph node, spleen, lung, heart, liver, and kidney were collected and placed in 10% formalin or frozen (-20˚C) for later testing.

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5.3.5 Rickettsial DNA detection

Genomic DNA was extracted from individual whole ticks or samples of larval clutches using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia CA). Extracted DNA samples were stored at -20˚C until PCR and quantitative (Q)PCR testing.

Prior to rickettsial PCR assays, DNA extracts for targeting rickettsial outer membrane protein A (*ompA*) gene were first tested in a PCR assay to amplify the tick mitochondrial 16S rRNA gene to confirm that DNA extraction was successful (Black and Piesman 1994) for quality control. To test for acquisition of *R. parkeri* in *A. maculatum* engorged nymphs and molted adults, we used a species-specific PCR assays targeting a portion of the *rompA* gene in *R. parkeri* (approximately 400-bp amplicon) (Varela-Stokes et al. 2011).

Rickettsial DNA detection for *R. parkeri*-infected adult *A. maculatum* (donor) and larvae (from donor's off-spring and recipient adult's larval mass) were performed by a TaqMan® multiplex quantitative QPCR. The QPCR consisted of one set of *Rickettsia*-wide primers, two species-specific probes targeting a unique sequence in the rickettsial outer membrane protein B (*rompB*) gene, one set of primers amplifying a portion of the *A. maculatum* macrophage migration inhibitory factor (MIF) gene and an MIF probe (sequences kindly provided by E. Harris and K. Macaluso, Louisiana State University). For each multiplex QPCR assay, 3µL of DNA sample was mixed with Brilliant Multiplex Master Mix 2X (Agilent Technologies, Santa Clara, CA, USA), and final concentrations of probes and primers as described in Table 1 in a final reaction volume of 25µL. Reference dye (ROX) was included at a 30 nM final concentration according to manufacturer recommendations for the instrument. We performed QPCR on a Stratagene

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Mx3005P with a two-step cycling profile consisting of 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and 60˚C for 1 min. Single samples were tested in assays alongside ten-fold dilutions (10^7 to 10^2) of a plasmid template mixture combining plasmids constructed for *R. parkeri* GFPuv Oktibbeha strain, "*Ca*. R andeanae" and *A. maculatum* MIF. Non-template (water) controls were also included in each run for quality control. Only data from multiplex QPCR reactions with efficiencies between 90% and 110% for all three targets, and R squared values above or equal to 0.985 were accepted for determining positive or negative samples. Amplification of tick MIF was evaluated for quality control.

Blood samples (whole blood and serum) from calves were collected on DPT 0, 4, 7 or 8, 12, 15, 19, 22, 26, 29 and 33 in both trials (Figure 1). We extracted DNA from DPT 0, 4, and 7 or 8, using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia CA). To test for rickettsial DNA, we performed a multiplex QPCR as above with the exception that, instead of tick MIF primers and probe, we included a set of primers and probe to detect genomic DNA (12S) for quality control (Table 1).

5.3.6 Histopathology

A full thickness section of skin, retropharyngeal lymph node, axillary lymph node, spleen, lung, heart, liver, and kidney, all removed on DPT 33, were fixed in 10% neutral buffered formalin, embedded in paraffin blocks by routine processing. Section (5µm thickness) was stained with hematoxylin and eosin for histopathologic examination.

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5.3.7 Indirect fluorescent antibody assay (IFA)

Serum samples from whole blood collected on DPT 0, 4, 7 or 8, 12, 15, 19, 22, 26, 29 and 33 were removed on sample days and stored at -80ºC. To evaluate exposure to rickettsial antigen, an indirect fluorescence antibody assay (IFA) was performed using 12-well slides coated with cultured *R. parkeri* (Portsmouth). The calf serum samples were initially tested at 1:32 and 1:64 serum dilutions, where reactivity at a 1:64 dilution was considered seropositive as previously described (Edwards et al. 2011; Moraru et al. 2013). After washing the slides with PBS, anti-bovine secondary antibody labeled with FITC (fluorescein isothiocyanate) was applied for 30 minutes at RT with 1:400 or 1:200 dilution (KPL Inc., Gaithersburg, MD). After washing with PBS, the slides were counterstained with Erichrome™ Black T (Fisher Scientific, Waltham, MA, USA), were mounted with Vectashield and DAPI (Vector Laboratories, Inc., Burlingame, CA, USA), and then were observed under UV light. For quality control, positive and negative plasmas were applied. Seropositive samples were diluted up to 1:1024 and titered.

5.4 Results

5.4.1 Tick recovery after co-feeding

The numbers of nymphal *A. maculatum* recovered after co-feeding on both trials were lower for the "*Ca*. R. andeanae"-infected group compared to the *"Ca.* R. andeanae"-free group. The efficiency of molting to adult ticks was similarly lower for the "*Ca*. R. andeanae"-infected nymphs than the "*Ca*. R. andeanae"-free nymphs on both trials (Table 2). Donor adult GCTs recovery rates between "*Ca*. R. andeanae"-infected group and the "*Ca*. R. andeanae"-free group in a trial 2 were similar which were 70.9% and 65.7%, respectively.

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5.4.2 Rickettsial DNA acquisition in recipient ticks after co-feeding

Transmission of *R. parkeri* to recipient *A. maculatum* nymphs (frozen) and molted adults is shown in Table 3. To evaluate rickettsial transmission to recipient adult *A. maculatum*, their offspring larvae were tested but each four tested larval masses (total 8) on "*Ca*. R. andeanae"-infected group and *"Ca.* R. andeanae"-free group were all negative for "*Ca*. R. andeanae" and *R. parkeri*. Overall, a greater percentage of ticks acquiring *R. parkeri* were observed in the "*Ca*. R. andeanae"-free group than in the "*Ca*. R. andeanae"-infected group. Rickettsial DNA was not detected in "*Ca*. R. andeanae"-infected nymphs and molted adults from this group. Additionally, co-feeding in close proximity to the *R. parkeri*-infected adult *A. maculatum* resulted in greater nymphal recovery and greater *R. parkeri* DNA detection in recovered ticks than when feeding in close proximity was minimized using an enclosure for donor ticks.

5.4.3 Gross lesions and histopathology

On gross examination, the pinnae were markedly thickened by edema and hyperemia. The epidermis was multifocally ulcerated, hemorrhagic and necrotic with serocellular crusts and inflammatory cells, especially on tick biting sites. The epidermis was mildly thickened at up to 5-7 cell layers with mild orthokeratotic hyperkeratosis and covered with cellular debris, fibrin and hemorrhage. The dermis was mildly to moderately thickened with small aggregates of lymphocytes, plasma cells, eosinophils, few macrophages, mildly dilated lymphatics, and reactive blood vessels. In the retropharyngeal lymph nodes, the lymphoid follicles were mildly hyperplastic. The subcapsular and medullary spaces were slightly expanded with small numbers of lymphocytes, macrophages, and erythrocytes, and small amount of edema.

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Retropharyngeal lymph nodes were reactive but no other changes were identified. The major organs such as kidney, liver, spleen, heart and lungs were within normal limits.

5.4.4 Indirect fluorescent antibody assay (IFA) and rickettsial DNA detection

All calves' sera of both experimental groups were serially diluted and tested by fluorescent antibody assay (Figure 1). The minimum positive and maximum antibody titers measured were ≥ 64 and ≥1024, respectively. For close proximity experiment, antibody titers on DPT 0 and 4 of both "*Ca*. R. andeanae"-infected group and "*Ca*. R. andeanae"-free group were negative. The seroconversion of "*Ca*. R. andeanae"-infected group was mildly increased on DPT 12 but no more than 128 until DPT 33. For "*Ca*. R. andeanae"-free group, one calf showed the increase of seroconversion titer on DPT 8 and peaked at ≥1024 on DPT 15 to DPT 29. However, the titers of other two calves were minimally or not seroconverted. For enclosure more than 1cm experiment, the seroconversion of "*Ca*. R. andeanae"-infected group and "*Ca*. R. andeanae"-free group were not detected.

All calf blood samples were collected and tested early infection on DPT 0, 4, and 8 which were negative for rickettsial DNA.

5.5 Discussion

All calves, infested in one ear with *R. parkeri*-infected *A. maculatum* adult (donor) ticks co-feeding with naïve nymphal and adult *A. maculatum* (recipient) ticks, demonstrated markedly thickened pinnae with multifocal ulceration, serocellular crusts and hemorrhage on the tick biting sites, as well as a deformed ear shape consistent with 'gotch ear' (Edwards 2011). On histopathological examination of the aural skin with tick

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biting sites, performed on DPT 33 of the study, the epidermis was mildly hyperplastic and the dermis was thickened with multiple aggregates of lymphocytes, plasma cells, fewer macrophages, eosinophils and edema. However, characteristic lesions for rickettsial infection such as vasculitis, was not observed at this time point, and might have resolved since our tissue observation was over two weeks from final removal of all ticks. In addition, rickettsemia was not observed during early stages of tick feeding, as rickettsial DNA was not detected on all 12 calves on early infection at DPT 0, 4, and 8. Mild to moderate seroconversion was detected in the first trial, when ticks were allowed to feed in close proximity; interestingly, the "*Ca*. R. andeanae"-free group of calves showed higher titers. However, seroconversion in the second trial, when an enclosure minimized close feeding was not observed for calves with either "*Ca*. R. andeanae"-free or "*Ca*. R. andeanae"-free recipient ticks. Calves may have had lower titers, as the antibody cutoff titer we chose was 64 in this study, which is the standard cutoff for this serological assay; cattle have demonstrated low titers at 32 in field studies (Edwards et al., 2011). Mild to moderate seroconversion and no rickettsial detection might also be due to relatively low numbers of tick infestation in calves.

We found that recovery rates of nymphs after co-feeding from calf ears were higher for the "*Ca*. R. andeanae"-free group than "*Ca*. R. andeanae"-infected group. Transmission of *R. parkeri* after co-feeding with *R. parkeri*-infected *A. maculatum* adults was not identified in the limited number of "*Ca*. R. andeanae"-infected recipient *A. maculatum* nymphs, while transmission was successful in the "*Ca*. R. andeanae"-free group, especially for ticks in close proximity to donors. The transmission efficiency after co-feeding has been studied in various organisms and may vary depending on

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microorganisms, host interaction and experimental condition (Piesman and Happ 2001; Kocan and de la Fuente 2003; Matsumoto et al. 2005; Zemtsova et al. 2010). Recipient nymphal transmission rate after co-feeding in this study was lower than compared to previous similar rickettsial study where up to 100% transmission of *R. conorii israelensis* on seronegative dogs co-feeding with *R. sanguineus* was detected (Zemtsova et al. 2010). The calves used in this study were approximately 3 months old and, though raised outdoors during the spring prior to entering the study, did not demonstrate evidence of previous rickettsial exposure as they were seronegative on DPT 0. While we do not know whether they were previously exposed to uninfected *A. maculatum,* this tick species typically peaks in summer months in Mississippi and would not likely be questing while calves were outdoors; no ticks were found attached to calves on arrival. Interestingly, the recovery of nymphs was also lower in "*Ca*. R. andeanae"-infected group than "*Ca*. R. andeanae"-free group, along with lack of detecable *R. parkeri* in "*Ca*. R. andeanae"-infected group. During the study, approximately half of recovered engorged nymphs were allowed to molt. We found lower molting success in the "*Ca*. R. andeanae"-infected group than "*Ca*. R. andeanae"-free group suggesting exposure to the second rickettsial species, *R. parkeri,* could hinder nymphal molting to the adult stage in *"Ca.* R. andeanae"-infected ticks. Although numbers were low, we found that ticks feeding in close proximity more efficiently transmitted *R. parkeri* than those with distant co-feeding. Absence of "*Ca*. R. andeanae"-transmission to co-feeding ticks should be further explored as this may play a role in *R. parkeri* exclusion from "*Ca*. R. andeanae" at the population level (Paddock et al. 2015).

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A previous *R. parkeri* co-feeding study also showed exclusion of *R. parkeri* by pre-acquired *R. amblyommii* (Wright et al. 2015). However, Wright et al. performed co-feeding transmission between *A. maculatum* and *A. americanum*. Therefore, the exclusion of *R. parkeri* might be in different condition but their study was one of support of possible exclusion. We also tested larval masses from recipient *A. maculatum* adult in both "*Ca*. R. andeanae"-infected and "*Ca*. R. andeanae"-free groups. None of larval masses tested for both *R. parkeri* and "*Ca*. R. andeanae" were positive, which showed lack of transovarial transmission of rickettsiae in this study. However, only four larval masses were tested and further study would help better understand the extent that transovarial transmission may or may not occur.

We recovered low numbers of nymphs under conditions in this study, and believe most died in the ears during the feeding period. This may have been due to trauma from calf scratching, as we did not restrict movement. Another reason may have been desiccation of the nymphs shortly after infestation if they remained on the inner surface of the ear sock chamber, rather than moving to the ear skin quickly. Using cattle as a blood source for nymphal feeding may also have not been ideal for this stage because immature *A. maculatum* more typically feed on birds, rodents, small mammals (Semtner and Hair 1973; Teel et al. 2010). However, recovery of *"Ca.* R. andeanae"-free nymphs was higher, so the difference may have also related to "*Ca*. R. andeanae" having a potentially negative effect on tick fitness. Additional nymphs would be required to better evaluate whether exclusion of *R. parkeri* in initially infected "*Ca*. R. andeanae" occurs, as suggested (Paddock et al. 2015). With similar recovery rates for *"Ca.* R. andeanae" free nymph, we found that *R. parkeri* transmission was higher under conditions where

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nymphs were allowed to feed adjacent to donor adults (26.8%), compared to where this was limited (4.3%).In this study we did not detect both rickettsial species in an individual tick; this may have been due to insufficient numbers for testing or possible exclusion of *R. parkeri* in the presence of the sympatric rickettsia. Co-infection of *R. parkeri* and "*Ca*. R. andeanae" is rare but has been reported (Varela-Stokes et al. 2011; Ferrari et al. 2012; Leydet and Liang 2013; Budachetri et al. 2014).

In summary, this study first evaluated the acquisition of *R. parkeri* in naïve *A. maculatum* nymphs while co-feeding with *R. parkeri*-infected adult *A. maculatum,* in the absence of host rickettsemia in the vertebrate host. Similar to the co-feeding study on *R. sanguineous* and *A. americanum*, the transmission of *R. parkeri* and possible exclusion from "*Ca*. R. andeanae" were observed. Blood samples tested as an indicator of rickettsemia during early tick feeding were negative for rickettsial DNA. We did not test ear skin during tick feeding as an indicator of rickettsiae within the endothelial cells lining blood vessels at the feeding site, however, we used seroconversion to determine calf exposure to rickettsiae. In this study, transovarial transmission of both *R. parkeri* and "*Ca*. R. andeanae" was not detected in the larval masses of recipient group. One of the limitations in this study was the use of *R. parkeri* (Oktibbeha) modified to express GFPuv. While this strain was chosen in order to use additional techniques (fluorescence *in situ* hybridization and immunohistochemitry) developed in the lab to differentiate the two rickettsiae microscopically, transmissibility of this strain may have been affected. Future studies using low passage unaltered strains could be used to further evaluate the importance of co-feeding for maintenance and transmission of *A. maculatum*-associated rickettsiae. Still, this study successfully demonstrated horizontal acquisition of *R. parkeri*

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by co-feeding, particularly in ticks not already infected with *"Ca.* R. andeanae." We anticipate that taken together, these data add to our knowledge of rickettsial maintenance in this system and will contribute to a better understanding of the natural history of spotted fever rickettsiosis in the United States.

5.6 Acknowledgments

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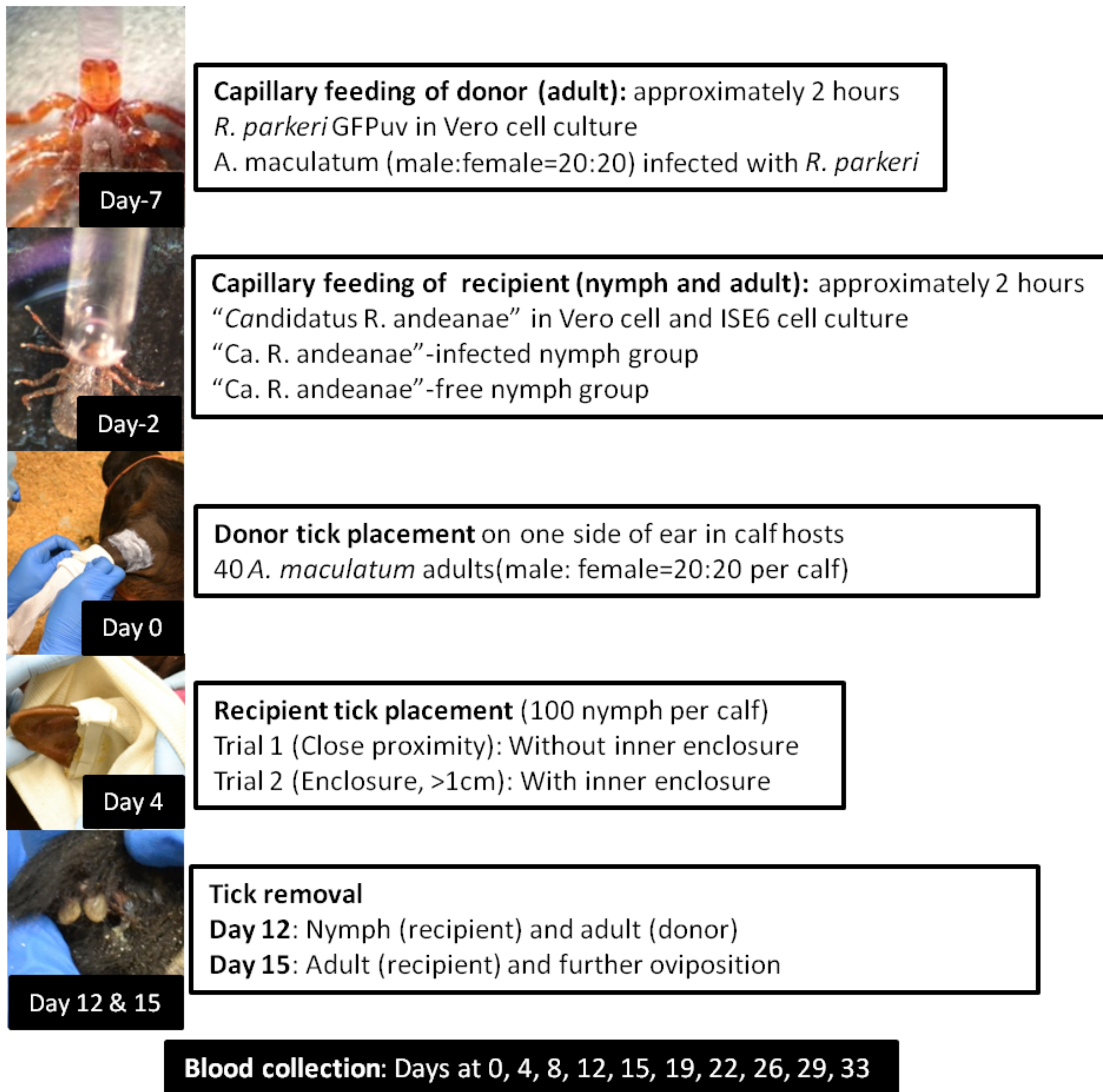

Figure 5.1 Overall study design for both trials, with capillary feeding for *R. parkeri* GFPuv-infected adult (donor) ticks and *"Ca.* R. andeanae"-infected and -free nymphs and adults (recipient) tick infestations on calves and blood collection.

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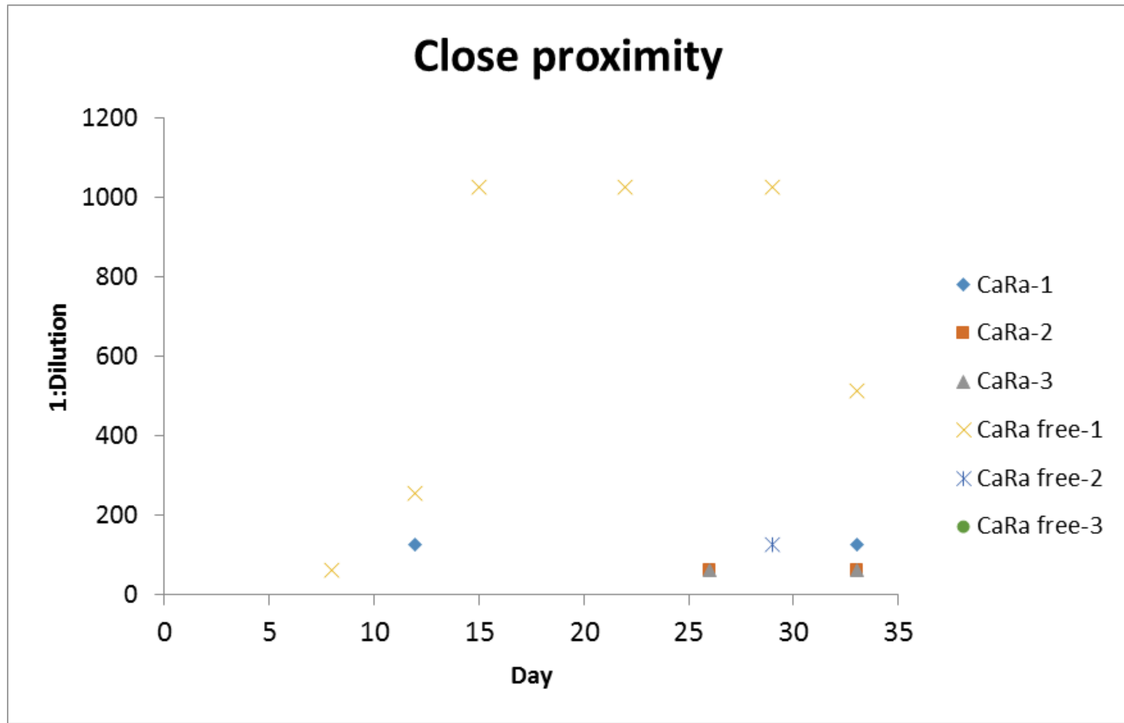

Figure 5.2 Individual antibody titers for six calve from trial 1 in "*Ca*. R. andeanae"-infected group (CaRa) and "*Ca*. R. andeanae"-free group (CaRa free).

The minimum positive and maximum antibody titers measured were ≥ 64 and ≥1024, respectively. The titers were determined by fluorescent antibody assay of calves host. Calves serum was serially diluted.

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Table 5.1 Primers and probes, with final concentrations, used in TaqMan® Multiplex QPCR assay for quantification of rickettsial levels.

Primer/Probe Name	Sequence (5'→ 3')	Final Concentration
QrompB_F	AAGTGGTACTTCAACATGGG	400 nM
QrompB_R	GCACCACCTTGGATTAAAG	400 nM
CaRa_probe_FAM	ATCGCGGAAGGTGCTCAAGTTAATG	50 nM
Rp_probe_HEX	ATTTTGGAAGGTGCGCAAGTTAATGC	400 nM
Amac MIF.18F	CCAGGGCCTTCTCGATGT	300 nM
Amac MIF.99R	CCATGCATTGCAAACC	300 nM
Amac MIF.63_Cy5	TGTTCTCCTTTGGACTCAGGCAGC	200 nM
Rab 12S-F	CTCTAAGAGCCAAAGGAGGATTT	300 nM
Rab 12S-R	TGTCACTTGAGGAGGGTGA	300 nM
Rab 12S Cy5	ATTGAACAAGGCCATGAAGCACGC	200 nM

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Table 5.2 Total numbers of engorged *A. maculatum* nymphs after co-feeding and molted adults from both trials, for nymphs with and without "*Ca.* R. andeanae."

	Close proximity (trial 1)		Enclosure at least 1cm (Trial 2)	
	Numbers of engorged Nymph	Numbers of recovered molted adults	Number of engorged Nymph	Numbers of recovered molted adults
"*Ca*. R. andeanae" -infected nymphs co-feeding with adult *A. maculatum*	32/250 (12.8%) Frozen: 0	2/32 (6.25%)	33/228 (14.5%) Frozen: 11	7/22 (31.8%)
"*Ca*. R. andeanae" -free nymphs co-feeding with adult *A. maculatum*	95/300 (31.7%) Frozen: 45	26/50 (52.0%)	121/300 (40.3%) Frozen: 57	35/64 (54.7%)

Both recovery of engorged nymphs and molting success were lower in "*Ca*. R. andeanae"-infected nymphs.

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Table 5.3 DNA detection of *R. parkeri* after co-feeding in recipient nymphs and adults.

	Close proximity	Enclosure at least 1cm
"*Ca*. R. andeanae" -infected nymph (recipient)	0/2 (0%) Molted: 0/2 Frozen: 0	0/18 (0%) Molted: 0/7 Frozen: 0/11
"*Ca*. R. andeanae" -free nymph (recipient)	19/71 (26.8%) Molted: 2/26 Frozen: 17/45	4/92 (4.3%) Molted: 1/35 Frozen: 3/57

Total numbers of recipient nymphs were included engorged nymphs, and molted adults. All larvae from adult recipient *A. maculatum* were negative for both *R. parkeri* and “*Ca*. R. andeanae”

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CHAPTER VI

OVERALL DISCUSSION AND CONCLUSIONS

Studies presented in this dissertation help provide a better understanding of *Amblyomma maculatum*-associated rickettsiae, specifically *R. parkeri*, a pathogen that contributes to spotted fever rickettsiosis in the Western Hemisphere, and "*Ca*. R. andeanae," a spotted fever group rickettsia of unknown pathogenicity. In particular, we focused on co-infection dynamics in ticks singly- infected or co-infected with *R. parkeri* and "*Ca*. R. andeanae." This work is meaningful in terms of public health because *A. maculatum* Koch (Acari: Ixodidae) is the primary vector for *R. parkeri*, the causative agent of American Boutonneuse fever, and a confounding agent in the diagnosis of Rocky Mountain spotted fever due to cross-reactivity of antibodies on diagnostic serological assays. Considering *A. maculatum* may also be infected with another rickettsia, "*Ca*. R. andeanae," the interactions between these rickettsiae as they are maintained in singly-infected ticks in nature, or occasionally in co-infected ticks, may impact infection rates of the known pathogen, which is important in terms of human risk of infection.

Overall, we found that infection rates of *R. parkeri* and "*Ca*. R. andeanae" in nature were 28.7% and 9.3%, respectively, with three additional *A. maculatum* (0.9%) co-infected, between 2013 and 2015. These infection rates varied annually even in the same sites. Rickettsial DNA levels, previously not reported for naturally infected *A.*

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maculatum, also varied between rickettsial species. *Rickettsia parkeri* levels were higher than "*Ca*. R. andeanae" levels in singly infected ticks while *R. parkeri* levels in co-infected ticks were similar to those of the sympatric rickettsial species, and lower than in singly-infected ticks. These results may have implications in terms of pathogen levels necessary for transmission to vertebrate hosts, and may affect the ability of *R. parkeri* to be maintained in ticks with "*Ca*. R. andeanae," or, exclusion of *R. parkeri*. Thus, monitoring the infections rates and levels are important to understanding human risk of infection with *R. parkeri*.

In experimental studies using artificially-infected ticks on a rabbit model, we found that *R. parkeri* levels using a modified strain of *R. parkeri* were relatively low in singly-infected ticks while higher in ticks co-infected with "*Ca*. R. andeanae," suggesting a possible synergistic effect under these conditions. Transovarial transmission of "*Ca*. R. andeanae" was a critical route for maintaining this *Rickettsia* species, which has no known vertebrate reservoir host. Both transmission electron microscopy and FISH/IHC were successful tools for visualization of rickettsial species. In addition, here we provide evidence of "*Ca*. R. andeanae" exposure in the rabbit host, although pathogenicity of "*Ca*. R. andeanae" is still unknown; no rabbits had clinical signs consistent with rickettsiosis in this study. Further studies using different animal hosts would be beneficial to understand pathogenicity of this *Rickettsia* species. In our co-feeding study on the transmission of *R. parkeri*, we found that *R. parkeri* was successfully transmitted to recipient nymphs without the presence of "*Ca*. R. andeanae" but survival diminished and transmission was not observed in recipient ticks with "*Ca*. R. andeanae." Additional

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studies to expand these results may consider the use other *R. parkeri* strains or naturally infected ticks.

Overall, this dissertation research contributed to a better understanding of co-infection dynamics for *R. parkeri* and "*Ca*. R. andeanae" and we anticipate further studies will continue to address additional questions generated in this study.

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