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Characterization of rickettsial infection dynamics within Dermacentor variabilis and Amblyomma maculatum

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CHARACTERIZATION OF RICKETTSIAL INFECTION DYNAMICS WTIHIN *DERMACENTOR VARIABILIS* AND *AMBLYOMMA MACULATUM*

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"The Building of the Ship"

"Like unto ships far off at sea,
Outward or homeward bound, are we.
Before, behind and all around,
Floats and swings the horizon's bound,
Seems at its distant rim to rise
And climb the crystal wall of the skies,
And then again to turn and sink,
As if we could slide from its outer brink.
Ah! It is not the sea,
It is no the sea that sinks and shelves,
But ourselves
That rock and rise
With endless and uneasy motion,
Now touching the very skies,
Now sinking into the depths of the ocean.
Ah! If our souls but poise and swing
Like the compass in its brazen ring,
Ever level and ever true
To the tail and the task we have to do,
We shall sail securely, and safely reach
The Fortunate Isles, on whose shining beach
The sights we see, and the sounds we hear,
Will to those of joy and not of fear!"
-Henry Wadsworth Longfellow

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ABSTRACT

Spotted fever group (SFG) *Rickettsia* are primarily associated with their reservoir host and vector, the tick. Rickettsial colonization and maintenance within the arthropod is a key component of vector competence and pathogen transmission to the mammalian host. Contemporary detection of novel tick hosts for rickettsial species, combined with an unprecedented rise in human cases of SFG rickettsiosis, necessitates a deeper understanding of tick/*Rickettsia* interactions. The hypothesis for this work is that if primary tick/*Rickettsia* pairings do not exist then rickettsial determinants account for primary vector/pathogen relationships. To this end, *Dermacentor variabilis* and *Amblyomma maculatum* ticks were exposed to *R. rickettsii, R. parkeri, R. montanensis, R. amblyommii* or *R. felis*. Rickettsial exposure negatively impacted the fitness of *A. maculatum*, but not *D. variabilis*. Transovarial and transtadial transmission of rickettsiae was most successful for *R. amblyommii* and *R. parkeri* in both, *A. maculatum* and *D. variabilis* eggs, larvae, and nymphs. Maintenance of rickettsiae in both tick species via transstadial transmission was diminished from unfed larvae to unfed F_1 adults. To further investigate the maintenance of *Rickettsia* in the arthropod host, an *in vitro* and *in vivo* model of *R. parkeri* infection was utilized. Rickettsial proteins implicated in intracellular actin-based motility (Sca2 and RickA) were shown to function similarly in mammalian and tick cell culture, suggesting conserved functionality in both hosts. *In vivo* dissemination of a wild-type strain of *R. parkeri* was measured against two strains deficient in Sca2 and RickA expression. Wild-type, RickA, and Sca2 deficient strains *R. parkeri* persisted in all tick organs organs at 7 days post-exposure. The findings suggest transovarial transmission specificity to be tick species dependent and vertical transmission is not sustainable.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Ticks: Arthropods of Importance and Variety

Ticks are highly specialized hematophagous ectoparasites of mammals and reptiles. These arthropods transmit a wider variety of bacterial, viral, and eukaryotic agents of disease than any other arthropod vectors of disease. Currently, over 900 species of ticks have been identified worldwide. These archaic arachnids, proposed to have arisen in the Cretaceous Period over 300 million years ago, tell the story of a lengthy history as arthropods of importance. The pages below, will describe the general characteristics of the tick life cycle, culminating in an introduction to ticks as vectors of disease causing agents.

1.1.1 Life History and Description: Ixodidae, Argasidae, and Nuttalliellidae

Generally, the tick life cycle consists of the egg, larval, nymphal, and adult stage (Sonenshine 1991). All ticks possess three pairs of walking legs at the larval stage and four pair at the nymphal and adult stage (Sonenshine and Roe 2013). Sexual dimorphism is only apparent at the adult stage (Sonenshine 1991). All stages, with some exceptions, require a host bloodmeal in order to further development. Phylogenetically, ticks are classified into three families: Nuttalliellidae, Argasidae, and Ixodidae.

1.1.1a Anatomy of the Ixodid tick

Morphology of the ixodid tick includes two main structures: 1) gnathosoma (or capitulum) which extends from anterior portion of the 2) idiosoma (Nicholson et al. 2009). The gnathosoma consists of four structures: the basis capituli, a paired 2-segmented chelicerae, a toothed hypostome, and a paired 4-segmented palps (Figure 1.1) (Sonenshine and Roe 2013). Directly anterior to the idiosoma is the basis capitulum which encases the mouth, pharynx, spinose shaft, and the expanded base of each chelicera. The chelicerae extend outward from the

dorsal aspect of the basis capituli and are situated medially to the palps (Figure 1.1) (Sonenshine 2005). Each extension has digits that have laterally directed cutting spines (Sonenshine and Roe 2013). The movement of these digits result in a ripping and tearing action, aiding in breaking the host skin barrier (Sonenshine and Roe 2013). The second segment (or inner digits) contain mechanosensory and chemosensory sensilla (Sonenshine and Roe 2013). These act to alert the tick to shear forces and also chemical composition of the host fluids and genital sex pheromones (Sonenshine and Roe 2013). The hypostome is located between the palps nearest to the ventral aspect of the capitulum (Figure 1.1). Decorating the hypostome are rows of recurved spines or denticles (Sonenshine and Roe 2013). Functionally, denticles help to anchor the tick to its mammalian host (Sonenshine and Roe 2013). They also act as distinguishing markers for tick identification (Sonenshine and Roe 2013). Situated in the mid-dorsal surface of the hypostome is a narrow food canal where fluids enter and exit the tick (Nicholson et al. 2009). Once the chelicerae have broken the skin, the hypostome is inserted into the opening. Located on either side of the chelicerae and hypostome is the 4-segmented palp (Figure 1.1). Located in the most distal segment are sensilla that function in chemosensory host recognition (Sonenshine 2005). As the tick inserts the chelicerae and hypostome into the host, both palps spread apart from the capitulum and remain outside of the host.

The idiosoma is covered in setae and is composed of 2 structures: 1) the podosoma and 2) opisthoma (Sonenshine and Roe 2013). The podosoma is the portion of the body immediately posterior to the gnathosoma. It is comprised of the area between the legs and the genital pore (Sonenshine 1991). The opisthoma is located posterior to the podosoma, including the area behind the last set of legs and the spiracular plates, extending to the end of the tick's body. Spiracular plates are located just below the last leg on either side of the ventral aspect of the tick.

They are the outward connection to the open respiratory system of the tick (Sonenshine and Roe 2013).

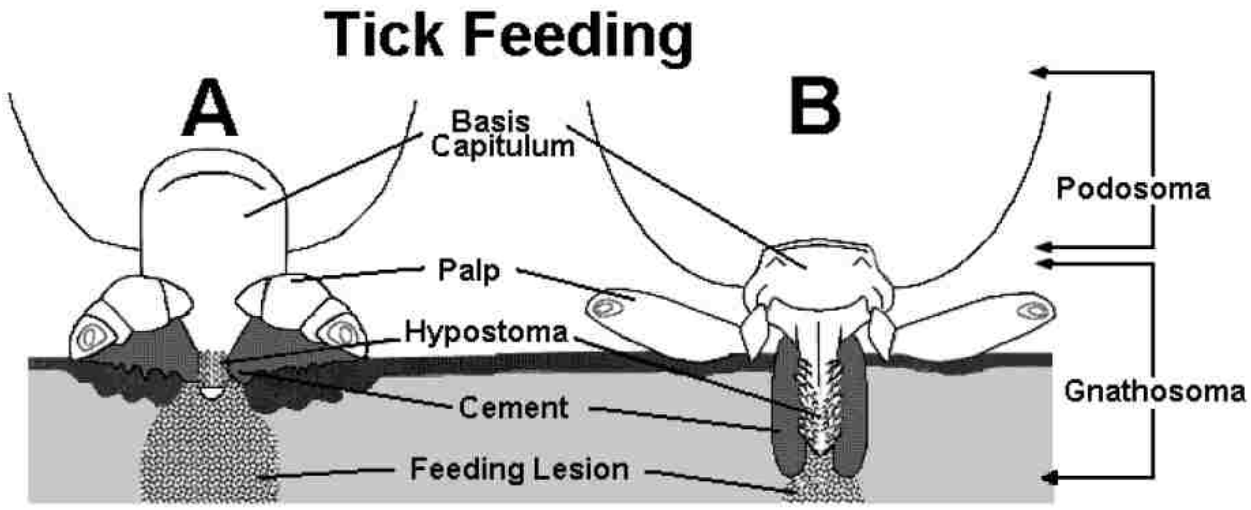

Figure 1.1. Cartoon of the gnathosoma and podosoma of the ixodid tick. A) Shows the ventral and B) the dorsal aspect of the imbedded tick (Courtesy of http://www.cvbd.org/en/tick-borne-diseases/about-ticks/tick-feeding/blood-feeding/).

Sclerotized plates cover the entire idiosomal surface. The scutum, located on the dorsal aspect of the tick, is the largest plate (Sonenshine 2005). In males, the entire opisthoma is covered in chitinous sclerotized plates (Sonenshine and Roe 2013). In females and immatures only half of the dorsum is covered with sclerotized plates (Sonenshine 2005). The rest of the dorsal surface is covered in cuticle (alloscutum) that expands during feeding (Sonenshine and Roe 2013).

1.1.1b Ixodidae

Ixodidae is the largest and most diverse tick family, with over 660 species (Marquardt 2005). Life stages consist of the egg, followed by a single larval, nymphal, and adult stage. At each of the active stages, a bloodmeal is required for molting and maturation. The diversity of "hard ticks" is due in part to their one-, two-, or three-host life cycle. The advantages and implications of these lifecycles are discussed below.

In a 1-host life cycle all stages of the tick feed on the same host (Figure 1.2). This is exemplified by the cattle tick, *Rhipicephalus microplus* (Sonenshine 1991). Larvae and nymphs feed to repletion and molt on the same host. Subsequent adult males and females will copulate, with the replete female dropping from the host into the natural environment (Sonenshine 1991). She produces a sizeable egg mass (3000-5000 eggs), yielding larvae that will seek out a new host.

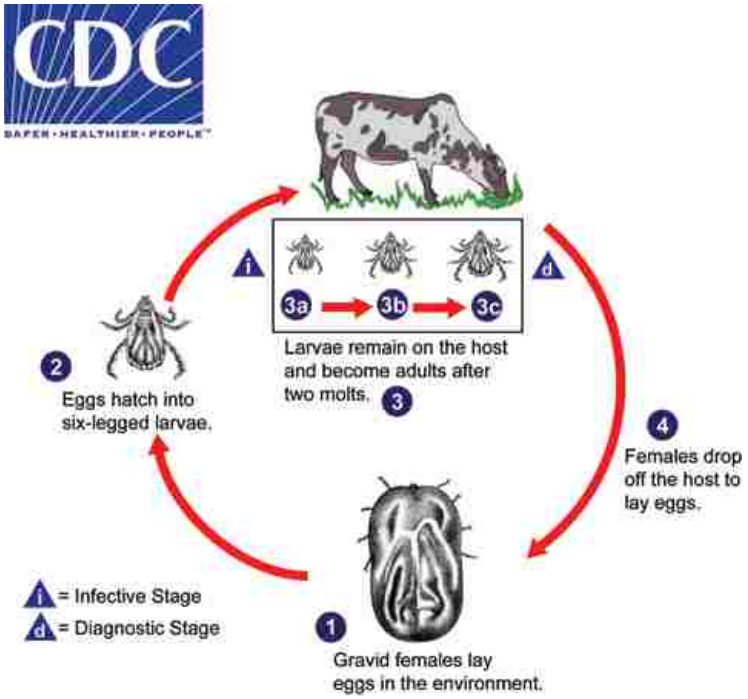

Figure 1.2. The life cycle of the one-host tick (Centers for Disease Control and Prevention-http://www.cdc.gov/dpdx/ticks/).

The camel tick, *Hyalomma dromedarii*, is an example of the 2-host life cycle (Figure 1.3) (Nicholson et al. 2009). Immature stages of this tick feed and molt on the same animal; while the adult stage must actively quest for and feed on a second host (Sonenshine 1991, Nicholson et al. 2009). Replete females will drop from the host into the natural environment. The life cycle of the one- or two-host tick is considered evolutionarily more advantageous because it affords shorter feeding and reproduction times (Sonenshine and Roe 2013).

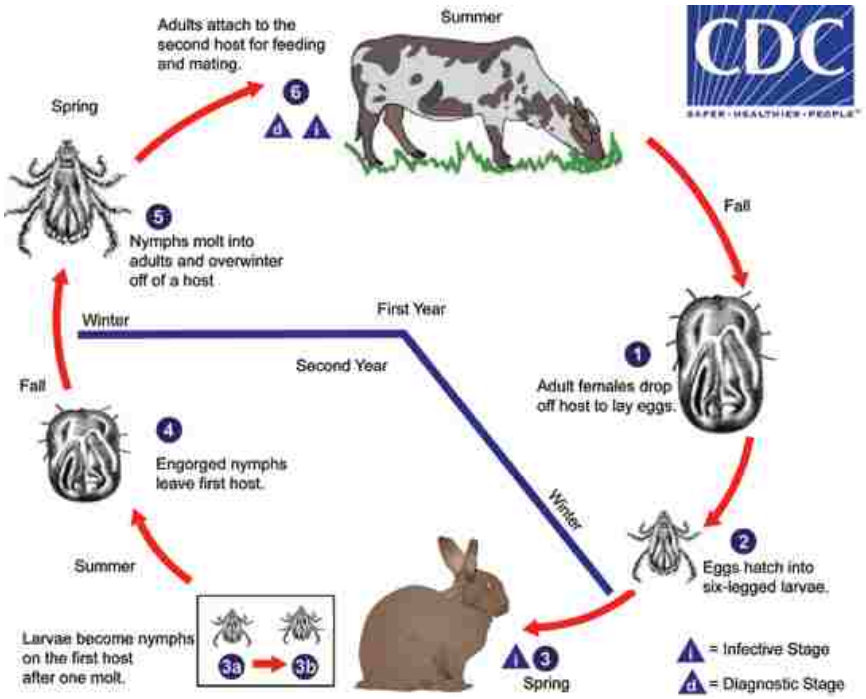

Figure 1.3. The life cycle of the two-host tick (Centers for Disease Control and Prevention- http://www.cdc.gov/dpdx/ticks/).

Despite this advantage, more than 90% of ixodid ticks have a 3-host life cycle (Figure 1.4) (Nicholson et al. 2009). Indirect evidence suggests that the 3-host developmental pattern is ancestral for all ixodid ticks (Sonenshine 1991, Sonenshine and Roe 2013). Larvae, nymphs, and adults must actively find a new host to parasitize. After feeding to repletion each stage will drop off the host into the surrounding environment to complete ecdysis (molting) or oviposit, in the case of the engorged, adult female. Following ecdysis the unfed life stage will seek out a new host. Newly emerged adults require 7-9 days after eclosion to begin feeding (Sonenshine and Roe 2013).

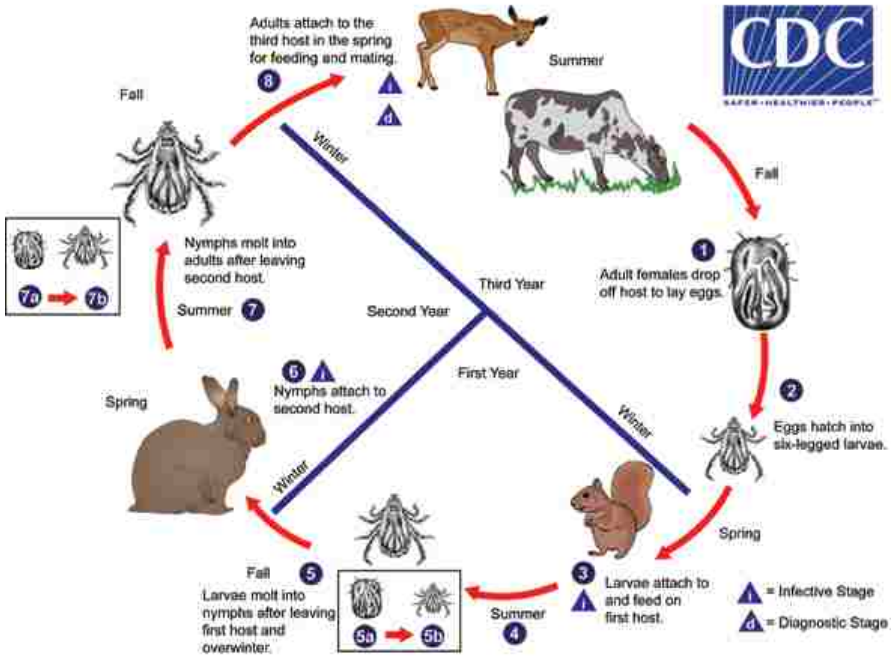

Figure 1.4. The life cycle of the three-host tick (Centers for Disease Control and Prevention-http://www.cdc.gov/dpdx/ticks/).

Ixodid ticks slowly acquire their bloodmeal. Larvae and nymphs on average require 3-6 days to reach repletion (Sonenshine 2005). Depending on the species, adult females can feed for 13 days (Sonenshine 2005). Aiding in these lengthy attachment times is the presence of a cement-like substance anchoring the tick to the host (Sonenshine and Roe 2013). Another identifying feature that makes ixodid ticks unique include the hard shell-like scutum present on the dorsal surface (Sonenshine 2005). In male ticks the scutum covers the entire dorsum (Sonenshine and Roe 2013). This restricts the amount of blood imbibed by male ticks. In females and immature stages the scutum is only present on the anterior portion of the dorsum (Sonenshine and Roe 2013). The remaining surface is covered by cuticular alloscutum (Sonenshine and Roe 2013). As the tick feeds the alloscutum expands and more cuticular surface is created (called neosomy) (Marquardt 2005). This allows for massive amounts of blood to be imbibed over the course of feeding. Larval ticks may engorge up to 20-times their unfed weight (Sonenshine 2005). Impressively, females can acquire a bloodmeal that is 120-times their unfed

weight (Sonenshine 2005). During feeding the tick will alternate between ingesting blood and injecting saliva (Sonenshine 2005). This allows the tick to concentrate its bloodmeal. Granular acini of the salivary glands actively secrete water and salt from the bloodmeal back into the parasitized host (Sonenshine 2005). This also includes a pharmacopeia of immune suppressing molecules (Sonenshine 2005). Thus, the true volume of blood consumed by a single tick is 2-3-times greater than that of the engorged tick itself (Sonenshine and Roe 2013). This is important as ixodid females undergo only one gonotrophic cycle and must acquire enough nutrients to lay 5,000-22,000 eggs (Sonenshine 1991). Nearly 50% of the engorged female's body weight is converted to egg mass (Sonenshine 1991). Production of large numbers of eggs is an evolutionary adaptation because only a small fraction of produced eggs will survive to the adult stage. The morphological and physiological characteristics of Ixodid ticks contribute greatly to the success of the tick lifestyle.

1.1.1c Argasidae

Argasidae is a family of multi-host ticks comprising four genera with 180 individual species (Marquardt 2005). Argasids, which are colloquially termed "soft ticks" due to their leathery appearance, have a global distribution. Life stages consist of the egg, larval, multiple nymphal instars, and adult stage. Exceptions include species of the Genus *Ornithodorus* which molt directly from the larval to nymphal stage without a bloodmeal, and *Otobius*, which do not feed as adults (Nicholson et al. 2009). Argasids exhibit a unique fast-feeding style in which bloodfeeding lasts 15-30 minutes (Sonenshine 1991). The only exception is larvae of the *Argas* and *Ornithodorus* species (Nicholson et al. 2009). The evolutionary development of this fast-feeding approach to obtaining a bloodmeal is considered more archaic than that of Ixodidae (Sonenshine and Roe 2013). Adults feed multiple times, depositing multiple clutches on average

of 500 eggs per gonotrophic cycle (Sonenshine 1991). As many as six gonotrophic cycles have been documented in argasids (Sonenshine 1991).

Morphologically, soft ticks possess a cuticular surface composed of multiple wrinkled folds (Sonenshine and Roe 2013). This structure unfolds during feeding, allowing limited amounts of blood to be imbibed at any one time (Sonenshine and Roe 2013). Adult, female argasids are able to expand their body mass up to 10-times their original unfed weight (Sonenshine 2005).

1.1.1d Nuttalliellidae

Nuttalliellidae is a monotypic family of which *Nuttalliella namaqua* is the only recognized species. Specimens have been collected across southern Africa (Mans et al. 2011). Genetic information from 16s and 18s mitochondrial analysis indicates that *N. namaqua* phylogenetically groups basal to all tick species (Mans et al. 2014).

Similar to ixodid ticks, *N. namaqua* immature stages, particularly larvae, undergo a slow feeding phase followed by fast feeding over a period of days concluding with engorgement. Larval stages appear to possess a scutum. Adult males also seem to have a scutum covering their dorsum (Mans et al. 2014).

Morphologically, this species resonates many aspects of the Argasidae. The adult and nymphal body is leathery and the prognathous (or mouthparts) are located anterior ventrally. Likewise, the feeding habits of adult, female *N. namaqua* is similar to many argasid species. This includes a fast feeding approach to ingesting a bloodmeal that may last anywhere from minutes to just over an hour (Mans et al. 2011). In between bloodmeals females will deposit hundreds of eggs before seeking another host.

1.1.1e Bloodmeal Digestion

The digestive system of the tick consists of 3 components: 1) preoral canal and foregut, 2) midgut, and 3) hindgut (Sonenshine and Roe 2013). Blood is drawn in through the preoral canal, which is located in the hypostome (Sonenshine and Roe 2013). The bloodmeal passes to the pharynx, through the esophagus, and into the midgut (Sonenshine and Roe 2013). A valve-like structure, the labium, prevents the mixture of blood with saliva (Sonenshine and Roe 2013). Additionally, the proventricular valve inhibits regurgitation of host fluids into the pharynx (Sonenshine and Roe 2013).

Of all the organs in the tick's body, the largest is the midgut. Though its primary function is to contain and digest the massive bloodmeal, this process can occur for months and even years (Sonenshine and Roe 2013). Anatomically, the midgut is composed of a central ventriculous or stomach and numerous diverticula that extend throughout the body cavity (Sonenshine and Roe 2013). The wall of the midgut is made of epithelium and a thin layer of elongated smooth muscles. There are two cell types present: 1) undifferentiated and 2) digestive (Sonenshine and Roe 2013). As blood moves into the midgut, cells are lysed in the lumen, making hemoglobin digestions entirely intracellular (Sonenshine and Roe 2013). After exposure to the bloodmeal, previously undifferentiated cell types now develop into digestive cells that begin to ingest hemoglobin (Sonenshine and Roe 2013). As hemoglobin is endocytosed by tick cells, the molecules bound to the digestive cells are internalized and become part of the phagolysosome (Sonenshine and Roe 2013). Aside from hemoglobin, free amino acids, monosaccharides, free fatty acids, and large amounts of water and salts are absorbed directly into the lumen (Sonenshine and Roe 2013). Waste bi-products, including dead and decaying digestive cells, will converge with guanine produced by the Malpighian tubules (Sonenshine and Roe 2013). Waste

is then excreted after moving through the hindgut. The hindgut is composed of the intestine, rectal sac, and the rectum (Sonenshine and Roe 2013). When filled the hindgut has a whitish appearance. Contents may include guanine crystals or red blood cells (Sonenshine and Roe 2013). These bi-products are excreted as the tick actively feeds.

1.1.1f Host-Finding Strategies

Ticks utilize a series of strategies to locate hosts. Nonnidiculous ticks (or exophiles) reside in exposed and open environments where they encounter passing hosts, a behavior exhibited by many ixodid ticks (Sonenshine 2005). Potential hosts are located either by ambush or hunter strategy (Sonenshine 2005). In the ambush strategy, a tick will climb to the top of vegetation and spread its first pair of legs in a waving fashion known as questing (Sonenshine and Roe 2013). Located on the dorsal surface of the first set of legs is the Haller's organ, is the primary organ for determining host location through odors, recognizing pheromones, and other sensory functions during questing (Sonenshine and Roe 2013). Hosts can be detected from distances as far as 1-2 meters away (Sonenshine and Roe 2013). When questing, the height a tick will climb is dependent on the amount of moisture in the environment and the size of the host (Sonenshine and Roe 2013). Additionally, the life stage of the tick is also important as the smaller, immature stages generally parasitize smaller hosts because they are only able to climb lower vegetation (Sonenshine and Roe 2013). To avoid water loss while questing, a tick may climb to lower points of vegetation where moisture is more abundant (Sonenshine and Roe 2013). As such, temperature and moisture are key determinants of the tick's daily routine (Sonenshine and Roe 2013). Ticks utilizing the hunter strategy will often remain buried in the soil or sand of their environment to avoid water loss (Sonenshine 2005). Once a host cue is

detected by the Haller's organ, the tick quickly emerges to race after its host up to 3 meters away (Sonenshine 2005).

Alternatively, some species of tick exhibit a nidiculous life style. These ticks typically are found in the burrows or nest of their hosts (Sonenshine 2005). Instead of actively seeking a host, nidiculous ticks will hide and wait for the host. Many argasids and ixodid ticks utilize this strategy (Sonenshine 2005).

1.1.2 Ticks as Vectors of Disease Causing Agents

Intimately associated with each of the aforementioned components is the tick's role in transmission of medically important agents of disease (Table 1.1). Ticks are vectors of an array of disease causing agents. Alternatively, ticks are also known to host to a multitude of agents with unknown or no diseases. Below is description of two key tick species present in the United States, including a brief description of the bacteria and protozoa they are capable of transmitting.

Table 1.1. Timeline of the identification of tick-borne diseases of North American by persons and year discovered (Nelson and Williams 2014).

Disease	**Disease Vector**	**Investigator**	**Year**
Babesiosis (Texas cattle fever)	Cattle tick	Smith and Kilbourne	1893
Rocky Mountain spotted fever	Wood tick	Ricketts, King	1906
Colorado tick fever	Wood tick	Topping, Cullyford, and Davis	1940
Lyme Disease	Deer tick	Burgdorfer	1982
Human monocytic ehrlichiosis	Dog tick and lone star tick	Madeo et al.	1986
Human granulocytic ehrlichiosis	Deer tick	Chen et al.	1994

1.1.3 *Amblyomma maculatum* Life History

Commonly known as the Gulf Coast tick, *A. maculatum* is a 3-host, Neotropical, Nearctic tick of veterinary importance (Figure 1.5) (Teel et al. 2010, Paddock and Goddard 2015). Established populations were initially documented only within 100-150 miles of the shores of the Gulf of Mexico (Teel et al. 2010). This led to the perception that *A. maculatum* was restricted to

environments with high humidity, rainfall, and temperature (Bishopp and Hixson 1936, Paddock and Goddard 2015).

Figure 1.5. Life stages of the Gulf Coast tick, *A. maculatum*. From left to right: larva, nymph, adult male, and adult female (Paddock et al. 2008).

However, in the 1970's stable populations of this tick expanded to Oklahoma and Kansas (Teel et al. 2010). Currently, the accepted geographic range of *A. maculatum* consists of 16 states, including the Gulf Coast region, the Midwest, the Northeastern portion of the United States (Figure 1.6) (Teel et al. 2010, Paddock et al. 2015, Paddock and Goddard 2015).

Biological reasons for the incursion of the arthropod into parts of the country where they had not previously been detected are extensive. One likely reason for the establishment of *A. maculatum* in Oklahoma and Kansas is due to the shipment of cattle from Texas as a part of trade (Teel et al. 2010). Additionally, multiple species of passerine birds are parasitized by immature stages of *A. maculatum* (Teel et al. 2010). Migratory bird routes encourage northward establishment by larval and nymphal *A. maculatum*. Expansion in populations of white-tailed deer (*Odocoileus virginanus*) and feral swine have also played a role in the distribution of adult *A. maculatum* into new regions (Paddock and Goddard 2015). Aside from these factors, the low transpiration rate of *A. maculatum* allows this species to be surprisingly xerophilic in the dry

climates of the Midwest prairies and savannas where little shade can be found for the tick to retreat and recover water loss (Paddock and Goddard 2015).

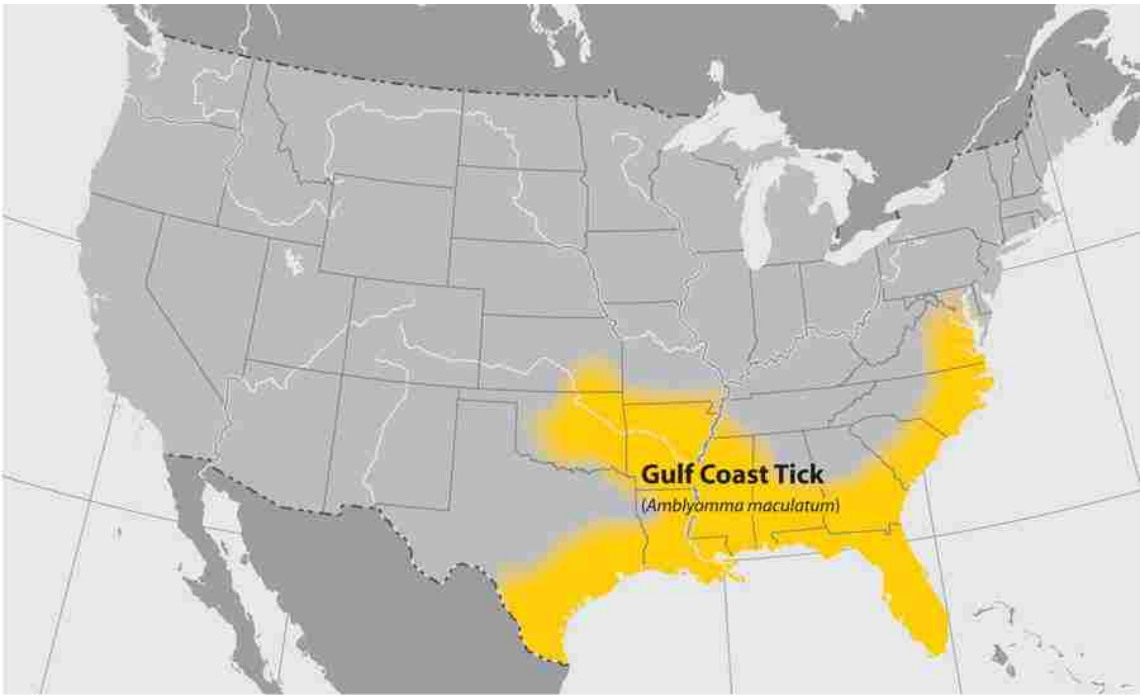

Figure 1.6. Approximate distribution of the Gulf Coast tick, *A. maculatum* **(**Centers for Disease Control and Prevention- http://www.cdc.gov/ticks/geographic_distribution.html).

1.1.3a Biology

Male *A. maculatum* initiate host attachment in large aggregates with other males by secreting attachment aggregation pheromones (Sonenshine 2004). Secondary female attachment occurs in response to these male chemical cues (Sleeba et al. 2010) Post-copulation and engorgement, the female lays over 9000 eggs (Drummond and Whetstone 1970). At the height of egg production over 1500 eggs will be laid in a single day (Drummond and Whetstone 1970). Oviposition typically lasts anywhere from 18-26 days (Drummond and Whetstone 1970, Teel et al. 2010) Bloodmeal conversion to egg mass is approximately 52% of the ticks engorged weight (Drummond and Whetstone 1970). Eggs will hatch after 14-28 days, or longer depending on the time of year in which eggs were deposited (Teel et al. 2010). Resulting unfed larvae can remain viable for 56-179 days before finding a host (Teel et al. 2010). Newly emerged larvae will be

most active during the afternoon hours, while older ticks will be most active in the morning and evening hours (Teel et al. 2010). Engorgement time for this life stage typically takes 3-10 days (Teel et al. 2010). In a laboratory setting ecdysis will take 11-17 days when stored at 27°C with a relative humidity of 90-98% (Koch and Hair 1975). Emergent unfed nymphs will remain viable for 154-168 days, requiring 5-8 days to reach engorgement (Hooker et al. 1912, Teel et al. 2010). Ecdysis typically lasts from 21-28 days at 27°C and high levels of relative humidity, but may take as long as 71 days in the natural environment (Teel et al. 2010). The longevity of unfed adults is approximately 2 and 4 times longer than larval and nymphal populations, respectively, lasting anywhere from 13-129 days (Teel et al. 2010). Peak seasonality for adult *A. maculatum* is in July and August, but varies based on region (Goddard and Paddock 2005, Goddard 2007).

1.1.3b Veterinary and Medical Importance

Prior to rigorous acaricide treatment implementation, cattle suffered greatly from parasitism by *A. maculatum*. Economic loss from infestations was estimated at 58 million dollars in 1987 (Teel et al. 2010). Gotch ear, a drooping of the ear in cattle results from heavy tick infestation can seriously affect the health of cows, horses, sheep, and goats. This can be exacerbated by secondary infestation by Myiasis flies, including the primary screwworm, *Cochliomyia hominovorax*. In humans, two separate cases of tick paralysis have been reported as a result of as few as one tick attaching to an individual (Paffenbarger 1951, Espinoza-Gomez et al. 2011).

The Gulf Coast tick is also a vector of medically important agents of human and animal disease. In 1998, it was definitively implicated as the vector for *Hepatozoon americanum*, the causative agent of American canine hepatozoonosis in dogs (Mathew et al. 1998). Acquisition of protozoa occurs as the tick receives a bloodmeal from a parasitemic dog (Mathew et al. 1998).

The infected tick will maintain *H. americanum* through molting, allowing for transmission by subsequent tick life stages to naïve hosts (Mathew et al. 1998). *Rickettsia* closely associated with *A. maculatum* include, *R. andeanae* and *R. parkeri*. *R. andeanae* is a newly identified species that is not currently associated with any human disease (Blair et al. 2004, Paddock et al. 2015). Field studies have demonstrated nearly 100% maintenance within tick populations (Paddock et al. 2015). Transmission of *R. andeanae* to human hosts has not been investigated. Alternatively, *R. parkeri* is a human pathogen that causes mild to moderate symptomatology (Paddock et al. 2004). Routes of *R. parkeri* maintenance within tick populations are a topic of recent investigation, with some publications observing nearly 100% maintenance between life stages and to offspring (Wright et al. 2015a). A more thorough description of *Rickettsia* and *R. parkeri* is discussed in later sections of this dissertation.

1.1.4 *Dermacentor variabilis* Life History

The American dog tick, *Dermacentor variabilis*, is a 3-host tick present throughout the eastern portion of the United States (from the Atlantic coast to the Midwest) and small portions of the California Pacific coast (Figure 1.) (Brown et al. 2005). This species is exemplary of a 2-cohort life cycle, in which adults active in the summer give way to adults the next spring (McEnroe 1985). Adults are active from April to September, with peak seasonality varying by region (Burg 2001). *Dermacentor variabilis* females are morphologically similar to *A. maculatum* (Figure 1.). *Amblyomma maculatum* are distinctive, however, because their capitulum is significantly longer than *D. variabilis*. Immature populations parasitize smaller animals such as mice, while adults infest medium sized animals such as coyotes and dogs (Goethert and Telford 2009).

Figure 1.7. Approximate distribution of the American dog tick, *D. variabilis* (Centers for Disease Control and Prevention- http://www.cdc.gov/ticks/geographic_distribution.html).

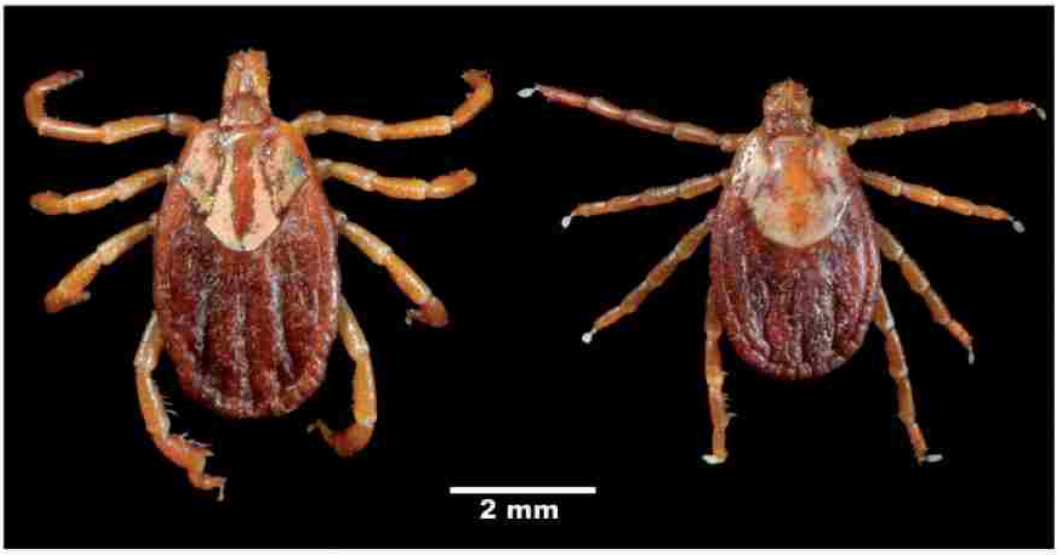

Figure 1.8. Morphological distinction of adult female *A. maculatum* (left) and *D. variabilis* (right) (Paddock and Goddard 2015).

1.1.4a Biology

In *D. variabilis* colonies maintained under laboratory conditions female ticks require 7-10 days for engorgement (Troughton and Levin 2007). Approximately 2-3 days later oviposition will begin, with eclosion of viable larvae in 5-8 weeks (Troughton and Levin 2007). Unfed

larvae may remain viable for 6 months (Troughton and Levin 2007). Larvae require 2-8 days to reach engorgement, with molting lasting up 2-3 weeks (Troughton and Levin 2007). Resulting unfed nymphs can remain viable for 2 months without a bloodmeal. Nymphal engorgement typically occurs 5-11 days post-attachment. Adults will emerge approximately 4-5 weeks later (Troughton and Levin 2007).

1.1.4b Medical and Veterinary Importance

American dog ticks are known to harbor multiple agents of human and animal disease. For instance, *D. variabilis* has been incriminated as a vector for *Francisella tularensis* (Bell 1945). The maintenance of this bacterium from mother to offspring is not well-defined; however, it is known that transmission appears to occur via tick saliva during feeding (Bell 1945, Goethert and Telford 2009). The causative agent of human monocytic ehrlichiosis, *Ehrlichia chafeensis* has been isolated multiple times from *D. variabilis* ticks collected in field studies (Roland et al. 1998, Steiert and Gilfoy 2002, Wright et al. 2014). Routes of infection and maintenance within tick populations have not been thoroughly investigated. *Rickettsia* transmitted and harbored by *D. variabilis* include *Rickettsia montanensis*, *Rickettsia peacockii*, and *Rickettsia rickettsii* (Azad and Beard 1998). Of the three, only *R. rickettsii* has definitively been shown to cause human disease. Human infections result in a highly virulent disease known as Rocky Mountain spotted fever (RMSF). An in-depth description of *Rickettsia* and its impact on human disease is discussed below.

1.2 The Genus *Rickettsia*

1.2.1 General information

Rickettsia are a diverse group of obligate intracellular bacteria that possess distinctive characteristics. They belong to the phylum Proteobacteria, class Alphaproteobacteria, order

Rickettsiales, and family Ricketttsiaceae. Morphologically, *Rickettsia* appear as pleomorphic rods or coccobacilli (Macaluso and Paddock 2014). Size can vary, but ranges from 0.7 to 2.0 μm by 0.3 to 0.5 μm (Figure 1.) (Hackstadt 1996). The cell membrane is composed of a trilaminar wall surrounded by a characteristic translucent zone or slime layer which is composed of polysaccharides (Figure 1.) (Hayes and Burgdorfer 1982, Macaluso and Azad 2005, Macaluso and Paddock 2014).

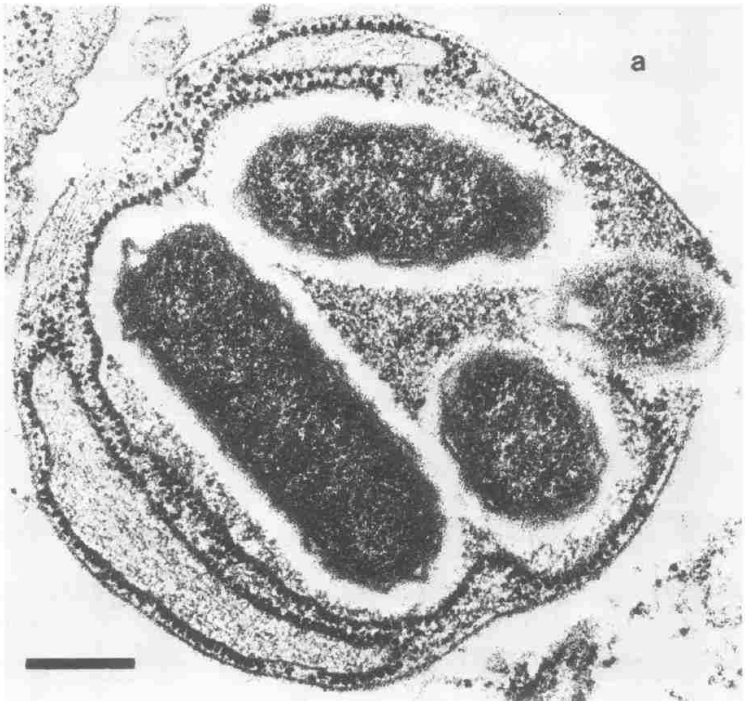

Figure 1.9. Thin section micrograph of *R. rickettsii* grown in chicken embryo with distinctive electron-lucent slime layer surrounding each organism. Bar=3μm (Silverman et al. 1978).

The obligate intracellular niche is the result of the loss of functional pathways involved in pentose phosphate, nucleotide metabolism, glycolysis, and amino acid biosynthesis pathways (Fuxelius et al. 2007, Audia 2012). Genome size across all *Rickettsia* ranges from 1.1-1.5 Mb, representing approximately 800-2000 genes (Fuxelius et al. 2007, Gillespie et al. 2008). The small size of genome and lack of key metabolic pathways is the result of reductive evolution (Audia 2012). Thus, *Rickettsia* are typically found in the cytoplasm or nucleus of the host cell

(Wolbach 1919, Burgdorfer et al. 1968). Intracellular movement of the bacterium is restricted. However, similar to *Shigella* and *Listeria*, some *Rickettsia* are capable of scavenging host cell actin to promote actin based motility (Bernardini et al. 1989, Tilney and Portnoy 1989, Heinzen et al. 1993, Jeng et al. 2004). Eukaryotic hosts for *Rickettsia* include plants (Davis et al. 1998), arthropods (Werren et al. 1994, Chen et al. 1996, Noda et al. 1997), annelids, and vertebrates (Weiss and Moulder 1984). *Rickettsia* exhibit an intimate relationship with hematophagous arthropod hosts such as ticks, fleas, lice, and mites.

Rickettsia are categorized into four main groupings based on genomic variations, surface proteins, disease manifestation, and host preferences: 1) ancestral group (AG), 2) typhus group (TG), 3) transitional group (TRG), and 4) spotted fever group (SFG) (Figure 1.) (Fuxelius et al. 2007, Gillespie et al. 2008, Walker and Ismail 2008). Within the ancestral group *R. canadensis* and *R. bellii* are primarily associated with ticks. Typhus group *R. prowazekii* and *R. typhus* are associated with lice and fleas, respectively. Transitional group members *R. akari* and *R. felis* are transmitted by mites and fleas, respectively. The largest grouping of rickettsial species is the spotted fever group. With over 25 individual species described, this group relies primarily on ticks for transmission to a vertebrate host (Macaluso and Paddock 2014). Increasing technology and surveillance have aided the ability to both, identify and track pathogenic species of *Rickettsia*.

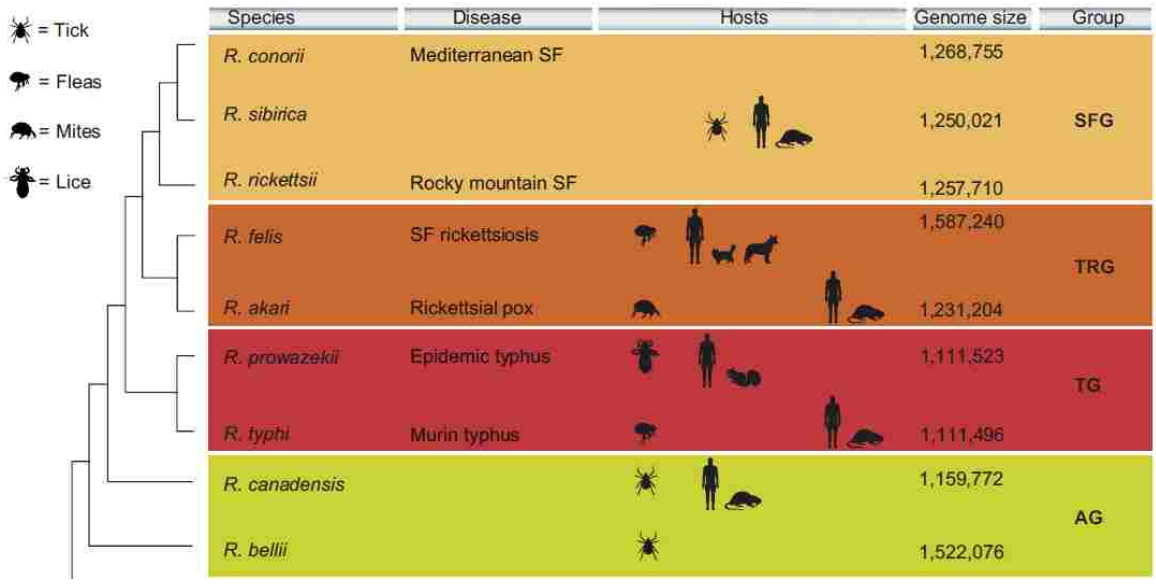

Figure 1.10. Summary of the four groupings of *Rickettsia* based on host preference, genomic variation, and disease manifestation. SF=spotted fever; SFG=spotted fever group; TRG=transitional group; TG=typhus group; AG=ancestral group (Fuxelius et al. 2007).

Within the last two decades cases of SFG rickettsioses have continued to rise dramatically. From 2000 to 2012 there was nearly a 7-fold increase in the number of reported cases of SFG rickettsiosis, representing the highest incidence since recording began in 1920 (Figure 1.1) (Dahlgren et al. 2016). Most cases of SFG rickettsiosis originate in Arkansas, Missouri, Oklahoma, North Carolina, and Tennessee (Figure 1.) (Dahlgren et al. 2016, Drexler et al. 2016). However, cases are detected in over the majority of the United States (Figure 1.). Additionally, multiple species within the SFG have not been clearly shown to be associated with human disease. Instead these species are classified as non-pathogenic endosymbionts of their tick host.

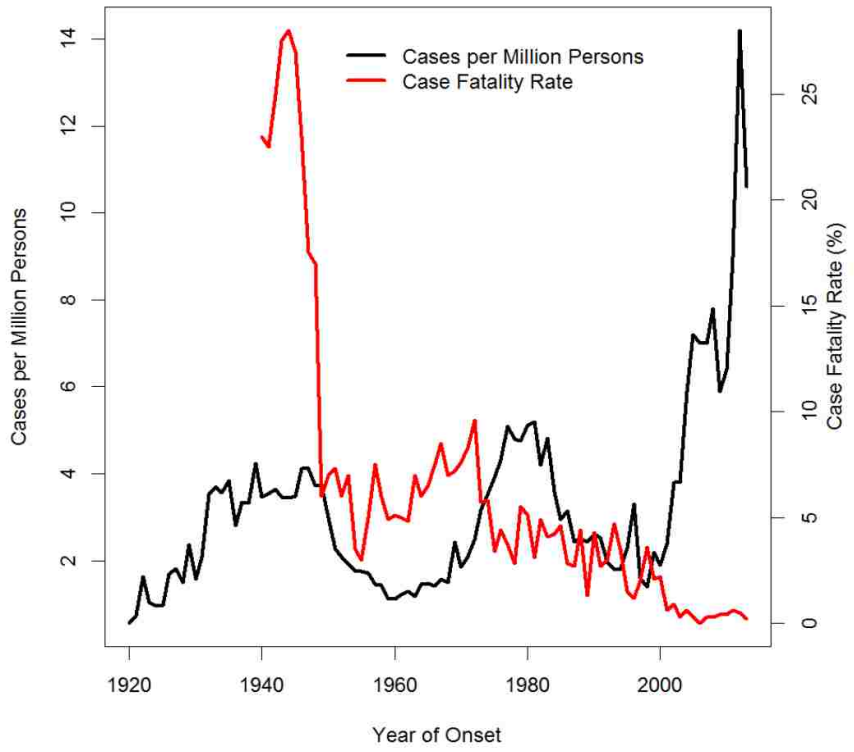

Figure 1.11. Incidence case fatality rate of SFG rickettsiosis in the United States from 1920-2013 (Dahlgren et al. 2016).

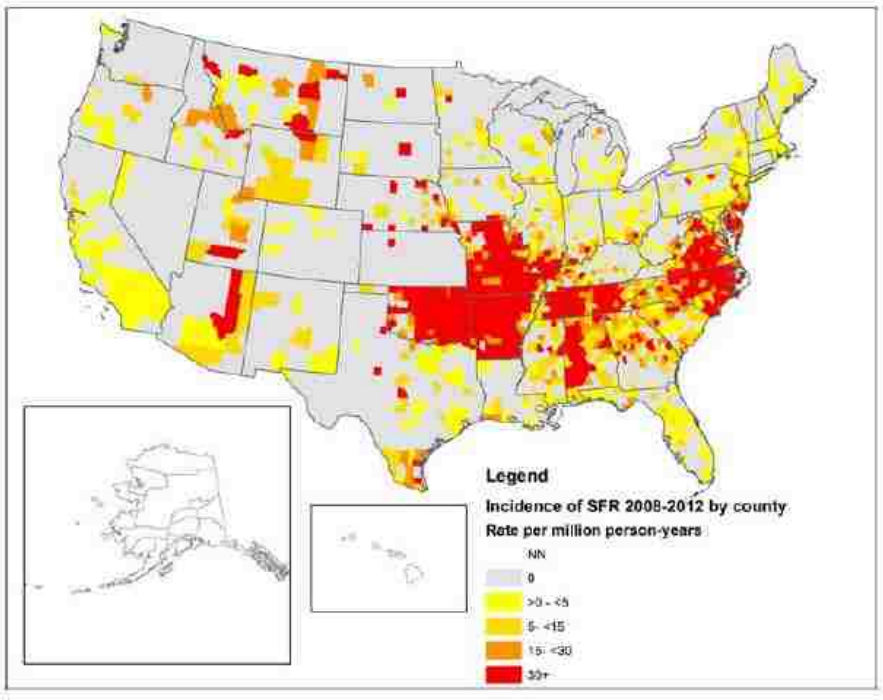

Figure 1.12. Incidence of SFG rickettsiosis by counties in each of the United States from 2008-2012 (Drexler et al. 2016).

1.2.2 Historical Perspective

The pathogenicity of *Rickettsia* should not be overlooked. Rickettsial agents of disease have enjoyed a long, deadly history worldwide. One of the first recorded outbreaks of rickettsial infection occurred in 16th Century Naples (Walker and Ismail 2008). The bacterium responsible was the agent of the epidemic typhus, *R. prowazekii*. Over 400 years later typhus continued to ravage populations. In Russia, 3 million people succumbed to epidemic typhus during 1917 to 1923 alone (Perlman et al. 2006, Walker and Ismail 2008).

In 1906 Howard T. Ricketts began investigating a typhus-like disease known as the "black measles" (Gross and Schafer 2011). Cases of afflicted individuals were highest in the Rocky Mountain region of the United States. Ricketts' first action was to collect samples from afflicted individuals and inject Guinea pigs and monkeys, a requirement of Koch's postulates (Ricketts 1907b). He further demonstrated that the agent causing this disease was transmitted through the bite of infected ticks (Ricketts 1906, 1907a) (Gross and Schafer 2011). Additionally, infected female ticks were shown to transmit infection to their offspring (Ricketts 1909, Gross and Schafer 2011). Altogether, this definitively demonstrated that the "black measles" was a tick-borne disease. At the same time, French researcher Charles Nicolle was working on a similar typhus-like disease. He focused primarily on how the agent of disease was transmitted, which was ultimately concluded to be the human body louse (Gross and Schafer 2011). Coincidentally, Ricketts had traveled to Mexico to investigate an outbreak of typhus. For his efforts, Ricketts was able to elucidate the causative agent of the illness, *R. prowazekii* (Gross and Schafer 2011). Tragically, this would also be the cause of Ricketts' own death. Stanislov von Prowazek would meet the same fate in the process of showing that not the bite but, rather, the feces of the body louse was the exact route of infection (Gross and Schafer 2011). Nearly a

decade later, S. Burt Wolbach, would name the collective genera of the organism after H.T. Ricketts in honor of his pioneering work (Wolbach 1919, Gross and Schafer 2011). Building on these early studies, several advancements have been made in the field of Rickettsiology.

1.2.3 SFG *Rickettsia* Disease Etiology

The onset of *R. rickettsii* infection and other disease-causing agents within the SFG of *Rickettsia* is marked by a number of symptoms. The intrinsic incubation period lasts 2-14 days post-infection, with an average of 7 for most patients (Lin and Decker 2012). Early symptoms include the clinical triad of fever, headache, and malaise (Lin and Decker 2012). Accompanying symptoms extend to arthralgia, myalgia, nausea, and vomiting (Lin and Decker 2012). A maculopapular rash develops as result of microvascular injury and immune cell infiltration (Walker and Ismail 2008, Lin and Decker 2012). The rash typically begins at the extremities, such as the wrists and ankles, spreading to the trunk, indicating the progress of systemic infection (Figure 1.) (Lin and Decker 2012). Eventually, the extensive microvascular injury results in a petechial rash (Figure 1.) (Lin and Decker 2012). This particular symptom develops 3-5 days after the onset of fever (Lin and Decker 2012). However, a small percentage of patients do not develop a petechial rash (Lin and Decker 2012).

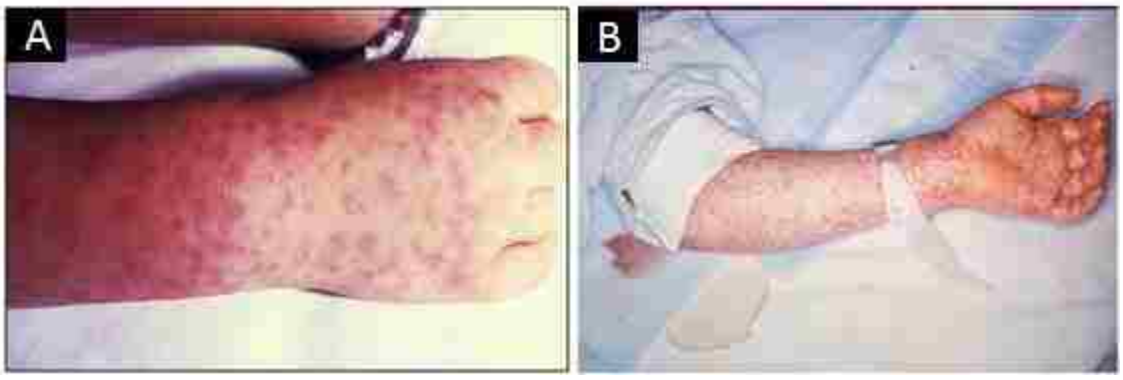

Figure 1.13. Image of a patient infected with a SFG *Rickettsia* presenting with A) an early stage rash and B) a late stage petechial rash (Centers for Disease Control and Prevention- http://www.cdc.gov/rmsf/symptoms/index.html).

Untreated systemic infection leads to increased vascular permeability, followed by edema, hypovolemia, and hypotensive shock (Lin and Decker 2012). Worsening condition is marked by meningitis, retinal vasculitis, renal failure, and pulmonary edema (Lin and Decker 2012). Failure to receive treatment often results in death within 7-15 days (Lin and Decker 2012). Infection with *R. parkeri* can be distinguished from *R. rickettsii* by the presence of an eschar at the tick attachment site (Paddock et al. 2004, Paddock et al. 2008). Eschars are small, non-pruritic ulcers surrounded by an erythematous halo where bacteria and immune cells are present (Figure 1.) (Paddock et al. 2004, Paddock et al. 2008).

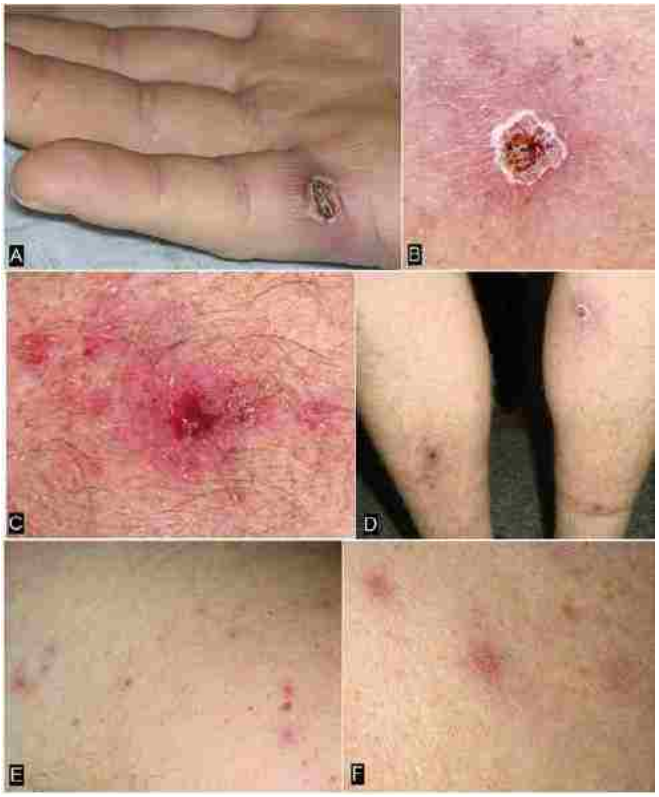

Figure 1.14. Cutaneous lesions from patients infected with *R. parkeri*. A-D) Eschars present at the site of tick attachment demonstrated by the typical ulcerated skin surrounded by an erythematous halo. E-F) Maculopapular or petechial rash present on the trunk and extremities of infected individuals (Paddock et al. 2008).

Serum titers of 1:64 indicate exposure to antigen, and a four-fold increase between samples a marker of active infection (Lin and Decker 2012). This can be followed by a Western

blot procedure as a confirmatory diagnosis (Lin and Decker 2012). Doxycycline is the drug of choice, however, chloramphenicol can also be administered (Lin and Decker 2012).

1.3 The Tick/*Rickettsia* Relationship

1.3.1 Vector Competence

For many zoonoses a requirement for maintenance of the life cycle is the transmission from one host to the next through a vector. Necessary for vector transmission is the establishment of competence. The tenants of a competent vector include multiple factors: 1) introduction of an agent to a susceptible arthropod, 2) development of infection within the arthropod, and 3) transmission of the agent to a naïve host by an infected arthropod (Reisen 2009). Demonstration of vector competence is a laboratory convention that is followed by a specific amount of vector incrimination, which implies that the agent of infection and the vector coexist in time and space (Reisen 2009). In fact, the journey from agent introduction into the vector to transmission is fraught with barriers, including: 1) the peritrophic membrane (which has only been described in *Ixodes*), 2) midgut barrier, 3) hemolymph barrier, 4) salivary gland barrier, and 5) salivary gland escape barrier (Edman 2004).

Invading *Rickettsia* are drawn into the tick from the wound site, through the feeding canal. As the tick lyses and digests erythrocytes, *Rickettsia* escape the tick immune response (Socolovschi et al. 2009). *Rickettsia* then invades epithelial cells of the midgut, where they are able to replicate (Popov et al. 2007). Pending ability to escape from the midgut, *Rickettsia* infect cells of the hemolymph (Socolovschi et al. 2009). Hemolymph, which is the gateway mechanism to dissemination in the tick, is a major barrier in and of itself. A multitude of antimicrobial peptides (AMPs) and hemocytes are present (Socolovschi et al. 2009). Hemocytes consist of prohemocytes, plasmatocytes, granulocytes, and spherulocytes (Munderloh and Kurtti 1995,

Socolovschi et al. 2009). Circulating cells transit *Rickettsia* to organs such as the ovaries and salivary glands. In the salivary glands *Rickettsia* has been shown to invade and divide within cells (Socolovschi et al. 2009). Infection of the salivary gland represents a crucial point as *Rickettsia* must escape during feeding to achieve salivary transmission. Infection in the ovaries can lead to transovarial transmission. Proteins produced in the fat body such as Vitellogenin can transit to the ovaries allowing for transovarial transmission (Munderloh and Kurtti 1995, Khalil et al. 2011).

A component of vertical maintenance is transovarial transmission, which is the passage of an agent from one life stage to the next (transtadial transmission) and also may consist of maintenance over multiple generations (Figure 1.) (Reisen 2009). Alternatively, horizontal transmission may occur through transmission of an agent from an infected tick to an uninfected tick through close proximity feeding on a mammalian host (Figure 1.) (Walker and Ismail 2008, Reisen 2009). Transmission of *Rickettsia* between tick and vertebrate hosts suggests that pathogenic species rely on horizontal transmission (Werren 1997). Therefore, any pathogenic effects the bacterium may have on the tick host populations may be overcome by renewed infection in susceptible populations of ticks. Conversely, non-pathogenic *Rickettsia* are maintained in the tick host mainly through vertical transmission (Werren 1997).

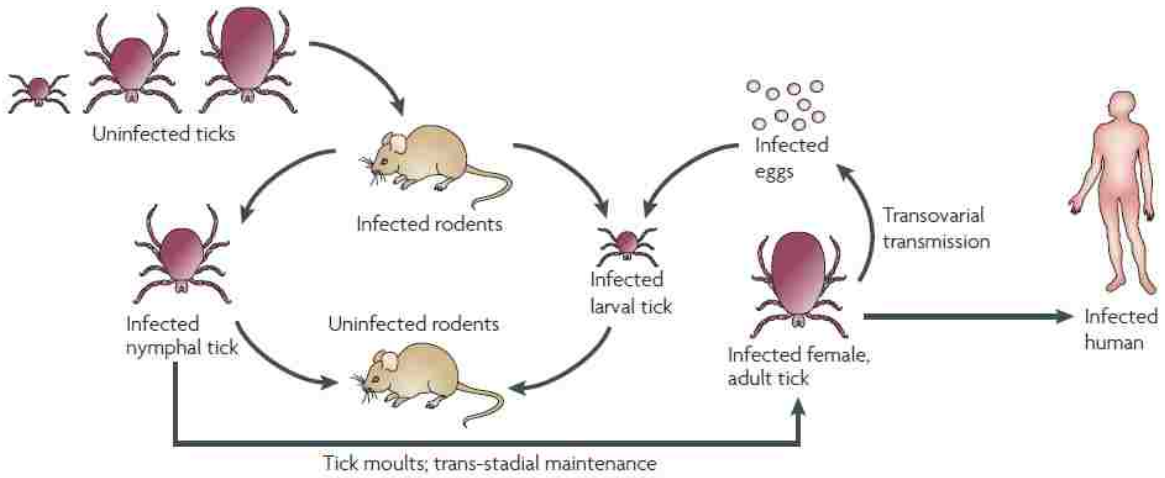

Figure 1.15. Mechanisms of vertical (transovarial and transtadial) and horizontal transmission used to maintain infection within populations of infected ticks (Walker and Ismail 2008).

The geographical distribution of SFG *Rickettsia* is bound by that of the tick host (Azad and Beard 1998). As the tick host is considered not only the vector but also the reservoir for many species of *Rickettsia*, intimate knowledge of the relationship between tick and pathogen is required. The following selected rickettsial species are of great importance in historical and modern Rickettsiology. All have been isolated from either *A. maculatum* or *D. variabilis*.

1.3.1 *Rickettsia rickettsii*

Rickettsia rickettsii is the prototypical SFG rickettsial species present in the United States. As discussed above it was first identified in *Dermacentor* ticks by Ricketts in a series of seminal studies (Ricketts 1907b). Following these studies, multiple strains of the bacteria were isolated for further characterization. Surprisingly, some of these *R. rickettsii* isolates were attenuated in a Guinea pig model of infection, implying strain-dependent variability in disease severity (Bell et al. 1963). Burgdorfer and Brinton 1975 demonstrated vertical transmission and fitness costs in cohorts of *D. andersoni*, *D. variabilis*, and *A. americanum* that were either naturally or artificially infected with *R. rickettsii* (Burgdorfer and Brinton 1975). The results of this study showed that infection with *R. rickettsii* was 1) not transmitted transovarially to 100% of the offspring, 2) infection with virulent strains of the bacterium leads to a negative impact on

tick fitness, and 3) transovarial transmission efficiency is reliant on development of *Rickettsia* in the ovaries (Burgdorfer and Brinton 1975). Additional work in *R. rickettsii*-infected *D. andersoni* bolstered this study, by also observing negative fitness effects on the tick (Niebylski et al. 1999). In 2005, an outbreak of Rocky Mountain spotted fever (RMSF) in Arizona resulted in the identification of a new vector for *R. rickettsii*, *Rhipicephalus sanguineus* (Demma et al. 2005). In recent years field studies have failed to identify substantial numbers of ticks infected with *R. rickettsii* (Stromdahl et al. 2011). This is interesting considering that case numbers of RMSF continue to rise in the United States (Drexler et al. 2016).

1.3.2 *Rickettsia parkeri*

Rickettsia parkeri was originally designated as a non-pathogen upon its initial isolation in 1937, despite its ability to cause clinical disease in Guinea pigs (Parker et al. 1939, Lackman et al. 1949). It was definitively identified as a human pathogen in 2002 when it was isolated from patients with symptoms reminiscent of RMSF (Paddock et al. 2004). Disease manifestation is distinctly milder than RMSF infection (Paddock et al. 2008). An eschar lesion is present at the tick attachment site in cases of *R. parkeri* rickettsiosis, whereas it is absent in RMSF (Paddock et al. 2008). Presumptively, this is due to the localized nature of infection residing at the inoculation site (Paddock et al. 2008). *Amblyomma maculatum* is the primary vector for *R. parkeri* (Paddock et al. 2004). Detection of *R. parkeri* in *A. maculatum* ranges from 5-52% (Edwards et al. 2011, Varela-Stokes et al. 2011, Nadolny et al. 2014, Gleim et al. 2016) The lone star tick, *A. americanum* has been implicated as an additional vector through laboratory studies (Goddard 2003). No natural incrimination of this tick in human cases has been identified (Wright et al. 2015b). A recently reported case of *R. parkeri* rickettsiosis from the western United States, outside of the normal range of *A. maculatum*, incriminated *A. triste* as an alternate vector

(Herrick et al. 2016). Field studies have also detected *R. parkeri* in *D. variabilis*, *R. sanguineus*, *Haemophysalis leporispalustru*s, and *Ixodes scapularis* (Williamson et al. 2010, Fornadel et al. 2011, Leydet and Liang 2013, Henning et al. 2014). *A. maculatum* infected with *R. parkeri* naturally incur no impact on fitness or ability to transmit the infection to successive life stages (Wright et al. 2015a). However, survival and fecundity has yet to be determined in ticks infected with known pathogenic isolates. To date 35 confirmed cases of *R. parkeri* rickettsiosis have been identified (Paddock and Goddard 2015)

1.3.3 *Rickettsia amblyommii*

Rickettsia amblyommii was initially isolated in multiple independent studies from *A. americanum* (Burgdorfer et al. 1974, Burgdorfer et al. 1975, Burgdorfer and Brinton 1975). Initial isolates were designated WB-8-2 agent and displayed no pathogenicity in Guinea pig models (Burgdorfer et al. 1974, Burgdorfer et al. 1981). Some cohorts of infected *A. americanum* maintain infection in 100% of their offspring (Zanettii et al. 2008). Moreover, laboratory assessment of rickettsial load in organs of infected adults showed that the salivary glands, midgut, and ovaries were all infected at comparable levels (Zanettii et al. 2008). Field collections have detected *R. amblyommii* in *A. americanum* at frequencies of 2.7-100% (Goddard and Norment 1986, Gleim et al. 2016, Hudman and Sargentini 2016). *Rickettsia amblyommii* has also been detected in *D. variabilis* and *A. maculatum* (Trout Fryxell et al. 2015, Gleim et al. 2016). In a 2004 study, 3 of 6 patients reporting symptomology of RMSF had discernably higher titers to *R. amblyommii* compared with *R. rickettsii* (Apperson et al. 2008). This suggested that *R. amblyommii* is a human pathogen. No isolates were derived from infected patients, which would more closely indicate pathogenicity. Another study demonstrated that an engorged *A. americanum* removed from a patient experiencing RMSF symptoms was positive for *R.*

amblyommii (Billeter et al. 2007). The patient developed a maculopapular rash; however, *R. amblyommii* was not isolated from the inoculation site (Billeter et al. 2007).

1.3.4 *Rickettsia montanensis*

Rickettsia montanensis (*R.* montana) was originally described in 1961 (Bell et al. 1963). A study of *D. variabilis* collected from the Bitterroot Valley in Montana failed to identify *R. rickettsii* and instead isolated an unknown non-pathogenic *Rickettsia* (Bell et al. 1963). A 2012 case report describes a case of RMSF in which a *R. montanensis*-positive tick was removed from the patient (McQuiston et al. 2012). However, no supporting evidence from the inoculation site was provided (McQuiston et al. 2012). *Rickettsia montanensis* has been detected in rodents (Raoult and Roux 1997). Though primarily associated with *D. variabilis*, contemporary field studies consistently detect low amounts (1.5-10%) of naturally infected ticks (Azad and Beard 1998, Nadolny et al. 2014, Hudman and Sargentini 2016, St John et al. 2016). Detection in *A. maculatum* and *A. americanum* has also been documented (Lee et al. 2014, Trout Fryxell et al. 2015). Laboratory models of *R. montanensis* have demonstrated that introduction of the rickettsiae, through artificial means, produces 100% transovarial infection in *D. variabilis* (Macaluso et al. 2001). Sustained infection at nearly 100% persisted for two generations (Macaluso et al. 2002). Infected cohorts effectively demonstrated transovarial transmission blocking, by preventing establishment of a second species of *Rickettsia* (*R. rhipicephali*) in the ovaries. Ultimately, this demonstrated that transovarial transmission blocking only required sufficient infection of the tick ovaries by the first *Rickettsia*, and that pathogenicity played no substantial role in the outcome of infection.

1.4 Rickettsial Factors Associated with Infection of Mammalian and Arthropod Host Cells

Rickettsial invasion of mammalian cells consists of a series of highly coordinated events: 1) adhesion to the host cell plasma membrane, 2) engulfment, 3) inclusion in a phagosome, 4) phagosome lysis, and 5) release into the cytosol (Figure 1.) (Teysseire et al. 1995).

1.4.1 Adhesion to the Host Cell Plasma Membrane

Rickettsia express a panel of surface proteins, some of which have correlated functions in adherence, while others have yet to be fully investigated in this context. The most highly studied of these outer membrane proteins (Omp) include 4 proteins: 1) OmpB (surface cell antigen 5 [Sca5]), 2) OmpA (Sca0), 3) Sca1, and 4) Sca2. Post-processing OmpB is located at the outer membrane of all rickettsial species (Hackstadt et al. 1992). Furthermore, it has been shown to bind to Ku70, a nuclear protein transported to the cell surface involved in adherence to and invasion of mammalian host cells (Figure 1.) (Martinez et al. 2005, Chan et al. 2009). However, ablation of rOmpB-Ku70 interactions does not completely inhibit rickettsial invasion into mammalian cells, suggesting a redundancy in invasion mechanisms (Chan et al. 2009, Chan et al. 2010). Additionally, high-throughput sequencing suggests that OmpB is not cleaved in at least one avirulent strain of *R. rickettsii* (Clark et al. 2015). OmpB has also been shown to bind to arthropod host cell receptors, namely Histone H2B (Thepparit et al. 2010). This was elucidated in a model of *R. felis* invasion into an *I. scapularis* embryonic tick cell line (ISE6) (Thepparit et al. 2010). Histone H2B is also a nuclear protein that may be trafficked to the cell surface (Khan et al. 1998, Herren et al. 2006, Das et al. 2007, Thepparit et al. 2010). The mammalian host cell receptor $\alpha 2\beta 1$ integrin is capable of binding to rickettsial OmpA (Sca0) allowing for bacterial adhesion and invasion (Hillman et al. 2013).

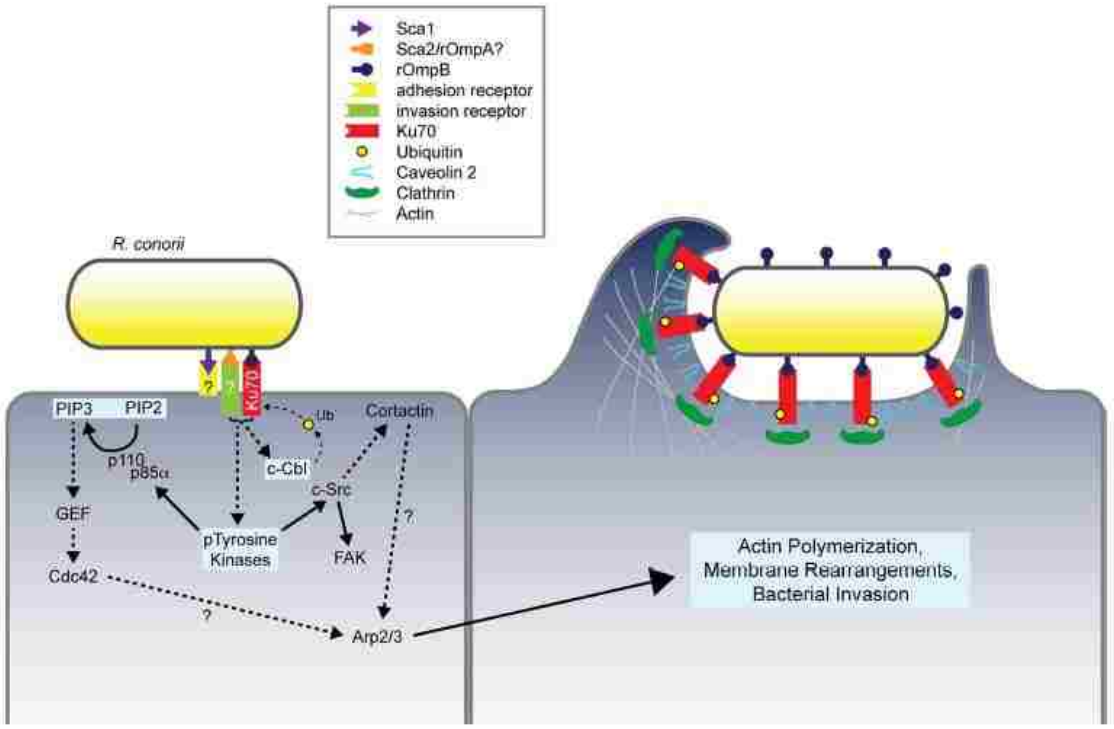

Figure 1.16. Model of host-cell interaction with *Rickettsia*. Interaction of *R. conorii* Sca1, Sca2, OmpA, and OmpB with host cell receptors leads to adherence and invasion of non-phagocytic mammalian cell types. The process of invasion by *Rickettsia* leads to the activation of multiple pathways and second messengers that result in the host cytoskeletal restructuring and rickettsial entry. Dashed lines with question marks indicate putative proteins roles (Chan et al. 2010).

OmpA is noteworthy as this protein is expressed only in SFG *Rickettsia*. It has been implicated as an adhesin in a model of *R. rickettsii* and *R. conorii* invasion into murine fibroblasts cells and an *Escherichia coli* system, respectively (Li and Walker 1998, Martinez 2012, Hillman et al. 2013). High-throughput sequencing has also shown that OmpA is truncated in an avirulent strain of *R. rickettsii*, but remains intact in highly virulent strains, suggesting a potential role for OmpA as a virulence factor (Clark et al. 2015). Sca1 is present in all *Rickettsia*, excluding *R. prowazekii* and *R. canadensis* (Martinez 2012). It has been shown to function in adherence to host cells, but not invasion (Riley et al. 2010). Lastly, Sca2 is another cell surface exposed protein present in nearly all *Rickettsia*, except for the TG (Blanc et al. 2005). Roles in both adherence and actin-based motility have been assigned to this protein (Cardwell and Martinez 2009, Haglund et al. 2010, Kleba et al. 2010, Reed et al. 2014).

1.4.2 Engulfment and Phagosomal Inclusion

Following rickettsial adhesion an array of second messengers are activated. Two proteins utilized include: 1) Arp 2/3 and 2) the Ras family GTP-binding protein, Cdc42 (Martinez 2012). These proteins are involved in actin cytoskeletal restructuring allowing for receptor-mediated endocytosis into non-phagocytic cells. The Arp 2/3 complex once activated by a nucleation promoting factor (NPF) is able to organize actin monomers, a vital component of rickettsial entry (Welch et al. 1997, Martinez 2012). Cdc42 has been shown to function in reorganization of actin-rich structures in cells and is able to act as a NPF for the Arp 2/3 complex (Hall 1998, Higgs and Pollard 2001, Martinez 2012). Additional immunofluorescence studies demonstrated that Cdc42 is present upon invasion of *R. conorii* into mammalian cells, though the mechanism of how it activates the Arp 2/3 complex is unknown (Martinez and Cossart 2004). Rickettsial invasion also induces tyrosine phosphorylation events among c-Src, cortactin, p125 (Focal adhesion kinase-FAK), and PI-3 kinase in mammalian cells (Martinez and Cossart 2004, Chan et al. 2010). As the rickettsiae invades the host cell, these proteins become phosphorylated and induce rearrangement of the cell cytoskeleton (Src), further phosphorylation of second messengers (Src and FAK activating cortactin), and activation of Arp 2/3 complex by cortactin (Weaver et al. 2002, Martinez 2012). The majority of the bacteria were able to be internalized and the rest of the inoculated *Rickettsia* had adhered to the membrane of the cell (Welch et al. 2012). At 20 minutes post-infection nearly all bacteria were internalized and either present in a phagosome or free in the cytosol (Welch et al. 2012). The entire process of rickettsial invasion occurs within 3 minutes of inoculation into culture conditions (Teysseire et al. 1995, Welch et al. 2012).

1.4.3 Phagosomal escape

A study of *R. prowazekii*, bacteria were observed to have escaped the phagosome and appear free in the cytosol 30 minutes post-infection (Whitworth et al. 2005). Phagosomal escape occurs early in invasion, before fusion with the lysosome (Welch et al. 2012). However, studies investigating this specific process are lacking, and, ultimately, the stage of phagosomal maturation at which *Rickettsia* escape is ill-defined.

Several proteins have been implicated in the escape of *Rickettsia* from the phagosome: 1) phospholipase A2 (PLA2), phospholipase D (PLD), and 2 different hemolysins (TlyA and TlyC). Inhibition of *R. rickettsii* PLA2 produced a decrease in infection and plaque forming units (Table 1.2) (Walker et al. 1983, Silverman et al. 1992). Phospholipase D is a potentially secreted protein that has been shown to hydrolyze phosphatidylcholine (Welch et al. 2012). Furthermore, PLD has been shown to mediate escape from the phagosome in an artificial system using *Salmonella enterica* serovar typhimurium (Whitworth et al. 2005). Alternatively, through site-directed mutagenesis of the gene encoding PLD had no effect on bacterial growth in host cells (Table 1.2) (Driskell et al. 2009). Hemolysins function to disrupt membrane proteins and are able to mediate rickettsial escape of the phagosome in artificial systems where a gain of function is imparted by the protein (TlyC) (Radulovic et al. 1999, Whitworth et al. 2005). These proteins have been identified in *R. prowazekii* and *R. typhi* (Table 1.2) (Radulovic et al. 1999, Whitworth et al. 2005). However, they have not been isolated in order to delineate their true function.

Table 1.2. Bacterial factors implicated in phagosome escape (adapted from (Welch et al. 2012)

Biochemical activity	Functional evidence for role in escape	Reference
PLA2	Inhibition reduced the frequency of plaque formation	(Walker et al. 1983, Silverman et al. 1992)
PLD	*RP819* enabled *S.* Typhimurium phagosomes escape, but *R. prowazekii Δpld* mutant showed no difference from wild-type in the timing of escape	(Whitworth et al. 2005, Driskell et al. 2009)
Hemolysin	*R. prowazekii tlyC* enabled *S.* Typhimurium escape from the phagosome	(Whitworth et al. 2005)

1.4.4 Eukaryotic Proteins Involved in Actin Polymerization

Eukaryotic host cells are composed of highly coordinated networks of actin cytoskeleton. the repertoire of cellular function in which actin processes are involved include: 1) membrane trafficking, 2) cell migration protrusions, and 3) cell division (Firat-Karalar and Welch 2011). These cytoskeletal networks can also be hijacked by bacterial and viral invaders for their own use in intracellular motility (Firat-Karalar and Welch 2011).

Host cell actin exists in two states: 1) globular (G-actin) or 2) filamentous actin (F-actin) (Welch et al. 2012). Globular actin is able to tightly bind to ATP allowing it to bind with neighboring G-actin molecules. When a trimer of G-actin comes together a nucleus is formed. Actin can continue to be added to this structure, however, this is kinetically unfavorable within the cell (Bugalhao et al. 2015). Thus, an actin nucleator (Arp 2/3, formin, or tandem-monomer-binding proteins) is required to stabilize this structure and allow for actin monomers to continue to be added to form a long chain of actin or F-actin (Figure 1.) (Campellone and Welch 2010). The structure of the growing F-actin chain consists of two ends: 1) the fast-growing (barbed or +) end and the slow-growing (pointed or -) end (Bugalhao et al. 2015). Addition of actin to the barbed end occurs more quickly than at the pointed end (Bugalhao et al. 2015). Moreover, as the

filament chain grows, pointed end monomers hydrolyze ATP releasing ADP whereby actin monomers are released (Truong et al. 2014). In addition to this process, multiple other cellular proteins can act on the actin filament through capping, cross-linking, severing, or bundling (Campellone and Welch 2010, Khaitlina 2014, Truong et al. 2014).

The Arp 2/3 complex is vital to this process (Figure 1.) (Welch et al. 1997). Identification of this complex has been made in multiple organisms including eukaryotic cells and, more recently, in the tick *D. variabilis* (Welch et al. 1997, Petchampai et al. 2014). Mediation of actin filament elongation occurs at a 70° angle to previous filaments when under the influence of Arp 2/3 (Firat-Karalar and Welch 2011). This results in a y-branched appearance of actin organization. Addition of actin monomers directs the movement of the bacterium within the host cell. Thus, speed of movement is controlled by how quickly proteins are able to gather actin monomers (Khaitlina 2014). Nucleation promoting factors (NPFs) activate actin nucleators such as Arp 2/3 and increase the speed of nucleation. Examples of NPFs include WASP, WHAM, and JMY (Welch and Way 2013). Characteristically, NPFs consist of a variable N-terminus, a C-terminus bearing a proline rich region (PRR) that interacts with profiling (a G-actin binding protein), 1 or 2 G-actin WASP homology 2 (WH2) motifs (W), and a C-terminal central and acidic region (CA) that binds the Arp 2/3 complex (Campellone and Welch 2010).

Formins are another class of actin nucleators (Figure 1.). They are typified by 2 main features: 1) a proline rich formin homology 1 (FH1) and 2) a formin homology 2 (FH2) domain (Truong et al. 2014). The FH2 domain forms a circular complex around the growing filaments. This complex is able to processively bind the barbed end of the developing actin filament (Campellone and Welch 2010, Bugalhao et al. 2015). This is advantageous because bound

formins are able to deter proteins which would cap or end filament elongation (Haglund et al. 2010).

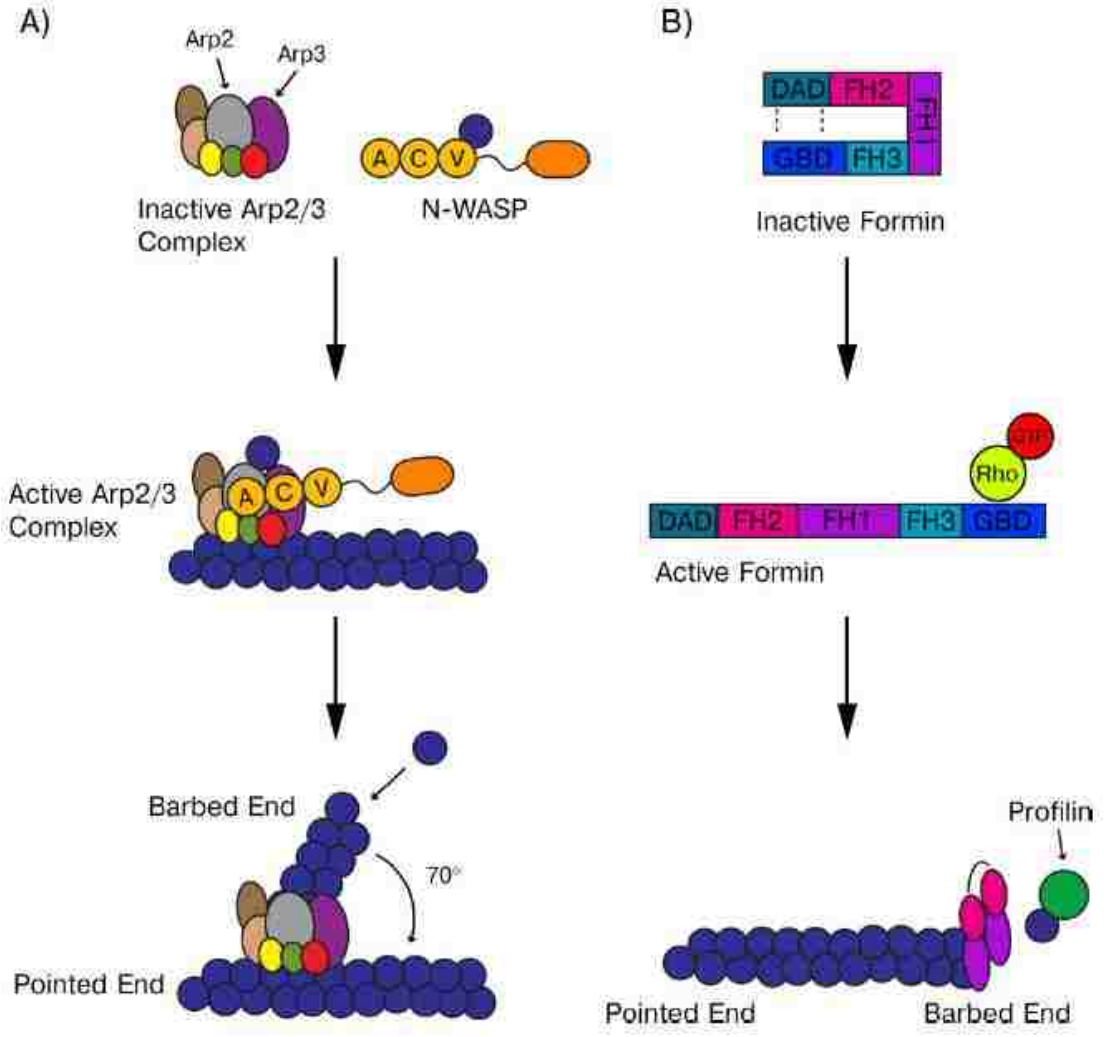

Figure 1.17. Alternative strategies in actin polymerization by either the Arp2/3 complex or host cell formins. A) Binding and activation of the Arp2/3 complex by nucleation promoting factors (N-WASP), which aids in barbed end actin filament elongation at a 70° angle. B) Rho GTPases binding N and C termini of inactive formins, disrupting formin intramolecular interactions. This allows for long, straight actin filaments to form (Truong et al. 2014).

1.4.4a Rickettsial Actin-Based Motility

Actin-based motility is found in many pathogenic bacteria, including *Listeria monocytogenes*, *Shigella flexneri*, *Burkholderia pseudomallei*, and *Mycobacterium marinum* (Bernardini et al. 1989, Tilney and Portnoy 1989, Kespichayawattana et al. 2000, Stamm et al.

2003). The first studies demonstrating *Rickettsia*'s association with actin motility utilized light microscopy to visualize host cell actin and invading *Rickettsia* (Heinzen et al. 1993, Teysseire et al. 1995). Actin polymerization was observed in two separate studies at 15 and 30 minutes and 24 hours (Figure 1.) (Heinzen et al. 1993, Teysseire et al. 1995). Presence of actin tail formation at time points coinciding with host cell invasion suggested that *Rickettsia* may enter the host environment primed for polymerization (Van Kirk et al. 2000). Additional ultrastructural analysis showed that, unlike *L. monocytogenes* and *S. flexneri*, *Rickettsia* polymerized long and unbranched actin tails (Van Kirk et al. 2000). Knockdown of host nucleation factors resulted in no distinct effect on actin polymerization and motility, suggesting that a bacterial protein, or group of proteins, aided in nucleation (Heinzen et al. 1993, Campellone and Welch 2010, Serio et al. 2010).

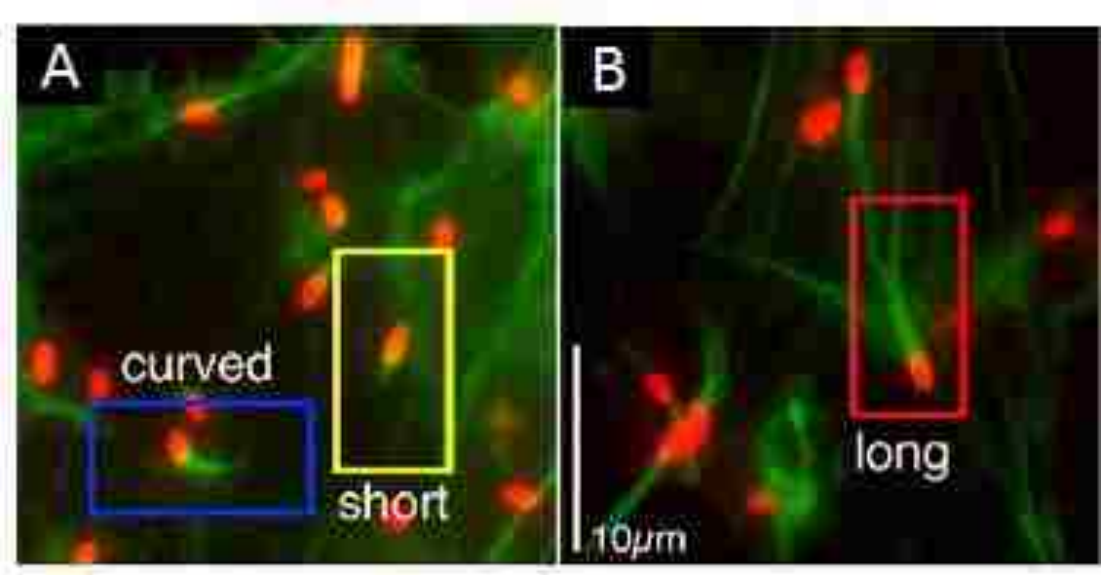

Figure 1.18. Actin-based polymerization by *R. parkeri* in HMEC-1 cells. A) Early phase polymerization (15 minutes post-infection) occurring under the control of RickA producing tails with short (yellow box) and curved (blue box) phenotypes. B) Late phase polymerization (24 hours post-infection) under the control of Sca2 producing elongated tails (red box). *Rickettsia*=red; phalloidin=green (Reed et al. 2014).

1.4.4b The Function of Rickettsial Sca2 in Actin-Based Motility and Adhesion

Sca2 is an autotransporter that localizes to the surface of *Rickettsia* (Figure 1.) (Ngwamidiba et al. 2005). Presentation at the surface level occurs through a series of events initiated by the signal sequence or N-terminal region's interaction with the Sec secretion system, which mediates passage of the protein across the inner bacterial membrane (Dautin and Bernstein 2007, Madasu et al. 2013).

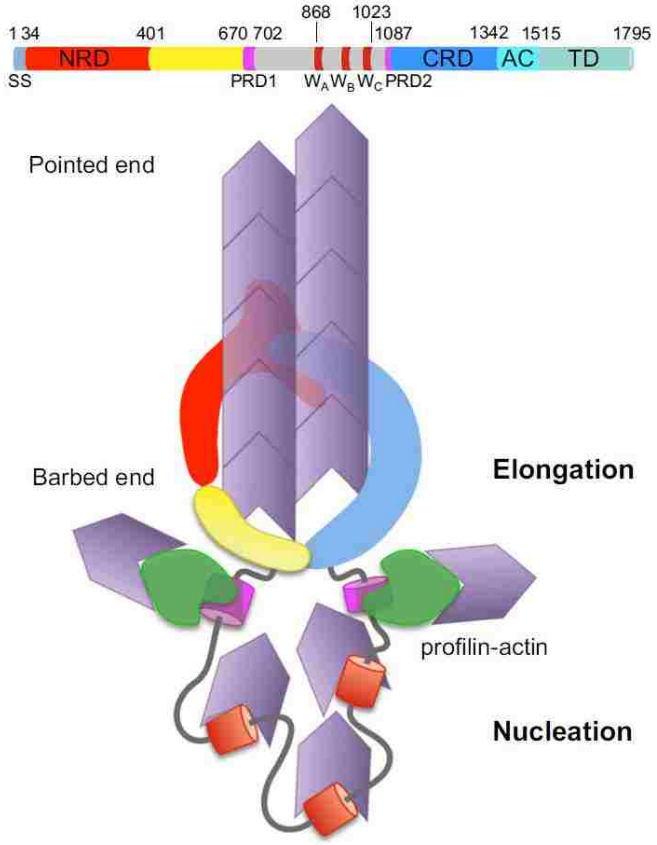

Figure 1.19. Nucleation and elongation mediated by rickettsial Sca2. Interaction between the N- (NRD, red) and C-terminus (CRD, light blue) produces an FH2-like processive cap present at the barbed end of actin elongation. Proline-rich regions (PRD1 and 2, magenta) are in charge of incorporation of actin bound to profiling. The WH2 domains ($W_{A,B,C}$, light red) recruit actin monomers. AC=autochaperone domain; TD=translocator domain (Madasu et al. 2013).

After localization to the gram-negative periplasm, the translocator or C-terminal region is inserted to the outer membrane (Dautin and Bernstein 2007, Madasu et al. 2013). The translocator region then forms a pore allowing the passenger domain to be transported outside of the cell, whereby this portion of the protein is exposed to the extracellular milieu (Dautin and

Bernstein 2007, Madasu et al. 2013). Four-putative WH2 and 2-proline-rich domains are present within the surface-exposed Sca2 passenger domain (Haglund et al. 2010, Kleba et al. 2010). The proline-rich domains (PRDs) function in actin nucleation by binding profilin-bound actin molecules, a role similar to formin homology 1 (FH1) domains (as discussed above) (Madasu et al. 2013). Secondary structure of the passenger domain is indicative of a FH2 domain (Welch et al. 2012). Additionally, the N-terminal and C-terminal ends of the Sca2 passenger domain interact to form a domain that is functionally similar to FH2 (as discussed above) (Madasu et al. 2013). Polymerization of actin has been shown to occur cooperatively with the N- and C-terminal regions of Sca2 forming a donut-like structure around the barbed end of the actin tail (Madasu et al. 2013). This provides functionality as a tracking unit, of which both termini are required (Madasu et al. 2013). Sca2 is distinctive in that unlike other formin-related proteins it is able to function as a monomer instead of a dimer (Madasu et al. 2013).

Replication patterns of a *R. rickettsii sca2* mutant were similar to the virulent wild-type strain (Kleba et al. 2010). However, disruption of Sca2 resulted in a loss of actin tail formation in mammalian cells, and an overall reduction in plaque sizes compared to wild-type *R. rickettsii* (Kleba et al. 2010). Furthermore, Guinea pigs inoculated with the mutant strain did not develop disease or symptomology associated with typical rickettsial infection (Kleba et al. 2010). Additional studies have shown that mutagenesis of *R. parkeri sca2* results in a similar loss of actin-based motility at 24 hours post-infection into host mammalian cells (Figure 1.) (Reed et al. 2014). Cell-to-cell spread was also diminished compared to wild-type *R. parkeri* (Reed et al. 2014). Early actin tail formation (15 and 30 minutes post-infection) was significantly shorter than those formed 24 hours post-infection (late tails) (Reed et al. 2014). Early intracellular movement was also slower and less directionally focused than bacteria forming late tails (Reed et

al. 2014). Further investigation into the mechanism directing early and late phase motility revealed that another protein (RickA) localized to the barbed end of filaments in early tails (Reed et al. 2014). Moreover, disruption of *rickA* in *R. parkeri* resulted in a loss of early phase actin-based motility; however, late phase movement still occurred (Reed et al. 2014). Thus, it has been postulated that early phase motility is guided by RickA and late phase actin tails are directed by Sca2 (Reed et al. 2014).

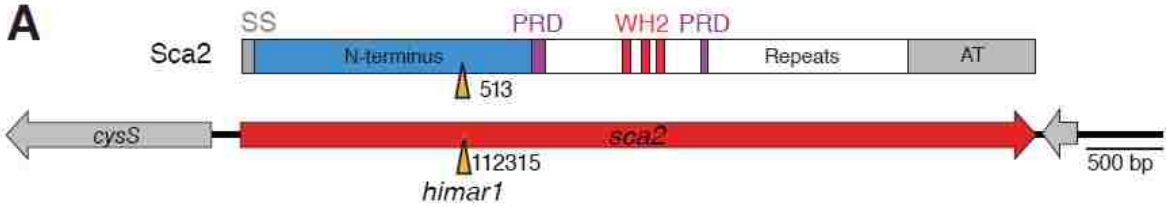

Figure 1.20. A) Schematic of *R. parkeri sca2* corresponding to Sca2. SS=signal sequence; PRD=proline-rich domain; WH2=WASP homology 2 domain; AT=autotransporter domain. The arrowhead indicates insertion of *himar1* transposon (Reed et al. 2014).

Aside from its role in actin-based motility, Sca2 has been shown to dually function as an adhesin (Cardwell and Martinez 2009). When expressed in a non-invasive strain of *E. coli* Sca2 mediates adherence of the bacterium to host cells (Cardwell and Martinez 2009). Closer study showed that this activity was controlled by a portion of the N-terminus (Cardwell and Martinez 2012).

1.4.4c The Function of Rickettsial RickA in Actin-Based Motility

Mechanistically, RickA mediates actin tail formation through interactions with the Arp2/3 complex. The amino acid sequence of RickA contains canonical domains indicative of Arp 2/3 complex nucleation promoting factors (Figure 1.) (i.e. WCA domains-as discussed above) (Gouin et al. 2004, Jeng et al. 2004, Welch et al. 2012). Unlike other proteins of similar

function and identity, RickA lacks sequence that would indicate its mode of transportation to the outer bacterial surface (i.e. Sec-dependent signal, TAT, or transmembrane domain) (Welch et al. 2012). Thus it is unclear how the protein is transported to the bacterial surface (Welch et al. 2012).

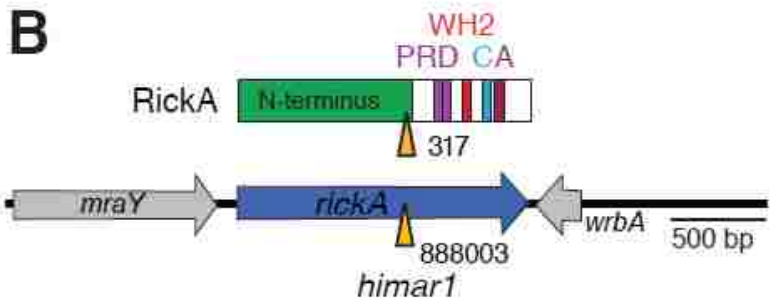

Figure 1.21. B) Cartoon of *R. parkeri rickA*. PRD=proline-rich domain; WH2=WASP homology 2 domain; C=central domain; A=acidic domain. The arrowhead indicates insertion of *himar1* transposon (Reed et al. 2014).

A clearly defined role for RickA has been difficult to elucidate. Initially, it was identified in multiple genomes within the ancestral group (*R. canadensis* and *R. bellii*), transitional group (*R. felis*), and spotted fever group (*R. conorii*, *R. parkeri*, *R. rickettsii*, and *R. montanensis* among others) (Ogata et al. 2005, Ogata et al. 2006, Balraj et al. 2008). Despite expressing RickA, *R. canadensis* does not polymerize actin (Jeng et al. 2004). Typhus group *Rickettsia*, such as *R. prowazekii* and *R. typhi* do not encode RickA (Ogata et al. 2001, McLeod et al. 2004).

Recombinant *R. conorii* and *R. rickettsii* RickA has been shown to promote y-branched actin polymerization via an activated Arp 2/3 complex (Gouin et al. 2004, Jeng et al. 2004). These studies showed the protein's ability to utilize the Arp 2/3 complex for organization of actin tails and motility. Additionally, colocalization of the Arp 2/3 complex to rickettsial actin tails was identified using fluorescence microscopy (Gouin et al. 2004). Bacteria that were actively polymerizing actin were observed to exhibit weak signal at one pole of the organism

(Gouin et al. 2004). Alternatively, bacteria without actin tails were heavily labeled with Arp 2/3 around the entire periphery (Gouin et al. 2004). Additional work completed in *R. rickettsii* showed that overexpression of Arp 2/3 exhibited no change in actin polymerization and movement of bacteria (Harlander et al. 2003). In a separate study *R. bellii* was engineered to overexpress *R. monacencis* RickA (Oliver et al. 2014). Overexpression produced an increase in cell adhesion, suggesting a potential role in adherence for initial actin polymerization (Oliver et al. 2014). Actin tail formation was observed to decrease in comparison to wild-type *R. bellii*, but only at times greater than 16 hours post-infection (Oliver et al. 2014). Bacterial movement also became more erratic in *R. bellii* containing ectopically expressed *R. monacencis-rickA* (Oliver et al. 2014).

Table 1.3. Summary of assigned function of Sca2 and RickA (adapted from (Welch et al. 2012).

Protein	Function/role in rickettsial pathogenicity	References
Sca2	Nucleates and elongates actin filaments; involved in late phase rickettsial motility; role in adherence to mammalian cells	(Haglund et al. 2010, Cardwell and Martinez 2012, Madasu et al. 2013, Reed et al. 2014)
RickA	Promotes actin nucleation with host Arp2/3 complex; involved in early phase rickettsial motility	(Gouin et al. 2004, Jeng et al. 2004, Reed et al. 2014)

1.4.4d Additional Aspects and Hypotheses Concerning Actin-Based Motility of *Rickettsia*

RickA has additionally been hypothesized to promote spread from infected to naïve cells, which has been described for Vaccinia virus (Doceul et al. 2010, Reed et al. 2014). Until recently, no data supported a clearly defined role for RickA in rickettsial motility. The most recent model suggests that RickA is the driving force behind early phase rickettsial motility (Reed et al. 2014). Thus, all preceding publications may have not explored the protein within these contexts, leading to misattributions of function.

One aspect of Rickettsiology that has yet to be investigated is the role actin-based motility of *Rickettsia* plays in invasion, infection, and dissemination of the tick host. Much of the rickettsial life cycle is spent in the arthropod. Since the reductive genome profile of *Rickettsia* highlights that only necessary genes are maintained it is unusual that the bacterium maintains two genes to promote actin-based motility, whereas other bacteria dependent on this mode of intracellular spread appear to possess only one. Movement within host eukaryotic cells has been shown to be reliant on expression of both Sca2 and RickA (Figure 1.). Invasion of *Rickettsia* into embryonic cells derived from *D. variabilis* has been shown to depend on machinery involved in formation of actin filaments (Petchampai et al. 2014, Petchampai et al. 2015). This system shows that proper machinery required for actin-polymerization and movement in the host cell is present. The convergence of these proteins with the known machinery should also be important for dissemination in the arthropod host.

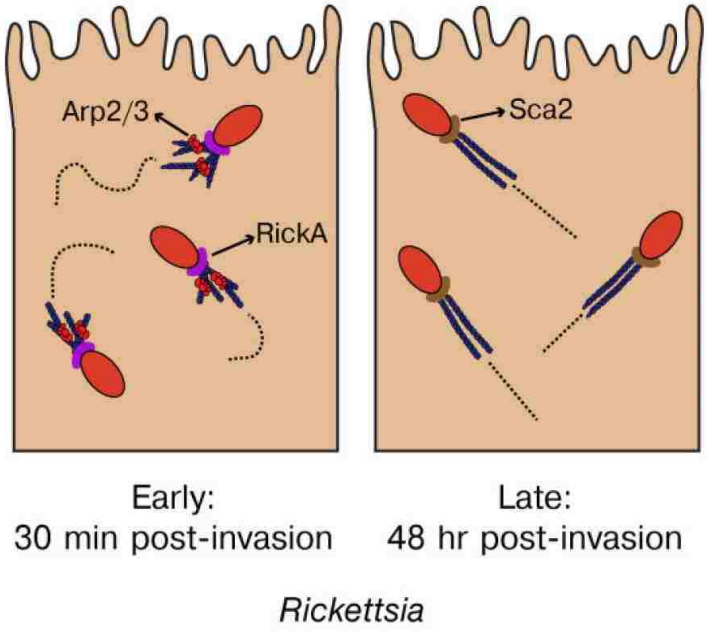

Figure 1.22. Schematic of the current model of proteins controlling rickettsial actin based motility (Truong et al. 2014).

1.5 Summary

Ticks are hematophagous arthropods of medical and veterinary importance. Their capacity to serve as vectors for agents of human and veterinary disease is second only to mosquitoes. This includes species within the SFG of *Rickettsia*. This collective group of gram-negative obligate intracellular bacteria, range from severe human pathogens to non-pathogenic endosymbionts. The highly pathogenic *R. rickettsii* is the prototypical SFG *Rickettsia* and the etiological agent of RMSF. Less pathogenic species such as *R. parkeri*, also contribute to human disease. Representative non-pathogenic species include *R. amblyommii* and *R. montanensis*. Specificity in tick host preference is a common across all SFG *Rickettsia*. This suggests that vector competence drives maintenance and transmission by select tick hosts. Mechanisms of vertical (transovarial and transtadial) and horizontal transmission are key aspects in defining competent tick/*Rickettsia* relationships. The distribution of multiple tick species present in the United States is rapidly expanding, highlighting the need to characterize transmission patterns among tick populations. Additionally, recognition of novel vectors for SFG *Rickettsia* have been identified through outbreaks of human rickettsiosis and field detection. Despite this, understanding of the tick/*Rickettsia* relationship is ill-defined. Defining the maintenance strategies of both pathogenic and non-pathogenic *Rickettsia* within tick populations is essential to enhanced epidemiology and diagnosis of rickettsial diseases.

Furthermore, current comprehension of rickettsial factors contributing to dissemination and persistence within the arthropod host remain uncharacterized. *Rickettsia* must overcome multiple barriers to infection in order to achieve vertical transmission. This includes infection and escape of tick midgut, ovaries, and salivary glands. Rickettsial movement from cell-to-cell is vital to forming multi-organ infection within the tick. Actin polymerization is the primary

method of rickettsial motility. The two rickettsial proteins implicated in this capacity include Sca2 and RickA. However, their functions have only been characterized in mammalian models of infection, where a loss of either protein results in reduced dissemination *in vitro*.

The overall hypothesis for the following studies was that if primary tick/*Rickettsia* relationships do not exist, then rickettsial determinants account for primary vector/pathogen relationship. To contribute to the understanding of TBRD and to test the above hypothesis, two objectives were designed to identify, 1) vertical transmission and fitness of ticks infected with *Rickettsia*, 2) significance of proteins mediating actin-based motility in arthropod models of infection *in vitro* and *in vivo*. The experiments in this dissertation were designed to examine the biology of rickettsial interactions. In Chapter 2, the ability of ticks (*D. variabilis* and *A. maculatum*) to serve as reservoirs for different SFG *Rickettsia* was examined. The specific hypothesis tested was that if tick/*Rickettsia* specificity exists, then this will facilitate sustained vertical transmission. The results suggest specificity did not occur, and no sustained vertical transmission occurred. Subsequent experiments in Chapter 3, were completed in order to elucidate specific rickettsial factors that may contribute to dissemination in the arthropod host. The specific hypothesis was that if that Sca2 and RickA are key components of intracellular motility in arthropod cells, they will contribute to the dissemination of *R. parkeri* within its tick host, *Amblyomma maculatum*. The results of these studies suggested that conserved mechanisms of actin polymerization *in vitro* were utilized in mammalian and arthropod cells. However, neither protein contributed to *in vivo* dissemination of *Rickettsia*, suggesting compensatory mechanisms or additional factors are primarily utilized in *A. maculatum*. Results of these studies will expand comprehension of the eco-epidemiology of *Rickettsia*, its persistence in tick populations, and its ability to cause human disease.

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CHAPTER 2
VERTICAL TRANSMISSION OF *RICKETTSIA* AFTER ARTIFICIAL FEEDING IN *DERMACENTOR VARIABILIS* AND *AMBLYOMMA MACULATUM*

2.1 Introduction

Ticks are hematophagous arthropods known for their ability to act as host, reservoir, and vector of a multitude of infectious and non-infectious agents, including those within the spotted fever group of *Rickettsia* (SFG). *Rickettsia* are gram-negative, obligate intracellular bacteria that vary in their ability to cause human disease. Many *Rickettsia* are maintained within tick populations by either a vertical (i.e. transovarial from female to offspring or transtadially from immature stage to proceeding life stage) or a horizontal (i.e. acquired during feeding) transmission route. However, sustained vertical transmission within tick populations is not successful for all rickettsial species. Models of vertical transmission in naturally and laboratory infected ticks demonstrate variability in stable maintenance (Burgdorfer and Brinton 1975, Niebylski et al. 1999, Macaluso et al. 2002). Rickettsial pathogenicity is one key factor to observed transmission patterns, as vertical maintenance favors non-pathogenic SFG *Rickettsia*, while distribution of horizontal transmission events are required for pathogenic maintenance (Macaluso and Paddock 2014).

Increased comprehension of competent tick/*Rickettsia* interactions is vital as the distribution of multiple tick species is expanding (Paddock and Goddard 2015, Dahlgren et al. 2016). Coinciding with the expansion of tick distribution, human cases of SFG rickettsioses continue to rise to unprecedented levels (Dumler 2010, Drexler et al. 2016). The sympatric distribution of among multiple tick and rickettsial species indicates that transmission routes and maintenance are highly complex. Indeed, field studies suggest that primary tick/*Rickettsia* relationships predominate. However, experimental evidence examining these interactions is

limited and it is important to anticipate tick transmission potential for *Rickettsia* in terms of vector competence to further understand the overall eco-epidemiology of SFG rickettsioses.

Implications of the encroachment of the Gulf Coast tick, *Amblyomma maculatum*, are multifactorial within the context of tick-borne rickettsial diseases (TBRD) (Teel et al. 2010, Paddock and Goddard 2015). This aggressive human-biting tick is recognized as the primary vector for *R. parkeri*, the agent of *R. parkeri* rickettsiosis which is detected in approximately 5-52% of *A. maculatum* (Hooker et al. 1912, Edwards et al. 2011a, Varela-Stokes et al. 2011, Nadolny et al. 2014, Paddock and Goddard 2015, Gleim et al. 2016). Contemporary field studies frequently detect alternative rickettsial species in field-collected ticks. This includes *R. amblyommii*, another emerging *Rickettsia*, detected in 5% of *A. maculatum* (Gleim et al. 2016). Also, *R. montanensis*, a non-pathogenic endosymbiont, detected in 14% of *A. maculatum* (Lee et al. 2014). However, whether *A. maculatum* is transmission competent for any of these additional *Rickettsia* remains unknown. Another tick species that also contributes to transmission of SFG *Rickettsia* within the United States is *Dermacentor variabilis*, the primary vector for the etiological agent of Rocky Mountain spotted fever, *R. rickettsii*. However, field surveys indicate that less than 1% of *D. variabilis* are infected with this *Rickettsia* (Stromdahl et al. 2011). As with *A. maculatum*, additional SFG *Rickettsia* are often detected in free-living *D. variabilis*. For example, *R. parkeri* and *R. amblyommii* have been detected in 28% and 1-50% of field-collected *D. variabilis* (Lee et al. 2014, Gleim et al. 2016). Lastly, *R. montanensis* is primarily associated with *D. variabilis*, however, incidence in ticks is relatively low (1.5-33%) (Lee et al. 2014, Nadolny et al. 2014). Laboratory models of vertical transmission suggest *R. montanensis* is maintained at relatively high levels in *D. variabilis* (Macaluso et al. 2001, Macaluso et al. 2002). Thus, although *D. variabilis* and *A. maculatum* are known to be infected with a variety of SFG

rickettsial species, the transmission biology for these additional species has not been thoroughly investigated.

The objective of the current study was to assess vertical transmission of *Rickettsia* by two sympatric tick species. The hypothesis of this study is that if emergent tick vectors are a major component of rising rickettsial cases then tick can transmit *Rickettsia* indiscriminately. To test this hypothesis, female *A. maculatum* and *D. variabilis* were infected with individual rickettsial species including *R. rickettsii*, *R. parkeri*, *R. montanensis*, *R. amblyommii*, and *R. felis*. *Rickettsia felis* was included as a negative control because this species is primarily flea-associated. The subsequent filial generation was monitored in order to determine infection within established cohorts. Additionally, we measured the fitness of each experimental cohort was measured in order to better understand how tick populations are affected by rickettsial exposure and infection. Host animal exposure to *Rickettsia* was also evaluated in order to determine the potential of infected cohorts to transmit bacteria to the host during feeding.

2.2 Materials and Methods

2.2.1 Rickettsial Strains and Culture

Vero E6 (African Green monkey kidney) cells were routinely cultured as previously described (Pornwiroon et al. 2009). The *Ixodes scapularis* (ISE6) embryonic tick cell line routinely cultured in supplemented L15B medium (Pornwiroon et al. 2006). All *Rickettsia*, excepting *R. montanensis* (M5/6) and *R. rickettsii* (Sheila Smith), were low passage (passage 5 or under). Isolates of *R. parkeri* (Portsmouth), *R. amblyommii* (Darkwater), and *R. montanensis* (M5/6) were routinely maintained in Vero cells; *R. felis* (LSU) was maintained in ISE6 cells. Rickettsiae were semi-purified and enumerated as previously described (Sunyakumthorn et al. 2008). Briefly, infected host cells were aspirated with a 27g needle, followed by low-speed

centrifugation at 275 x g for 10 minutes at 4°C to pellet unlysed host cells. The *Rickettsia*-containing supernatant was then passed through a 2μm filter (Whatman) to remove host cell debris. Total concentration of *Rickettsia* was quantified via Live/Dead BacLight Bacterial Viability kit (Invitrogen) on a Petroff Hausser counting chamber (Hausser Scientific Company) and viewed with a fluorescence microscope (Leica) (Kurtti et al. 2005).

2.2.2 Ticks and Capillary Feeding Technique

Rickettsia-free *D. variabilis* (originally provided by Dr. Daniel Sonenshine of Old Dominion University) were maintained in a controlled environmental chamber at 27°C, with 92% relative humidity, and a 12:12 hour (light: dark) cycle at Louisiana State University. Adult, female *Rickettsia*-free *A. maculatum* were obtained from BEI resources (https://www.beiresources.org/). Propagation of ticks was as previously described (Troughton and Levin 2007, Grasperge et al. 2014, Banajee et al. 2015). Experimental groups were created by designating 7 groups of 20 *D. variabilis* (10 females and 10 males) and 7 groups of 15 *A. maculatum* (10 females and 5 males). Groups of male and female ticks were encapsulated on individual Sprague Dawley (SD) rats and allowed to pre-feed for 3-4 days, after which females were forcibly removed with forceps. Females that had not attached were excluded from experimentation. Individual ticks were restrained dorsal side down to adherent tape secured to the bottom of a petri dish (Macaluso et al. 2001). Capillary tubes containing *Rickettsia* or media control were fitted over the palps of each tick and the open end of the capillary was embedded in modeling clay. Each of the 5 treatment groups and two media control groups were prepared at a final concentration of 5 X 10^7 *Rickettsia*/μl in appropriate host cell medium mixed 3:1 with 0.1% Rhodamine-B in 0.85% NaCl (w/v). Immobilized ticks were then placed in a humidified incubator and stored overnight (≈14 hours) at 37°C to allow feeding and acquisition of bacteria.

Subsequently, ticks were separated from the capillary tube, rinsed twice with 70% ethanol, and once in deionized water to remove residual medium. Each treatment group was observed under fluorescent light to assess Rhodamine-B labeling. Those not positively labeled, were excluded from further experimentation. Ticks positive for Rhodamine-B labeling were returned to their respective animal host and allowed to feed to repletion. Replete females were weighed to calculate engorgement weight after they had been cleaned, as described above. All vials containing engorged females and mated males in a controlled environmental chamber as listed above. After tick removal, skin from the site of tick attachment was collected for genomic DNA (gDNA) extraction.

2.2.3 Assessment of Transovarial and Transtadial Transmission

All ticks were analyzed for rickettsial infection via species-specific quantitative real-time PCR (qPCR) assays. At the beginning of oviposition 50 eggs were subsampled from each egg clutch (cohort) for analysis of transovarial transmission. Post-eclosion, 50 unfed larvae were subsampled from each cohort to test for transtadial transmission. Larval cohorts that had tested positive as eggs were allowed to feed on naïve Balb/c mice. Engorged larvae were collected and stored until molting was complete. A portion of the resulting unfed nymphs were then assessed for transtadial transmission. Nymphal cohorts that tested positive were then fed on Balb/c mice or SD rats and allowed to molt to the adult life stage. Adults were then assessed for infection.

2.2.4 Fitness Metrics

Fitness associated with rickettsial exposure was determined by calculating the nutrient index (NI or real conversion index) as well as the egg production index (EPI or apparent conversion index) (Bennett 1974). The NI (equation 1) is a measurement of efficiency in bloodmeal conversion to egg mass. The EPI (equation 2) is measured to determine the efficiency

with which ticks oviposit egg mass. One sample of 100 eggs was also taken from each egg clutch for the calculation of egg hatch percentage. Total mass from all eggs sampled was taken and added back to the total mass of the egg clutch for assessment.

Equations:

1) $Nutrient\ index = \frac{Weight\ of\ eggs}{Initial\ weight\ of\ engorged\ tick - residual\ weight\ of\ tick} \times 100$

2) $Egg\ producion\ index = \frac{Weight\ of\ eggs}{Initial\ weight\ of\ engorged\ tick} \times 100$

3) $\%\ Egg\ hatch = \frac{Number\ of\ viable\ life\ stage}{Total\ number\ of\ life\ stage\ prior\ to\ eclosion/molt} \times 100$

2.2.5 DNA Extraction and qPCR

Genomic DNA was extracted from eggs, unfed larvae, unfed nymphs, and unfed adults. Eggs and unfed larvae were pooled into groups of 50. Five groups of 10 nymphs were pooled for gDNA extraction. Adults were cut in half with a sterile scalpel blade; half was subjected to gDNA extraction, while the other half was stored individually at -20°C. All extractions were carried out using the Zymo Quick g-DNA Miniprep kit (Zymo, Irvine, CA). Briefly 2-sterile, glass 3mm beads were added to an Eppendorf Safe-Lock microcentrifuge tube containing sample. Next, 50µl of DNase/RNase-free H_20 was added. All samples were placed in a TissueLyser (Qiagen) for 2 cycles of 30 Hz for 3 minutes. After lysis, gDNA was extracted according to the manufacturer's protocol for Cell Suspensions and eluted in 40µl of DNase/RNase-free water. Isolation of gDNA from rat skin samples was performed using a Qiagen DNeasy protocol for the Purification of Total DNA from Animal Tissues Samples were eluted in 100µl DNase/RNase-free water (Grasperge et al. 2012). All samples were analyzed on a Roche LightCycler 480II instrument (Roche) using the primers listed in (Table 2 1). Reactions included of 8µl of 5X 1536 Probes Master (Roche), 0.8µl of 10µM primers, 1.2µl of 10µM

probe, 4.2µl of DNase/RNase-free water, and 25µl of template to give a total reaction volume of 45µl. Template consisted of unknown sample, internal standard, or a water (negative) control. Samples were loaded onto a 96-plate which was then aliquoted into 7 replicates on a 384-well plate. Cycling parameters are as listed in Thepparit et al with the modification of the pre-incubation step to 95°C for 1 minute (Thepparit et al. 2011). Amplicons for gene-specific primers were incorporated into pCR4 TOPO and serially diluted in order to serve as internal standards for each rickettsial assay and were included along with experimental samples in each set of reactions.

2.2.6 ELISA

Indirect ELISA to detect anti-rickettsial IgM or IgG was performed as previously reported with minor modifications (Banajee et al. 2015). Briefly, *R. parkeri* was sucrose purified, followed by sonication, and protein quantification via DC assay (BioRad) (Ammerman et al. 2008). Maxisorp plates (Nunc) were coated overnight with 0.5 ng/µl *R. parkeri* lysate in carbonate bicarbonate coating buffer (Sigma). Sera or plasma from host animals were diluted 1:32 and 1:64 in blocking buffer (5% skim milk/0.1% Tween-20 in PBS), and added to either a well coated with *R. parkeri* antigen or a non-antigen well coated with only carbonate bicarbonate buffer. Anti-rat IgG and IgM or anti-mouse IgG and IgM conjugated to horse radish peroxidase diluted in blocking buffer were used as secondary antibodies. Positive

1 Table 2. 1**.** Primers and Probes utilized in qPCR analysis.

Primer Set (5'-3') **Probe (5'-3')**	**Partial Gene Amplified**	**Citation**
ompBRm2832F-GCGGTGGTGTTCCTAATAC ompBRm2937R-CCTAAGTTGTTATAGTCTGTAGTG RmompB-HEX/CGGGGCAAAGATGCTAGCGCTTCACAGTTACCC CG/IABkFQ	*R. montanensis ompB*	(Petchampai et al. 2014)
RamompBEHF-CCGTTAACACCATTAACTATTAAAGCA RamompBEHR-GTGCTGCGGCTTCTACATTA RamompB-FAM/AGAGGCGCCTTTTGAGTTGTAGGATTTGC/BHQ_1	*R. amblyommii ompB*	This paper
RpompB129FJJ-CAAATGTTGCAGTTCCTCTAAATG RpompB224RJJ-AAAACAAACCGTTAAAACTACCG RparompB-FAM/TTTG+A+G+C+A+G+CA/IABkFQ	*R. parkeri ompB*	(Banajee et al. 2015)
Rf17KD135F-ATGAATAAACAAGGKACNGGHACAC Rf17KD249R-AAGTAATGCRCCTACACCTACTC R17Kbprobe- FAM/CGCGACCCGAATTGAGAACCAAGTAATGCGTCGCG/BHQ	*Rickettsia* 17kDa	(Jiang et al. 2004)
ompBRr1370F-ATAACCCAAGACTCAAACTTTGGTA ompBRr1494R-GCAGTGTTACCGGGATTGCT RrompB-FAM/CGCGATCTTAAAGTTCCTAATGCTATAACCCTT ACCGATCGCG/IABkFQ	*R. rickettsii ompB*	(Jiang et al. 2005)
AmacMIF.18F-CCAGGGCCTTCTCGATGT AMACmif.99R-CCATGCGCAATTGCAAACC AmacMIF.63-Hex-TGTTCTCCTTTGGACTCAGGCAGC/BHQ	*A. maculatum mif*	This paper
CRTDv321F-AGGAGAAAAGCAAGGGACTG CRTDv452R-CAATGTTCTGCTCGTGCTTG DvCRT_TYE665-TYE665/TGGAGAAGGGCTCGAACTTGGC/IAbRQsp	*D. variabilis* citrate synthase	(Petchampai et al. 2014)
+ denotes the use of a locked nucleic acid (LNA).		

and negative control serum was added to each plate along with experimental samples. Serum was considered positive for exposure if mean OD_{414} values were greater than two times the standard deviation for all samples.

2.2.7 Animal Statement

All experimental animals were obtained from the LSU Division of Laboratory and Animal Medicine (DLAM) and monitored in accordance with LSU IACUC 13-034.

2.2.8 Statistical Analysis

Data were analyzed in SAS version using the GLM procedure. A Levene's test of Homogeneity was performed for all data. Engorgement weigh was analyzed using a one-way ANOVA and a Dunnett's post hoc test. Egg production index and nutrient index data were analyzed using a Kruskall Wallis test. A p-value of ≤ 0.05 was considered significant.

2.3 Results

2.3.1 Effect Of *Rickettsia* Infection On Tick Engorgement Weight

To determine the influence of rickettsial exposure on tick fitness, we assessed engorgement weight of *D.* variabilis and *A. maculatum* post-exposure to *R. rickettsii*, *R. parkeri*, *R. amblyommii*, *R. montanensis*, or *R. felis*. Previous publications have shown that the engorgement weight of females can be negatively impacted by rickettsial infection (Burgdorfer and Brinton 1975, Niebylski et al. 1999, Schumacher et al. 2016). As shown in Table 2, engorgement weight of replete females was compared to *Rickettsia*-free *D. variabilis* and *A. maculatum*, exposed to vehicle control consisting of cell growth media. All *Rickettsia*-exposed *D. variabilis* resulted in a reduction in engorgement weight (6.4- 23.3%); however, none were significantly reduced in comparison to media control cohorts (Table 2.2).

Alternatively, *A. maculatum* exposed to *R. parkeri*, *R. amblyommii*, *R. montanensis*, or *R. felis* had significantly lower engorgement weights compared to media control-exposed ticks (Table 2.2). Engorgement weight for *R. montanensis* and *R. parkeri*-exposed groups was approximately 37.1% less than that of media control-exposed *A. maculatum* (Table 2.2). A reduction of over 29.6% was observed in *A. maculatum* exposed to *R. amblyommii* and *R. felis* (Table 2.2). Exposure to *R. rickettsii* did not have a significant impact on the engorgement weight of *A. maculatum*. Though there was a decrease in engorgement weight, it was surprising considering that this was not significant for two reasons. Firstly, all other *Rickettsia* exposure led to a significant decrease. Secondly, *R. rickettsii* strain Sheila Smith is highly pathogenic. Highly virulent *Rickettsia* have been shown to significantly reduce engorgement weight of ticks in previous studies (Schumacher et al. 2016). Overall, these results demonstrate introduction of *Rickettsia* to *D. variabilis* has no effect on engorgement weight. However, *A. maculatum* engorgement weight is severely impacted by *Rickettsia*.

2.3.2 Rickettsial Exposure Significantly Affects Bloodmeal Conversion in *A. maculatum* exposed to *R. amblyommii*, *R. montanensis*, and *R. parkeri*

Efficient bloodmeal conversion in the engorged female is vital for the production of viable progeny. To measure this conversion post-rickettsial exposure, we utilized the nutrient index (NI). This metric is designed to determine how well the engorged female is able to utilize a bloodmeal and convert it to egg mass. All *Rickettsia*-exposed *D. variabilis* had statistically comparable NI (Table 2.2). Conversely, *A. maculatum* exposed to *R. amblyommii*, *R. montanensis*, or *R. parkeri* had a significant reduction in nutrient index, which was on average 23.6% lower than control ticks (Table 2.2). Nutrient index values for *R. rickettsii* and *R. felis* on average displayed a decrease of 15.5% compared to media control ticks (Table 2.2). Consistent

with engorgement weight, *A. maculatum* biology was impacted significantly by rickettsial exposure as compared to *D. variabilis*.

2.3.3 Egg Production Index Is Negatively Impacted In Both Tick Species Post-Rickettsial Exposure

Although the NI is a powerful metric in its ability to calculate bloodmeal conversion for the production of eggs, it does not account for the ability of the tick to oviposit eggs. It is necessary for the engorged female to deposit a large egg mass to promote the continuation of the overall population. If this is not carried out efficiently due to infection, a negative impact will be seen on the tick population. Thus, to further quantify the effects of *Rickettsia* on exposed ticks, we next looked at the egg production index (EPI) of engorged females was calculated. The EPI of *D. variabilis* exposed to *R. montanensis* showed a statistically significant reduction of 11% as compared to the control group (Table 2.2). In addition, ticks exposed to *R. amblyommii, R. felis, R. parkeri,* and *R. rickettsii* produced EPI values that were, on average, 6% lower than that of the media control group, however, were not statistically significant (Table 2.2). Alternatively, exposure of *A. maculatum* to *R. amblyommii, R. parkeri*, and *R. montanensis* conferred a significant reduction in oviposited egg mass. This was consistent with a reduction in nutrient index for all groups. Additionally, *R. felis* and *R. rickettsii*-exposed *A. maculatum* produced 4.1% smaller egg masses, but not significantly in comparison to media control ticks (Table 2.2). Altogether, the egg output of *R. montanensis*-exposed *D. variabilis* was significantly reduced, suggesting a negative impact on the overall fitness of the population. Additionally, *A. maculatum*-exposed to *R. amblyommii, R. montanensis,* and *R. parkeri* were unable to produce the same amount of eggs as those exposed to media alone. This indicates fitness is severely

impacted in relation to engorgement weight, nutrient conversion, and egg output, affecting the total number of viable, infected offspring capable of being produced.

2.3.4 Exposure to *Rickettsia* in *D. variabilis* and *A. maculatum* Produce Viable Larvae and Nymphs at Levels Comparable to Control Groups

It is biologically imperative for ticks to not only generate large numbers of eggs, but also for a large portion of those to produce viable larvae. Negative fitness effects caused by rickettsial infection can greatly impact the fate of progeny. In fact, it is has been demonstrated that pathogenic rickettsial infections manifest in a decrease in viable offspring over multiple filial generations (Burgdorfer and Brinton 1975, Niebylski et al. 1999, Schumacher et al. 2016). Thus, percent hatch rates for all exposed cohorts of ticks was determined. The percent of resulting viable unfed larvae was calculated for all experimental groups which successfully oviposited (Table 2.3). Overall, no exposed group for either *D. variabilis* or *A. maculatum* showed a significant decrease in viable larvae. Furthermore, there was no correlation between infection and a change in viability through the F_1 adult stage. Most cohorts produced approximately 90-100% molt success, with the exception of cohorts exposed to *R. parkeri* (Table 2.3). Statistical significance could not be assigned to emergent nymphal and adult cohorts due to smaller numbers of positive cohorts.

2.3.5 Exposure To *Rickettsia* Leads To Successful Transovarial Transmission and Variable Transstadial Transmission

Subsequent to rickettsial exposure, the vertical transmission of each rickettsial species in *D. variabilis* and *A. maculatum* was characterized. Engorged females were allowed to oviposit; a subsample of eggs was then removed from the egg mass, followed by gDNA extraction.

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Table 2. 2. Engorgement weight, nutrient index, and egg production index values for *D. variabilis* and *A. maculatum* exposed to a panel of either rickettsial species or media control. * Denotes significance of $p \leq 0.05$. Values in brackets represent the SEM values for each group.

	D. variabilis			*A. maculatum*		
Rickettsia	Engorgement Weight (mg)	Nutrient Index (NI)	Egg Production Index (EPI)	Engorgement Weight (mg)	Nutrient Index (NI)	Egg Production Index (EPI)
R. amblyommii	497.6 [±52.0]	28.0 [±1.12]	55.2 [±2.6]	744.6* [±59.6]	31.7* [±4.0]	43.5* [±3.2]
R. felis	509.4 [±42.9]	26.7 [±2.8]	55.6 [±1.7]	716.4* [±37.0]	34.9 [±2.4]	50.6 [±1.7]
R. montanensis	543.0 [±14.3]	27.7 [±1.1]	48.1* [±3.0]	652.4* [±92.4]	24.4* [± 5.8]	34.0* [±4.9]
R. parkeri	505.7 [±38.6]	26.4 [±3.0]	53.6 [±4.4]	651.7* [±57.7]	28.1* [±4.4]	43.1* [±3.0]
R. rickettsii	445.0 [±60.7]	25.3 [±4.6]	49.7 [±4.8]	739.8 [±59.8]	37.3 [±4.4]	52.0 [±1.7]
Media Control	580.8 [±24.4]	33.2 [±1.4]	59.5 [±0.7]	1037.0 [±84.9]	51.6 [±7.1]	55.4 [±2.1]

Table 2. 3. Percentages for hatching and molting of *D. variabilis* and *A. maculatum* exposed to either *Rickettsia* or media control.

	D. variabilis			*A. maculatum*		
Rickettsia	% Hatch	% Molt-nymph	% Molt-adult	% Hatch	% Molt-nymph	% Molt-adult
R. amblyommii	76	97	100	81	94	100
R. felis	59	100	ND	92	ND	ND
R. montanensis	62	98	ND	77	ND	ND
R. parkeri	73	96	81	69	88	100
R. rickettsii	69	ND	ND	87	ND	ND
Media Control	74	86	98	90	95	100

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.

Next, qPCR was utilized to determine rickettsial burden via species-specific assays (Table 2.4 and 2.5). For media control-exposed ticks, a previously published assay allowing for pan-*Rickettsia* detection (Jiang et al. 2004). Cohorts that produced eggs negative for *Rickettsia* were discarded from further analysis. Eggs that were determined to be infected with *Rickettsia* were subsampled at the F_1 larval stage. Here both positive and negative cohorts were allowed to molt to the nymphal stage. Unfed nymphs were sampled and only those that were positive for infection were included in the analysis of subsequent F_1 adults.

Of the eight females exposed to *R. amblyommii* only two females (25%) produced eggs with detectable infection. Neither of these cohorts produced larvae with detectable infection. However, 50% (1/2) of the cohorts was positive for infection at the nymphal stage which was again not detected in the subsequent adult stage. Out of six females exposed to *R. felis* only one (17%) produced eggs with detectable infection. *Rickettsia montanensis* exposed *D. variabilis* produced infected eggs from 14% (1/9) of initially exposed females. This cohort did not produce detectable levels of infection at either the larval or the nymphal stage. Lastly, of the six *D. variabilis* exposed to *R. parkeri* five females (83%) produced infected eggs. Subsequently *R.*

parkeri was only detected in 20% (1/5) cohorts at the larval and nymphal stage, and was not detectable in adults.

Although *D. variabilis* females were susceptible to most (4/5) rickettsial species used in this study, opposing results were noted for exposed *A. maculatum*. Only females exposed to *R. amblyommii* and *R. parkeri* produced detectable infection in the tested eggs (Table 2.5). All other females produced eggs negative for *Rickettsia*. Of the seven females exposed to *R. amblyommii* only two (29%) produced infected eggs. No infection was detected at the larval stage. However, *R. amblyommii* was detected in 50% (1/2) cohorts at the nymphal stage. No infection in this singular cohort was detected at the adult stage. Of the eight females exposed to *R. parkeri* only three (38%) produced infected eggs. One out of 3 (33%) cohorts produced positive results at the larval stage and 67% (2/3) of the cohorts were positive at the nymphal stage. Infected nymphs from the two remaining cohorts did not test positive after maturation to the adult stage.

Table 2. 4. Vertical transmission of *Rickettsia* in *D. variabilis* through one generation.

	D. variabilis			
	Transovarial transmission (TOT)	Transstadial transmission (TST)		
Rickettsia	Egg n=50	Larva n=50	Nymph n=50	Adult n=29-40
R. amblyommii	25% (2/8)	0% (0/2)	50% (1/2)	0% (0/1)
R. felis	17% (1/6)	(0/1)	(0/1)	ND
R. montanensis	14% (1/9)	0% (0/1)	0%(0/1)	ND
R. parkeri	83% (5/6)	20% (1/5)	20% (1/5)	0% (0/1)
R. rickettsii	0% (0/8)	ND	ND	ND
Media Control	0% (0/10	0% (0/10)	0%(0/2)	0% (0/2)

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.

Table 2. 5**.** Vertical transmission of *Rickettsia* in *A. maculatum* through one generation.

	A. maculatum			
	Transovarial transmission (TOT)	Transtadial transmission (TST)		
Rickettsia	Egg n=50	Larva n=50	Nymph n=50	Adult n=29-40
R. amblyommii	29% (2/7)	0% (0/2)	50% (1/2)	0% (0/1)
R. felis	0% (0/7)	ND	ND	ND
R. montanensis	0% (0/9)	ND	ND	ND
R. parkeri	38% (3/8)	33% (1/3)	67% (2/3)	0% (0/2)
R. rickettsii	0% (0/5)	ND	ND	ND
Media Control	0% (0/8)	0% (0/8)	0%(0/2)	0% (0/2)

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.

2.3.6 Mammalian Hosts Parasitized by *R. parkeri*- and *R. amblyommii*-infected Cohorts Leads to Detectable Host IgM Responses

In order to further characterize the transmission patterns of *Rickettsia* exposed ticks we assayed for antibody responses in the host animals used to perpetuate the positive cohorts of the F_1 generation (Table 2.6). Transmission to the mammalian host is an important marker for vector competence (Reisen 2009). All rats on which initially exposed adult, females were fed had negative titers for exposure against *Rickettsia* IgM. Larval cohorts infesting mice only produced detectable titers of IgM at 1:64 in serum collected from two *D. variabilis* cohorts exposed to *R. amblyommii* and one cohort exposed to *R. parkeri*. All other serum collected from host animals of positive cohorts tested negative at 1:64 for IgM. No detectable titers for IgG were noted for any animals tested in this portion of the experimentation.

Table 2. 6. Vertebrate host exposure to *Rickettsia* post tick feeding. Sera collected post tick feeding was assayed for anti-*Rickettsia* IgM and IgG**.** Results are displayed as IgM/IgG.

	D. variabilis			*A. maculatum*		
Rickettsia	F_0 adults	F_1 larvae	F_1 nymphs	F_0 adults	F_1 larvae	F_1 nymphs
R. amblyommii	-/-	+ /-	-/-	-/-	-/-	-/-
R. felis	-/-	-/-	ND	-/-	ND	ND
R. montanensis	-/-	-/-	ND	-/-	ND	ND
R. parkeri	-/-	+/-	-/-	-/-	-/-	-/-
R. rickettsii	-/-	ND	ND	-/-	ND	ND
Media Control	-/-	-/-	-/-	-/-	-/-	-/-

ND (not determined) denotes a set not included in evaluation based on negative qPCR result at the preceding life stage.

2.4 Discussion

Acquisition of rickettsial infection by ticks occurs through horizontal routes via feeding on infected hosts, or by vertical transmission in which rickettsiae are maintained through transtadial and transovarial transmission. The incidence of *Rickettsia*-infected ticks suggests that competent vector/pathogen relationships predominate, despite geographically overlapping populations of ticks with multiple identified rickettsial species, non- and pathogenic alike. The susceptibility of any given tick species for acquisition of a novel infection is not well-defined. Some rickettsial species are efficiently vertically maintained over multiple filial generations. This is often associated with selective pressure resulting in maintenance of less virulent species (Fine 1975, Yamamura 1993, Mather and Ginsberg 1994, Werren 1997). Little to no decrease in fitness is observed for these populations resulting in sustained levels of infected ticks. Alternatively, pathogenic rickettsial species have been shown to decrease the fitness of their arthropod host, a selection method which favors horizontal routes of maintenance (Burgdorfer and Brinton 1975, Niebylski et al. 1999). In order to explore the ecology of *Rickettsia* within tick species which may serve as vector competent hosts, *A. maculatum* and *D. variabilis* were chosen.

When assessing fitness of exposed ticks, a variety of metrics including, engorgement weight, nutrient conversion, egg production, and offspring viability was measured. In comparison to media control-exposed ticks, *Rickettsia*-exposed *D. variabilis* had no significant decrease in engorgement weight or in nutrient conversion. A significant decrease in egg production was recorded for *D. variabilis* exposed to *R. montanensis*. The percent viability of subsequent larval *D. variabilis* was not significantly different than media control. Numbers of viable F_1 nymphs were slightly higher in *Rickettsia*-exposed cohorts. A decrease in viable F_1 adults was noted for *R. parkeri*-exposed ticks. Statistical significance could not be assigned to the viability of infected nymphs or adults due to low numbers of individuals within cohorts.

Comparatively, the engorgement weight of *R. amblyommii*-, *R. felis*-, *R. montanensis*-, and *R. parkeri*-exposed *A. maculatum* was significantly lower than media control-exposed ticks. Fitness was further impacted by a significant decrease in nutrient conversion and egg production for *R. amblyommii*-, *R. montanensis*-, and *R. parkeri*- exposed *A. maculatum*. The percent viability of F_1 larvae was decreased, except for *R. felis*-exposed cohorts, but not significantly. A decrease in viability was observed for F_1 *R. parkeri*-infected nymphs. Comparable viability was observed for F_1 *R. amblyommii*-exposed nymphs. The F_1 adult cohorts did not show a similar decrease in viability, however. Again, due to low numbers of individual cohorts no statistical significance could be assigned at nymphal and adult stages.

Transovarial infection was detected in *D. variabilis* exposed to *R. amblyommii*, *R. montanensis*, and *R. parkeri*. Continued vertical maintenance of rickettsial infection through F_1 larval and nymphal stages was detected in portions of cohorts that had been exposed to *R. amblyommii* and *R. parkeri*. However, infection was not detectable for either *Rickettsia* in F_1 adults. Cohorts of *A. maculatum* exposed to *R. amblyommii* and *R. parkeri* were positive for

transovarial and further transtadial transmission at both F_1 larval and nymphal stages. Here again, infection was not detectable for either *Rickettsia* in F_1 adults. Host animal exposure, as assessed by ELISA, was positive for IgM in animals parasitized by F_1 *D. variabilis* larvae infected with *R. amblyommii*.

Dermacentor variabilis inhabits much of the eastern portion of the United States from the Atlantic coast to the Midwest (Brown et al. 2005). It is the primary vector for *R. rickettsii*, a pathogen which has been previously demonstrated to have adverse effects on its alternate tick host, *D. andersoni* (Burgdorfer and Brinton 1975, Niebylski et al. 1999). In seminal work completed by Burgdorfer and Brinton, adult *D. andersoni* were infected with tick or human isolates either by feeding on rickettsemic hosts or via injection (Burgdorfer and Brinton 1975). Ticks infected for multiple generations demonstrated 100% filial infection rates, regardless of the introduction method (Burgdorfer and Brinton 1975). This contrasts our results as no observed transovarial transmission post-exposure in the present research. In a separate study larval and nymphal *D. andersoni* acquired infection via rickettsemic hosts (Niebylski et al. 1999). Multiple strains of *R. rickettsii* propagated with different methods were utilized (Niebylski et al. 1999). This resulted in 100% filial infection rates, except for two strains which notably had been passaged in Vero cells as compared to chicken embryo or Guinea pigs (Niebylski et al. 1999). The *R. rickettsii* isolate used in the current study was passaged in Vero cells and undetermined number of times. Perhaps, this contributed to the lack of transovarial transmission. Interestingly, ticks that acquired infection as naïve adults did not transmit infection to their offspring, much the same as was observed in our own study (Niebylski et al. 1999). For both of the abovementioned studies, transtadial maintenance was successful but the fitness of the tick was severely impacted by infection (Burgdorfer and Brinton 1975, Niebylski et al. 1999). Indeed, large portions of

infected cohorts failed to complete ecdysis or feed efficiently (Burgdorfer and Brinton 1975, Niebylski et al. 1999). More contemporary work has shown that *D. variabilis* exposure to *R. rickettsii* isolated from an opossum results in marked decreases in engorgement weight, bloodmeal conversion, and egg production (Schumacher et al. 2016). Larval and nymphal cohorts of *D. variabilis* acquired infection via inoculated mammalian hosts, producing post-molt infection rates of 64-100% (Schumacher et al. 2016). Approximately half of the resulting adult females failed to transmitted infection to F_2 larvae (Schumacher et al. 2016). No effects on longevity of infected ticks were observed (Schumacher et al. 2016). Overall, these data contrast current work in that no significant decrease in measured fitness metrics was recorded.

Owing to its expansive geographic range, a multitude of *Rickettsia* have been detected in free-living *D. variabilis.* This includes *R. montanensis,* which is associated with high rates of transovarial transmission, but is only detected in approximately 1.5-8% of collected *D. variabilis* (Macaluso et al. 2001, Macaluso et al. 2002, Ammerman et al. 2004, Pagac et al. 2014, Hudman and Sargentini 2016). This study shows that low amounts of transovarial transmission are possible but that continued transtadial transmission is not efficient. Transovarial transmission followed reduced engorgement weight and NI, though not significantly. Egg production was significantly impacted. This mirrors previous publications in which exposed *D. andersoni* failed to produce infected progeny despite visualization of *Rickettsia* in the ovaries (Niebylski et al. 1999). However, artificial feeding mechanisms have previously been able to produce stably infected cohorts of *R. montanensis*-infected *D. variabilis* (Macaluso et al. 2001, Macaluso et al. 2002). The results of this indicate that *D. variabilis* is able to transovarially and transtadially sustain *R. amblyommii* and *R. parkeri* infection are novel, and corroborate previously identified

associations from field collected ticks (Williamson et al. 2010, Fornadel et al. 2011, Leydet and Liang 2013, Henning et al. 2014, Lee et al. 2014, Gleim et al. 2016).

Transovarial transmission of *R. felis* in exposed *D. variabilis* was also detected. This transitional group of *Rickettsia* species (TRG) is frequently associated with intermittent feeding hematophagous arthropods, such as the cat flea, *Ctenocephalides felis* (Adams et al. 1990, Brown and Macaluso 2016). Though the cat flea is considered the primary vector for *R. felis*, it has been detected sporadically in ticks (Ishikura et al. 2003, Jiang et al. 2012, Abarca et al. 2013, Soares et al. 2015). Recent experimental transmission has also been shown in *Anopheles* mosquitoes (Dieme et al. 2015). This research shows that possible transmission of *R. felis* by ticks can likely contribute to the incidence of human infections. Also, this may be a route by which *R. felis* is maintained in the natural environment. Tick-borne transmission of *R. felis* has not been investigated but should be a topic of future research.

Populations of *A. maculatum* were originally restricted to the high humidity regions of the Gulf Coast regions of the United States. However, recent studies have documented the geography of this species encroaching into regions of the Northeast and Midwest, contributing to populations of newly susceptible arthropod hosts and the changing epidemiological landscape of rickettsial diseases (Paddock 2005, Paddock et al. 2015, Paddock and Goddard 2015). To date, *A. maculatum* has been implicated as the primary vector for *R. parkeri*, with incidence varying regionally. Infection percentages have been reported to range from 5-52% (Edwards et al. 2011b, Varela-Stokes et al. 2011, Nadolny et al. 2014, Gleim et al. 2016). The results of the current study demonstrate that transovarial transmission could be a viable method, albeit one that reduces fitness, of maintaining infected cohorts in nature. These results, however, are contrasted by a recent study analyzing three filial generations of naturally infected *R. parkeri-A. maculatum*

(Wright et al. 2015). Infected adults were shown to transovarially transmit *R. parkeri* to subsequent eggs at 66.7% for the F_1 generation and increasing to 100% by the F_3 generation (Wright et al. 2015). Furthermore, there was no appreciable decrease in fitness as noted in the present study. In our work the sustained transtadial transmission was shown through the unfed larval and unfed nymphal stages. Transtadial maintenance at immature stages highlights the role larval and nymphal ticks possess in transmission to mammalian hosts. The previous study may differ from the current for a number of reasons. For example, the strain of *R. parkeri* used in this work was a human isolate. Comparatively, the strain used by Wright et al. is an uncultivated strain of unknown pathogenicity. Sequencing analysis in Wright et al. (2015) showed that their *R. parkeri*-infected cohort was highly similar to that of the Portsmouth strain. Further, they showed that feeding ticks were able to transmit *Rickettsia* during the process of feeding, as they were able to detect gDNA in some skin samples tested. However, the animals showed no outward signs of infection and seroconversion was not assayed. Like other species of *Rickettsia* there could exist strain variation based on geographic origin. Also, the life stage or time ticks initially acquired infection is unknown. Maintenance within the tick over multiple generations could lead to attenuation of virulence (Burgdorfer and Brinton 1975, Mather and Ginsberg 1994, Niebylski et al. 1999).

Contemporary field studies have also detected additional SFG *Rickettsia* in adult *A. maculatum*. Our study also demonstrates transovarial and transtadial transmission of *R. amblyommii*, but not *R. rickettsii*, *R. montanensis*, or *R. felis*. Twenty-eight percent of 18 *A. maculatum* collected in a Georgia survey were positive for *R. amblyommii* (Gleim et al. 2016). The flea-borne *R. felis* has also been identified in pools of collected *A. maculatum* originating from Mississippi and Virginia (Jiang et al. 2012). Although *R. amblyommii* is routinely detected

in multiple species within the *Amblyomma* genera, including *A. maculatum*, our study is the first to assess maintenance of this association (Burgdorfer et al. 1981, Stromdahl et al. 2008, Zanettii et al. 2008, Trout et al. 2010, Saraiva et al. 2013, Medlin et al. 2015). In this study, 28.5% (2/7) exposed ticks produced detectable infection in eggs. Fitness was decreased for *R. amblyommii*-exposed *A. maculatum*, with exposed ticks producing engorged females of lower weight, decreased bloodmeal conversion, and overall egg output. This highlights the likelihood that this association is maintained naturally, but with negative health benefits for the tick. Furthermore, horizontal transmission may be required to maintain infected populations of ticks. Biologically, this is not a beneficial association.

Taken together, this data suggests that *D. variabilis* is more susceptible to infection by multiple *Rickettsia*. However, its competence as a host is narrow, which in this study includes only *R. parkeri* and *R. amblyommii*. This data perhaps sheds light on low numbers of naturally infected *D. variabilis* with *R. rickettsii* and *R. montanensis*, while associations with *R. parkeri* seem frequent (Williamson et al. 2010, Fornadel et al. 2011, Stromdahl et al. 2011, Leydet and Liang 2013, Henning et al. 2014). Our data suggest that *D. variabilis* can act as a competent vector of *R. parkeri* transmission by immature ticks. Also demonstrated in this study is that *D. variabilis* could be a competent vector for *R. amblyommii*. Alternatively, *A. maculatum* displays a more specific range that details its role as a competent vector for *R. parkeri* and *R. amblyommii*, which is accompanied by decrease in tick fitness. Within the confines of this study, the ability to maintain infection likely necessitates horizontal transmission between infected ticks or repeated exposures by infected animal hosts.

The ability to detect infection at immature stages but not adult stages can be interpreted in multiple ways. Firstly, this could highlight the importance of immature ticks in transmission as

opposed to feeding adults. Secondly, rickettsial exposure occurred at the adult stage. Larval or nymphal exposure may have resulted in more successful transtadial maintenance to adults and over multiple generations. Niebylski et al. reported a similar phenomenon post-exposure of *D. variabilis* adults to *R. rickettsii* (Niebylski et al. 1999). In fact, successful sustained infection only occurred when larval and nymphal populations were the initial exposed life stage. Lastly, because transmission was not 100% for all infected ticks and due to destructive sampling methods, detection of infection may have not selected at the time of analysis (Ricketts 1907, Burgdorfer and Brinton 1975). Underlying morbidity, causing death of infected ticks and survival of non-infected individuals could have also hampered our ability to detect infected ticks at any life stage.

Factors not explored in this study undoubtedly open many avenues for future work. The tipping point at which rickettsial exposure either results in infection or clearance in the tick is not well-defined. The rickettsial load required may vary based on species of *Rickettsia*. Thorough infection of the ovaries is a requirement, but how *Rickettsia* traverse multiple barriers within the tick to achieve this has yet to be completely characterized (Burgdorfer and Brinton 1975). Successful introduction of rickettsial infection may be life stage dependent, as has already been suggested by previous studies (Burgdorfer and Brinton 1975, Niebylski et al. 1999). This would imply that immature stages of ticks are more susceptible to infection. Downstream implications of this may include higher transtadial maintenance and the ability to transmit infection. Indeed, it is possible that immature life stages (i.e. larvae and adults) play a bigger role in rickettsial transmission than is currently conceived. Furthermore, the amount of *Rickettsia* required to establish tick infection is not well-defined and may vary by species. Here the method of infection was via capillary feeding. Although this is an established method, it differs from the route of

infection used in previous works. For example, ticks feeding on rickettsemic animals are likely exposed to *Rickettsia* over the entire course of feeding, as compared to capillary feeding which only lasts for 16 hours. Thus, the dose and time at which the tick receives *Rickettsia* that successfully establishes infection is inherently different. Undoubtedly, this could play a role in success with which ticks contract infection. Ultimately, further work is needed to analyze the infection dynamics displayed by these two tick species. This study broadens our understanding of the eco-epidemiology of *Rickettsia* in relation to two key vectors of disease.

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CHAPTER 3
THE ROLE OF SCA2 AND RICKA IN THE DISSEMINATION OF *RICKETTSIA PARKERI* IN *AMBLYOMMA MACULATUM*

3.1 Introduction

Rickettsia are gram-negative, obligate intracellular bacteria capable of causing an array of severe human diseases. Members of the spotted fever group (SFG) of *Rickettsia* include *R. parkeri*, a newly emergent human pathogen. Within the past decade alone there have been over 35 confirmed cases, not including patients misdiagnosed with classic Rocky Mountain spotted fever (RMSF) (Paddock and Goddard 2015). This, combined with the unprecedented rise in incidence of SFG rickettsiosis, necessitates a deeper understanding the biology of such pathogens as *R. parkeri* (Paddock et al. 2008, Paddock 2009, Drexler et al. 2016). Indeed, the rickettsial factors contributing to colonization, maintenance, and transmission within its primary vector and reservoir, *Amblyomma maculatum*, have not been identified.

It has recently been reported that loss of *R. parkeri* actin-based motility (ABM) impacts dissemination of bacteria from cell-to-cell during *in vitro* models of mammalian infection (Reed et al. 2014). A nucleation promoting factor (NPF) implicated in rickettsial motility is RickA, which activates the Arp2/3 complex to mediate actin-tail filamentation (Gouin et al. 2004, Reed et al. 2014). A second protein, surface cell antigen 2 (Sca2), has also been shown to act as a formin-like mediator of actin-based motility and contributes to mammalian cell adhesion (Haglund et al. 2010, Cardwell and Martinez 2012, Madasu et al. 2013). The current model of *R. parkeri* actin-based motility suggests that RickA coordinates early phase motility (15-30 minutes post-infection) giving rise to short actin tails and slow bacterial movement (Reed et al. 2014). However, late phase motility (24-48 hours post-infection) is mediated by Sca2, resulting in more elongated actin tails and increased rickettsial velocity within the cell (Reed et al. 2014). This has

been determined through transposon mutagenesis of *R. parkeri*, yielding two strains, one lacking full length expression of Sca2 and the other lacking RickA (Reed et al. 2014).

The goal of the current study was to assess the *in vitro* phenotypes of RickA and Sca2 deficient *R. parkeri* in an arthropod model of infection to determine if similar strategies of actin-based motility are utilized in the tick host. It is hypothesized that Sca2 and RickA are key components of intracellular motility in arthropod cells and will contribute to the dissemination of *R. parkeri* within its tick host, *Amblyomma maculatum*. To experimentally determine this, two previously published strains of *R. parkeri* were utilized, one lacking expression of Sca2 and the other RickA (Reed et al. 2014). Because *in vitro* studies are limited in their ability to mimic the actively feeding tick host, dissemination and infection dynamics of *R. parkeri* wild-type, *R. parkeri rickA::Tn*, and *R. parkeri sca2::Tn* in *A. maculatum* during bloodmeal acquisition were observed.

3.2 Methods and Materials

3.2.1 *Rickettsia*

Rickettsia parkeri (Portsmouth strain), *R. parkeri sca2::Tn*, and *R. parkeri rickA::Tn* were derived and propagated as previously published (Paddock et al. 2004, Pornwiroon et al. 2009, Reed et al. 2014). For *in vitro* and *in vivo* experimentation, low passage (passage 5 or below) *Rickettsia* were semi-purified as previously published (Sunyakumthorn et al. 2008). Briefly, infected cells were lysed using a 27 gauge needle, followed by centrifugation to separate *Rickettsia* from cell debris, and, finally, supernatant was passed through a 2μm syringe filter (Whatman). Rickettsial enumeration was performed using a BacLight Live/Dead viability kit (Molecular Probes) with a Petroff-Hausser counting chamber (Hauser Scientific Company) and viewed on a fluorescent microscope (Leica).

3.2.2 *In Vitro* Infection Assays

Vero and *Ixodes scapularis* endosymbiotic cells (ISE6) were cultured as previously described (Pornwiroon et al. 2006, Pornwiroon et al. 2009). For microscopic examination, Vero and ISE6 cells were seeded onto glass coverslips in 24-well plates at a density of $5x10^4$ and $2x10^5$ cells per well, respectively. Vero and ISE6 cells were also seeded into 96-well plates at a density of $1x10^3$ and $5x10^4$ cells per well. Multiwell plates were incubated at 32°C for 48 hours or 34°C for 24 hours prior to experimentation for ISE6 and Vero cells, respectively. Cells were infected at a multiplicity of infection (MOI) of 50. Contact between the bacteria and host cells was induced by centrifugatiion at 500 x g for 5 minutes at room temperature (RT). One hour post-inoculation (hpi) media was replaced to remove unbound bacteria. Vero cells were removed from 96-well plates by washing cells with PBS, incubating with trypsin-EDTA, adding fresh media, and dislodging the cells. ISE6 cells were removed by dislodging with PBS. Samples were centrifuged at 21000 x g for 10 minutes at 4°C, the supernatant removed, and pellets stored at -20°C until processed for genomic DNA (gDNA) extraction.

3.2.3 *Amblyomma maculatum*

Rickettsia-free *A. maculatum* were either propagated at the Louisiana State University School of Veterinary Medicine (LSU-SVM) using methods previously described, or purchased from BEI resources (https://www.beiresources.org/)(Troughton and Levin 2007, Grasperge et al. 2014, Banajee et al. 2015). All ticks were stored in a controlled environmental chamber at 27°C with 92% relative humidity and a 12:12 hour (light:dark) cycle prior to experimentation.

3.2.4 *In vivo A. maculatum* Dissemination Assays Via Capillary Feeding Technique

For dissemination assays, 20 female and 6-10 male *A. maculatum* were pre-fed on host animals for 3-4 days within a containment apparatus. Subsequently, female ticks were forcibly

removed with forceps, and restrained in a petri dish dorsum side down. Capillary tubes (25µl) (Kimble Chases Life Science and Research Products) containing 2µl of Rhodamine B and *Rickettsia*, at a concentration of $5x10^{10}$ *Rickettsia*/ml, were fitted over the hypostome. Ticks were placed in a humidified incubator set at 37°C overnight (≈14 hours) to allow for imbibement of infectious dose. Post-incubation ticks were removed from petri dishes, and serially washed in 70% ethanol and deionized water to remove non-imbibed media from the tick surface. Rhodamine B positivity was determined by microscopic examination using a fluorescent dissecting microscope. Non-fluorescently labeled *A. maculatum* were excluded from further study. Positively labeled ticks were separated into three groups: 1) 14 hours post-exposure (hpe) 2) 3 days post-exposure (dpe), and 3) 7 (dpe). Group 1 was dissected immediately after capillary feeding. Groups 2 and 3 were returned to host and allowed to feed for the allotted number of days, at which point they were removed, dissected, and processed for gDNA as previously described (Sunyakumthorn et al. 2012). A portion of the midgut, salivary glands, ovaries, and hemolymph was removed for immunofluorescence assay. For all tick feeding assays, adult *A. maculatum* were fed on Hartley Guinea pigs (Charles River Laboratories) in accordance with the LSU-SVM Institutional Care and Use Committee (IACUC) under the approved protocol number 15-115.

3.2.5 gDNA Extractions and Quantitative Real-Time PCR (qPCR)

All gDNA was extracted via Qiagen DNeasy Blood and Tissue kit (Qiagen) with some modification. Briefly, tick tissues were snap frozen in liquid nitrogen and ground with a pestle. Buffer ATL and Proteinase K were added, followed by incubation overnight at 56°C. Further steps were completed according to the manufacturer's protocol and eluted into 35µl of RNase/DNase-free water. Samples were analyzed on a Roche LightCycler 480II (Roche) using

gene-specific primers and probes (Table 3.1). Amplicons for each primer set were incorporated into pCR4 TOPO, and the resultant plasmids were serially diluted to serve as internal standards for each rickettsial assay. Standards were included along with unknown samples in each set of reactions.

Table 3.1. List of primers and probes used for qPCR.

Primer Set (5'-3') **Probe (5'-3')**	**Partial Gene Amplified**	**Citation**
RpompB129FJJ-CAAATGTTGCAGTTCCTCTAAATG RpompB224RJJ-AAAACAAACCGTTAAAACTACCG RparompB-FAM/TTTG+A+G+C+A+G+CA/IABkFQ	*R. parkeri ompB*	(Banajee et al. 2015)
AmacMIF.18F- CCAGGGCCTTCTCGATGT AmacMIF.99R- CCATGCGCAATTGCAAACC AmacMIF.63- Hex-TGTTCTCCTTTGGACTCAGGCAGC	*A. maculatum mif*	This paper
Vero b-actin.61F-TGAAGTGTGACGTGGACATCCATA Vero b-actin.170 R-GGCAGTAATCTCCTTCTGCATCCT Vero b-actin.116-TGGCACCACCATGTACCCTGGCATTGCT	Vero *β-actin*	(Riley et al. 2016)
ISE6_calFW-AGCAGGGAACTTTCAAGCTG ISE6_calREV-AGAAAGGCTCGAACTTGGTG ISE6 cal.67 -HEX/AGACCTCTGAAGATGCCCGCTTT	Calreticulin	This paper

+ denotes the use of a locked nucleic acid (LNA).

3.2.6 Immunofluorescence Staining

All incubations were completed at room temperature in a humidified chamber. *In vitro* and tick samples were washed once in PBS and fixed with 4% paraformaldehyde, (PFA), 4% sucrose in PBS for 15 minutes. Samples were permeabilized with 0.1% Triton X-100 diluted in PBS for 15 minutes and then blocked with 3% BSA for 1 hour. Antibodies were diluted in 1% bovine serum albumin (BSA) (Sigma Aldrich) in PBS. For detection of *Rickettsia*, RcPFA was utilized followed by goat anti-rabbit Alexa Fluor 488 (Molecular Probes) (Chan et al. 2011). Samples were washed 3 times with PBS to remove unbound antibody. 4',-diamindino-2-phenylindole, dihydrochoride (DAPI) was utilized for nuclear staining. Cytoskeletal structure of host cells was visualized using Phalloidin-X-AlexaFluor568 for *in vitro* samples or Phalloidin-X-

AlexaFluor647 for tick tissues. No secondary controls were stained along with experimental samples. All samples were mounted with MOWIOL, sealed, and stored at 4°C until imaged.

3.2.7 Imaging and Analysis

A Leica TCS SPS confocal microscope (Leica) was utilized for analysis of actin polymerization and *Rickettsia* infection *in vivo* and *in vitro*. Ten random images were taken for each treatment group for *in vitro* analysis. Images were adjusted for brightness, contrast, smoothness, and analyzed in Fiji software (Schindelin et al. 2012).

3.2.8 Statistical Analysis

Statistical analysis consisted of a one-way ANOVA with a Tukey's post-hoc test as administered in GraphPad Prism software. A p-value of <0.05 was considered significant in all studies. Data were expressed as means ± standard error of the mean (SEM).

3.3 Results

3.3.1 Actin polymerization of *Rickettsia parkeri* in Mammalian Cells is Comparable to Arthropod Cells

To define the temporal a pattern of *R. parkeri* motility, ISE6 cells were infected across a range of times. Tandem experiments in Vero cells were completed to act as a positive control for previously established actin polymerization patterns (Reed et al. 2014). Fluorescent microscopy was utilized to visualize actin polymerization by staining rickettsiae and F-actin. Bacteria that were actively polymerizing actin were then quantified against non-polymerizing. Less than 5% of *R. parkeri* were observed to be polymerizing actin after 30 minutes of infection in ISE6 cells Figure 3.1 and Figure 3.2). This matched observations made in Vero cells, and those previously published (Reed et al. 2014). Early phase polymerization is marked by lower amounts of motile bacteria, coinciding with initial invasion into host cells. Motility of *R. parkeri* ISE6 and Vero

cells decreased to its lowest level at 2 hours post-infection (hpi) Figure 3.2). This is consistent with previous observations which hypothesized this juncture in infection marked the beginning of the bacterial division period (Reed et al. 2014). Additionally, maximum polymerization was observed at late time points post-infection (24-48 hpi). These results show that *R. parkeri* actin polymerization in tick cells in a manner similar to that of mammalian cells (Figure 3.2). Thus, this mechanism of intracellular motility is likely to occur during arthropod infection.

3.3.2 *rickA* Deficient *R. parkeri* Infection of Arthropod Cells Results in a Lack of Early Phase Actin Motility

Next, to characterize the contribution of RickA to the intracellular motility of *R. parkeri* in the arthropod system, a strain of *R. parkeri* deficient in expression of RickA (*R. parkeri rickA::tn*) was utilized (Reed et al. 2014). ISE6 and Vero cells were infected simultaneously, followed by collection of infected cells at 30 mpi and 48 hpi. Previous analyses in mammalian cells suggested that early (15-30 mpi), but not late phase ABM (24-48 hpi) is driven by RickA (Reed et al. 2014). As such, time points were chosen that would represent these two phases of ABM. cells. Utilizing fluorescent microscopy to visualize infected cells, it was determined that *R. parkeri rickA::tn* lacks actin polymerization actin at 30 mpi in both, ISE6 and Vero cells (Figure 3. 3). However, ABM was restored at 48 hpi, demonstrating that like mammalian *in vitro* infection, early, but not late, phase rickettsial motility in arthropod cells is coordinated by RickA (Figure 3. 3).Furthermore, replication of *R. parkeri::rickATn* is similar to that of *R. parkeri* wild-type and *sca2::Tn* strains in Vero and ISE6 cells (Figure 3.4).

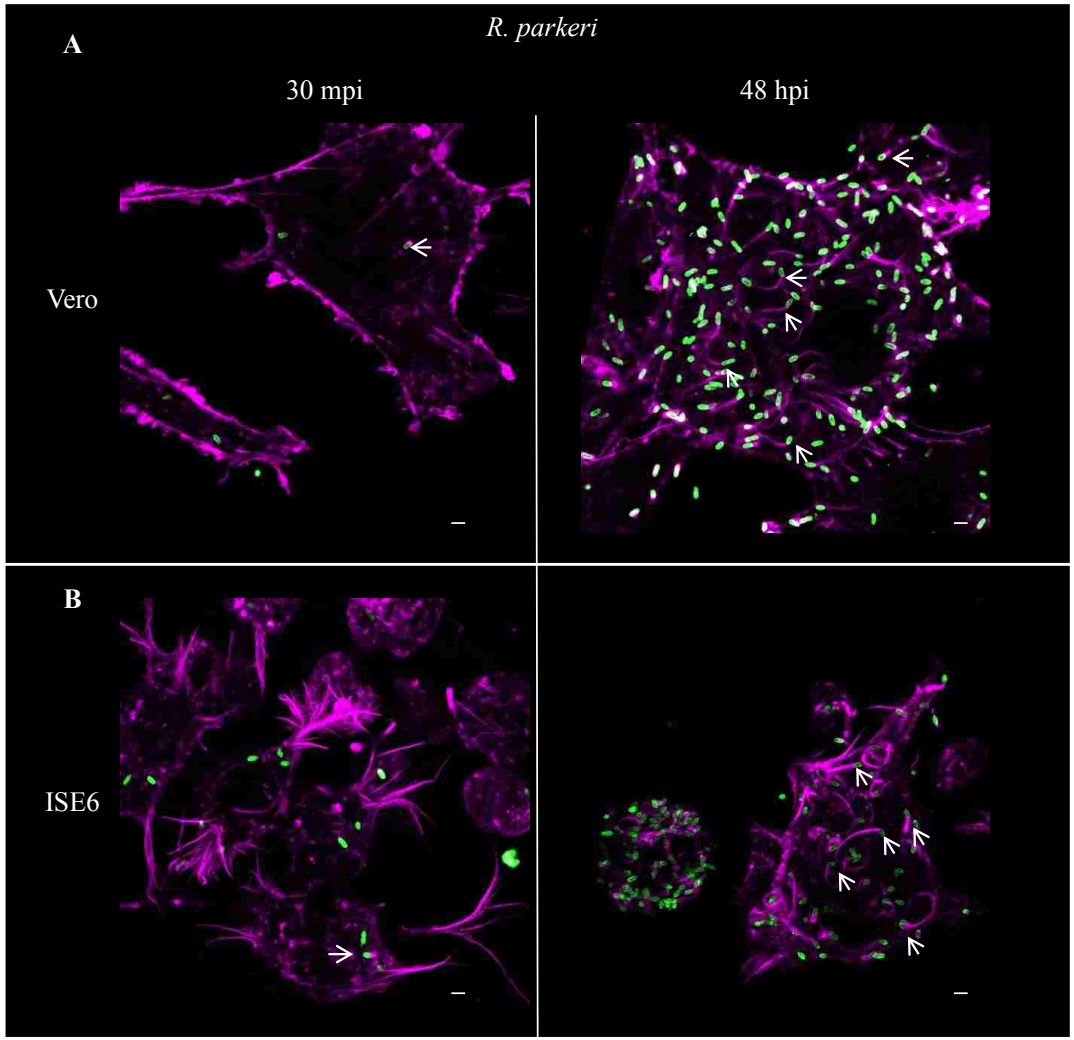

Figure 3. 1. Actin polymerization of *R. parkeri* in Vero and ISE6 cells. A) *Rickettsia parkeri* (green) polymerizing actin (red) in Vero cells at 30 mpi and 48 hpi. B) *Rickettsia parkeri* (green) polymerizing actin (magenta) in ISE6 cells 30 mpi and 48 hpi White scale bar=3μm. Arrows indicate *Rickettsia* polymerizing actin. Arrows indicate *Rickettsia* polymerizing actin.

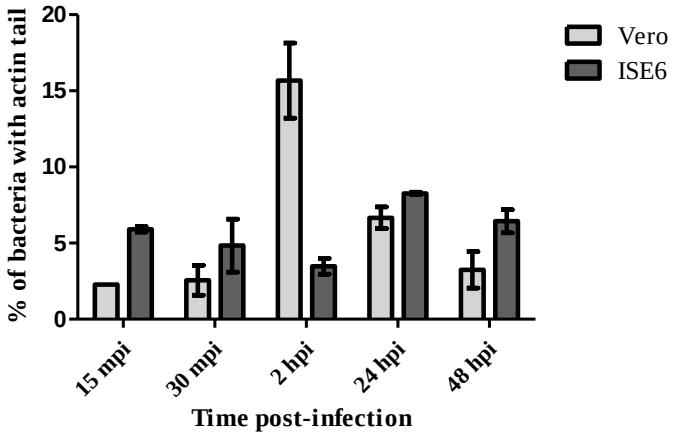

Figure 3.2. Percent of *R. parkeri* present in Vero and ISE6 cells with an actin tail at specific times post-infection. Data was collected over a range of time points post-infection into host cells. Error bars represent the standard error of the mean. Data are representative of two replicates per experiment and two independent experiments. Ten images taken across all experimental replicates were used in analysis.

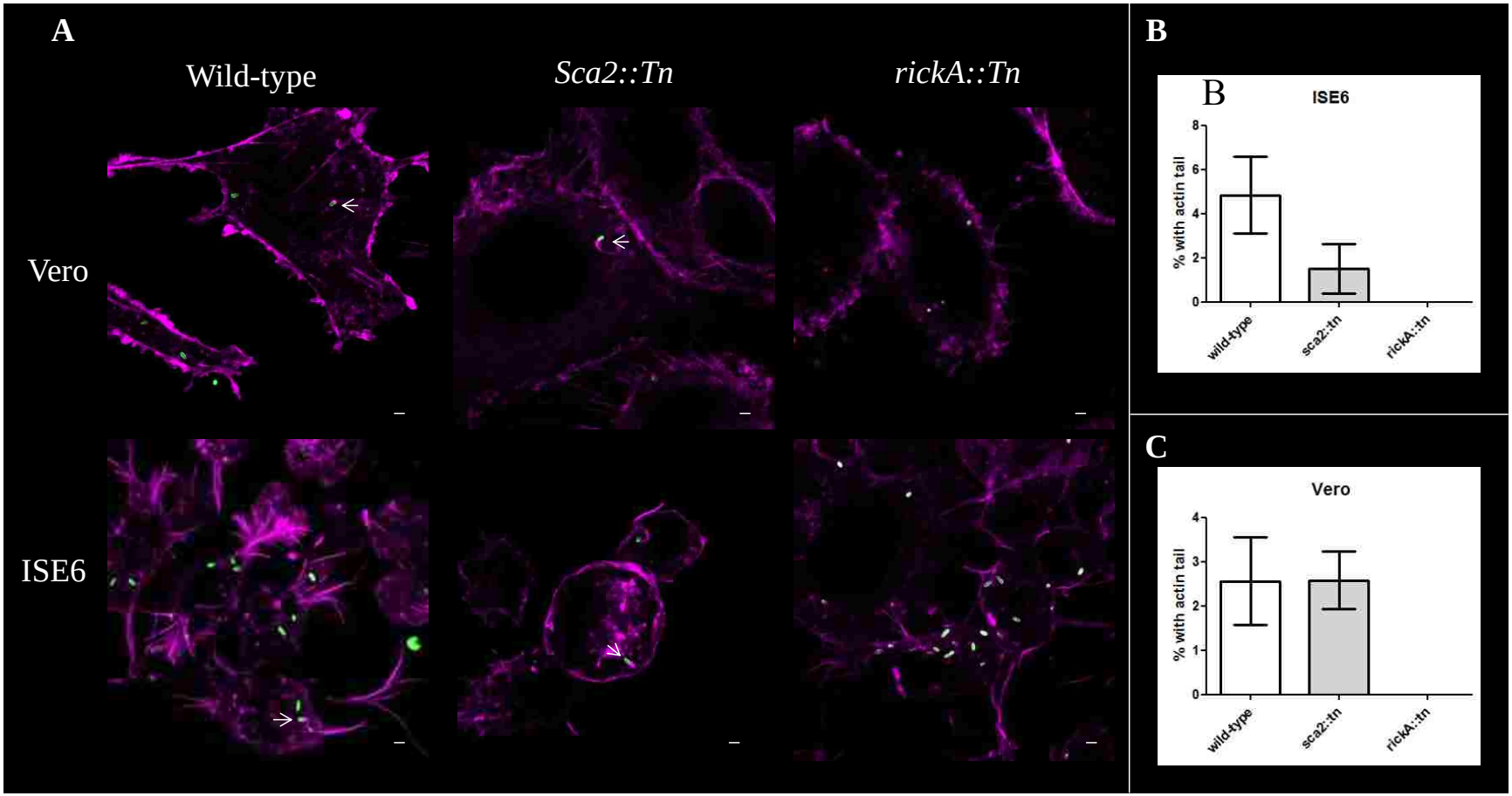

Figure 3. 3. Actin polymerization profile of *R. parkeri* compared to *R. parkeri sca2::Tn* and *R. parkeri rickA::Tn* in Vero and ISE6 cells at 30mpi. A) Shows *Rickettsia* (green) actively polymerizing actin (magenta) in Vero (top panel) and ISE6 (bottom panel). This assay was repeated for *R. parkeri* (left panel), *R. parkeri sca2::Tn* (middle panel), and *R. parkeri rickA::Tn* (right panel). B) and C) Graphical representation of % of *Rickettsia* with an actin tail in Vero (B) and ISE6 (C) cells. Data are representative of two replicates per experiment and two independent experiments. Statistical analysis consisted of a t-test. $p<0.05$. White scale bar=3μm. Arrows indicate *Rickettsia* polymerizing actin. Arrows indicate *Rickettsia* polymerizing actin.

3.3.3 *sca2* Deficient *R. parkeri* infection in Arthropod Cells Results in a Lack of Late Phase Actin Motility

To further demonstrate that rickettsial ABM in arthropod cells is controlled by two separate proteins, *R. parkeri sca2::Tn* was utilized to query the actin dynamics in ISE6 cells (Reed et al. 2014). Infected ISE6 and Vero cells were collected at 30 mpi and 48 hpi, and visualized for actin polymerization. Early phase (30 mpi) ABM was noted in ISE6 and Vero cells (Figure 3.5). Conversely, Sca2-deficient *R. parkeri* were unable to polymerize actin at 48 hpi (late phase) in ISE6 and Vero cells (Figure 3.5). As such, late phase ABM in ISE6 cells is dependent on Sca2 activity. These data recapitulate the model of rickettsial actin polymerization previously established (Reed et al. 2014). Additionally, replication of *R. parkeri sca2::Tn* is similar to that of the wild-type strain in Vero and ISE6 cell lines (Figure 3.4). Thus, *in vitro* actin polymerization in arthropod cells is similarly controlled by two separate proteins corresponding to times post-infection of host cells.

3.3.4 *In vivo* Dissemination of *R. parkeri* in *A. maculatum* 12 Hours Post-exposure

Analysis of *in vitro* ABM resulted in the observation of temporal control of polymerization by two different rickettsial effectors. This led to the hypothesis that Sca2 and RickA will contribute to the dissemination of *R. parkeri* within its tick host, *Amblyomma maculatum*. Thus, the *in vivo* function of RickA and Sca2 was investigated by infecting *A. maculatum* ticks, the primary host for *R. parkeri*, was inoculated via a previously published capillary feeding technique (CFT) to deliver a dose of $5x10^{10}$ *R. parkeri*/ml (Macaluso et al. 2001). Ticks were separated into three groups: *R. parkeri*, *R. parkeri sca2::Tn*, or *R. parkeri rickA::Tn*. Pre-fed adult, female ticks were allowed to imbibe an infectious dose overnight (≈12 hours). Positively labeled ticks were dissected immediately post-exposure to remove midgut,

salivary glands, and ovaries. Rickettsial load was quantified for all organs. A portion of each dissected tissue was stained to visualize *Rickettsia* and actin.

Within each treatment group 75-100% of ticks became infected post-CFT, demonstrating the effectiveness of the inoculation route (Figure 3.6). At 12 hours post-exposure (hpe), all organs were positive for rickettsial gDNA. Rickettsial load in the midgut was statistically similar across all treatment groups with *R. parkeri* wild-type strain having the highest overall load at $7.7x10^5 \pm 5.2x10^5$ (±SEM) (Figure 3.6). *R. parkeri* wild-type infection load in the ovaries was significantly greater than, both, *R. parkeri sca2::Tn* or *rickA::Tn*. The overall load for *R. parkeri* in this organ was $6.4x10^5 \pm 2.4x10^5$ (±SEM) (Figure 3.6). The salivary glands had the lowest rickettsial loads at 12 hpe compared to the midgut and ovaries. All rickettsial treatment groups in the salivary glands were statistically similar. The highest load in the salivary glands was detected in the *R. parkeri* wild-type treated group with $1.2x10^5 \pm 9.7x10^4$ (±SEM) *Rickettsia* (Figre 3.6).

The data presented demonstrate that rickettsial dissemination into all major organs occurs after 12 hours of capillary feeding. Multi-organ dissemination is a key aspect of establishing an intracellular niche within the arthropod. It is important to define a timeline for when and which organs become infected. Additionally, *sca2::tn* and *rickA::tn* are not deficient in infecting the midgut or salivary glands when compared to the parent strain. However, their ability to infect the ovaries was significantly diminished compared to the wild-type strain. While slight differences in bacterial load were observed, it can be concluded that neither protein plays a vital role at early infection *in vivo*.

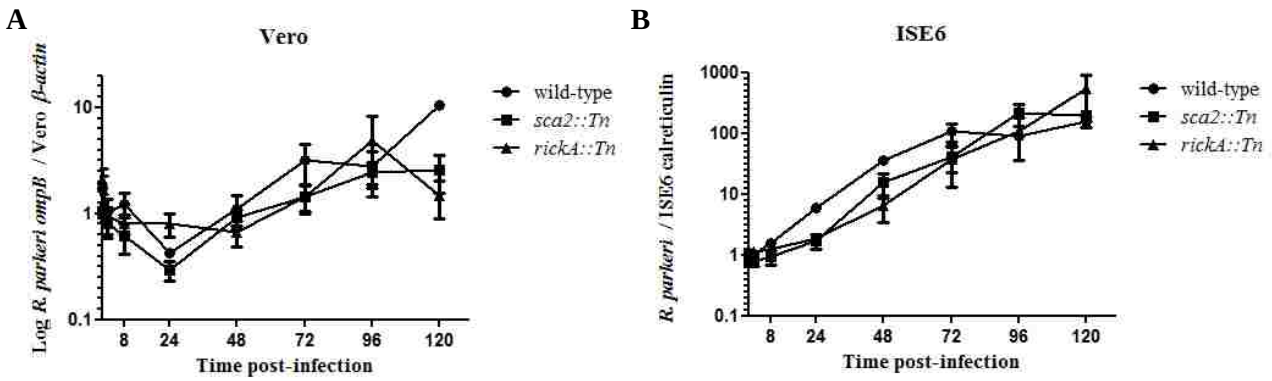

Figure 3.4. Growth curves for *R. parkeri* wild-type, *sca2::TN*, and *rickA::Tn* in Vero (A) and ISE6 (B). Data is fold change compared to initial imput. Collection times: 15 and 30mpi; 2, 8, 24, 48, 72, 96, 120 hpi. Repeated measures analysis completed in Graphpad Prism software. A $p<0.05$ was considered significant.

3.3.5 *In vivo* Dissemination of *R. parkeri* in *A. maculatum* 3 Days Post-exposure

Since no appreciable differences in rickettsial loads were noticed at 12 hpe, the contributing to role of Sca2 and RickA in bacterial persistence was investigated. Therefore, post-exposure to *R. parkeri* strains, a second feeding group was created. Female ticks positive for *R. parkeri* imbibement were returned to host and allowed to feed with previously attached male ticks. After three days of additional feeding, all ticks were forcibly removed, and the females were dissected for analysis.

At 3 days post-exposure (dpe), 50-100% of analyzed ticks were infected with *R. parkeri* strains (Figure 3.7). Overall, the *Rickettsia* in each organ for all treatment groups decreased from 12 hpe to 3 dpe. All strains of *R. parkeri* variably infected the midguts, salivary glands, and ovaries. Therefore, no significant difference was found between each treatment group. The wild-type strain produced the highest infection load in the midgut with a mean of $1.5x10^4 \pm 1.4x10^4$ (±SEM) (Figure 3.7).

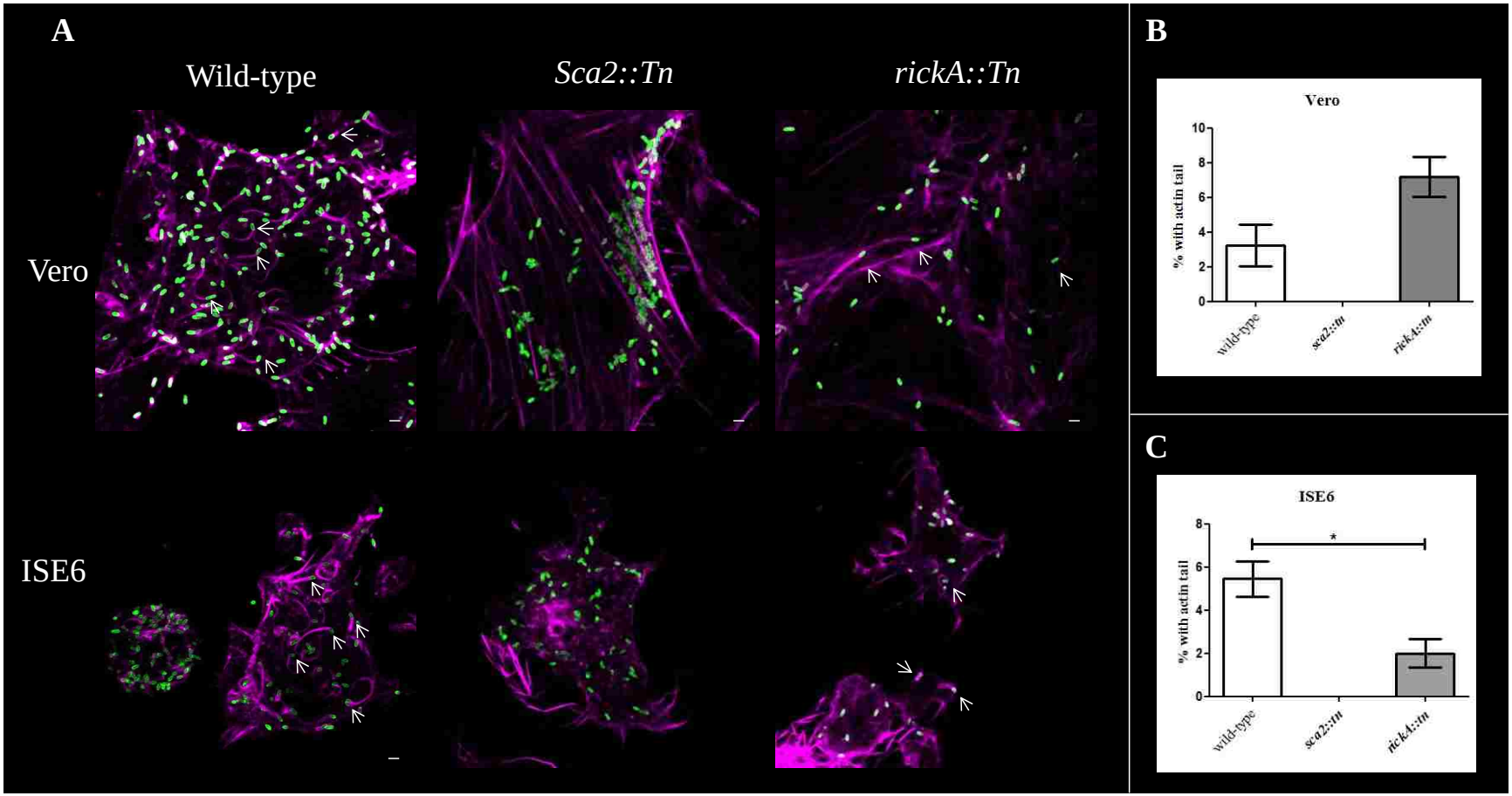

Figure 3. 5. Actin polymerization of *R. parkeri* compared to *R. parkeri sca2::Tn* and *R. parkeri rickA::Tn* in Vero and ISE6 cells at 48hpi. A) Shows *Rickettsia* (green) actively polymerizing actin (red) in Vero (top panel) and ISE6 (bottom panel). *Rickettsia parkeri* (left panel), *R. parkeri sca2::Tn* (middle panel), and *R. parkeri rickA::Tn* (right panel). B) and C) Graphical representation of percent *Rickettsia* with an actin tails in Vero (B) and ISE6 (C) cells. Data are representative of two replicates per experiment and two independent experiments. Statistical analysis consisted of a t-test. $p<0.05$ White scale bar=3μm. Arrows indicate *Rickettsia* polymerizing actin.

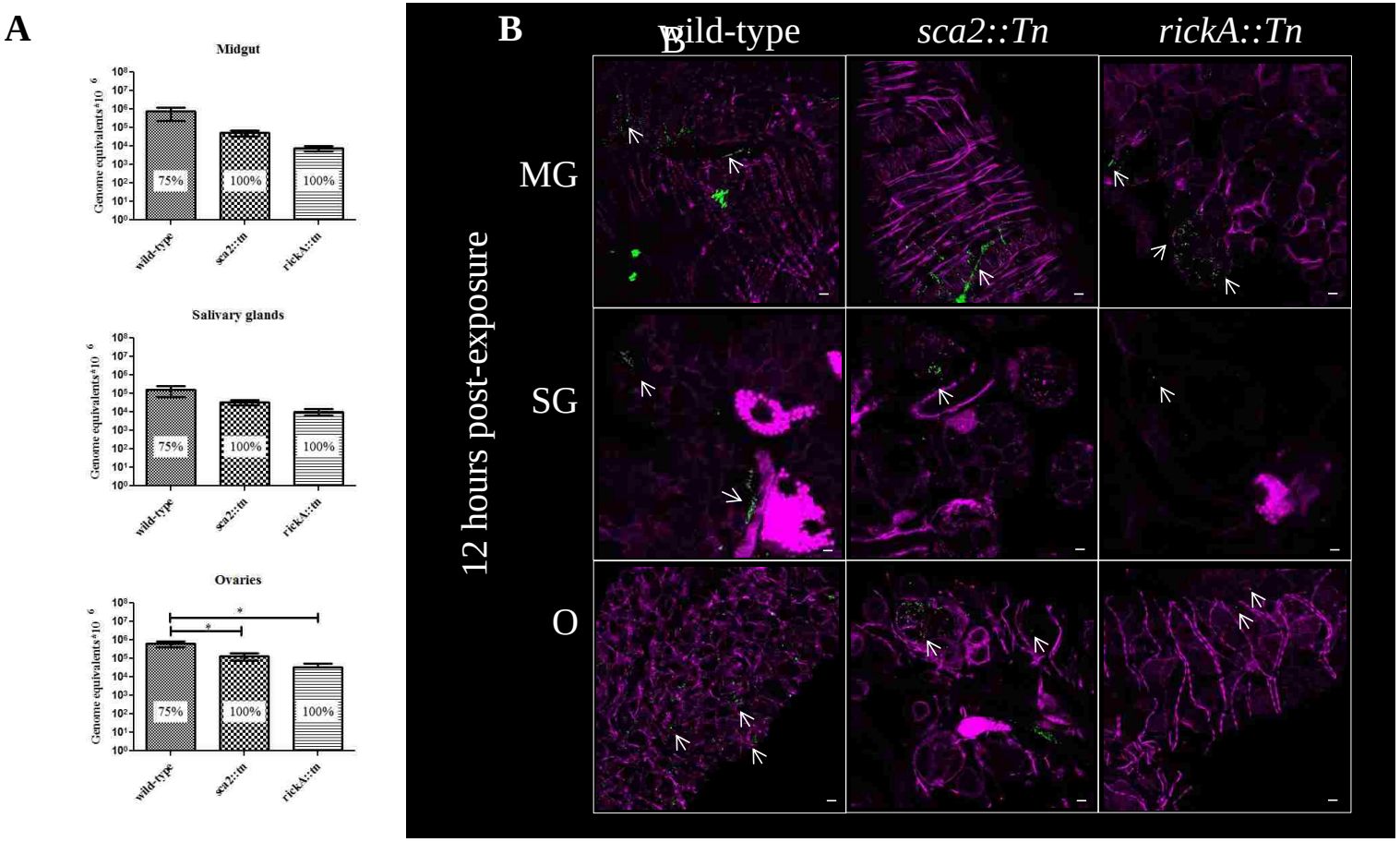

Figure 3. 6. Dissemination of *R. parkeri* wild-type, *R. parkeri sca2::Tn*, and *R. parkeri rickA::Tn* in *A. maculatum* at 12 hpe A) Mean rickettsial load as quantified by qPCR for the midgut (top graph), salivary glands (middle graph), and ovaries (bottom graph). B) Confocal microscopy of midgut (top panel), salivary glands (middle panel), and ovaries (bottom panel) corresponding to the data presented in A) for *R. parkeri* wild-type (left panel), *sca2::Tn* (center panel), and *rickA::Tn* (right panel). All tissues were stained for *Rickettsia* (green) and actin (magenta). Statistical analysis consisted of a one-way ANOVA with $p<0.05$ considered significant, denoted by *. Data are representative of 2 independent experiments. White scale bar=3μm. Arrows indicate rickettsiae. Arrows indicate rickettsiae.

Rickettsia parkeri rickA::Tn infection in the midgut was higher than *R. parkeri sca2::Tn*, though not significantly. *R. parkeri rickA::Tn* had the highest infection load in the salivary glands $1.7x10^4 \pm 6.6x10^3$ (±SEM), followed by successively decreasing for *R. parkeri*. *sca2::Tn* and *R. parkeri* wild-type (Figure 3.7). A similar pattern of infection was noted in the ovaries where the highest titer was seen in ticks exposed to *R. parkeri rickA::Tn*. The load here was $2.3x10^3 \pm 3.2x10^2$ (±SEM) mean *Rickettsia* (Figure 3.7). Infection levels for *R. parkeri* wild-type and *R. parkeri sca2::Tn* were 1 log lower than the *R. parkeri rickA::Tn*. The overall rickettsial load was, again, lowest in ticks exposed to *R. parkeri sca2::Tn* (Figure 3.7).

These data demonstrate that disseminated *R. parkeri* infection is sustained 3 days after exposure and initial detection. However, infection decreased 1-2 logs at 3 dpe compared with 12 hpe. A decrease in rickettsial load could likely be a result of clearance within *A. maculatum*. Overall, rickettsial infection across all organs and treatment groups varied greatly and lacked significance. Taken with the previous *in vivo* data, this demonstrates that *Rickettsia* invading *A. maculatum* are easily able to establish infection, but that rickettsial load decreases as feeding progresses. This provides valuable insight to how rickettsial infections are established *in vivo*. Also, at 3 dpe *sca2::tn* and *rickA::tn* do not appear to contribute to enhanced clearance of *Rickettsia*.

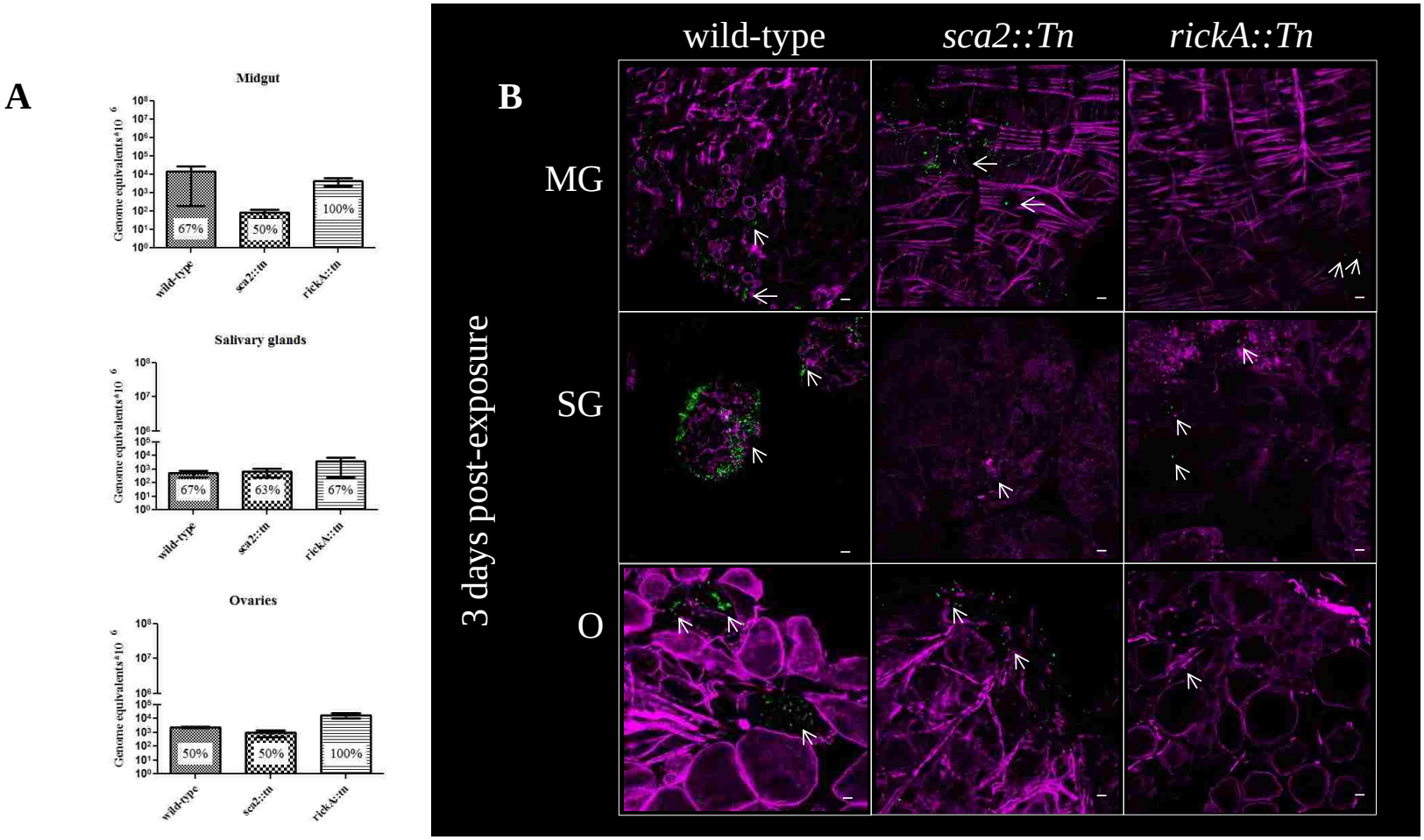

Figure 3.7. Dissemination of *R. parkeri* wild-type, *R. parkeri sca2::Tn*, and *R. parkeri rickA::Tn* at 3 dpe. A) Mean rickettsial load as quantified by qPCR for the midgut (top graph), salivary glands (middle graph), and ovaries (bottom graph). B) Confocal microscopy of midgut (top panel), salivary glands (middle panel), and ovaries (bottom panel) corresponding to the data presented in A) for *R. parkeri* wild-type (left panel), *sca2::Tn* (center panel), and *rickA::Tn* (right panel). All tissues were stained for *Rickettsia* (green) and actin (magenta). Statistical analysis consisted of a one-way ANOVA with $p<0.05$ considered significant, denoted by *. Data are representative of 2 independent experiments. White scale bar=3μm. Arrows indicate rickettsiae.

3.3.6 *In vivo* Dissemination of *R. parkeri* in *A. maculatum* 7 days post-exposure

To further investigate the dissemination and persistence pattern of wild-type *R. parkeri* in relation to *sca2::tn* and *rickA::tn*, a third group of exposed ticks that were allowed to feed for seven days after capillary feeding. This is a critical time point for the feeding tick, as the female nears engorgement. The tick has consumed large amounts of blood at this point; the ovaries and salivary glands have enlarged as well. This is ultimately in preparation for conversion of bloodmeal to progeny.

Infection of *A. maculatum* at 7 dpe ranged from 0-67% of exposed ticks (Figure 3.8). Wild-type infection in the midgut decreased approximately 2 logs from its levels at 3 dpe, with a mean rickettsial load of $6.4x10^2 \pm 4.7x10^2$ (±SEM) (Figure 3.8). Statistical inferences could not be made regarding the *R. parkeri sca2::Tn* and *R. parkeri rickA::Tn* strains. Mean rickettsial load for both groups diminished so much so that only 1 tick per treatment was positive for *Rickettsia*. The values for both are 1-2 logs lower than when measured at 3 dpe. Despite the lack of statistical analysis, it is observed *Rickettsia* persists in the midgut for 7 dpe, albeit at much decreased levels. Salivary glands across all treatments sustained infection at 7 dpe. The highest mean rickettsial load was observed in ticks that had been exposed to *R. parkeri* wild-type at $5.5x10^2 \pm 2.2x10^2$ (±SEM) (Figure 3.8). This was a full 1 log lower than values detected at 3 dpe. Mean rickettsial load in the salivary gland of *A. maculatum* infected with *R. parkeri sca2::Tn*, was slightly lower than those exposed to wild-type. Nonetheless, sustained, low level maintenance of the rickettsiae in the absence of Sca2. A similar level of *R. parkeri rickA::Tn* was detected in the 7 dpe salivary glands. However, only 1 tick was *Rickettsia*-positive. Surprisingly, no wild-type *R. parkeri* was present in the ovaries at 7 dpe. *R. parkeri* infection in the ovaries decreased consistently at each time point, reaching undetectable levels at 7 dpe. Alternatively,

detectable *R. parkeri sca2::Tn* and *R. parkeri rickA::Tn*, was detected at all time points. The highest mean rickettsial load was detected in the *R. parkeri sca2::Tn*-exposed group at $4.1x10^3$ $\pm 3.9x10^3$ (±SEM) (Figure 3.8). These data show that *R. parkeri* lacking expression of Sca2 and RickA are still able to achieve and sustain mutli-organ infection in *A. maculatum*. Though the overall mean rickettsial load decreases post-CFT, complete clearance does not occur. This data implies that though Sca2 and RickA play an important role *in vitro*, their expression by *R. parkeri* is not vital to infection in *A. maculatum*.

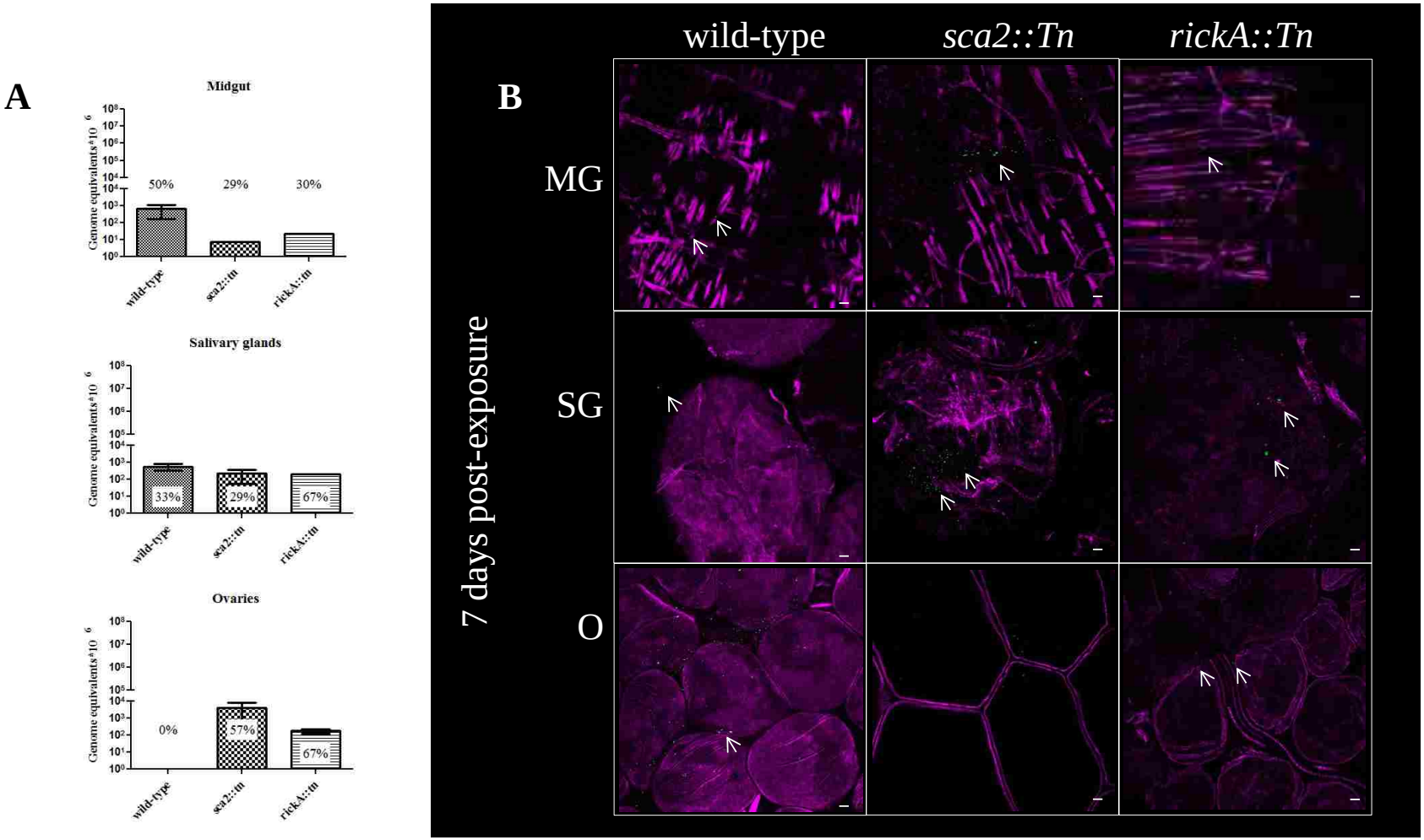

Figure 3. 8. *Rickettsia parkeri* wild-type, *R. parkeri sca2::tn*, and *R. parkeri rickA::Tn* 7 dpe . A) Mean rickettsial load as quantified by qPCR for the midgut (top graph), salivary glands (middle graph), and ovaries (bottom graph). B) Confocal microscopy of midgut (top panel), salivary glands (middle panel), and ovaries (bottom panel) corresponding to the data presented in A) for *R. parkeri* wild-type (left panel), *sca2::Tn* (center panel), and *rickA::Tn* (right panel). All tissues were stained for *Rickettsia* (green) and actin (magenta). Statistical analysis consisted of a one-way ANOVA with $p<0.05$ considered significant, denoted by *. Data are representative of 2 independent experiments. White scale bar=3μm. Arrows indicate rickettsiae.

3.4 Discussion

Rickettsia parkeri is an emerging human pathogen primarily transmitted by the Gulf Coast tick, *Amblyomma maculatum*. Molecular characterization of such tick-borne rickettsial diseases (TBRD) is occurring at a rapid pace. However, the intracacies of the tick/*R. parkeri* relationship have yet to be fully elucidated. Several pan-rickettsial proteins have been implicated as key players in cellular infection within mammalian *in vitro* systems. However, their potential role within the arthropod host is unknown. Relatively little is known concerning the factors that contribute to *R. parkeri* colonization and dissemination of *A. maculatum*.

The *R. parkeri* proteins Sca2 and RickA have been implicated in mediating actin polymerization (Haglund et al. 2010, Reed et al. 2014). This work has contributed to a working model for how *Rickettsia* propel themselves intracellularly. This includes RickA mediating early phase ABM, coordinated with initial invasion of the host cell. Actin tails appear short and curved in length and structure. Movement at this time is slow and non-directional. This phase of motility is hypothesized to last approximately 15-30 mpi. Bolstering this model is the fact that anti-RickA antibodies colocolize to actin tails most intensely during this time. At 2 hours post-infection (hpi), the majority of rickettsiae observed do not polymerize actin, signifying a shift in the the rickettsial mediator of ABM. Measurements of Sca2 localization to late actin tails is most intense at 8 and 48 hpi. Additional aspects of this late phase ABM under the control of Sca2 include, longer, unbranched tails and increased velocity and directionality of bacterial movement (Reed et al. 2014). The role of each protein was independently verified in two mutagenized strains of *R. parkeri*, each lacking expression of RickA or Sca2. A cell-to-cell spread assay also demonstrated that the lack of RickA or Sca2 resulted in a significant decrease in the overall number of infected foci when compared to the wild-type strain (Reed et al. 2014).

A series of experiments were designed to determine if Sca2 and RickA possessed similar functionality in arthropod cells as compared to mammalian, and, also, if either Sca2 or RickA contributed to a specific infection and dissemination profile in *A. maculatum*. These results suggest that in a tick embryonic cell line (ISE6), actin polymerization occurs in a similar manner to that described in mammalian cells. At 30 mpi of *R. parkeri* to ISE6 cells actin tails were short. Though some rickettsiae were observed to polymerize long tails as well, categorization based on tail length was not defined in this study. Small amounts of long tail formation during early phase infection was noticed previously (Reed et al. 2014). Conversely, at 48 hpi with the wild-type strain of *R. parkeri*, actin tails were elongated in congruence with the previous study and the present observations with infected mammalian cells (Reed et al. 2014). Additionally, bacterial polymerization was tracked for *R. parkeri* strains lacking RickA and Sca2 functionality. A lack of RickA resulted in a loss of actin-based polymerization in both ISE6 and Vero cells at 30mpi. However, abundant ABM was observed at 48 hpi. Alternatively, cells infected with *R. parkeri sca2::Tn* displayed early ABM but no late phase polymerization in both ISE6 and Vero cells.

It can be concluded that RickA and Sca2 display conserved functionality in arthropod cells. However, models of arthropod infection *in vitro* don't include the multitude of arthrpod host factors, nor do they mimic changes within the tick during bloodfeeding. Tick-derived cell lines are developed from embryonic tick cells and often contain multiple cell types. Ultimately, the *in vitro* observations will be strengthened by modeling infection *in vivo*. Thus, making their contribution to infection of *R. parkeri* in *A. maculatum* an appropriate objective.

This study utilized an established capillary feeding technique to infect *A. maculatum* with three strains of *R. parkeri* to produce an infection in the tick which could be tracked temporally (Macaluso et al. 2001). Ticks were allowed to imbibe a dose of *R. parkeri* that has been

previously shown to produce infection within *A. maculatum* (see Chapter 2). Post-inoculation, ticks were split into three groups: 1) 12 hpe, 2) 3 dep, or 3) 7 dpe. All groups excepting the 12 hpe were returned to host and allowed to continue bloodfeeding post-exposure.

At 12 hpe, *R. parkeri* was detected in all organs across all treatment groups. Though this observation was unexpected it highlights a possibly important aspect of tick acquisition of *Rickettsia*. Previous stuides have discussed the contributing role of the tracheal system, which extends throughout nearly all aspects of the tick body (Baldridge et al. 2007). Significantly higher levels of wild-type *R. parkeri* were present in the ovaries, compared to both mutant strains. At 3 dpe, all ticks had detectable levels of *Rickettsia* present in all organs. Howwever, rickettsial load had decreased from the first that detected at 12 hpe. The highest rickettsial load was observed in ticks exposed to wild-type *R. parkeri*. At 7 dpe, levels of *Rickettsia* were the lowest of all the time points. Number of infected ticks was also the lowest compared to 12 hpe and 7 dpe. The wild-type strain retained the highest infection load in the midgut and the salivary glands. Surprisingly, clearance of wild-type *R. parkeri* was observed in the ovaries at 7 dpe. The *R. parkeri Δsca2* strain had the highest infection load compared to *R. parkeri rickA::Tn*. The mechanism for persistence of these two strains, and clearance of the wild-type is not clear. Tick immune molecules may have led to clearance of the wild-type, which displays no defect in motility. A similar hypothesis has been proposed for *R. peacockii* (Mattila et al. 2007). This rickettsial species lacks expression of RickA, which was originally thought to impart persistence in the ovary of its tick host, *D. andersoni* (Mattila et al. 2007). However, genome sequencing has also shown that *R. peacockii* possesses deletions in genes leading to the disruption of *ompA,* and *Sca1,* among other candidate genes (Felsheim et al. 2009). Thus, the reason for *R. peacockii*'s persistence in tick ovaries is unexplored at the molecular level.

An additional model of *Rickettsia* infection within ticks has assessed multi-organ distribution of the bacterium post-capillary feeding. However, observations were made 7 dpe, upon which large but non-quantified amounts of *Rickettsia* were observed in midguts only of adult *A. americanum* and *D. variabilis* (Baldridge et al. 2007). These ticks were not pre-fed and did not receive a bloodmeal post-exposure, thus these conditions are slightly different from the present study.

The current study creates a model for assessing rickettsial factors that have the potential to contribute to the infection dynamics of *Rickettsia* within an actively feeding tick host. The data show that *R. parkeri* RickA and Sca2 do not inhibit infection and persistence of *Rickettsia* in the tick host. Both mutant strains were diminished at each time point similar to that observed in the wild-type strain. Both mutant strains were able to maintain infection even a week after capillary feeding, whereas the wild-type was noticeably cleared from all exposed *A. maculatum*. The mechanims contributing to this result is unknown but warrants further study. Importantly, though all observed tissues were stained to visualize actin in tick organs, no evidence of rickettsial ABM was recorded. This is in contrast with te *in vitro* data. Though there are many differences between the *in vitro* and *in vivo* systems, the lack of ABM is currently unexplained. It can be concluded, however, that *R. parkeri* Sca2 and RickA are vital to mammalian infection and dissemination, but not do not overwhelmingly contribute to tick infection.

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CHAPTER 4
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

4.1 Discussion of Results and Future Directions

There are over 25 recognized species within the spotted fever group (SFG) of *Rickettsia* (Macaluso and Paddock 2014). All share an intimate relationship with their tick reservoir and vector host. Contributing to the complexity of tick/*Rickettsia* relationship is the observation that sustained rickettsial maintenance in the tick is not successful for all invading rickettsiae. Investigations assessing these relationships are limited in both, the rickettsial and tick species evaluated (Burgdorfer and Brinton 1975, Niebylski et al. 1999, Macaluso et al. 2001, Wright et al. 2015, Schumacher et al. 2016). This includes emerging *Rickettsia* whose eco-epidemiology and capacity for vector competence is impartially characterized. Moreover, rickettsial mechanisms leading to successful colonization and dissemination into multiple tick organs are unknown. The unprecedented rise in human cases of SFG rickettsioses, along with the recognition of novel tick vectors for *Rickettsia*, allows these studies to provide insight into the incidence of tick-borne rickettsial diseases (TBRD) (Demma et al. 2005, Drexler et al. 2016). The hypothesis for this work is that if primary tick/*Rickettsia* pairings do not exist then rickettsial determinants account for primary vector/pathogen relationships. To test the above hypothesis, and thereby contribute to the understanding of TBRD, two objectives were designed to identify, 1) vertical transmission and fitness of ticks infected with *Rickettsia*; and 2) significance of rickettsial proteins mediating actin-based motility in arthropod models of infection *in vitro* and *in vivo*.

In nature, SFG *Rickettsia* are maintained via vertical transmission, either transovarially (infected female to offspring) or transstadially (infected life stage to the next) within tick populations. *Amblyomma maculatum* was selected for this study because of its role as an

emerging vector of human disease causing agents, including *R. parkeri* (Paddock et al. 2004). Likewise, *D. variabilis* is an important vector in the contemporary ecology of TBRD, including *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF). Additional SFG *Rickettsia* have been detected in both tick species. However, tick vector competence concerning additional species is unknown. To define the parameters of vertical transmission, female ticks were exposed independently to five rickettsial species: 1) *R. rickettsii*, 2) *R. parkeri*, 3) *R. amblyommii*, 4) *R. montanensis*, or 5) *R. felis*. Post-engorgement four biological indices were recorded: 1) engorgement weight, 2) nutrient index, 3) egg production index, and 4) percent egg hatching. Each was designed to characterize utilization of bloodmeal resources to produce progeny post-rickettsial exposure. Molting rates for immature life stages (i.e. larval and nymphal) were recorded to determine if vertically maintained infection impacted the overall tick population.

Transovarial transmission rates within *D. variabilis* ranged from 17-25%. Only *R. amblyommii*, *R. felis*, *R. montanensis*, and *R. parkeri* were detected in eggs. Fitness of exposed ticks was not affected by rickettsial infection; except for the egg production of *R. montanensis*-exposed ticks, which was negatively impacted. Sustained transtadial maintenance for F_1 larvae and nymphs was documented for only *R. amblyommii* and *R. parkeri*. Detectable infection ranged between 20-50% of the tested subpopulation. Adults resulting from these cohorts were negative for rickettsial infection. Interestingly, all resultant infected F_1 life stages hatched (egg) or molted (larval and nymphal) at levels similar to media controls. These data suggest that *D. variabilis* is susceptible to infection by a wide-range of *Rickettsia*. However, its ability to maintain infection vertically is not sustainable. Furthermore, maintenance of *Rickettsia* does not

negatively impact host fitness. These results suggest amplifying vertebrate hosts are likely necessary for continued maintenance of *Rickettsia* in populations of tick species.

Comparatively, *A. maculatum* transovarial transmission was only successful for *R. amblyommii* and *R. parkeri* at rates of 29 and 38%, respectively. Both species were maintained in F_1 larvae and nymphs at rates of 20-50%. However, rickettsial infection was not detectable in F_1 adult cohorts. Ticks exposed to *R. amblyommii, R. montanensis*, and *R. parkeri* displayed significant decreases in engorgement weight, nutrient index, and egg production. *R. felis*-exposed *A. maculatum* were negatively impacted in their ability to reach full engorgement weight but maintained comparable nutrient and egg production indices. Eggs resulting from exposed females had reduced levels of hatching to the larval stage, however, molting to nymphal and adult stages returned to nearly 100%. Overall, the fitness of *A. maculatum* was significantly impacted by exposure to *R. amblyommii, R. montanensis, R. parkeri*, and *R. felis*; but not *R. rickettsii*. Together, this data suggests that *A. maculatum* can harbor a more narrow range of *Rickettsia* than *D. variabilis*. In this case, assault by invading *Rickettsia* significantly reduces the fitness of the female tick, so amplifying vertebrate hosts must be necessary to maintain rickettsial infection within tick populations. Consistent with observations made for *Rickettsia*-infected *D. variabilis*, vertical transmission of *Rickettsia* in *A. maculatum* is not alone sustainable.

Seminal studies have demonstrated that vertical maintenance of *Rickettsia* in ticks does not extend to 100% of the tick's progeny (Ricketts 1907, Price 1954). Additionally, strain variation between rickettsial isolates also dictates maintenance in ticks (Price 1954). This concept was expanded to suggest that the fitness of ticks infected with pathogenic *Rickettsia* was negatively impacted (Burgdorfer and Brinton 1975, Niebylski et al. 1999). Furthermore, immature life stages are possibly more susceptible to rickettsial infection (Niebylski et al. 1999).

Niebylski noticed that upon infecting adult ticks, rickettsial infection transovarially was not successful (Niebylski et al. 1999). However, attempts to infect immature stages yielded infection that was maintained transstadially and transovarially. Studies have yet to compare the immune system of immature ticks in relation to their adult counterparts. Adult ticks may have enhanced ability to clear infection, whereas larval or nymphal ticks may be susceptible to thorough colonization of multiple organs. Established infection may effectively facilitate maintenance over multiple generations. Previous studies have shown that tick genes in adult *D. variabilis* are differentially expressed in an organ-specific manner, suggesting that the tick response to rickettsial infection is species-specific (Sunyakumthorn et al. 2013). Ultimately, the ability of the tick to respond to invading rickettsiae controls competent tick/*Rickettsia* relationships. The lack of transovarial transmission presented in Chapter 2 could likely be due to repeated passages in cell culture, strain variation (human isolates versus animal isolates), tick life stage infected, the use of laboratory tick colonies, and tick infection route (capillary feeding versus infection acquired on a rickettsemic host). Future studies should include the susceptibility of immature ticks to establishment of infection. Recent studies have also given more importance to utilizing rickettsial and tick strains from similar geographies (Schumacher et al. 2016). Haplotypes variation in ticks is related to origin of geographical isolation, which could be a defining factor in tick vector competence (Krakowetz et al. 2010, Schumacher et al. 2016).

Furthermore, the required number of *Rickettsia* sufficient to infect a tick host is unknown within the context of SFG *Rickettsia*. Ticks naturally infected with rickettsial concentrations ranging from 10^3-10^7 copies within a single tick have been observed (Zanettii et al. 2008, Monje et al. 2014). Whether the tick was initially infected with a higher or lower concentration to produce infection is also ill-defined. The work presented in Chapter 2 utilized a fixed

concentration of *Rickettsia* across five species. However, the concentration of *Rickettsia* required to produce sustainable infection in the tick may differ by rickettsial species. Future work should include determination of the rickettsial concentration necessary to establish infection within the tick by utilizing multiple rickettsial concentrations. Burgdorfer and Brinton hypothesized that a key factor to tick transovarial infection was heavy infection of the ovaries (Burgdorfer and Brinton 1975). Additionally, whether a tick would encounter a host so highly infected as to deliver 10^7 rickettsial organisms has not been demonstrated. Defining animal reservoir hosts for *Rickettsia* have been difficult. For example, no animal reservoir for *R. parkeri* has been identified, despite intensive studies in geographies were infected ticks are common (Edwards et al. 2011, Moraru et al. 2013). This suggests that the tick is the reservoir and vector, or, alternatively, mechanisms such as co-feeding are involved (Schumacher et al. 2016). The work presented in Chapter 2 did not investigate horizontal transmission; however, future work should investigate this mechanism.

If *Rickettsia* are maintained among tick populations, this would require both vertical and horizontal (co-feeding) transmission routes. Horizontal transmission seems to favor maintenance of pathogenic rickettsial species within tick populations (Werren 1997). Alternatively, vertical transmission selects non-pathogen associations (Werren 1997). Multiple studies have shown that pathogenic *Rickettsia* are able to be maintained transovarially without horizontal transmission (Burgdorfer and Brinton 1975, Niebylski et al. 1999). An interesting addition to these observations is that repeated vertical transmission of *Rickettsia* within the tick host leads to attenuation (Yamamura 1993, Mather and Ginsberg 1994, Paddock et al. 2015). Indeed, *R. rickettsii* exhibits strain variation and complete attenuation of pathogenicity (Price 1954 , Burgdorfer and Brinton 1975, Niebylski et al. 1999). Sequencing of virulent and avirulent strains

of *R. rickettsii* has identified differences in rickettsial outer membrane proteins (Omps) (Clark et al. 2015). This suggests that changes in evolutionary pressure results in changes between the *Rickettsia*/tick host biochemical interface. The roles of rickettsial proteins during infection of the tick host remain ill-defined, and warrant intensive study.

To elucidate rickettsial factors that contribute to arthropod infection, the role of *R. parkeri* Surface cell antigen 2 (Sca2) and RickA was determined. Both proteins have been implicated in intracellular motility of *Rickettsia* through ABM (Reed et al. 2014). Importantly, ABM has only been examined in mammalian cells *in vitro*, so these studies are the first to explore the contribution of Sca2 and RickA to tick infection (Reed et al. 2014). In order to investigate the profile of rickettsial movement in the arthropod host, an embryonic tick cell line derived from *Ixodes scapularis* (ISE6) was utilized. Tandem experiments in Vero cells acted as a positive control for previously established mammalian infection patterns (Reed et al. 2014). Results from Chapter 3 demonstrate that *R. parkeri* actin polymerization in tick cells are similar to mammalian cells. Infection of ISE6 cells with *R. parkeri rickA::Tn* demonstrated that ABM was abolished at 30 mpi but returned at 48 hpi. Alternatively, cells infected with *R. parkeri sca2::Tn*, resulted in a loss of ABM at 48 hpi, but was similar to wild-type at 30 mpi. This bi-modal approach to ABM in arthropod cells is identical to that previously shown in the mammalian system, thus, suggesting conserved functionality (Reed et al. 2014).

Results gathered from *in vitro* experiments suggest that ABM of *R. parkeri* would play a key role in its dissemination within *A. maculatum*. To query the *in vivo* role of actin polymerization in the arthropod host, ticks were capillary fed doses of *R. parkeri* wild-type, *R. parkeri rickA::Tn*, or *R. parkeri sca2::Tn*. Ticks were sampled at 12 hours, 3 days, and 7 days post-exposure (hpe or dpe), individual organs (midgut, salivary glands, and ovaries) were

dissected, and the rickettsial load quantified. All strains disseminated to the midgut, salivary glands, and ovaries at 12 hpe, demonstrating that Sca2 and RickA do not contribute to initial rickettsial colonization *in vivo*. The highest mean rickettsial concentration for all treatment groups was detected at 12 hpe, and decreased serially at 3 and 7 dpe. Salivary gland infection was statistically similar across all treatment groups at 12 hpe, 3 and 7 dpe. Rickettsial load in midgut tissues was similar across all treatment groups at 12 hpe and 3 dpe. However, identifying differences at later time points will require additional experimentation. Ovarian rickettsial infection at 12 hpe was significantly higher in wild-type-exposed *A. maculatum* compared with *sca2::Tn* and *rickA::Tn* treatment groups. Rickettsial loads were similar across treatment groups at 3 dpe, but were not comparable at 7 dpe because infection was not detectable in the wild-type-exposed ticks. Confocal microscopy of tissues collected at all time points recorded no evidence of ABM. In summary, this portion of the data suggests that Sca2 and RickA are not vital to maintenance of rickettsial infection in the midgut and salivary glands. However, ovarian clearance of the wild-type strain while persistence of *sca2::Tn* and *rickA::Tn* may suggest a role for these proteins in rickettsial persistence.

The *in vitro* data put forth demonstrates that, like mammalian models, Sca2 and RickA contribute to a pattern of rickettsial ABM. Early infection into host cells is affected by RickA. Conversely, late cellular infection is guided by Sca2. While this distinction is able to be teased apart *in vitro*, the impact of these two proteins is not easily noted *in vivo*. No implications for early versus late movement of *Rickettsia in vivo* could be discerned in the present study. Interestingly, clearance of the wild-type *R. parkeri* was observed 7 dpe, while both mutant strains were still detectable. One hypothesis is that due to defect in movement from cell-to-cell, imparted by the lack of Sca2 and RickA, the *Rickettsia* was able to escape immune molecules

present in the ovaries. Overall, the data suggest that whilst ABM driven by Sca2 and RickA may be important in *in vitro* models of rickettsial infection, no evidence put forth by the current experiments supports the role of ABM in *in vivo* infection of the tick host. Thus, alternative methods/factors may contribute to rickettsial dissemination *in vivo*. The *in vitro* data gathered suggests that less than 20% of *Rickettsia* polymerize actin at any one time post-infection. Therefore, it is possible that the microscopy employed within the current study failed to capture rickettsial ABM events.

Previous data has shown that actin polymerization is the primary mode of rickettsial movement intracellularly. However, in this study no evidence of this method of dissemination was observed *in vivo*. Thus, the contribution to intracellular movement by actin polymerization may not be a mechanism utilized *in vivo*. A potential reason for the disparate findings may be because *in vitro* infection models are artificial in nature. Arthropod cell cultures are often derived from tick embryos, which do not mimic conditions present in the actively feeding tick, such as active digestion and onslaught of immune molecules. Importantly, these systems do not require the bacterium to invade and infect multiple cell types as is seen in the midgut, salivary glands, and ovaries. Perhaps a better *in vitro* model would consist of primary cultures derived from individual tick organs. This is often troublesome due to bacterial contamination. However, such a model would aid in identification of organs that could act as barriers to infection in the tick host. The *in vivo* system of infection used in this study is highly relevant because it avoids the artificiality and limitations of *in vitro* models.

The data gathered in these studies demonstrate the complex nature of the tick/pathogen interaction. The knowledge gained will move the field of Rickettsiology further by increasing the understanding of novel tick/*Rickettsia* relationships. This will help in detection and diagnosis of

TBRDs. Also, the model for identifying rickettsial factors important for persistence within the tick host can be utilized beyond this study. This is increasingly important as genetic manipulation of *Rickettsia* becomes more common.

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APPENDIX A: COMMONLY USED ABBREVIATIONS

ABM-actin-based motility

Arp 2/3-actin related proteins 2/3

AG-ancestral group

AT-autotransporter domain

BSA-bovine serum albumin

CFT-capillary feeding technique

Dpe-days post-exposure

F-actin-filamentous actin

FAK-focal adhesion kinase

FH1-formin homology 1

FH2-formin homology 2

G-actin-globular actin

HPE-hours post-exposure

HPI-hours post-inoculation

JMY-junction-mediating and regulatory protein

MPI-minutes post-inoculation

NPF-nucleation promoting factor

OMP-outer membrane protein

PBS-1x phosphate buffered saline

PLA2-phospholipase A2

PLD-phospholipase D

SS-signal sequence

SFG-spotted fever group

Sca-surface cell antigen

TBRD-tick-borne rickettsial diseases

TRG-transitional group

TG-typhus group

WASP-Wiscott-Aldrich syndrome family protein

WH2-WASP homology 2

WHAM-WASP-homologue associated protein with actin, membranes and microtubules

VITA

Emma Kate Harris is the daughter of Autamus P. Harris and Deborah A. Harris. She was born and raised in Kiln, Mississippi. She attended Pearl River Community College where she obtained an Associate of Arts degree in 2007. Upon graduating, Emma then moved to Columbus, Mississippi to attend Mississippi University for Women. In 2010, she earned her Bachelor of Science degree in Microbiology with a minor in Chemistry. At this time she began her doctoral career, joining the laboratory of Dr. Kevin Macaluso. Her research work included elucidations of rickettsial interactions with their tick hosts. Emma expects to graduate in August 2016, after which she will join the lab of Dr. Robert Gilmore at the Centers for Disease Control and Prevention in Fort Collins, Colorado. All should wish her luck as she will most likely freeze.