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Drivers of Rickettsial Pathogen Transmission and Spillover in Local Tick Populations in Southeastern Virginia

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DRIVERS OF RICKETTSIAL PATHOGEN TRANSMISSION AND SPILLOVER IN LOCAL TICK POPULATIONS IN SOUTHEASTERN VIRGINIA

by

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ABSTRACT

DRIVERS OF RICKETTSIAL PATHOGEN TRANSMISSION AND SPILLOVER IN LOCAL TICK POPULATIONS IN SOUTHEASTERN VIRGINIA

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Cases of spotted fever group rickettsiosis are becoming more prevalent in the United States. In Virginia, there are three human-biting ticks which are largely responsible for the spread of rickettsial pathogens and the increase in disease cases. These species include *Dermacentor variabilis, Amblyomma americanum*, and *Amblyomma maculatum*; all of which are vectors of rickettsial agents to vertebrate hosts. These species are sympatric as adults and have the potential to share large and small mammal hosts. Their interactions on and off host and their associated rickettsiae were the focus of this dissertation work. *Amblyomma americanum* is the vector of *R. amblyommatis*. *Amblyomma maculatum* is the vector of *R. parkeri*. *Dermacentor variabilis* is a vector of *R. rickettsii.* The purpose of this dissertation was to better understand and identify the potential drivers of rickettsial pathogen spillover and transmission between these species and in local host populations.

The first aim of this research was to determine the small mammals acting as hosts to immature *A. maculatum,* and to assess the role these hosts may play as potential reservoirs or amplifying hosts of *R. parkeri*. Six small mammal species were identified as hosts of immature *A. maculatum*; three of which potentially play a role in the enzootic cycle of *R. parkeri* as both carriers of infected ticks and systemically infected hosts.

The second aim of this research was to assess spillover of *R. parkeri* and *R.*

amblyommatis into local *D. variabilis* populations. Wild-caught *D. variabilis* were found to harbor *R. montanensis, R. parkeri*, and *R. amblyommatis* at all life stages. Laboratory studies found horizontal and transstadial transmission of *R. parkeri* during the larval and nymphal life stages of *D. variabilis* when co-feeding with infected *A. maculatum*.

The third aim of this research was to determine the potential role of the microbiome in the high local prevalence of *R. parkeri* seen in *A. maculatum* populations from Virginia. Microbiome analyses were performed on lab-raised and wild-caught *A. maculatum*. Overall, site and host appear to play a role in the microbiota seen in both lab-raised and wild-caught individuals.

This work is dedicated to my Lord and Savior Jesus Christ, without whom I am nothing.

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CHAPTER I

INTRODUCTION AND BACKGROUND

Over the past 15 years, arthropod-borne diseases have been increasing in incidence in the United States and globally (Sutherst, 2004; Rosenberg et al., 2018). Arthropod-borne pathogens can be bacterial, viral, or protozoal in nature and require an arthropod vector in order to be transmitted to a new host (Jongejan and Uilenberg, 2004). Mosquito-borne and tick-borne diseases are the most commonly studied and wide-spread of the arthropod-borne diseases (de la Fuente et al., 2008, WHO, 2014). In the United States, there is a higher incidence of tick-borne diseases than any other arthropod-borne diseases (Rosenberg et al., 2018). The spread of tick-borne pathogens has continued despite increased control efforts and pathogen detection technology (White and Gaff, 2018; Institute of Medicine (US) Committee on Lyme Disease and Other Tick-Borne Diseases, 2011). Tick control is different to mosquito control because ticks live in the environment and on hosts for longer periods of time (Lighton and Fielden, 1995) and require a blood-meal during all life stages (de la Fuente et al., 2008). Dependent upon species and environmental conditions, the lifespan of a mosquito is typically for days to months, while ixodid ticks can live for many years (Clements and Paterson, 1981). The immature life stages of ticks require a blood meal to transition to the next life stage whereas mosquito larvae and juvenile instars do not require a blood meal to become adult mosquitos. Requiring a blood meal at each life stage allows multiple opportunities over a longer period of time for ticks to acquire pathogens from different vertebrate hosts. For mosquitoes, the adult female life stage is the only blood-

feeder, and although she can take multiple blood meals, her overall lifespan and time in which she is able to infect susceptible hosts is shorter than that of an ixodid tick (Clements and Paterson, 1981; Styer et al., 2007). The difference in life history characteristics between vectors of disease can affect the way disease outbreaks are predicted as well as the effectiveness of control methods (Ogden and Lindsay, 2016; White and Gaff, 2018).

A better understanding of the vector ecology of local tick populations can be used to assess risk and pathogen spread in tick-endemic areas. Most tick-borne pathogens are transmitted by a specific tick, and the specific association between one tick and a tick-borne pathogen is often the focus in tick-pathogen surveillance studies (Table 1). The tick that is most commonly associated with acquisition and transmission of a specific pathogen is referred to as a principal tick vector (Sonenshine, 2005). On the other hand, a non-target species is a tick that can acquire a pathogen but may or may not be able maintain the pathogen through subsequent life stages (transstadially) or transmit the pathogen to a susceptible host (Sonenshine, 2005). Non-target tick vectors typically acquire pathogens by taking a bloodmeal from an infected host or by co-feeding with infected ticks on the same host. This interaction occurs in areas where local tick populations overlap and is referred to as pathogen spillover (Power and Mitchell, 2004).

Pathogen spillover occurs during the enzootic cycle of pathogens when they are circulating in the tick vector and in local host populations (Mwangi et al., 1991). In regard to pathogen spillover, host populations can consist of reservoir, amplifying, and non-reservoir hosts. A reservoir host is an organism that can become systemically

infected with a pathogen and maintain this pathogen while showing no ill effects. An amplifying host is not only systemically infected with the pathogen but creates an environment where the pathogen can replicate to high concentrations. Non-reservoir hosts are parasitized by ticks, but do not become systemically infected with a pathogen. Tick vectors may also serve as reservoirs of pathogens allowing them to remain circulating enzootically even in the absence of infected host populations (de la Fuente et al., 2008). Often these pathogens are commensal in their tick vector, but can cause disease once they enter a vertebrate host, such as a human. These pathogens have the potential to be acquired by multiple ticks, but in many cases cannot be transmitted to a vertebrate host (Sonenshine, 2005). *Borrelia burgdorferi*, the causative agent of Lyme disease, has been observed in *Amblyomma americanum*, the lone star tick, and *Dermacentor variabilis*, the American dog tick, but it is not transmitted transstadially nor is it transmissible to a susceptible host from the non-target ticks (Piesman and Sinsky, 1988). In other circumstances, pathogen transmission can occur from non-target tick vector. A case study in Arizona in 2002 reported 16 cases of Rocky Mountain spotted fever (RMSF), caused by bites from a non-target tick vector, *Rhipicephalus sanguineus*, the brown dog tick (Demma et al., 2005). Historically the RMSF pathogen, *R. rickettsii*, was transmitted by *D. variabilis* as its principal tick vector (Philip et al., 1978).

Table 1
Examples of principal tick vectors and their associated pathogens. Transmission traits include transstadial and transovarial transmission. The associated host(s) which are important in the enzootic cycle of the pathogen are listed.

Tick Species	Pathogen	Disease	Transmission	Reservoir or Amplifying Host	References
Blacklegged tick (*Ixodes scapularis*)	*Borrelia burgdorferi*	Lyme disease	Transstadial	White-footed mouse (reservoir and amplifier)	Piesman and Sinsky, 1988; Levin et al., 1999
	Anaplasma phagocytophilum	Anaplasmosis	Transstadial	White-footed mouse, short-tailed shrew, and eastern chipmunk (reservoirs)	Telford et al., 1996; Keesing et al., 2012
	Babesia microti	Babesiosis	Transstadial	White-footed mouse (reservoir)	Walter and Weber, 1981; Spielman et al., 1981
American dog tick (*Dermacentor variabilis*)	*Rickettsia rickettsii*	Rocky Mountain spotted fever	Transstadial Transovarial	American dog tick (reservoir)	Burgdorfer and Varma, 1967; McDade and Newhouse, 1986
	Francisella tularensis	Tularemia	Transstadial Transovarial	Voles (amplifier)	Reese et al., 2010; Philip and Jellison, 1934; Rossow et al., 2014
Gulf Coast tick (*Amblyomma maculatum*)	*Rickettsia parkeri*	*R. parkeri* rickettsiosis	Transstadial Transovarial	Unknown; potentially rice rats (amplifier)	Wright et al., 2015; Krawczak and Labruna, 2018
Lone star tick (*Amblyomma americanum*)	*Ehrlichia chaffeensis*	Ehrlichiosis	Transstadial	White-tailed deer (reservoir)	Long et al., 2003; Lockhart et al., 1997
	Ehrlichia ewingii		Transstadial	White-tailed deer (reservoir)	Anziani et al., 1990; Yabsley et al., 2002

In areas where tick-borne diseases occur, humans are usually not the target host for pathogens, but are incidental hosts following the bite of an infected tick (Dennis and Piesman, 2005). Humans are considered dead-end hosts as the pathogen no longer has the capacity to spread or cycle enzootically (Dennis and Piesman, 2005). Most tick-borne pathogens cycle enzootically in an animal reservoir host or are maintained in the tick vector as an endosymbiont (Mwangi et al., 1991). There are many factors which may contribute to tick-borne pathogens persisting in the environment and contribute to an increase in tick-borne disease incidence year after year. Host and tick community composition, tick life history, and opportunistic pathogens can all be components contributing to a specific niche in which the host and vector can persist, creating specific conditions that determine whether a pathogen can be transmitted.

Niches where hosts, tick vectors, and pathogens persist can be classified in broad terms as macrohabitats and microhabitats (Sonenshine and Mather, 1994). The macrohabitat and microhabitat can play either direct or indirect roles in pathogen introduction, maintenance, and spillover.

The macrohabitat can be defined as a large area dominated by specific environmental conditions (including temperature, humidity, and weather), as well as the plant and animal communities that live there (Sonenshine and Mather, 1994; Stitch et al., 2014) (Fig. 1). The macrohabitat can additionally be defined in the context of its proximity to other macrohabitats, such as populated towns and cities, or wildlife and domestic livestock. For example, in Tennessee in the macrohabitat of a golf-oriented retirement community, 11 people contracted cases of ehrlichiosis, a tick-borne illness

caused by the pathogen, *Ehrlichia chaffeensis,* due its close proximity to another macrohabitat, an adjacent wildlife reserve (Standaert et al., 1995).

Proximity to other macrohabitats as well as plant diversity and abundance influence what animal hosts are present and their relative abundance in the macrohabitat. For the purpose of this dissertation, small mammal host interactions with *A. maculatum*, the Gulf Coast tick, and its pathogen *R. parkeri* in southeastern Virginia were studied, but proximity to other macrohabitats and plants were not considered.

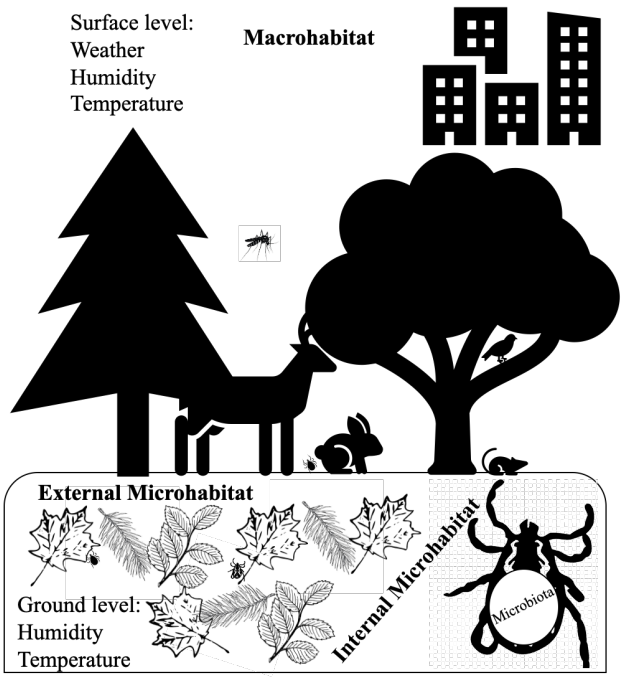

Fig. 1. Graphical depiction of each habitat level which may influence pathogen transmission.

Ticks are considered part of the animal community that exists in any macrohabitat and can interact with each other in different ways, including co-feeding on a single host or multiple hosts (Sonenshine and Mathers, 1994). Ticks acting as both vectors and reservoirs play a major role in pathogen persistence in an environment, and are influenced by other habitat conditions, including the external and internal microhabitats (Fig. 1). The external microhabitat encompasses the environmental conditions (including ground level humidity and temperature) and habitat structure at the level of the tick (often in leaf litter) which is influential for the tick's survival off host and its ability to quest effectively for a new host (Sonenshine and Mathers, 1994). Different microhabitats exist for ticks depending on whether they are off-host and inactive (potentially in diapause), off-host and actively questing, or on-host taking a blood meal (Sonenshine and Mathers, 1994; Randolph, 2004).

The movement of ticks between different microhabitats is important for understanding pathogen transmission and spillover. Ticks become 'activated' into questing behavior in the presence of chemical (pheromones, urine, sweat, CO_2, etc.) and non-chemical cues (light, temperature, movement, etc.) (Carroll et al., 2003; Carr et al., 2017). Changes in behavior states are also influenced by the life history of all ticks, which may follow phenological patterns as well as specific host preferences and questing behavior (Randolph, 2004). In the case of *D. variabilis* each of these life history factors may play a role in the maintenance and spillover of rickettsial pathogens into host populations and potentially humans.

Dermacentor variabilis is a hard-bodied tick that has a large home range in the eastern half of the United States and also on the West Coast (CDC, 2020a). This tick is

historically associated with being the primary vector of *Rickettsia rickettsii*; it has also been shown to harbor other *Rickettsia* species of unknown pathogenicity (Piesman and Gage, 1996). In Virginia, the phenology and host preference of this tick overlaps with two other ticks, *A. maculatum* and *A. americanum* as adults (Sonenshine and Stout, 1971; Merten and Durden, 2000; Nadolny and Gaff, 2019). This overlap may contribute to pathogen spillover.

Rickettsia species, like *R. rickettsii* and *R. parkeri,* are classified into a subgroup referred to as the spotted fever group rickettsiae (SFGR) (Fournier et al., 1998). This group encompasses over 20 species (Fournier et al., 1998). The SFGR group name is based on symptoms that occur during human infection; these include a spotted rash, eschar formation, and a high fever (Raoult and Paddock, 2005). Infections with SFGR are reportable to the CDC and referred to as spotted fever group rickettsioses. In Virginia, SFGR incidence has increased over the past decade, but recent reports show leveling out of cases at ~300-400 cases consistently each year (VDH, 2020). Prior to 2010, cases of spotted fever group rickettsiosis were classified as Rocky Mountain spotted fever (RMSF) which is caused by the virulent *R. rickettsii*. RMSF has a high case fatality rate compared to other spotted fever group rickettsioses, but as cases per million increased from 1920 to 2010 and case fatality decreased, *R. rickettsii* didn't appear to be the only pathogenic *Rickettsia* species involved (CDC, 2020b). In 2010, the case definition of SFGR was updated to include all pathogenic spotted fever group rickttsiae (CDC, 2020c). For Virginia, the case fatality rate is decreasing even with new cases of SFGR, suggesting that *R. rickettsii* is not the only pathogen responsible (Raoult and Paddock, 2005).

Amblyomma maculatum, the Gulf Coast tick, is another hard-bodied tick whose historical range extends from Central America to the Gulf Coast region of the United States (Nadolny and Gaff, 2018). *Amblyomma maculatum* is expanding its range up the eastern seaboard bringing *R. parkeri* to new areas (Nadolny and Gaff, 2018). In these new areas, particularly Virginia, prevalence of *R. parkeri* is higher than in the historical range of *A. maculatum,* reaching over 60% at some sites (Wright et al., 2011; Nadolny et al., 2014; Wright et al., 2015).

The introduction of *R. parkeri* with *A. maculatum* into Virginia may be one of the causes of the increased incidence of SFGR cases (Nadolny et al., 2014). *Rickettsia parkeri* is not only present in high prevalence in its target tick vector, *A. maculatum*, but has also been detected in other local ticks including *A. americanum* (Wright et al., 2015), *D. variabilis* (Henning et al., 2014), and in less common ticks, like *Ixodes brunneus* (Cumbie et al., 2020),

The life history traits of *A. maculatum, A. americanum*, and *D. variabilis* may facilitate the spillover of rickettsial pathogens into multiple tick species. Life history traits include questing behavior, human biting behavior, host preference, and phenology. These traits can be used to estimate tick bite risk and pathogen infection risk to humans. Questing is a behavior exhibited by ticks when looking for a host blood meal (Sonenshine and Mathers, 1994). Questing behavior of adult *D. variabilis* towards humans is markedly different than that of *A. maculatum* and *A. americanum* (Sonenshine et al., 1966; Carroll and Grasela, 1986; Nadolny and Gaff, 2018; Childs and Paddock, 2003). *Amblyomma maculatum* is often referred to as a cattle pest (Paddock and Goddard, 2015) and is less often reported on humans compared to *D.*

variabilis and *A. americanum* (Merten and Durdan, 2000). Both *A. maculatum* and *A. americanum* display questing behavior in which they actively move towards their host (Nadolny and Gaff, 2018; Childs and Paddock, 2003). *Dermacentor variabilis*, on the other hand, uses passive questing in which it 'sits and waits' for a host to walk by (Sonenshine et al., 1966; Carroll and Grasela, 1986). The host biting behavior of these ticks is also different. *Amblyomma maculatum* and *A. americanum* are found to bite on multiple areas of the body, often in plain sight (Childs and Paddock, 2003). Their bites can be painful or itchy, which can aid in their detection and removal. In contrast, *D. variabilis* are more often found on the head or neck without such reactions which allows them to avoid detection and removal (Slaff and Newton, 1993).

Dermacentor variabilis is of interest for its role in acquiring pathogens through spillover because of its life history and its ability to feed on humans with less detection than *Amblyomma* species (Merten and Durdan, 2000; de la Cruz et al., 1984; Slaff and Newton, 1993). Chapters two and three of this dissertation will examine the interactions of *D. variabilis* both in the context of its macrohabitat interactions with host populations and sympatric ticks as well as external microhabitat interactions on host with *A. maculatum* and the transmission of *R. parkeri*.

Pathogen maintenance and transmission can not only be influential on a tick-host level, but also on a tick-pathogen level. Once on-host and consuming a blood meal, the tick will be altering its internal microhabitat including its microbiota, which may influence pathogen acquisition and maintenance in the tick vector (Clay and Fuqua, 2010; Menchaca et al., 2013; Swei and Kwan, 2017). Pathogen maintenance inside the tick vector can be affected by different factors including host bloodmeal, the tick immune

response, and potentially the microbial communities acquired or passed from life stage to life stage or from female to offspring (transovarially). The microbial communities present inside the tick, including pathogens, make up the internal microbiome. The fourth chapter of this dissertation focuses on the role of the microbiome in the maintenance of *R. parkeri* in *A. maculatum* from southeastern Virginia.

In human studies, gut microbiota has a great influence on their health (Ding et al., 2019) and in many cases, healthy gut microbiota provides competition against pathogenic bacteria (Kamada et al., 2013). In ticks, the microbiota may play a similar role either through competition with pathogens (Paddock et al., 2015), or by facilitating success of a pathogen (Budachetri et al., 2018). The effect of microbial communities on tick fitness as well as the ability of specific tick-borne pathogens to persist and be transmitted is an important area of research when examining pathogen transmission and prevalence in tick populations (Clow et al. 2018, Macaluso et al. 2002).

There are a few microbiome studies that have been completed for *A. maculatum* collected from their historical range in Mississippi and Louisiana (Budachetri et al. 2014; Varela-Stokes et al. 2018), but there is no data about the tick microbiome in *A. maculatum* from other states where the prevalence of *R. parkeri* is higher. Most tick microbiome studies give basic information regarding major and minor phyla and genera of bacterial species present in the tick (Narasimhan and Fikrig, 2015), but not much is known of how the relative abundance or presence of each of these may affect pathogen maintenance and transmission.

To understand if the tick microbiota is playing a role in the higher prevalence of *R. parkeri* in *A. maculatum* collected in Virginia, we first need to know if there are

general patterns in microbiota composition that can be observed and used for comparing differences. The purpose of this study was to establish baseline microbiota composition and to determine differences in *A. maculatum* raised in a laboratory colony and in unrelated, field-caught *A. maculatum* adults. This information can be used to direct further research into the role of microbiome in pathogen maintenance and transmission as well as give potential insight into the causes of the higher prevalence of *R. parkeri* seen in *A. maculatum* populations in Virginia.

Targeted studies into the enzootic cycling of rickettsial pathogens in the scope of host population research, sympatric tick species, and the microbial communities present within the tick can be useful in determining potential drivers of rickettsial pathogen maintenance and transmission. Host populations are an important component of the macrohabitat in which ticks are found, and they additionally provide the microhabitat where ticks will blood-feed and acquire or transmit pathogens. Other sympatric ticks that actively quest during the same seasons or co-feed on the same hosts warrant further investigation as they are subject to pathogen spillover. The primary goal of this dissertation is to examine the potential drivers of rickettsial pathogen maintenance and transmission in southeastern Virginia using these both field and laboratory studies targeting host and tick populations.

Research Aims

In the Commonwealth of Virginia and across the United States, ticks and tick-borne pathogens are continuing to spread, even in the presence of directed control efforts and advances in medicine. Understanding more about the tick vector itself can contribute to overall understanding of how pathogen transmission occurs and persists. This dissertation examines potential drivers of tick-borne pathogen introduction, maintenance, and spillover in tick populations in southeastern Virginia. The research objectives are specifically directed toward rickettsial pathogen spillover in the context of: (1) host reservoir populations, (2) non-target tick vectors, and (3) microbiome influences.

CHAPTER II

SURVEY OF *RICKETTSIA PARKERI* IN SMALL MAMMALS AND *AMBLYOMMA MACULATUM* IN SOUTHEASTERN VIRGINIA

Introduction

The *Amblyomma maculatum* species complex encompasses a group of hard-bodied tick that have medical and veterinary importance in the Gulf and Atlantic regions of the United States (Sumner et al., 2007) and in over 10 countries in Central and South America (Santos Dias, 1993; Guglielmone et al., 2006). This species complex was originally comprised of 7 major species: *A. maculatum, A. triste*, *A. tigrinum, A. neumanni, A. parvitarsum, A. ovale,* and *A. aureolatum* (Camicas et al., 1998; Santos Dias, 1963; Santos Dias, 1993), all of which are known to bite humans and domestic livestock. Subsequently this species complex was redefined to encompass only 3 lineages: *A. maculatum, A. triste*, and *A. tigrinum* (Estrada-Pena et al., 2005); more recently it was suggested that *A. maculatum* and *A. triste* be synonymized (Lado et al., 2018). *Amblyomma maculatum*, *A. triste*, and *A. tigrinum* are known vectors of *Rickettsia parkeri* sensu stricto, the causative agent of *Rickettsia parkeri* rickettsiosis (Paddock et al., 2004; Nieri-Bastos et al., 2018). The other *Amblyomma* species (except *A. neumanni*) can harbor different strains of *R. parkeri* that may or may not be pathogens capable of causing a human rickettsiosis (Nieri-Bastos et al., 2018). Other

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important veterinary pathogens associated with these *Amblyomma* species include *Hepatozoon americanum* (Ewing and Panciera, 2003) and *Ehrlichia ruminantium* (Mahan et al., 2000).

In the U.S., *A. maculatum* has recently expanded geographically from its historical range in the southern states of the Gulf Coast into North Carolina and other Mid-Atlantic states, including Virginia, Maryland, and Delaware (Sumner et al., 2007; Wright et al., 2011; Fornadel et al., 2011; Varela-Stokes et al., 2011; Jiang et al., 2012; Florin et al., 2014). This range expansion has not only resulted in intermittent populations of *A. maculatum* in these states, but also the introduction of *R. parkeri* to these areas (Nadolny and Gaff, 2018).

For humans and large domestic animals, *R. parkeri* infection usually follows the bite of an infected adult tick (Teel et al., 2010). Infected populations of adult *A. maculatum* in their historical range show a prevalence of *R. parkeri* ranging from ~20%-40% (Sumner et al., 2007, Nadolny et al., 2014; Mays et al., 2016); however, in southeastern Virginia, *R. parkeri* prevalence can reach upward of 60% at individual sites (Wright et al., 2011; Varela-Stokes et al., 2011; Fornadel et al., 2011; Nadolny et al., 2014; Wright, 2015). Because of this high pathogen prevalence, *A. maculatum* is of particular medical importance in the Mid-Atlantic region.

Although first reported in Virginia as early as 1898 (Cooley and Kohls, 1944), established populations of *A. maculatum* have only been studied since 2010 (Fornadel et al., 2011; Nadolny and Gaff, 2018). These populations could be considered transitory or fragmented because the introduction, establishment, and die off usually occurs in less than five years (Nadolny and Gaff, 2018). Habitat and host community structure

could play a role in this phenomenon. Small mammals are abundant in early secondary successional habitat, but once the habitat transitions to later stages of succession with more woody plant species, small mammal abundance and diversity decreases (Rose et al., 2018). One possible reason for the decline in *A. maculatum* populations may be the necessity for specific rodent hosts, which disappear with the loss of herbaceous vegetation (Nadolny and Gaff, 2018). In many natural systems involving rickettsial pathogens, small mammals living in early successional habitat are often the primary hosts for immature arthropods, including ticks (Azad and Beard, 1998); such small mammals could be the primary hosts for immature *A. maculatum* in Virginia.

Collection of immature *A. maculatum* from vegetation or secondary successional environments is extremely difficult (Portugal and Goddard, 2015; Nadolny and Gaff, 2018). The most reliable methods for collecting *A. maculatum* species complex ticks are host-targeted techniques such as small mammal trapping (Barker et al., 2004; Portugal and Goddard, 2015; Colombo et al., 2018) or avian sampling (Teel et al., 1998; Gonzalez-Acuña et al., 2004; Moraru et al., 2012; Colombo et al., 2018).

The recorded small mammal hosts for immature *A. maculatum* in South Carolina (Clark et al., 1998; Clark et al., 2001), northwestern Florida (Durden et al., 2000), Mississippi (Moraru et al., 2013), and western Tennessee (Mays et al., 2016) are from two subfamilies: Sigmodontinae (includes hispid cotton rats and marsh rice rats) and Neotominae (includes woodrats, white-footed mice, and cotton mice). Overall, the studies have resulted in the collection of very few immature *A. maculatum* from small mammals, the exception being the South Carolina study where 179 *A. maculatum*

larvae and 29 *A. maculatum* nymphs were collected over a one-year period; however, 116 of these larvae came from one rodent (Clark et al., 1998).

The goal of our study was to identify small mammal hosts of immature *A. maculatum* in southeastern Virginia, and to identify hosts that could be reservoirs or amplifying hosts of *R. parkeri* and to provide information for future studies focusing on the interactions between the pathogen, the tick, and these small mammal species.

Materials and Methods

Small mammal live trapping

Modified Fitch live traps (Rose, 1994) were set along pre-marked transects at 13 trap sites (Fig. 2) across southeastern Virginia from 2011-2018. The traps were baited with a mixture of birdseed and sunflower seeds and supplemented with mealworms when insectivore populations were high. Polyester fiberfill was added to traps when nighttime temperatures had the potential to go below freezing. Traps were set in the evenings and checked every morning for a 2-4 day period per trapping session. Trapping sessions were conducted monthly or seasonally depending on the sampling year. Species, sex, weight, and reproductive condition of trapped mammals were recorded, and each small mammal was given an individually numbered metal ear tag. All small mammal handling was conducted in accordance with ODU IACUC permit #11-012, 16-003, 17-006 using guidelines set forth by the American Society of Mammalogists (Sikes et al., 2016). Additionally, a number of the small mammals from 2017 in our study were donated by local pest control companies operating in southeastern Virginia.

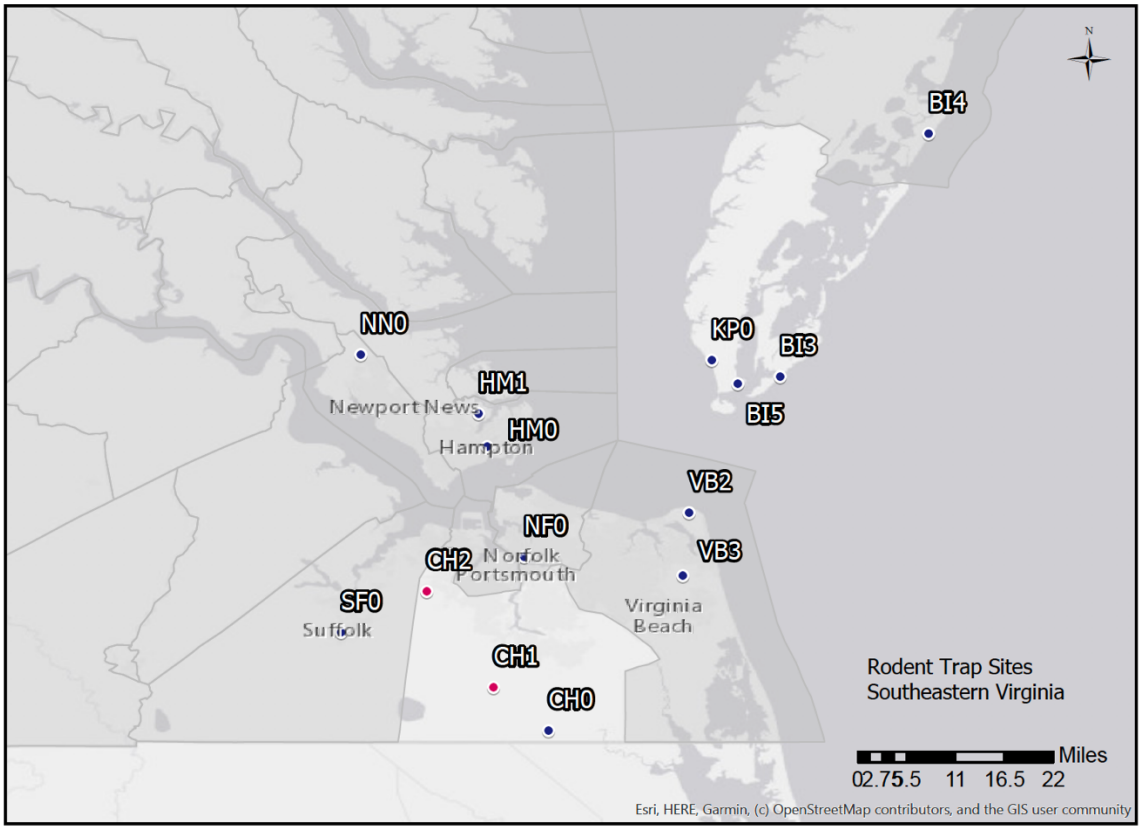

Fig. 2. Map of small mammal live-trapping sites in southeastern Virginia. Long-term monthly surveillance was done at CH1 and CH2, which area located within the City of Chesapeake. Trapping was also done at HM1 in the City of Hampton and SF0 in the City of Suffolk. Additional samples were donated from other contracted work from locations in the City of Newport News (NN0), the City of Hampton (HM0), the City of Virginia Beach (VB2 and VB3), the City of Chesapeake (CH0), in Northampton County (KP0, BI3, BI5), and in Accomack County (BI4).

Tick and tissue sampling

Trapped, small mammals were ear-tagged and given a full-body examination for ticks, with specific attention given to the face and ears. Ticks were removed and placed in a vial with a label corresponding to the mammal's ear tag number. A 2mm ear punch taken from the other ear and placed in the same vial as the ticks, if ticks were found attached to the host, or in its own vial if no ticks were present. The forceps and ear punch tool were cleaned with an alcohol swab after each use. All vials were transported back to the lab at ambient temperature then stored at -20℃ for future processing.

Tick and tissue extraction

DNA from ticks and associated mammal tissues was extracted using the GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, Pittsburgh, PA) following the manufacturer's instructions. Immature ticks were extracted whole following an initial pulverization using approximately the same volume (as the tick) of 1mm glass beads and one 5mm glass bead in a Mini Beadbeater (BioSpec, Inc. Bartlesville, OK, USA) to break apart the hard tick cuticle. After pulverization, there was an initial digestion in 180 µL of digestion buffer and 20 µL of Proteinase K at 57℃ overnight. DNA was eluted from columns in 100 µL of elution buffer. A subset of 183 ticks, collected prior to 2015 and used in a previous study identifying ticks on small mammals, were extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, CA) and DNA was eluted in 200 µL of elution buffer.

Tick identification

All ticks collected from rodents were immature life stages (larvae or nymphs), and thus could not be easily identified morphologically because of engorgement or

distortion that occurred during removal. Immature *A. maculatum* were identified by real-time PCR that amplified a variable region in the ITS2 gene of the tick genome (Appendix A). A real-time PCR assay based on the *A. maculatum* actin gene which was developed in this study (Appendix A) was used to evaluate the integrity of the tick DNA before testing for rickettsial pathogens.

A 74 bp fragment was amplified in a 20 µL reaction composed of 10 µL 2X EconoTaq PLUS (Lugien Corp., Middleton, WI), 1 µL of each *A. maculatum* actin primer (10 µM final concentration), 0.5 µL of *A. maculatum* actin probe (10 µM final concentration), and 5 µL of DNA at extraction concentration. Thermocycler conditions for the actin assay consisted of 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The *A. maculatum* actin assay primers and probe were created and modified based on the *Ixodes* actin assay developed by Graham et al. (2016). Other common tick species from our area, including *I. scapularis*, *A. americanum*, and *D. variabilis* controls, were used to determine accuracy of the assay for detecting only *A. maculatum* DNA. Twelve samples, representing ticks from different years, were confirmed by sequencing the tick mitochondrial 16S rRNA gene using the 16S+1 and 16S-1 primers (Appendix A).

Pathogen testing

All immature *A. maculatum* (collected 2011-2018) and mammal tissues (collected 2015-2018) were tested for *R. parkeri* using a real-time PCR assay that amplifies a fragment of the *omp*B gene using the Rpa129F and Rpa224R primers (Appendix A). All *R. parkeri*-positive ticks and a total of six randomly selected *R. parkeri*-positive tissues taken from each year were confirmed by sequencing a portion of the *omp*A gene using the RR190.70, RR190.701, and RR190.622n primers (Appendix A). Mammal tissue extracts were concentrated as needed by ethanol precipitation before *omp*A amplification when DNA concentration was low as determined by a C(q) value between 34 and 38 with a good peak from the real-time assay. The ethanol precipitation was performed by adding 0.10 times the sample volume (25 µL of DNA) of 3 M sodium acetate and 2.5 volumes of 100% ethanol, followed by centrifugation at 13.2 x g for 20 min, and resuspend in 10 µL of water. Sequences were initially aligned and analyzed in Geneious (Biomatters, NZ, https://www.geneious.com/) with sequence identification determined using NCBI BLAST (http://blast.ncbi.nlm.nih.gov).

Results

During our study, we collected 1,486 ticks (490 of which were *Ixodes* spp.) from 833 rodents and 217 shrews (*Blarina* spp.); shrews were only parasitized by *Ixodes* ticks. The 833 rodents included: 226 hispid cotton rats (*Sigmodon hispidus*), 16 golden mice (*Ochrotomys nuttalli*), 79 house mice (*Mus musculus*), 76 eastern harvest mice (*Reithrodontomys humulis*), 164 marsh rice rats (*Oryzomys palustris*), 182 meadow voles (*Microtus pennsylvanicus*), 68 white-footed mice (*Peromyscus leucopus*), 9 pine voles (*Pitymys pinetorum*), 9 black rats (*Rattus rattus*), 1 Eastern flying squirrel (*Glaucomys sabrinus*), and 3 small mammals that were not identified due to age or body condition. Of these small mammals, 37% (n=315) had ticks with 7% (n=22) parasitized by immature *A. maculatum*. The majority of immature ticks collected in this study were *Dermacentor variabilis*, the American dog tick (n=965 ticks), but 31 immature *A. maculatum* (16 larvae and 15 nymphs) were collected. No adult *A. maculatum* were found on small mammals in this study, and no *A. maculatum* of any life stage were found on shrews.

Six rodent species were parasitized by immature *A. maculatum* including 12 hispid cotton rats, 1 golden mouse, 3 house mice, 1 eastern harvest mouse, 1 marsh rice rat, and 4 meadow voles (Table 2). No immature *A. maculatum* were found on any white-footed mouse, pine vole, black rat, or Eastern flying squirrel. The prevalence of immature *A. maculatum* for each small mammal species by tick life stage was determined by dividing the number of ticks per life stage by the number of hosts captured per species (Table 3). The golden mouse, eastern harvest mouse, and

meadow vole were parasitized by only *A. maculatum* nymphs, whereas the house mouse and marsh rice rats only by *A. maculatum* larvae. Hispid cotton rats were parasitized by both immature *A. maculatum* life stages; most had only nymphs or larvae, but one rat had both.

Of the 31 immature *A. maculatum* collected from small mammals, 8 (25.8%) were positive for *R. parkeri*. These were from three small mammal species: 5 hispid cotton rats, 1 marsh rice rat, and 2 meadow voles. The majority of infected immatures were nymphs (6 of the 8 ticks). All eight infected ticks were confirmed, by sequencing, to be *R. parkeri*; these were 99.6-100% identical over 448-546 bp to *R. parkeri* str. Portsmouth.

Rodent ear punches collected (108 samples) from 2015 to 2018 were tested for *R. parkeri,* regardless of whether immature *A. maculatum* were present. Ear punches from 17 animals were positive for *R. parkeri* by real-time PCR. Marsh rice rats were the dominant species found with *R. parkeri*-infected tissues with 12 positive rats out of 108 tested (11%). *Rickettsia parkeri* was also detected in the tissue in 1 of 34 white-footed mice, 2 of 17 house mice, 1 of 82 meadow voles, and 1 of 9 black rats. A subsample of each of these species was sequence-confirmed for the presence of *R. parkeri*. Six *R. parkeri*-positive tissue punches were amplified using a portion of the *omp*A gene and were 99.8-100% identical over 437-546 bp to *R. parkeri* str. Portsmouth. None of the 42 cotton rats nor the one pine vole tested were positive for *R. parkeri.*

Table 2
Small mammal live trapping results by rodent species including numbers of rodents captured with *R. parkeri*-infected and un-infected immature *A. maculatum* in southeastern Virginia collected from 2011 to 2018.

Rodent species	Scientific name	Total rodents caught	Total rodents with *Dermacentor* and *Amblyomma* ticks	Total rodents with *A. maculatum*	Total rodents with *R. parkeri*-infected *A. maculatum*
Hispid cotton rat	*Sigmodon hispidus*	226	97	12	5
Golden mouse	*Ochrotomys nuttalli*	16	8	1	0
House mouse	*Mus musculus*	79	13	3	0
Eastern harvest mouse	*Reithrodontomys humulis*	76	16	1	0
Marsh rice rat	*Oryzomys palustris*	164	59	1	1
Meadow vole	*Microtus pennsylvanicus*	182	81	4	2
White-footed mouse	*Peromyscus leucopus*	68	29	0	0
Pine vole	*Microtus pinetorum*	9	8	0	0
Black rat	*Rattus rattus*	9	1	0	0
Eastern flying squirrel	*Glaucomys sabrinus*	1	0	0	0

Rodent ear punches collected (108 samples) from 2015 to 2018 were tested for *R. parkeri,* regardless of whether immature *A. maculatum* were present. Ear punches from 17 animals were positive for *R. parkeri* by real-time PCR. Marsh rice rats were the dominant species found with *R. parkeri*-infected tissues with 12 positive rats out of 108 tested (11%). *Rickettsia parkeri* was also detected in the tissue in 1 of 34 white-footed mice, 2 of 17 house mice, 1 of 82 meadow voles, and 1 of 9 black rats. A subsample of each of these species was sequence-confirmed for the presence of *R. parkeri*. Six *R. parkeri*-positive tissue punches were amplified using a portion of the *omp*A gene and

were 99.8-100% identical over 437-546 bp to *R. parkeri* str. Portsmouth. None of the 42 cotton rats nor the one pine vole tested were positive for *R. parkeri.*

Most *Rickettsia parkeri*-positive ticks came from sites with known infected adult *A. maculatum* populations (CHS1, CHS2, BI3). The other site, where immature *A. maculatum* were detected on small mammals (VB3), was not flagged so no information is available regarding infected adult *A. maculatum* populations. Interestingly, two sites (HM1, NN0) that did not yield immature *A. maculatum* did have small mammals with *R. parkeri*-infected tissues (Table 4).

Table 3
Immature *A. maculatum* collected from rodents sampled 2011-2018. The number of ticks collected from all rodents is shown in the second column and is listed by tick life stage. The percent of the rodents that represents is shown in the third column along with the total number of rodents of that species that were examined. All small mammal species not listed did not have any *A. maculatum*.

Rodent species Common name	Life stage (Number of ticks)	Percentage parasitized (Number of rodents examined)
Hispid cotton rat	Larva (8) Nymph (7)	3.5% (226) 3.1% (226)
Golden mouse	Larva (0) Nymph (1)	0 (16) 6.3% (16)
House mouse	Larva (6) Nymph (0)	7.6% (79) 0 (79)
Eastern harvest mouse	Larva (0) Nymph (3)	0 (76) 3.9% (76)
Marsh rice rat	Larva (2) Nymph (0)	1.2% (164) 0 (164)
Meadow vole	Larva (0) Nymph (4)	0 (179) 2.2% (179)

Discussion

In most enzootic cycles involving rickettsial pathogens, small mammals play a critical role as either reservoirs or amplifying hosts (Azad and Beard, 1998). To understand vector-pathogen dynamics, it is important to identify the key mammalian hosts for immature *A. maculatum*, because this tick and its associated pathogen, *R. parkeri*, are expanding into the mid-Atlantic states. Our study has identified two species of rodents (the hispid cotton rat and marsh rice rat) as potential reservoirs or amplifying hosts for *R. parkeri* in the geographical expansion zone of *A. maculatum*. Further studies into the pathogen's ecology should focus on an assessment of reservoir competency of these two species in controlled laboratory experiments.

In Central and South America, *Amblyomma* spp. are widespread, and are of particular interest because they transmit numerous rickettsial pathogens endemic to the area, including *R. parkeri* (Guglielmone et al., 2006). Studies of small mammal populations in Central and South America show that *Amblyomma* spp., specifically *A. triste*, have an affinity for feeding on sigmodontine rodents (Guglielmone et al., 2011). This same preference is seen with *A. maculatum* collected from rodents in the U.S. (Clark et al., 1998; Clark et al., 2001; Durden et al., 2000; Moraru et al., 2013; Mays et al., 2016) where rodent species hosting immature *A. maculatum* were from two rodent subfamilies: Sigmodontinae (includes hispid cotton rats and marsh rice rats) and Neotominae (includes woodrats, white-footed mice, and cotton mice). Muroid rodents (including house mice and black rats), however were not found to act as hosts for immature *A. maculatum*.

The rodent sub-family harboring the majority of immature *A. maculatum* in our study was Sigmodontinae. *Rickettsia parkeri*-infected *A. maculatum* and *R. parkeri*-infected rodent tissues were collected from hispid cotton rats and marsh rice rats, suggesting that these rodents may play an important role as reservoirs or amplifying hosts of *R. parkeri*. Alternatively, these rodents may only be acting as hosts to immature infected *A. maculatum* ticks but not be systemically infected with *R. parkeri*. In addition, *R. parkeri*-infected *A. maculatum* were collected from two meadow voles (subfamily Arvicolinae). One meadow vole tissue sample tested positive for *R. parkeri*, which may be indicative of meadow voles as another important species in the ecological maintenance of *R. parkeri*. Additional studies are required to explore the relationship between immature *A. maculatum* and rodents from these subfamilies.

The anthropophilic house mouse yielded the highest infestation rate with immature *A. maculatum* (Table 3), suggesting that there may be potential hosts thriving in habitats close to human settlements where other successional (and native) species of rodents may be absent. We also found *R. parkeri*-infected tissues from a white-footed mouse and a black rat, even though neither of these species, in our study, harbored immature *A. maculatum*. It is possible that immature *A. maculatum* ticks had previously parasitized these animals and dropped off; the vector-pathogen dynamics of *R. parkeri* in these species warrants additional investigation.

Table 4
Numbers of immature *A. maculatum* collected at small mammal live-trapping sites in southeastern Virginia.

Trapping site	Year	Number of *A. maculatum* removed from rodents	Life stage	Number of *R. parkeri* infected *A. maculatum*	Proportion of *R. parkeri* infected tissues
CH1	2011	4	1L, 3N	1	Not tested
CH1	2012	14	5L, 9N	6	Not tested
CH2		0	N/A	0	Not tested
VB2		0	N/A	0	Not tested
CH1	2013	0	N/A	0	Not tested
CH2		7	4L, 3N	1	Not tested
SF0		0	N/A	0	Not tested
KP0		0	N/A	0	Not tested
BI3		0	N/A	0	Not tested
CH1	2014	0	N/A	0	Not tested
KP0		0	N/A	0	Not tested
HM1		0	N/A	0	Not tested
HM1	2015	0	N/A	0	0%
BI3		2	2L	0	12%
BI4		0	N/A	0	0%
BI5		0	N/A	0	0%
HM1	2016	0	N/A	0	0%
HM1	2017	0	N/A	0	0.9%
HM0		0	N/A	0	0%
NN0		0	N/A	0	0.9%
HM1	2018	0	N/A	0	0%
CH0		0	N/A	0	0%
VB3		4	4L	0	1.9%

*The site designations that correspond to those given in Fig. 2.
**L = larva and N = nymph.

Positive rodent tissues were collected at sites without a known established population of adult *A. maculatum*, suggesting *A. maculatum* is potentially present but not detected or the *R. parkeri* is coming from some other source. It is possible that other ticks, such as *D. variabilis* and/or *A. americanum*, could be playing cryptic roles in the

enzootic cycle of *R. parkeri*. Although these ticks are not currently known to transmit *R. parkeri* to humans, they have been found to be naturally infected with *R. parkeri* in field studies (Cohen et al., 2009; Fornadel et al., 2011; Henning et al., 2014; Wright et al., 2015). Furthermore, there is laboratory evidence of their ability to acquire *R. parkeri* transovarially, maintain *R. parkeri* transtadially, and infect animals (Harris et al., 2017; Wright et al., 2015).

It is important to note the rarity of finding immature *A. maculatum*: only 31 immature *A. maculatum* were found on 833 rodents in 8 years of study. Continued studies at our sites in southeastern Virginia, as well as at other sites where *A. maculatum* populations become established, will provide insight into the hosts, life history and phenology of the immature stages of *A. maculatum* and a better understanding of the introduction and establishment of this tick species as it continues to expand its current range.

CHAPTER III

DERMACENTOR VARIABILIS IN SOUTHEASTERN VIRGINIA AND ITS ASSOCIATED RICKETTSIAE INFLUENCED BY PATHOGEN SPILLOVER FROM LOCAL SYMPATRIC TICK SPECIES

Introduction

Dermacentor variabilis, the American dog tick, is a hard-bodied tick (family Ixodidae) present on the east and west coasts of the United States, with its range expanding into the entire region east of the Rocky Mountains (CDC, 2020a). This species is characterized by uniquely decorated dorsal scuta (Keirans and Litwak, 1989; Dryden and Payne, 2004), relatively sedentary or short-distance host-seeking behavior (Sonenshine et al., 1966; Carroll and Grasela, 1986), and specific edge habitat usage (Sonenshine and Stout, 1968a; Sonenshine and Levy, 1972). Despite their passive questing behavior relative to more tenacious questing hard-bodied ticks in the US, including *A. americanum* and *A. maculatum*, *D. variabilis* are active human biters and often go undetected when feeding, because they attach in low visibility areas such as around the head and neck (de la Cruz et al., 1984; Slaff and Newton, 1993). Adult *D. variabilis* feed on medium to large mammals and frequently parasitize humans (Smith et al., 1946; Sonenshine et al., 1971; Merten and Durden, 2000). The immatures of this species do not typically bite humans, but primarily feed on small mammals such as rodents and lagomorphs (Smith et al., 1946; Sonenshine, 1972).

In Virginia and across the US, *D. variabilis* is historically known as the vector of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF), but it has

been observed to harbor a variety of other rickettsiae, including *R. montanensis* and *R. bellii* (McDade and Newhouse, 1986; Piesman and Gage, 1996; Stromdahl et al., 2011; Gleim et al., 2016; Philip et al., 1983; Carmichael and Fuerst, 2010). Rocky Mountain spotted fever was detected in the US as early as the 1880s (Maxey, 1899), but *R. rickettsii* was not recognized as the causative agent until 1906 (Ricketts, 1906). This virulent pathogen was and is still known for a high case fatality rate (Paddock et al., 1999). From the 1940s to the 1980s, *R. rickettsii* case-fatality rates in the US ranged from 5% to 10% (Hattwick et al., 1978; D'Angelo et al., 1978; Bernard et al., 1978). Presently, case-fatality rates associated with RMSF are declining (Dalton et al., 1995). The prevalence of this pathogen in *D. variabilis* populations has also declined and higher prevalences of other spotted fever group rickettsiae (*R. montanensis*, *R. bellii*, *R. amblyommatis*, and *R. parkeri*) have been observed (Hecht et al., 2019).

Spotted fever group rickettsiae (SFGR), comprised of over 20 species of obligate intracellular bacteria, include many human pathogens (Fournier et al., 1998). At least two other tick species, besides *D. variabilis,* are vectors of SFGR in Virginia: *A. maculatum* and *A. americanum*. These hard-bodied ticks are known vectors of *R. parkeri* and *R. amblyommatis*, respectively (Paddock et al., 2004; Apperson et al., 2008).

Amblyomma maculatum populations were first detected in Virginia in 2010 and had a higher prevalence of *R. parkeri*, the causative agent of *R. parkeri* rickettsiosis, than in historical populations along the Gulf Coast of the US (Sumner et al., 2007; Fornadel et al., 2011; Wright et al., 2011). On the other hand, *A. americanum* populations were detected much earlier, with establishment records dating back to the

1960s (Sonenshine and Levy, 1971). *Amblyomma americanum* is an aggressive human-biting tick which has increased in numbers in the coastal Mid-Atlantic region of the US over the past century due an increase in white-tailed deer populations (Paddock and Yabsley, 2007). Presently, *A. americanum* is the most dominant tick in southeastern Virginia, surpassing both *D. variabilis* and *A. maculatum* populations (Nadolny et al., 2014). This species is a vector of many tick-borne pathogens, including *Ehrlichia chaffeensis*, *E. ewingii*, *R. amblyommatis* (its associated endosymbiont; pathogenicity unknown), and *R. parkeri*, potentially the result of spillover from *A. maculatum* (Ewing et al., 1995; Anziani et al., 1990; Jiang et al., 2010, Wright et al., 2015).

There is little evidence of shared hosts among the three ticks during their immature life stages; *D. variabilis* and *A. maculatum* feed on small mammals during different times of year, while *A. americanum* prefers medium to large mammals for all life stages. However, as adults, all three ticks share similar phenology and host preferences (Sonenshine and Stout, 1971; Zimmerman et al., 1988; Wright et al., 2015; Nadolny and Gaff, 2018); this overlap could facilitate “pathogen spillover” between these species.

Pathogen spillover of rickettsiae has been studied in some detail for *A. americanum* (Wright et al., 2015), but is lacking for *D. variabilis,* with the exception of a few observational studies (Fornadel et al., 2011; Nadolny et al., 2014). Experimentally, *D. variabilis* has been observed to acquire *R. parkeri* through capillary tube feeding and to transmit it transovarially and transstadially (Harris et al., 2017), but little is known about natural transmission from an infected host or whether *D. variabilis* is a competent

vector of *R. parkeri*. The purpose of this study was to assess phenological and host overlap between these sympatric ticks and assess changes in rickettsial pathogen prevalence and spillover in *D. variabilis* from local *A. americanum* and *A. maculatum* populations.

Material and Methods

Study area

Dermacentor variabilis, *A. americanum,* and *A. maculatum* were collected from twelve field sites in southeastern Virginia between 2011 and 2018 (Fig. 3). Blue represents sites where vegetation was sampled between 2011 to 2018. Red represents sites with established *A. maculatum* populations. Yellow represents sites with reported *A. maculatum* populations. *Amblyomma maculatum* populations were considered established if >6 ticks of the same life stage were collected in the county over a 12-month period (Dennis et al., 1998). *Amblyomma maculatum* populations were considered reported if <6 ticks of the same life stage were collected over a 12-month period (Dennis et al., 1998). *Amblyomma maculatum* populations were observed infrequently at sites YRK1, PT1, PT2, HM1, SF1, VB3, and KP0. All sites had established *D. variabilis* populations and the majority of sites (11/12) had established *A. americanum* populations. These sites were not designated by a specific color.

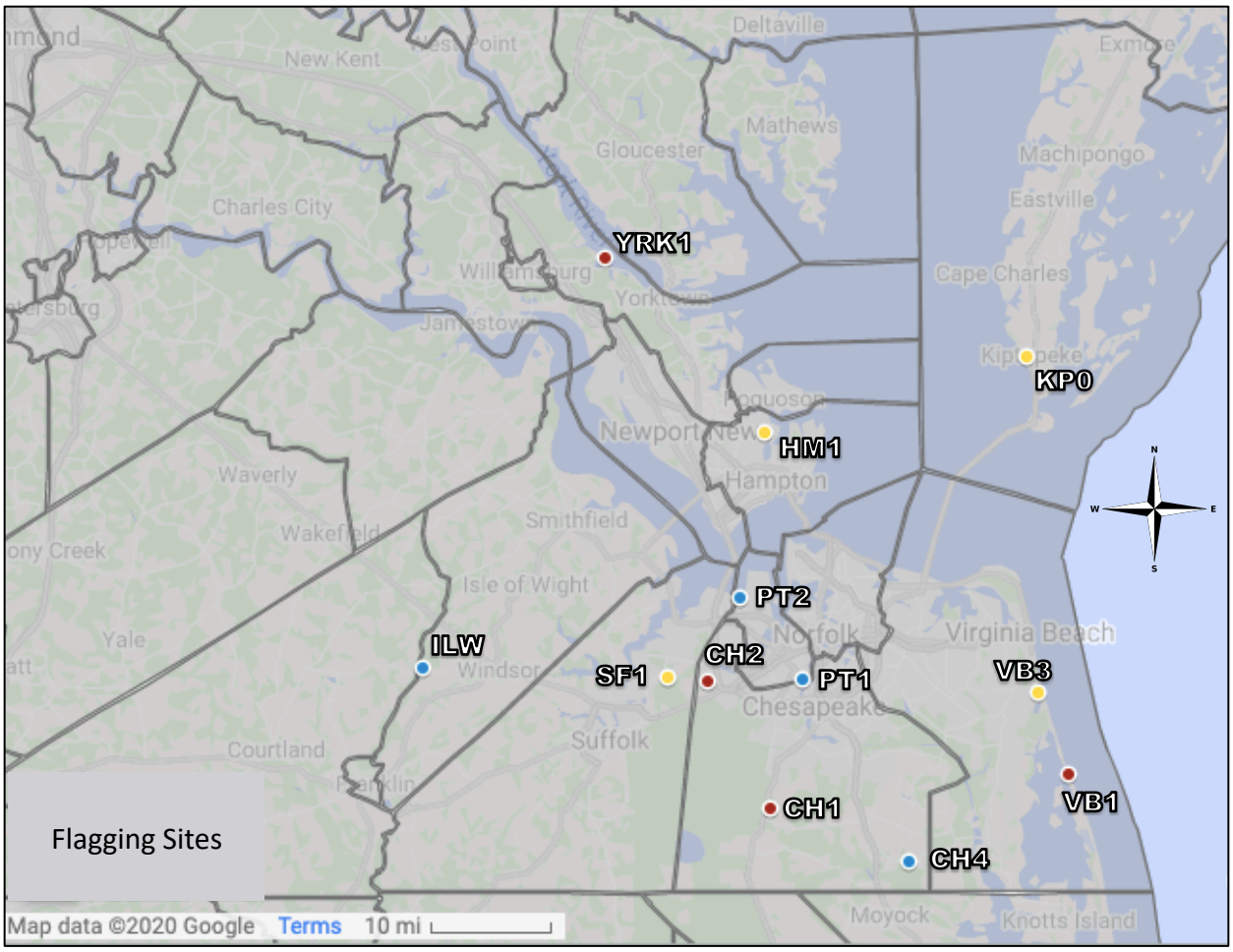

Fig. 3. Regular flagging sites in southeastern Virginia for collection of all three ticks from vegetation. Blue markers represent any site where vegetation was sampled between 2011 and 2018. Red markers represent sites with established *A. maculatum* populations. Yellow markers represent sites with reported *A. maculatum* populations. This map was generated using GoogleMyMaps.

Collecting adult ticks from vegetation

Ticks were collected from vegetation at each site by sweeping a 1m^2 white denim flag attached to a dowel rod (Ginsberg and Ewing, 1989). Tick collections were conducted year-round on a weekly or bi-weekly basis during their active questing season, between early spring and late summer, and monthly during the fall and winter

months. Ticks were placed in plastic vials in the field, kept at ambient temperature, and returned to the lab for storage in a -20°C freezer prior to processing.

Collection of immature ticks from small mammal hosts

Modified Fitch live traps (Rose, 1994) were used at five field sites (CH1, CH2, KP0, HM1, VB3) between 2011 and 2018. Trapping sessions were conducted monthly or seasonally depending on the sampling year. Site CH1 was sampled from 2011 to 2013 when established *A. maculatum* populations were present. Site CH2 was sampled from 2012 to 2013 while established *A. maculatum* populations were present. Sites KP1, HM1, and VB3 were sampled infrequently by small mammal trapping. Trapping sessions typically lasted 2-3 trap nights per trapping session. Traps were baited with birdseed and sunflower seeds, and supplemented with mealworms when insectivorous populations were present. When temperatures had the potential to drop below freezing, polyester fiberfill was added to each trap. Small mammals were examined for ticks and tagged with a unique metal ear tag. A tissue sample (ear punch) was also taken from their ear. All ticks and tissues were kept at ambient temperature and returned to the lab for storage in a -20°C freezer prior to processing. All small mammal handling was conducted in accordance with ODU IACUC permit #11-012, 16-003, and 17-006.

Collection of ticks from medium to large animal hosts

Ticks were collected from animals between 2010 and 2019. Some ticks used were donated by wildlife rehabilitators across the state of Virginia, veterinarians, groomers, and pet owners. Other methods of tick collection from animals included: serendipitous sampling from roadkill and removal of ticks from animals at hunt check stations. During sampling from roadkill or at hunt check stations, ticks were removed using forceps and placed in individually labeled vials or plastic bags with the date, location, and host animal species. All samples were transferred to the lab for storage in a -20°C freezer within a week of collection.

DNA extraction and tick identification

Dermacentor variabilis were extracted for DNA and tested for rickettsiae. Adult *D. variabilis* were identified morphologically (Keirans and Durdan, 1989) then cut in half bilaterally with one half placed in site-specific pools of 1 to 20 ticks per pool. Pools were extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, CA).

Immature *D. variabilis* collected from small mammals were extracted whole using the GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, Pittsburgh, PA) following the manufacturer's instructions with an initial digestion overnight at 57ºC. Both larvae and nymphs were identified using a real-time PCR assay amplifying a variable region in the ITS2 gene of *D. variabilis* (Shone et al., 2006). The primers and probe used in this PCR were DvalTS2b-F (CTGAAGATTCTTTGCGAGGAGCGG), DvalTS2b-R (GCGTCAGCTCGGCCAAC), and DvarITS2-P (FAM/AGAAGGGCGTGCCCGAAAGCGG/BHQ1). Each PCR (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1 µL of

each primer (10 µM final concentration), 1 µL of probe (10 µM final concentration), and 2 µL of DNA at extraction concentration. The thermocycler conditions consisted of 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s, 60ºC for 1 min.

A subset of *D. variabilis* were confirmed to species by sequencing the tick mitochondrial 16S rRNA gene (de la Fuente et al., 2001; Nadolny et al., 2011) using the primers 16S+1 and 16S-1 (Appendix A). PCR products for use in sequencing reactions were purified using Wizard PCR preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sequencing reactions were performed using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were initially aligned and analyzed in Geneious (https://www.geneious.com/) with sequence identification determined using the BLAST tool in the NCBI database (http://blast.ncbi.nlm.nih.gov; Altschul et al., 1990).

Rickettsia identification

Four real-time PCR assays were used to determine the presence of *Rickettsia* spp. in both adult and immature *D. variabilis* (Appendix A). All samples were initially analyzed for the presence of a portion of the rickettsial 17-kDa gene to detect the presence of any *Rickettsia* species in the sample (Jiang et al., 2012). Each rickettsial 17-kDa gene real-time PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1.25 µL of each primer (10 µM final concentration), 1 µL of probe (10 µM final concentration), 2 µL $MgCl_2$ (25 mM final concentration), 2 µL of nuclease-free water, and 5 µL DNA at extracted concentration. Any positives from the rickettsial 17-kDa assay were tested for three *Rickettsia* species: *R. montanensis, R. amblyommatis,* and *R. parkeri*. No samples were

tested for *R. bellii* as it is not a common rickettsial species in this study region. The *R. montanensis*, *R. amblyommatis,* and *R. parkeri* real-time PCR assays amplified a portion of the *omp*B gene (Appendix A). The *R. montanensis* real-time PCR reaction (final volume 20 µL) consisted of 10 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1 µL of each primer (10 µM final concentration), 1 µL of probe (10 µM final concentration), 4 µL $MgCl_2$ (25 mM final concentration), and 3 µL DNA. The *R. parkeri* real-time PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1.75 µL of each primer (10 µM), 1 µL of probe (10 µM), 42.5µL $MgCl_2$ (25 mM), 0.5 µL of nuclease-free water, and 5 µL DNA at extracted concentration. The *R. amblyommatis* real-time PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1.25 µL of each primer (10 µM), 1 µL of probe (10 µM), 3 µL $MgCl_2$ (25 mM), 4µL of nuclease-free water, and 2 µL DNA. The thermocycler conditions for all four assays included a pre-hold step of 50°C for 2 min, a denaturing step of 95°C for 2 min, followed by 45 cycles of: 95°C for 15 s and 60°C for 30 s, and a plate read after each cycle.

A subset of *R. parkeri*-positive samples was confirmed by sequencing of a portion of the *omp*A gene (Blair et al., 2004; Regnery et al., 1991). All *R. parkeri*-positive adult pools were confirmed using the *omp*A gene by sequencing individuals from the positive pools. Three *R. parkeri*-positive immature *D. variabilis* were also sequence confirmed. Sequences were initially aligned and analyzed in Geneious (https://www.geneious.com/) with sequence identification determined using the BLAST tool in the NCBI database (http://blast.ncbi.nlm.nih.gov; Altschul et al., 1990).

Pathogen prevalence estimation

Maximum likelihood estimates (MLE) were used for pooled adult ticks to calculate the prevalence of *Rickettsia* species by year. Pooled infection rates were calculated using the binGroup package in R (Zhang et al., 2018). For immature ticks that were extracted and tested individually, prevalence was calculated by dividing the total number of positive ticks by the total number of ticks tested.

Results

Phenological overlap

From 2011 to 2018, over 11,000 adult (2,364 *D. variabilis*, 396 *A. maculatum*, and 8,419 *A. americanum*) and over 72,000 (931 *D. variabilis*, 52 *A. maculatum*, 71,052 *A. americanum*) immature ticks were collected from vegetation and host sampling (Table 5). The majority of ticks captured at all life stages were *A. americanum*, but for this study, we focused on only the adults of this species, because as adults they share hosts with both *D. variabilis* and *A. maculatum*. No immature *A. americanum* were found on small mammals thus small mammal cofeeding was only analyzed for immature *D. variabilis* and *A. maculatum*.

Phenological overlap was observed between the three tick species on vegetation during the adult life stage (Fig. 4). Adult *A. americanum* had the highest abundance peaking in March through June. Adult *D. variabilis* were active from April to August with peak activity in May and June. Adult *A. maculatum* were active from May to September with peak activity in June. All species were observed less frequently from vegetation starting in September.

Table 5
Number of ticks collected from 2011-2018 by tick species, life stage, and origin.

Tick Species	Life Stage	# of Ticks from Vegetation	# of Ticks from Medium to Large Mammals	# of Ticks from Small Mammals
	Larva	0	0	733
D. variabilis	Nymph	3	9	186
	Adult	1569	795	0
	Larva	6	0	16
A. maculatum	Nymph	12	3	15
	Adult	383	13	0
	Larva	58,429	238	0
A. americanum	Nymph	11,753	632	0
	Adult	7008	1,411	0

Phenological overlap of all three ticks was also seen on host with all life stages of each tick represented in the phenology (Fig. 5). *Amblyomma americanum* were found on medium to large mammals hosts throughout the entire year with peak activity in May and June as well as in October. *Dermacentor variabilis* were found on medium to large mammals during the spring and summer with peak activity in May and July. Overall, *A. maculatum* were scarce when surveying medium to large mammals for ticks with variable collections throughout the year.

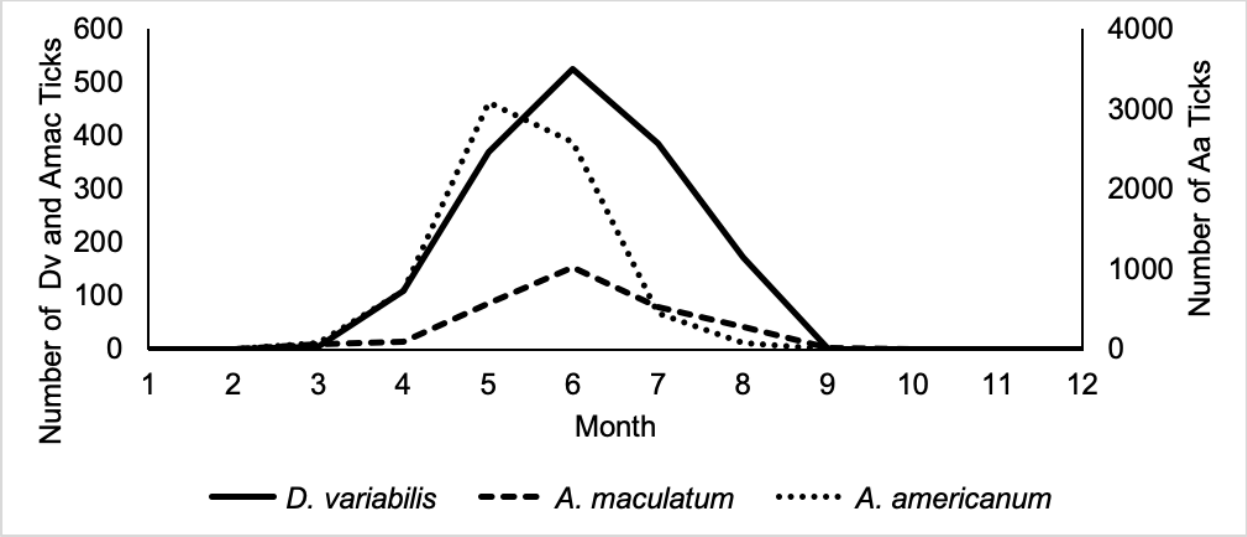

Fig. 4. Number of adult ticks within each species by month of the year over all years from tick collections on vegetation. The primary y-axis represents numbers of *D. variabilis* and *A. maculatum*. The secondary y-axis is numbers of *A. americanum*.

Phenological overlap was assessed for immature *D. variabilis* and *A. maculatum* across all sites where small mammal trapping occurred (Fig. 6A&B). *Dermacentor variabilis* larvae and nymphs appear to be active intermittently throughout the year with peak activity in the winter months for larvae, and peak activity in May and September for nymphs. Very few immature *A. maculatum* were collected from small mammals in this study. Based on very small sample sizes, peak activity for larvae was in August and November, and peak activity for nymphs was in April, August, and September. The months of April and September through November are time periods when all ticks appear to have overlapping phenologies.

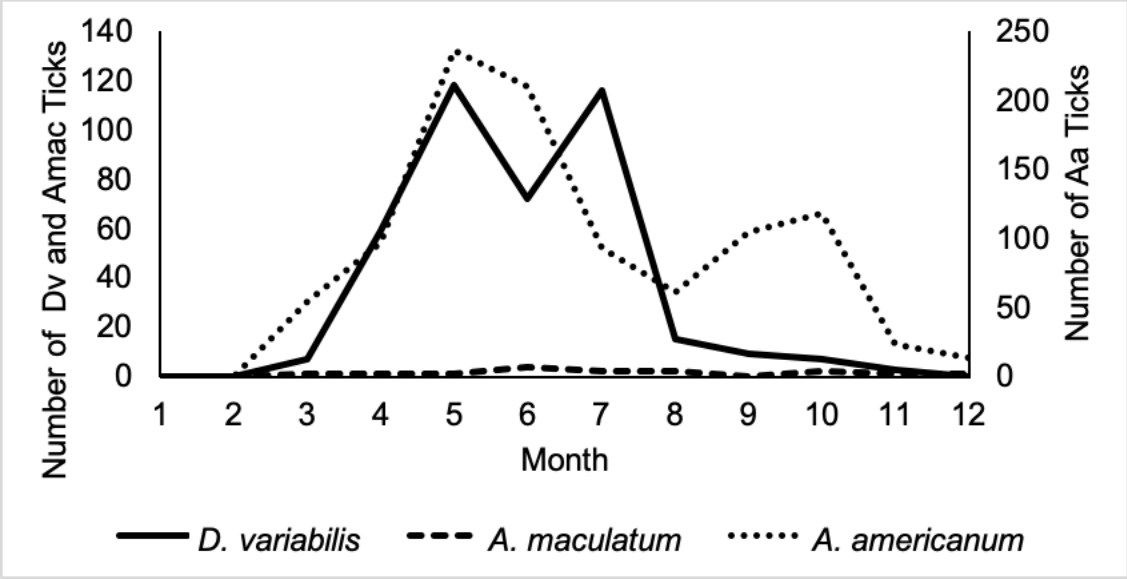

Fig. 5. Number of ticks within each species by month of the year over all years from tick collections on medium to large mammals. The primary y-axis represents numbers of *D. variabilis* and *A. maculatum*. The secondary y-axis is numbers of *A. americanum*.

Host overlap

As adults, *D. variabilis*, *A. americanum*, and *A. maculatum* were found parasitizing many of the same medium to large mammal hosts (Table 6). All three ticks were found parasitizing coyotes (*Canis latrans*), dogs (*Canis lupus familiaris*), cats (*Felis catus*), deer (*Odocoileus virginianus*), red foxes (*Vulpes vulpes*), gray foxes (*Urocyon cinereoargenteus*), and feral hogs (*Sus scrofa*). *Amblyomma americanum* were the most abundant tick on any medium to large mammal host, especially on *O. virginianus* and *C. lupus familiaris*. *Dermacentor variabilis* were collected most frequently from *U. americanus* and *C. lupus familiaris*. Albeit a low sample size, *A. maculatum* were found parasitizing *O. virginianus*, *S. scrofa*, and *C. latrans* simultaneous to *D. variabilis* and *A. americanum*.

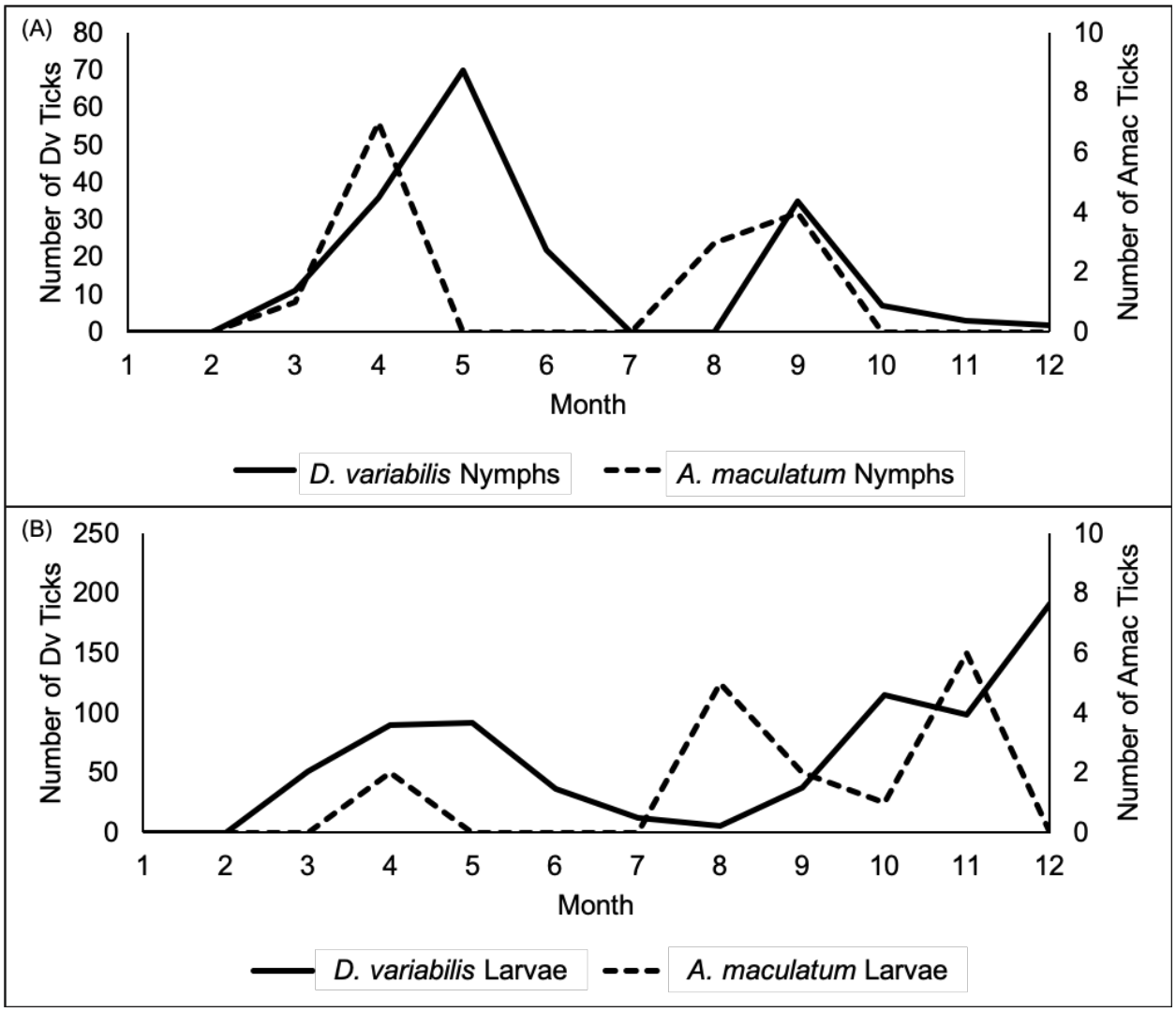

Fig. 6. (A) Phenology for larval *D. variabilis* and *A. maculatum* collected from small mammal hosts by month of the year over all years. The primary y-axis represents numbers of *D. variabilis.* The secondary y-axis is numbers of *A. maculatum.* (B) Phenology for nymphal *D. variabilis* and *A. maculatum* collected from small mammal hosts by month of the year over all years. The primary y-axis represents numbers of *D. variabilis.* The secondary y-axis is numbers of *A. maculatum.*

As immatures, *D. variabilis* and *A. maculatum* fed on mostly small mammals and were rarely found on medium to large mammals (Table 7). As nymphs, *D. variabilis* and *A. maculatum* parasitized hispid cotton rats (*Sigmodon hispidus*), meadow voles (*Microtus pennsylvanicus*), eastern harvest mice (*Reithrodontomys humulis*), and golden mice (*Ochrotomys nuttalli*). As larvae, *D. variabilis* and *A. maculatum* were found parasitizing hispid cotton rats (*Sigmodon hispidus*), marsh rice rats (*Oryzomys palustris*), and house mice (*Mus musculus*). These numbers include rodents parasitized by both ticks upon capture as well as rodents parasitized by either tick at any point during trapping.

Rickettsia prevalence and spillover

Dermacentor variabilis collected from vegetation and small mammal sampling were tested for *R. montanensis, R. parkeri*, and *R. amblyommatis* by real time PCR (Table 8). *Rickettsia montanensis* was the most prevalent rickettsia present with prevalence fluctuating from year to year. In D. variabilis populations, *R. montanensis* pooled prevalence over all years was 2.3% (95% CI: 1.6%-3.1%) in adults and 6.1% (95% CI: 4.6%-7.9%) in immatures. For adult *D. variabilis*, *R. amblyommatis* pooled prevalence was <1% (95% CI: 0.0%-0.4%) over all years. Interestingly, immature *D. variabilis* showed *R. amblyommatis* prevalence at 1.1% (95% CI: 0.6%-2.1%) over all years. Adult and immature *D. variabilis* were observed to harbor *R. parkeri* during 2012, 2013, and 2016. *Rickettsia parkeri* pooled prevalence was 0.5% (95% CI: 0.2%-0.9%) over all years in adult *D. variabilis* and 3.2% (95% CI: 2.2%-4.6%) over all years in immature *D. variabilis*.

Table 6
Medium to large mammal sampling from 2010-2019. The medium to large mammal hosts were surveyed through convenience sampling from veterinarian donation, pet owner donation, wildlife rehabilitation donation, hunt check surveys, and roadkill surveys. The last three columns labeled with tick species represent the number of hosts found parasitized by that tick.

Host Category	Host	Scientific Name	Hosts Surveyed	*D. variabilis*	*A. americanum*	*A. maculatum*
Large Mammals	Deer	*Odocoileus virginianus*	195	3	87	6
	Black bear	*Ursus americanus*	37	21	15	0
	Feral hog	*Sus scrofa*	26	6	19	1
	Coyote	*Canis latrans*	11	9	7	4
	Horse	*Equus caballus*	4	0	4	0
	Bobcat	*Lynx rufus*	1	1	0	0
Meso-mammals	Dog	*Canis lupus familiaris*	321	109	164	1
	Cat	*Felis catus*	65	8	28	1
	Eastern cottontail	*Sylvilagus floridanus*	39	0	2	0
	Raccoon	*Procyon lotor*	29	16	10	0
	Opossum	*Didelphis virginiana*	16	13	3	0
	Red Fox	*Vulpes vulpes*	10	4	4	1
	Gray Fox	*Urocyon cinereoargenteus*	9	1	2	1
	Groundhog	*Marmota monax*	2	1	1	0

Table 7
Small mammal hosts of immature *D. variabilis*, *A. americanum*, and *A. maculatum* collected between 2011 and 2018. The last three columns labeled with tick species represent the number of hosts found parasitized by that tick.

Host	Scientific Name	Hosts Surveyed	*D. variabilis*	*A. americanum*	*A. maculatum*
Hispid cotton rat	*Sigmodon hispidus*	226	90	0	12
Meadow vole	*Microtus pennsylvanicus*	182	80	0	4
Marsh rice rat	*Oryzomys palustris*	164	59	0	1
House mouse	*Mus musculus*	79	12	0	3
Eastern harvest mouse	*Reithrodontomys humulis*	76	14	0	1
White-footed mouse	*Peromyscus leucopus*	68	28	0	0
Golden mouse	*Ochrotomys nuttalli*	16	6	0	1
Pine vole	*Microtus pinetorum*	9	8	0	0
Black rat	*Rattus rattus*	9	1	0	0
Gray Squirrel	*Sciurus carolinensis*	3	0	0	0

Table 8
Prevalence (percentage) of *Rickettsia* spp. present in *D. variabilis* collected from vegetation across all sites and analyzed separately by year. Adults were tested in pools and prevalence was measured using maximum likelihood estimation using the R package binGroup. Immatures were tested individually.

Year	# of ticks	*R. montanensis*		*R. amblyommatis*		*R. parkeri*	
		Adults	Juveniles	Adults	Juveniles	Adults	Juveniles
2012	n_A=416 n_I=241	0.5%	0.4%	0.0%	0.8%	0.0%	4.6%
2013	n_A=379 n_I=251	1.4%	11.2%	0.0%	2.4%	1.7%	5.6%
2014	n_A=162 n_I=106	1.2%	12.3%	0.6%	0.0%	0.0%	0.0%
2015	n_A=175 n_I=123	1.8%	3.3%	0.6%	0.0%	0.0%	0.0%
2016	n_A=158 n_I=69	1.3%	1.5%	0.0%	1.5%	0.6%	0.0%
2017	n_A=95 n_I=12	2.1%	0.0%	0.0%	0.0%	0.0%	0.0%
2018	n_A=63 n_I=23	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

*n_A = number of adult ticks and n_I = number of immature ticks

During 2012 and 2013, established *A. maculatum* populations were present at three sites (VB1, CH1, and CH2). *Rickettsia parkeri* spillover into *D. variabilis* populations occurred at these sites as indicated by the higher prevalence of *R. parkeri* during these years (Table 8). Site VB1 had no *Rickettsia* positives during any years of sampling regardless of the presence of sympatric ticks. Sites CH1 and CH2 were analyzed further to observe potential trends in the prevalence of *R. parkeri* and *R. montanensis* in *D. variabilis* populations in the presence of established *A. maculatum* populations (Fig. 7). *Rickettsia parkeri* was more prevalent than *R. montanensis* in adult *D. variabilis* during these years. Once the established *A. maculatum* populations were

no longer observed at these sites, the dominant rickettsial species in adult *D. variabilis* became *R. montanensis* (Fig. 7).

For immature *D. variabilis* collected at these sites from 2011 to 2013, *R. parkeri* and *R. montanensis* were both prevalent in the ticks collected from small mammals. *Rickettsia montanensis* prevalence in immature *D. variabilis* at site CH1 was 2.9% in 2011, 0.6% in 2012, and 17.6% in 2013. From 2011 to 2013, *R. parkeri* prevalence in immature *D. variabilis* at site CH1 was from 2.0% to 2.9%. At site CH2, *R. montanensis* prevalence in immature *D. variabilis* was 0.0% in 2012 and 10.9% in 2013. *Rickettsia parkeri* prevalence at this same site was 3.1% in 2012 and 6.2% in 2013. In 2013 the established *A. maculatum* populations were starting to die out and *R. montanensis* became more prevalent in immature *D. variabilis* than *R. parkeri*. Sites CH1 and CH2 were not sampled through small mammal trapping in the years following 2013 so additional analysis was rickettsial prevalence was not observed.

Rickettsia parkeri presence was detected in 30 immature *D. variabilis* during small mammal tick sampling. The majority of *R. parkeri*-positive ticks were nymphs (20/30). Five small mammal host species were parasitized by *R. parkeri*-positive immature *D. variabilis*: eastern harvest mouse, hispid cotton rat, marsh rice rat, meadow vole, and white-footed mouse (Fig. 8). Hispid cotton rats, marsh rice rats, and meadow voles were also parasitized by *R. parkeri*-infected immature *A. maculatum* making these small mammal species the most likely hosts for pathogen spillover. The other two species, eastern harvest mouse and white-footed mouse, were not parasitized by any immature *A. maculatum,* but harbored *R. parkeri*-positive *D. variabilis*.

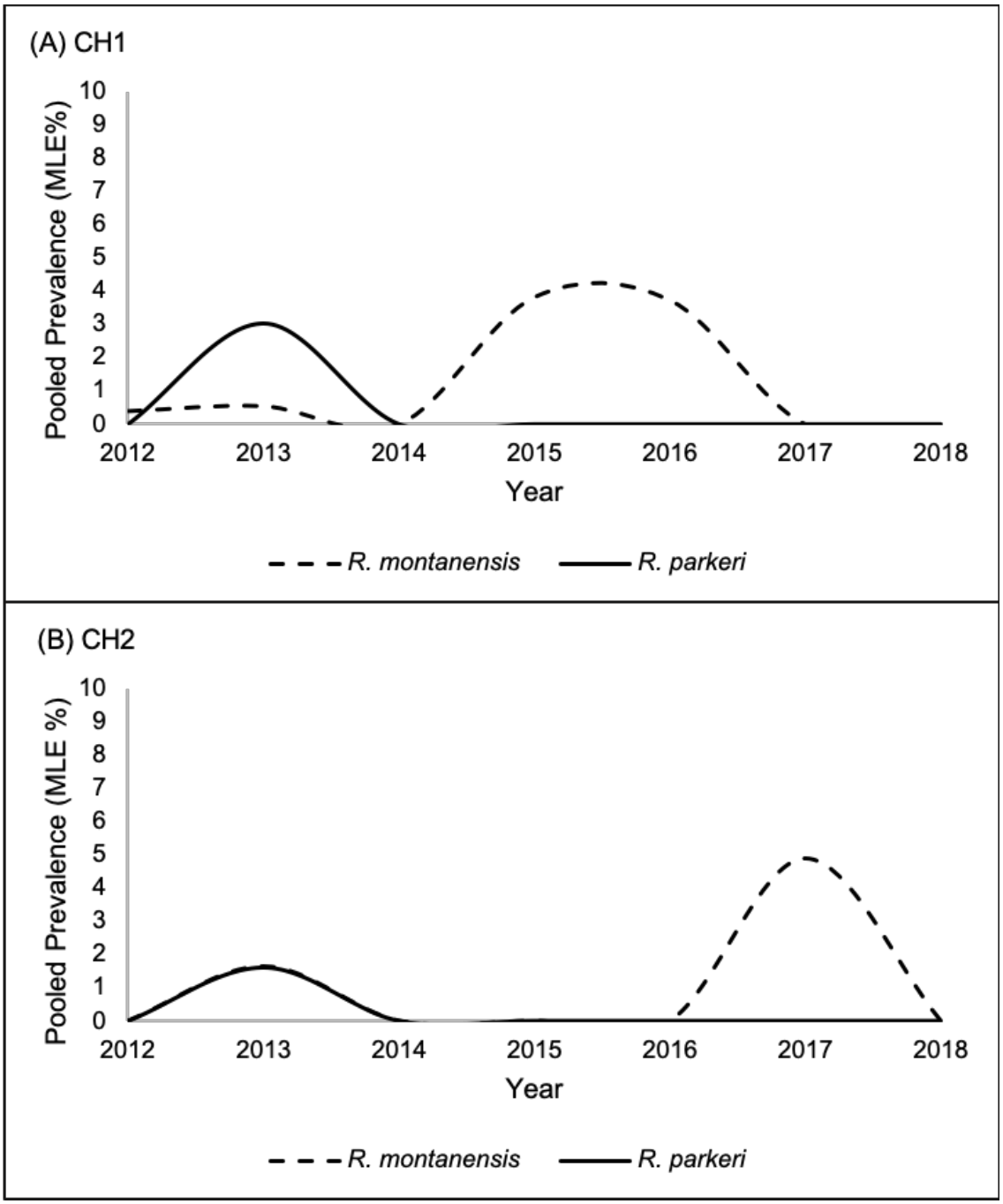

Fig. 7. Percent pooled prevalence (MLE) of *R. montanensis* and *R. parkeri* in adult *D. variabilis* collected from vegetation from 2012 to 2018. (A) Percent pooled prevalence (MLE) of *R. montanensis* and *R. parkeri* in adult *D. variabilis* at site CH1. (B) Percent pooled prevalence (MLE) of *R. montanensis* and *R. parkeri* in adult *D. variabilis* at site CH2.

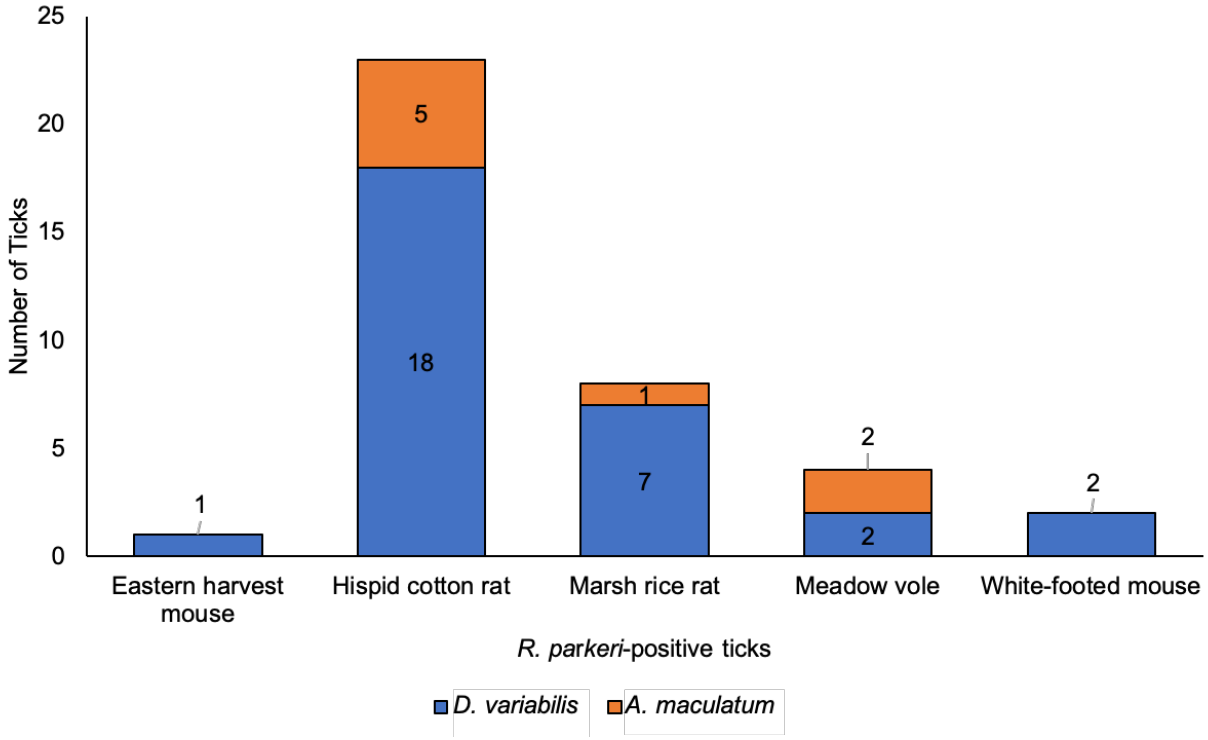

Fig. 8.
Number of *R. parkeri*-positive *D. variabilis* and *A. maculatum* by small mammal species.

Discussion

In southeastern Virginia, *D. variabilis* was observed to harbor multiple rickettsiae, indicative of host sharing behavior and phenological overlap with *A. americanum* and *A. maculatum*. *Rickettsia montanensis* was the most prevalent rickettsia in both adult and immature *D. variabilis*, followed by *R. parkeri*, and *R. amblyommatis*. *Rickettsia rickettsii* was not detected in any adult or immature *D. variabilis* in this study. The absence of *R. rickettsii* was not surprising as other *D. variabilis* populations have shown the same absence of this pathogen (Nadolny et al., 2014; Miller et al., 2016, Hecht et al., 2019). The prevalence of *R. montanensis* in *D. variabilis* of 2.3% across all years was similar to the prevalences reported in other surveillance studies in this region (Ammerman et al., 2004; Pagac et al., 2014; Nadolny et al., 2014; Hecht et al., 2019). Overall *R. parkeri*

and *R. amblyommatis* had a lower prevalence across all years at <1%. The prevalences of these rickettsiae are also similar to previous surveillance studies (Fornadel et al., 2011; Hecht et al., 2019).

At sites CH1 and CH2, when established *A. maculatum* populations were present, site prevalence of *R. parkeri* in adult *D. variabilis* was 0.5% (95% CI: 0.2%-0.9%). In adult *D. variabilis* populations, *R. parkeri* was maintained at low levels in 2013, but as *A. maculatum* populations started to dwindle at these sites, *R. montanensis* became the most prevalent rickettsia. This same trend was also observed in the immature *D. variabilis* where *R. parkeri* was detected in the immatures from 2011 to 2013 along with *R. montanensis*.

With regard to *R. amblyommatis* prevalence, the majority of field sites (16/17) had established *A. americanum* populations which probably contributed to the spillover of low levels of *R. amblyommatis* in both adult and immature *D. variabilis*. The higher prevalence of *R. amblyommatis* in immature *D. variabilis* versus adult *D. variabilis* was interesting as immature *A. americanum* do not feed on small mammals in southeastern Virginia. *Rickettsia amblyommatis* prevalence in the immature *D. variabilis* could potentially highlight transovarial transmission of this rickettsia. As adults, *D. variabilis* and *A. americanum* had high host overlap, but *R. amblyommatis* was the least prevalent rickettsia detected in this study. In additional to their shared hosts (*C. lupus familiaris*, *U. americanus*, and *P. lotor*), *D. variabilis* appeared to have a disinclination to *O. virginianus* which were highly parasitized by *A. americanum*. This host preference may contribute to lack of spillover of *R. amblyommatis* between these sympatric ticks.

Odocoileus virginianus, C. latrans, and *S. scrofa* were parasitized by adults of all three species; in some instances all three ticks were present on the same animal. These animal species warrant further investigation into their role in rickettsial pathogen spillover as they were hosts to the adult life stage of all three ticks. It is important to note that the collection of adult *A. maculatum* from medium to large mammal hosts was sparse. *Canis lupus familiaris* (n=321 surveyed for ticks), *F. catus* (n=65 surveyed for ticks), and *O. virginianus* (n=195 surveyed for ticks) appeared to be well represented in our sampling yield, whereas *C. latrans* (n=11) and *S. scrofa* (n=26) were underrepresented and more difficult to sample. Further investigation will be needed to determine if adult *D. variabilis* are acquiring pathogens from these medium to large mammal hosts.

For immature *D. variabilis*, there was high host overlap but low phenological overlap with immature *A. maculatum*. Because of this pattern, the host itself may be a potential source for *R. parkeri* in the environment. Small mammals from the family Cricetidae (hispid cotton rats, marsh rice rats, and meadow voles) were found parasitized by *R. parkeri*-infected *A. maculatum* and also had *R. parkeri*-infected tissues (Cumbie et al., 2020). Infected *D. variabilis* were also found on these small mammals demonstrating the need for further investigation into their roles as reservoirs for *R. parkeri* and other rickettsiae. There is also evidence of other small mammals being parasitized by *R. parkeri*-infected *D. variabilis*: Eastern harvest mice and white-footed mice (both family Cricetidae, subfamily Neotominae), but neither species were parasitized by *R. parkeri*-infected *A. maculatum* and their competencies as reservoirs of *R. parkeri* is currently unknown.

In conclusion, *D. variabilis* harbors multiple rickettsiae including *R. montanensis*, *R. parkeri,* and *R. amblyommatis.* The impact of rickettsial pathogen spillover between these sympatric tick in relation to public health, laboratory infection studies involving target host sampling and mimicking natural co-feeding systems are future directions for this work.

CHAPTER IV

EXPERIMENTAL HORIZONTAL TRANSMISSION OF *RICKETTSIA PARKERI* IN *DERMACENTOR VARIABILIS* THROUGH CO-FEEDING WITH INFECTED *AMBLYOMMA MACULATUM*

Introduction

There are four common human-biting ticks in Virginia: *Ixodes scapularis*, *Amblyomma americanum*, *Amblyomma maculatum*, and *Dermacentor variabilis*. Each of these ticks is capable of transmitting tick-borne pathogens and all are known to harbor at least one rickettsial agent (Socolovschi et al., 2009). *Dermacentor variabilis*, the American dog tick, is a frequent human-biter widely associated with transmission of multiple rickettsial pathogens and is the historical vector of *R. rickettsii*, the causative agent of Rocky Mountain spotted fever (Piesman and Gage, 1996). In the eastern US, *D. variabilis* harbors multiple rickettsial species in addition to *R. rickettsii*, including *R. montanensis*, *R. bellii*, *R. parkeri,* and *R. amblyommatis* (Stromdahl et al., 2011; Gleim et al., 2016; Philip et al., 1983; Carmichael and Fuerst, 2010; Hecht et al., 2019).

Spotted fever group rickettsioses are increasing in incidence in the US and without proper follow up with specific pathogen detection by PCR, most *Rickettsia* are not identified to the species level (Paddock et al., 1999; Adem, 2019; Snowden et al., 2020). In Virginia, rickettsial infections have been observed more frequently (Nadolny et al., 2014). *Rickettsia parkeri*, the causative agent of *R. parkeri* rickettsiosis (Paddock et al., 2004) has also been more frequently observed in Virginia over the past 10 years (Wright et al., 2011). The increase in *R. parkeri* prevalence coincides with the

movement of its tick vector *A. maculatum* into this area during their range expansion northward over the past 10 years (Sumner et al., 2007; Fornadel et al., 2011; Nadolny et al., 2018).

Rickettsia parkeri has been detected in adult *D. variabilis* populations in Virginia since this range expansion (Fornadel et al., 2011; Nadolny et al., 2014). As adults, both *D. variabilis* and *A. maculatum* bite humans, but *D. variabilis* has been reported on humans more frequently (Merten and Durden, 2000). As immatures, *D. variabilis* and *A. maculatum* preferentially feed on small mammals and are not typically found biting humans (Smith et al., 1946; Sonenshine, 1972; Teel et al., 2010). In southeastern Virginia, immature *D. variabilis* and *A. maculatum* have been observed parasitizing the same small mammal hosts (see Ch. III) and immature *D. variabilis* collected from those hosts have tested positive for *R. parkeri* (see Ch. III).

Amblyomma maculatum and other *Amblyomma* species have been studied in some detail regarding *R. parkeri* transmission (Sumner et al., 2007; Wright et al., 2015; Wright et al.; 2015), but other tick species, such as *D. variabilis*, have not been fully considered. Previous tick surveillance studies have shown a small percentage of wild-caught adult *D. variabilis* harbor *R. parkeri* (Fornadel et al., 2011; Nadolny et al., 2014; Hecht et al., 2019). Lab experimentation has shown that *D. variabilis* can acquire *R. parkeri* following capillary feeding and transmit it between life stages (Harris et al., 2017). To our knowledge, no studies have examined the transmission of *R. parkeri* from *A. maculatum* to *D. variabilis* through co-feeding on a small mammal host which is likely the way *R. parkeri* transmission from *A. maculatum* to *D. variabilis* naturally occurs.

Natural acquisition of *R. parkeri* by *D. variabilis* would be through one of two routes: (1) through a blood meal from a systemically infected host or (2) non-systemic transmission by co-feeding with infected ticks at a localized infection site on a host (Randolph and Nuttall, 1996). Both of these transmission routes involve *D. variabilis* acquiring *R. parkeri* during its immature life stages in order to molt into an infected adult. In this study we investigated horizontal transmission of *R. parkeri* through co-feeding experiments using immature infected *A. maculatum* and immature non-infected *D. variabilis*.

Materials and Methods

Tick collection and propagation

Adult *A. maculatum* and *D. variabilis* were collected from vegetation in southeastern Virginia in June 2019 and used as progenitors for tick colonies at Old Dominion University. *Amblyomma maculatum* and *D. variabilis* mating pairs were separated by species and fed in small capsules attached to the back of retired breeding rats (*Rattus norvegicus*). Once engorged and detached, female ticks were removed and allowed to lay eggs. Each female and a pool of ten eggs from each egg mass were tested for the presence of rickettsiae, and then tested for the presence *R. parkeri*. Larval and nymphal progeny from these groups were fed on mice and rats, and allowed to molt to the next life stage. Larvae were infested on laboratory mice (*Mus musculus*) using a “sock method” (Graham Hickling, unpublished data) where they were placed in a sock with ticks for a duration of 45 min to an hour. Larvae were allowed to feed freely on the mouse and drop off through mesh wiring in bottom of the cage for collection. Nymphs

were fed on retired breeding rats in small capsules as described for the adults. When not on a host, all ticks were housed in an incubator at 26°C, with 93% relative humidity for a photoperiod of 14:10 (L:D) hours.

Nymphs from these colonies were used in the initial co-feeding experiments. Due to complications involved with university shut-downs in 2020 due to SARS-CoV-2, these colonies did not survive for future experiments, and new progenitors were collected in June 2020. A subsample of adult ticks from these collections were used directly in the adult co-feeding studies; another portion were fed separately on laboratory rats to produce egg masses for larval co-feeding experimentation. Additionally, after each co-feeding session and euthanization, host tissue samples were taken at the bite site of the ticks (on the ears for mice and skin from the capsule area for rats) and away from the bite site (toe clips). All experiments involving laboratory mice and rats were conducted in accordance with ODU IACUC permit #17-015 and #20-016.

DNA extraction and pathogen testing

Extraction of DNA from *A. maculatum* and *D. variabilis* from all life stages was completed using the GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, Pittsburgh, PA) as specified in the manufacturers' instructions with an initial digestion overnight at 57°C. *Amblyomma maculatum* DNA extracts were tested for the presence of *R. parkeri* using a real-time PCR assay targeting the *ompB* gene using Rpal29F, Rpa224R, and FAM/BHQ-labeled Rpal88probe (Appendix A). *Dermacentor variabilis* DNA extracts were tested for the presence of *Rickettsia* spp., *R. montanensis*, and *R. parkeri* using real-time PCR assays targeting the 17kDa gene (for *Rickettsia* spp.) and the *omp*B gene for *R. montanensis* and *R. parkeri* (Appendix A). All reactions were

carried out in 20 µL volumes using 10 µL of EconoTaq PLUS 2x Master Mix (Lucigen Inc., Middleton, WI), 1 µL of each primer (10 µM final concentration), 1 µL of probe (10 µM final concentration), and 2-5 µL of DNA at extraction concentration. The PCR protocol for all assays consisted of a 2 min denaturation at 95°C followed by 45 cycles of 95°C for 15 s and 60°C for 30 s.

Experimental design for testing for co-feeding transmission of R. parkeri

Dermacentor variabilis were co-fed with *R. parkeri*-infected *A. maculatum* at each life stage. There were 8 larval cohorts consisting of approximately 100 *D. variabilis* larvae and 100 *A. maculatum* larvae per cohort. Larvae fed freely on laboratory mice for 2-8 days, and were allowed to fully engorge, drop off, and molt. Newly molted *D. variabilis* and *A. maculatum* nymphs were separated morphologically using mouth parts (Brinton et al., 1965; Keirans and Durden, 1998) and color. Flat *A. maculatum* nymphs had elongated palps, a u-shaped anal groove, and appeared gray-ish in color (Fig. 8). *Dermacentor variabilis* nymphs had shorter and wider palps in comparison to *A. maculatum* and were red-brown in color (Fig. 9). In order to confirm the difference between *D. variabilis* and *A. maculatum* newly molted nymphs, 10 *D. variabilis* nymphs from each of the 8 cohorts (80 nymphs total) were tested for the presence of *D. variabilis* DNA using a real-time PCR assay amplifying a variable portion of the *D. variabilis* ITS2 gene (Shone et al., 2006). Each PCR reaction (final volume 25 µL) consisted of 12.5 µL EconoTaq PLUS 2X Master Mix (Lucigen, Middleton, WI), 1 µL of each primer (10 µM), 1 µL of probe (10 µM), and 2 µL of DNA at extraction concentration. The thermocycler conditions consisted of 95ºC for 10 min, followed by 40 cycles at 95ºC for 15 s, 60ºC for 1 min.

Nymphs were fed on rats using capsules made from 50 mL conical tubes attached to the rat's back. These capsules were 1.2 inches in diameter which created a small co-feeding area. Four nymphal cohorts were tested. Each cohort consisted of 10-15 *D. variabilis* and 10-15 *A. maculatum* co-fed on a single rat. Nymphs fed on laboratory rats for 4-10 days and were removed from the capsule once fully engorged. All newly molted adults from the nymphal cohorts were identified morphologically.

Fig. 9. Magnified image (20X magnification) of an *A. maculatum* nymph (left) and a *D. variabilis* nymph (right).

Pathogen prevalence estimation

Larvae were grouped into pools for DNA extraction. Larvae were pooled into 106 pools ranging in size from 1 to 10 based on the number of ticks obtained from each cohort feeding. Maximum likelihood estimates (MLEs) were used to calculate the pooled infection rates for larvae. These were calculated using the binGroup package in R (Zhang et al., 2018). Nymphs were extracted individually and their prevalences calculated by dividing the total number of positive ticks by the total number of ticks tested.

Results

Rickettsia parkeri acquisition through co-feeding was detected in *D. variabilis* in both the larval and nymphal life stages, with 7.18% of larvae and 23.3% of nymphs being positive for *R. parkeri* (Table 9). Of the 800 *D. variabilis* larvae infested, only 296 (37%) survived and molted successfully into nymphs. Similar survival was seen with the co-feeding *A. maculatum* larvae; 312 (39%) successfully molting into nymphs. A subset of 80 *D. variabilis* nymphs were tested by real-time PCR assay to identify to species. All were confirmed as *D. variabilis*. No mouse tissues from the bite site (ear punch) or away from the bite site (toe clip) were positive for *R. parkeri* by to the *R. parkeri* real-time PCR assay.

The majority of *D. variabilis* nymphs (43/67) survived co-feeding and successfully molted into adults (Table 9). Thirty-four out of the 67 co-feeding *A. maculatum* nymphs survived and molted into adults. The *D. variabilis* adults that molted from nymphs were tested for the presence of *R. parkeri*; 10 were determined positive by real-time PCR

assay. Tissues from the rat hosts were also tested; 3 out of the 4 tissue samples from the site of co-feeding were positive for *R. parkeri*. No rat toe clips were positive.

Discussion

Dermacentor variabilis are able to acquire *R. parkeri* as larvae and nymphs through horizontal transmission by co-feeding with infected *A. maculatum*. Historically, *D. variabilis* have not been associated with *R. parkeri* infection, but recent *Rickettsia* surveillance studies in Virginia *D. variabilis* populations have observed 0.46% prevalence of *R. parkeri* in adult populations and up to 5.58% infection in immature populations (see Ch. III). Immature *D. variabilis* have been observed on the same small mammal hosts as immature *A. maculatum* (Cumbie et al., 2020) which creates a plausible scenario for natural co-feeding infection to occur.

Table 9
Rickettsiae infection of *D. variabilis* through co-feeding at each life stage.

Life stage	Systemically infected hosts based on toe clips	Locally infected hosts at bite site	# of ticks used for infestation	Total fed to completion and molted (%)	Total # of pools tested	# of pools infected with *R. montanensis.*	# of pools infected with *R. parkeri*	Pooled prevalence (MLE)
Larva	0/8	0/8	800	296 (37%)	106	0	20	7.18%
Nymph	0/4	3/4	67	43 (64%)	43	0	10*	23.3%

*Tested after molting into adult ticks.

The estimated prevalence of *R. parkeri* for the nymphs (larval cohorts) and adults (nymphal cohorts) were 7.18% and 23.3%, respectively. The prevalence of *R. parkeri* is relatively high in *D. variabilis* raised the laboratory colonies than is seen in local populations (see Ch. III). The prevalence of *R. parkeri* is lower in wild populations due to short periods of phenological overlap between immature *D. variabilis* and *A. maculatum* (see Ch. III).

This study presents evidence of non-systemic transmission of *R. parkeri* from *A. maculatum* to *D. variabilis*. Research assessing the natural infection of co-feeding ticks through non-systemic transmission of rickettsial pathogens is generally understudied with only a few co-feeding transmission studies having been implemented (Kocan and Fuente, 2003; Zemtsova et al., 2010; Lee et al., 2018). For the nymphal cohorts, host tissue at the bite site in 3 out of the 4 rats tested positive for *R. parkeri*, but systemic infection, although possible, was not apparent, as no toe clips were positive. For the larval cohorts, no mouse tissues at or away from the bite sites were positive for *R. parkeri*, although every cohort yielded infected *D. variabilis*. Systemic infection of *R. parkeri* in laboratory-raised mice and rats is variable and not fully characterized (Grasperge et al., 2012; Moraru et al., 2013). Further characterization and laboratory-based studies are needed to assess the role that systemic infection may play in *R. parkeri* transmission to sympatric tick such as *D. variabilis*. Until definitive reservoir hosts are identified for *R. parkeri* in Virginia, localized infection on host through co-feeding appears to be a likely mechanism for *D. variabilis* infection.

The overall results of this study indicate that *D. variabilis* are able to acquire *R. parkeri* at both immature life stages. A higher percentage of nymphs were infected with

R. parkeri than larvae which lends support to having *R. parkeri*-positive unfed adult *D. variabilis* in natural tick populations. Further studies into infection by life stage and the infectivity of *R. parkeri* at each life stage are necessary to understand the enzootic cycle of *R. parkeri* and *R. parkeri* infection in sympatric ticks, like *D. variabilis*.

CHAPTER V

BACTERIAL COMMUNITY ANALYSIS IN *AMBLYOMMA MACULATUM* TICKS FROM SOUTHEASTERN VIRGINIA

Introduction

The microbiome has been shown to play a functional role in the overall health of many organisms, including arthropod vectors (Moloney et al., 2014; Saldaña et al., 2017). The microbiota present in arthropods can be influential in the acquisition, maintenance, and transmission of pathogens. Microbiota can be separated into two groups: the external (living on the surface) and the internal (living in the host). In the case of arthropods that act as vectors, such as ticks and mosquitos, the internal microbiota interacts with pathogens during blood-feeding and transmission (de La Fuente et al, 2017; Saldaña et al., 2017). Internal microbial communities of an arthropod host can have two non-neutral functions with respect to pathogen acquisition, maintenance, and transmission: (1) they can be harmful to the pathogen, for example by outcompeting the pathogen for survival in the arthropod host (Paddock et al., 2015; Dutra et al., 2016) or (2) they are commensal with the pathogen, as is seen with many *Francisella* spp. commonly found in hard-bodied ticks (Bonnet et al., 2017).

In southeastern Virginia, *Amblyomma maculatum*, the Gulf Coast tick, is a hard-bodied tick that has undergone a geographical range expansion northward along the eastern US. This tick is the primary vector of *Rickettsia parkeri*, the causative agent of *R. parkeri* rickettsiosis. Within its historical range along the Gulf Coast of the United States, *R. parkeri* prevalence in *A. maculatum* populations vary between 1% and 40%

(Sumner et al., 2007, Nadolny et al., 2014; Mays et al., 2016), but in Virginia, *R. parkeri* prevalence is 40-60% across all field sites (Wright et al., 2011; Nadolny et al., 2014; Wright, 2015).

High *R. parkeri* prevalence could be correlated with many factors, including variation in the internal microbiota of the ticks. For arthropods, such as ticks, vertical and horizontal transmission influence pathogen acquisition adding to their internal microbiota (Kwan et al., 2017). Vertical transmission occurs when the tick maintains its microbial communities through subsequent life stages (transstadial transmission) or through eggs (transovarial transmission). In blood-sucking arthropods, acquisition of pathogens through taking a blood meal is an example of horizontal transmission. Secondarily, during feeding, ticks may co-feed and mate, creating an additional opportunity to share pathogens and other organisms.

To understand if the internal microbiota of *A. maculatum* from Virginia might be influencing the prevalence of *R. parkeri* seen in this area, “baseline” patterns of the microbial composition of *A. maculatum* were determined, then the possible interactions between study location, internal microbial diversity, and *R. parkeri* infection were examined. In this study, microbial composition and diversity were identified and quantified from two generations of lab-raised *A. maculatum* and from thirty wild-caught *A. maculatum* collected from three geographically distinct study locations. Similarities and dissimilarities between *R. parkeri*-infected ticks and un-infected ticks were examined to characterize differences in microbiota composition depending on the presence or absence of *R. parkeri*.

This information will be used to direct future research into the associations between tick microbiota and *R. parkeri* and the possible mechanisms by which the microbiome may contribute to pathogen maintenance and transmission.

Materials and Methods

Sample collection

Adult *A. maculatum* were collected from 4 field sites in southeastern Virginia and 1 field site at the Virginia-North Carolina border (Fig. 10). Ticks were identified morphologically (Keirans and Durden, 1998) and either separated for use as progenitors of the laboratory colony or stored at -80°C for use in next-generation Illumina sequencing. Lab-raised *A. maculatum* colony progenitors were a *R. parkeri*-negative female from TP1 and a *R. parkeri*-positive male from MH1 collected from vegetation in 2018. Their progeny were raised through two generations. Larval ticks were fed on pathogen-free mice (*Mus musculus*). Nymphs and adult ticks were fed on pathogen-free rats (*Rattus norvegicus*). All experiments involving laboratory mice and rats were conducted in accordance with ODU IACUC permit #17-015 and #20-016. For the wild-caught *A. maculatum*, thirty adults representing 10 ticks from each of the other sites: BI3, CH3, and TP1 were used as representative samples for each site for next generation Illumina sequencing.

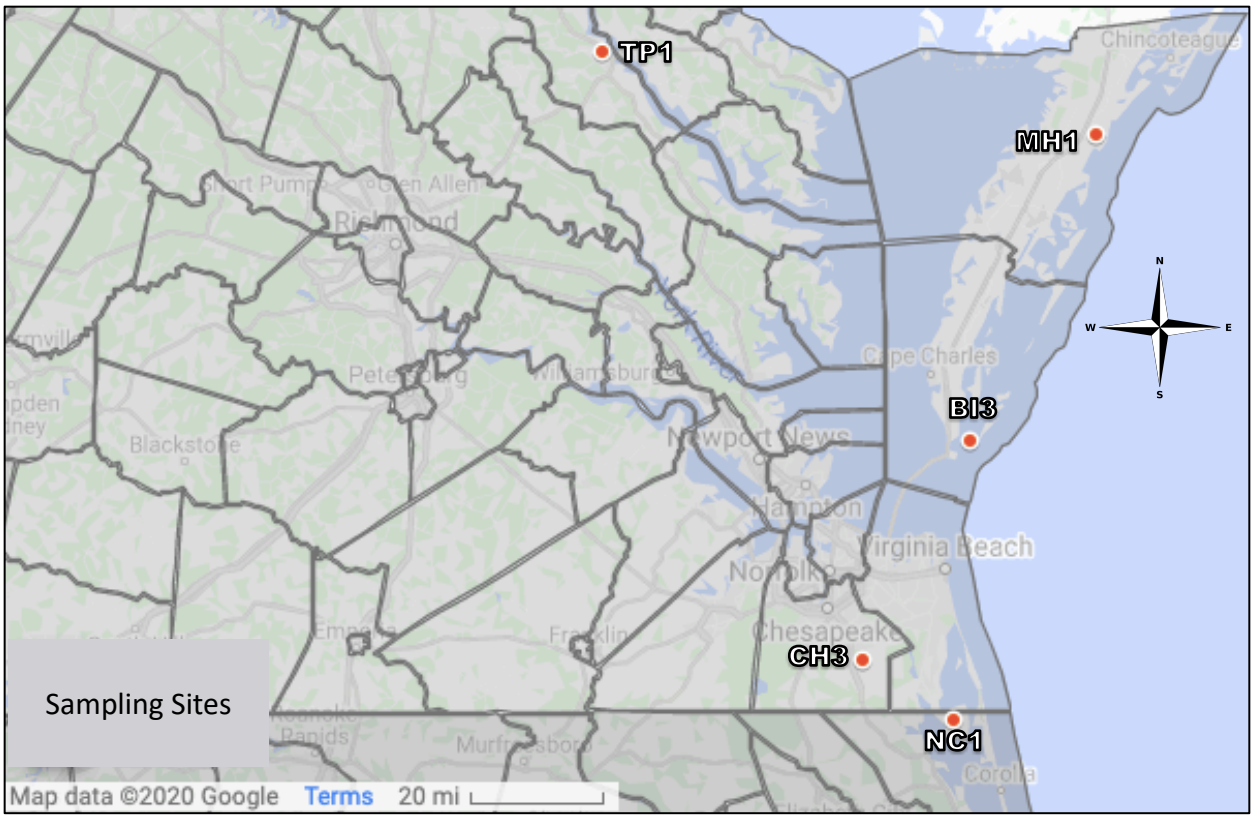

Fig. 10. Sampling sites for wild-caught *A. maculatum* adult ticks in southeastern Virginia and North Carolina: TP1(Tappahannock county, VA), MH1 (Accomack county, VA), BI3 (Somerset county, VA), CH3 (Chesapeake City, VA), and NC1 (Currituck county, NC). This map was generated using GoogleMyMaps.

Sample preparation

Wild-caught and lab-raised *A. maculatum* were surface sterilized using 0.5% sodium hypochlorite, 70% ethanol, and three washes with nuclease-free water. All samples pre-extraction were stored at -80°C after surface sterilization. Adult ticks were cut in half prior to DNA extraction. One half was extracted; the other half was stored at -80°C for future use. Immature ticks from the colony were extracted whole.

DNA extraction

For the lab-raised colony ticks, 22 larval samples (10 individual larvae and 1 pool of 10 larvae from each generation), 22 nymphal samples (10 individual nymphs and 1 pool of 10 nymphs from each generation), and 20 adults (10 individuals from each generation) were extracted. The wild-caught *A. maculatum* consisted of 30 individuals representing 10 adult ticks from each site. Ticks were pulverized using approximately the same volume (as the tick) of 1 mm glass beads and one 5 mm glass bead in a Mini Beadbeater (BioSpec, Inc. Bartlesville, OK, USA) and extracted individually either using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) for generation 1 and the GeneJet Genomic DNA Purification Kit (ThermoFisher Scientific, Pittsburgh, PA) for generation 2 as specified in the manufacturers' instructions. Extracted DNA was stored at -80°C until used for library preparation and sequencing.

Library preparation

Separate 16S rRNA libraries were prepared for each tick sample as well as two negative controls of nuclease-free water following the guidelines in the Illumina 16S Metagenomic Sequencing Library Preparation manual (Illumina, Inc., San Diego, CA, USA). Amplicon PCR was performed using primers targeting the 16S rRNA V3-V4 hypervariable region; each amplicon was purified using the Mag-Bind TotalPure NGS kit (Omega Bio-tek Inc., Norcross, GA, USA). Purified amplicons were run on a 1% agarose gel to confirm retainment of amplicon during purification. For the index PCR, dual indices were attached to the purified amplicons using primers from an NGS Index Kit set (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Indexed amplicons were purified using a Mag-Bind TotalPure NGS kit before library quantification. Library

concentrations were initially quantified using an AccuBlue quantification kit (Biotium, Inc., Fremont, CA, USA) followed by quantification with a KAPA Library Quantification kit (Kapa Biosciences,Woburn, MA, USA). All samples were diluted to a 4 nM concentration and pooled to form a multiplexed library.

Library sequencing

The final pooled library was sequenced on an Illumina MiSeq using a MiSeq Reagent Kit v3 (600-cycle, 300 base pair, paired-end) (Illumina, Inc., San Diego, CA, USA).

Sequence processing

Illumina MiSeq output (fastq files) were processed using mothur v1.44.1 (Schloss and Westcott, 2011). Operational taxonomic units (OTUs) were assigned to a taxonomic group based on the silva reference database (Quast et al., 2013; Yilmaz et al., 2014; Glockner et al., 2017). Downstream processing, visualization, and statistics were completed in R v. 3.5.1 using vegan (Oksanen et al., 2019), ampvis2 (Andersen et al. 2018), and SpadeR packages (Chao et al., 2016).

Diversity analyses

Hill numbers (Chao et al., 2010) were used to determine OTU richness, abundance, and diversity in the tick microbial communities. Hill numbers are ‘the effective number of species’ in a community. They take into account species richness and species abundance measures in order to quantify species-neutral diversity. Diversity is measured by Hill numbers using orders of q. The q orders account for diversity similarly to commonly used diversity indices, like Shannon diversity and Simpson diversity (Shannon et al., 1948; Simpson et al., 1949; Chao et al., 2010;). At

q=0, relative abundances are not taken into account and only species richness is measured. As the q order increases, species richness and abundance of OTUs per group are taken into account. At q=1 (Shannon diversity), OTUs are weighted by species abundance and richness. At q=2 (Simpson diversity), OTUs are additionally weighted by common or dominant species to determine diversity.

Results

Species (OTU) abundance, richness, evenness, and diversity

For the lab-raised colony, sixty-two individuals and two pooled groups generated 249,689 unique species (OTUs). Pooling of unique OTUs by genus resulted in 336 representative genera. In the initial analysis, the female progenitor for the colony was found to be weakly positive for *R. parkeri* based on a real-time PCR assay but since her offspring were all negative for *R. parkeri*, it was determined that her positive test likely occurred by infection during mating with the highly positive male progenitor. All progeny were *R. parkeri*-negative by real-time PCR. One nymph in generation 1 was removed from the analysis due to low sequence read counts.

For the thirty wild-caught ticks representing 3 field sites, a total of 1,747,390 sequences representative of 1,544 unique species (OTUs) were detected. Further pooling of unique species by genus resulted in 250 representative genera. The two negative controls from the sequence run generated 101,296 sequences which appeared to be the result of contamination. The top two OTUs had ~10,000 reads per OTU. All other OTUs had 1 to 5000 reads per OTU.

To determine OTU evenness and richness, Hill numbers were calculated for each individual, and rank abundance curves (Whittaker plots) were used to visualize OTU richness and evenness between generations and life stage in the colony. Based on abundance rank, all lab-raised individuals followed a normal trend for species richness and evenness with top OTUs ranking in the highest relative abundance category and low abundance OTUs ranking the lowest (Fig. 11). There was no sharp decline in the curves indicating that all OTUs are well-represented in these samples.

Whittaker plots were used to look at OTU richness and evenness at each site. Based on the abundance ranks, site BI3 had lower observed species richness and evenness compared to sites CH3 and NC1 (Fig. 12). Rank abundance curves also show that common species for each site are different especially at site BI3 compared to sites CH3 and NC1.

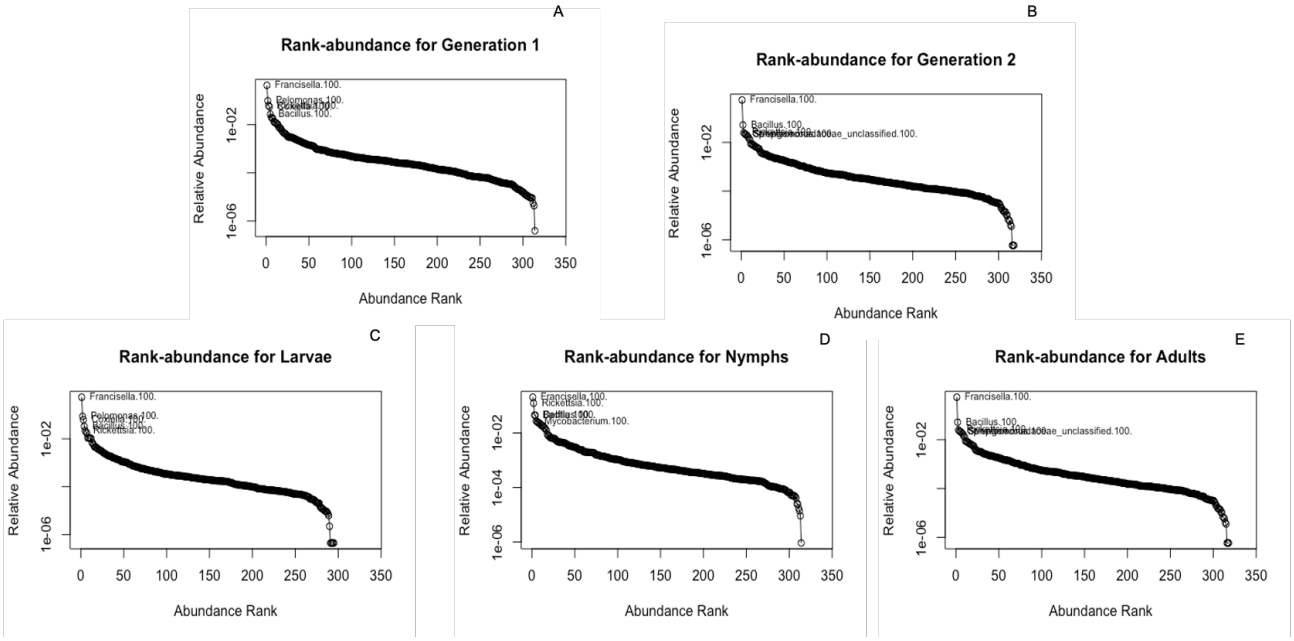

Fig. 11. Rank abundance plots for the lab-raised *A. maculatum* colony. (A) Rank abundance plot for generation 1 of the lab-raised *A. maculatum* colony, (B) rank abundance plot for generation 2 of the lab-raised *A. maculatum* colony, (C) rank abundance plot for all larvae in the lab-raised *A. maculatum* colony, (D) rank abundance plot for all nymphs in the lab-raised *A. maculatum* colony, (E) rank abundance plot for all adults in the lab-raised *A. maculatum* colony.

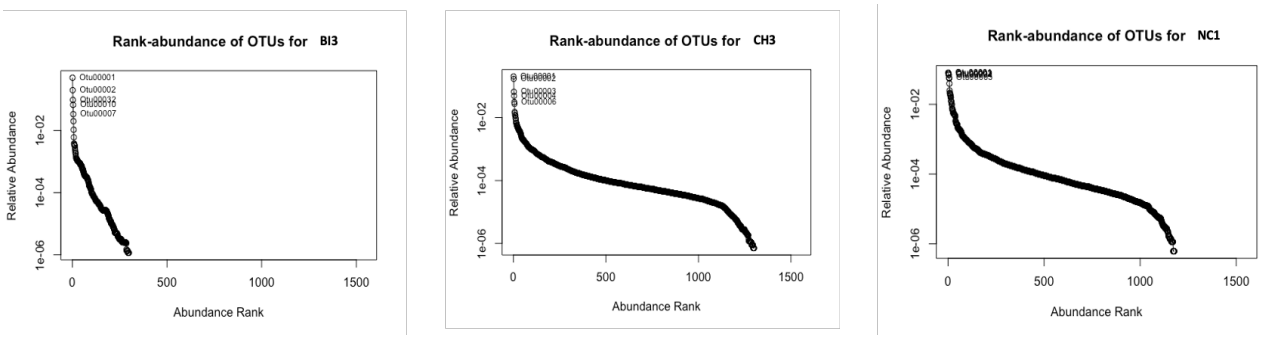

Fig. 12. Rank abundance plots for wild-caught *A. maculatum* from BI3, CH3, and NC1.

OTU diversity between groups was measured by calculating Hill numbers at different orders of q. Since all data was non-parametric, significant differences between groups were determined using permutational multivariate analysis of variance (PERMANOVA) on the Hill numbers. Lab-raised ticks were compared amongst each other within generation and life stage. There was a significant difference between individuals from the F1 generation larvae, but no significant difference between individuals in the F1 nymphal groups or the F1 adult groups (larvae: p=0.02; nymphs: p=0.56; adults: p=0.39). For generation 2, there was no significant different between individuals in any life stage group (larvae: p=0.21; nymphs: p=0.23; adults: p=0.21). Colony diversity based on relative abundances of OTUs observed that local or Shannon diversity among related individuals was only somewhat similar (q=1, Horn estimate=0.4877), but when weighted by common or dominant OTUs, individual microbial communities were not similar (q=2, Morisita-Horn estimate=0.1937). Overall, larval groups in either generation were more similar to each other than both nymphal or adult groups based on shared OTUs. Further, nymphal groups in either generation shared more OTUs than adult groups even among individuals who had low quality reads and less reads per OTU total. The microbial communities in the lab-raised ticks were significantly different from each other when measured between groups by generation and life stage (Table 10). At q=0, the microbial communities in lab-raised ticks were significantly different by generation (p=0.0001), but not by life stage (p=0.0998). At q=1 and q=2, the microbial communities in lab-raised ticks were significantly different by both generation (p=0.0001) and life stage (p=0.0001). Potential drivers of these differences were analyzed using non-metric multi-dimensional scaling

(NMDS). Individuals from the same life stage cluster together but dissimilarities between generation and between each life stage are apparent (Fig. 13).

For wild-caught individuals, diversity was measured based on relative abundances of unique OTUs at each site resulting in many shared OTUs (q=1, Horn estimate=0.6034). Diversity across all sites weighted to consider only common or dominant OTUs showed less similarity (q=2, Morisita-Horn estimate=0.2723). PERMANOVAs were run on Hill numbers for all sites (Table 11). Diversity across all sites showed that observed number of OTUs were significantly different from each other at all q orders. Based on Bray-Curtis dissimilarity, the drivers for these differences were the bacterial communities from ticks collected at site BI3 which were significantly different from those at both site CH3 and site NC1 (Fig. 14). Individuals from sites CH3 and NC1 share a similar bacterial community structure.

Table 10
Significance table for colony individuals factored by both generation and life stage. Significance was preset as P < 0.05. The 'Combined' group represents both generation and life stage together.

	q=0					**q=1**					**q=2**				
	Df	SumOf Sqs	R2	F	P-value	Df	SumOf Sqs	R2	F	P-value	Df	SumOf Sqs	R2	F	P-value
Generation	1	0.38047	0.24655	19.633	0.0001	1	2.5595	0.36048	33.82	0.0001	1	2.8363	0.42324	44.029	0.0001
Life stage	2	0.09869	0.06395	2.0156	0.0998	2	1.4573	0.20525	7.6186	0.0001	2	1.2646	0.18871	6.8617	0.0001
Combined	5	0.63052	0.40858	7.7374	0.0001	5	4.8691	0.68576	24.442	0.0001	5	5.1200	0.76403	36.263	0.0001

*Df = degrees of freedom, R2 = coefficient of determination, and F = F statistic

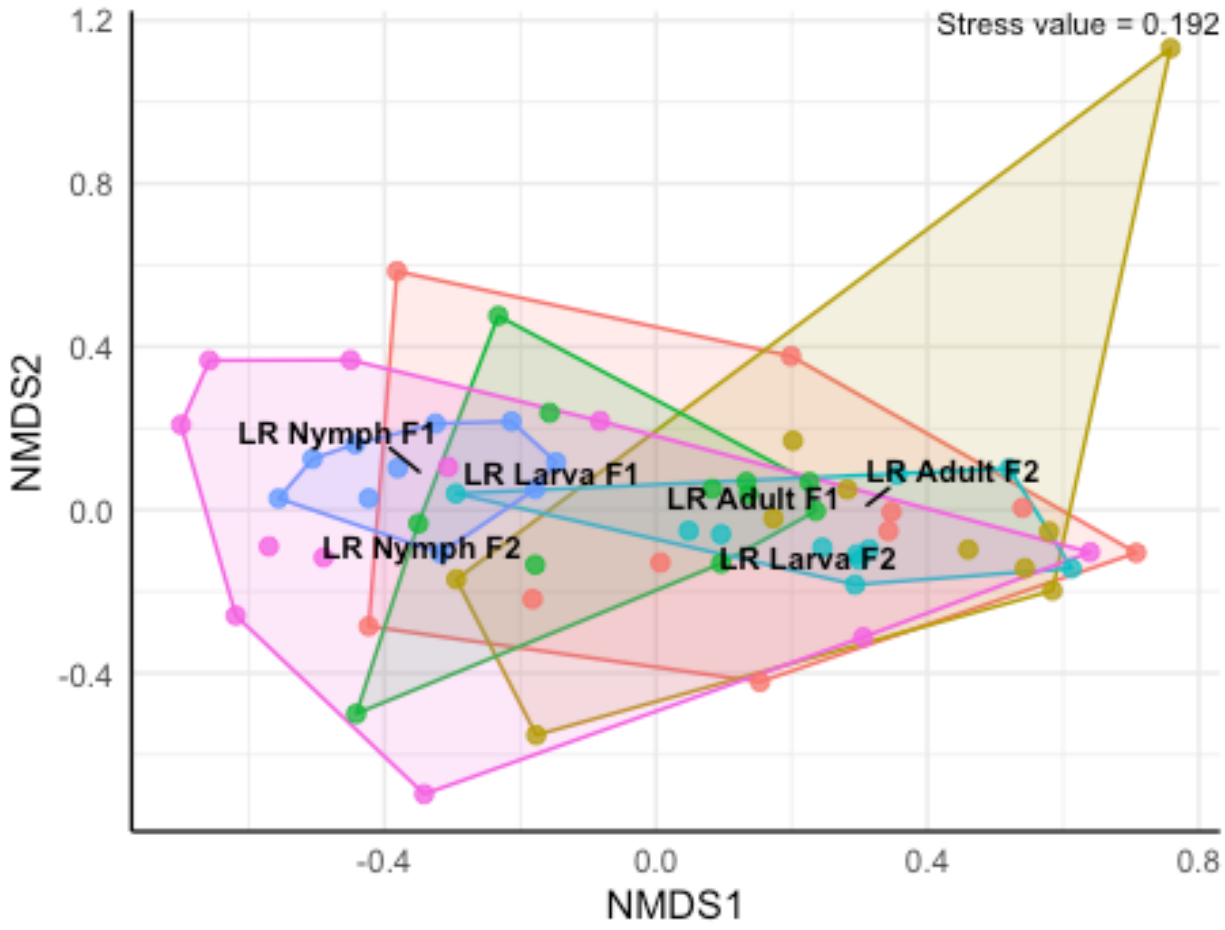

Fig. 13. NMDS plots of lab-raised individuals separated by generation and life stage based on Bray-Curtis dissimilarity metrics.

Table 11
Significance table for beta diversity by field site and infection with *R. parkeri* among wild-caught adult *A. maculatum.* Significance was preset as P < 0.05.

	q=0					q=1					q=2				
	Df	SumOf Sqs	R2	F	P-value	Df	SumOf Sqs	R2	F	P-value	Df	SumOf Sqs	R2	F	P-value
Site	2	1.32369	0.74966	40.427	0.0001	2	1.7605	0.53843	15.748	0.0001	2	1.6434	0.55008	16.506	0.0001
Infection	1	0.04202	0.0238	0.6826	0.429	1	0.2866	0.08764	2.6898	0.0807	1	0.24427	0.08176	2.4932	0.0866

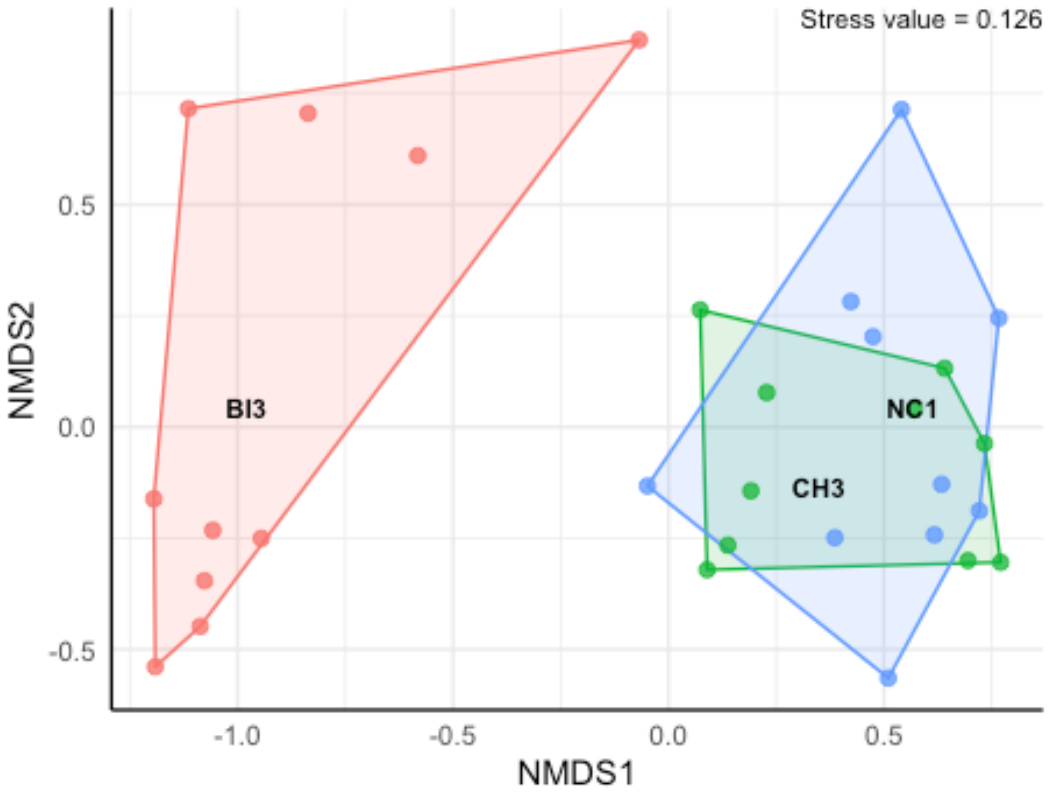

Fig. 14. NMDS plots of wild-caught individuals separated by site based on Bray-Curtis dissimilarity metrics.

Species (OTU) composition

For lab-raised individuals, there were 24 common or dominant genera with an abundance of >10,000 reads per OTU shared by all colony members (Fig. 15). These OTUs made up 89.4% of all reads. Many of these species have been previously identified in tick microbiota surveys (Appendix B). Genus 1174-901-12 (family Beijerinckiaceae), *Amnibacterium*, and *Sciscionella* were not previously reported.

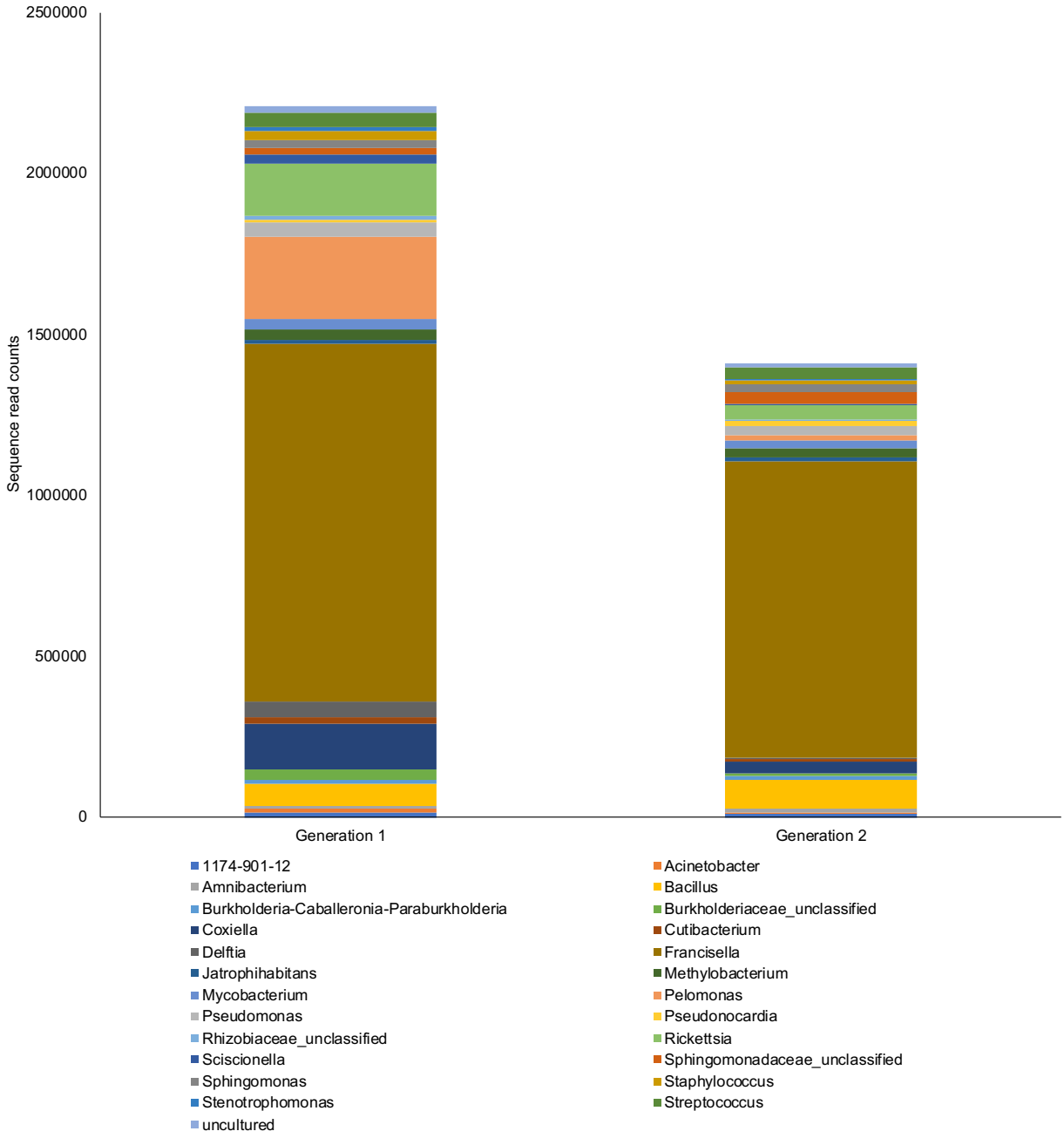

Fig. 15. Top 25 genera based on >10,000 reads per OTU shared between the lab-raised colony.

The top 10 most common genera represented in the colony-raised ticks (based on relative abundance of reads) were *Francisella, Rickettsia, Pelomonas, Bacillus, Coxiella, Streptococcus, Pseudomonas, Sphingomonadaceae, Methylobacterium*, and

Pseudonocardia (Fig. 16). A higher relative abundance of *Rickettsia* and *Pelomonas* was noted in generation 1 nymphs of the colony relative to generation 2. Generation 2 had a higher relative abundance of *Francisella* when compared to generation 1.

For wild-caught individuals, there were 17 common or dominant genera with an abundance of >10,000 reads per OTU shared by individuals at each of the three sample sites (Fig. 17).

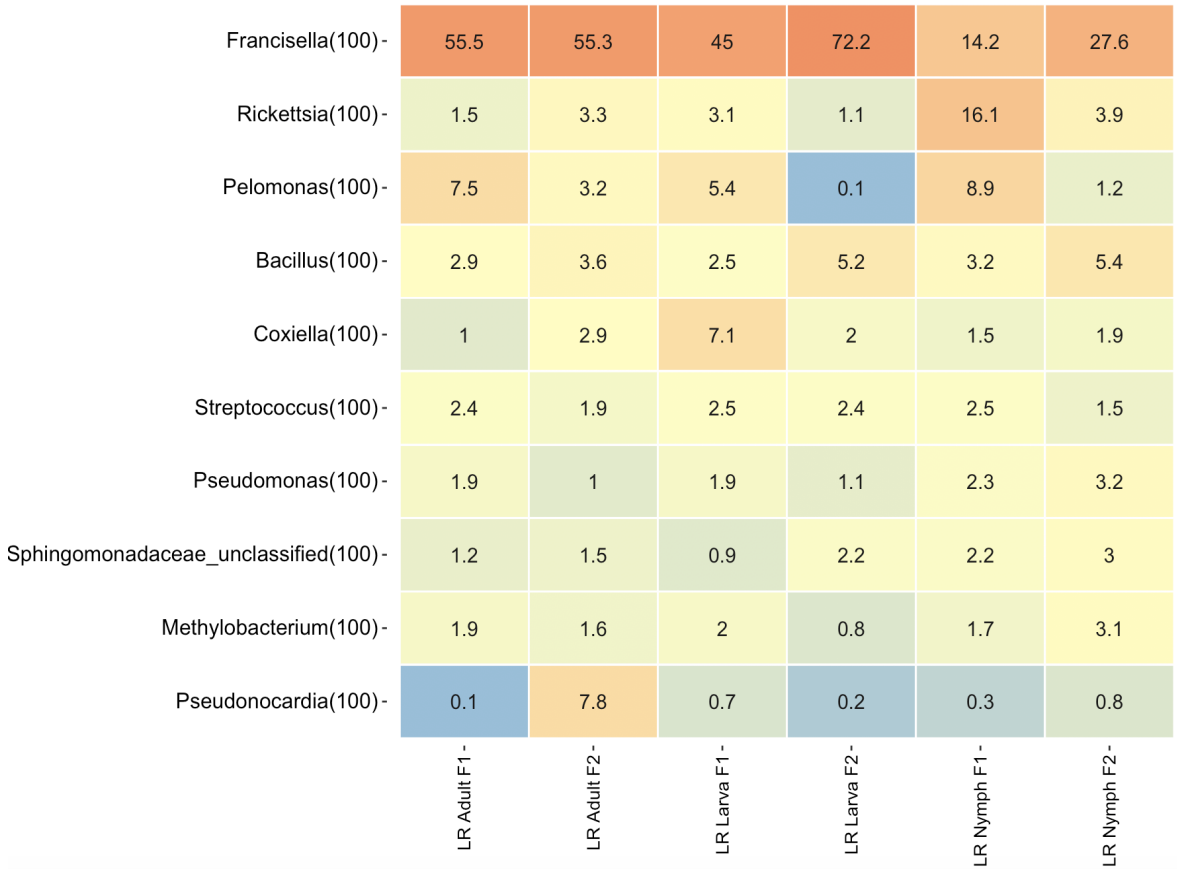

Fig. 16. Read abundance percentages of top genera between related individuals from the lab-raised (LR) colony separated by generation 1 (F1) and generation 2 (F2) in the colony.

The relative abundance of these common species made up roughly 83% of all reads. All common species are presenting previously reported tick bacterial communities (Appendix B) except genus 1174-901-12. Read abundance of *Rickettsia* and *Francisella* were high at all sites, with site BI3 having the highest abundances for both (Fig. 18).

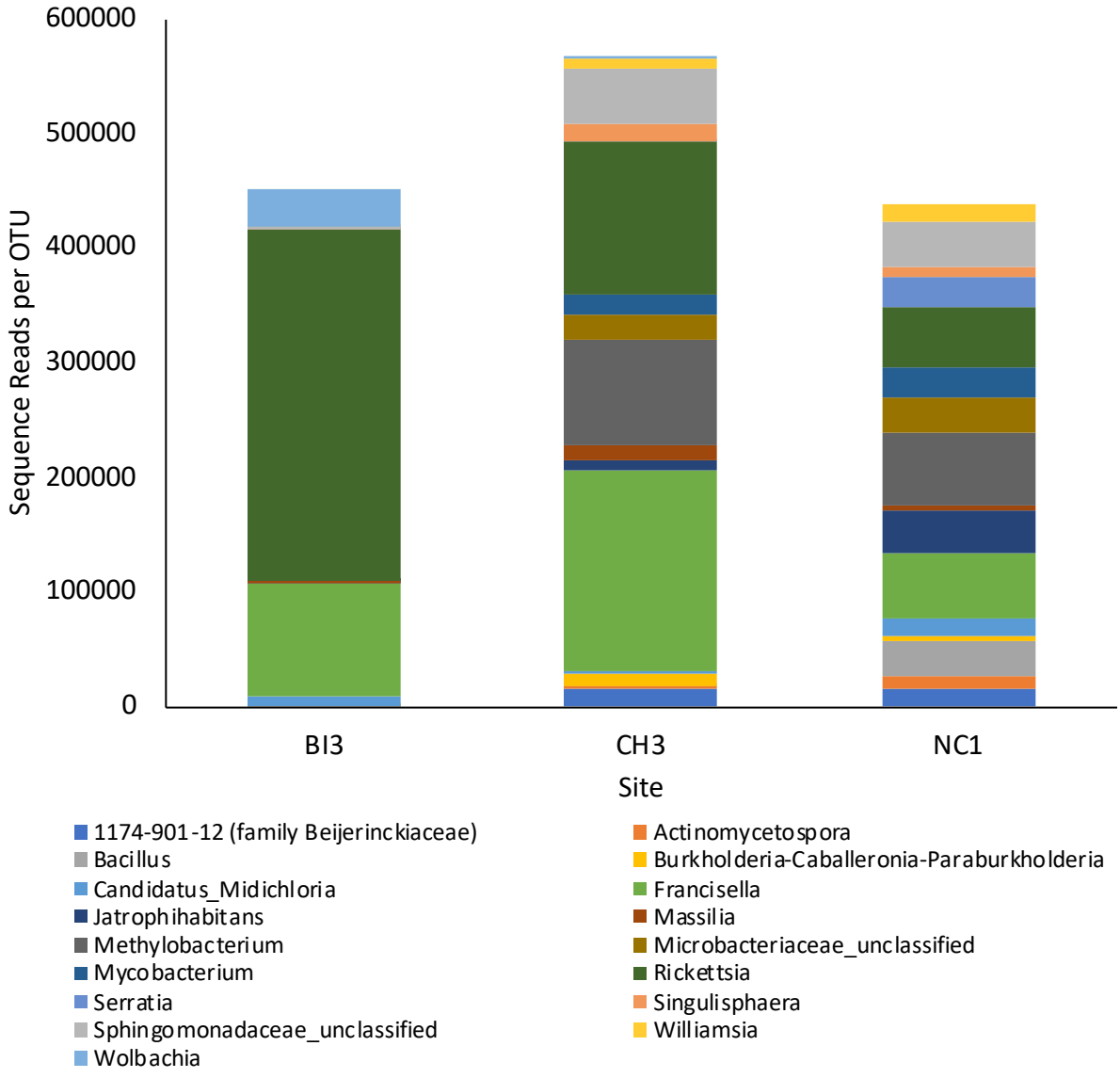

Fig. 17. Top 17 bacterial communities factored by genus based on >10,000 reads per OTU shared between BI3, CH3, and NC1.

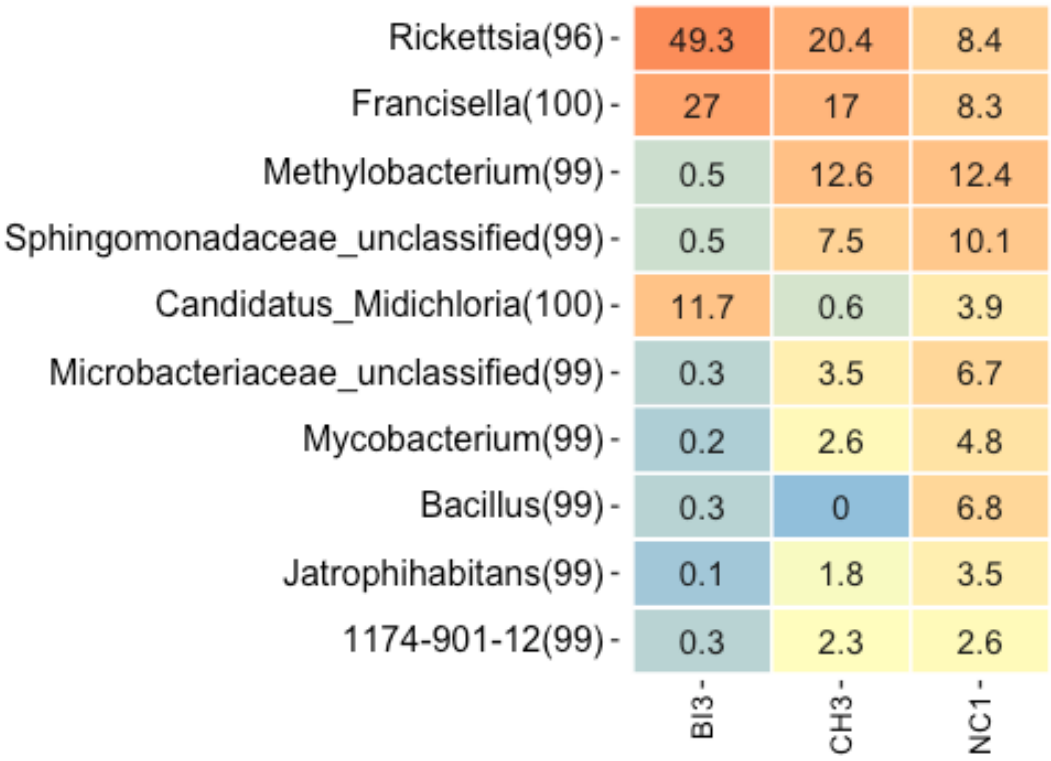

Fig. 18. Read abundance percentages of top genera between BI3, CH3, and NC1.

Insight into bacterial communities based on infection with R. parkeri

The site-specific prevalence of *R. parkeri,* based on two years of field collection data were already assessed at the beginning of this study (Benham et al., unpublished data). At site BI3, *R. parkeri* prevalence was 46.15% (n=78). Five of the ten samples from BI3 were positive for *R. parkeri* by real-time PCR. The most common OTUs in *R. parkeri*-positive samples at site BI3 were *Francisella, Candidatus Midichloria, Rickettsia*, and *Wolbachia* (Fig. 19). At site CH3, *R. parkeri* prevalence was 48.15% (n=27). Three of the ten samples from CH3 were positive for *R. parkeri*. The most common OTUs in *R. parkeri*-positive samples from site CH3 were *Francisella, Burholderia, Jatrophihabitans, Massilia, Methylobacterium, Microbacteriaceae, Mycobacterium, Rickettsia*,

Singulissphaera, and *Sphingomonadaceae*. At site NC1, *R. parkeri* prevalence was 36.00% (n=25). Two of the ten samples from NC1 were positive for *R. parkeri*. The most common genera in these samples were *Actinomycetospora, Bacillus, Candidatus Midichloria, Francisella, Jatrophihabitans, Methylobacterium, Microbacteriaceae, Mycobacterium, Rickettsia, Serratia, Singulisphaera, Sphingomonadaceae*, and *Williamsia*.

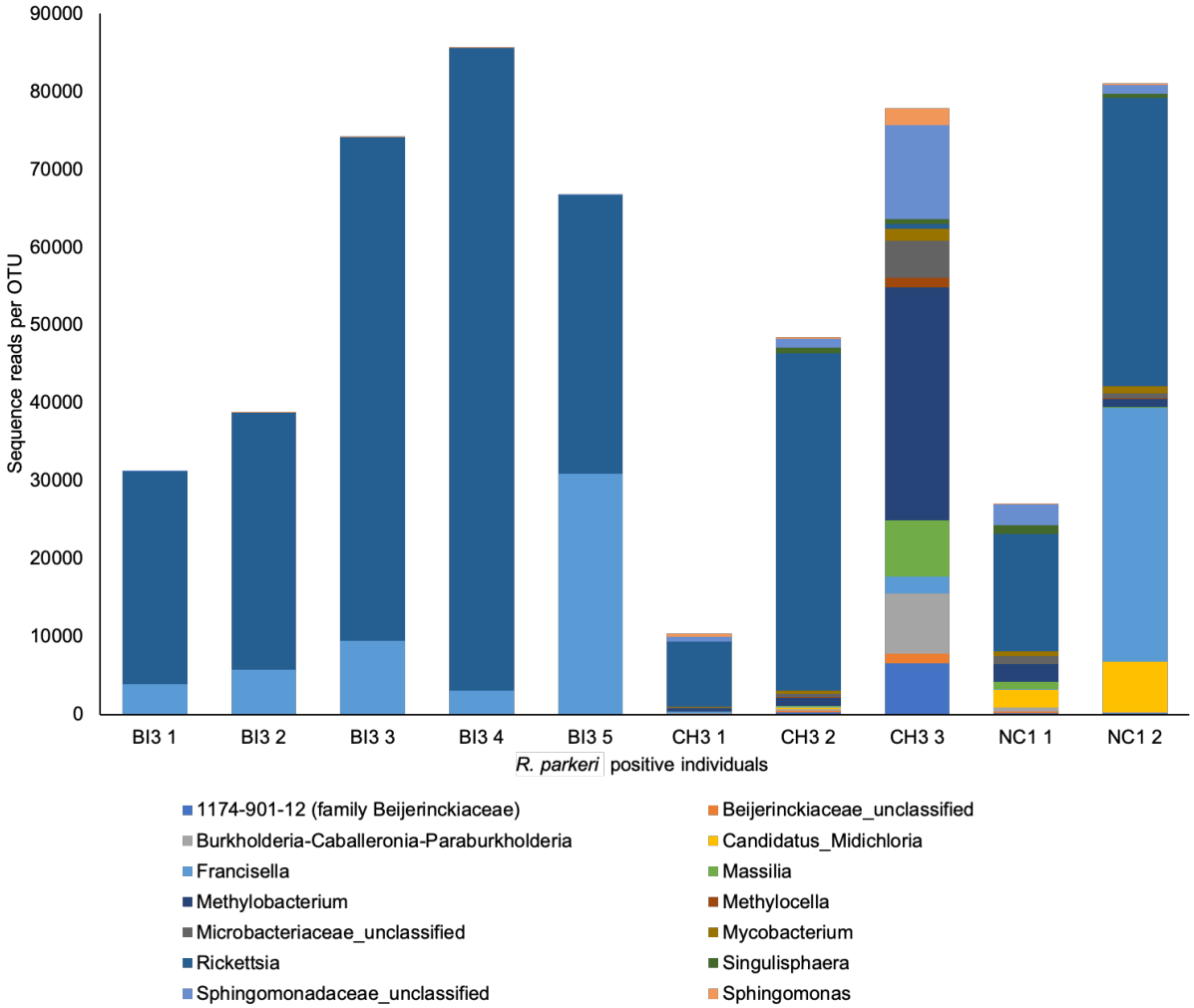

Fig. 19. Common OTUs (genera) among *R. parkeri* positive samples from BI3, CH3, and NC1.

Overall bacterial community composition and diversity were similar between infected and non-infected ticks, with *Rickettsia* read abundances higher in infected ticks versus non-infected ticks (Fig. 20). Read abundances per OTU were higher for environmental bacterial communities than tick-borne bacterial communities in the non-infected ticks.

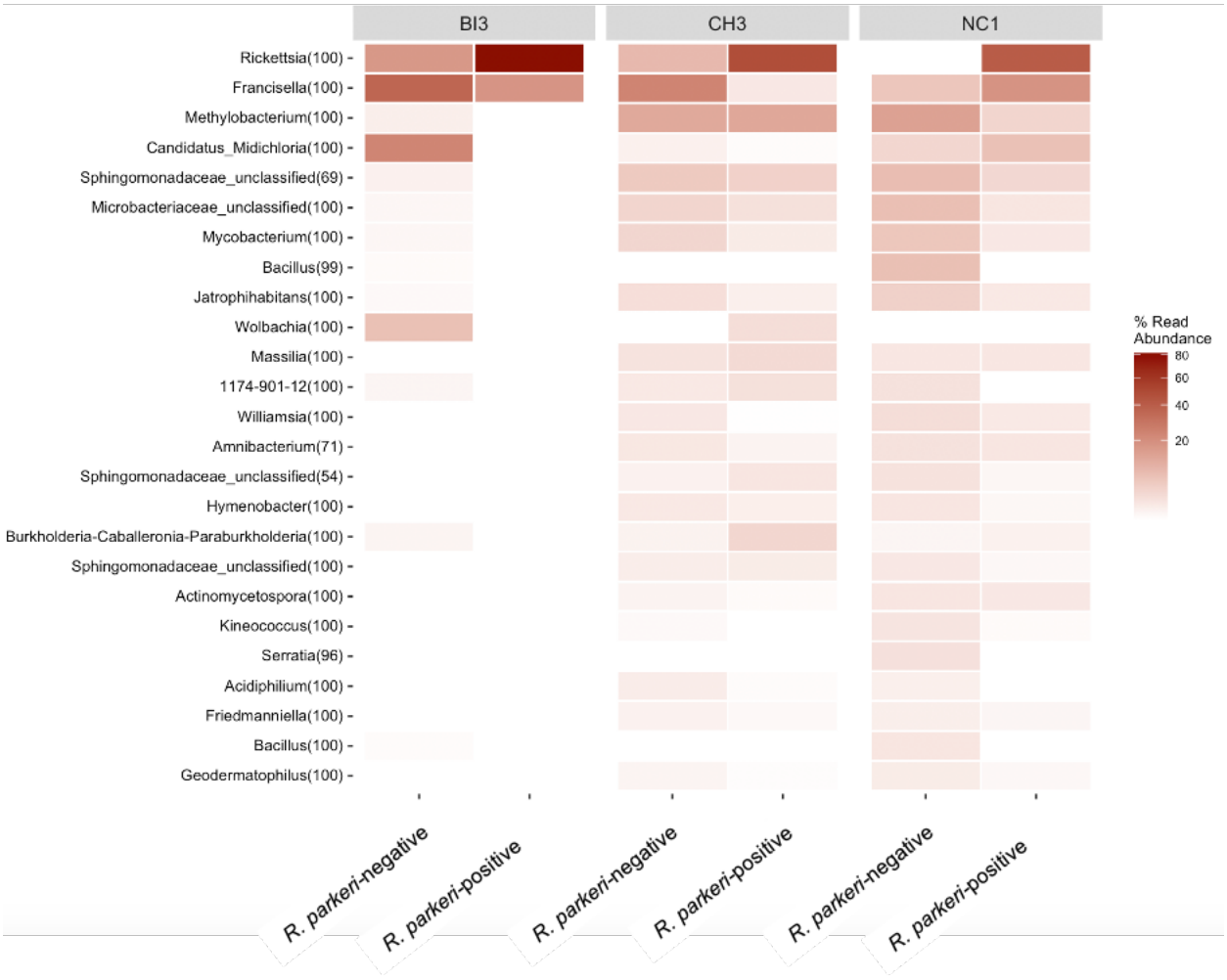

Fig. 20. Read abundance percentages of top genera between *R. parkeri*-infected individuals and non-infected individuals.

There was no significant difference between the bacterial communities in infected and non-infected samples based on the PERMANOVA results (Table 11). When visualizing individuals by *R. parkeri* infection status in an NMDS plot, there is overlap between individuals based on infection, but also some separation in the groups (Fig. 21).

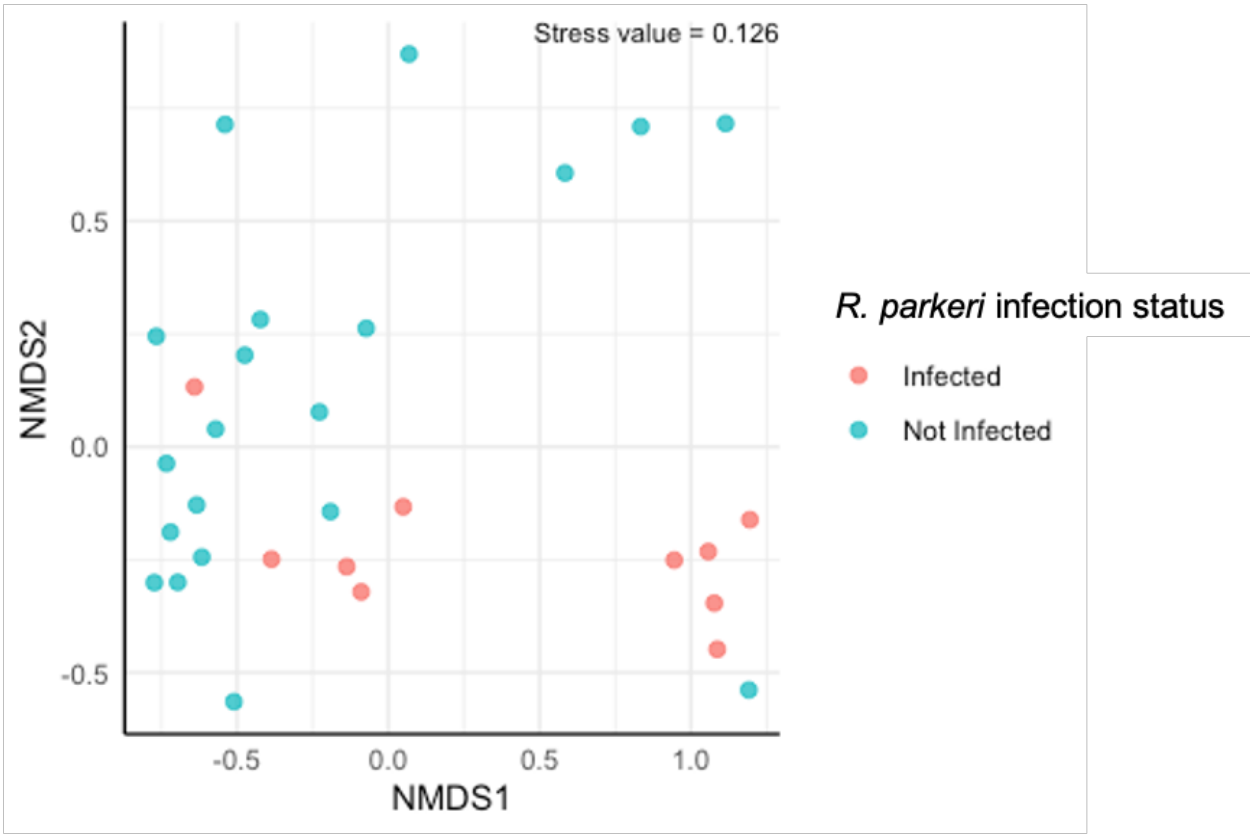

Fig. 21. NMDS plot of wild-caught individuals separated by *R. parkeri* infection based on Bray-Curtis dissimilarity metrics.

Analysis of negative controls

The negative controls from the 16S rRNA amplification appeared to be contaminated, thus further analysis was warranted. When comparing to the sequence read counts between the tick samples and the negative controls, the lab-raised ticks had 2.5x more sequence reads than the negative controls and the wild-caught ticks had 17x more sequence reads than the negative controls. During the amplicon PCR, no bands were present for the negative controls during gel electrophoresis. After the index PCR, the purified PCR products of the negative controls were run on a 1% agarose gel. Each negative control had a band indicating that the index primers were bound to the samples. Based on abundance rank, the negative controls showed low species richness and evenness (Fig. 22). The top ranked OTUs in these controls were *Coxiella* and *Rickettsia* with ~10,000 reads per OTU each. The overall read abundances for these genera were 11.6% and 10.5% (Fig. 23). All other OTUs were the same environmental genera detected in the other tick samples, but in much lower numbers.

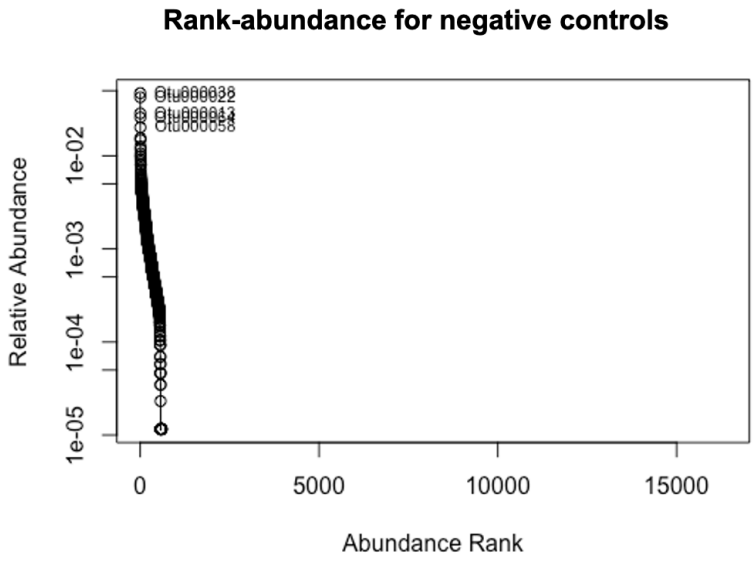

Fig. 22. Rank abundance plot for the 16S rRNA amplification PCR negative controls.

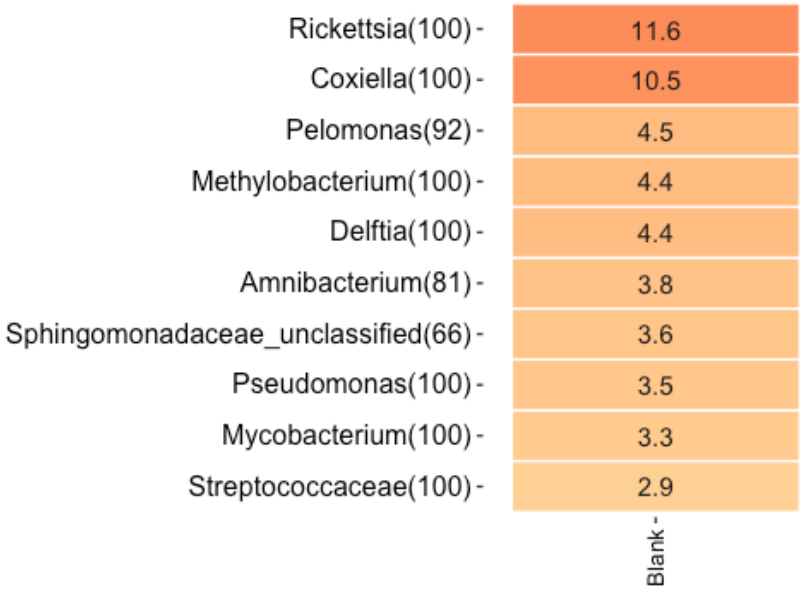

Fig. 23. Read abundance percentages of the top genera for the 16S rRNA amplification PCR negative controls (blanks).

Discussion

In this study, we used 16S V3-V4 region rRNA sequencing to identify bacterial genera in related and unrelated *A. maculatum*. Additionally, we sought to identify and qualify the internal microbiota of wild-caught *A. maculatum* to determine any relationship between the microbiome and the higher prevalence of *R. parkeri* seen in these tick populations collected from Virginia.

The bacterial communities in Virginia *A. maculatum*, identified from 16S rRNA sequencing, were similar to bacterial genera reported in other microbiome studies of *A. maculatum*, *A. americanum*, and *I. ricinus* (Appendix B). Budachetri et al. (2014) and Varela-Stokes et al. (2018) both completed microbiome studies using *A. maculatum* from Mississippi and noted an absence of *Coxiella* species in their samples, likely due to a high abundance of *Francisella*. We did not see this trend in our lab-raised individuals, most being infected with *Coxiella* in the presence of a high abundance of *Francisella*. Additionally, we did not find *Candidatus* Midichloria in high abundance in our *R. parkeri* positive samples. This species had been previously reported as essential for *R. parkeri* replication in *A. maculatum* (Budachetri et al., 2018). Based on our findings, it does not appear necessary for *R. parkeri* presence in *A. maculatum*.

Many of the bacteria identified in our ticks and other tick species could be partially the result of extraction kit contamination (Salter et al., 2014). Many of these bacterial genera fall into the category of “environmental contaminants” according to Salter et al. (2014); unfortunately we did not sequence our extraction negative controls to account for this issue. We did however include two blanks during the 16S rRNA

amplicon PCR. Common or dominant OTUs were represented in these samples from 2.9-11.6% read abundance per OTU which included potential reagent contaminants: *Pelomonas*, *Methylobacterium*, *Delftia*, *Amnibacterium*, *Sphingomonadaceae*_unclassified, *Pseudomonas*, and *Streptococcaceae* as well as some tick-borne contaminants: *Rickettsia*, *Coxiella*, and *Mycobacterium* (Fig. 23). The contamination of the negative controls appears to occur during the index PCR due to the presence of bands on the 1% agarose gel, even though unique adaptor primer pairs were used in each well. Based on the lower number of sequence reads and low species richness, the microbial communities present in the negative controls are likely due to spillover from samples in adjacent tubes during the index PCR and are not indicative of a mass contamination issue in all samples.

In the colony-raised ticks, adults were mated to each other to ensure congruence of bacterial communities between related individuals. Additional bacteria into their internal microbiota came from their host blood meal. Host blood meal appears to play a role in the type of microbial communities present in the colony-raised ticks. The microbial communities in the larvae and adults were more similar to each other compared to the nymphs, likely due to the larvae coming from adults that fed on lab rats and the adults coming from nymphs that fed on lab rats. Although the lab rats were different individual animals, the bacterial communities in both larvae and adults were similar across these life stages and generations. Nymphs would perhaps have different bacterial communities because although they were related to the larvae and adults, they came from larvae that fed on lab mice.

The greatest similarity between bacterial communities was observed between siblings of the same life stage. These siblings would have fed on the same animal and raised in the same environmental conditions. Larval siblings shared the most bacterial communities compared to other life stages. The similarity between larval siblings may be attributed to an 'inherited' microbiome derived from the female (Brinkerhoff et al., 2020).

In wild-caught ticks, infection with *R. parkeri* was not a significant factor in determining the presence of specific bacterial populations; this could be due in part to a low sample size. For infected individuals, site (geographic location) appeared to be the largest contributing factor to the relative abundance of particular bacterial communities and overall bacterial community diversity. Due to a low sample size, determining the bacterial genera associated with *R. parkeri* infection requires additional study. It is important to note that the mammalian host communities at each site likely play a role in the bacterial communities present in the individual ticks. Prevalence of *R. parkeri* was highest at site BI3; this site also had the lowest species diversity and evenness among OTUs. Sites CH3 and NC1 were geographically closer to each other than to site BI3 and had an observed higher species diversity and evenness. Site BI3 is an island; physical isolation may contribute to host community diversity and thus bacterial community diversity compared to the inland sites CH3 and NC1. Alternatively, *Rickettsia* spp., like *R. parkeri*, may be acting as a dominant genera at site BI3, thus precluding other bacterial species from becoming established in this *A. maculatum* population.

Metagenomic analysis using the 16S rRNA gene was useful in identifying trends in bacterial community composition in genetically related individuals (siblings) and in

individuals relative to their geographical location. Individuals that live in the same environment and geographic location have similar microbiomes. Additionally, individuals that are genetically related also have very similar microbiomes. It appears likely that most of the internal bacterial communities of the tick are host-driven. Changes in bacterial communities between tick life stages should not be significantly different if the host bloodmeal did not play a role in changing the bacterial community composition. This trend was seen not only in the colony-raised ticks but also possibly those from sites CH3 and NC1 since individuals from these sites cluster together. Future directions for this work will focus on the host microbiome and the microbiome of the ticks feeding on the host using both lab-raised and wild-caught *A. maculatum*.

CHAPTER VI

CONCLUSIONS

The first aim of this research was to determine which small mammals are being used by immature *A. maculatum* as hosts and to assess such hosts as potential reservoirs of *R. parkeri*. Small mammals are often parasitized by the immature stages of hard-bodied ticks (family Ixodidae) and may serve as reservoir hosts for tick-borne pathogens. *Amblyomma maculatum*, the Gulf Coast tick, is the primary vector of *Rickettsia parkeri*, the causative agent of *R. parkeri* rickettsiosis. This hard-bodied tick is expanding its range from the Gulf Coast of the U.S. up the Mid-Atlantic coast. In Mid-Atlantic states, such as Virginia, *R. parkeri* prevalence in *A. maculatum* is higher than found in its historical range. This high prevalence may be explained in part by the small mammal populations present. In this study, small mammals were trapped and checked for the presence of immature *A. maculatum*. The ticks as well as tissue samples from these mammals were tested for *R. parkeri*. This study found six rodent species acting as hosts to immature *A. maculatum* (the hispid cotton rat, the golden mouse, the house mouse, the eastern harvest mouse, the marsh rice rat, and the meadow vole), and three species (the hispid cotton rat, the marsh rice rat, and the meadow vole) that may play a role in the enzootic cycle of *R. parkeri* in Virginia.

The second aim of this research was to assess spillover of *R. parkeri* from local *A. maculatum* populations into local *D. variabilis* populations. *Dermacentor variabilis* populations surveyed from 2011-2018 had a *R. parkeri* prevalence of 0.46% (95% CI: 0.23%-0.91%) in adult ticks and 3.15% (95% CI: 2.16%-4.58%) in immature ticks from

small mammal hosts. In controlled co-feeding infection studies, both *D. variabilis* larvae and nymphs were found to acquire *R. parkeri* through horizontal transmission. The prevalence of *R. parkeri* infection in this controlled setting was higher than is naturally seen in immature *D. variabilis* removed from small mammals and in adult *D. variabilis* surveyed from vegetation. The results for the adult D. variabilis co-feeding groups were inconclusive due to low sample size.

The third aim of this research was to assess the potential role of the microbiome in the high prevalence of *R. parkeri* seen in *A. maculatum* populations from Virginia. This research is ongoing and requires larger numbers *R. parkeri* positive *A. maculatum*. Our study found no significant difference in *R. parkeri*-infected individuals when compared to non-infected individuals, but our sample size was small. Site played a role in the differences seen in bacterial populations of wild-caught individuals. In *R. parkeri*-infected individuals, common OTUs overlapped, but were largely site dependent.

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APPENDIX A

PRIMERS AND PROBES USED TO IDENTIFY RICKETTSIAE AND TICK SPECIES

Primer/Probe	Sequence (5'-3')	Gene target	Amplicon length (bp)	Reference
AmacITS2F	TTGTGCGGGAAACGACCGGGTGT	ITS2	193	Zemtsova et al., 2014
AmacITS2R	AACGCTCGTAACGAGATACGCG			Zemtsova et al., 2014
AmacITS2Pr	/56-FAM/ACAATGCTTGAGCAGA+G+AGAC/3IABkFQ/			Zemtsova et al., 2014
Amac_actin_F	GCCCTGGACTTCGAGCAG	Actin	74	This study
Amac_actin_R2	CCCGTCAGGAAGTTCGTAGG			This study
Amac_actin_Pr2	/5HEX/ACCGCCGCCT/ZEN/CGTCCTCCTC/3IABkFQ/			This study
16S-1	GTCTGAACTCAGATCAAGT	16S rDNA	454	de la Fuente et al., 2001
16S+1	CTGCTCAATGATTTTTTAAATTGCTGT			Nadolny et al., 2011
Rpa129F	CAAATGTTGCAGTTCCTCTAAATG	*omp*B	96	Jiang et al., 2012
Rpa224R	AAAACAAACCGTTAAAACTACCG			Jiang et al., 2012
Rpa188Probe	/56-FAM/CGCGAAATTAATACCCTTATGAGCAGCAGTCGCG/BHQ-1/			Jiang et al., 2012
RR190.70 (primary)	ATGGCGAATATTTCTCCAAAA	*omp*A	590	Blair et al., 2004
RR190.701	GTTCCGTTAATGGCAGCATCT			Blair et al., 2004

Primer/Probe	Sequence (5'-3')	Gene target	Amplicon length (bp)	Reference
RR190.70 (nested)	ATGGCGAATATTTCTCCAAAA		540	Regnery et al., 1991
RR190.622n	AGTGCAGCATTCGCTCCCCCT			Regnery et al., 1991
R17K128F2	GGGCGGTATGAAYAAACAAG	17-kDa	111	Jiang et al., 2012
R17K238R	CCTACACCTACTCCVACAAG			Jiang et al., 2012
R17K202TaqP	6FAM- CCGAATTGAGAACCAAGTAATGC-TAMRA			Jiang et al., 2012
RMF2832	GCGGTGGTGTTCCTAATAC	*omp*B	106	Jiang et al., 2012
RMR2937	CCTAAGTTGTTATAGTCTGTAGTG			Jiang et al., 2012
RMB2875	FAM- CGGGGCAAAGATGCTAGCGCTTCACAGTTACCCCG-BHQ-1			Jiang et al., 2012
Rpa129F	CAAATGTTGCAGTTCCTCTAAATG	*omp*B	96	Jiang et al., 2012
Rpa224R	AAAACAAACCGTTAAAACTACCG			Jiang et al., 2012
Rpa188probe	6-FAM- CGCGAAATTAATACCCTTATGAGCAGCAGTCGCG-BHQ-1			Jiang et al., 2012
Ra477F	GGTGCTGCGGCTTCTACATTAG	*omp*B	142	Jiang et al., 2010
Ra618R	CTGAAACTTGAATAAATCCATTAGTAACAT			Jiang et al., 2010
Ra532Probe	6FAM- CGCGATCTCCTCTTACACTTGGACAGAATGCTT ATCGCG-BHQ-1			Jiang et al., 2010

APPENDIX B

BACTERIAL GENERA PRESENT

IN HARD-BODIED TICKS

Genus	Associated tick	Reference
Acinetobacter	*Ixodes ricinus*	Moutailler et al., 2016
Actinomycetospora	*Ixodes ricinus*	Moutailler et al., 2016
Bacillus	*Amblyomma maculatum*	Budachetri et al., 2014
Burkholderia-Caballeronia-Paraburkholderia	*Ixodes ricinus*	Stojck and Dutkiewicz, 2004 Estrada-Pena et al., 2018
Burkholderiaceae_unclassified	*Ixodes ricinus*	Stojck and Dutkiewicz, 2004 Estrada-Pena et al., 2018
Candidatus_Midichloria	*Amblyomma maculatum*	Budachetri er al., 2014
Coxiella	*Ixodes ricinus*	Moutailler et al., 2016
Cutibacterium	*Ixodes scapularis*	Chauhan et al., 2020
Delftia	*Amblyomma maculatum*	Budachetri er al., 2014
Francisella	*Amblyomma maculatum*	Budachetri er al., 2014
Jatrophihabitans	*Ixodes ricinus*	Estrada-Pena et al., 2018
Massilia	*Amblyomma americanum*	Ponnusamy et al., 2013
Methylobacterium	*Amblyomma maculatum*	Varela-Stokes et al., 2018
Microbacteriaceae_unclassified	*Amblyomma maculatum*	Budachetri er al., 2014
Mycobacterium	*Amblyomma maculatum*	Varela-Stokes et al., 2018
Pelomonas	*Ixodes ricinus* *Amblyomma americanum*	vanOverbeek et al., 2008 Yuan, 2010
Pseudomonas	*Amblyomma maculatum*	Budachetri er al., 2014
Pseudonocardia	*Amblyomma americanum*	Willis, 2016
Rhizobiaceae_unclassified	*Amblyomma americanum*	Clay et al., 2008
Rickettsia	*Amblyomma maculatum*	Budachetri er al., 2014
Serratia	*Ixodes ricinus*	Stojek and Dutkiewicz, 2004

Genus	Associated tick	Reference
Singulisphaera	*Amblyomma americanum*	Trout Fryxell and DeBruyn, 2016
Sphingomonas	*Amblyomma maculatum*	Varela-Stokes et al., 2018
Staphylococcus	*Amblyomma maculatum*	Budachetri er al., 2014
Stenotrophomonas	*Amblyomma maculatum*	Budachetri er al., 2014
Streptococcus	*Amblyomma maculatum*	Budachetri er al., 2014
Williamsia	*Dermacentor spp.* *Ixodes ricinus*	Zhang et al., 2019 Estrada-Pena et al., 2018
Wolbachia	*Amblyomma maculatum*	Budachetri er al., 2014

APPENDIX C

PROTOCOLS

All research was carried out under the guidelines of the ODU IBC protocol #s 17-001 and 17-001 and the ODU IACUC protocol #s 17-015, 20-016, 11-012, 16-003, and 17-006. Tick surveillance and collection from vegetation and wild animals were permitted under protocol VADGIF #041740, #047118, and #053333.

APPENDIX D

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American Society for Microbiology- Virginia Branch Travel Award, $75 2018
ODU Biology Graduate Student Organization Research Award, $200 2017
ODU SEES Graduate Research Award, $200 2016

SELECTED PUBLICATIONS

Cumbie A., E. Walters, H. Gaff, and W. Hynes. 2020. First report of *Candidatus* Rickettsia mendelii, in *Ixodes brunneus* ticks collected in the United States. Ticks and Tick-Borne Disease. 11(1), 101309.

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Cumbie A., E. Heller, Z. Bement, A. Phan, E. Walters, W. Hynes, and H. Gaff. 2020. Possible role of passerine birds in the spread and maintenance of *Borrelia burgdorferi* in *Ixodes* tick in the mid-eastern United States. Ticks and Tick-Borne Disease. *Under review.*