Survey of *Rickettsia parkeri* and *Amblyomma maculatum* associated with small mammals in southeastern Virginia

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Abstract

Small mammals are often parasitized by the immature stages of hard-bodied ticks (family Ixodidae) and may serve as reservoir hosts of 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 species is expanding its historical range from the Gulf Coast of the U.S. up the Mid-Atlantic coast. In Mid-Atlantic states, such as Virginia, *R. parkeri* prevalence is higher in these ticks than those found in its historical range. This high prevalence may be explained in part by small mammal populations. 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 the presence of *R. parkeri*. This study found six rodent species acting as hosts to immature *A. maculatum* and three species that may play a role in the enzootic cycle of *R. parkeri* in Virginia.

Keywords

Amblyomma maculatum, *Rickettsia parkeri*, hosts, rodents, immature, enzootic cycle

Introduction

The *Amblyomma maculatum* species complex encompasses a group of hard-bodied tick species 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 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 (Figure 1) 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.

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 2 mm 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 (Table 1). *Amblyomma maculatum* were additionally confirmed using a real-time PCR assay based on the *A. maculatum* actin gene developed in this study (Table 1).

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), 0.5 µL of *A. maculatum* actin probe (10 µM), and 5 µL of extracted DNA. Thermocycler conditions for the actin assay consisted of 95℃ for 3 min, followed by 40 cycles of of 95℃ for 10 s and 60℃ 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 (Table 1).

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 (Table 1). 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 (Table 1). 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 3M 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.*

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).

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.

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 tick species, such as *D. variabilis* and/or *A. americanum*, could be playing cryptic roles in the enzootic cycle of *R. parkeri*. Although these tick species 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.

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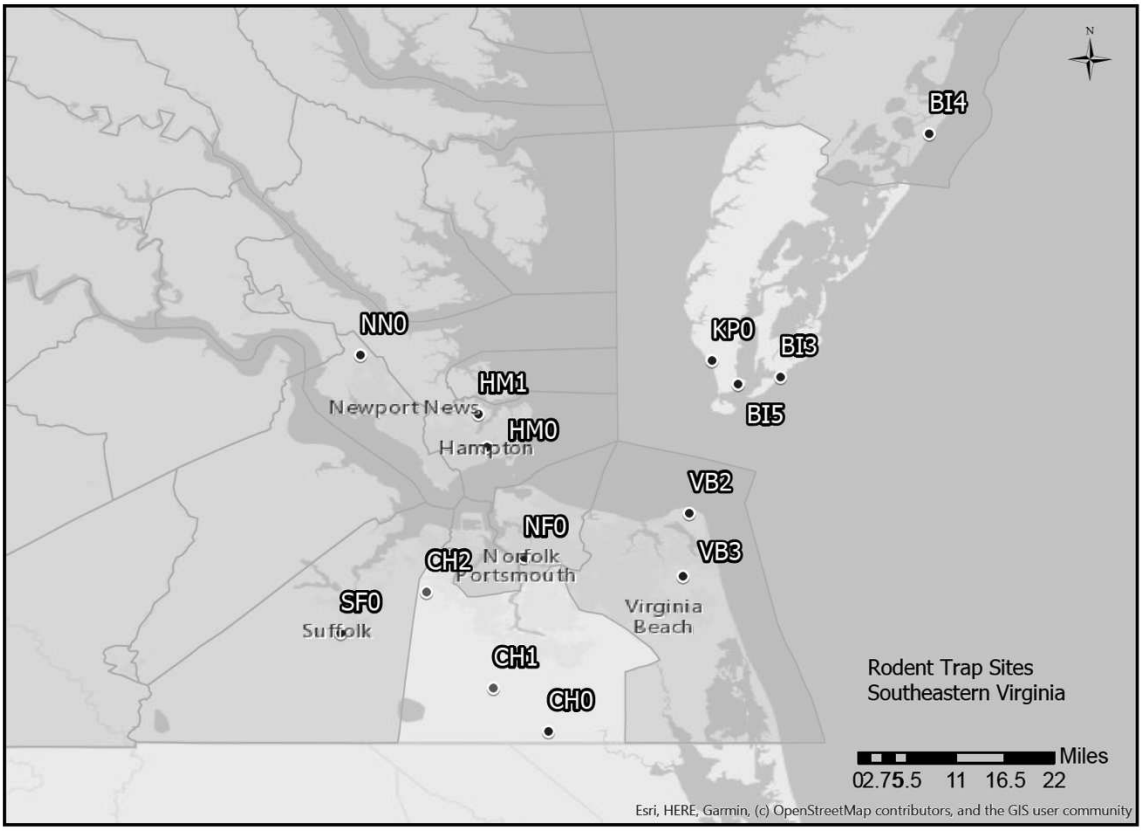

Figure 1. Map of small mammal live-trapping sites in southeastern Virginia. Long-term monthly surveillance was done at CH1 and CH2, which area located within the City of Chesapeake. Trapping was also done at HM1 in the City of Hampton and SF0 in the City of Suffolk. Additional samples were donated from 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).

Table 1. Primer pairs and probes used for tick and pathogen identification.

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
RR190.70 (nested)	ATGGCGAATATTTCTCCAAAA		540	Regnery et al., 1991
RR190.622n	AGTGCAGCATTCGCTCCCCCT			Regnery et al., 1991

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

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% (16)
House mouse	Larva (6) Nymph (0)	8% (79) 0 (79)
Eastern harvest mouse	Larva (0) Nymph (3)	0 (76) 4% (76)
Marsh rice rat	Larva (2) Nymph (0)	1.2% (164) 0 (164)
Meadow vole	Larva (0) Nymph (4)	0 (179) 2.2% (179)

Table 4. Numbers of immature *A. maculatum*, ticks and rodent tissues, and number infected with *R. parkeri* collected at from small mammal live trapping sites in southeastern Virginia and number of *R. parkeri* infected *A. maculatum* for those areas

Trapping site*	Year	Number of *A. maculatum* removed from rodents	Life stage**	Number of *R. parkeri* infected *A. maculatum*	Number of *R. parkeri* infected rodent 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	13
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	1
HM0		0	N/A	0	0
NN0		0	N/A	0	1
HM1	2018	0	N/A	0	0
CH0		0	N/A	0	0
VB3		4	4L	0	2

* The acronyms for the trapping sites correspond to those given in Figure 1;

** L means larva and N means nymph.

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