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Tick Burdens in a Small-Mammal Community in Virginia

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Abstract - Virginia has seen dramatic increases in reported cases of Lyme disease and Rocky Mountain spotted fever, but basic knowledge on the community ecology of these tick-borne diseases is poor. We examined the tick burdens of 5 small-mammal species in northwest Virginia from October 2011 to December 2012. We live-trapped individuals, quantified the tick burdens, assessed the burden structure, and tested a subset of the ticks for tick-borne pathogens. We found the tick burdens to be composed predominantly of *Ixodes scapularis* (Black-Legged Tick), and *Ixodes* sp. ticks, with *Amblyomma americanum* (Lone Star Tick) and *Dermacentor variabilis* (American Dog Tick) also present at lower densities. We detected *Borrelia burgdorferi* (prevalence 15%), *Rickettsia* spp. (4%), *Anaplasma phagocytophilum* (4%), and *Hepatozoon* spp. (1%). Black-Legged Ticks, a species which has shown range expansion in recent decades, tested positive for *B. burgdorferi* (17%) and for multiple pathogens in individual ticks. For better predictions of tick-borne disease risk across the Mid-Atlantic region, we recommend tracking changes in tick communities by continuous monitoring of tick burdens, densities of questing ticks, and prevalence of tick-borne pathogens.

Introduction

Many of the tick-borne diseases affecting humans have increased in geographic range and number of reported cases in the United States in recent years (Dantas-Torres et al. 2012). This increase has been especially strong in Virginia, which has seen dramatic increases in reported cases of Lyme disease and Rocky Mountain spotted fever over the last decade (Brinkerhoff et al. 2014, CDC 2017, Nadolny et al. 2014). Of the 10 states with the highest reports for Lyme disease from 2006 to 2016, Virginia has had the second highest rate of increase, following Pennsylvania (CDC 2018). While no single factor seems primarily responsible, increases in disease prevalence have been attributed to tick range expansion, increases in human–tick interactions, and changes in biodiversity and habitat (Gubler et al. 2001, Nadolny et al. 2014).

Small mammals play an important role in the prevalence of tick-borne pathogens, as they are both hosts to feeding ticks and reservoir hosts for pathogens

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(Dallas et al. 2012). While not adversely affected by the pathogens (Tilly et al. 2008), small-mammal species differ in quality as reservoir hosts (Ostfeld and Keesing 2012), with some being major carriers of pathogens (e.g., *Peromyscus leucopus* (Rafinesque) [White-Footed Mouse] and the Lyme disease pathogen *Borrelia burgdorferi* (Johnson)), and others as poor quality hosts, a "dead end" for the pathogen. LoGiudice et al. (2003) found that high vertebrate biodiversity and community composition can lead to lower prevalence of Lyme disease. Small-mammal hosts can also impact overall tick populations as many immature ticks feed primarily on such hosts (Smart and Caccamise 1988).

Due to their importance, tick burdens (all of the ticks attached to an individual host) have been the focus of many studies. Past research on tick burdens has often been limited to examinations of a single tick species, mammal host species, or tick-borne pathogen. Recent tick research in Virginia and the surrounding region focused on pathogen prevalence in host-seeking, or "questing", ticks (Henning et al. 2014; Herrin et al. 2014; Nadolny et al. 2011, 2014), and have not assessed the tick burdens of small mammals. All studies that have assessed tick burdens of small mammals in Virginia and surrounding states are decades old (Levine et al. 1991, Sonenshine and Haines 1985, Sonenshine and Stout 1968, Zimmerman et al. 1987). Therefore, there is a strong need for tick-burden research in Virginia and the surrounding region, to provide researchers and the medical community with important updated information on ticks and tick-borne disease risk.

This study aims to address this deficiency. We surveyed ticks and tick-borne pathogens within a small-mammal community in northwest Virginia. We examined the tick burdens on small mammals for the following: tick abundance, tick species composition, and tick-borne pathogen prevalence. In addition, we examined the abundance, diversity, and pathogen prevalence of questing ticks.

Field-site Description

This study was conducted in forests and fields at the Smithsonian Conservation Biology Institute (SCBI), near Front Royal, VA (Warren County; 38°53′15.6″N, 78°9′54.6″W). SCBI features long-term ecological monitoring projects including a Smithsonian Institution Forest Global Earth Observatory (ForestGEO), a 25.6-ha monitoring plot surveyed since 1990 (Bourg et al. 2013), and has been a National Ecological Observatory Network core site since 2014. These features make SCBI an ideal location for conducting a baseline tick survey, as the long-term data collected at this site could provide ecological context for understanding any future changes in the tick, pathogen, or host communities.

Methods

Small-mammal captures and tick collection

We trapped small mammals from October to November 2011 and April to October 2012 (5460 trap-nights) using 8 cm × 9 cm × 23 cm live traps (Sherman, Tallahassee, FL). Each trapping session for the project consisted of 4 consecutive nights of trapping. The trapping period during fall of 2011 and from spring to fall

in 2012 coincided with tick phenology, thus capturing activity and abundance of larval and nymphal ticks (Gatewood et al. 2009). For 2012, trapping in the forest took place within the ForestGEO grid (680 m × 420 m), which was divided into 8 sections. The sections included 4 smaller sections (210 m x 160 m) with 88 trap grid-points, and 4 larger sections (210 m x 180 m) with 99 trap grid-points. Trapping took place at 2 of the 8 sections (including 1 of each size) with a total of 374 traps set at 187 trap locations. We set 2 traps facing opposite directions near each trap grid-point site, with 20 m between locations. We randomly selected the 2 grids (1 from each size) for trapping, which allowed for each section to be trapped for multiple sessions across the trapping period, varying from 2 to 8 trapping sessions.

We also conducted trapping along 10 transects (100 m each), with 2 traps set every 10 m, for 1 session each in 3 fields and 3 forests in 2011 and in 2 fields in 2012. Trapping along transects with a total of 22 traps per transect enabled us to survey small mammals outside of the ForestGEO grid, in field and forest sections across SCBI that varied in area and shape.

All captured animals were identified to species and sex, marked with an ear tag if unmarked, weighed, searched thoroughly for ticks, and then released at the point of capture. We did not use anesthesia during processing. We counted and collected all observed, attached ticks, which we then placed into vials filled with ethanol (Hersh et al. 2014, Schmidt et al. 1999). For all ticks, we also recorded the location of the tick attachment upon the host using 3 location categories (ear, face [including chin], and torso). All *Peromyscus* mice were identified as White-Footed Mice based on tail characteristics (Reid 2006). However, we recognize that the study may have included *P. maniculatus* (Wagner) (Deer Mice) as these *Peromyscus* species are difficult to differentiate in the field (Rich et al. 1996). Procedures for all animal captures were approved by the Smithsonian Institution's Animal Use and Care Committee (#11-30).

Tick collection from the environment

To measure the density of questing ticks in the environment, we sampled ticks opportunistically from October to November 2011 and from May to October 2012 at SCBI using standard drag cloths (90 cm × 185 cm) for dragging at ground level (Brunner and Ostfeld 2008, Goddard 1993, Henning et al. 2014). We dragged the area around each mammal trap for 40 m^2 in each individual survey, with a total survey area of 20,650 m^2. These surveys provided a measure of questing tick density and diversity in the area surrounding each trap site. We dragged at each trap site as soon as logistically possible after mammal trapping, and sampling was completed within 1 week following mammal captures.

Tick morphological identification

All collected ticks were observed under a 6.3:1 (5–378x magnification) microscope (Olympus CHA, Tokyo, Japan). Adult and nymphal ticks were identified to species using characteristics (scutum, basis capituli, spurs, etc.) as shown in 2 pictorial keys (Keirans and Durden 1998, Keirans and Litwak 1989). We did not morphologically identify the larval ticks to species as few keys exist for larvae, and

identification is difficult and prone to error. Preliminary species identification was checked by taxonomist R. Robbins (Air Forces Pest Management Board, US Army Garrison-Forest Glen, Silver Spring, MD).

Pathogen screening and tick molecular identification

As we were unable to test all collected ticks due to time and budget constraints, we selected a subsample (n = 250) of collected ticks, including both attached and questing, for genetic testing. Attached ticks were selected randomly but stratified by host and tick species across the study period. We randomly selected questing ticks from each dragging location across the study period. This subsample was genetically tested to verify tick species identification and screen for tick-borne pathogen groups including *Anaplasma phagocytophilum* (Foggie) Dumler et al., *Ehrlichia* spp., *Theileria microti* (França) (= *Babesia microti*), *Borrelia burgdorferi*, *Coxiella burnetii* Derrick, *Rickettsia* spp., and *Hepatozoon* spp. We chose these pathogens because they have been reported in the region (CDC 2017) and have had well-tested protocols designed for their detection using PCR-based methods (Campana et al. 2016). We refer to genera that include pathogenic and non-pathogenic species, such as *Rickettsia* spp., as pathogen groups with the understanding that the group may include pathogenic and/or non-pathogenic species.

We homogenized whole ticks using 1.0-mm silica beads in a BeadBeater (BioSpec Products, Inc., Bartlesville, OK). Each individual tick was processed (Appendix 1). We identified morphologically unidentified ticks, mostly larvae, and pathogens by previously published conventional PCR assays (primer pairs listed in Appendix 1; detailed methodology listed in the appendix of Campana et al. 2016).

Rarefaction curves for community composition

We computed rarefaction-type curves, based on the Shannon–Weaver diversity index (Dumas et al. 2011, Gauthier et al. 2010) to assess whether the sizes of our subsamples of identified ticks were sufficient to characterize the tick communities we sampled. We first divided our identified ticks into 4 groups: (1) attached adults, (2) attached nymphs, (3) questing adults, and (4) questing nymphs. For each of these groups with at least 2 species identified, we then randomly resampled (without replacement) 15,000 times, and computed the Shannon–Weaver index, as a function of sample size, for each re-sampling. For each sample size (from 1 individual to the total number of individuals in each group), we then averaged over the 15,000 re-samplings to obtain the rarefaction curve and its standard deviation. Finally, we plotted the rarefaction curve for each group, together with ± 1 SD error bars, against the full-data Shannon–Weaver diversity estimate for the focal group. This latter value is simply the Shannon–Weaver estimate computed from all of the data for a given group. We developed a small R package, shannonRarefy (https://github.com/jmcalabrese/shannonRarefy), to automate these calculations. The package can be installed using the devtools package via: devtools::install.github("jmcalabrese/shannonRarefy").

These rarefaction curves allowed us to assess the extent to which species diversity in each of the above-defined groups could be expected to change as sample sizes

decreased. A small decrease in expected diversity produced by a large decrease in sample size would suggest that the actual sample size of the focal group was sufficient to characterize diversity. At the other extreme, a large decrease in expected diversity produced by a small decrease in sample size would suggest a group-level sample size that was likely too small to adequately characterize diversity.

Results

Small-mammal captures

We captured 471 small mammals representing 5 species, including *Blarina brevicauda* (Say) (Short-Tailed Shrew), White-Footed Mice, *Zapus hudsonius* (Zimmermann) (Meadow Jumping Mouse), *Microtus pennsylvanicus* (Ord) (Meadow Vole), and *Tamias striatus* (L.) (Eastern Chipmunk) (Table 1), all of which were identified, measured, and examined for ticks. Most mammals (75%) were White-Footed Mice. A total of 87 White-Footed Mice and 4 Eastern Chipmunks were recaptured and reexamined for ticks.

Tick collection and identification

We collected 1114 ticks attached to the mammals, the majority of which were larvae (Table 1). Most ticks were attached on ears (86%), with less on the face region (9%) and the torso (5%). Ticks were not observed on 36% of all small mammals, including the majority of Short-Tailed Shrews, a third of Eastern Chipmunks and White-Footed Mice, and 1 Meadow Vole (Table 1). We identified a total of 164 ticks (115 genetically, 49 morphologically) to the following 3 species: *Amblyomma americanum* (L.) (Lone Star Tick), *Dermacentor variabilis* (Say) (American Dog Tick), and *Ixodes scapularis* (Say) (Blacklegged Tick). Tick abundance varied over time for each life stage (Fig. 1), with peak abundance of adults in October (n = 12 ticks), nymphs in June (n = 66), and larvae in July (n = 234). Tick burdens were lowest in November (91%) and peaked in July (16%). In addition, we collected 2389 questing ticks (Table 2). A subsample of 428 was identified (25 genetically, 403 morphologically) to the same 3 species. Most (57%) were Blacklegged Ticks.

Table 1. Small mammals captured in forests and fields in Virginia, and the ticks collected from them, by species and life stage: Adult (A), Nymph (N), and Larvae (L). Average tick burdens (± 1 SD) are given. Unidentified ticks are not listed except for those in *Ixodes* genus. *A. a.* = *A. americanum* (Lone Star Tick), *D. v.* = *D. variabilis* (American Dog Tick), and *I. s.* = *I. scapularis* (Blacklegged Tick).

					I. s.			Unknown *Ixodes*			
Host species	Total hosts	Average tick burden	*A. a.* N	*D. v.* L	A	N	L	A	N	L	Totals
Blarina brevicauda	55	0.51 (± 2.04)	0	0	0	0	6	0	0	8	26
Microtus pennsylvanicus	7	1.71 (± 1.60)	0	2	2	0	1	0	0	0	11
Peromyscus leucopus	353	3.04 (± 4.24)	6	11	16	34	54	1	55	527	930
Tamias striatus	55	2.85 (± 4.05)	0	0	6	11	12	0	14	95	144
Zapus hudsonius	1	3	0	0	0	0	3	0	0	0	3
Total ticks			6	13	24	45	76	1	69	630	1114

The density of questing ticks peaked in June for adults (0.05 individuals/m^2) and for nymphs (0.14 individuals/m^2), and in August for larvae (14.28 individuals/m^2) (Fig. 1).

We identified an additional 700 of the 1114 ticks collected from small mammals (Table 1) and an additional 663 of the 2389 questing ticks to the *Ixodes* genus (Table 2). Ten ticks were identified to the *Ixodes* genus using molecular techniques,

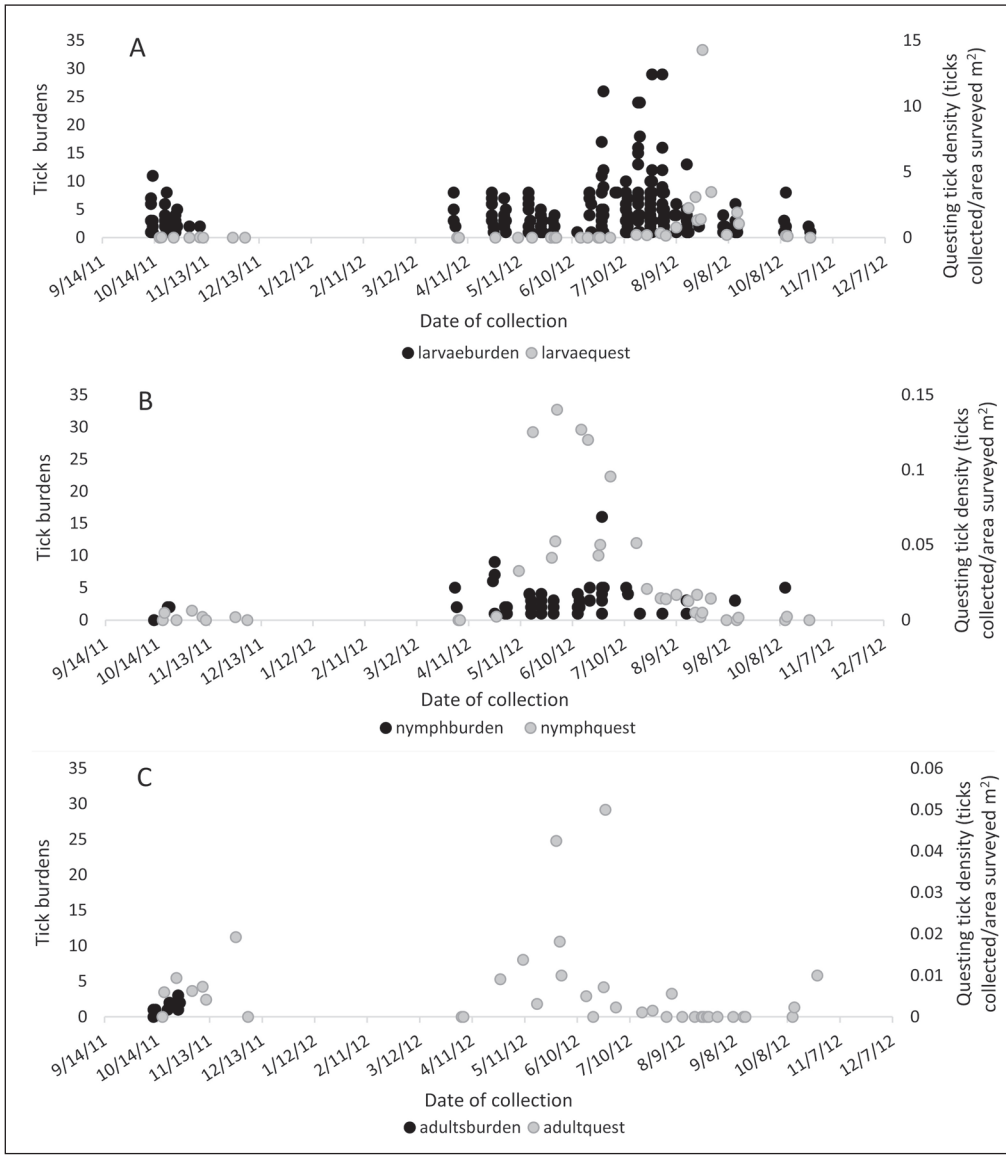

Figure 1.Tick burdens (number of ticks collected) and questing tick density (ticks/m^2) over time for: (A) larvae, (B) nymphs, and (C) adults. Sampling efforts across trapping and dragging sessions were constant over time. No trapping took place between December 2011 and March 2012. No dragging for questing ticks took place between January and March 2012.

while the remaining 1353 ticks were identified morphologically. We were unable to identify the remaining 1548 collected ticks (250 attached, 1298 questing) because these specimens were either too damaged or non-*Ixodes* larvae, which are difficult to identify due to their morphological traits being less conspicuous.

Taxonomist R. Robbins verified samples of adult and nymphal ticks that we had identified morphologically. The ticks selected for genetic identification were those that had not been morphologically identified to species, the majority of which were larval ticks or too damaged to identify. We verified the morphological identification of ticks to genus through genetic testing. All ticks that were genetically identified as Blacklegged Ticks, or at least to the *Ixodes* genus, were morphologically identified to the *Ixodes* genus. All larvae that were genetically identified as American Dog Ticks and a single Lone Star Tick were morphologically identified as non-*Ixodes*. These results indicates that visual identification was accurate.

We computed Shannon–Weaver diversity-based rarefaction curves for attached nymphs ($n = 51$), questing adults ($n = 105$), and questing nymphs ($n = 320$) (Fig. 2). We were unable to compute a rarefaction curve for attached adults, as only 1 species (Blacklegged Tick) was identified in this group. For the 3 groups of identified ticks with ≥ 2 species per group, the rarefaction curves suggested that the estimated species diversity of each group was not sensitive to changes in sample size. Specifically, our analysis suggested that a 50% reduction in sample size of identified ticks would lead to reductions in Shannon diversity of only 5.0% for attached nymphs (Fig. 2A), 1.4% for questing adults (Fig. 2B), and 0.3% for questing nymphs (Fig. 2C).

Pathogen detection

Using molecular methods, we tested 250 ticks (218 from small mammals and 32 questing) for 7 pathogens (Table 3). We detected varying prevalence of 4 pathogen groups in tested ticks: *A. phagocytophilum*, *B. burgdorferi*, *Hepatozoon* spp., and *Rickettsia* sp. (Table 3). Overall, 26% of the larvae ($n = 33$), 22% of the nymphs ($n = 19$), and 26% of the adults ($n = 9$) tested positive for pathogens. Five Blacklegged Ticks tested positive for multiple pathogens.

Discussion

Virginia has seen significant increases in reported cases of tick-borne diseases over the past decade (CDC 2017), but basic knowledge on the community ecology of these tick-borne diseases is poor. We examined the tick species and tick-borne

Table 2. Questing ticks captured in forests and field in Virginia, grouped by species and life stage. Unidentified ticks are not listed except for those in *Ixodes* genus.

	Amblyomma americanum	*Dermacentor variabilis*	*Ixodes scapularis*	Unknown *Ixodes*	Total
Adult	45	20	40	1	106
Nymph	119	0	201	82	402
Larvae	1	0	2	580	583
Total	165	20	243	663	1091

pathogens in questing ticks and within tick burdens of a small-mammal community in forests and fields from October 2011 to November 2012 in northwest Virginia, in order to generate valuable information regarding tick burdens and pathogen prevalence that can be used as a baseline for future studies.

Among ticks collected from 5 small-mammal species, we found that Blacklegged Tick was the most abundant tick species and *Ixodes* the most abundant genus at each tick life stage. Lone Star Ticks and American Dog Ticks were present in the burdens at much lower levels of abundance. It is likely that the unidentified portion of attached larval ticks consisted of Lone Star Ticks and American Dog Ticks, but even with these additional ticks, the 2 species remain at low abundances when compared to *Ixodes*. These findings reflect that the range of

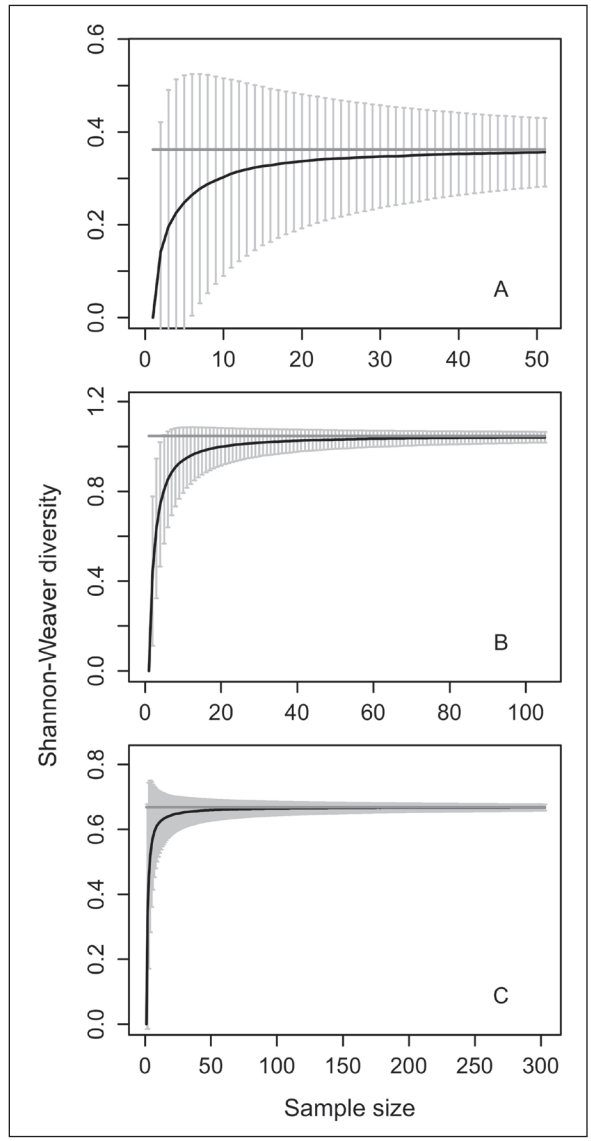

Figure 2. Rarefaction-type curves representing Shannon-Weaver diversity vs sample size (black curves) for identified: (A) attached nymphs (n = 51), (B) questing adults (n = 105), and (C) questing nymphs (n = 320). The (vertical) error bars are ±1 SD, and the horizontal line is the Shannon-Weaver diversity estimate from the full dataset for the focal group. Notice that the full sample sizes noted above for each group are reflected in the maximum sample size value on the x-axis of each panel. In all cases, cutting the full sample size in half resulted in no more than a 5% decrease in Shannon diversity.

Table 3. Pathogen prevalence in ticks collected from 3 small mammal species—*Peromyscus leucopus* (PELE; *n* = 166 ticks tested), *Tamias striatus* (TAST; *n* = 38), *Microtus pennsylvanicus* (MIPE; *n* = 5)—and directly from the environment (q) in forests and fields in Virginia, grouped by species and life stage: Larvae (L), Nymph (N), and Adult (A). Tested ticks from *Blarina brevicauda* (BLBR; *n* = 6) and *Zapus hudsonius* (ZAHU; *n* = 3) were negative for all pathogens and are not listed below. Totals equal total number tested (number positive).

Tick species	Tick life stage	*Anaplasma phagocytophilum*			*Borrelia burgdorferi*				*Hepatozoon* spp.		*Rickettsia* spp.	
		PELE	TAST	q	PELE	TAST	MIPE	q	PELE	q	PELE	q
A. americanum	L	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)	0 (0)	1 (1)
	N	1 (0)	0 (0)	3 (0)	1 (0)	0 (0)	0 (0)	3 (0)	1 (0)	3 (1)	1 (0)	3 (2)
	A	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)	0 (0)	1 (0)
D. variabilis	L	11 (1)	0 (0)	0 (0)	11 (0)	0 (0)	2 (0)	0 (0)	11 (0)	0 (0)	11 (0)	0 (0)
	A	0 (0)	0 (0)	2 (0)	0 (0)	0 (0)	0 (0)	2 (0)	0 (0)	2 (0)	0 (0)	2 (0)
I. scapularis	L	54 (4)	12 (0)	2 (0)	54 (11)	12 (6)	1 (1)	2 (0)	54 (0)	2 (0)	54 (4)	2 (0)
	N	34 (2)	11 (1)	13 (1)	34 (2)	11 (4)	0 (0)	13 (2)	34 (0)	13 (0)	34 (1)	13 (0)
	A	16 (0)	6 (0)	6 (1)	16 (0)	6 (0)	2 (0)	6 (3)	16 (0)	6 (0)	16 (0)	6 (3)
Unknown *Ixodes*	L	19 (0)	13 (0)	1 (0)	19 (4)	13 (0)	0 (0)	1 (0)	19 (1)	1 (0)	19 (0)	1 (0)
	N	24 (0)	1 (0)	0 (0)	24 (1)	1 (1)	0 (0)	0 (0)	24 (1)	0 (0)	24 (0)	0 (0)
	A	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Totals			250 (10)			250 (37)			250 (3)		250 (11)	
% of ticks testing positive			4.0			14.8			1.2		4.4	

the Blacklegged Tick has been expanding geographically over the past 20 years, and is moving from the East Coast inland to the west (Brinkerhoff et al. 2014, Brownstein et al. 2003). Our findings confirm Brownstein et al. (2003), who modeled future distributions of Blacklegged Ticks, and predicted prominent increases in Virginia. We found that the Blacklegged Tick has become the dominant tick species in burdens in northwest Virginia.

Blacklegged Ticks tested positive for *Borrelia burgdorferi*, *Anaplasma phagocytophilum,* and *Rickettsia* spp., and some specimens even tested positive for multiple pathogens. Given the major roles that Blacklegged Ticks and White-footed Mice have in the transmission and maintenance of Lyme disease (*B. burgdorferi*; Brunner and Ostfeld 2008, Schmidt et al. 1999), the dominance of Blacklegged Ticks in tick burdens can help explain the drastic increases in Lyme disease and other tick-borne diseases in Virginia (Brinkerhoff et al. 2014). Monitoring of the expansion of Blacklegged Ticks across North America would be needed to track and predict increases in associated tick-borne pathogen prevalence and resulting disease risks to human health.

Our survey of questing ticks showed high abundances of Blacklegged Ticks and Lone Star Ticks; however, many of the larvae could not be identified. In surveys in southeastern Virginia, Lone Star Ticks represented 95% of the questing ticks collected (Nadolny et al. 2014). As Lone Star Ticks do not use small mammals as primary hosts (Kollars et al. 2000), studies should include larger-sized hosts to better understand the interactions of this species. Our rarefaction analyses suggested that our sampling efforts were sufficient to characterize the tick assemblages we studied. For the specific samples we collected, these analyses demonstrated that halving the sample size would be expected to cause a negligible change in estimated species diversity for attached nymphs, questing adults, and questing nymphs. However, we caution that there is no guarantee employing such reduced sample sizes in future studies would sufficiently characterize diversity in these groups. We also caution that no method can "see" what was not in a sample. However, based on the data we do have, the rarefaction results lend some confidence that our results would not have changed much even if we had collected substantially smaller samples. We detected 4 tick-borne pathogens: *Rickettsia* spp., *B. burgdorferi*, *A. phagocytophilum*, and *Hepatozoon* spp., the agents of *Rickettsia* diseases (i.e., Rocky Mountain Spotted Fever), Lyme disease, human granulocytic anaplasmosis, and hepatozoonosis, respectively (CDC 2017). While pathogen presence in ticks collected from mammalian hosts does not necessarily imply that the mammal is infected, it does provide information regarding the infection risk to the host species. With at least a quarter of the tested ticks having one or more pathogen, these data demonstrate the high risk for tick-borne disease in the study area.

Our study provides a snapshot of the tick burdens and tick-borne disease risk in Virginia, and can provide a baseline for future research. Given the range expansion of various tick species across the Mid-Atlantic region and the possible consequences for human health, we recommend further monitoring of the changes in both the tick community and variation in prevalence and disease risk over time.

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Appendix 1. We genetically identified ticks to species using the cytochrome c oxidase subunit 1 (*cox1*) barcode region and the 16S rRNA gene (Folmer et al. 1994, Hebert et al. 2004, Ivanova and Grainger 2006, Nadolny et al. 2011). The Apicomplexan primer pair BT-1F/BT-1R (Criado-Fornelio et al. 2003) sometimes amplified the tick 18S rRNA gene, permitting identification of ticks to genus. We identified pathogens by previously published diagnostic regions (Criado-Fornelio et al. 2003, Ghafar and Eltablawy 2011, Levin and Fish 2000, Mediannikov et al. 2010, Shih and Chao 2002, Ujvari et al. 2004). All PCR set-ups included an extraction negative control and a PCR negative control (containing water rather than DNA). Positive controls were obtained for the 8 pathogens from known infected animals. Therefore, all non-Apicomplexan PCR set-ups included positive controls. PCRs were conducted in 25-µl volumes containing 1× AmpliTaq Gold PCR buffer (Life Technologies, Carlsbad, CA), 2 mM of $MgCl_2$, 1 mM of dNTPs, 0.4 µM of each primer, 20 µg of BSA, 1U of AmpliTaq Gold (Life Technologies) and 2–3 µl of DNA.

Thermocycling for the ectoparasite cytochrome c oxidase subunit I (*cox1*) reactions included the following: an initial 5-min denaturation step at 95 °C; 5 cycles of 30 s at 95 °C, 40 s at 45 °C, and 1 min at 72 °C; 35 cycles of 30 s at 95 °C, 40 s at 51 °C, and 1 min at 72 °C; and a final 10-min extension step at 72 °C. Thermocycling for *B. burgdorferi*, *C. burnetii*, *Rickettsia* sp., and ectoparasite 16S rRNA assay programs consisted of an initial 5-min denaturation of 95 °C; 40 (*B. burgdorferi, Rickettsia* sp.) or 35 (*C. burnetii*, ectoparasite) cycles of 1 min at 94–95 °C, 1 min at annealing temperature (60 °C for *B. burgdorferi* and *C. burnetii*, 55 °C for *Rickettsia* sp., 50 °C for ectoparasites), and 1 min at 72 °C; and a final 5-min extension step of 72 °C. For the *A. phagocytophilum* and *Ehrlichia* sp. assays, thermocycling consisted of an initial 5-min denaturation of 95 °C; 35 cycles of 30 s at 94 °C, 30 s at annealing temperature (55 °C for *Ehrlichia* sp., 58 °C for *A. phagocytophilum*), and 30 s at 72 °C; and a final 5-min extension step of 72 °C. Thermocycling for the apicomplexan assays consisted of an initial 5-min denaturation of 95 °C; 40 (BTH-1F/BTH-1R primer pair) or 35 (HepF300/HepR900 primer pair) cycles of 30 s at 94 °C, 30 s at 60 °C, and 60 s (BTH-1F/BTH-1R) or 45 s (HepF300/Hep4900) at 72 °C; and a final 5-min extension step of 72 °C. Sequencing was done on an ABI 3130 (Life Technologies) for representative subsamples of positive PCR products following standard protocols.

PCR products were visualized on a 1.5% agarose gel stained with GelRed (Biotium Inc., Freemont, CA). We purified and sequenced representative samples of positive PCR products on an ABI 3130 sequencer (Life Technologies) following standard protocols (sequences available in FASTA format upon request from the authors). Sequences were edited using Sequencher® 5 (Gene Codes Corporation, Ann Arbor, MI) and then aligned against the GenBank non-redundant nucleotide database using Megablast to determine tick and pathogen identities (Zhang et al. 2000). We identified pathogen and tick species by their best Megablast matches. In the case of multiple best (or near-best) matches, we conservatively identified sequences to genus. All pathogens identified to species were at least 97% identical with publicly available reference sequences.

The following table provides the primer pairs used to identify tick and pathogen species by polymerase chain reaction. Published primer names are given with the marker in parentheses.

Organism	Marker	Forward (5′→3′)	Reverse (5′→3′)	Reference
Tick	*cox1* (HC02198/LC01490)	TAA CTT CAG GGT GAC CAA AAA TCA	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer et al. 1994
Tick	*cox1* (LEPF1/LEPR1)	ATT CAA CCA ATC ATA AAG ATA TTG G	TAA ACT TCT GGA TGT CCA AAA ATC A	Hebert et al. 2004
Tick	*cox1* (dgLEPF1/dgMLEPR1)	AYT CAA CYA ATC AYA AAG AYM TTG G	CCW GTY CCA GCW CCA KWT TC	Ivanova and Grainger 2006
Tick	16S rRNA (16s + 1/16s - 1)	CTG CTC AAT GAT TTT TTA AAT TGC TGT	GTC TGA ACT CAG ATC AAG T	Nadolny et al. 2011
Anaplasma	16S rRNA (E1/E2)	GGC ATG TAG GCG GTT CGG TAA GTT	CCC CCA CAT TCA GCA CTC ATC GTT TA	Ghafar and Eltablawy 2011
Apicomplexa	18S rRNA (HepF300/HepR900)	GTT TCT GAC CTA TCA GCT TTC GAC G	CAA ATC AAG AAT TTC ACC TCT GAC	Ujvari et al. 2004
Apicomplexa	18S rRNA (BT-1F/BT-1R)	GGT TGA TCC TGCC AGT AGT	GCC TGC TGC CTT CCT TA	Criado-Fornelio et al. 2003
Borrelia	*ospA* (SL_F/SL_R)	AAT AGG TCT AAT AAT AGC CTT AAT AGC	CTA GTG TTT TGC CAT CTT CTT TGA AAA	Shih and Chao 2002
Borrelia	Flagellin (FLA297/FLA652)	CGG CAC ATA TTC AGA TGC AGA CAG	CCT GTT GAA CAC CCT CTT GAA CC	Levin and Fish 2000
Coxiella	IS1111 (CbISF/CbISR)	CAA GAA ACG TAT CGC TGT GGC	CAC AGA GCC ACC GTA TGA ATC	Mediannikov et al. 2010
Ehrlichia	16S rRNA (HE1F/HE3R)	CAA TTG CTT ATA ACC TTT TGG TTA TAA AT	TAT AGG TAC CGT CAT TAT CTT CCC TAT	Ghafar and Eltablawy 2011
Rickettsia	*ompB* (BG1-21/BG2-20)	GGC AAT TAA TAT CGC TGA CGG	GCA TCT GCA CTA GCA CTT TC	Eremeeva et al. 1994