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Passerine birds as hosts for *Ixodes* ticks infected with *Borrelia burgdorferi* sensu stricto in southeastern Virginia

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ABSTRACT

The ecology of vector-borne diseases in a region can be attributed to vector-host interactions. In the United States, tick-borne pathogens are the cause of the highest number of reported vector-borne diseases. In the mid-Atlantic region of the eastern United States, tick-borne diseases such as Lyme disease, have increased in incidence, with tick-host-pathogen interactions considered a contributing factor to this increase. Ticks become infected with pathogens after taking a blood meal from a systemically infected host or through a localized infection while co-feeding on a host with other infected ticks. The host not only plays a role in pathogen acquisition by the tick, but can also facilitate dispersal of the tick locally within a region or over greater distances into new geographical ranges outside of their historical distributional range. In this study conducted in southeastern Virginia (USA), we examined the interaction between both resident and migratory bird species and *Ixodes* ticks, the primary vectors of *Borrelia burgdorferi* sensu stricto (s.s.) the main causative agent of Lyme disease on the East coast of the United States. Over a two-year period (2012–2014), 1879 passerine birds were surveyed, with 255 *Ixodes* ticks tested for the presence of *Borrelia* spp. Eighty passerine birds (4.3 %) representing 17 bird species were parasitized by at least one *Ixodes* tick, but only three bird species were parasitized by *Ixodes* ticks that tested positive for *B. burgdorferi* s.s. Twenty *Ixodes* ticks (7.8 %) tested positive for *B. burgdorferi* s.s. with nearly all collected from resident bird species including the Carolina wren (*Thryothorus ludovicianus*) and brown thrasher (*Toxostoma rufum*). Given that millions of birds pass through southeastern Virginia during migration, even with the low number of *Ixodes* ticks parasitizing passerine birds and the low prevalence of *B. burgdorferi* s.s. found within *Ixodes* ticks collected, the sheer volume of passerine birds suggests they may play a role in the maintenance and dispersal of *B. burgdorferi* s.s. in southeastern Virginia.

1. Introduction

The eastern half of the United States is known to harbor many hard tick species (family Ixodidae) and their associated pathogens. Most notably on the east coast, where *Ixodes* ticks are populous, there is a high incidence of Lyme disease cases (Kugeler et al., 2015). The blacklegged tick (*Ixodes scapularis*) is one of several *Ixodes* species established in southeastern Virginia and is a vector of *Borrelia burgdorferi* sensu stricto (s.s.), the primary causative agent of Lyme disease in North America (Keirans et al., 1996). The relapsing fever group spirochete *Borrelia miyamotoi*, the causative agent of *Borrelia miyamotoi* disease (CDC, 2015), is also spread by *Ixodes* ticks in the United States and is of interest because it can be associated with co-infections of *B. burgdorferi* sensu lato (s.l.), including *B. burgdorferi* s.s. (Barbour et al., 2009).

Lyme disease incidence in North America is changing (CDC, 2018), particularly in the northern mid-Atlantic region of the United States, where the number of infections has increased substantially over the past 20 yrs (Brinkerhoff et al., 2014). This increase is likely mediated, at least in part, by tick-host interactions (Levy, 2013). Because *B. burgdorferi* s.s. is not transmitted transovarially (Patrican, 1997a), *Ixodes* ticks become infected through a blood meal from an infected host or through localized infection caused by another infected tick feeding on the same host (States et al., 2017). Once infected, an *Ixodes* tick will retain *B. burgdorferi* s.s. through subsequent life stages and can transmit the

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pathogen to additional hosts (Gauthier-Clerc et al., 1998; Gatewood et al., 2009).

Passerine birds are frequently parasitized by ticks, including several *Ixodes* species, but the role of such hosts in the maintenance and transmission of *B. burgdorferi* in southeastern Virginia has, to our knowledge, not been examined previously. This region serves as a major stopover area to millions of migratory birds each autumn and spring (Hinshaw et al., 1985; Eggeman and Johnson, 1989). Thus, understanding the potential role of birds in tick-borne pathogen maintenance and transfer has far-reaching implications given the large concentration of birds in the area and their propensity to move long distances in short periods of time. Migrating birds likely play the greatest role among vertebrate hosts as long-distance dispersal agents of ticks and their associated pathogens when compared with mammalian, reptilian, or amphibian hosts that disperse ticks across much shorter distances (Smith et al., 1996; Scott et al., 2001; Loss et al., 2016; Norte et al., 2020). The goal of this study was to assess the role of passerine birds, both resident and migratory, as hosts of *B. burgdorferi* s.l.-infected ticks in a region of the eastern United States where passerine birds are concentrated during autumn and spring migration.

2. Methods

2.1. Host collection and tick removal

Birds were caught and sampled at 10 locations across southeastern Virginia from August 2012 to August 2014 (Fig. 1) using 12 m x 2.5 m mist nets erected for 4 h, typically beginning at local sunrise. Mist net sites consisted of 5 core locations that were sampled on one day every other week over the two years of this study (750+ net hours per site) and 5 sites that were sampled on an ad hoc basis at least once per month (≤350 net hours per site). Each bird was banded and checked for ticks, primarily around the head and vent. Ticks were removed using forceps, placed in individually labeled tubes, and stored at −20 °C prior to identification and DNA extraction. We calculated the mean infestation of *Ixodes* per individual host species by taking the sum of all *Ixodes* ticks found on a particular host species divided by the total number of individual hosts of that species.

We defined migratory birds as those that had seasonal movement from one region to another; however, we categorized some birds as "resident" even though they may exhibit some seasonal movement throughout parts of their range as our recapture data suggested some individuals did not exhibit such seasonal movements (e.g., American robin and brown thrasher); therefore, we conservatively classified them as residents.

2.2. Tick identification and DNA extraction

Ticks were identified morphologically to genus and life stage (Keirans and Clifford, 1978). All ticks identified as *Ixodes* sp. were separated from other tick species, labeled individually, and DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. Larval and nymphal ticks were extracted whole, while adult ticks were cut bilaterally (one half of the tick was stored at −80 °C and the other half pulverized for DNA extraction). Pulverization was carried out using 1 mm and 5 mm glass beads in a Mini Beadbeater (BioSpec, Inc., Bartlesville, OK, USA); extracted DNA was eluted in 200 µL elution buffer according to the manufacturer's instructions.

Each individual *Ixodes* tick was identified to species by first using an

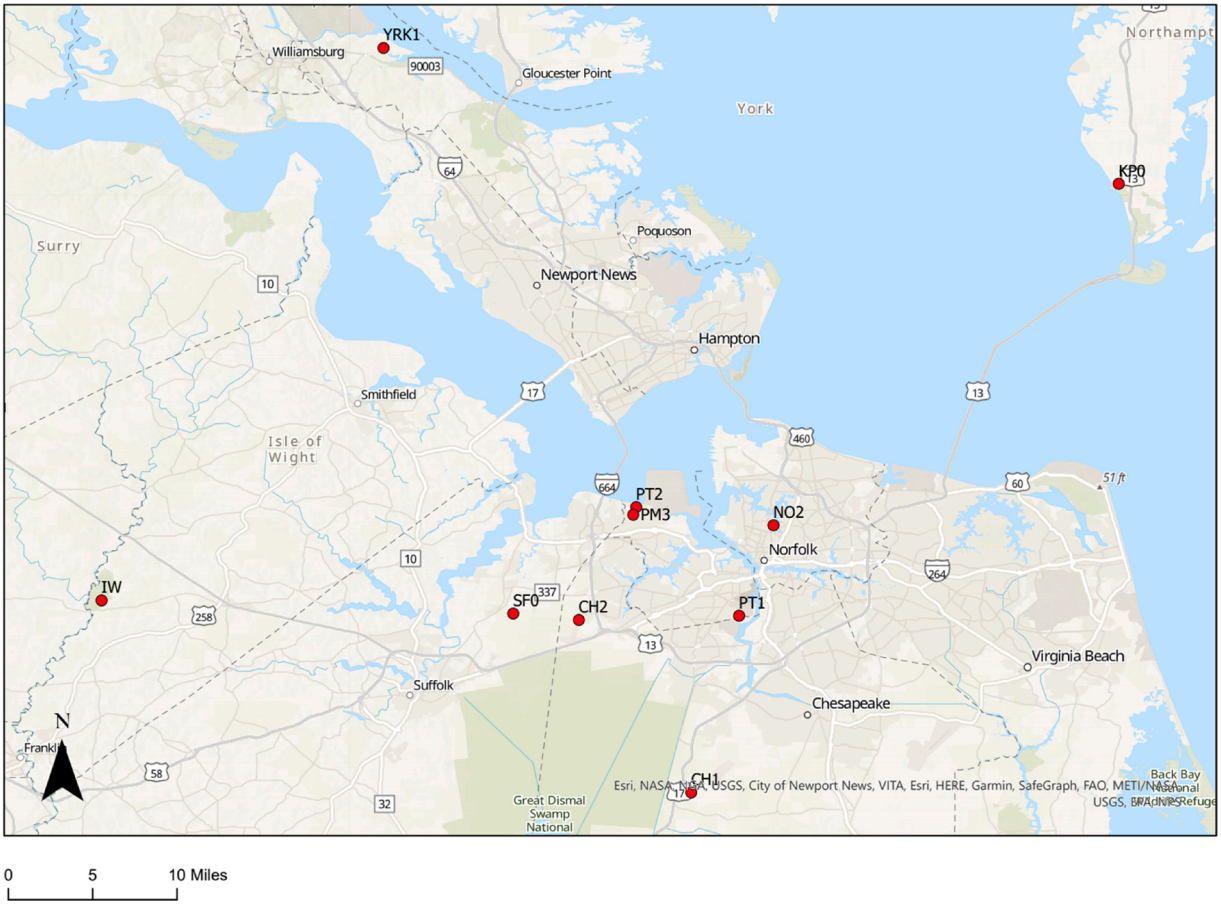

Fig. 1. Map showing location of mist-netting sites sampled during the 2-yr study period (2012–2014) in southeastern Virginia, United States.

I. scapularis / Ixodes affinis real-time PCR assay (Wright et al., 2014). The *I. scapularis / I. affinis* real-time PCR assay amplified a fragment of the tick internal-transcribed spacer 2 (ITS2) region of the nuclear rDNA transcriptional unit; amplification methodology followed that described by Wright et al. (2014). Melt curve real-time PCR reactions were carried out using 15 μL reaction volumes consisting of 1X Bio-Rad iQ SYBR green supermix, 0.5 μL of each primer (10 μM) (Table 1), and 2 μL of template DNA. Melt temperatures of samples with C(q) values $\leq$ 30 were used to separate the ticks into groups: *I. scapularis*, *I. affinis*, or *Ixodes* sp. for those that did not fit into either the *I. scapularis* or *I. affinis* groups. A 454-bp fragment of tick mitochondrial 16S rRNA gene was amplified using primers 16S+1 and 16S-1 (Table 1) to identify any *Ixodes* ticks that were neither *I. scapularis* nor *I. affinis*. PCR reactions were carried out using 12.5 μL of 2X EconoTaq PLUS (Lugien Corp., Middleton, WI, USA), 2 μL of each primer (10 μM), and 5 μL template DNA (Fuente et al., 2001; Nadolny et al., 2011). Samples not identified through mitochondrial 16S rRNA gene sequencing were further characterized by sequencing the 12S rDNA gene and the tick internal transcribed spacer (ITS) region (Table 1). 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). DNA sequences were assembled using Geneious Bioinformatics Software for Sequence Analysis (https://www.geneious.com/) and compared against known DNA sequences using NCBI BLAST (http://blast.ncbi.nlm.nih.gov).

2.3. *Borrelia* identification and sequencing

To verify the quality of the extracts, an *Ixodes* actin real-time assay was performed as described by Graham et al. (2018). The real-time assay protocol was 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 45 s, with a plate reading after the extension step. Real-time PCR reactions were carried out using 20 μL aliquots consisting of 10 μL of 2X EconoTaq PLUS master mix, 1 μL of each primer (10 μM), 0.5 μL of probe (10 μM) (Table 1), and 5 μL of template DNA. The quality of all extracts was verified as good with C(q) values below 30 for every sample. Tick DNA was screened for *Borrelia* sp. using a real-time PCR assay detecting the 23S rRNA gene as described in Courtney et al. (2004). Real-time PCR reactions were carried out using 20 μL aliquots consisting of 10 μL of 2X EconoTaq PLUS master mix, 1.4 μL of each primer (10 μM), 0.35 μL of probe (10 μM) (Table 2), 1.2 μL of $MgCl_2$ (50 μM), and 5 μL of template DNA.

Samples positive for the 23S real-time assay were also screened for *Borrelia* sp. using the *Borrelia* 16S real-time PCR assay described in Graham et al. (2018) but modified by running the samples as single reactions (using FAM-labeled probes) rather than as multiplex reactions (Table 2). Real-time PCR reactions were carried out in 20 μL aliquots consisting of 10 μL of 2X EconoTaq PLUS master mix, 1 μL of each primer (10 μM), 0.5 μL of probe (10 μM) (Table 2), and 5 μL of template DNA. The real-time assay was performed using the same protocol as the *Ixodes* actin assay (Graham et al., 2018). *Borrelia*-positive samples were further screened for *B. burgdorferi* s.s. and *B. miyamotoi* using the *B. burgdorferi* s.s. *opp*A2 real-time PCR assay modified as above (Graham et al., 2018) and a *B. miyamotoi glp*Q real-time PCR assay (Reiter et al., 2015), respectively. Each reaction for the *B. burgdorferi* s.s. *opp*A2 assay was carried out in 20 μL aliquots consisting of 10 μL of 2X EconoTaq PLUS master mix, 1 μL of each primer (10 μM), 0.5 μL of probe (10 μM) (Table 2), and 5 μL of template DNA. Each reaction for the *B. miyamotoi glp*Q was conducted using the same protocol as the *Borrelia* 16S assay. The positive control used in each *B. burgdorferi* assay was DNA from *B. burgdorferi* s.s. cell culture. The positive control used in the *B. miyamotoi* assay was tick DNA from a *B. miyamotoi*-infected adult *I. scapularis*. All negative controls used in these assays consisted of nuclease-free water.

Samples positive for *B. burgdorferi* s.s. from real-time screening were amplified using standard end point PCR for *B. burgdorferi* s.s.-specific genes: *fla*B, *osp*C, and IGS (Table 2). Amplified DNA was visualized following gel electrophoresis and purified using a Wizard PCR preps DNA Purification System (Promega) according to the manufacturer's instructions. Sequencing reactions were performed using a BigDye Terminator v.3.1 Cycle Sequencing Kit using the primers for the specific genes (Table 2). DNA sequences were assembled in Geneious and compared against known sequences using NCBI BLAST.

3. Results

3.1. Host-tick interactions

During this study (2012–2014), 1879 birds were banded and checked for ticks at field sites in southeastern Virginia (Supplemental Table 1). The number of banded birds per bird species provided a relative measure of abundance at each sampling site, given that sampling effort was roughly equivalent among sites. Eleven percent of the birds sampled (207 of 1879) were parasitized by ticks (*Ixodes* and non-*Ixodes*) with approximately 4.3 % of birds (80 of 1879) being parasitized by at least one *Ixodes* tick. Host species where >10 % of individuals were parasitized by at least one *Ixodes* tick (Table 3) included Carolina wrens (*Thryothorus ludovicianus*; 32 of 186), brown thrashers (*Toxostoma*

Table 1
Primers and probes used to determine *Ixodes* species.

Primer/Probe	Primer/Probe Sequence	Amplicon Size	Reference
Tick 16S gene			
16S-1	5′-GTCTGAACTCAGATCAAGT-3'	454 bp	de la Fuente et al. (2001)
16S+1	5′-CTGCTCAATGATTTTTTAAATTGCTGT-3'		Nadolny et al. (2011)
Tick 12S gene			
85F	5′-TTAAGCTTTTCAGAGGAATTTGCTC-3'	140 bp	Williamson et al. (2010)
225R	5′-TTTWWGCTGCACCTTGACTTAA-3'		Williamson et al. (2010)
Ixodes ITS2 region			
IsSeq 5.8F	5′-TCGATGAAGAACGCAGCCAG-3'	800 bp	Zahler et al. (1995)
IsSeq 28SR	5′-TTCTATGCTTAAATTCAGGGGGTTGTC-3'		McLain et al. (1995)
I. scapularis / I. affinis assay			
aff_f8	5′-TGGAAATCCCGCAAATCT-3'	75 bp	Wright et al. (2014)
aff_r8	5′-CCGTTCCAATCTCCGTTTA-3'		Wright et al. (2014)
Scap_f2.2	5′-GCGTTAGAAACGGAGATTTGA-3′	142 bp	Wright et al. (2014)
Scap_r2.2	5′-CCACGAGATTTACATTTGCC-3′		Wright et al. (2014)
Ixodes actin			
Actin-R	5′-CCGTCGGGAAGCTCGTAGG-3'	76 bp	Hojgaard et al. (2014)
Actin-F	5′-GCCCTGGACTCCGAGCAG-3'		Hojgaard et al. (2014)
Actin-Pr	5′-HEX-705-CCACCGCCGCCTCCTCTTCTTCC-BHQ3−3'		Hojgaard et al. (2014)

Table 2
Primers and probes used to identify *Borrelia* spp.

Primer/Probe	Primer/Probe Sequence	Amplicon Size	Reference
Borrelia 23S			
Bb23SF	5′-CGAGTCTTAAAAGGGCGATTTAGT-3’	75 bp	Courtney et al. (2004)
Bb23SR	5′-GCTTCAGCCTGGCCATAAATAG-3’		Courtney et al. (2004)
Bb23SPr	5′-FAM-AGATGTGGTAGACCCGAAGCCGAGTG-3BHQ-3’		Courtney et al. (2004)
Borrelia 16S			
16S-F	5′-AGCYTTTAAAGCTTC GCTTGTAG-3’	148 bp	Kingry et al. (2018)
16S-R	5′-GCCTCCCGTAGGAGTCTG G-3’		Kingry et al. (2018)
16S-Pr	5′-FAM-CCGGCCTGAGAGGGTGAWCGG-3IABkFQ-3’		Kingry et al. (2018)
Borrelia oppA2			
Bb-F	5′-AATTTTTGGTTCCATACCC-3’	162 bp	Graham et al. (2018)
Bb-R	5′-CTGTCAATAGCAAGAGTTAA-3’		Graham et al. (2018)
Bb-Pr	5′-FAM-CGTTCAATACACACATCAAACCACT-3IABkFQ-3’		Graham et al. (2018)
B. miyamotoi glpQ			
BmglpQ-F	5′-GACCCAGAAATTGACAAACCACAA-3’	108 bp	Graham et al. (2018)
BmglpQ-R	5′-TGATTTAAGTTCAGTTAGTGTGAAGTCAGT-3’		Graham et al. (2018)
BmqlpQ-Pr	5′-FAM-CAATCGAGCTAGAGAAAACGGAAGATATTACG-3IABkFQ-3’		Graham et al. (2018)
Borrelia flagellin			
*fla*B outer 1	5′-AAGTAGAAAAAGTCTTAGTAAGAATGAAGGA-3’	475 bp	Johnson et al. (1992)
*fla*B outer 2	5′-AATTGCATACTCAGTACTATTCTTTATAGAT -3’		Johnson et al. (1992)
*fla*B inner 1	5′-CACATATTCAGATGCAGACAGAGGTTCTA- 3’	437 bp	Johnson et al. (1992)
*fla*B inner 2	5′-GAAGGTGCTGTAGCAGGTGCTGGCTGT- 3’		Johnson et al. (1992)
Borrelia ospC			
ospC outer-forward	5′-ATGAAAAAGAATACATTAAGTGC- 3’	658 bp	Tsao et al. (2013)
ospC outer-reverse	5′-ATTAATCTTATAATATTGATTTTAATTAAGG- 3’		Tsao et al. (2013)
ospC inner-forward	5′-TATTAATGACTTTATTTTTATTTATATCT- 3’	618 bp	Tsao et al. (2013)
ospC inner-reverse	5′-TTGATTTTAATTAAGGTTTTTTTGG- 3’		Tsao et al. (2013)
Borrelia IGS			
rrs-rrlA IGS F	5′-GTATGTTTAGTGAGGGGGGTG- 3’	1028 bp	Bunikis et al. (2004)
rrs-rrlA IGS R	5′-GGATCATAGCTCAGGTGGTTAG- 3’		Bunikis et al. (2004)
rra-rrlA IGS Fn	5′-AGGGGGGTGAAGTCGTAACAAG- 3’	812 bp	Bunikis et al. 2004)
rra-rrlA IGS Rn	5′-GTCTGATAAACCTGAGGTCGGA- 3’		Bunikis et al. (2004)

rufum; 12 of 75), gray-cheeked thrushes (*Catharus minimus*; 1 of 5), hermit thrushes (*Catharus guttatus*; 4 of 37), ovenbirds (*Seiurus aurocapilla*; 2 of 8), swamp sparrows (*Melospiza georgiana*; 3 of 15), and winter wrens (*Troglodytes hiemalis*; 1 of 9). The other bird hosts surveyed (10 additional species) with *Ixodes* ticks had infestation prevalences $\leq$ 10 % (Fig. 2).

Two-hundred fifty-five *Ixodes* ticks, representing 4 *Ixodes* species, were collected during this study. Of the *Ixodes* collected, 84 were *I. scapularis*, 77 *Ixodes brunneus*, 64 *I. affinis*, and 30 *Ixodes dentatus*. Only three adult *Ixodes* ticks were detected on birds in this study, i.e., two female *I. brunneus* from an American robin (*Turdus migratorius*) and one *I. brunneus* female from a Carolina wren. Larvae from all four species were collected while nymphs were only collected for *I. scapularis, I. brunneus,* and *I. affinis*. Each *Ixodes* species was found parasitizing multiple bird species (Table 4), both resident and migratory. All four *Ixodes* species were found parasitizing Carolina wrens and northern cardinals (*Cardinalis cardinalis*), both of which are resident to the study area. American robins and brown thrashers, also resident bird species, were parasitized by three of the four *Ixodes* species. Migratory hosts were only parasitized by one or two of the four *Ixodes* species found.

Phenologies of immature tick life stages (larvae and nymphs) on birds were determined during the 2-yr sampling period based on the number collected each calendar month for each *Ixodes* species. *Ixodes scapularis* larval feeding activity on bird hosts occurred from April through October with a peak in July; *I. affinis* larvae from June to December with peaks in August and October; *I. dentatus* larvae from July to November with the peak in October and November; and *I. brunneus* larvae in November and December with the peak in November (Fig. 3). The number of *Ixodes* nymphs found was much lower than the number of larvae (55 nymphs versus 197 larvae). The peak feeding time for *I. scapularis* nymphs (representing the majority of nymphs collected) was during July and August, *I. affinis* nymphs had peaks in July and December, and *I. brunneus* nymphs had peaks in March and December (Fig. 4); no *I. dentatus* nymphs were collected. *Ixodes scapularis* nymphal

Table 3
Mean infestation of *Ixodes* per bird by host bird species; host bird species listed had >10 % of individuals from that species parasitized by *Ixodes* spp. The number of birds examined parasitized by *Ixodes* spp. is listed as well as the range of the number of ticks parasitizing individual hosts from a species.

Host Bird Species	Number of birds examined	Number of birds parasitized by *Ixodes* ticks	Total number of *Ixodes* ticks from each host species (range of ticks per bird)	Mean infestation of *Ixodes* per bird (+/- SD)
Carolina wren *Thryothorus ludovicianus*	186	32	180 (1–50)	0.97 (+/- 4.58)
Brown thrasher *Toxostoma rufum*	75	12	28 (1–8)	0.37 (+/- 1.16)
Gray-cheeked thrush *Catharus minimus*	5	1	1 (N/A)	0.20 (+/- 0.45)
Hermit thrush *Catharus guttatus*	37	4	7 (1–3)	0.19 (+/- 0.62)
Ovenbird *Seiurus aurocapilla*	8	2	5 (2–3)	0.50 (+/- 0.93)
Swamp sparrow *Melospiza georgiana*	15	3	4 (1–2)	0.27 (+/- 0.59)
Winter wren *Troglodytes hiemalis*	9	1	2 (N/A)	0.22 (+/- 0.67)

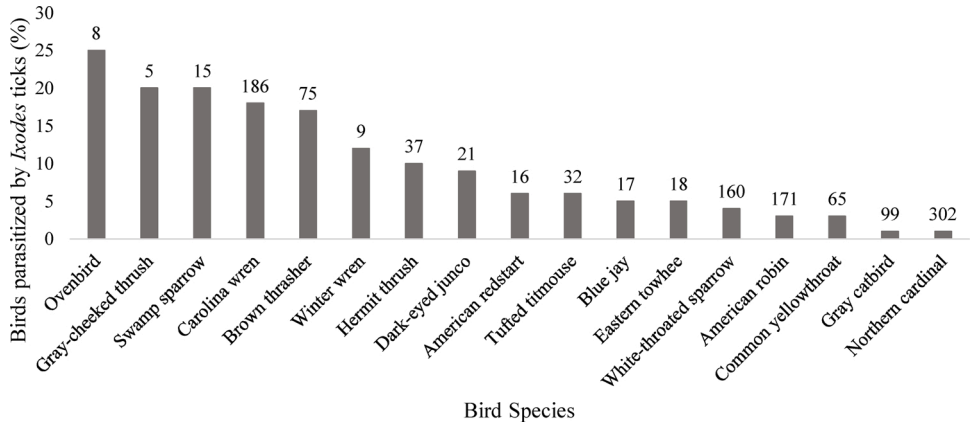

Fig. 2. Percentage of birds with *Ixodes* ticks in southeastern Virginia by species. Number above each bar represents the number of individual birds surveyed for each bird species.

Table 4
Ixodes species collected from various avian hosts.

Tick Species	Host Bird Species	Migratory Status	Individuals Infested (%)	Mean number of *Ixodes* per bird (+/- SD)
Ixodes scapularis	American robin	resident	1.2	0.01 (+/- 0.11)
	Brown thrasher	resident	9.3	0.15 (+/- 0.51)
	Carolina wren	resident	11.8	0.35 (+/- 1.67)
	Northern cardinal	resident	0.3	0.00 (+/- 0.06)
	Ovenbird	migratory	25.0	0.50 (+/- 0.93)
	Swamp sparrow	migratory	6.7	0.07 (+/- 0.26)
Ixodes affinis	American robin	resident	0.6	0.01 (+/- 0.08)
	Brown thrasher	resident	2.7	0.05 (+/- 0.36)
	Carolina wren	resident	5.4	0.28 (+/- 2.14)
	Common yellowthroat	migratory	0.3	0.03 (+/- 0.17)
	Dark-eyed junco	migratory	4.8	0.05 (+/- 0.22)
	Eastern towhee	resident	5.6	0.06 (+/- 0.24)
	Northern cardinal	resident	0.3	0.00 (+/- 0.06)
	White-throated sparrow	migratory	0.6	0.01 (+/- 0.08)
Ixodes brunneus	American robin	resident	1.8	0.02 (+/- 0.13)
	Brown thrasher	resident	2.7	0.03 (+/- 0.16)
	Carolina wren	resident	4.3	0.25 (+/- 2.94)
	Dark-eyed junco	migratory	4.8	0.05 (+/- 0.22)
	Hermit thrush	migratory	10.8	0.19 (+/- 0.62)
	Northern cardinal	resident	0.3	0.00 (+/- 0.06)
	Swamp sparrow	migratory	13.3	0.20 (+/- 0.56)
	Tufted titmouse	resident	6.3	0.06 (+/- 0.25)
	White-throated sparrow	migratory	4.4	0.06 (+/- 0.33)
Ixodes dentatus	Carolina wren	resident	2.7	0.08 (+/- 0.75)
	Brown thrasher	resident	4.0	0.15 (+/- 0.95)
	Gray-cheeked thrush	migratory	20.0	0.20 (+/- 0.45)
	Gray catbird	resident	1.0	0.01 (+/- 0.10)
	Northern cardinal	resident	0.3	0.00 (+/- 0.06)
	Winter wren	migratory	11.1	0.22 (+/- 0.67)

feeding overlapped with *I. scapularis, I. affinis, and I. dentatus* larval feeding from April to October.

3.2. Tick-Borrelia interactions

Borrelia spp. DNA was detected in 20 of the 255 immature *Ixodes* ticks screened by real-time PCR detecting the 23S rRNA region (Table 5); 14 were *I. scapularis,* 5 *I. affinis*, and 1 *I. brunneus*. Based on real-time PCR, only 5 ticks were positive for *B. burgdorferi* s.s. (Table 5), while no ticks tested positive for *B. miyamotoi*. The majority of infected *Ixodes* were larvae. All *I. affinis* and *I. brunneus* infected with *Borrelia* sp. were larvae. For *I. scapularis*, 7 larvae and 7 nymphs were infected.

Eighteen of the twenty real-time positive samples were amplified and confirmed by sequencing of the *fla*B gene in both directions (Table 5); one was only sequenced in one direction for the *fla*B gene; the other ran out of template. All sequences were homologous with *B.burgdorferi* s.s. with no other *Borrelia* species detected by sequence identity. Five *Borrelia* from *I. scapularis* were sequenced in at least one direction for the 3 genes tested and two *Borrelia* from *I. affinis* were sequenced in at least one direction for 2 of the 3 genes tested. The *Borrelia* from the single *I. brunneus* larva was only amplified and sequenced for the *fla*B gene. Sequencing indicated that these ticks carried *B. burgdorferi* s.s., with sequence identity over 570 bp for the *osp*C gene, 260 bp for flaB, and 308 bp for IGS.

There were many instances of co-feeding on birds with *Borrelia*-infected ticks. The Carolina wren from site KP0 was found parasitized by three *I. scapularis* nymphs in August; one of which was positive for *Borrelia* sp. by real-time PCR; we were unable to determine the species of *Borrelia*. At site CH2, four birds were parasitized by *Borrelia*-infected ticks (one each in April, May, July, and August). Two birds from site CH2 had co-feeding ticks; one bird had two *I. scapularis* larvae (one of which was positive for *B. burgdorferi* s.s.); the other bird had one *I. scapularis* nymph (negative for *Borrelia* spp.) and 27 *I. affinis* larvae (five of which were positive for *B. burgdorferi* s.s.). At site CH1, the swamp sparrow was

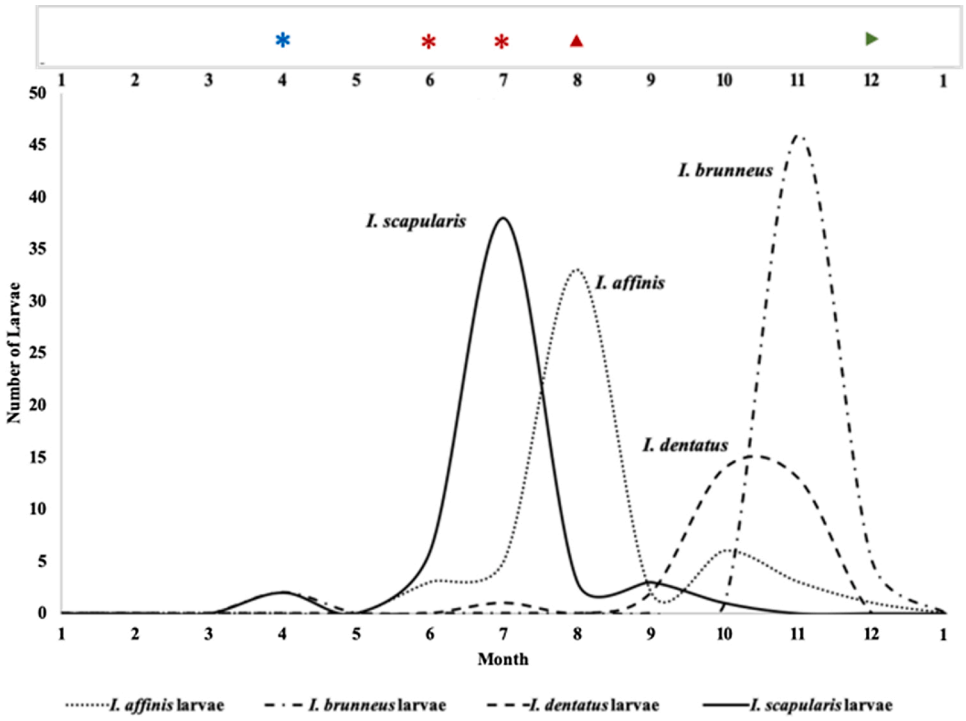

Fig. 3. The phenology of larval *Ixodes* spp. on birds in this study over a 2-yr period (2012–2014). Individual data points were fitted to a cubic spline curve. Symbols above the graph indicate the month when *Borrelia burgdorferi* sensu stricto-infected *Ixodes* ticks were collected. Infected *I. scapularis* larvae occurred at different points in the year. *: infected *I. scapularis* larvae from Carolina wrens; *: infected *I. scapularis* larvae from brown thrashers; ▲: infected *I. affinis* larvae from Carolina wrens; and ▶: infected *I. brunneus* larvae from swamp sparrows.

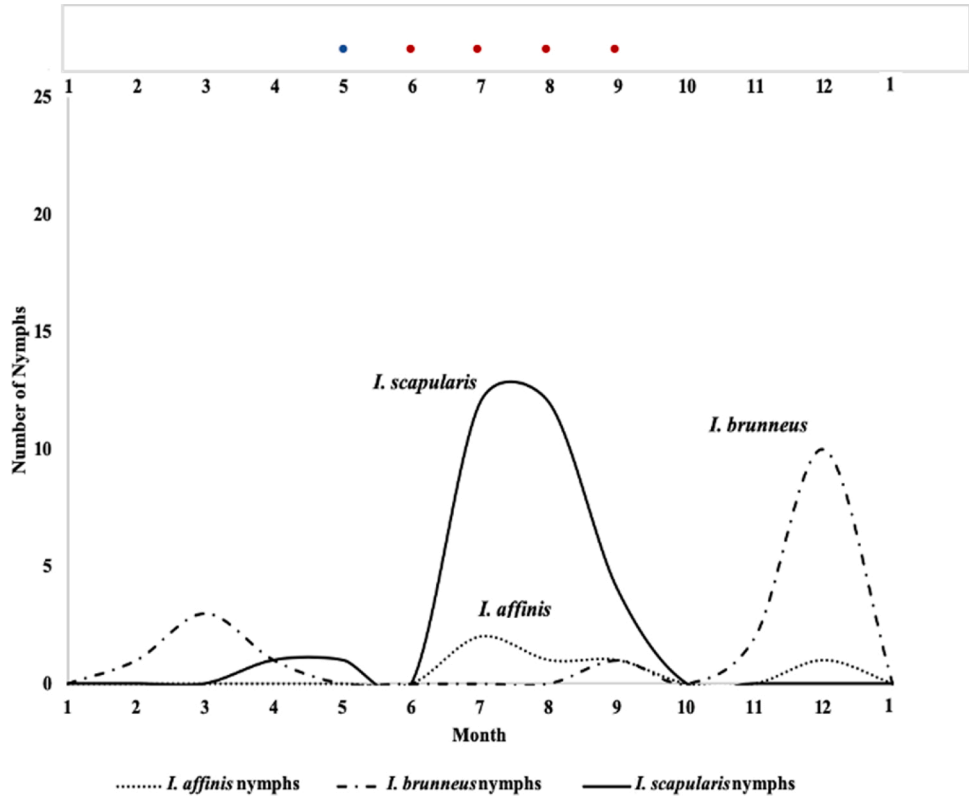

Fig. 4. The phenology of nymphal *Ixodes* spp. on birds in this study over a 2-yr period (2012–2014). Individual data points were fitted to a cubic spline curve. Symbols above the graph indicate the month when *Borrelia burgdorferi* sensu stricto-infected ticks were collected. Infected *I. scapularis* nymphs occurred at different points in the year. Bull infected *I. scapularis* nymphs from Carolina wrens; bull;: infected *I. scapularis* nymphs from brown thrashers.

Table 5
Bird host and life stage associated with *Ixodes* ticks that tested positive for *Borrelia burgdorferi* sensu stricto.

				Real-time PCR assay (0-negative, 1-positive)				Gene targeted sequencing (Yes-able to amplify, No-did not amplify)		*Borrelia* species detected
	Bird Species	*Ixodes* spp.	Tick Life Stage	Bb 23S	Bb 16S	Bb s.s.	*fla*B	*osp*C	IGS	
KP0	CARW	*I. scapularis*	Nymph	1	[b]	[b]	[b]	No	[b]	*B. burgdorferi* s.l.
CH2	BRTH	*I. scapularis*	Larva	1	1	1	Yes	Yes	Yes	*B. burgdorferi* s.s.
CH2	BRTH	*I. scapularis*	Nymph	1	1	0	Yes	No	No	*B. burgdorferi* s.s.
CH2	CARW	*I. scapularis*	Larva	1	1	0	Yes	No	No	*B. burgdorferi* s.s.
CH2	CARW[a]	*I. affinis*	Larva	1	1	0	Yes	No	Yes	*B. burgdorferi* s.s.
CH2	CARW[a]	*I. affinis*	Larva	1	1	0	Yes	No	Yes	*B. burgdorferi* s.s.
CH2	CARW[a]	*I. affinis*	Larva	1	0	0	Yes	No	No	*B. burgdorferi* s.s.
CH2	CARW[a]	*I. affinis*	Larva	1	1	0	Yes	No	No	*B. burgdorferi* s.s.
CH2	CARW[a]	*I. affinis*	Larva	1	0	0	Yes	No	No	*B. burgdorferi* s.s.
CH1	SWSP	*I. brunneus*	Larva	1	0	0	Yes	No	No	*B. burgdorferi* s.s.
PT2	CARW	*I. scapularis*	Nymph	1	[b]	[b]	Yes	No	[b]	*B. burgdorferi* s.s.
PT2	CARW	*I. scapularis*	Nymph	1	[b]	[b]	Yes	No	[b]	*B. burgdorferi* s.s.
PT2	CARW	*I. scapularis*	Nymph	1	0	0	Yes	No	No	*B. burgdorferi* s.s.
SF0	CARW[a]	*I. scapularis*	Larva	1	1	1	Yes	Yes	Yes	*B. burgdorferi* s.s.
SF0	CARW[a]	*I. scapularis*	Larva	1	1	1	Yes	Yes	Yes	*B. burgdorferi* s.s.
SF0	CARW[a]	*I. scapularis*	Larva	1	1	1	Yes	Yes	Yes	*B. burgdorferi* s.s.
SF0	CARW[a]	*I. scapularis*	Nymph	1	1	1	Yes	Yes	Yes	*B. burgdorferi* s.s.
YRK1	CARW	*I. scapularis*	Larva	1	[b]	[b]	Yes	No	[b]	*B. burgdorferi* s.s.
YRK1	CARW[a]	*I. scapularis*	Larva	1	[b]	[b]	Yes	No	Yes	*B. burgdorferi* s.s.
YRK1	CARW[a]	*I. scapularis*	Nymph	1	[b]	[b]	Yes	No	[b]	*B. burgdorferi* s.s.

CARW = Carolina wren, BRTH = brown thrasher, SWSP = swamp sparrow.
[a] Same bird.
[b] Ran out of DNA.

parasitized by two *I. brunneus* larvae in December, but only one was positive for *B. burgdorferi* s.s. At site PT2, three birds were parasitized by *Borrelia*-infected *I. scapularis* nymphs in July, August, and September. Two birds had other *Ixodes* ticks co-feeding with the infected *I. scapularis* nymphs (one bird with a second *I. scapularis* larva; one bird with one *I. scapularis* nymph and one *I. affinis* larva), but none of these co-feeding ticks were positive for *B. burgdorferi s.s*. At site SF0, one Carolina wren was parasitized by three *Borrelia*-infected *I. scapularis* larvae and one nymph in June. This bird was also parasitized by three other *I. scapularis* larvae and one *I. brunneus* nymph. At site YRK1, two Carolina wrens were parasitized by *I. scapularis* larvae and nymphs in June. One bird had 11 larvae and one nymph but only one larva tested positive for *B. burgdorferi* s.s.; the other bird had 12 larvae and five nymphs with one larva and one nymph testing positive for *B. burgdorferi* s.s.

4. Discussion

Few *Ixodes* ticks were found parasitizing passerine birds over the 2-yr period of this study in southeastern Virginia, with a subset of ticks being positive for *B. burgdorferi* s.s. Four *Ixodes* species were found parasitizing birds: *I. scapularis* was the most common species detected, a species considered medically important as it is the only human-biting vector of *B. burgdorferi* s.s. in Virginia (Piesman and Sinsky, 1988). *Ixodes affinis* and *I. brunneus* have not been observed to bite humans (Luttrell et al., 1996; Merten and Durden, 2000; Nadolny et al., 2011), while *I. dentatus* has rarely been reported to feed on humans (Kollars and Oliver, 2003). Despite *I. affinis* and *I. brunneus* not feeding on humans and the rarity with which *I. dentatus* feeds on humans, these three tick species contribute to the sylvatic cycle of *B. burgdorferi* s.s. in animals, including avian hosts (Heller et al., 2016). *Ixodes affinis,* in particular, serves as a vector and plays an important role in the sylvatic cycle of *B. burgdorferi* s.s. involving small mammals (Nadolny et al., 2011).

The seasonal activity (phenology) of immature *I. scapularis* varies geographically, with debate over whether spring active nymphs and late summer active larvae (as occurs in the northeastern United States) or synchronous phenology of nymphs and larvae based on computer models is better for transmission of *B. burgdorferi* (Ogden et al., 2018). A reversed pattern, with larvae active earlier in the year and nymphs in the autumn, seen in the southeastern United States may explain the lower Lyme disease incidence in this part of the country (Ogden et al., 2018). Additionally, there is an East-West gradient (northeast to upper Midwest) that results in a bimodal larval phenology in the Midwest. Two states in which data were collected by Ogden et al. (2018) closest to Virginia showed differences in immature *I. scapularis* phenologies; Tennessee having nymphs feeding before larvae, while North Carolina had larvae before nymphs. Stromdahl et al. (2014), using information from *I. scapularis* collected from humans over a 15-yr period from 1997 to 2012, reported that in Pennsylvania the nymphal peak was in June, while in Minnesota both nymphs and adult *I. scapularis* peaked in June. Phenologies of the immatures of most *Ixodes* species in Virginia have not been fully characterized. Our study provides important insight into the timing of host-seeking activity for immature *I. scapularis*, *I. affinis*, *I. brunneus*, and *I. dentatus* on avian hosts in this region. Host-seeking activity of *I. affinis* in Virginia has been previously examined (Nadolny and Gaff, 2018); on small mammals, *I. affinis* larval feeding activity peaks in winter, but on birds, we found *I. affinis* larval feeding activity peaked later in August. We observed this same trend for nymphs, where peak feeding activity is in April on small mammals but much later, July to September, on birds. Despite these differences, phenological data for all immature *Ixodes* ticks from any host are useful in understanding the disease ecology of an area. Phenological data for immature *Ixodes* ticks collected from birds are particularly important for determining dispersal of potentially infected ticks into novel areas. The phenologies we observed do not match those reported for immature *I. scapularis* from nearby states (Stromdahl et al., 2014; Ogden et al., 2018); therefore, determining the phenology of *Ixodes* spp. in southeastern Virginia is important in understanding the role of these ticks in the prevalence of pathogenic *B. burgdorferi* and the risk of human infection.

Finding larval ticks infected with *B. burgdorferi* s.s. is suggestive that some birds were infected with *B. burgdorferi* s.s. Host reservoir competence for *B. burgdorferi* s.s. is variable among avian taxa, as well as within individuals of a given species (Richter et al., 2000; Ginsberg et al., 2005). Within the United States, up to 20 % of *Ixodes* ticks, and up to 50 % of nymphs, collected from birds have been reported to be infected with *B. burgdorferi* s.l. (Smith et al., 1996; Hamer et al., 2012a, 2012b; Dingler et al., 2014; Newman et al., 2015; Heller et al., 2016), indicating that birds may serve as reservoirs of *Borrelia burgdorferi* s.s. Avian reservoir competency studies found American robins, song sparrows

(*Melospiza melodia*), northern cardinals, and gray catbirds (*Dumetella carolinensis*) are reservoir-competent for *B. burgdorferi* s.s. (Ginsberg et al., 2005). We found *Ixodes* species parasitizing American robins, gray catbirds, and northern cardinals, but none of these ticks were infected with *B. burgdorferi* s.s. or any other *Borrelia* species; however, it is noted that our sample size of *Ixodes* from these birds was relatively low.

As an alternative to a systemic host infection, the *Ixodes* larvae in our study could have become infected with *B. burgdorferi* s.s. on hosts by concomitant feeding with other infected ticks through a localized infection (Patrican, 1997b). Two of the infected ticks were from different life stages feeding on the same Carolina wren host; in both incidences, we found an *I. scapularis* nymph and larva (both infected) feeding. The wren may have served as a host in which a localized infection of *B. burgdorferi* s.s. could have occurred following the bite from an infected nymph that then allowed the larvae to acquire the pathogen. The phenological data obtained in this study demonstrated that *I. scapularis* nymphs actively seek hosts and feed from April to October, overlapping with *I. scapularis, I. affinis*, and *I. dentatus* larval feeding. These findings suggest the other infected *I. scapularis* and *I. affinis* larvae in our study may have encountered birds parasitized by infected *I. scapularis* nymphs during their blood meals, even if co-feeding was not documented at the time of collection.

The proportion of birds parasitized by *Ixodes* ticks in general, but *B. burgdorferi* s.s.-infected *Ixodes* ticks in particular, was low in comparison to that seen with small mammal sampling in other regions of southeastern Virginia (Anderson et al., 1985, 1987). Although small mammals may not have the capacity for long-distance dispersal, they are host to *Ixodes* ticks and amplify reservoirs in the enzootic cycle of *B. burgdorferi* s.s. (Levine et al., 1985). For brown thrashers and Carolina wrens, prevalence rates of individuals parasitized by infected ticks varied from 2.7 to 4.8 %, respectively. The brown thrasher and the Carolina wren are both ground foragers; however, the lower prevalence in the brown thrasher is not unexpected since this species is known to be reservoir-incompetent for *B. burgdorferi* s.s. (Ginsberg et al., 2005). Swamp sparrows had the highest prevalence of individuals parasitized by *B. burgdorferi* s.s.-infected ticks (6.7 %, 1 of 15 birds surveyed) albeit based on a small sample size. Systemic *B. burgdorferi* s.s. infection (i.e. reservoir competency) in Carolina wrens and swamp sparrows has not been studied to date.

We did not find evidence that one bird species was more likely to harbor *B. burgdorferi* s.s.-infected *Ixodes* ticks than another. The mean infestation number of ticks per host species was low for all bird species parasitized by *Ixodes* juveniles, with numbers typically <1 *Ixodes* tick per host (Table 3). However, the mean infestation number of infected ticks per host species may have been skewed because of small sample sizes for some bird species. For instance, 25 % (2 of 8) of ovenbirds were parasitized by *Ixodes* ticks, but none were positive for *B. burgdorferi* s.s. The risk of encountering a *B. burgdorferi* s.s.-infected tick may change with a larger host sample size, but for our study region, the low number of captures per host species likely represents the relative abundance and presence of hosts in this region and does provide an assessment of host roles and parasitism by infected *Ixodes* ticks.

It is important to note the locations on the bird where the *Ixodes* ticks were attached. We predominantly surveyed the head and vent region, where ticks regularly congregate; however, a recent paper by Roselli et al. (2020) noted other body regions that were parasitized by non-*Ixodes* ticks including the flank, thigh, and wing. These areas were not routinely checked for ticks in our study, which may have contributed to a lower number of ticks detected per bird and/or the overall number of ticks detected on certain bird species.

Our results indicate that passerine birds harbor both larval and nymphal life stages of *B. burgdorferi* s.s.-infected *Ixodes* ticks, albeit in low numbers and on a small number of hosts. Host species that were resident year-round were parasitized by more *B. burgdorferi* s.s.-infected *Ixodes* ticks than migratory species, suggesting long-distance dispersal of infected ticks initially parasitized in the southeastern region of Virginia is not common. Resident bird species parasitized by *Ixodes* ticks (e.g. Carolina wrens) may explain movement of *B. burgdorferi*-infected ticks within localized regions and may help in assessing the human risk of encountering infected ticks. We acknowledge that several of the species we categorized as "resident" (e.g. American robin, brown thrasher, Eastern towhee, and gray catbird) do exhibit some form of seasonal movement throughout parts of their range (Cavitt and Haas, 2020; Greenlaw, 2020; Smith et al., 2020; Vanderhoff et al., 2020); however based on our recapture data indicating some individuals did not exhibit such seasonal movements we classified them as resident. Moreover, given the timing of parasitism, we suspect the source of the feeding ticks was locally obtained, rather than being a function of seasonal movement. However, we acknowledge that some individuals sampled during the winter could be temperate migrants wintering at our field sites. Recognizing that many of the resident host species in this study are found associated with urban areas (Heller et al., 2019), we cannot determine if *Borrelia burgdorferi* s.s. infections in our region can be explained by the dispersal of infected ticks on passerine birds. We also do not know if our findings scale up to explain patterns exhibited at much larger continental scales. This, due in part, to the particular community of tick species found in our study area; in particular, the relative abundance of *I. affinis* to *I. scapularis*.

CRediT authorship contribution statement

Alexandra N. Cumbie: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Erin L. Heller:** Investigation, Writing - original draft. **Zachary J. Bement:** Investigation, Writing - original draft. **Anna Phan:** Investigation, Writing - original draft. **Eric L. Walters:** Investigation, Resources, Writing - review & editing, Funding acquisition. **Wayne L. Hynes:** Conceptualization, Methodology, Writing - review & editing, Project administration, Supervision. **Holly D. Gaff:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Project administration, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2021.101650.

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