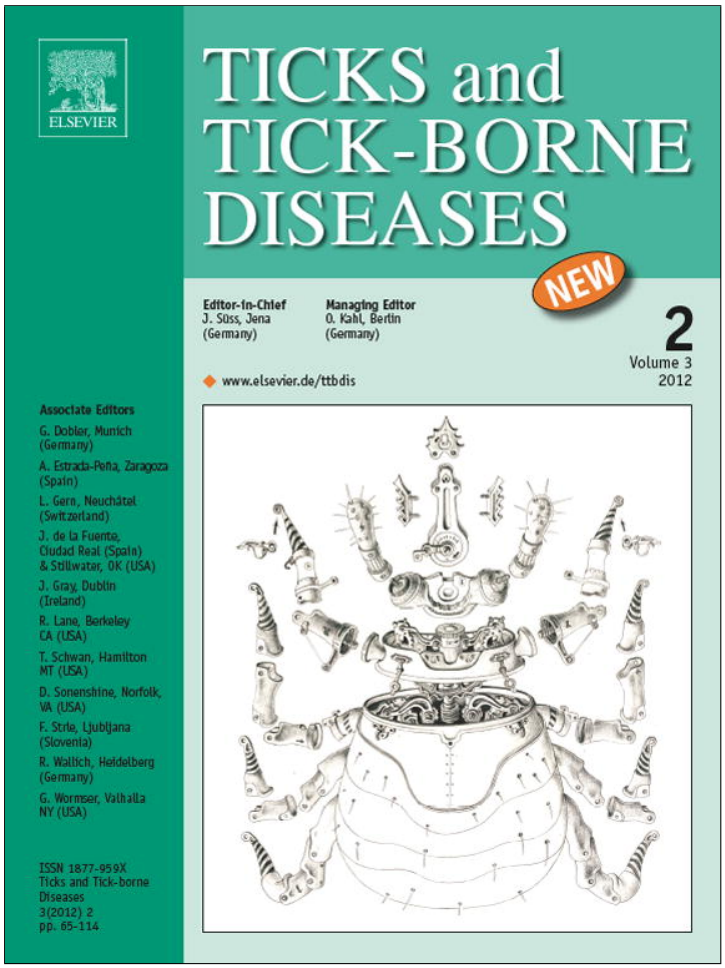

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Original article

Synchronous phenology of juvenile *Ixodes scapularis*, vertebrate host relationships, and associated patterns of *Borrelia burgdorferi* ribotypes in the midwestern United States

Sarah A. Hamer[a,*], Graham J. Hickling[b], Jennifer L. Sidge[c], Edward D. Walker[d], Jean I. Tsao[a,e]

[a] Dept. Fisheries and Wildlife, 13 Natural Resources Building, Michigan State University, East Lansing, MI 48824, USA
[b] Dept. Forestry, Wildlife, and Fisheries and National Institute for Mathematical and Biological Synthesis (NIMBioS), University of Tennessee, Knoxville, TN, USA
[c] College of Veterinary Medicine, Michigan State University, East Lansing, MI, USA
[d] Dept. Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA
[e] Dept. Large Animal Clinical Sciences, Michigan State University, East Lansing, MI, USA

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ABSTRACT

To elucidate features of enzootic maintenance of the Lyme disease bacterium that affect human risk of infection, we conducted a longitudinal study of the phenology of the vector tick, *Ixodes scapularis*, at a newly invaded site in the north-central United States. Surveys for questing ticks and ticks parasitizing white-footed mice and eastern chipmunks revealed that *I. scapularis* nymphal and larval activity peaked synchronously in June and exhibited an atypical, unimodal seasonality. Adult seasonal activity was bimodal and distributed evenly in spring and fall. We discuss implications of these phenology data for the duration of the *I. scapularis* life cycle. Densities of *Borrelia burgdorferi*-infected, questing nymphs were comparable to those found in endemic areas elsewhere in the midwestern and northeastern U.S. Molecular genetic diversity of *B. burgdorferi* infecting these ticks and rodents was assessed by analysis of the ribosomal spacer types (RSTs). RST 1, a clade that includes strains with highly pathogenic properties, was relatively uncommon (3.4%) in contrast to the northeastern U.S., whereas less pathogenic ribotypes of the RST 2 and 3 clades were more common. These features of the ecology of this midwestern Lyme disease system likely contribute to the lower incidence of Lyme disease in humans in the Upper Midwest compared with that of the Northeast owing to reduced exposure to pathogenic strains of *B. burgdorferi*.

Introduction

Lyme disease is the most frequently reported vector-borne disease in the United States, with over 20,000 cases reported annually (Bacon et al., 2008). The disease is caused by infection with the bacterium *Borrelia burgdorferi*, which is transmitted by the bite of ticks. In the eastern and midwestern United States, the blacklegged tick, *Ixodes scapularis*, is the primary vector of *B. burgdorferi*. In these regions, white-footed mice (*Peromyscus leucopus*) and eastern chipmunks (*Tamias striatus*) are important maintenance hosts for the bacterium (Anderson, 1988).

I. scapularis and *B. burgdorferi* continue to invade new areas (Klich et al., 1996; Ogden et al., 2008). In Michigan's lower peninsula, a new population of *I. scapularis* was first noted in 2002 (Foster, 2004), and progressive northward and inland expansion of both the tick and pathogen has been documented across the subsequent 8 years (Hamer et al., 2010). Tick and pathogen invasions present new disease risk to residents and outdoor recreationalists in areas where the medical and veterinary communities are not accustomed to screening for this disease. Appropriate public health interventions and educational campaigns require knowledge of where and when ticks are active, and such data presently are largely based on studies conducted where the tick is heavily endemic. The dynamics and processes that affect tick and pathogen densities and distributions in recently-invaded zones may be different from those in endemic areas, due to geographic variation in extrinsic factors such as photoperiod, climate, habitat suitability as well as unequilibrial population dynamics as the organisms become established.

Genetic variation in *B. burgdorferi* sensu stricto is both ecologically and epidemiologically meaningful. A molecular typing system based on restriction fragment length polymorphisms in the 16S–23S ribosomal DNA (rDNA) spacer region yielded 3 ribosomal spacer type (RST) groups: RST 1, 2, and 3 (Liveris et al., 1995), each of which is comprised of many ribotypes/strains. Based on analysis of clinical isolates, disseminated infection (spirochetemia and secondary erythema migrans) was more strongly associated with RST

* Corresponding author. Present address: Dept. of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX 77843-4458, USA.
Tel.: +1 517 775 4360; fax: +1 517 432 1699.
E-mail address: hamer@msu.edu (S.A. Hamer).

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1 spirochetes than RST 2 or 3 (Wormser et al., 1999). Using a laboratory mouse model, Dolan et al. (2004) found that the duration and intensity of spirochetemia induced by the disseminated (RST 1) strains were greater than that induced by the non-disseminated (RST 3) strains and that the host genetic background also influenced infection. Furthermore, an RST 1 strain was more efficiently transmitted to xenodiagnostic ticks by *P. leucopus* mice than was an RST 3 strain (Derdakova et al., 2004; Hanincova et al., 2008). Recently, a standardized field survey of questing *I. scapularis* nymphs found RST 1 spirochetes to be more prevalent in ticks, relative to RST 2 and 3 spirochetes, in the Northeast versus in the Midwest (Gatewood et al., 2009). In this study, the Northeast was characterized by an approximate lag time of 2 months between nymphal and larval activity, whereas the immature stages were found to be more synchronous in the Midwest (Gatewood et al., 2009). The difference in ribotype distribution was attributed to the greater longevity of RST 1 spirochetes in hosts, which may confer a selective advantage in areas where there is a longer period of time between the nymphal and larval activity periods (Gatewood et al., 2009). Knowledge of the interaction between pathogen genetic diversity and the phenology of different life stages of the tick therefore may be useful in explaining regional variation in human risk.

Here, we use a longitudinal study design and trapped select vertebrate hosts that are known to be important players in the ecology of this tick and bacterium to address the following objectives in a focal area of the midwestern United States: (1) to determine the activity periods of larval, nymphal, and adult *I. scapularis* in the study area as they relate to rodent population dynamics; and (2) to characterize the molecular diversity of *B. burgdorferi* ribosomal spacer types in ticks and select vertebrate hosts. We hypothesized that (i) *I. scapularis* larvae and nymphs would exhibit synchronous host-seeking activity; (ii) *B. burgdorferi* genetic diversity would be characterized by a predominance of strains that group with RST 2 and 3; and (iii) RST 1 strains would be most prevalent in hosts at the end of the transmission season due to their greater longevity in hosts relative to non-disseminated strains.

Materials and methods

Van Buren State Park in South Haven, Van Buren County, MI, encompasses 162 ha of sandy beaches, high dunes, and oak woodlands on Lake Michigan's eastern shore. Following statewide surveillance from 1985 to 1996 that detected few *I. scapularis* in this region (Walker et al., 1998), an established population of *I. scapularis* was first documented at Van Buren State Park in 2002 (Foster, 2004). Tick density has since increased (Hamer et al., 2010) with current densities comparable to those in other areas with long-established populations (Diuk-Wasser et al., 2010). Within the closed canopy, oak-dominated deciduous forest habitat of the park, six 250-m linear transects were established for sampling at monthly intervals from summer June 2008 through summer July 2009 (exceptions noted below).

Sampling questing ticks

At each monthly site visit, each of the 6 transects was sampled for questing ticks by dragging a 1-m^2 white corduroy cloth (Falco and Fish, 1992) for a total average of 2500 m^2 of sampling. Drag sampling was performed on a total of 17 rain-free days in the late morning or late afternoon to avoid the hottest and least humid times of day (Diuk-Wasser et al., 2006). The cloth was inspected every 10–20 m, and attached ticks were stored in 70% ethanol. Drag sampling was not performed on excessively hot or wet days or when there was dense snow cover.

Small mammal trapping

Small mammals were trapped along 4–6 transects using 25 Sherman live traps (H.B. Sherman Traps, Tallahassee, FL) spaced 10 m apart and baited with sunflower seed for 1–2 trap nights per month. Small mammals were anesthetized using isoflurane (IsoFlo, Abbot Laboratories, Abbott Park, IL). Animals were identified to species and sex by inspection. Age (juvenile or adult) of mice was determined based on weight, in which individuals <15 g were recorded as juveniles, and individuals >15 g were recorded as adults. Each animal was examined for ticks, biopsied in both ears using a 2-mm biopsy punch (Miltex Instruments, York, PA), and marked with a uniquely numbered metal ear-tag (National Band and Tag, Newport, KY). Ticks and ear biopsies were stored separately in 70% ethanol. A single biopsy was obtained from animals recaptured after an interval of 2 weeks or more; recaptures that were already processed the previous day were simply rechecked for ticks. All animals were released at the site of capture. Capture success was calculated as the number of mammals captured divided by the number of effective trap nights, where the total number of trap nights was discounted by the number of empty, tripped traps as follows: number traps set $- 0.5 \times$ number tripped traps. This expression assumes that on average, tripped traps were open for half the night. Wildlife procedures were approved through Michigan State University's Institutional Animal Use and Care Committee permit #02-07-13-000.

The monthly population sizes of white-footed mice and eastern chipmunks were estimated from the mark-recapture data using the Chapman modification of the Lincoln–Petersen method (Jones and Kitron, 2000), which is more accurate than other methods when unequal trappability is suspected (Mares et al., 1981). In computing population density for the second year, no marked animals were assumed to have successfully overwintered. To estimate the size of our trapping area, we assumed that each trap covered an area equal to the average home range size of the mammal [white-footed mouse, 1000 m^2 (Lackey et al., 1985); eastern chipmunk, 3400 m^2 (Elliot, 1978)]. The area trapped was calculated by assuming that each trap provided a circular trapping area of 0.1 ha (i.e., radius 17.8 m). The 25 traps were only 10 m apart on the transects, so areas of overlap were counted only once, giving a trapped area of 9360 m^2 for each transect (=56,200 m^2 in total for the 6 transects) for mice and 19,300 m^2 for each transect (=115,200 m^2 in total for the 6 transects) for chipmunks. In the 131 recapture events of mice in which the transect of capture was identified on both captures, there are 12 instances in which the same mouse was captured on different transects (9.1% of recapture events), and therefore the actual sampling area may be larger than suggested above. Monthly small mammal population densities were calculated as the estimated mammal population size per 10,000 m^2 (hectare).

B. burgdorferi detection

All ticks were identified to species and stage (Durden and Keirans, 1996; Keirans and Durden, 1998; Sonenshine, 1979). Total DNA from a random subset of ticks and ear biopsies was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as described in Hamer et al. (2010). We tested both questing *I. scapularis* and *Dermacentor variabilis* – the latter species, though not a vector for *B. burgdorferi* (Piesman and Sinsky, 1988), can serve as a bio-indicator for pathogen presence (Walker et al., 1994; Hamer et al., 2010). Furthermore, we included *D. variabilis* in order to provide more samples for looking at genetic variation in *B. burgdorferi*. Ear biopsies (one per animal), adult and nymphal ticks were extracted individually, and conspecific larvae from the same individual animal or drag transect were pooled for extraction. When more than 5 nymphs parasitized the same animal, 5 nymphs were

selected at random for testing. DNA from *B. burgdorferi* strain B31-infected ticks from the Centers for Disease Control and Prevention served as a positive extraction control, and water served as a negative extraction control.

B. burgdorferi was detected using one of 2 assays with comparable sensitivities: (1) a nested polymerase chain reaction (PCR) for the 16S–23S rRNA intergenic spacer region (IGS) of *Borrelia* spp. (Bunikis et al., 2004), or (2) a quantitative PCR (qPCR) of a region of the 16S rRNA of *B. burgdorferi* (Tsao et al., 2004) as previously described (Hamer et al., 2010); the latter assay was adopted to facilitate higher-throughput screening of samples. In addition to the positive and negative controls used in the extraction, we also used separate PCR controls that consisted of *B. burgdorferi* DNA extracted from infected laboratory ticks and water.

Ribotyping of B. burgdorferi

DNA was sequenced from a random subset of *B. burgdorferi*-positive ticks and ear biopsies to evaluate molecular variation. The IGS PCR product was purified (Qiagen PCR Purification Kit; Qiagen, Valencia, CA), and sequences were determined in both directions using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). IGS ribosomal spacer type (RST) groups were inferred based on alignment with a set of reference strains published in Bunikis et al. (2004) using the program MEGA (Liveris et al., 1995; Tamura et al., 2007).

Statistics

Comparisons of infestation and infection between host sexes were made by calculating the *z*-ratio and associated 2-tail probabilities for the difference between 2 independent proportions. Chi-squared tests were used to test if observed frequencies of coinfestations and pathogen ribotypes differed from expectations. To assess trends in tick burdens on animals over the sampling period, linear mixed effects models were computed with the host identification and year as random factors to account for potential autocorrelation among observations in the time series. Statistics were performed using Program R (R Development Core Team, 2008). To achieve a normal distribution of model residuals, transformations of data included log $(X+1)$ on larval burdens and square-root $(X+0.5)$ on nymphal burdens. We used Geepack in Program R to implement the generalized estimating equations approach for fitting marginal generalized linear models to clustered data (suitable for longitudinal data and repeated measures). This equation yields a logistic regression in which the host identification was used as the identity of the cluster.

We calculated the monthly 'feeding density' of each host species for each tick life stage as the product of the estimated host density and the average *I. scapularis* burden per host. In the early summer months in which host density could not be calculated due to a lack of recaptures in the dataset (see above), we estimated host density as the same density as calculated for the subsequent month (which may be an overestimate). Monthly feeding density can be interpreted as the density of ticks expected to be fed by a given host species, and this parameter can be compared among hosts to determine relative contributions of hosts to feeding of ticks. It is equivalent mathematically to the unnamed products 'm' used by Giardina et al. (2000) and 'L_sD_s' used by Mather et al. (1989).

We calculated the product of (i) the feeding density (as defined above) and (ii) the proportion of hosts infected with *B. burgdorferi*, and refer to this as the 'modified reservoir potential' for a given host species. This parameter is similar to the 'reservoir potential' as defined by Mather et al. (1989), which is synonymous with 'relative reservoir capacity' as defined by Kahl et al. (2002), except that the infection component of our parameter is based on PCR of host ear biopsies instead of xenodiagnosis. Modified reservoir potential is also similar to the term I_i introduced by LoGiudice et al. (2003), which is the product of the number of molted nymphs from larvae that fed on a given host species and the host's reservoir competence/specific infectivity, except that our field approach did not allow ticks to molt, and we calculate our parameter on a monthly basis to show change across the season.

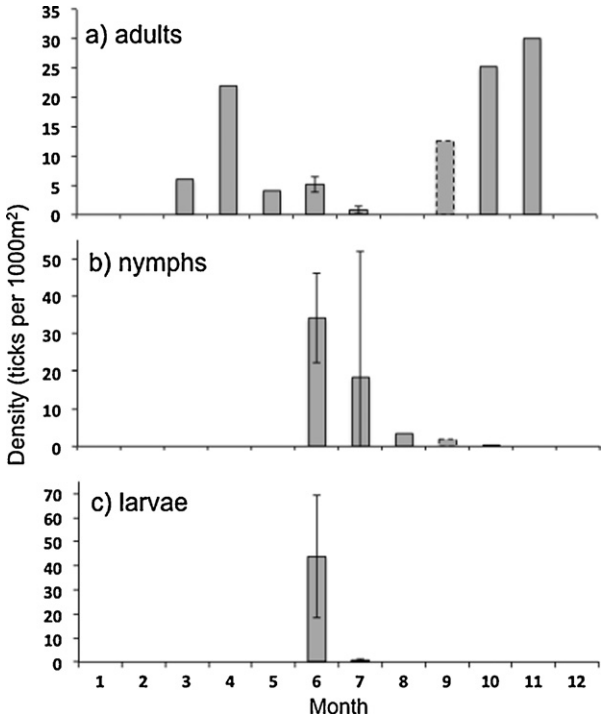

Fig. 1. Phenology of questing *I. scapularis* in June 2008–July 2009 based on monthly drag sampling at Van Buren State Park in southwestern Michigan. No sampling was conducted in January and February (snow) and September (heavy rain); September tick activity is an interpolation of the August and October estimates (indicated by the dashed bars). Data from both 2008 and 2009 were pooled for June and July, and error bars represent standard error of the mean.

Results

Questing ticks

A total of 28,250 m^2 of drag sampling were conducted during the 14-month study, ranging from 1750 m^2 to 5400 m^2 per sampling month. No drag sampling was conducted in January and February due to snow cover, and none was conducted in September due to heavy rainfall. A total of 867 ticks of all life stages were collected; the majority was *I. scapularis* (93.4%) and the balance was *D. variabilis*. Seasonal activity of *I. scapularis* (Fig. 1) was characterized by bimodal peaks in adult activity, with peak densities of 22.0 and 30.1 adults/1000 m^2 in April and November, respectively. Totals of 176 males and 155 females were collected; the only month with a sex bias was November, in which significantly more males ($n=84$) were collected than females ($n=59$; $P=0.003$). Totals of 240 nymphs and 239 larvae were collected; nymphal and larval questing activity both peaked in June, with 34.1 and 43.9 ticks/1000 m^2, respectively. A total of 90.2% of nymphal activity occurred in June and July, and nymphs exhibited a low level of activity through October. In contrast, 98.3% of larval questing activity occurred in June with the remaining activity in July.

Table 1
Infection of ticks and hosts with *B. burgdorferi* by month at Van Buren State Park in southwestern Michigan, 2008–2009. Data from June and July of 2008 and 2009 are pooled. Infection prevalence of larvae on hosts is expressed as the proportion of larval pools that tested positive, where each pool represents the conspecific larvae from one individual host. Sample size tested for infection is indicated in parenthesis.

Month	Proportion positive (no. tested)									
	Drag-sampled *I. scapularis*		Ear biopsies		*I. scapularis* on mice		*I. scapularis* on chipmunks		*D. variabilis* on mice	
	Adults	Nymphs	Mice	Chipmunks	Larval pools	Nymphs	Larval pools	Nymphs	Larval pools	Nymphs
3	0.43 (21)									
4	0.20 (10)		0 (2)						0 (2)	
5	0.56 (9)		0.14 (7)		0.50 (2)				1.0 (1)	
6	0.52 (21)	0.12 (41)	0.22 (45)	0.33 (18)	0.25 (44)	0.23 (26)	0.50 (6)	0.46 (13)	0.15 (52)	0.05 (38)
7	1.00 (2)	0.07 (15)	0.17 (76)	0.58 (12)	0.33 (43)	0.45 (11)	0.25 (4)	0.59 (34)	0.15 (20)	0 (12)
8		0 (7)	0.28 (50)	1.0 (1)	0.39 (23)				0 (1)	0 (1)
9			0.57 (49)	0 (1)	0 (12)	0 (1)	1.0 (1)	1.0 (1)		
10	0.35 (20)	0 (1)	0.38 (58)		0 (13)			0.5 (2)		
11	0.68 (25)									
Total	0.49 (108)	0.09 (64)	0.31 (287)	0.44 (32)	0.25 (137)	0.29 (38)	0.45 (11)	0.56 (50)	0.16 (76)	0.04 (51)

B. burgdorferi infection of questing ticks

A total of 200 questing *D. variabilis* and *I. scapularis* adults, nymphs, and larval pools was tested for *B. burgdorferi* by PCR. Of 24 *D. variabilis*, the 20 adults, 2 nymphs, and 2 larval pools all tested negative. Of 176 *I. scapularis*, 53 of 108 adults (49.1%), 6 of 64 nymphs (9.4%), and 0 of 4 larval pools tested positive. There was no significant difference in infection prevalence in female *I. scapularis* (54.9%) versus males (43.9%; $P = 0.25$). Monthly adult infection prevalence ranged from 20% to 68% in months where more than 2 ticks were tested, and nymphal infection prevalence ranged from 0% to 12% (Table 1). The density of infected nymphs (i.e., acarological risk = the product of the monthly drag-sampled nymphal density and the monthly nymphal infection prevalence) peaked in June (4.2 per 1000 m^2) and was 1.2 per 1000 m^2 in July.

Small mammal trapping

A total of 1800 nights' trapping were undertaken during the study period, with no trapping from November 2008 to March 2009 due to low temperatures that would have put trapped animals at risk. This total was discounted by the number of tripped traps to yield 1693 effective trap nights with 388 captures (22.9% overall capture success). Captures per 100 effective trap nights increased progressively from April (1.4% capture success) to October (54.7% capture success; Fig. 2). The 388 capture events comprised 232 individual small mammals, of which the majority was white-footed mice ($n = 180$ individuals) followed by eastern chipmunks ($n = 33$), northern short-tailed shrews (*Blarina brevicauda*, $n = 12$), southern flying squirrels (*Glaucomys volans*, $n = 6$), and a Virginia opossum (*Didelphis virginiana*, $n = 1$).

Monthly mouse population density, estimated from the mark-recapture data, increased from July to October 2008 with estimates of 30.3 ± 5.3, 32.6 ± 4.5, 51.7 ± 4.4, and 83.9 ± 5.7 mice per ha $\pm$ standard error, respectively. In the second summer of the study, the density of mice in July was estimated to be only 6.4 ± 2.6 mice per 10,000 m^2. Chipmunk population densities remained fairly stable across the months for which it was able to be determined and was always one order of magnitude less than mouse densities, with 2.3 ± 0.2 and 2.5 ± 0.4 chipmunks per ha in August and September 2008, respectively, and 2.1 ± 0.6 per ha in July 2009.

One hundred eighty white-footed mice were captured, with individuals captured 1–6 times each in 320 total captures. Three (1.7%) mice were known to have overwintered (i.e., captured in 2008 and again in 2009); this is a violation of the mark-recapture method to estimate population density (reported above), but the effect was likely negligible. There was no sex bias present in the trapped population of mice (79 females, 95 males, 6 unknown sex; $P = 0.25$). Upon first capture, 80.3% of mice were categorized as adults, 17.9% were juveniles, and 1.7% were of unknown age. A total of 63 (19.7%) mouse captures had visible infestations with 1–3 botfly larvae. All botfly infestations occurred from July to October, with a majority (74.6%, including all multiple infestations) occurring in September and October.

A total of 33 eastern chipmunks were captured, with individuals captured 1–3 times each in 48 total captures. Four (12%) chipmunks were known to have overwintered, and therefore the July 2009 population size estimate reported above may be a slight overestimate. Males were over-represented within the trapped population of chipmunks (9 females, 22 males, 2 unknown sex; $P = 0.031$). A total of 12 northern short-tailed shrews were captured including 3 females, 1 male, and 8 of unknown sex. Five individual Southern

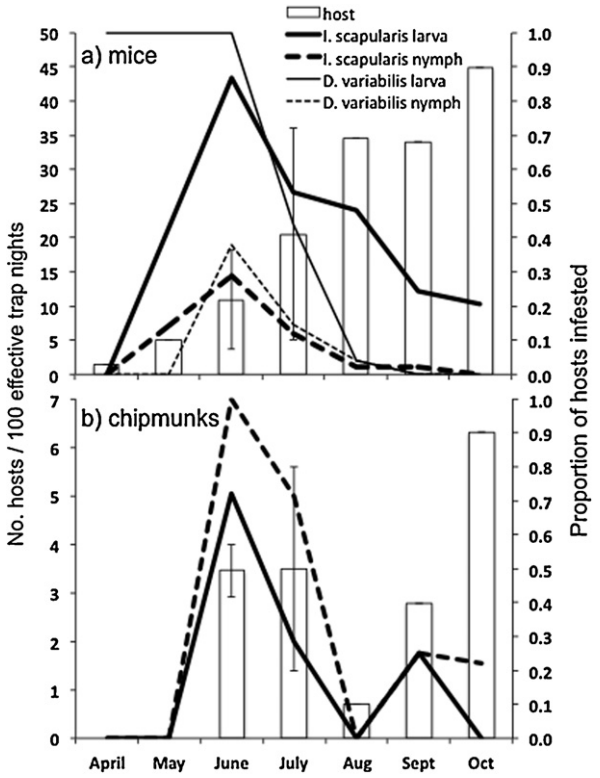

Fig. 2. Seasonal dynamics of tick infestation of (a) white-footed mice and (b) eastern chipmunks. The relative host population size is plotted as the standardized capture success. Data from 2008 and 2009 were pooled for June and July, and error bars represent standard error of the mean.

Table 2
Monthly feeding density of larval and nymphal ticks of white-footed mice and eastern chipmunks at Van Buren State Park in southwestern Michigan, 2008–2009. Data from June and July of 2008 and 2009 are pooled. Feeding density is the product of host density and the average *I. scapularis* burden per host. Mark-recapture data did not allow for determination of mouse host density in May and June or chipmunk host density in June or October; the values used were equal to the subsequent or previous month's calculated density. Modified reservoir potential (no. of ticks fed by infected hosts per 1000 m^2) is the product of the feeding density and host infection (see Table 1). Due to small sample sizes of tested ear tissues in August–October, chipmunk infection prevalence was assumed to be the same as in July (58%). The aggregate feeding density and modified reservoir potential are the sums of the monthly estimates of each parameter across the study period.

Host species	Month	Host density	Proportion infested		Average burden		Feeding density		Modified reservoir potential	
Animals per 1000 m^2	Larvae	Nymphs	Larvae	Nymphs	Larvae	Nymphs	Larvae	Nymphs		
White-footed mouse	5	3.0	0.43	0.14	1.7	1.0	2.21	0.43	0.31	0.06
	6	3.0	0.87	0.29	6.5	1.7	17.10	1.51	3.76	0.33
	7	3.0	0.53	0.12	4.0	1.2	6.46	0.44	1.10	0.07
	8	3.3	0.48	0.02	1.8	1.0	2.74	0.07	0.77	0.02
	9	5.2	0.24	0.02	2.2	1.0	2.75	0.11	1.57	0.06
	10	8.4	0.21	0.00	1.5	0.0	2.67	0.00	1.01	0.00
	Aggregate						33.92	2.55	8.51	0.55
Eastern chipmunk	6	0.2	0.72	1.00	4.5	6.7	0.68	1.41	0.22	0.47
	7	0.2	0.29	0.71	1.5	3.4	0.09	0.51	0.05	0.30
	8	0.2	0.00	0.00	0.0	0.0	0.00	0.00	0.00	0.00
	9	0.3	0.25	0.25	1.0	1.0	0.06	0.06	0.04	0.04
	10	0.3	0.00	0.22	0.0	1.0	0.00	0.06	0.00	0.03
	Aggregate						0.83	2.04	0.31	0.83

flying squirrels were captured 6 times, including 4 females and 1 of unknown sex.

Ticks on mammals

A total of 1703 ticks were removed from all mammals, consisting of 809 larval and 67 nymphal *D. variabilis* and 626 larval and 201 nymphal *I. scapularis*. A subset of the study mammals ($n = 25$ mice and 1 chipmunk) was recaptured the night after being initially processed. From these recaptures, a total of 76 ticks (range: 0–16 per mouse; median of 1) were removed on the second day, indicating recent acquisition of ticks by the host or overlooked ticks from the previous day; data from these next-day recaptures are not considered further. Six mammals (4 mice, 1 chipmunk, 1 flying squirrel) escaped prior to checking for ticks; these mammals are not included in the denominator for calculations of infestation or infection prevalences. Across the study period, 47.1, 67.4, 25.0, 0, and 100% of mice, chipmunks, shrews, flying squirrels, and opossum, respectively, were infested with *I. scapularis*; 32.3, 2.2, and 16.7% of mice, chipmunks, and flying squirrels were infested with *D. variabilis*.

Mouse infestations with larval *I. scapularis* were present in May through October and peaked in June at 86.7% (Fig. 2a). Mouse infestations with nymphal *I. scapularis* were present from May through September and peaked in June at 28.9%, coincident with the larval peak. There was significant variation in mouse larval and nymphal burdens among months (df = 6, 130; $F = 13.17$; $P < 0.001$; df = 6, 130; $F = 5.5$; $P < 0.001$, respectively; Table 2). When the highest proportion of mice was infested (in June), tick burdens were also at their peak – in June, median burdens of larval and nymphal *I. scapularis* on infested mice were 4 and 2, and the peak burdens were 37 and 4, respectively. Significantly more male than female mice were infested with both larvae (males, 52.8%; females, 32.1%; $P = 0.0002$) and nymphs (males, 13.1%; females, 3.7%; $P = 0.004$). Coinfestation of larvae and nymphs was noted on 6.5% of mice; this is more frequent than expected based on chance ($\chi^2 = 9.5$; df = 3; $P = 0.023$). Larval *D. variabilis* were found on mice from April through August, with 100% infestation in April through June. Nymphal *D. variabilis* were found on mice from June through August, peaking at 37.8% infestation in June. When the highest proportion of mice was infested in June, tick burdens were also at their peak – in June, median burdens of larval and nymphal *D. variabilis* on infested mice were 9 and 2, with maximum burdens of 56 and 7, respectively. There was no significant difference in infestation by mouse sex (27.3% of females and 36.6% of males; $P = 0.09$). Coinfestations of mice with both *I. scapularis* and *D. variabilis* were present on 23.7% of captures; this is more frequent than expected by chance ($\chi^2 = 39.12$; df = 3; $P < 0.0001$).

Chipmunk infestations with larval *I. scapularis* were present in June, July, and September with the largest peak of activity in June and a smaller activity peak in September (72.2%; Fig. 2b). Chipmunk infestations with nymphal *I. scapularis* were present in June through October with the exception of August. Peak nymphal infestation was in June (100%), coincident with the larval peak. There was significant variation in chipmunk larval and nymphal burdens among months (df = 4, 6; $F = 5.42$; $P = 0.034$; df = 4, 6; $F = 7.97$; $P = 0.014$, respectively; Table 2). When the highest proportion of chipmunks was infested in June, tick burdens were also at their peak – in this month, median burdens of larval and nymphal *I. scapularis* on infested chipmunks were 3 and 5.5, and the peak burdens were 18 and 17, respectively. There was no significant difference in *I. scapularis* infestations by chipmunk sex for larvae (males, 35.5%; females, 40.0%; $P = 0.767$) or nymphs (males, 73.3%; females, 61.3%; $P = 0.421$). Coinfestation of larvae and nymphs was noted on 39.1% of chipmunks, and every chipmunk that harbored larvae also simultaneously harbored at least one nymph; this is more frequent than expected based on chance ($\chi^2 = 14.8$; df = 3; $P = 0.002$). Only a single chipmunk in our study was found to be infested with *D. variabilis*; this chipmunk was captured in July and harbored a single larval *D. variabilis*. Larval *I. scapularis* infestations of shrews were present in June to August, with median and peak burdens of 2 and 12, respectively; no shrews harbored nymphs.

Summation of the monthly feeding densities across the study period (the aggregate feeding density; Table 2) provides an index of the relative contribution of different host species to feeding *I. scapularis*. The aggregate feeding density of larvae was over 40 times greater for mice (33.9) than for chipmunks (0.8). In contrast, the feeding density for nymphs was nearly equal for the 2 host species (2.6 and 2.0, respectively), despite the order of magnitude greater density of mice. Our data suggest that the proportion of all *I. scapularis* larvae and nymphs that are fed by mice and chipmunks is small. For example, in June the feeding density of larvae on mice plus chipmunks was 17.8 ticks/1000 m^2 (Table 2). The whole population of larvae in the environment at that time included those on mice and chipmunks (17.8 ticks/1000 m^2), those on alternative hosts (unknown), those that we drag-sampled (43.9 ticks/1000 m^2), and those in the vegetation that we did not drag due to drag sampling inefficiency or because they were not questing at

that time (unknown). We therefore conclude that mice and chipmunks feed at maximum 29% of larvae that are in the environment. The nymphal data follow a similar pattern; in June, mice and chipmunks feed at maximum 6% of nymphs that are in the environment.

B. burgdorferi infection of small mammals

A total of 287 mouse ear biopsies were tested for infection with *B. burgdorferi*, of which 30.7% tested positive. These samples represent 170 individual mice that were each sampled 1–5 times. Of the 67 mice associated with 2 or more ear samples, 29 (43.3%) were negative on all testing dates, 3 (4.5%) were positive on all testing dates, 18 (26.9%) became infected (all infections occurred from July to October), 10 (14.9%) changed from positive to negative, and 7 (10.4%) were sampled 3 or more times in which status changed from both positive to negative and negative to positive. Mouse infection prevalence increased across the calendar year (df = 6; $P < 0.001$) greatest in September with 57.1% of ear samples testing positive (Table 1); year did not explain significant variation in the model. Significantly more male mice were infected as compared to female mice (35.4 and 24.6%, respectively; $P = 0.049$). A total of 32 chipmunk biopsies were tested for infection with *B. burgdorferi*, of which 43.8% tested positive (Table 1). These samples represent 27 individual chipmunks that were each sampled 1–2 times. Of the 5 individuals with repeated samples, one chipmunk became infected between initial capture in June 2008 and subsequent capture in June of 2009, and one chipmunk was positive on both capture events, and 3 chipmunks were negative on both capture events. There was no significant difference in infection between male and female chipmunks (47.6 and 40%, respectively; $P = 0.69$). While monthly samples sizes were too small to assess for trends in chipmunk infection across the season, infection trended upward in June and July (33.3 and 58.3%, respectively; $P = 0.09$).

The modified reservoir potential for mice and chipmunks – i.e., the density of ticks fed by infected hosts – was greatest in June for both larvae and nymphs, this month alone accounted for nearly half (47.0%) of the aggregate modified reservoir potential that we documented across the year (Table 2). Summing across the June-to-October time period, the months for which modified reservoir potential estimates were able to be computed for both host species, infected mice are expected to feed to 27.3 times more larvae than are infected chipmunks; conversely, infected chipmunks are expected to feed 1.5 times more nymphs than do infected mice.

B. burgdorferi infection of ticks removed from small mammals

Among the subset of the *I. scapularis* removed from hosts and tested for infection, higher infection prevalence was present among the larval pools and nymphs removed from chipmunks (45% of 11 larval pools and 56% of 50 nymphs) versus mice (24.8% of 137 larval pools and 29% of 38 nymphs; Table 1). The monthly pattern of infected larval pools removed from mice and chipmunks generally followed the trend of host infection based on ear biopsy, with the exception of no infected larval pools on mice among our samples in September and October ($n = 25$ pools tested), when 46.7% of mice had infected ear tissue (Table 1).

Of the 2 *I. scapularis* larval pools removed from shrews that were tested, one was positive (50%). Among the *D. variabilis* removed from hosts, infection prevalence in *D. variabilis* removed from mice was less than that of *I. scapularis* removed from mice (Table 1): 15.8% of 76 larval pools tested and 3.9% of 51 nymphs tested positive (infection in *D. variabilis* likely reflects an infected blood meal as this species is not a vector; Piesman and Sinsky, 1988). The single *D. variabilis* pool of 6 larvae removed from a flying squirrel was negative, and the single larva from the chipmunk was not tested.

There were 40 instances of animals harboring infected *I. scapularis* larval pools, representing 34 mammals, 5 of which had infected larvae on 2 or 3 separate occasions. Of these, 19 (47.5%) also had a positive ear biopsy on the same date, 20 (50%) had a negative ear biopsy on the same date, and 1 animal's ear biopsy was not tested. Across the entire season, among those 36 mammals associated with at least one positive *I. scapularis* larval pool, 68% also had at least one positive ear biopsy taken on the same or a different date. Regarding *D. variabilis*, 70% of mammals associated with at least one positive larval pool ($n = 10$ mammals) also had a positive ear biopsy taken on the same or a different date.

B. burgdorferi ribotypes

Ribotypes were determined for a total of 147 samples, including 45 infected questing *I. scapularis* ticks (41 adults and 4 nymphs), 54 infected mammal ear biopsies, and 48 infected larval pools or nymphs removed from hosts (all were *I. scapularis* with the exception of 2 *D. variabilis* larval pools removed from mice). All 3 RST groups were represented, with RST 1 strains being the least common (3.4, 49.0, and 47.6% of samples were RST 1, 2, and 3, respectively; $\chi^2 = 59.3$; df = 2; $P < 0.0001$). The proportions of the 3 RST groups differed among the 3 sample types ($\chi^2 = 11.48$; df = 4; $P = 0.02$), with host ears having higher prevalence of infection with spirochetes of RST 1 and 2 and lower prevalence of RST 3 as compared to drag-sampled ticks and ticks removed from mammals (percent of samples with RST 1, 2, and 3 was 5.6, 64.8, and 29.6% for host ears; 2.2, 37.8, and 60% for questing ticks; and 2.1, 41.7, and 56.3% for ticks removed from mammals, respectively). Only 5 samples were infected with RST 1 strains, including one questing adult female *I. scapularis,* one pool of 4 larval *I. scapularis* removed from a mouse, and 3 mouse ear biopsies. The single RST 1-infected questing tick was collected in March; there were no statistically significant trends in RST groups in questing nymphs and adults over time (in part because RST 1 was so rare). The single RST 1-infected larval pool removed from a mouse was collected in July and comprised 14% of infected larval pools in that month ($n = 7$). Larval pools infected with RST 2 and 3 spirochetes were also removed from mice in May ($n = 1$), June ($n = 3$), and October ($n = 8$). The 3 RST 1-infected mice were collected in May, July, and August, comprising 100% ($n = 1$), 9% ($n = 11$), and 10% ($n = 10$) of genotyped host samples from those months, respectively, with no significant trend over the May-to-October sampling period ($n = 51$ total genotyped mouse samples; logistic regression $R^2 = 0.15$; $P = 0.30$). Among the ribotypes determined for questing ticks, RST 2 and 3 were equally represented within the nymphs ($n = 4$ each), and RST 3 predominated among the adults (61% of 41 samples), relative to RST 2 (37%) and RST 1 (2%). Of the 14 instances in which ribotypes were determined from a nymph or larval pool and from the ear biopsy removed simultaneously from the mammal, 8 sample pairs (57%) were comprised of identical ribotype. The majority of these ($n = 7$ pairs) consisted of larval pools and ear biopsy, and the minority ($n = 1$ pair) consisted of a nymph and ear biopsy. Conversely, 6 sample pairs (43%) were comprised of different ribotypes, in which the majority ($n = 4$ pairs) consisted of nymphs and ear biopsy, and the minority ($n = 2$ pairs) consisted of larval pools and ear biopsy.

Discussion

Synchronous juvenile I. scapulars phenology: causes and consequences

An understanding of the tick life cycle is a prerequisite for predicting the changing risk of human infection with tick-borne zoonotic pathogens and developing effective tick control methods.

Combining data from questing and on-host ticks at our southwest Michigan site, we describe 2 features of the *I. scapularis* phenology that are markedly different than that seen in the Lyme disease-endemic northeastern United States: (i) larval *I. scapularis* exhibit a single peak of activity in June; and (ii) this larval peak is completely coincident with nymphal activity. The pattern of seasonal synchrony of the juvenile life stages was also described as characteristic of the Midwest in our large-scale standardized drag sampling study of *I. scapularis* across its distributional range (Gatewood et al., 2009). In contrast, the Northeast is typically characterized by bimodal larval activity, in which there is limited larval activity in early summer, followed by peak nymphal activity, which precedes peak larval activity in the late summer/fall by at least one month (Daniels et al., 1996; Fish, 1993; Gatewood et al., 2009; Ostfeld et al., 1996; Yuval and Spielman, 1990).

Some earlier midwestern studies reported similar juvenile activity to the northeastern patterns; for example, in northwestern Illinois, Kitron et al. (1991) and Jones and Kitron (2000) found that the prevalence and intensity of larvae on mice was highest in August, with a smaller peak in early June – mimicking the pattern of drag-sampled larvae from the same area (Siegel et al., 1991). Furthermore, our previous and current research at this Michigan site suggests that at least in some years, there is also larval activity in late July–August (Hamer, Sidge, unpublished data). Thus, we posit that a continuum of phenologies exists not just across geographic space (Gatewood et al., 2009), but also over time within a region due to temporally-variable environmental conditions, as is apparently true for *I. ricinus* in Europe, Asia, and North Africa (Gray, 1991, 2008).

The variation in timing of juvenile *I. scapularis* activity has been attributed to climatic differences in the Midwest versus Northeast, in which the magnitude of the difference between summer and winter daily temperature maximums (greater in the Midwest) was positively correlated with the degree of seasonal synchrony of the 2 immature stages of *I. scapularis* (Gatewood et al., 2009). The rate of spring warming has been identified as an important environmental factor that influences patterns of *I. ricinus* nymphal and larval cofeeding in nature, which is prerequisite for transmission of tick-borne encephalitis virus (Randolph and Sumilo, 2007). The ways in which the climate at our study site, which is buffered by the adjacent Lake Michigan (Eichenlaub et al., 1990), may contribute to the seasonal synchrony of *I. scapularis* ticks requires further investigation.

The efficiency of *B. burgdorferi* maintenance in nature has long been attributed to the sequential feeding of nymphs followed by larvae, allowing for nymphs to infect hosts that later serve as sources of infection to the larvae. In that respect, synchronous activity of the 2 juvenile stages may serve to dampen pathogen maintenance, as some larvae may feed on hosts that have not yet been infected or become systemically infected. A counter-argument, however, is that synchrony may allow opportunity for cofeeding transmission (larval infection due to concurrent feeding on the same host with infected nymphs without the need for systemic host infection), which has not largely been recognized as important for *B. burgdorferi* enzootic maintenance, but has been shown to occur (Ogden et al., 1997). Furthermore, during the time lag between nymphal and larval activity in an asynchronous phenology, hosts undergo natural mortality, and the pool of infected hosts is diluted by the recruitment of uninfected juveniles into the population. Thus, from the pathogen perspective, seasonal synchrony may minimize these otherwise negative effects.

A three-year life cycle for I. scapularis in southwestern Michigan?

The bimodal *I. scapularis* larval activity pattern reported by others (reviewed above) is thought to represent the activity of 2 consecutive cohorts of ticks. The early peak consists of larvae that hatched in the previous year that did not find a host, but successfully overwintered, and the later peak consists of larvae that hatched from eggs laid by adults that were either active in the spring of the current year or fall of the previous year, as oviposition and larval eclosion are synchronized regardless of when adult females feed (Daniels et al., 1996; Yuval and Spielman, 1990). A 2-year life cycle is thought to predominate in the Northeast (Steere et al., 2004), though *I. scapularis* is known to exhibit variable length of life cycles from 2 to 4 years depending on environmental conditions including summer drought, winter temperatures (Daniels et al., 1996; Lindsay et al., 1995, 1998), and host abundance (Yuval and Spielman, 1990).

The source of the single peak of larvae at our study site could be: (1) eggs of females that overwintered as fed adults (see Lindsay et al., 1995; Daniels et al., 1996; Yuval and Spielman, 1990), (2) larvae that hatched in July and August of the previous year followed by a period of quiescence without host seeking until the next year (see Daniels et al., 1996; Yuval and Spielman, 1990), or (3) eggs laid the previous year that diapaused as eggs (see Gray, 1982). The single larval peak is unlikely to arise from eggs laid by adults that fed in the spring of the same year due to insufficient time for larval development. If scenario 1 occurs, then the cues for delaying the peak of larval questing until August in the Northeast are not operating in this study region. If the second and/or third scenarios predominate, then eggs laid in year 0 may yield larvae that quest in June of year 1, nymphs that quest in year 2, and adults that quest in the fall of year 2 or spring of year 3. A consequence of this reasoning is that we now hypothesize that *I. scapularis* in lower Michigan can exhibit a 3-year life cycle (Fig. 3), similar to that which is known for *I. pacificus* in the western United States (Padgett and Lane, 2001). The implications of this for tick population growth and pathogen transmission are currently unknown, and warrant further studies.

Density of infected nymphs, relative abundance of pathogen ribotypes, and implications for disease incidence

At our Van Buren study site, the density of infected nymphs (DIN; the product of the monthly nymphal density estimate and

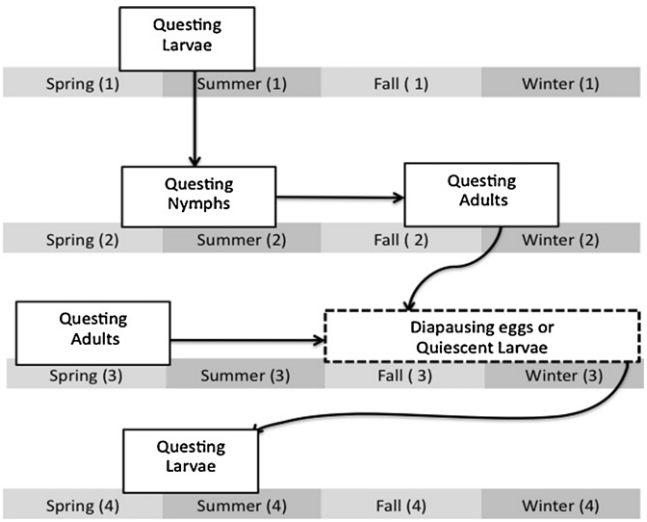

Fig. 3. Hypothesized 3-year life cycle of *I. scapularis*. The 4 seasons of each of 4 sequential years are represented by horizontal shaded bars. The seasonal activity periods of *I. scapularis* life stages observed in the present study are represented by boxes with solid lines, with the hypothesized cohort of ticks traced by the arrows.

the monthly nymphal infection prevalence) – a measure of Lyme disease risk to humans (Falco and Fish, 1989) – was greatest at 4.2 per 1000 m^2 in June and was 1.2 per 1000 m^2 in July. The DIN can alternatively be computed as the product of the mean area under the curve of nymphal activity and the overall nymphal infection prevalence, as described in Diuk-Wasser et al. (in press). Calculated in this manner for the June–August curve of nymphal activity, the mean DIN at our study site was 1.7 per 1000 m^2. In comparison, the mean DIN at 86 sites that occurred in the Lyme disease-endemic reference states (Connecticut, Delaware, Maryland, Massachusetts, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin; Bacon et al., 2007) was 1.5 ± 2.9 (Diuk-Wasser et al., in press). Despite the comparable DINs at our study site and the Lyme disease endemic states, Lyme disease incidence surrounding our study site in 2009 was only 1.4 cases per 100,000 people (Michigan Dept. of Community Health). In contrast, the average annual incidence in the reference states 2003–2005 was 29.2 cases per 100,000 population (Bacon et al., 2007). Thus, the DIN we report in the recently-invaded zone in lower Michigan is similar to that reported in endemic areas across the country, yet reported Lyme disease incidence is much lower.

One of several possible explanations for this discrepancy may relate to increased pathogenicity of RST 1 spirochetes relative to RST 2 and 3 spirochetes (Jones et al., 2006; Wormser et al., 1999), the former of which are relatively more abundant in the northeastern versus midwestern United States (Gatewood et al., 2009). Our data support this hypothesis, as RST 1 spirochetes comprised only 3.4% of our Michigan samples. The relative paucity of RST 1 in the Midwest has been attributed to a phenology difference in which there is an approximate 2-month lag time between peak nymphal and larval activity in the Northeast and near-synchronous (no lag) activity of larvae and nymphs in the Midwest (Gatewood et al., 2009). A selective advantage may therefore be conferred to the spirochetes with greater longevity in hosts (RST 1 strains) in the Northeast where there is a time lag during which spirochetes must survive in the host between nymphal infection of hosts and larval acquisition of infection from hosts (Gatewood et al., 2009). Additionally, there is likely low contact between the local residents and the ticks around our study site because the ticks presently are confined to a narrow band of landscape along the coast with Lake Michigan (Hamer et al., 2010), whereas in the Northeast they are ubiquitous and Lyme disease risk is often peridomestic. Further work is needed to relate pathogen genotype, tick life cycle, and human behavior to epidemiological risk.

We did not find a higher proportion of RST 1 spirochetes in hosts at the end of the transmission season, which we had predicted based on the reported longevity of these spirochetes relative to others (Dolan et al., 2004), though the overall paucity of RST 1 spirochetes would make any such temporal trend challenging to detect. While it has been emphasized that RST 1 strains appear be more long-lived versus certain RST 2 and 3 strains in mice in laboratory experiments, our field study demonstrates that mouse populations at the end of the summer are infected with RST 2 and 3 despite the nymphal peak months earlier. Similarly, at an endemic site in the Northeast, Tsao et al. (2004) found approximately 50% of infected mice harbored RST 2 and 3 spirochetes at the end of summer, a finding that also indicates non-RST 1 strains can be long-lived in natural populations. The larger diversity of strains in RST 2 and 3, relative to RST 1, likely encompasses heterogeneity in persistence in vertebrate hosts and accounts for these findings. Mouse infection prevalence peaked at 57% at the end of the transmission season in September. This infection prevalence is lower than that seen in an endemic field site in the Northeast, which was 76% at the end of summer (Barbour et al., 2009). We suggest that the lower infection prevalence may be a result of the relative paucity of RST 1 strains.

Small mammal host ecology, infestation, and infection

White-footed mice are efficient reservoirs for *B. burgdorferi* and excellent hosts for juvenile *I. scapularis*, so enzootic maintenance of the pathogen should be most efficient when white-footed mice feed the highest proportion of ticks (Fish, 1993). Ostfeld et al. (1996) found that ticks had higher success in attaching to mice at higher densities of mice, likely because higher densities of mice occupy a greater proportion of the landscape, and host-seeking ticks can therefore be more successful in finding a host. In contrast to the Northeast, in our study area, when larvae were at the peak of their host-seeking activity period, mouse densities were at their lowest. Conversely, when mouse densities were at their highest in the late summer/fall, larval host-seeking had waned or ceased. We therefore posit that larval activity starts too early in the season to match the population dynamics of mice, and so the overall feeding success of ticks is reduced relative to other areas where the larval host-seeking period is more congruent with recruitment of vertebrate juveniles.

Sampling of white-footed mice and eastern chipmunks for *I. scapularis* ticks was more sensitive than drag sampling, as during late summer ticks were often found on mammals, but not on drag cloths (comparing Figs. 1 and 2). Given mortality of each sequential life stage, many more larvae than nymphs occur in nature, yet we collected almost identical numbers using drag cloths. In contrast, approximately 3 times as many larvae as nymphs were collected from small mammals. Our data support the notion that drag cloths are relatively less efficient for collection of larvae (as also suggested for *I. ricinus* by Tack et al. (2011) and Gray (1985)), which may also help to explain why larvae were present on hosts until October, in contrast to June only for drag sampling. The relative inefficiency of drag cloths for larval sampling should not affect our assessment of the timing of infestations of mice with larvae and nymphs.

More male than female mice were infested by nymphal *I. scapularis*; this finding predicted our finding of greater infection prevalence in males. In addition, male mice fed more larvae than female mice, and we therefore conclude that male mice are more important for both feeding and infecting *I. scapularis*. The mechanistic basis for this sex-biased parasitism likely relates to a sexual difference in home range (Brei and Fish, 2003). This relationship, however, was not observed in chipmunks in our study.

Our data emphasize the differential importance of mice versus chipmunks for feeding larvae and nymphs, as also found by Schmidt et al. (1999) and Slajchert et al. (1997). We found that mice contribute 40 times more than chipmunks to feeding of larvae, and infected mice feed 27 times more larvae than infected chipmunks. Conversely, chipmunks and mice feed nearly equal numbers of nymphs, and infected chipmunks feed 1.5 times more nymphs than do infected mice, despite the order of magnitude greater densities of mice. Using data from the month of June, we highlight that mice and chipmunks alone feed a maximum of 29% of the density of larvae and 6% of the nymphs in the environment. Given that drag sampling is not completely efficient and that many ticks are in the environment, but not questing, and thus not able to be quantified, our estimates are likely to be markedly below the maximum. While many of the ticks will not successfully find a host, our results do highlight the potentially significant role of alternative hosts in this system, as also suggested by Brisson et al. (2008) who estimated that mice alone fed only 10% of ticks. Not only are alternative hosts important for providing blood meals to ticks, but also for infecting ticks. Based on vaccination trials of mice, Tsao et al. (2004) concluded that non-mouse hosts contributed more to infecting ticks than previously expected.

We observed that over 50% of infected larval pools were removed from hosts that did not at that time have infected ear tissue; yet some of these animals on subsequent capture

events were found to have infected ear tissue. In the absence of transovarial transmission in this system and ignoring potential effects of cofeeding transmission, we conclude that xenodiagnoses of mice provides a more sensitive measure of current infection status than does direct PCR of ear tissue.

The relationships among tick phenology, host population dynamics, and vertebrate community structure are likely to sculpt tick population size, infected tick population size (DIN), and pathogen genetic diversity. Future longitudinal studies that span different geographic regions with variable climates, host communities, and other biotic and abiotic feature will allow us to understand those features of Lyme disease ecology that are generalities versus those that are conditional. Importantly, these studies describe the enzootic components that comprise human risk, such that ecological and medical interventions to protect public health can be better targeted.

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