



Original article

Nymphal *Ixodes scapularis* questing behavior explains geographic variation in Lyme borreliosis risk in the eastern United StatesIsis Arsnoe^{a,b,1}, Jean I. Tsao^{a,b,c}, Graham J. Hickling^{d,*}^a Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI, 48824, USA^b Program in Ecology, Evolutionary Biology, and Behavior, Michigan State University, East Lansing, MI, 48933, USA^c Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, MI, 48933, USA^d Center for Wildlife Health, University of Tennessee Institute of Agriculture, Knoxville, TN, 37996, USA

ARTICLE INFO

Keywords:

Blacklegged tick
Ixodes scapularis
 Host-seeking behavior
 Disease risk
 Lyme borreliosis
 Lyme disease
 Tick-borne disease

ABSTRACT

Most people who contract Lyme borreliosis in the eastern United States (US) acquire infection from the bite of the nymphal life stage of the vector tick *Ixodes scapularis*, which is present in all eastern states. Yet < 5% of Lyme borreliosis cases are reported from outside the north-central and northeastern US. Geographical differences in nymphal questing (i.e., host-seeking behavior) may be epidemiologically important in explaining this latitudinal gradient in Lyme borreliosis incidence. Using field enclosures and a ‘common garden’ experimental design at two field sites, we directly tested this hypothesis by observing above-litter questing of laboratory-raised nymphal *I. scapularis* whose parents were collected from 15 locations (= origins) across the species’ range. Relative to southern nymphs from origins considered to be of low acarologic risk, northern nymphs from high-risk origins were eight times as likely to quest on or above the surface of the leaf litter. This regional variation in vector behavior (specifically, the propensity of southern nymphs to remain under leaf litter) was highly correlated with Lyme borreliosis incidence in nymphs’ counties of origin. We conclude that nymphal host-seeking behavior is a key factor contributing to the low incidence of Lyme borreliosis in southern states. Expansion of northern *I. scapularis* populations could lead to increased incidence in southern states of Lyme borreliosis and other diseases vectored by this tick, if the ‘northern’ host-seeking behavior of immigrant nymphs is retained. Systematic surveillance for *I. scapularis* nymphs questing above the leaf litter in southern states will help predict future geographic change in *I. scapularis*-borne disease risk.

1. Introduction

An estimated 300,000 Lyme borreliosis cases occur annually in the United States (US), where the disease is generally referred to as ‘Lyme disease’ (LD). Yet < 5% of these cases are reported from outside of the north central, northeastern and mid-Atlantic states (Mead, 2015; Schwartz et al., 2017) despite wide distribution of the vector, the blacklegged tick (*Ixodes scapularis*), throughout southeastern and south central states (Eisen et al., 2016a,b). In North America, LD is caused by the bacterium *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* s.s.) and less commonly by *B. mayonii* (Schwartz et al., 2017). It is a multisystem disease, with diagnosis and prophylactic measures guided in part by the likelihood that the patient was bitten by an *Ixodes* tick in a high LD incidence state (Wormser et al., 2006). Northern *I. scapularis* populations are expanding into previously non-endemic areas (Eisen et al., 2016a,b), so understanding how ecological factors contribute to

regional variation in LD incidence will assist in prevention, diagnosis, and prompt treatment of this disease in areas where it is newly emerging and unfamiliar.

Geographic variation in LD incidence in the eastern US has been attributed variously to regional differences in vertebrate host biodiversity (Ostfeld and Keesing, 2000; Schmidt and Ostfeld, 2001; Keesing et al., 2000; Levi et al., 2012), climate (Brownstein et al., 2005; Ogden et al., 2008; Eisen et al., 2016a,b), and biological differences associated with tick population genetics (Humphrey et al., 2010; Van Zee et al., 2015). Differences in how nymphal ticks ‘quest’ for hosts (i.e., their host-seeking behavior) is one such biological difference that may play a key role. Nymphal tick behavior is highly relevant to human disease risk because nymphs are the life stage responsible for the large majority of human LD infections (Falco et al., 2009).

Several lines of evidence from prior ecological studies have led us to suspect that the extent to which *I. scapularis* nymphs quest above the

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leaf litter varies profoundly with latitude. First, while this species is widespread in the South, records of nymphal bites in southern states have been extremely rare (Felz et al., 1996; Stromdahl and Hickling, 2012). Second, in southern locations where researchers observe nymphs parasitizing wildlife, or readily collect questing adults by dragging a white cloth over the vegetation, questing nymphs are rarely collected (Diuk-Wasser et al., 2006; Goddard and Piesman, 2006; Goltz and Goddard, 2013). Third, a LD risk map for the eastern US (Diuk-Wasser et al., 2012) – based on the density of infected nymphs collected by drag cloth (Ginsberg and Ewing, 1989; Mather et al., 1996) – classified areas in the north-central and northeastern US as ‘high risk’, with ‘transitional’ areas of increasing tick numbers and increasing LD risk on their margins. In contrast, very low numbers of questing nymphs were collected throughout the southern US, which consequently was classified as ‘low risk’ despite the known presence of *I. scapularis* in most counties surveyed (Eisen et al., 2016a,b). Fourth, in a pilot study (Arsnøe et al., 2015), we found that nymphs originating from a northern state (Wisconsin) were 12 times more likely than nymphs originating from two southern states (North and South Carolina) to quest above the leaf litter.

We suspect that the paucity of collectable (and human-biting) host-seeking nymphs in southern states is due in large part to their distinctive questing behavior. As a test of this hypothesis, we expanded upon our pilot study to investigate variation in the behavior of nymphal *I. scapularis* whose mothers were collected from 15 states (= ‘origins’) throughout the eastern US (Fig. 1). These states varied in LD risk status as classified by Diuk-Wasser et al. (2012) and in LD incidence as reported to the Centers for Disease Control and Prevention (CDC, 2016a). Specifically, we tested the prediction that a quantitative measure of nymphs’ propensity to quest on or above the leaf litter (which greatly increases their opportunity to attach to passing humans) would correlate with LD risk status, and LD incidence, of the counties from which the nymphs originated.

2. Materials and methods

We raised nymphs from engorged female *I. scapularis* collected throughout the eastern United States. These nymphs were released into outdoor arenas and their questing behavior observed over the spring-summer period corresponding to natural questing of wild nymphs. Observation in arenas removed several confounding factors – such as regional differences in vegetation structure, tick phenology, and removal by hosts – that affect detection of wild nymphs by standard field survey methods.

2.1. Nymphs for experiments

The nymphs used in our experiment originated from engorged female *I. scapularis* collected October–December 2012 from hunter-harvested deer in 15 states (= ‘origins’) across the range of *I. scapularis* in the eastern United States (Fig. 1A). Collection locations were classified as high risk (n = 4), transitional (n = 3), or low risk (n = 8) based on the LD risk map published by Diuk-Wasser et al. (2012). We reclassified one location (Nelson County, Virginia) from low risk to transitional because of an increased abundance of infected nymphs collected at this location (Brinkerhoff et al., 2014) subsequent to the field surveys reported by Diuk-Wasser et al. (2012).

At Michigan State University, the engorged females were allowed to oviposit in individual vials in humidity chambers (21 °C, > 95% relative humidity, 16:8 h light:dark photoperiod). At 1–9 weeks of age, the resulting larvae were fed on laboratory mice (ICR CD-1 *Mus musculus*) and allowed to molt into nymphs. Species identification was confirmed using taxonomic keys (Durden and Keirans, 1996).

2.2. Field arenas

We observed our lab-reared nymphs in field arenas at a northern site in Barry County, Michigan (MI, 42.49 °N) and ~700 km to the south in Anderson County, Tennessee (TN, 36.01 °N). The TN field site was dominated by upland oaks (*Quercus* spp.), hickory (*Carya* spp.) and tulip tree (*Liriodendron tulipifera*). The MI site was dominated by oaks (*Quercus* spp.), maples (*Acer* spp.), and beech (*Fagus* spp.). We confined our observations to two sites because our pilot study (Arsnøe et al., 2015) indicated that testing site had minimal effect on questing differences among nymphs from different origins.

Our experimental design consisted of four blocks of four arenas (n = 16) in MI, and eight blocks of four arenas (n = 30, 2 arenas were not used) in TN. The number of blocks differed between the two field sites because low numbers of nymphs in some clutches limited our ability to replicate arenas for every clutch at both sites. In those cases, replicates were assigned to the TN field site because of its location in the middle of *I. scapularis*’ natural latitudinal range. Blocks were located in uniform habitat, separated from each other by a minimum of 10 m. Each arena (Fig. 2A) contained wooden dowels emerging from natural leaf litter (Fig. 2B), mimicking the twigs and stems upon which northern nymphs commonly quest. Arena construction is described in Arsnøe et al. (2015); briefly, the arenas consisted of 0.5 m circular 25 cm high aluminum flashing sunk 7 cm into the ground, containing leaf litter and 15 bamboo skewers (0.3 cm diameter), five each of emerging to three heights (5 cm, 10 cm, 20 cm) above the leaf litter (Fig. 2B). Any naturally-occurring ticks were removed by heat-treating locally-obtained leaf litter before adding it to each arena. A 6 cm wide band of Tree Tanglefoot Insect Barrier (Ortho, Marysville OH) was applied to the interior top edge of the flashing to prevent nymphs from escaping.

Each block of arenas was surrounded by an outer wire-mesh barrier that excluded wildlife but exposed the nymphs to ambient environmental conditions. Temperature and relative humidity were recorded hourly at both sites using pairs of iButton® data loggers (EDS, Lawrenceburg KY) placed on and above the surface of the leaf litter (0 cm and 10 cm height, respectively). One pair of loggers was placed inside a randomly selected arena, a second pair was placed outside of the same arena.

Forty-five of the arenas each received 40–59 nymphs from a single female from one of the 15 origins. Three arenas received 17, 26 and 28 nymphs from clutches with low larval survival. Having two testing sites enabled us to incorporate within- and between-site replication in our study design; siblings from eight origins were tested in replicate arenas (2 per site) in both MI and TN; these origins, listed in order of decreasing latitude, were Wisconsin (WI), Massachusetts (MA), Michigan (MI), Virginia (VA), North Carolina (NC), Tennessee (TN), South Carolina (SC) and Florida (FL). The remaining seven origins were tested in replicate arenas only in TN due to limited availability of nymphs: Minnesota (MN), Maine (ME), Illinois (IL), Alabama (AL), Georgia (GA), Mississippi (MS) and Texas (TX).

2.3. Nymphal questing observations, recovery, and LD incidence estimates

We released our lab-reared nymphs into the field arenas in May 2013, at 3–6 weeks of age. Their questing behavior was recorded thereafter by observers who were blind to tick origin. Observations were made in the morning (approx. 0800 h) and late afternoon (approx. 1600 h) at weekly or biweekly intervals from May – October 2013; these months encompassed the natural nymphal questing period in both MI and TN. We assume that nymphs that host-seek below the surface of the leaf litter have limited opportunity to attach to a passing human. Therefore, we recorded the number of nymphs visible on the surface of leaf litter (Fig. 2C), on dowels (Fig. 2D), and on arena walls during 2-min observation periods. We then used the sum of these counts to calculate the probability that nymphs were questing in ways that pose

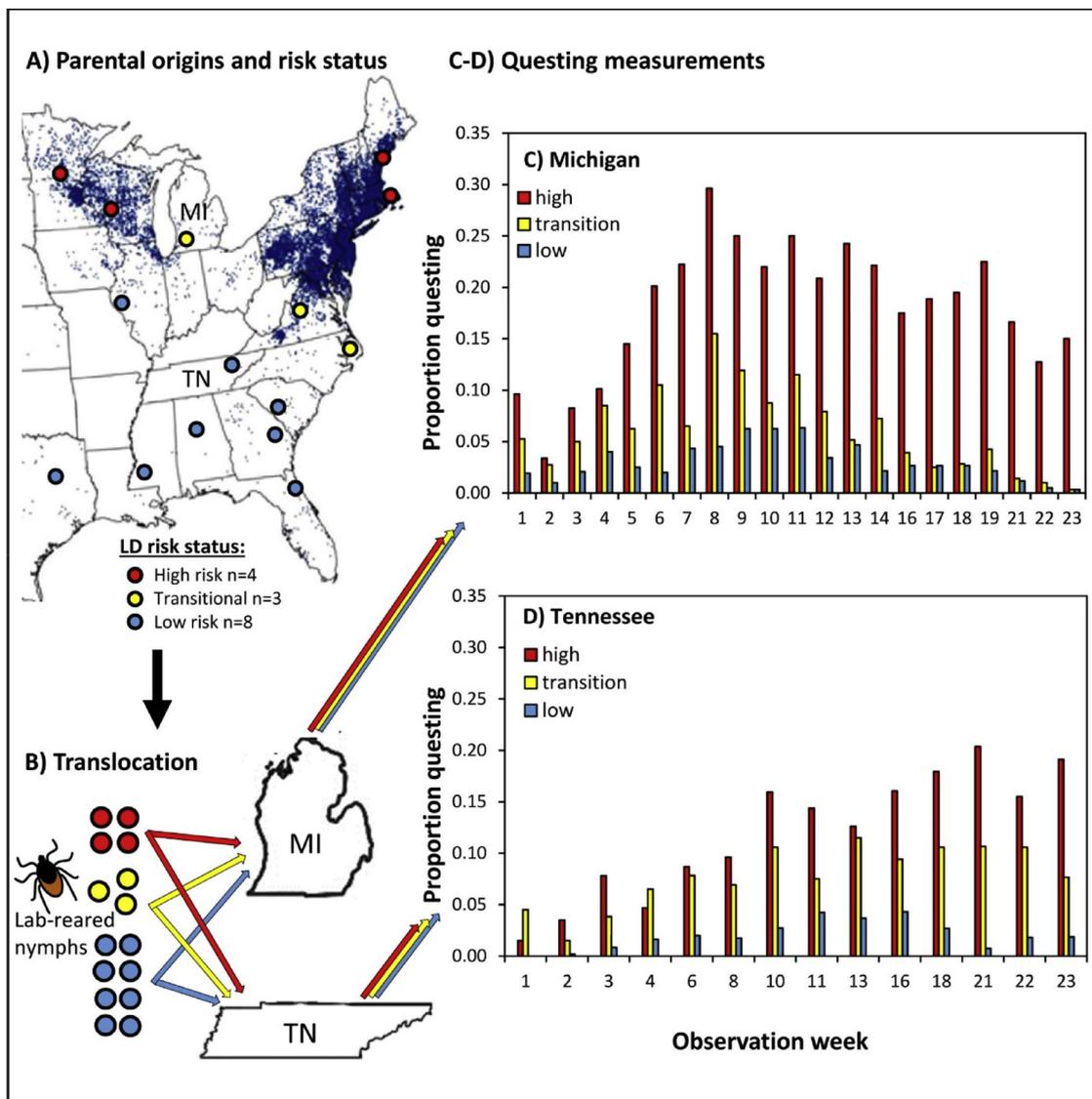


Fig. 1. Diagram of the experiment’s design. A) Engorged female *Ixodes scapularis* were collected from wild deer at 15 ‘origins’ in the eastern US; origins are shown overlain on a map of Lyme disease case locations reported to the Centers for Disease Control in 2012 (CDC, 2016a) and are color-coded by county LD risk status (high, transitional, low) from (Diuk-Wasser et al., 2012). Nelson County VA was re-classified as transitional risk based on Brinkerhoff et al. (2014). B) In May 2013, nymphs raised from one female from each origin were translocated into field arenas in Michigan and Tennessee. C–D) The proportion of nymphs visible on or above the leaf litter in each arena was recorded weekly or biweekly for 23 weeks (May to October 2013); the data are summarized here as mean proportions for each of the three LD risk categories for each observation week at each field site.



Fig. 2. Examples of experimental arenas and questing *Ixodes scapularis* nymphs. (A) Arenas (0.5 m² diameter) were exposed to ambient weather but inaccessible to hosts. (B) Wooden dowels emerging from local leaf litter placed in arenas. (C) Nymphs questing on leaf litter. (D) Nymph questing on a dowel.

an increased risk of pathogen transmission to humans. All arenas were observed during each visit; the MI and TN arenas were assessed 40 and 23 times respectively. Survival of the nymphs was quantified by calculating the proportion of nymphs recovered alive in each arena by

repeated, intensive searches of the litter at the end of the experiment in October 2013.

Human Lyme borreliosis incidence for each county from which nymphs were derived was calculated from a national surveillance

Table 1

Variation in nymphal *Ixodes scapularis* questing probability, and LD incidence in their county of parental origin, ranked by LD risk status and ascending latitude of the county of parental origin. Probability of questing (bolded) was estimated as a function of the proportion of ticks observed questing in field arenas over 23 weeks in Michigan and Tennessee. Standard errors of questing probabilities are shown in parentheses.

State of origin	State abbreviation	County of origin	Latitude (decimal degrees)	LD risk status of county ¹	LD annual incidence ²	Mean probability of questing (SE)
Florida	FL	St. John's	30.02	Low	1.8	0.0155 (0.0105, 0.0229)
Texas	TX	Freestone	31.71	Low	< 0.001	0.0112 (0.0072, 0.0175)
Mississippi	MS	Jefferson	31.74	Low	< 0.001	0.0100 (0.0063, 0.0160)
Georgia	GA	Bulloch	32.42	Low	< 0.001	0.0116 (0.0075, 0.0180)
Alabama	AL	Bibb	33.04	Low	< 0.001	0.0112 (0.0079, 0.0158)
South Carolina	SC	Aiken	33.25	Low	< 0.001	0.0082 (0.0055, 0.0122)
Tennessee	TN	Anderson	35.93	Low	0.9	0.0654 (0.0457, 0.0926)
Illinois	IL	Adams	39.97	Low	< 0.001	0.0087 (0.0055, 0.0137)
North Carolina	NC	Hyde	35.5	Transitional	< 0.001	0.0166 (0.0113, 0.0242)
Virginia	VA	Nelson	37.79	Transitional ³	29.1	0.1154 (0.0822, 0.1596)
Michigan	MI	Cass	41.92	Transitional	1.9	0.0625 (0.0437, 0.0886)
Massachusetts	MA	Plymouth	41.98	High	119.1	0.1294 (0.0926, 0.1779)
Maine	ME	York	43.38	High	106.2	0.1189 (0.0821, 0.1691)
Wisconsin	WI	Monroe	44.01	High	100.3	0.1706 (0.1238, 0.2304)
Minnesota	MN	Morrison	46.09	High	60.5	0.0603 (0.0407, 0.0885)

¹ From Diuk-Wasser et al. (2012).

² Average annual reported incidence per 100,000 for county, 2011–2013 (25, 26); see methods.

³ Reclassified from low risk to transitional based on Brinkerhoff et al. (2014).

dataset of probable and confirmed cases (CDC, 2016b) and US census population estimates (United States Census Bureau, 2013). Reported incidence rates fluctuate, so we calculated a 3-year average (2011–2013; Table A1) to represent each county's annual incidence. LD incidence and risk classification of the 15 origin counties are listed in Table 1.

2.4. Statistical analysis

We created four statistical models to assess: 1) the log-odds of questing by nymphs from the three risk categories (high, transitional, and low risk areas); 2) the log-odds of nymphal survival by risk status; and 3) a two-step model approach to assess the predictive value of questing activity of nymphs from a single origin and the incidence of Lyme borreliosis in that origin. We used a linear mixed modeling approach for 1) and 2) because this type of model accounts for clustered responses in the data (e.g., observations clustered by arenas, arenas clustered by site). For 3), we first used a linear mixed model to estimate the log-odds of questing for each origin ($n = 15$) and then used a linear regression to regress the incidence of Lyme borreliosis from each origin onto these questing probabilities. All log-odds were back-transformed to probabilities for ease of interpretation. To compare the questing behavior among sibling nymphs in different arenas, we utilized a Pearson's product moment correlation.

All statistical analyses were performed using R 3.1.0 (<http://www.r-project.org>). We began by modeling the log-odds of observing nymphs questing as a function of the LD risk region (i.e., low, transitional, and high) from which they were derived. Function *glmer* in the R package *lme4* (Bates et al., 2014) was used to implement a Generalized Linear Mixed Model (GLMM) within which LD risk status was a fixed factor, while state, site, arena, and week were modeled as random factors. Possible effects of relative humidity and survival on the number of nymphs observed questing were assessed by including each in the questing behavior model and examining the fit statistics. Abiotic variables were centered prior to statistical analysis to facilitate interpretation and model fitting. Fit statistics were calculated using the function *r.squaredGLMM* in the R package *MuMIn* (Barton, 2014). This function calculates conditional ($R^2_{(c)}$) and marginal ($R^2_{(m)}$) coefficients of

determination (R^2) for GLMMs (Nakagawa and Schielzeth, 2013); the difference between $R^2_{(c)}$ and $R^2_{(m)}$ describes the explanatory power of the random effects in the model. To assess the change in variance components when a predictor was added, we calculated the proportion change in variance (PCV) between the null (intercept) model and full model fitted for the dataset. Positive PCV values indicate that the addition of a predictor has reduced variance, whereas negative values indicate an increase in variance. The estimated log-odds and their associated standard errors were then back-transformed to probabilities using the function *logistic* in the R package *rethinking* (McElreath, 2014). Model estimates and fit statistics are available in Appendix B.

To assess the importance of nymphal questing behavior as a predictor of human disease risk, we used *glmer* to model the log-odds of observing nymphs questing as a function of the origin from which they were derived. The estimated log-odds and their associated standard errors were then back-transformed to probabilities using the function *logistic* in the R package *rethinking* (McElreath, 2014). These estimates – to which we refer as 'probability of questing' – describe the mean probability of a nymph being visible in an arena during a given 2-min observation period. We then used linear regression to examine the relationship between probability of nymphs questing and the log-transformed estimate of LD incidence in the nymphs' county of origin. We also used *glmer* to analyze the log-odds of recovery of nymphs (survival) from arenas as a function of LD risk status (fixed factor), with arena, site, and nymph origin modeled as random factors for the reasons described above; fit statistics were calculated as above.

To evaluate the influence of shared genetics on questing behavior, we used a Pearson's product moment correlation to compare the proportion questing of sibling groups that we tested at both sites ($n = 8$ between sites MI and TN) and in replicate arenas within each site ($n = 8$ within MI; $n = 15$ within TN).

Descriptive statistics (mean, standard deviation) for abiotic conditions at the sites were calculated from 15,153 iButton® readings obtained (7521 in MI; 7632 in TN). A total of 111 entries were removed due to iButton® malfunction during the course of the study. All removed entries came from the MI site; 5 readings from one ambient (10 cm) RH logger and 106 readings from one leaf litter (0 cm) RH logger.

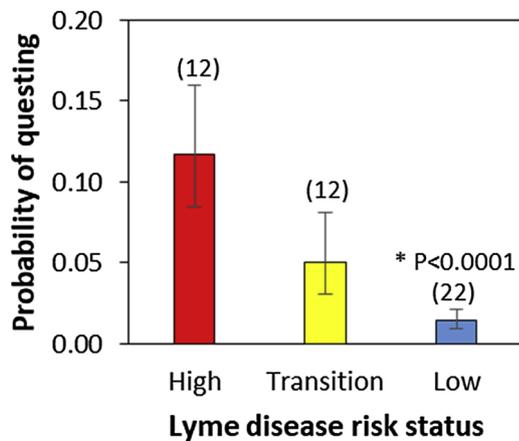


Fig. 3. Relationship between mean probability of nymphal questing (\pm SE) and LD risk status of the county from which the mothers of lab-reared nymphs were collected. Questing probability estimated by observing questing behaviors of nymphs in outdoor arenas in Michigan and Tennessee from early May through mid-October in 2013. LD risk status and bar colors correspond to those of Diuk-Wasser et al.'s (2012) LD risk map. Number of arenas tested for each risk category in parentheses. Statistical significance determined by Wald's test.

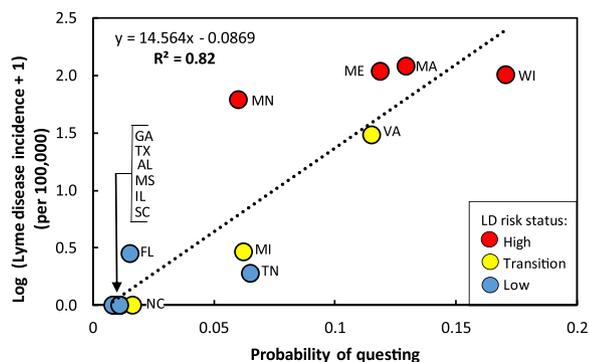


Fig. 4. Relationship between county-level LD incidence and probability of questing for nymphs raised in the lab from engorged females from 15 geographic origins (denoted by state abbreviations) in the eastern US. County LD risk status was assigned based on Diuk-Wasser et al. (2012), with VA reclassified from low to transitional based on Brinkerhoff et al. (2014). Regression equation and line of best fit are shown; $\beta = 14.56$, 95% CI [10.4, 18.7], $R^2 = 0.82$, $P < 0.0001$.

3. Results

Over our 23-week study, the mean probability of observing an individual nymph on or above the leaf litter was low (2.8%, standard error (SE) 2.0%–3.9% in MI arenas; 3.5%, SE 3.0%–4.1% in TN arenas). Questing activity in MI arenas peaked in early summer (Fig. 1C), which is broadly consistent with nymphal phenology recorded in the Northeast and upper Midwest (31, 32). Thereafter activity in MI declined, perhaps reflecting reduced survival as fall approached. In Tennessee arenas, questing peaked mid-summer and showed no consistent trend thereafter (Fig. 1D).

Questing probability, averaged across the 23 weeks of observations, varied markedly by state of origin, ranging from 0.8% for SC nymphs to 17.1% for WI nymphs (Table 1). Nymphs originating from high-risk and transitional counties quested far more frequently than nymphs from low-risk counties (Fig. 3 and Table B1; Wald's $z = -5.3$, $P < 0.0001$). This pattern was evident at both observation sites during every week of

the study (Fig. 1C–D). Ticks originating from transitional counties exhibited a wide range of questing probabilities (from 1.7% for NC ticks to 11.5% for VA ticks; Table 1). Questing behaviors exhibited by siblings from each origin were highly correlated among replicate arenas within sites ($r = 0.92$, $P = 0.001$ for MI; $r = 0.78$, $P = 0.024$ for TN) (Fig. D1) and between sites ($r = 0.93$, $P < 0.001$).

Nymphal survival, measured as the log-odds of recovering nymphs at the end of the experiment, was significantly higher in high-risk arenas than in transitional arenas ($z = -2.9$, $P = 0.004$) or low-risk arenas ($z = -4.2$, $P < 0.0001$) (Table B2; mean probability of recovery for high-risk = 19.9%, SE 14.3%–27.0%; transitional = 5.7%, SE 3.6%–9.0%; low-risk = 4.1%, SE 2.7%–6.1%). Differential survival did not, however, explain the differences in questing probability observed among nymphs from different LD risk regions ($z = -0.75$, $P = 0.46$) (Table B1).

Average abiotic conditions for the two sites are reported in Table C1. Our TN site was hotter and slightly more humid than our MI site, however the surface of the leaf litter remained close to 100% RH at both sites throughout the study. Temperature and ambient RH values were highly collinear; with temperature excluded, RH provided very little explanatory power in the questing model ($R^2_{(m)} = 0.003$, Table B3). Questing tended to decrease slightly when relative humidity was very high, although this effect reached significance only in the transitional arenas (significant interaction of LD risk (transition) * RH; $z = -2.7$, $P = 0.007$).

3.1. Questing behavior as a predictor of LD risk

We assessed the utility of tick questing behavior as a predictor of human LD risk by quantifying the relationship between our nymphal behavior index (probability questing for ticks from each origin) and human LD incidence in the county where parent ticks were collected (Table 1). We observed a strong positive relationship between nymphal questing probability and reported LD incidence in nymphs' county of origin (Fig. 4; $R^2 = 0.82$, $P < 0.0001$).

4. Discussion

Our experiment has documented pronounced geographic differences in nymphal *I. scapularis* questing behavior: in outdoor field arenas, nymphs derived from 'high LD risk' states were 8 times as likely as nymphs derived from 'low LD risk' regions to quest on or above the leaf litter. Furthermore, the probability of nymphal questing was highly positively correlated with LD incidence in the counties from which the nymphs originated. This demonstrates a tight linkage between nymphal questing behavior and geographic variation in measurements of acarological risk (Diuk-Wasser et al., 2012) that are predictive of LD incidence (Pepin et al., 2012). Taken together, these findings support the hypothesis that very low LD incidence in the southeastern US is largely a consequence of geographic differences in the host-seeking behavior of nymphal blacklegged ticks. These results are consistent with, and expand upon, our pilot study (Arsnoe et al., 2015), which investigated nymphs from just three origins (Wisconsin, North Carolina and South Carolina) over a shorter observation period (19 weeks). The broad geographic representation of nymphal origins investigated here increases our confidence that the link we have identified between questing behavior and LD risk can be generalized to *I. scapularis* populations throughout the geographic range of this species.

Regardless of origin, the *I. scapularis* nymphs in our study spent the majority of their time below the surface of the leaf litter; the overall probability of above-litter questing in our arenas was only 2.8%. This finding is consistent with the behavior of nymphs in our pilot study

(Arsnoe et al., 2015) and with previous mark-recapture and behavior studies undertaken in the Northeast (e.g., Falco et al., 1999; Daniels et al., 2000). Remaining within the leaf litter will help nymphs avoid desiccation and may increase their success in attaching to hosts moving within litter. That this behavior is more pronounced among nymphs from the South than from the North may reflect adaptation to hotter, more desiccating environmental conditions in southern habitats (Ginsberg et al., 2014). Since *I. scapularis* nymphs readily feed on several fossorial lizard species (Apperson et al., 1993; Durden et al., 2002), there also may be greater host availability within the leaf litter in the South than in the North.

A limitation of our study is that, for logistical reasons, our experimental nymphs were all derived from larvae fed on mice rather than skinks, which are an important host for southern *I. scapularis*. Feeding success, engorgement weights, and molting success of larvae not differ significantly between larvae derived from mothers collected in the north versus south (pers. obs.); nevertheless, we cannot discount the possibility that southern larvae fed on skinks might have produced nymphs that behaved differently from the ones we observed. It is also possible that nymphs infected with *B. burgdorferi* s.s. would have exhibited questing behavior different from that of our uninfected nymphs; release of infected nymphs would not however have been permitted at either of our field sites.

There was a modest trend for more per capita questing in arenas containing more nymphs ($R^2 = 0.15$). We cannot discount the possibility that this trend resulted from a 'crowding' effect, however the more likely explanation is that under laboratory condition larval clutches from southern origins had slightly lower survival than northern larvae (Ginsberg et al., 2014). Consequently, we had fewer nymphs available to stock some southern-origin arenas.

A further limitation of our study was that observations were made at only two field sites that differed somewhat in vegetation type and other environmental conditions. It was also not feasible to hold constant stimuli from passing wildlife hosts that might alter the nymphs' behavior. Questing patterns in TN and MI arenas were nevertheless broadly similar (compare Fig. 1C and D), which reiterates the finding of our pilot study that questing patterns of translocated nymphs were similar at four widely-separated observation sites (WI, RI, TN, and FL) (Arsnoe et al., 2015). Our TN field-site was hotter on average than our MI site, but also more humid (Table C1); unexpectedly, we found a small, but statistically significant, negative relationship between relative humidity and questing probability. Our highest RH measurements corresponded to periods with recent rainfall, so we speculate that nymphs may reduce their above-litter questing while it is raining.

It was infeasible to track the survival of individual nymphs during the course of the experiment; a single survival estimate was obtained at the end of the experiment. More nymphs were recovered from high-risk arenas than from transitional and low-risk arenas, which is consistent with the suggestion that northern nymphs have a modest survival advantage over southern nymphs (Ginsberg et al., 2014). Nevertheless, variation in survival was not a significant predictor of the proportion of nymphs seen questing in arenas. Variation in behavior, rather than survival, is the important attribute underlying differences in questing probability by origin. Nevertheless, from the perspective of evaluating LD risk, these two attributes act synergistically – if high-questing nymphs also survive for longer periods, this will amplify the difference in LD risk they pose relative to low-questing nymphs.

In the Northeast and upper Midwest, questing activity of nymphs typically peaks in early summer and declines rapidly thereafter (Fish, 1995; Gatewood et al., 2009). Nymphal phenology is less well described in the South, but collections of southern nymphs from trapped wildlife suggest their seasonal activity peak is broadly similar to that in the

North (Kollars et al., 1999; Goltz and Goddard, 2013). In our arenas, the increase in questing over the first several weeks of the study was similar to that seen in the wild, however questing levels thereafter remained relatively high until the experiment terminated in October (Fig. 1C–D). This suggests that the typical mid-summer decline in drag-cloth counts of wild nymphs is driven in part by their removal from vegetation by hosts. The extended period of questing seen in our arenas is consistent with observations made in Canada, where *I. scapularis* nymphs protected from hosts in outdoor mesocosms remained active from April until November (Lindsay et al., 1995).

Our experimental design allowed us to begin investigating genetic vs. environmental contributions to nymphal questing behavior, as all the nymphs from each origin were siblings derived from a single clutch of eggs. Highly correlated questing behavior was observed between replicate sibling groups within each site; and the behaviors of the 8 sibling groups split between MI and TN were also highly correlated (Fig. D1). Since all nymphs were raised simultaneously under the same laboratory conditions, this implies a genetic component to the observed variation in questing behavior. Future molecular analysis using diverse nuclear markers could provide insight into the genetic basis for the variation in behavior that we observed (Van Zee et al., 2013).

Our study was motivated by the prospect that a better understanding of tick questing behavior could help explain current, and perhaps future, regional variation in human LD risk. Past studies have found that local *I. scapularis* abundance and *B. burgdorferi* s.s. infection prevalence both correlate positively with LD risk (Mather et al., 1996; Pepin et al., 2012). Present *I. scapularis* densities in southern states are typically around one-tenth of those reported in the Northeast (e.g., Stromdahl and Hickling, 2012; Goddard and Goddard, 2008). There are no published records of the prevalence of infection in questing southern nymphs (in part due to the difficulty in collecting them), but for comparison, adult tick infection prevalence is typically < 5% in southern versus > 40% in northern states (Mead, 2015; Piesman et al., 1999). These differences in tick abundance and infection prevalence tend to intensify human LD risk in northern regions and to abate risk in southern regions. It is important to recognize, however, that these two factors are important only if nymphs bite people, which at the present time is rare in southern states (Felz et al., 1996; Stromdahl and Hickling, 2012).

The propensity of southern nymphs to remain nearly exclusively below the surface of the leaf litter provides a compelling explanation for the low reported incidence of locally acquired LD in southern states. As noted in the Introduction and above, multiple factors have been proposed to help explain the latitudinal gradient Lyme incidence. Our experimental findings highlight the significance of geographic variation in nymphal questing behavior, leading us to conclude that the importance of these behavioral differences has been under-appreciated. Conversely, explanations focusing on low infection prevalence in southern *I. scapularis* may have been over-emphasized.

Looking to the future, use of incompetent hosts by southern nymphs will reduce the force of transmission of *B. burgdorferi* s.s. In the North, immature *I. scapularis* often feed on abundant small mammals that are efficient reservoirs for *B. burgdorferi* s.s., whereas in the South, immatures feed primarily on abundant skink species (e.g., Apperson et al., 1993; Kollars et al., 1999) that are poorly competent for *B. burgdorferi* s.s. (Levin et al., 1996). It is possible that under-litter questing is primarily a tactic for southern nymphs to acquire skink hosts. If so, this implies a causal link between under-litter questing and low *B. burgdorferi* s.s. prevalence in southern nymphs. There are, however, plausible alternative explanations for under-litter questing in the South (e.g., desiccation avoidance), so the precise nature of the linkage between nymphal questing and *B. burgdorferi* s.s. transmission and

prevalence remains uncertain.

As northern *I. scapularis* populations expand southwards, changes in abiotic factors and host species availability may alter the questing behavior of these immigrants, mediated by phenotypic plasticity and/or natural selection. For example, when given the opportunity, northern *I. scapularis* immatures readily feed on skinks in the lab and in the field (Giery and Ostfeld, 2007). This flexibility in host selection raises the possibility that questing behavior of northern immigrant ticks could become more similar to that of southern nymphs. Furthermore, northern and southern *I. scapularis* populations can interbreed (Oliver et al., 1993), which could produce hybrid offspring with questing behaviors different from either of their parents.

5. Conclusions

Assessing regional variation in tick-borne disease risk is an ongoing challenge because climate, habitat, and land use are continually changing, and tick populations continue to expand. At present, public health efforts in southern regions primarily target tick-borne pathogens transmitted by the American dog tick (*Dermacentor variabilis*), lone star tick (*Amblyomma americanum*), and Gulf Coast tick (*A. maculatum*). We are concerned that LD incidence could increase in southeastern states as northern, human-biting populations of *I. scapularis* expand their range, as appears to have happened in western Virginia over the past decade (Brinkerhoff et al., 2014). Improved understanding of tick behavior in transitional zones should therefore be of high priority for public health officials seeking to predict future patterns of disease risk. We observed wide variation in questing behavior of nymphs in transitional zones (questing probabilities ranged from 1.7% for NC nymphs to 11.5% for VA nymphs). Two of the three transitional origins tested in this study (MI and VA) are at the edge of expanding northern *I. scapularis* populations (Hamer et al., 2010; Kelly et al., 2014); the third (NC) is near the northern limit of a southern population (Qiu et al., 2002). Interestingly, one of our low-risk groups (TN) exhibited elevated questing probability (6.5%) relative to other low-risk groups (all < 1.6%). Since our experiment, *I. scapularis* populations infected with *B. burgdorferi* s.s. have been detected in four eastern TN counties (Hickling et al., 2018); we speculate that the eastern part of that state has become a transition zone

and that the engorged TN female used in this study may have been a northern or north-south hybrid biotype originating from nearby high-questing populations in southwestern VA.

The behavioral pattern evident from our results may similarly help to explain north-south gradients in LD incidence and questing activity of *I. pacificus* nymphs in California (Lane et al., 2013). In addition to *B. burgdorferi* s.s., *I. scapularis* and *I. pacificus* are responsible for transmitting at least six other etiological agents of known human diseases that have distinctly northern distributions (Eisen et al., 2017). Although our analyses here have focused on *I. scapularis* and LD, our findings may also help to explain the latitudinal distributions of these other diseases. Expansion of northern *I. scapularis* populations could lead to increased incidence in southern states of LD and other diseases, if the ‘northern’ host-seeking behavior of immigrant nymphs is retained. Systematic surveillance for *I. scapularis* nymphs questing above the leaf litter in southern states is recommended to help predict future geographic change in *I. scapularis*-borne disease risk.

Ethical statement

All use of animals was approved by Michigan State University IACUC 06-12-103-0. Inter-state transport and containment of ticks was approved by USDA Veterinary Permits for Importation 121368 and 124136.

Acknowledgements

We thank members of the Lyme Gradient Consortium for their participation, the many individuals who provided female ticks, and Howard Ginsberg and Nicholas Ogden for helpful critiques of earlier versions of this manuscript. We acknowledge the University of Tennessee’s Forest Resources AgResearch and Education Center and the W.K. Kellogg Biological Station for access to our field sites. This research was supported by the National Science Foundation [grant numbers EEID EF-0914476 and DDIG 1405840], Michigan State University’s College of Agriculture and Natural Resources, and the Hal and Jean Glassen Foundation.

Appendix A. County Lyme borreliosis incidence estimates

Table A1

Reported Lyme borreliosis cases (confirmed and probable), county population, and county Lyme borreliosis incidence estimates. Number of LD cases for each county from 2011 to 2013 obtained from a national surveillance database (5). County population estimates obtained from US census estimates (6). Estimated county incidence was calculated per 100,000 individuals by dividing the mean LD cases in the county of origin for 2011–2013 by the county population estimate for 2011–2013 and multiplying by 10⁵.

Origin	County	Cases 2011	Cases 2012	Cases 2013	LD Cases 2011-2013 (mean)	Population 2011	Population 2012	Population 2013	Population 2011-2013 (mean)	est. county incidence
AL	Bibb	0	0	0	0.00	22,770	22,662	22,512	22,648	0.00
FL	St. John's	3	4	4	3.67	196,052	202,328	209,647	202,676	1.81
GA	Bulloch	0	0	0	0.00	72,780	72,737	71,214	72,244	0.00
IL	Adams	0	0	0	0.00	67,183	67,243	67,130	67,185	0.00
MA	Plymouth	269	691	825	595.00	498,039	498,393	501,915	499,449	119.13
ME	York	198	217	219	211.33	198,239	199,041	199,431	198,904	106.25
MI	Cass	2	1	0	1.00	52,474	52,117	51,910	52,167	1.92
MN	Morrison	24	13	23	20.00	33,230	33,102	32,872	33,068	60.48
MS	Jefferson	0	0	0	0.00	7,603	7,659	7,629	7,630	0.00
NC	Hyde	0	0	0	0.00	5,822	5,739	5,721	5,761	0.00
SC	Aiken	0	0	0	0.00	161,978	163,299	164,176	163,151	0.00
TN	Anderson	0	2	0	0.67	75,195	75,401	75,542	75,379	0.88
TX	Freestone	0	0	0	0.00	19,607	19,483	19,646	19,579	0.00
VA	Nelson	5	3	5	4.33	15,026	14,813	14,789	14,876	29.13
WI	Monroe	76	30	30	45.33	45,154	45,123	45,298	45,192	100.31

Appendix B. Model summaries

Table B1

Model summaries for the effects of LD risk status (high, transitional, low), survival (% recovered), and survival x LD risk interaction on probability of questing of nymphs originating from mothers collected from 15 locations across the eastern US. Intercept and coefficients are listed for the effect of Lyme borreliosis risk status on log-odds of observing questing nymphs in arenas (main research question); $N_{[site]} = 2$, $N_{[week]} = 21$, $N_{[arena]} = 46$, $N_{[origin]} = 15$. Asterisks indicate significant difference from intercept at $P \leq 0.05$, Statistical significance determined by Wald's test. CI, confidence interval; inxn, interaction; VC, variance component; PCV, proportion change in variance; $R^2_{(m)}$, marginal R^2 (quantifies the explanatory power for fixed effects); $R^2_{(c)}$, conditional R^2 (quantifies the explanatory power of the fixed and random effects of the model).

Questing Models				
	Null Model	LD risk	Survival x LD risk	Survival
Fixed effects	[95%CI]	[95%CI]	[95%CI]	[95%CI]
Intercept ¹	-3.41 [-4.06,-2.76]	-2.02 [-2.72,-1.32]	-1.87 [-2.66,-1.08]	-3.52 [-4.17,-2.87]
LD risk (low)	-	-2.23 [-3.05,-1.42]*	-2.49 [-3.43,-1.55]*	-
LD risk (trans)	-	-0.92 [-1.92, -0.08]	-1.32 [-2.41,-0.23]*	-
Survival	-	-	-0.01 [-0.02, 0.01]	0.01 [-0.00, 0.02]
LD risk (low) inxn	-	-	0.02 [-0.01, 0.04]	-
LD risk (trans) inxn	-	-	0.04 [-0.01, 0.07]*	-
Random effects	VC	VC	VC	VC
Site	0.010	0.003	0.000	0.000
Week	0.220	0.219	0.219	0.221
Arena	0.207	0.205	0.159	0.205
Origin	1.299	0.377	0.395	1.238
PCV [Site]	-	0.670	1.000	1.000
PCV [Week]	-	0.008	0.006	0.000
PCV [Arena]	-	0.009	0.231	0.012
PCV [Origin]	-	0.709	0.696	0.047
$R^2_{(m)}$	-	17.6%	18.8%	0.3%
$R^2_{(c)}$	-	33.8%	34.2%	33.8%

¹ The intercept represents 'high' Lyme risk status. 95% CI was estimated by assuming infinite degrees of freedom ($t = 1.96$).

Table B2

Model summaries for the effect of LD risk status on log-odds of recovery of nymphs from arenas vs. null (intercept) model; $N_{[site]} = 2$, $N_{[arena]} = 46$, $N_{[state]} = 15$. Asterisk indicates significance at $P \leq 0.01$. CI, confidence interval; VC, variance component; PCV, proportion change in variance, AIC, Akaike Information Criterion; BIC Bayesian Information Criterion. AIC and BIC values estimated using Maximum Likelihood.

	Survival Models	
	Null Model	LD risk model
Fixed effects	b [95%CI]	b [95%CI]
Intercept ¹	-2.64 [-3.43,-1.86]	-1.39 [-2.18,-0.61]
LD risk (trans)	-	-1.41 [-2.36,-0.45]*
LD risk (low)	-	-1.76 [-2.59,-0.94]*
Random effects	VC	VC
Site	0.134	0.105
Arena	0.517	0.531
State	0.921	0.178
PCV [Site]	-	0.217
PCV [Arena]	-	-0.028
PCV [State]	-	0.806
$R^2_{(m)}$	-	11.8%
$R^2_{(c)}$	-	29.3%
AIC	257	249
BIC	264	260

¹ In LD risk model, the intercept represents “high” Lyme risk status. 95% CI was estimated by assuming an infinite degree of freedom ($t = 1.96$).

Table B3

Model summaries for the effects of LD risk status, ambient relative humidity (RH10, centered at the mean), and RH10 x LD Risk interaction on probability of questing of nymphs originating from mothers collected in 15 locations across the eastern US Intercept and coefficients listed for modeling effect of Lyme borreliosis risk status on log-odds of questing of nymphs in arenas (main research question); N[site] = 2, N[week] = 21, N [arena] = 46, N[state] = 15. Asterisk indicates significant difference from intercept at $P \leq 0.05$, statistical significance determined by Wald’s test. CI, confidence interval; VC, variance component; PCV, proportion change in variance; $R^2(m)$, marginal R^2 (quantifies the explanatory power for fixed effects); $R^2(c)$, conditional R^2 (quantifies the explanatory power of the fixed and random effects of the model).

Questing Models			
	Null Model	RH10 x LD risk	RH10
Fixed effects	[95%CI]	[95%CI]	[95%CI]
Intercept ¹	-3.408 [-4.059,-2.756]	-2.052 [-2.801,-1.302]	-3.424 [-4.119,-2.729]
LD risk (low)	-	-2.220 [-3.055,-1.385]*	-
LD risk (trans)	-	-0.898 [-1.916, 0.121]	-
RH10	-	-0.005[-0.007,-0.002]*	-0.007 [-0.009,-0.005]*
LD risk (low) inxn	-	-0.003 [-0.009, 0.002]	-
LD risk (trans) inxn	-	-0.006 [-0.0101,-0.002]*	-
Random effects	VC	VC	VC
Site	0.010	0.027	0.035
Week	0.220	0.252	0.253
Arena	0.207	0.213	0.207
State	1.299	0.390	1.319
PCV [Site]	-	-1.868	-2.680
PCV [Week]	-	-0.142	-0.149
PCV [Arena]	-	-0.030	-0.001
PCV [State]	-	0.699	-0.016
$R^2(m)$	-	17.9%	0.3%
$R^2(c)$	-	35.2%	35.8%

¹ In modes with LD Risk as interaction term, the intercept represents “high” Lyme risk status. 95% CI was estimated by assuming infinite degrees of freedom (t = 1.96).

Appendix C. Environmental conditions at the study sites

Table C1

Environmental conditions at the Michigan and Tennessee field sites in 2013. Mean temperature and relative humidity (SD in parentheses) at leaf litter level (0 cm) or above leaf litter level (10 cm) at each field site from May 10, 2013 through October 16, 2013. Readings were collected at hourly intervals and pooled for two loggers placed at each level at each site.

Field site location	Latitude (°N)	Months sampled	Temp (°C) 0 cm	Temp (°C) 10 cm	Relative humidity (%) 0 cm	Relative humidity (%) 10 cm
Barry County, MI	42.49	May-Oct.	17.5 (3.53)	18.4 (5.23)	99.5 (8.87)	86.4 (15.4)
Anderson County, TN	36.01	May-Oct.	20.1 (3.29)	20.3 (3.76)	98.9 (6.93)	96.1 (9.49)

Appendix D. Nymphal questing in sibling arenas

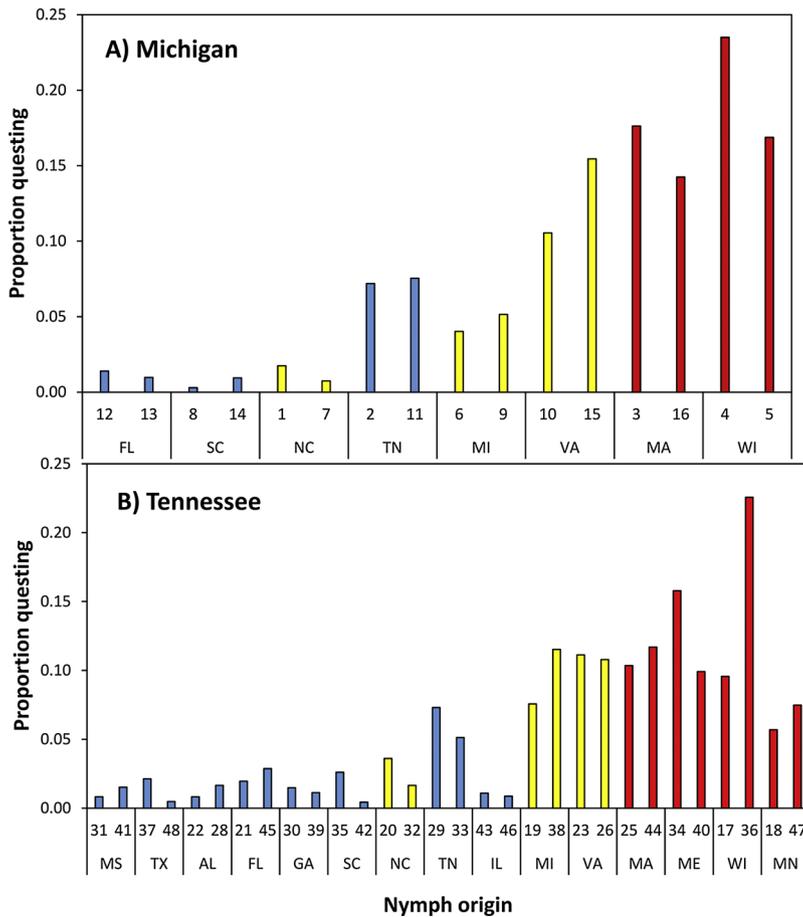


Fig. D1. Comparison of nymphal questing in sibling arenas at each site. Mean proportion of questing nymphs observed in arenas at MI (top) and TN (bottom) sites in 2013. Nymphs from each origin (= state abbreviation) were placed in replicate at each site (MI and TN). Numbers on x-axis indicate anonymous arena IDs for sibling arenas during the study. Red bars represent nymphs classified as ‘high risk’, yellow bars ‘transitional’, and blue bars ‘low risk’ as per the LD risk map from Diuk-Wasser et al. (2012). Pearson’s product-moment correlation yielded significant positive correlations for replicate arenas within sites ($r = 0.92$, $P = 0.001$ for MI; $r = 0.78$, $P = 0.024$ for TN) and between sites ($r = 0.93$, $P < 0.001$).

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