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Questing Behavior and Analysis of Tick-Borne Bacteria in *Ixodes scapularis* (Acari: Ixodidae) in Oklahoma

Trisha R. Dubie,[1] Justin Turner, and Bruce H. Noden

Department of Entomology and Plant Pathology, Oklahoma State University, 127 Noble Research Center, Stillwater, OK 74078 and [1]Corresponding author, e-mail: trishadubie@gmail.com

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Abstract

The blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), is an economically important tick that affects veterinary and public health, but it can be difficult to collect in Oklahoma. The primary goal of this research was to examine the diel activity of each species to help improve collection methods for future field research and test field-collected *I. scapularis* for endemic and nonendemic tick-borne bacterial genera in the southern Great Plains region. Questing behavior was observed using caged bioassays over 24-h periods throughout fall and spring, and field collections were conducted throughout the afternoon and evening in different locations across Oklahoma. Blacklegged ticks were found to be more active during late afternoon and evening hours, and more ticks were recovered in pastures in the evening. None of the pools of adult *I. scapularis* tested positive for *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae) or *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae) DNA. Of the 46 pools of *I. scapularis* tested, 27 (58.7%) were positive for *Rickettsia* sp. with ticks collected from the same location infected with the same species of rickettsial endosymbionts. Results suggest that sampling times later in the day may benefit off-host recovery of *I. scapularis* in Oklahoma ecosystems.

Key words: blacklegged tick, Lyme disease, *Rickettsia*, diel pattern, host-seeking behavior

The biology, ecology, and phenology of the blacklegged tick, *Ixodes scapularis* Say (Acari: Ixodidae), in Oklahoma need further exploration. While information regarding the response of this species to specific climate variables and habitat structure has improved in other regions of the United States, conditions in Oklahoma could impact them differently. Immature and adult stages are difficult to collect off-host, even throughout traditionally active months of the year and in areas where they are known to be established. Although *I. scapularis* is much more common in the northeastern United States, collecting at certain times of day to avoid sampling bias has also been suggested for northern populations as they have been shown to be more active in the early morning and evening hours in this region (Carroll et al. 1998, Schulze et al. 2001, Madden and Madden 2005, Lee and Paskewitz 2015). One study conducted in the southeastern United States also reported greater blacklegged tick activity during evening hours (Durden et al. 1996), but this has not been replicated across the region with southern populations of *I. scapularis*. A more targeted approach for field collection may be a necessity in Oklahoma and other areas of the southern Great Plains where *I. scapularis* are more difficult to sample off-host.

Lyme disease is one of the most prevalent vector-borne diseases in temperate holarctic regions, and in the United States, it is associated primarily with the blacklegged tick (Stanek et al. 2012, Burtis et al. 2016, Gray et al. 2016). Most cases of Lyme disease (95%) are reported from northern and northeastern states (CDC 2018). Consequently, considerable research has been dedicated to *I. scapularis* in those regions, but research on this important species in the southern United States and Great Plains remains limited (Goddard 1992, Goltz and Goddard 2012). In Oklahoma, Lyme disease is less significant than other endemic tick-borne diseases, such as Spotted Fever group rickettsiosis and ehrlichiosis (Biggs et al. 2016). However, *Borrelia burgdorferi* has previously been isolated from *I. scapularis* (Kocan et al. 1992), and several cases of Lyme disease have been reported (18 reported cases, CDC 2018) despite the presumed low vectorial capacity of blacklegged ticks in the region (Oliver 1996, Bacon et al. 2008, Garvin et al. 2015). Recent reports in the Oklahoma media indicate a notion of rise in Lyme disease and granulocytic anaplasmosis cases by the general population but with limited published evidence (Dahlgren et al. 2015, Schwartz et al. 2017). The last survey to molecularly examine *I. scapularis* ticks collected in Oklahoma for *B. burgdorferi* DNA was in 1992 (Kocan et al. 1992). While serological tests indicate a low probability for exposure to these pathogens in the state, it is important to examine field-collected adult ticks for potential pathogens. Therefore, the aims of the study were to determine the diurnal and nocturnal questing habits of adult blacklegged ticks in Oklahoma and test field-collected samples for tick-borne bacterial genera.

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Materials and Methods

Bioassays

Observations of adult *I. scapularis* activity throughout 24-h intervals were conducted using black mesh cages set in an outdoor area throughout the fall, winter, and spring seasons in Central Oklahoma. Bioassays were conducted on four separate occasions during months when *I. scapularis* is traditionally active. Blacklegged tick assays were performed in October and November to cover the adult peak activity period, and in the months of February and March to cover the small activity period known to occur in southern *I. scapularis* populations (Kollars et al. 1999, Goltz and Goddard 2012). Assay dates were chosen randomly, with varying ambient conditions. Cylindrical mesh cages approximately 33 × 43 cm (wide by tall; Bioquip, Rancho Dominguez, CA) were set up along a tree line in a partially shaded area with moderately dense vegetation. Each cage contained a mixture of wood chips and sawdust covering the bottom portion of the cage, and leaf litter was added on top for a total depth of approximately 2 cm. Clumps of taller vegetation were added to provide substrate for the ticks to climb.

Adult *I. scapularis* were obtained from Oklahoma State University's Tick Rearing Facility 3 d prior to each assay (n = 30). The ticks were approximately 3-mo old and unfed. Colonies are supplemented with wild, native ticks on an annual basis. Each tick was marked with one of five different colors of fluorescent powder (Day-glo ECO pigments, Day-glo Color Corp., Cleveland, OH) and returned to their respective plastic containers to rest. These markers have been previously evaluated in the laboratory (Pike 2016). After a period of 24 h, one tick of each color for each sex was placed in each of six mesh cages. Each cage contained a total of five ticks, and males and females were kept in separate cages (three cages with males, n = 15, and three cages with females, n = 15). Temperature and humidity were also recorded using HOBO U23 Pro v2 data loggers placed at ground level and at approximately 43 cm high near the assay arena (U23-002, Onset Computer Corp., Bourne, MA). These data were used to calculate a saturation deficit value for each hourly interval using methods adapted from Randolph and Storey (1999).

Ticks were allowed to acclimate to the outdoor observation area for a period of 18–24 h before beginning each round of observations. Ticks were introduced to the arena and observations were initiated at varying times for each replicate to eliminate the timing of these procedures as an artifact affecting the measured response. Tick activity and location within the cage were monitored every hour for 24 h. Individuals that were moving along the cage or vegetation, positioned with forelegs extended, were considered active. Ticks that were observed crawling or sitting on the litter surface without forelegs extended or unable to be seen were considered inactive. A handheld UV lamp was used to detect marked ticks during dark hours. After completion of each assay, ticks were removed from the cages and disposed of in 70% ethanol.

Field Collections

Native *I. scapularis* were collected from field sites in central Oklahoma where the species has previously been recovered. These included two areas in Payne Co., one area in Logan Co., and one area in Rogers Co. Flagging (36 × 27 in flannel flags attached to poles from primarily wooded areas at each site) took place a total of seven separate occasions throughout the months of October and November, 2016 by two of the authors. Flagging was performed for 30 min, every 2 h between 12:00 and 20:00 h on each occasion. Flagging after sunset was aided by LED head lamps (Dorcy International, Columbus, OH). All specimens recovered were preserved in 70% ethanol and subsequently identified in the laboratory.

Molecular Testing

Pools of adult field-collected *I. scapularis* were tested for *B. burgdorferi*, *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Ehrlichia Ewingii*, and *Rickettsia* spp. DNA using modified PCR protocols (Mays et al. 2014, Salazar 2015, Noden et al. 2017). Ticks from the same date and location were grouped into pools of one to five ticks. To limit DNA contamination, all tick DNA extractions were conducted using site-specific reagents and equipment in a biosafety cabinet in a different laboratory from where the PCR assays were run. Individual adult *I. scapularis* ticks were washed in deionized water and 70% ethanol and then bisected with one half used for DNA extraction and the other half stored at −80°C. After bisection, adult ticks were grouped into pools of one to five ticks in 2-mL vials (Sarstedt) (Biospec, Bartlesville, OK) containing 100 µL of DNAzol Direct (Molecular Research Center, Cincinnati, OH) sample processing reagent based on date and location of collection. After heating at 80–90°C for 15 min, zirconia/silica beads (BioSpec Products) were added, and the vials were placed in a Mini-Beadbeater-16 (BioSpec Products) for 3 min. After bead-beating, resulting supernatant was collected and stored at −20°C until DNA testing. Pooled samples of *I. scapularis* were screened by endpoint PCR for the presence of *B. burgdorferi* using ospA-specific primers (ospA2/ospA4; Scott et al. 2012) and *Rickettsia* sp. using specific primers for citrate synthase (*gltA*; CS-78/CS-323; Labruna et al. 2004) and 190 kDA protein antigen (*ompA*; Regnery et al. 1991). Nested PCR assays were used to test for the presence of *A. phagocytophilum* (16S rDNA: outer primers [GE3a/GE10R], inner primers [GE9F/GE2; Chen et al. 1994, Massung et al. 1998]), and *E. chaffeensis* and *E. ewingii* (16S rDNA: outer primers [ECC/ECB], inner primers [*E. chaffeensis* (HE3/HE1) and *E. ewingii* (HE3/EE72)]; Heise et al. 2010) as well as a nested PCR assay that simultaneously detects *Ehrlichia* and *Anaplasma* species targeting the *groEL* gene (Tabara et al. 2007, Takano et al. 2009). All pools were intially screened using all five of the assays described, and all *Rickettsia gltA*-positive amplicons were bidirectionally sequenced at the Oklahoma State University Core Facility. Each resulting sequence was verified using BioEdit (Ibis Therapeutics, Carlsbad, CA), aligned to create consensus sequences using Clustal Omega (EMBL-EBI, Cambridgeshire, United Kingdom), and divided into different groups-based sequence differences. Resulting consensus sequences were compared with GenBank submissions using default conditions on NCBI BLAST (highly similar sequences [megablast]) where the highest % sequence identity was used to determine species similarity. All of the individual ticks from five different pools with noisy sequences from the first *gltA* screening were retested using both *gltA* and *ompA* assays, and all positive amplicons were sequenced and assessed as already detailed.

Statistical Analysis

Mean number of actively questing individuals was compared at each hourly interval from 6 a.m. to 5 a.m. for *I. scapularis* adults using a randomized complete block procedure, PROC MIXED with an LSMEANS test, with each date representing a block (SAS 9.4, SAS Institute 2015). The mean number of questing individuals at different heights from 2 to 43 cm was also compared using this procedure.

Results

Questing Bioassays and Field Collections

Bioassay results showed a higher number of adult *I. scapularis* were active during later hours in the day (n = 4; Fig. 1). The hours of 4 p.m. through 6 a.m. were significantly different from earlier times

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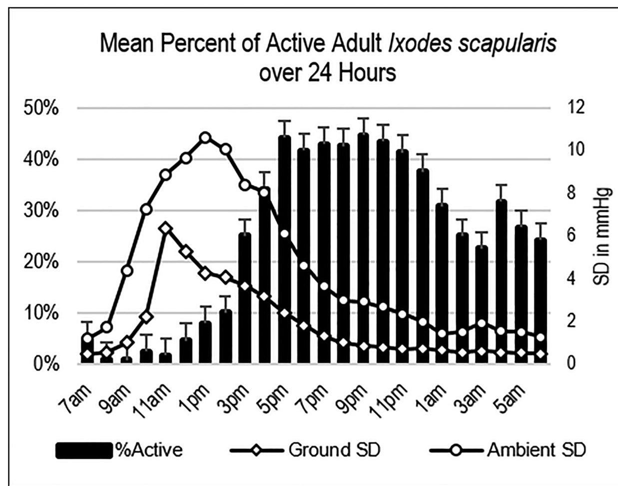

Fig. 1. Mean number of questing adult blacklegged ticks observed in cages at each hourly interval for 24 h with saturation deficit (SD) values at ground level and near the top of the mesh cage ('Ambient'). The hours of 4 p.m. through 6 a.m. were significantly different from earlier times of day for cage bioassays (P = 0.0201 for 4 p.m., P = 0.0003 for 5 p.m., P < 0.0001 for intervals from 6 p.m. through 1 a.m., P = 0.0018 for 2 a.m., P = 0.0115 for 3 a.m., P = 0.0003 for 4 a.m., P = 0.0025 for 5 a.m., and P = 0.0064 for 6 a.m.).

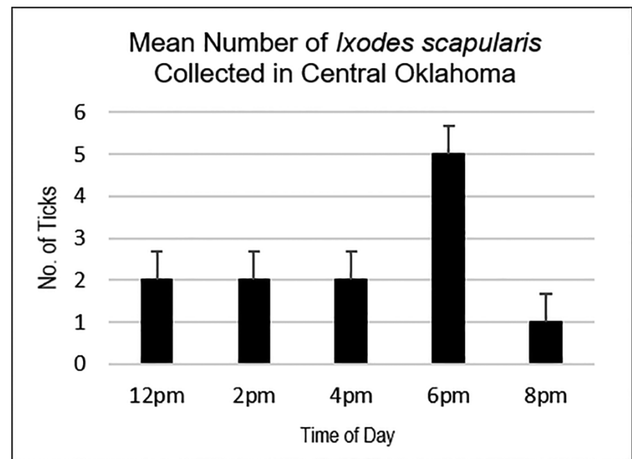

Fig. 2. Mean number of adult blacklegged ticks collected from flagging at each sampling interval between 12 p.m. and 8 p.m. at field sites in central Oklahoma. The number of ticks collected at the 6 p.m. interval was significantly greater than other sampling times (P = 0.0002).

of day for cage bioassays (F = 5.49, df = 69, P = 0.0201 for 4 p.m., P = 0.0003 for 5 p.m., P < 0.0001 for intervals from 6 p.m. through 1 a.m., P = 0.0018 for 2 a.m., P = 0.0115 for 3 a.m., P = 0.0003 for 4 a.m., P = 0.0025 for 5 a.m., and P = 0.0064 for 6 a.m.). The greatest number of adult *I. scapularis* were collected from the field sites (n = 7) at 6 p.m. (F = 4.78, df = 24, P = 0.0002; Fig. 2). Questing height of *I. scapularis* individuals in cages ranged from 2 to 43 cm with majority occurring near 22–24, 40–42, and 43 cm (F = 1.35, df = 21, P = 0.0005, 0.0032, and 0.0298, respectively; Table 1).

Table 1. Active *Ixodes scapularis* adult males and females observed at each height within mesh cages

	Overall proportion of active ticks observed at each height		
	Males (%)	Females (%)	Total (%)
2 cm	29 (11.4)	17 (5.4)	46 (8.1)
10–12 cm	18 (7.1)	39 (12.3)*	57 (10.0)
14–16 cm	23 (9.1)	22 (7.0)	45 (7.9)
22–24 cm	84 (33.1)*	57 (18.0)*	141 (24.7)*
28–30 cm	28 (11.0)	31 (9.8)	59 (10.4)
32–34 cm	22 (8.7)	33 (10.4)	55 (9.6)
40–42 cm	40 (15.7)	74 (23.4)*	114 (20.0)*
43 cm†	37 (14.6)	43 (13.6)*	80 (14.0)*

* Indicates significant differences in the number of questing ticks with a P value of 0.05 or less.

† Indicates the height at which ticks were observed questing on the lid of the mesh cages.

Molecular Testing

None of the pools of adult *I. scapularis* tested positive for the presence of *B. burgdorferi*, *A. phagocytophilum*, or *Ehrlichia*sp, DNA. Of the 46 pools of *I. scapularis* tested, 31 (67.4%) were positive for *Rickettsia* sp. *gltA* and 28 (60.9%) tested positive for *Rickettsia* sp. DNA. Twenty-seven (58.7%) pools were positive for *Rickettsia* sp. for both assays. Positive *gltA* sequences from pooled ticks were grouped into three distinctive groups of endosymbiont *Rickettsia* sp. DNA. The majority (26/31; 84%) of the *gltA* samples were 99% identical to known sequences of rickettsial endosymbionts of *Ixodes* ticks (GenBank KU529481.1) and *Rickettsia tamurae* (GenBank KT753265.1). Three samples (10%) were 97% identical with sequences involving a *Rickettsia* clone (GenBank KU255716.1) and *Rickettsia asemboensis* (GenBank KY445723.1) while two samples (7%) aligned with sequences similar to *Rickettsia siberica* (GenBank HM050296.1) and *Rickettsia monogolotimonae* (GenBank DQ097081.1). To confirm these results at the level of individual ticks, 21 ticks from five pools (pools 4, 8, 17, 28, and 31) were tested using *gltA* and *ompA* assays. While at least one tick from all pools (pools 4 [n = 4/4], 8 [n = 2/5], 17 [n = 1/3], 28 [n = 5/5] and 31 [n = 1/4]) were positive using *gltA*, only ticks from two pools (pools 4 [n = 4/4] and 28 [n = 4/5]) were positive with the *ompA* primers. The sequences from the *gltA*-positive ticks formed three distinct groups: pools 4 and 28 were 99% identical to rickettsial endosymbionts of *Ixodes* ticks and *R. tamurae* (Genbank KU529481.1 and KT753265.1), two ticks from pool 8 were 100% identical to known sequences for *R. amblyommatis* (Genbank MG674587.1 and KY273595.1), and one tick each from pools 17 and 31 were 97% identical to known sequences of *R. asembonensis* and uncultured *Rickettsia* sp. clones (GenBank KY445723.1 and KU255716.1). The sequences from the *ompA* positive ticks from pools 4 (n = 4/4) and 28 (n = 4/5) were 100% identical to known sequences of *Rickettsia* endosymbionts in *I. scapularis* (GenBank KP172259.1 and KX772759.1).

Discussion

Results from this study indicate that *I. scapularis* in Oklahoma may become more active later in the day and early morning (4 p.m. to 6 a.m.) which is similar to questing behavior patterns reported for *I. scapularis* larvae and nymphs in the northern United States (Carroll et al. 1998, Schulze et al. 2001, Madden and Madden 2005, Lee and Paskewitz 2015). One report from two islands off of the coast of Georgia in the southern United States also indicated increased activity of adult *I. scapularis* during nighttime hours (Durden et al. 1996). Interestingly, of the studies that have included times of day in their

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methods (Goddard 1992, Schulze et al. 2001, Lubelczyk et al. 2004, Schulze and Jordan 2005), none reported early morning or evening collections despite reports in the literature of increased activity peaks during certain times of day.

Adult *I. scapularis* in Oklahoma were also observed questing primarily between 22 and 43 cm. While differences in questing of adult *I. scapularis* between northern and southern populations have not been investigated, the questing behavior of nymphal and larval *I. scapularis* substantially differs between northern and southern populations in the United States (Arsnoe et al. 2015). Nymphs from northern clade *I. scapularis* were much more likely to quest and be active within the testing environment while those in the southern clade tended to stay in the leaf litter. This may be due, in part, to the preference of southern clade *I. scapularis* for reptile hosts in contrast to northern populations which tend to actively seek and parasitize small rodents (Durden et al. 2002, Garvin et al. 2015). Differences between northern and southern *I. scapularis* have been further documented through genetic analyses of each clade, and they were found to represent distinctly different genetic lineages (Sakamoto et al. 2014). While adult ticks were shown in this study to readily move upward within mesh cages, research focussing on the nymphal stage in this region would be beneficial. Additionally, valuable information concerning the diel activity of both adult and nymphs could be obtained through further laboratory and field research. Overall, these data provide insight into the potential host-seeking activity of adult *I. scapularis* in Oklahoma and support the notion that more efficient means of collection would be advantageous in future field-based experiments with this species.

Since most Lyme disease cases occur in the northern and northeastern United States, most ecological and behavioral studies involving *I. scapularis* have occurred in regions where northern clade populations dominate (Goddard 1992, Goltz and Goddard 2012, Sakamoto et al. 2014, Goddard et al. 2015, Mead 2015, Eisen et al. 2016). A nation-wide survey for *I. scapularis* nymphs utilizing traditional dragging methods in habitats suitable for this species was unsuccessful in collecting any samples from four separate sites in Oklahoma and documented only 21 specimens from sites in the southern United States overall (Diuk-Wasser et al. 2006). This regional bias ensures that most reported off-host collection of all life stages can easily be accomplished through traditional collection methods, mostly during daylight hours, in the northern and northeastern United States. In Oklahoma, traditional collection methods employed during daylight hours have proven insufficient, and a more targeted approach exploiting potential behaviors such as nocturnal activity could increase overall collection efficiency. Another interesting component that may play a role in tick questing behavior is the recent discovery of photoreception by the Haller's organ in *Dermacentor variabilis* ticks (Mitchell III et al. 2017). Similar phenomena regarding the relationship between darkness and increased mobility have been observed in select *Ixodes* species. Experiments examining questing and parasitism by *Ixodes pacificus* determined increases in both movement and host acquisition in shaded versus sunlit areas and nocturnal periods (Lane et al. 1995). Perret et al. (2003) also reported increases in activity in nymphal *Ixodes ricinus* during dark conditions in the laboratory. While humidity is known to impact blacklegged tick movement (Goddard 1992, Schulze et al. 2001, Vail and Smith 2002, Perret et al. 2003), the tendencies of some *Ixodes* species to become more active under darker and nocturnal conditions suggest that saturation deficit may not be the only variable influencing host seeking. It is also possible that dramatic differences in ambient temperature and relative humidity contribute to these observations of increased nocturnal activity in the southern Great Plains region. The results in the current study suggest that our understanding of the ecology and vector competence of these southern clade populations may be based on only a small proportion of the population collected during 'normal' hours. The dramatic shift in activity to later afternoon and early evening means that sampling protocols of researchers desiring to learn more about the ecology and vector competence of this important species of tick may need to be adjusted in this region.

As the first Oklahoma-based study to test adult *I. scapularis* ticks for *B. burgdorferi* DNA in 25 yr (Kocan et al. 1992), none of the pools of *I. scapularis* tested positive for *B. burgdorferi*. While Lyme disease has been reported and has been diagnosed in various patients by several Oklahoma-based media outlets, there is limited proof via serology of sentinel animals or through the current study in which adult *I. scapularis* collected from various regions of the state were tested. These observations differ from an earlier study by Kocan et al. (1992) in which *B. burgdorferi* was isolated from two separate pools of 10 *I. scapularis* and one pool of 10 *D. albipictus* that were recovered as partially fed adults from white-tailed deer caught in Oklahoma. Other studies from that same period indicated potential *B. burgdorferi* infections in Oklahoma-based deer, dogs, and mice (Mukolwe et al. 1992a,b). Since those reports, however, there have been no further reports of *B. burgdorferi* found in Oklahoma ticks in the peer-reviewed literature. In addition to *B. burgdorferi*, no pools of *I. scapularis* tested positive for *A. phagocytophilum*. Between 2008 and 2014, over 70 reported cases of granulocytic anaplasmosis (caused by *A. phagocytophilum*) were reported in Oklahoma (Dahlgren et al. 2015). However, only four of the cases met the laboratory criteria for a confirmed case, one of which was a travel-acquired case and two of the cases had unknown travel history (Dr Kristy Bradley, Oklahoma State Epidemiologist, personal communication). These data, together with the results from our tick samples, indicate a relatively low risk for acquiring granulocytic anaplasmosis in Oklahoma.

A majority of pooled and individual *I. scapularis* tested positive for endosymbiotic *Rickettsia* species. While not known to transmit a pathogenic *Rickettsia* species (Nelder et al. 2016), the strongest homology was with endosymbiotic rickettsial species reported in *Ixodes* sp. Grouped in three different sequence groups, these results align with the reports of others which are beginning to catalog a wide variety of *Rickettsia* sp. in *I. scapularis* from different geographical areas (Swanson et al. 2007, Steiner et al. 2008, Leydet and Liang 2013, Mays et al. 2014, Kurtti et al. 2015, Van Treuren et al. 2015). Steiner et al. (2008) also reported a high rate of infection with an *Ixodes* endosymbiont in samples obtained from various state parks in the northern United States. With the reports of *E. ewingii* and *Ehrlichia* sp. 'Panola Mountain' in *I. scapularis* collected in Tennessee (Mays et al. 2014), there is a need for continued surveillance to monitor *I. scapularis* as potential vectors of tick-borne pathogens.

In conclusion, this study highlights the questing activity of an important tick species and results of PCR testing for human pathogens in *I. scapularis* in Oklahoma. Due to the high prevalence of spotted fever group rickettsiosis and ehrlichiosis in humans and dogs in Oklahoma, much of the focus on tick species has centered on *Amblyomma americanum* (lone star ticks) and *D. variabilis* (the American dog tick). However, this study highlights the need to continue to study the ecology and vector potential of tick species not normally thought to be involved in the epidemiology of disease within a given region. By not knowing when these species are most active or in how they behave in field setting while seeking a host, it is very possible to miss times most likely to encounter these ticks, therefore, resulting in lost work effort. Enhancing experimental

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designs for research targeting native tick species will help to better understand these aspects of local vector populations and regional tick-borne disease epidemiology. A more targeted approach when sampling for *I. scapularis* could contribute to the overall efficiency of collecting samples for field-based research examining blacklegged ticks in the southern Great Plains region of the United States.

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