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Searching for the Immature Stages of *Ixodes scapularis* (Acari: Ixodidae) in Leaf Litter and Soil in Texas

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Abstract

The standard tick collection methods of flagging and dragging are successful for collecting all stages of the blacklegged tick, *Ixodes scapularis* (Say) (Acari: Ixodidae), in the northern United States. However, for unknown reasons, these methods are unsuccessful for collecting the immature stages of *I. scapularis* in the southern United States. Thus, a different collection strategy was employed to search for the immature stages of *I. scapularis* in the southern state of Texas. Monthly sampling of three types of microhabitats potentially harboring ticks was conducted for 17 mo at the Big Thicket National Preserve. Samples of leaf litter, topsoil, and subsoil were placed within Berlese funnels to determine if the immature stages of *I. scapularis* are residing in these layers. No ticks were found in any of the 600 substrate samples examined. Along nearby trail edges in the same area, 656 adult *I. scapularis* (an average of 22.6 per 1,000 m^2), as well as 268 immatures of other species (i.e., *Amblyomma americanum* (Linnaeaus) (Acari: Ixodidae) and *Dermacentor variabilis* (Say) (Acari: Ixodidae)) were collected using flagging and dragging. These results suggest that unlike speculations from previous studies in the southern United States, the immature stages of *I. scapularis* may not be residing in the leaf litter and soil layers in Texas. We hypothesize that they may be residing in their host's nests and burrows. Perhaps *I. scapularis* in the south is exhibiting a stage specific mixed host-seeking strategy by residing in nests and burrows as immatures, contributing to the geographical difference in Lyme disease prevalence between the northern and southern United States.

Key words: *Ixodes scapularis*, blacklegged tick, leaf litter, Berlese funnel

Cases of Lyme disease (LD) in the United States are reported at 30,000 a year, however the Centers for Disease Control and Prevention estimate that over 300,000 people become infected annually (Hinckley et al. 2014, Nelson et al. 2015). The most prevalent vector-borne illness in the United States is considered to be LD (Schwartz et al. 2017). The causative agent of LD is *Borrelia burgdorferi* (Johnson, Schmid, Hyde, Steigerwalt, and Brenner) (Spirochaetales: Spirochaetaceae), which is transmitted by *Ixodes scapularis* (Say) (Acari: Ixodidae). The majority of LD cases in the United States occur in the Northeast and Northcentral states. However, *B. burgdorferi* has been found in *I. scapularis* and vertebrate hosts in the Southeast and Southwest states (i.e., states south of 37° N latitude and 100°W longitude) (Teltow et al. 1991, Kocan et al. 1992, Oliver et al. 1993b, Oliver et al. 1995, Levine et al. 1997, Williamson et al. 2010, Feria-Arroyo et al. 2014). The factors explaining this geographic variation in LD prevalence are not fully understood. One hypothesis is that geographic differences in *I. scapularis* behavior could be contributing to this difference (Ginsberg et al. 2014, Arsnoe et al. 2015, Ginsberg et al. 2017). Behavioral differences among ticks from different regions may also impact field collection success.

In the North, *I. scapularis* immatures are easily collected using the standard methods of flagging and dragging; where a 1 m^2 piece of cloth is flagged across the vegetation or dragged along the ground (Falco and Fish 1992, Diuk-Wasser et al. 2006). Multiple ecological studies have noted that immature *I. scapularis* are rarely collected using these same methods in the south even where adults are abundant and reasons for this are unknown (Diuk-Wasser et al. 2006, Goddard and Piesman 2006, Goltz and Goddard 2013). There are climate differences between these two geographic areas which could be contributing to this phenomenon. *I. scapularis* has a higher risk of desiccation in the south as temperatures are higher in the summer months and it has been shown that *I. scapularis* larvae kept in southern environmental conditions die faster than larvae kept in northern conditions (Ginsberg et al. 2017). Thus, climate could affect the behavior and life history of *I. scapularis* in the south perhaps leading *I. scapularis* to seek refuge in the soil and leaf litter to avoid desiccation (Arsnoe et al. 2015, Ginsberg et al. 2017). Immature *I. scapularis* ticks in the south therefore could be seeking hosts in the leaf litter or in different soil layers rather than on the understory vegetation.

In the south, lizards, rather than small mammals, are the most frequently and heavily parasitized hosts of *I. scapularis* immatures (Apperson et al. 1993, Oliver et al. 1993a, Oliver 1996, Levine et al. 1997, Kollars et al. 1999, Durden et al. 2002, Clark et al. 2005). Because of this difference in host use, perhaps southern *I. scapularis* are behaving as other tick species that also feed on lizards. For example, in Australia, the ticks *Amblyomma limbatum* (Neumann) (Acari: Ixodidae), and *Bothriocroton hydrosauri* (Denny) (Acari: Ixodidae) feed on lizards and it was found that *A. limbatum* was most frequently found at the interface between leaf litter and soil while *B. hydrosauri* was most commonly found within the leaf litter (Chilton and Bull 1993, Chilton et al. 2009). Thus, it would be reasonable to speculate that immature *I. scapularis* in the southern United States may also occur within leaf litter and/or soil layers.

In order to determine if immature *I. scapularis* in Texas are living and seeking hosts in the leaf litter or soil, monthly sampling was conducted for a full year and 5 mo. Samples of leaf litter, topsoil, and subsoil were placed into Berlese funnels to collect ticks from these substrates.

Methods

Field Site Characterization

Leaf litter, topsoil, and subsoil were collected at the Beech Woods Trail, Big Thicket National Preserve in Tyler County, Texas (30°43′11.63″ N, 94°13′34.74″ W). Leaf litter on the forest floor was composed of the tree species present at this field site: *Fagus grandifolia, Magnolia grandiflora, Pinus taeda, Quercus alba*, and *Acer saccharum*. Topsoil and subsoil samples were analyzed by the Soil, Water and Forage Testing Laboratory in the Department of Soil and Crop Sciences at Texas AgriLife Extension Service College Station, Texas. The topsoil was determined to be 3% clay, 17% silt, and 81% sand, and the subsoil was determined to be 7% clay, 18% silt, and 75% sand. Topsoil was visibly different from subsoil with the subsoil having a higher percentage of clay and being densely packed. This visible change occurred at a depth of 25 cm thus, topsoil samples were collected from the top 25 cm of soil and subsoil samples were collected from the next 25 cm of soil.

Substrate Collection

Monthly samplings were conducted every fourth week of the month, starting in June 2016 and ending in October 2017, for a full year and 5 mo (to sample one full year and two summer seasons). Samples were collected at dawn and dusk in accordance with northeastern *I. scapularis* immature peak activity times (Schulze and Jordan 2003). Additionally, from May to October 2017 noon samples were also collected. To reduce variability of vegetation and geography, a single 250 × 125 m plot was sampled for the duration of the study. Within this plot, a new 200 m transect was sampled every month. The experimental plot sampled was selected because it was located at the site that had the highest flagging and dragging success for collecting adult *I. scapularis* (with a density of 22.6 per 1,000 m^2) compared to all of the sites we sampled in Texas (15 field sites total). Five samples of each substrate (leaf litter, topsoil, and subsoil) were collected every 50 m within each transect (East to West). All selected transects were at least 25 m apart (North to South) to avoid transect resampling. Each sample was taken using a 25 × 20 cm shovel resulting in ~4,000 cm^3 of leaf litter and ~2,000 cm^3 of topsoil and subsoil per sample. As 600 substrate samples were taken the total volume of substrate sampled was ~3,600,00 cm^3. Samples were brought to Texas A&M University and placed within Berlese funnels (Precision Calibration and Equipment, Florence, Ontario, Canada) for 4 d, as preliminary trials showed that no additional arthropods were recovered after this time.

Tick Collection

Each sample was placed within Berlese funnels which consisted of a metal funnel with a 12.7 cm diameter at the top where a petroleum jelly barrier was added as an extra precaution to stop ticks from escaping. At the bottom was a 7.62 cm diameter opening with a metal screen to keep substrate samples in funnel but allow arthropods through. Funnels rested on collection vials at the bottom and above each funnel was a light source (50 W). This collection method exploits the tendency of most soil arthropods to be photophobic, crawling down the funnel to avoid light and landing into a vial filled with ethanol at the bottom for collection. This method has been successful for collecting the seabird tick *Ixodes uriae* (White) (Acari: Ixodidae) (Barton 1995). To ensure that most ticks were collected by the Berlese funnels, random samples were also examined by floatation. This was accomplished by placing a substrate sample in a water tub to allow soil to sink and organic matter to float which can then be examined by hand sorting. Floatation of samples after Berlese funnel extraction has been shown to be an effective and comprehensive sampling method (Sabu et al. 2010). All arthropods present in the Berlese funnel collection tubes were transferred to vials filled with 80% ethanol. Arthropods were counted and sorted using a dissection microscope. Standard flagging and dragging (Rulison et al. 2013) was also conducted each month from June 2016 to October 2017 at the same field site where substrate samples were collected using the site's hiking trail as the transect for a total of 4,282 m.

Results

No ticks were found in any of the 600 substrate samples that were collected and placed in Berlese funnels. Using flotation to examine the substrate samples also returned no ticks. Similarly, no immature *I. scapularis* were collected by flagging and dragging in the same forest along the trail edges. In contrast, 656 adult *I. scapularis* (an average of 22.6 per 1,000 m^2) were collected using flagging and dragging along the trail edges (Fig. 1). Adult *I. scapularis* were found from November 2016 to April 2017 and peaked in abundance in February. Interestingly, flagging and dragging also yielded *Dermacentor variabilis* (Say) (Acari: Ixodidae) and *Amblyomma americanum* (Linnaeaus) (Acari: Ixodidae) immatures, even though these collection methods did not yield any *I. scapularis* immatures (Fig. 1).

Discussion

It has been proposed that the geographic difference in drag and flag collection success for the immature stages of *I. scapularis* in the northern and southern United States may be due to ticks remaining below the leaf litter surface in the south (Arsnoe et al. 2015, Ginsberg et al. 2017). The results of this study suggest that the immature stages of *I. scapularis* in Texas may not be residing in the leaf litter, topsoil or subsoil layers, at least not in a uniformly distributed fashion allowing for collection with the sampling effort in this study. In the northern United States, *I. scapularis* females lay egg masses in the leaf litter and the resulting larvae usually quest together in a clumped distribution on top of the leaf litter or on the vegetation (Daniels and Fish 1990). However, later in the season, a limited number of larvae disperse up to 3 m and their distribution becomes relatively less clumped (Daniels and Fish 1990, Stafford 1992). Nymphs are more uniformly distributed than larvae, in part as a result of their dropping off their hosts at different times

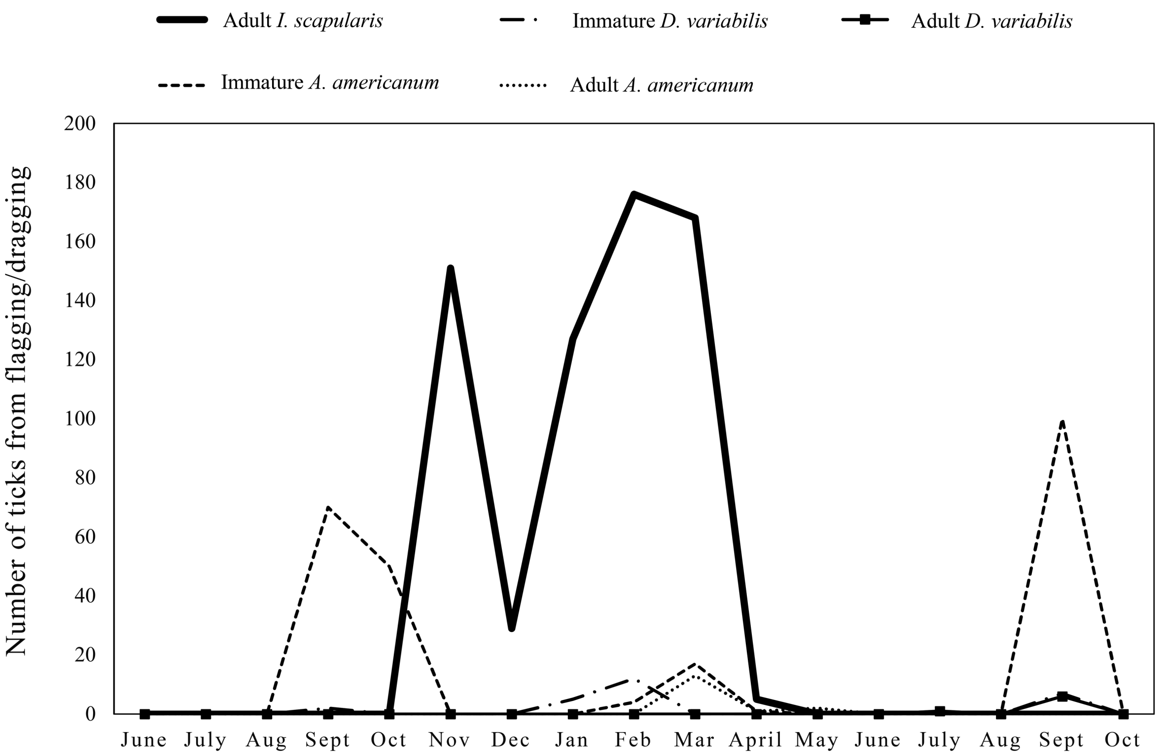

Fig. 1. Seasonality and abundance of tick species present on the vegetation collected by flagging and dragging June 2016 to October 2017.

in different places. Thus, if southern *I. scapularis* behave the same as their northern counterparts one would have expected that even though egg masses may have been clustered in the environment, at least nymphs should occur on leaf litter, topsoil, or subsoil samples at some point of the year.

Interestingly, the methods used in this study were successful for collecting mites and documenting their seasonality and abundance, but ticks were not present in the samples. It was found that Berlese funnels were 90% effective for recovering *I. scapularis* nymphs and larvae from field microcosms in the northern United States (in samples of leaf litter with known amount of *I. scapularis*) (Burtis 2017). Thus, if *I. scapularis* were present in our samples they should have been recovered using Berlese funnels.

Also, flagging and dragging at the same field site produced many adult *I. scapularis* as well as the immature stages and adult stages of *A. americanum* and *D. variabilis* but failed to produce immature *I. scapularis*. This finding is consistent with many studies, conducted in various southern states, by different research groups (Rogers 1953, Diuk-Wasser et al. 2006, Goddard and Piesman 2006, Goltz and Goddard 2013). At our field site, *A. americanum* larvae were collected by flagging and dragging but none were found using Berlese funnels. This is probably due to the fact that *A. americanum* adult densities were much lower than *I. scapularis* adult densities and therefore expectations to find *A. americanum* immatures using Berlese funnels were low. Therefore, if the immature stages of *I. scapularis* in Texas are not on the vegetation or in the leaf litter, topsoil, or subsoil layers, where can they be? One possibility is the dwellings of their hosts (e.g., lizard nests and/or mammal burrows).

Ticks have two categories of host-seeking behavior. Nidicolous ticks live in their host's nests or burrows while non-nidicolous ticks live in open environments and actively quest to seek out their hosts. *I. scapularis* has been traditionally categorized as a non-nidicolous tick because all three stages in the North can be collected from the vegetation. However, because only the adults can be collected from the vegetation in the south, perhaps southern *I. scapularis* possesses both behaviors acting as a non-nidicolous tick during the adult stage, and as a nidicolous tick in the immature stages to ensure survival during hot and dry seasons. This type of stage specific mixed host-seeking behavior has been characterized in the tick *Hyalomma asiaticum* (Schulze and Schlottke) (Acari: Ixodidae) which quests in the open for large ungulates while the immature stages feed on burrowing animals and are characterized as nidicolous (Sonenshine 2013). Also, it is thought that *Hyalomma dromedarii* (Koch) (Acari: Ixodidae) live in burrows while the adult stage quests openly for camels and other ungulates (Alahmed and Kheir 2003). The life history of these ticks is thought to be a response to dry desert conditions (Balashov 1960).

However, a yet unanswered and intriguing questions is how the larvae of a mixed host-seeking strategy would find their host's nest or burrows as the adult females drop from their hosts in the open environment. Most adult *I. scapularis* feed on white-tailed deer and thus, the majority of females drop from deer and oviposit their eggs in the leaf litter. Unlike tick habitats in the northern United States, southern United States habitats have a higher density and diversity of lizard species (Pianka 1977). For example, in one study in a southern state (i.e., North Carolina), it was found that lizards were two times more abundant than small mammals (Apperson et al. 1993). Since leaf litter constitutes a major portion of the habitat of several lizard species that are hosts of *I. scapularis*, perhaps successful larvae quickly encounter and attach to lizard hosts in the leaf litter while unsuccessful larvae quickly desiccate and die, making it less likely for researchers to encounter them. Successful larvae would then later detach within their host's nests and burrows which is how they may have become nidicolous.

If *I. scapularis* populations in the south are using a mixed host-seeking strategy this could allow the physically smaller stages, that are more prone to desiccation, to avoid the hot southern climates by

dropping within their hosts' burrows as engorged larvae and remaining in the burrows as nymphs, resulting in the absence of nymphs from collection efforts using flagging and dragging. If immature *I. scapularis* are dwelling in nests and burrows, this would be of public health significance because it would result in immatures being less likely to come into contact with humans resulting in less tick bites. Thus, this mixed host-seeking strategy would contribute to the geographic difference in LD prevalence observed between the northern and southern United States.

Future studies are needed to survey hosts dwellings to explain the difference in collection success of immature *I. scapularis* between the northern and southern United States. The lack of a successful collection method for *I. scapularis* immature stages is an obstacle for tick-borne disease surveillance in the south as the immature stages cannot be collected for pathogen testing apart from labor-intensive host trapping and inspection.

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