Environmental Factors Affecting Nymphal *Ixodes pacificus* Abundance Under Field and Laboratory Conditions

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Abstract The prevalence of human cases of Lyme disease in the United States has been steadily increasing since 1982, and in northern California, the seasonal occurrence of human Lyme disease cases coincides with the peak activity period of nymphal *Ixodes pacificus* Cooley and Kohls 1943 (Eisen and Lane 2000). This study examined nymphal *I. pacificus* in Marin County in attempt to elucidate what biotic and abiotic factors influence their abundance. Field and laboratory studies were conducted to determine both the correlations and effects of soil pH, air and soil temperature on nymph abundance. Vegetation association among nymphs was also determined in the field. In the field, it was observed that nymphs were significantly associated with areas consisting predominately of oak trees and showed highest abundance in areas with air temperatures of 14°C, soil temperatures of 9°C and a soil pH of 6.7. Nymph abundance was significantly correlated with soil pH in the field but no significant correlation was observed between soil and air temperature. Under experimental conditions, it was found that nymphs had a significant association to a maintained soil temperature of 9°C and a significant association to maintained air temperatures of 14°C and 11°C. No significant association of nymphs was observed to maintained soil pH. The significant association of nymphs with conditions in the field and laboratory may provide an explanation as to which natural conditions determine nymphal abundance in certain locations.

Introduction

The western black-legged tick, *Ixodes pacificus* Cooley and Kohls 1943, is the primary vector of Lyme disease in the western United States, transmitting the disease causing spirochete, *Borrelia burgdorferi* Johnson, Hyde, Steigerwalt and Brenner 1984, in two of its three life stages (Padgett and Lane 2001). Larval *I. pacificus* hatch in the end of spring to midsummer and are typically uninfected with *B. burgdorferi* as transovarial transmission between adult female and larvae is rare (Schoeler and Lane 1993). Larvae seek western fence lizards, *Sceloporus occidentalis* Baird & Girard 1852, western scrub jay's, *Aphelocoma californica* Grinnell 1901 and dusky-footed woodrats, *Neotoma fuscipes* Baird 1858, for a blood meal and attach to these hosts for several days (Brown 1992). Larvae drop off the hosts and proceed to the nymphal life stage which may remain inactive for several months. Nymphs will seek the same host species as larvae for a blood meal and molt into adults who seek Columbia black tailed deer, *Odocoileus hemionus columbianus* Richardson 1829, dusky-footed woodrats, and western gray squirrels, *Sciurus griseus* Ord 1818 as hosts (Brown 1992).

Both nymphs and adults are competent vectors for Lyme disease but a discrepancy exists between the infection and transmission rates of nymphs and adults. Adults have been shown to have lower infection rates because of borreliacidal factors in the blood of western fence lizards that kill *B. burgdorferi* during the feeding of *I. pacificus* nymphs (Lane and Quistad 1998). In addition, the adults larger size and activity during winter months when people wear more protective clothing causes their transmission of Lyme disease to be low (Xiahong *et al.* 2000). Due to the more inconspicuous size of nymphs, they are less likely to be noticed and removed promptly and are thus primarily responsible for transmitting Lyme disease causing spirochetes to humans (Vredevoe 1998).

Lyme disease is the most frequently reported vector-borne disease in the U.S. (Bacon *et al.* 2005). Systemic symptoms of Lyme disease include headache, neck stiffness and heart palpitations while chronic symptoms in untreated cases include arthritis, joint swelling and neurological problems (Zhang *et al.* 2006). In 2005, 23,305 cases of Lyme disease were reported, yielding a national average of 7.9 cases for every 100,000 individuals (Bacon *et al.* 2005). The cost to treat Lyme disease has been decreasing since 1997, but the loss of productivity to an individual may still be very high (Zhang *et al.* 2006). In 2000, the expected mean total cost attributable to Lyme disease was $1,965 per patient due to the cost of hospitalization,

consultations, office visits and prescription of antibiotics. However, the loss of productivity and suffering accrued by an individual with late-stage Lyme disease could be high as $9,108 due to days missed from work (Zhang *et al.* 2006). Control measures to decrease the transmission of Lyme disease to humans may result in the alleviation of financial costs associated with disease treatment. The implementation of such measures requires a strong understanding of the factors that promote nymphal abundance.

Prior studies regarding the biotic and abiotic factors that influence the prevalence of ticks have focused mainly on the distribution of vertebrate hosts and climatic fluctuations (Brownstein *et al.* 2005). These findings indicated that landscape features such as the presence of logs, topographic exposure and the amount of vegetation affected population sizes and tick physiological processes, such as their potential to move to find either hosts or mates (Eisen 2000). *I. pacificus* nymphs predominantly occur in areas with oak trees as opposed to grassland areas. This association with oak trees may be because oak trees and surrounding leaf litter create areas with high humidity and low air temperature which has been shown to be necessary for tick survival in the lab (Xiahong *et al.* 2000). In addition, oak habitats may also be suitable habitats for vertebrate hosts which would in turn cause the presence of nymphs. In laboratory experiments, tick mortality has been greater under high temperatures and low humidity suggesting a physiological process must be affected by temperature and humidity (Needham 1991). As a result, it is believed that high temperatures in the spring and summer months reduce the activity and abundance of Lyme disease carrying *I. pacificus* (Brownstein *et al.* 2005).

Ticks spend approximately 95% of their lives on the forest floor, either seeking a host or digesting a blood meal and are subject to the conditions associated with the ground (Brownstein *et al.* 2005). The living environment of *I. pacificus* is mostly the lower layer of vegetation, the upper layer of soil, dead leaf litter and upper layers of wood humus (Xiahong *et al.* 2000). Because of the decomposition of leaves and wood, soil temperature and pH may change depending on the upper material. Exposure to low temperatures and oak habitats with high densities of leaf litter has been shown to create microhabitats suitable to tick physiological processes such as questing and finding a mate or host (Xiahong *et al.* 2000).

Studies investigating developmental processes under field and laboratory conditions have been conducted (Padgett and Lane 2001) but none have assessed temperature, soil pH, and habitat association simultaneously. In a study combining field and laboratory data, Padgett and

Lane (2001) found that under laboratory conditions, accelerated developmental rates of all three life stages were correlated with warm temperatures and environmental conditions. Assessments of the many variables which may control tick abundance have only focused on certain conditions reserved only to the field or laboratory (Schauber *et al.* 2005). The tolerances of temperature extremes have both been examined in the field and in laboratory experiments but the two have existed independently of each other (Canham *et al.* 2006). In addition, studies altering vegetation to demonstrate the impact on tick abundance have only occurred in the field (Wilson 1986).

This study was done to monitor the parameters of soil temperature, air temperature and soil pH in both a field and a laboratory setting, an aspect that has not been previously studied in tandem. The objective of this study this study is to develop a better understanding of the distribution and abundance of *I. pacificus* nymphs. While ecologically relevant, this information has direct applications for counties and governmental entities that wish to model and predict regions which pose the greatest risk to humans for contracting Lyme disease. Because little is known about the specific biotic and abiotic control of nymph abundance, I address in my study the following question: What is the relationship between habitat type, soil pH, soil temperature, air temperature and nymph abundance? My hypothesis is that *I. pacificus* nymphs will be spatially clustered in habitats comprised of oak trees, a slightly acidic soil pH, a low soil temperature and a low air temperature. Data collected from the field that is associated with the highest abundance of nymphs will be used in laboratory experiments to see how nymphs respond to various conditions.

Methods

   Field Collection Field work was conducted at China Camp State Park which is located on the southwestern shore of San Pablo Bay, near the city of San Rafael (Marin County, CA, USA). The dominant forest trees are California black oak, *Quercus kelloggi* (Newb) and Coast live oak, *Quercus agrifolia* (Nee). Other trees that constitute significant portions of the forest flora are California buckeye, *Aesculus californica* (Spach), Douglas-fir, *Pseudotsuga menziesii* (Mirb), and Coast Redwood, *Sequoia sempervirens* (Lamb). Common shrubs and small trees include Manzanita, *Arctostaphylos manzanita* (Wells), and Hazel, *Corylus cornuta* (Sharp). The study site has an area of $7500m^2$ and is fairly heterogeneous. It is comprised of open fields, dense shrubs, and closed canopy forests. The study sight was divided into four different habitat types

based on the dominant vegetation. The habitat classifications were the following: open grassland, shrub land, oak and mixed conifer stands. Classifying and stratifying samples by specific vegetation types ensured equal sampling of the site without bias of site selection.

Prior to each sampling effort, it was randomly determined which specific habitat types were sampled. Nymphs were collected using a 1 x 1 m^2 drag cloth along 15 meter transects. Nymphs present on the ground, or any substrate in contact with the cloth were caught in the netting. Drags were done and analyzed visually every 15 meters in order to limit underestimation due to tick drop-offs (Li and Dunley 1998) and ensure collection at the nymphal life stage.

Soil pH was measured at five points within the sampling area and at a depth of two centimeters in order to mimic the area of soil that nymphs are in contact with. Soil pH was monitored during each sampling effort with a Rapitest pH meter (Luster Leaf Products, Inc., Woodstock, IL, USA) that was inserted directly into the soil. Soil and air temperature were measured using the same procedure as soil pH and temperature was measured using a digital thermometer (Outset, Inc., Minneapolis, MN, USA). The timing of these measurements was done at 10:00 am every week from December 28th until February 7th. Field data was analyzed using linear regression and ANOVA with Tukey's HSD test.

Laboratory Data In order to create separate environments to observe tick habitat preference, a model environment was constructed where conditions were adjusted on one side ("variable side") while conditions on the other side remain constant and similar to observed field conditions ("constant side") (Figure 1). Slits were cut in the barrier that separated the two sides, allowing ticks to move between environments (Figure 1). The dimensions of the enclosure are length: 25cm, width: 25cm and height: 7cm. From the middle point where the nymphs were placed, the distance to each side was five centimeters. Each condition was analyzed individually in relation to the environmental condition observed in the field. Twenty nymphs were randomly selected from a total population of 85 and were placed in the middle of the enclosure. Movement to the variable or constant side indicated a preference for the specific condition. Twenty nymphs were used per trial and each analyzed condition was replicated for three trials.
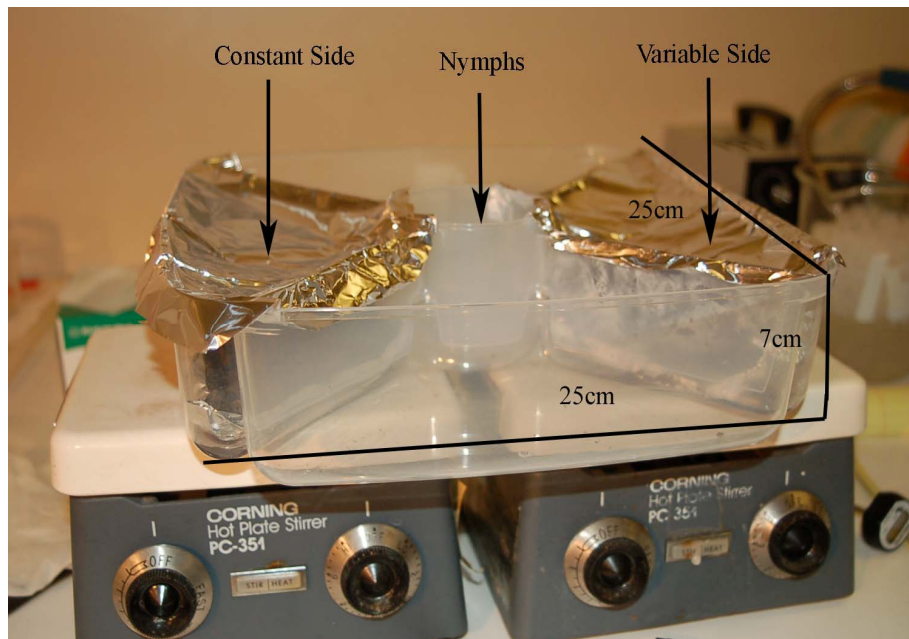
Figure 1. Experimental design with two separate environments in which nymphs can have a preference toward. Dimension of the enclosure are height: 25cm width: 25cm height: 7cm.

Soil and air temperatures in 3°C increments above and below the average temperature observed in the field were analyzed and replicated for three trials. In order to fluctuate and maintain a certain soil temperature above the temperature associated with the highest abundance, a standard hot plate was used. Air temperature was also controlled by using a hot plate. To maintain a temperature below the observed average, an ice water bath and a hot plate were used in conjunction. Soil pH was manipulated by one pH unit above and below the average soil pH observed in the field. Manipulation was accomplished by saturating the soil with pre-made pH buffers of 4, 7 and 10 (Transcat, Inc., Rochester, NY, USA). Laboratory data analysis was done using a two tailed *t*-test and the JMP statistical program.

Results

Field Soil pH in each of the habitats sampled ranged from pH 6.0 to 7.0, showing a slightly acidic to neutral range. Peak nymph abundance of 17 nymphs was observed in habitats with a soil pH of 6.7. A positive correlation was observed between soil pH and nymph abundance (Figure 2). Air temperature values recorded in each habitat type showed a positive association with nymph abundance but were not statistically significant (Figure 3). Peak nymph abundance
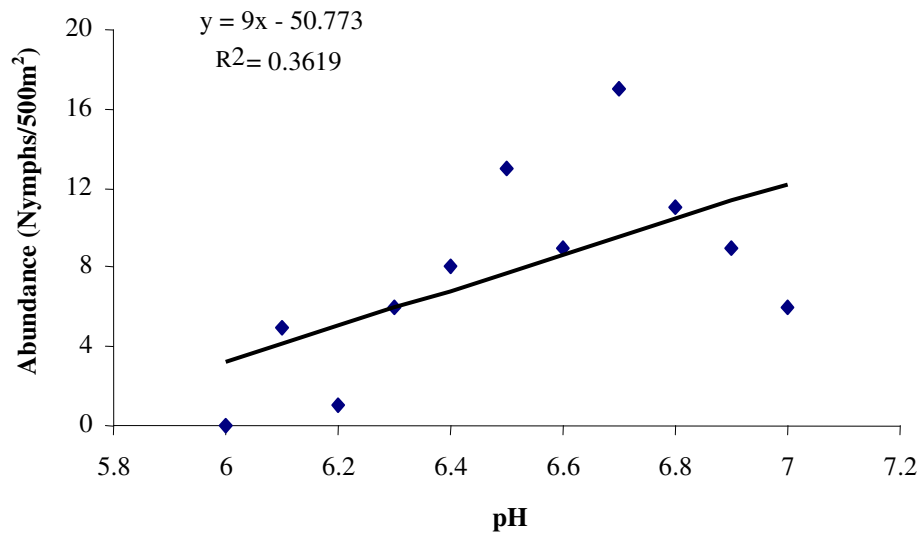
Figure 2. Nymphal abundance measured at each pH value. Trend line indicates a positive regression of nymphal abundance over soil pH (p =0.05) n=85

of 35 nymphs was observed in habitats with an air temperature of 14^oC. Soil temperature values recorded in the field showed a negative association with nymph abundance but was not statistically significant (Figure 4). Peak nymph abundance of 35 nymphs was observed in habitats with a soil temperature of 9^oC. Air and soil temperatures were rounded to the next whole number to facilitate an easier manipulation of laboratory conditions.
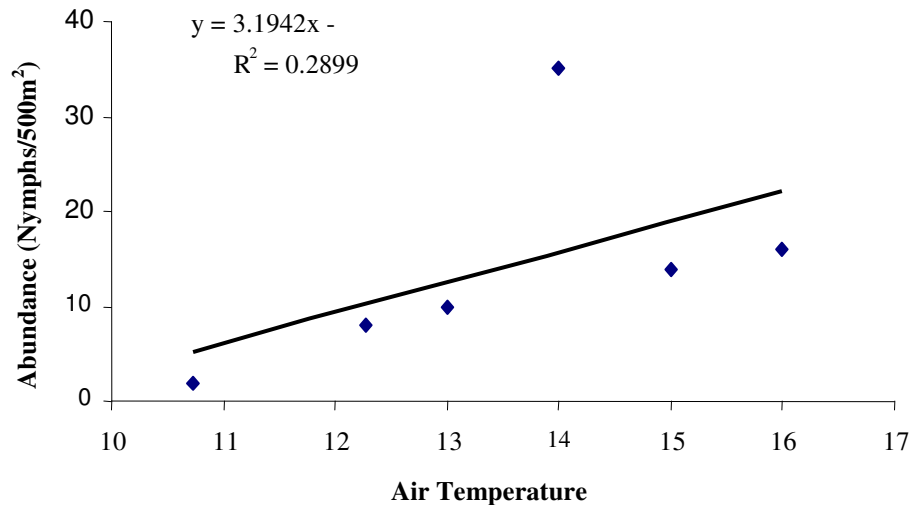

Figure 3. Nymphal abundance at each measured Air Temperature (p =0.27) n=85. Trend line indicates a positive regression of nymphal abundance over air temperature.

At the habitat level, nymph abundance in oak habitats was significantly greater than in common shrub and open grassland habitats (Fig. 5), with an abundance of 28 nymphs as compared to 18 and 13 nymphs in common shrub and open grasslands respectively. No significant difference was observed between mixed conifer and the other habitat categories (Figure 5).
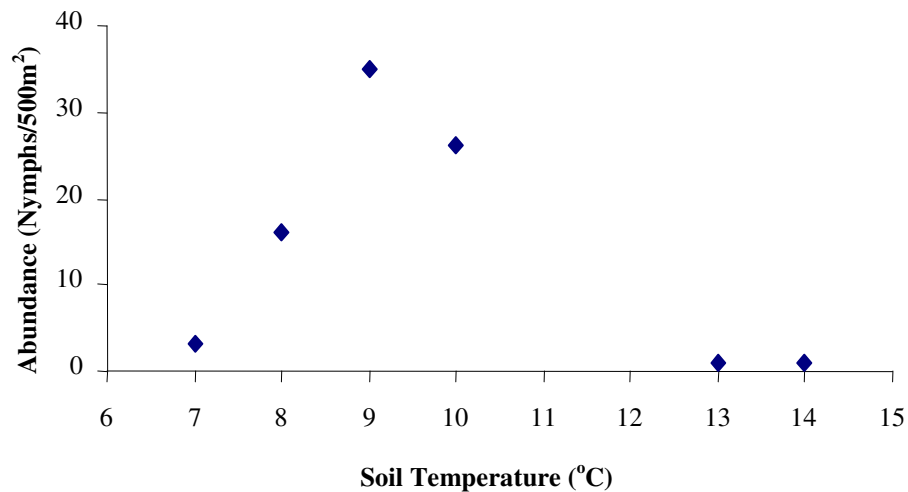

Figure 4. Nymphal abundance at each measured soil temperature (p=0.41) n=85.
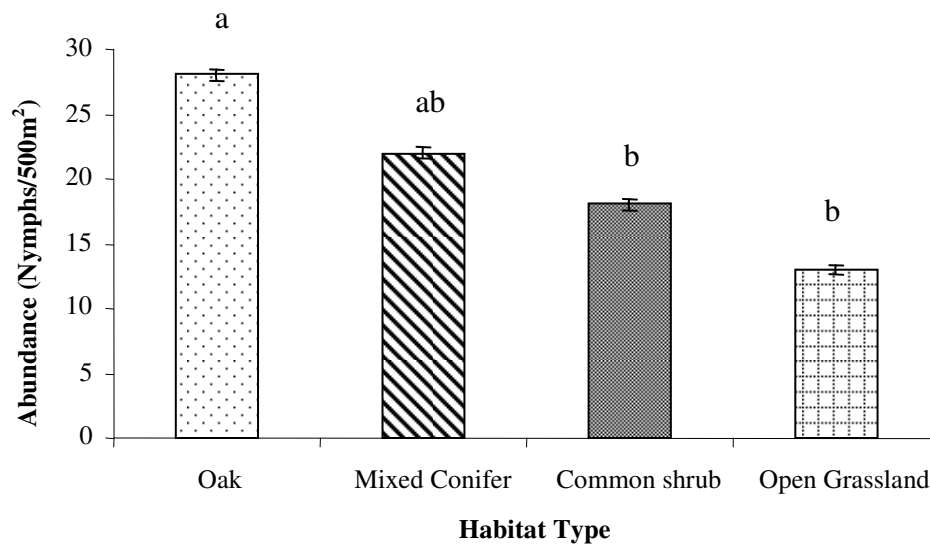

Figure 5. Abundance (± 1 S.E.) of nymphs in each vegetation type sampled at China Camp State Park, December 28 to February 7th. Letters indicate significant differences based on a Tukey's HSD test at the p ≤ 0.05 level. F=6.32. df= 3. Oak species include California black oak and Coast live oak. Species of mixed conifers are California buckeye and Douglas fir. Common shrubs include Manzanita and Hazel.

Laboratory Experiments   Under laboratory conditions, a significant difference was observed between nymph abundance at soil temperatures above the constant soil temperature. At temperatures of 12°C and 15°C, 78% and 93% of nymphs migrated to the constant soil temperature of 9°C, p= 0.05 and p=0.05 (Table 1). At 6°C, three degrees below the constant soil temperature, no significant difference was observed, p=0.87. Fluctuations in air temperature 3°C above the constant temperature of 14°C showed a significant difference as 67% and 95% of nymphs migrated to the constant temperature. Air temperatures 3°C below the constant temperature also resulted in a significant difference as 85% of nymphs migrated to the variable side p=0.003 (Table 1).

No significant preference was observed in regard to soil pH p=0.50 and p=0.71 (Table 1). The constant soil pH was maintained at 7 while changing the variable pH to 8 and 6. At both of these values, nymph preference was not observed as 58% and 43% of nymphs were found in the constant region (Table 1).

Table 1. Experimental Data and Percentage of Nymphs on Each Side of the Enclosure. Three trials were done for each value analyzed, n=20 nymphs per trial.

Condition	Value (Constant)	Value (Variable)	Number of Nymphs per Condition	% of Nymphs on Constant Side	P value
Air Temperature (^{o}C)	14	11	60	15	0.003
	14	17	60	67	0.005
	14	20	60	95	0.002
Soil Temperature (^{o}C)	9	6	60	52	0.87
	9	12	60	78	0.05
	9	15	60	93	0.05
Soil pH	7	8	60	43	0.50
	7	6	60	58	0.71

Discussion

Based on my research, several biotic and abiotic variables have been proposed as influential factors of nymphal *I. pacificus* abundance. The goal of this study was to determine if specific field variables related to nymph abundance and create several of these conditions in a controlled lab environment to determine direct regulation. By observing which conditions in both the field and lab were associated with the highest number of ticks, a better understanding of the relationship between nymphs and biotic and abiotic factors was gained. The observed association of *I. pacificus* nymphs to oak habitats, air temperatures below 14°C and a soil temperature of 9°C is supported through both field and laboratory data (Figures 2-4 and Table 1). These findings

support the hypotheses that nymphs would be primarily found in habitats comprised of oak trees and would be influenced by a low air and soil temperature. Laboratory experiments did not support the hypothesis that soil pH is a contributing factor to nymphal abundance in specific habitats although field results showed that a correlation exists between nymph abundance and soil pH (Figure 2).

The present study shows that in an area with mixed vegetation, fewer nymphs are found in areas outside of oak habitats (Figure 5). Oak forests have been shown to support developmental and physiological processes of nymphs by creating lower temperatures and humidity which increases locomotor activity and reduces desiccation (Ostfeld *et al.* 2006). In oak forests, leaf litter from California black oak and Coast live oak provides a buffer against solar radiation, wind, rain and other environmental extremes (Degraff and Rudis 1990). Oak and other old growth forests have been shown to be habitat for dusky-footed woodrats, a primary host of nymphal *I. pacificus* (Sakai and Noon 1993). The distribution of ticks is dependent on the distribution of their hosts so it can be expected that habitats which favor the abundance of a primary host would also favor the presence of host seeking nymphs requiring a blood meal.

In the field, no significant correlation was seen between soil and air temperatures and nymph abundance (Figures 3 and 4). The high abundance of nymphs at the air temperature of 14°C and two nymphs at soil temperatures of 13°C and 14°C caused deviations in the linear regression which may explain the non-significant correlation. It is possible that a larger sample size would reveal a significant correlation and remains an area for future research. Also in the field, it was observed that specific conditions were associated with the highest amount of nymphs sampled. The highest abundance was associated with a soil pH of 6.7, a soil temperature of 9°C and an air temperature of 14°C (Figures 2-4). The measured soil pH in each of the vegetation classes where nymphs were collected differed only slightly from 6.0 to 7.0 (Figure 2). Soils with a pH greater than 7.0 were not observed, indicating that the geochemistry of the soil sampled appears to be maintained at a neutral or slightly acidic pH. It is tempting to think that since a specific soil pH was associated with the highest nymph abundance, it would be contributing to nymphal abundance. It is important to remember that no terrestrial habitat is homogeneous in regards to its soil constituents. Various processes including organic decomposition and microbial activity contribute to a soils pH (Martius *et al.* 2004) and operate at a scale finer than my sampling scale. Although a possibility remains that nymph abundance is associated with soil pH, further efforts

are needed to account for soils inherent pH variability.

The high abundance associated with the low soil temperature of 9°C and air temperature of 14°C are consistent with previous studies that have shown that lower temperatures are required for tick survival and mobility (Nosek 1978). For several different *Ixodes* species, active movement is still possible at temperatures below 3°C and their mobility is sustained until reaching a maximum temperature of 23°C (Daniel 1978). It is interesting to note that the measured temperatures associated with peak nymphal abundance are both below the maximum recorded temperature limit. It is inferred that by living in habitats associated with temperatures slightly lower than the 23°C suggested maximum temperature, nymphal mobility may be maximized. This temperature dependent mobility correlates with peak nymph activity during the early morning when nymphs are actively searching for a blood meal (Brownstein *et al.* 2005).

It may be possible that other unmeasured factors such as the biotic components of the environment may influence the spatial distribution of *I. pacificus* nymphs. *I. scapularis* in the northeastern United States appears to be related to fluctuations in acorn production in oak forests, such that the density of nymphal *I. scapularis* is highest in oak forests after high acorn production (Ostfeld *et al.* 2006). The influence of biotic factors on the distribution of *I. pacificus* has not been significantly investigated to date and would require a study of longer duration and increased factors than just those associated in oak habitats.

Laboratory experiments further supported several of the observations in the field. Nymphs placed in the center of the exclusion migrated away from soil and air temperature that were greater than the constant condition. The implications of this study suggest that a temperature threshold exists which may provide insight into natural conditions that could promote or decrease nymph abundance. For soil temperature, the temperature limit appears to be at or close to 9°C as temperatures greater resulted in ticks relocating to the lower temperature (Table 1). A possible limit for air temperature may be close to 14°C. Temperatures below 14°C were shown to be preferred while temperature greater than 14°C appear to be disfavored (Table 1). As shown under field and laboratory conditions, association of nymphs to low soil and air temperatures may be a significant factor influencing their abundance. Further analysis is needed to understand if an exact soil and air temperature upper limit exists.

Limitations to this methodology include using mobility as an indicator of preference. Some

nymphs may have been more fit than others and were more able to move to one side of the enclosure. It is possible that the conditions over a broad range are suitable for nymphs and that their mobility between different conditions isn't dependent on a specific condition. It is also possible that when analyzing air temperature, humidity may have been the factor that determined nymph preference. Because hot and cold temperatures were used on both sides of the enclosure, a humid environment was created that was difficult to control. Nymph abundance has been shown to be positively correlated with increased humidity so this increase in humidity may have been the preferred factor and not the change in air temperature (Needham 1991). Although it is possible to monitor and create environmental conditions in the laboratory, such conditions are kept constant for a period of time which isn't representative of the actual conditions experienced by nymphs in nature (Padgett *et al*. 2001).

The strong relationship found between nymph abundance in oak woodlands, air temperatures of 14°C and soil temperatures of 9°C under both field and laboratory conditions strengthens the possibility that these conditions determine the prevalence of ticks in certain areas. Understanding the factors which determine the presence of nymphal *I. pacificus* is highly important to understanding the prevalence and transmission of Lyme disease. By knowing the extent that biotic and abiotic factors play in regards to nymph abundance, focused safety and control measures can be implemented to areas where nymphs are known to be prevalent.

Acknowledgements

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