Exploration of Stable Isotope Analysis to Identify Prior Host in *Ixodes scapularis*

By

Imogene Clara Welles

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Abstract

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One of the most enigmatic concepts in tick-borne disease ecology is how to identify the prior host of a questing tick. The ability to do so would provide predictions to directly aid in controlling the spread of the many tick-borne pathogens, including the bacterial spirochete *Borrelia burgdorferi*, which causes Lyme disease in humans. I explored the application of a novel technique, stable isotope analysis (SIA), to identify the most recent host in molted *Ixodes scapularis* (black-legged tick). The common reservoir and feeding host, *Peromyscus leucopus* (white-footed mice; n = 46), were trapped, infested with nymphal ticks, and fed restricted diets, simulating feeding guilds, to confirm previous findings regarding the temporal enrichment of $\delta^{13}C$ and $\delta^{15}N$ in molted adults. Over a feeding period of up to seven days, $\delta^{13}C$ was found to be significantly higher in molted ticks that fed on animals on a corn diet than wild ($p = 0.014$), standard ($p = 0.013$), and meat diets ($p = 0.002$), but was not significantly different in $\delta^{15}N$ (Tukey HSD). To directly test the feasibility of SIA to identify prior hosts, I used isotopic data from multiple years of research to generate a k-means cluster analysis model using isotopic signatures from ticks fed on standard-fed and wild-fed hosts, organized by both feeding guild and species. I then tested the model using field-collected ticks. Seventy-two percent of field-collected ticks fell into the model's five 95% confidence ellipses. I propose the potential application of SIA to the identification of a prior host in questing ticks as an alternative or enhancement to DNA-based methods in the trophic ecology of tick-borne diseases.

Table of Contents

Introduction

Introduction to Lyme Borreliosis and Ixodes scapularis

Ticks are recognized as an opportunistic arthropod vector of a wide variety of aggressive pathogens (Sonenshine, 1991). Diseases caused by tick-borne pathogens include anaplasmosis, babesiosis, ehrlichiosis, Rocky Mountain spotted fever, tularemia, Colorado tick fever, tick-borne relapsing fever, Powassan disease, and others worldwide (CDC, 2017). Causing widespread disease, infection by the bacterial spirochete *Borrelia burgdorferi* creates a cascade of uncomfortable symptoms in affected humans, infamously known as Lyme disease (Sonenshine, 1991). Although once thought to be a relatively new disease, Lyme disease has been associated with tick bites in Europe since the early twentieth century and has likely existed for thousands of years (Ostfeld, 1997). As the most prevalent vector-borne disease in the Northern Hemisphere, it is extremely necessary to identify the cause and effects of Lyme disease, and investigate the overall disease dynamics of this historically endemic yet enigmatic syndrome (Boulanger et al., 2019). Effectively managing the transmission of the disease must be done after completely assessing the ecology which makes the transmission of the spirochete successful.

Following transmission from an infected tick to a human through a bite, a one to three-week incubation period of *B. burgdorferi* occurs (Sonenshine, 1993). During this time, symptoms of Lyme manifest in humans and include sore throat, fever, headache, nausea, and fatigue (Sonenshine, 1993). However, erythema migrans is characteristic of Lyme disease and is often an identifying symptom (Sonenshine, 1993). Appearing before the onset of flu-like symptoms, this bullseye rash surfaces near the site of the tick's bite and persists for up to ten weeks (Sonenshine, 1993). Professionals diagnose Lyme disease using an Elisa assay in conjunction with a Western blot, confirming the presence of IgM or IgG antibodies in patients (Boulanger et al., 2019; CDC, 2017). Treatment includes multiple daily doses of doxycycline, cefuroxime axetil, or amoxicillin (CDC, 2017). Humans may experience more serious long-term effects of Lyme disease, including joint

swelling as well as severe neurological, nervous system, and cardiac maladies (Sonenshine, 1991). These begin to occur six months after infection and treatment, and are medically classified as Post-Treatment Lyme disease syndrome (Eldin et al., 2019).

It has been estimated that a minimum of 240,000 and maximum of 440,000 cases of Lyme disease occur each year in the United States, with the majority of cases going vastly unreported (Ostfeld, 1997; Hinckley et. al, 2014). Ninety-five percent of these cases occur in fourteen states, primarily in the Northeast, yet forty-eight states have reported cases historically (CDC, 2017; Ostfeld, 1997). The black-legged tick, *Ixodes scapularis*, is the primary vector of *B. burgdorferi* (CDC, 2017). In the Northeastern United States, roughly fifty to seventy percent of adult *I. scapularis* carry the bacteria, while twenty-five to thirty-five percent of nymphs do (Ostfeld, 1997). The black-legged tick acquires the bacteria in the larval instar while feeding upon an infected mammalian host, most commonly the white-footed mouse, *Peromyscus leucopus* (Ostfeld, 1997; Magnarelli, 2011). Other species of ticks may ingest the spirochete, but are incompetent vectors, resulting in the death of the bacteria and the unsuccessful infection of the subsequent host (Sonenshine, 1991). Evidently, tick abundance and infection incidence are positively correlated (Mather et al., 1996).

Life Cycle and Feeding of I. scapularis

The life cycle of a tick encompasses four stages: the embryonated egg, larva, nymph, and adult (Sonenshine, 1991). *I. scapularis*, once laid by a female, and hatched into its larval stage, quests for a host and feeds once before undergoing ecdysis and molting into a nymph (Sonenshine, 1991). Again, as a nymph, the tick repeats questing, feeding, and dropping (Sonenshine, 1991). As a sexually dimorphic unfed adult, *I. scapularis* seeks both a host and a mate to complete its life cycle (Sonenshine, 1991). The female will consume a blood meal over one hundred times her unfed body weight during a pre-oviposition period (Sonenshine, 1991). She converts roughly half of her engorged body weight to produce thousands of eggs, and the cycle begins again (Sonenshine, 1991).

In order to feed and successfully molt, ticks must quest for a host, acting as an ambush predator. To do so, ticks passively expose their Haller's organ, located on their dorsal side near their first pair of legs (Rahlenbeck et. al, 2016; Sonenshine, 1991). Consisting of the posterior capsule and anterior pit, the Haller's organ's innervated sensilla provide functionality (Sonsenshine, 258-259). The sensilla detect carbon dioxide, odor, vibrations, and heat, and are crucial for host acquisition (Rahlenbeck et. al, 2016). More sensilla are located on the ventral surface of the tick and are used to identify host and feeding sites (Sonenshine, 1991). Ticks are hematophagous, consuming strictly blood (Boulanger et al., 2019). Tick questing behavior using these sensilla is opportunistic (Rahlenbeck et. al, 2016). However, it has been suggested that small mammals are preferred hosts by larvae and nymphs, larger mammals are preferred by adults, and humans are only accidentally fed upon when invading habitats occupied by ticks (Rahlenbeck et. al, 2016; Boulanger et al., 2019).

Ticks feed by embedding their mouthparts into their host, siphoning blood and returning plasma to the host, leading to transmission, infection, and persistence of *B. burgdorferi* (Rahlenbeck et al., 2016). The feeding process in *I. scapularis* lasts over seventy-two hours, allowing for transmission of *B. burgdorferi* and full engorgement of the tick (Piesman et al., 1986). Once the tick has identified a warm, moist area to initiate feeding, it uses its sharp mouthparts, collectively referred to as the hypostome, to attach to the host (Sonenshine, 1991). Teethlike chelicerae cut further into the host (Sonenshine, 1991). After penetrating the host, antigenic, immunosuppressant saliva acts as a binding cement, fully attaching the tick to the host (Sonenshine, 1991). Ticks have salivary protein variations for feeding on permissive and non-permissive hosts; white-footed mice do not develop a strong immune response to tick bites and do not develop resistance, even after repeated infection (Narasimhan et al., 2019). Additional epigenetic alterations in protein expression are caused by *B. burgdorferi* infection in tick hosts to improve feeding capability and success, and are an interest in disease prevention through the creation of targeted, novel vaccines

(Hovius et al., 2007). Current genomic research continues to investigate the parasite-vector-host relationship to improve human health.

Reservoir Competence and Transmission of B. burgdorferi

After the ingestion of *B. burgdorferi* by a tick from an infected host, the bacteria quickly multiply in the midgut fluids, before entering dormancy in epithelial gut cells (Sonenshine, 1991). This dormant state ends when the tick begins to feed; *B. burgdorferi* migrate to the salivary glands after a few hours of feeding (Sonenshine, 1991). Within twenty-four hours of attachment and feeding by the tick, *B. burgdorferi* can be transmitted to the tick's host (Piesman et al., 1987). The transmission of *B. burgdorferi* depends on the ability of the tick's juvenile host species to infect the tick. This concept, known as realized reservoir competence, describes the contribution to infection of the vector (reservoir potential), based on the probability the host is infected, the spirochete persists, and successfully infects the tick (Mather et al., 1989; Buskrik and Ostfeld, 1995; LoGiudice et al., 2003; Brunner et al., 2008). Further, the probability of transmission of *B. burgdorferi* in competent hosts increases by ten percent after forty-eight hours and seventy percent after seventy-two hours of attachment to the host (Eisen, 2018). This chance increases six-fold when fed upon by multiple infected ticks after forty-eight hours, with possible transmission occurring before twenty-four hours (Eisen, 2018).

Habitats and Hosts of I. scapularis

Both habitat and host availability are crucial aspects in determining tick density, and consequently, prevalence of Lyme disease. Temperature and water vapor pressure are known to indicate an environment's suitability to foster *I. scapularis* populations (Brownstein et. al, 2003). During questing, a tick can easily become desiccated, thus a moist climate is required (Gray, 1998). Incidentally, ticks are likely to quest after a rainstorm when the humidity is high (Rahlenbeck et. al, 2016). Most often, ticks inhabit dense deciduous forests with plentiful hosts, but populations can persist in wetter coniferous woodlands (Gray, 1998). Breakthrough studies conducted by Ostfeld et

al. in 1995 and 1997 revealed that tick populations follow oak and maple mast production due to the cyclical movement of deer seeking food; in this scenario, adult ticks lay eggs after feeding on deer, a non-reservoir host, directly affecting tick populations in defined vegetation (Ostfeld et al., 1995; Ostfeld, 1997). Thus, a combination of habitat and available hosts predict the dynamics of tick populations.

Available hosts in suitable habitats include rodents, small mammals, and birds, but each species has varied competency as both a reproduction host and/or a reservoir host (Gray, 1998). It has been suggested that larvae and nymphs will preferentially feed on rodents or small mammals while adult females will feed on larger mammals such as deer (Gray, 1998). However, it has also been found that *I. scapularis* will feed on a wide range of hosts, including species of raccoon, opossum, skunk, shrew, birds, and squirrels, which have a variety of reservoir competence (LoGiudice et al., 2003). Both survival and fecundity of ticks depends on host availability, while infection rates depend on host competency as reservoirs (Buskirk and Ostfeld, 1995).

Disease Ecology and the Parasite-Vector-Host Relationship

Vectors such as *I. scapularis* have complex ecological roles, occupying different niches and trophic levels of food webs at different stages of their lives by interacting with various host guilds (Lafferty et. al, 2008). Competency of the host ensures transmission of *B. burgdorferi* and is an essential aspect of the parasite-vector-host relationship. Hosts that cannot serve as a vector of the bacterium effectively increase tick population, consequently decreasing the density of competent hosts necessary for persistence of the pathogen (Norman, 1999). Because ticks are opportunistic in their host selection, *B. burgdorferi* infection prevalence depends on host availability and diversity (LoGiudice et al., 2003). These various hosts affect feeding, molting, and infection successes. In conjunction with density dynamics, the Dilution Effect model proposes that infection prevalence is negatively correlated with host diversity (LoGiudice et al., 2003).

To decipher the parasite-vector-host relationship in *I. scapularis*, multiple approaches have been suggested in past studies. DNA isolation methods, such as PCR, have been broadly applied to identify hosts' genus; mitochondrial genes have been exploited in tick blood meal to do so (Kirstein and Gray, 1996). Recently, specific host species have been identified using this method (Wodecka and Skotarczak, 2016). However, stable isotope analysis has emerged as a viable alternative to host species identification, especially if blood meal has degraded and traditional methods cannot be applied; this can assist in unraveling vector-host relationships (Gómez-Díaz and Figuerola, 2010). Stable isotope analysis measures naturally-occurring isotopes, most commonly ^{13}C and ^{15}N (Ben-David and Flaherty, 2012). Isotopes result from additional neutrons around an element's nucleus (Frye, 2006). The variation in isotopes can be exploited to trace element cycling in an ecosystem (Frye, 2006).

Application of Stable Isotope Analysis to the Parasite-Vector-Host Relationship

In stable isotope analysis, ratios of elements are used to describe the isotopic composition of a substance, and consequently, a sample's isotopic signature (Frye, 2006). The ratio of the heavy element to the light element in the sample is compared to the same ratio of a standard, producing $\delta^{13}C$ and $\delta^{15}N$, as used in this study (Frye, 2006; Ben-David and Flaherty, 2012). Natural processes such as photosynthesis and animal metabolic processes fractionate the isotopic content of an element that composes a material, creating a traceable pattern that can suggest an ecological relationship (Frye, 2006). For example, decreasing levels of $\delta^{13}C$ and $\delta^{14}C$ and increasing levels of $\delta^{12}C$ in atmospheric carbon dioxide have been correlated to the increased release of $\delta^{13}C$ and $\delta^{14}C$-depleted fossil fuels due to industrial burning (Frye, 2006; Keeling, 1979; Keeling et al., 2011). Mixing of elements occurs throughout an ecosystem through various interactions, further complicating the resulting identity of a sample's isotopic signature (Frye, 2006). At the producer level, C_3 and C_4 plants have differing carbon-nitrogen ratios and are eaten selectively by consumers (Kelly, 2000; Crawford et al., 2008). This effect reverberates as fractionations occur in chemical

reactions, resulting in various ratios in consumer tissues and biogeographic distribution (Kelly, 2000; Frye, 2006; Ben-David and Flaherty, 2012).

In past and current studies of parasite-vector-host interactions using stable isotope analysis, enrichment of carbon and nitrogen occurs in a positive stepwise function across increasing trophic levels of the food web, indicative of host diet (Layman et al., 2012). Enrichment of isotopes increases across trophic levels according to consumed foods, which are incorporated into tissues: thus, a species can successfully be identified using known ratios (Martínez del Rio and Wolf, 2005; Ben-David and Flaherty, 2012). A bivariate plot of both $\delta^{13}C$ and $\delta^{15}N$ signatures indicates an organism's isotopic niche, which is often, but not always, reflective of its realized niche (Layman et al., 2012; Fig. 1). Limitations in these assumptions originate in variation in carbon and nitrogen, indicating resource availability in a system or host diet preference (Layman et al., 2012). Hosts of the same guild are not distinguishable when consuming identical diets (LoGiudice et al., 2018). Additional limitations relate to the deposition of tissues, occurring at assorted times and at various turnover rates (Crawford et al., 2008). Tissues may be analyzed using a half-life to compensate when analyzing the isotopic niche (Crawford et al., 2008; Layman et al., 2012).

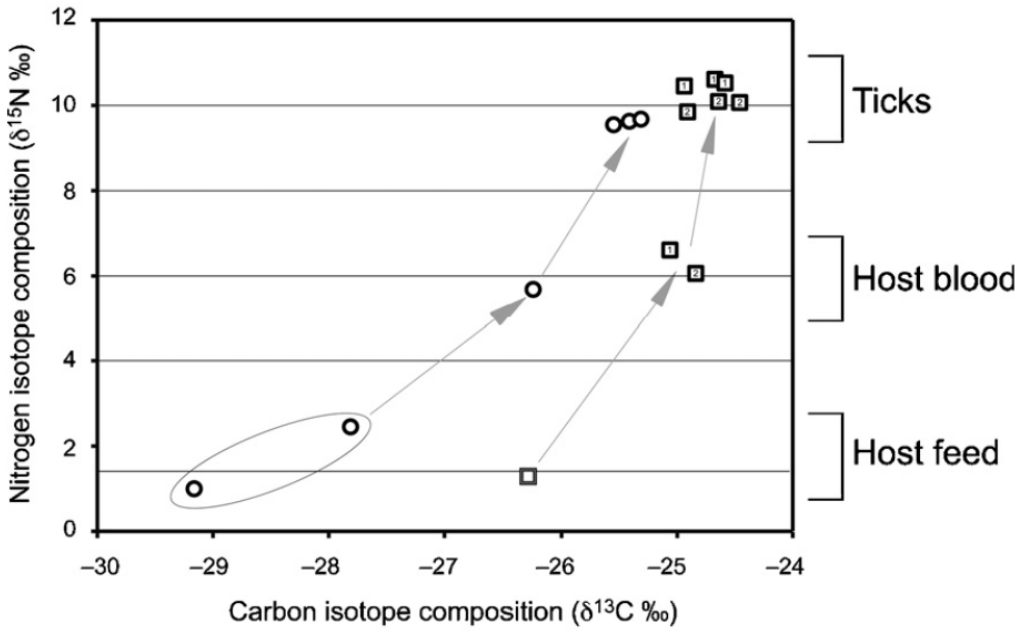

Figure 1: Stable-isotope composition of *I. ricinus* nymphs in relation to host blood and host feed (circles: rabbit; squares: gerbils, numbered 1 and 2). Rabbits were fed compound pellets and hay, gerbils were fed compound pellets only. Schmidt et al. 2011.

In vectors, such as *I. scapularis*, $\delta^{13}C$ and $\delta^{15}N$ obtained from host blood meal are enriched in unfed nymphs after molting from a larvae, and are indicative of the prior host (Schmidt et al., 2011). In particular, molted nymphs that fed on corn-fed hosts for 96 hours produced isotopic signatures indicating the rapid incorporation of ^{13}C into the blood (LoGiudice et al., 2018). The vagility of the isotopic signature, particularly the $\delta^{13}C$ signature, in relation to host diet, is subject for further research. Known patterns of enrichment can also be utilized to estimate tick age since the last molt using stable isotope analysis (Schmidt et al., 2011). Moreover, stable isotope analysis has been suggested as a more accurate method of indicating host species after molting than DNA-based PCR techniques (Hamer et al., 2015).

In this study, we continue the research of LoGiudice et al., 2018, in differentiating between host feeding guilds and ecologically similar host species using stable isotope analysis of *I. scapularis* blood meal. We investigate the timing and effects of enrichment of carbon and nitrogen in the parasite-vector-host relationship based on variation in diet.

Methods

Trapping Data

Two covered traps were set oriented in opposite directions along fallen logs at each of the 68 unique trap sites. Traps were prebaited with oats and left open for twenty-four hours before setting to increase trapping success. Traps were checked within 15 hours of setting. Captured animals were identified, sexed, weighed, aged (using weight and pelage), and ear-tagged. Pregnant and lactating females were released. All other animals were transported to the Union College Vivarium and held for no more than 144 hours, after which they were released at their capture site. Animals were released immediately at the point of capture if they exhibited signs of depression or sickness.

Infestation

Flat nymphs were collected from the Albany Pine Bush Preserve and the Reist Sanctuary in June of 2019. Nymphs were randomly assigned to individual hosts. Upon introduction to the animal care facility, mice were infested with an average of 4 flat nymphs and held for twenty minutes in a handling cone inside a holding bin to allow for attachment. If a flat nymph was in the holding bin after twenty minutes, the nymph was reapplied to the individual and infestation continued for another twenty minutes. Animals were kept in suspended metal cages in plastic holding bins lined with petroleum jelly to prevent tick escape. The bottom of the bin contained a moist paper towel, changed daily during morning checks. Bins were checked and food was refreshed twice daily. An afternoon check was conducted for animal welfare; engorged nymphs were collected during this time in addition to the morning check. Individuals were held for no more than six days. All animal handling and holding protocols were approved by the Institutional Animal Care and Use Committee at Union College, 807 Union St. Schenectady, NY 12308 and were in full compliance with the 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education (Sikes, 2016). Engorged larvae that were not needed for alternative data were

randomized by individual host and diet and allowed to molt in soil cores in the field as part of a separate experiment.

Diet Assignment

During the holding period, animals were randomly assigned to be fed a diet of *i.)* apple (*ad lib.* for hydration), walnut, and sunflower seeds (standard diet), or *ii.)* apple, restricted sunflower seeds, rodent chow, and dried and frozen corn kernels (corn diet), or *iii.)* apple, restricted sunflower seeds, and grasshoppers (*Schistocerca americana*) (insect diet), or *iv.)* apple, restricted sunflower seeds, and grass-fed beef (meat diet) (Table 1). Two forms of beef, ground and chuck, were provided to increase palatability. The corn, insect, and meat diets were also assigned as pulses, switching from the standard diet to the pulse diet on the third day of holding. All foods were provided *ad lib.* except where indicated. Engorged ticks that dropped within 24 hours of capture were assumed to be from a fifth, 'wild' diet. Foods from each assigned diet were stored at −70°C, dried in a drying oven at 60°C for 48 hours and ground to a powder in preparation for stable isotope analysis.

Table 1. 2019 diet treatments. All animals were provided apple (*ad lib.*) for hydration. Instead of *ad lib.*, sunflower seeds were restricted to 10 per day for non-standard diets. Pulse diets received treatment foodstuff on days 3-6 of holding.

Diet	Apple	Sunflower seeds	Rodent chow	Walnut	Dried corn	Frozen corn	Ground beef	Chuck beef	Grasshopper
Standard	x	x	x	x					
Corn	x	x			x	x			
Corn pulse	x	x			x	x			
Meat	x	x					x	x	
Meat pulse	x	x					x	x	
Insect	x	x							x
Insect pulse	x	x							x

Sample Preparation

One batch of molted adults (with a molting percentage of 55%) was prepared for stable isotope analysis. Thirty samples from 25 individuals were dried in a drying oven at 60°C for 48 hours, cooled in a desiccator, and weighed whole in tin cups. Twenty-four tick samples were accidentally destroyed due to drying at a high temperature. Molted nymphs were used as alternatives when no molted adult was produced from the individual host. Three standard diet samples and 5 wild diet samples used nymphs as alternatives to adults. Because the average nymph size (0.07-0.1 mg) is too small to be analyzed with most mass spectrometers, nymphs were used in batches of 2-6 (but see Langel and Dyckmans, 2014).

A batch of foodstuff and bird tissues was prepared for stable isotope analysis. Twenty-four samples of foodstuff (chuck grass-fed beef, ground grass-fed beef, sunflower seeds, rodent chow, dried corn, frozen corn, grasshoppers, and walnuts) were dried in a drying oven at 60°C for 48 hours, cooled in a desiccator, ground to a powder, and weighed whole in tin cups.

Stable Isotope Analysis

Samples were analyzed using a Thermo Delta Advantage mass spectrometer in continuous flow mode connected to a Costech Elemental Analyzer via a ConFlo IV at the Union College Stable Isotope Laboratory. Reference standards (sorghum flour, acetanilide, ammonium sulfate [IAEA-N-2] and caffeine [IAEA-600]) were used for isotopic corrections, and to assign the data to the appropriate isotopic scale. Percent C and N were calculated using additional acetanilide standards of varying mass. Corrections were done using a regression method. The analytical uncertainty for $\delta^{13}C$ (VPDB) is 0.05‰ and $\delta^{15}N$ (Air) is ± 0.1‰, based on 16 Acetanilide standards over four analytical sessions.

Data Selection

Historical data exclusive to the LoGiudice laboratory were included in the final analysis. All samples were ticks fed on known hosts on known diets, and processed comparably to this study. This included 9 wild-fed Eastern chipmunk (*Tamias striatus*) samples from 2017 (three samples were means of multiple ticks fed on the same individual), 70 samples from 2015 (19 corn-fed white-footed mouse samples, 18 standard-fed white-footed mouse samples, 22 corn-fed Eastern chipmunk samples, and 11 standard-fed Eastern chipmunk samples), and 30 samples collected in 2002 in the Ostfeld laboratory (R.S. Ostfeld, Cary Institute of Ecosystem Studies; (all standard-fed, 7 samples from 3 North American opossums (*Didelphis virginiana*), 6 samples from 2 striped skunks (*Mephitis mephitis*), 9 samples from 3 raccoons (*Procyon lotor*), 5 samples from 2 Eastern gray squirrels (*Sciurus carolinensis*), and 3 samples from 1 red squirrel (*Tamiasciurus hudsonicus*))) (Table 2). Standard-fed, wild-fed, and corn-fed samples were analyzed to test the findings of LoGiudice et al. (2018).

Data Analysis: Model Creation

Host feeding guild (herbivore, omnivore, fungivore) was analyzed separately from species. Herbivores were considered to be the Eastern chipmunk, the white-footed mouse, and the Eastern gray squirrel. Fungivores were considered to be the red squirrel. Omnivores were considered to be the North American opossum, the striped skunk, and the raccoon. Variations of a five, k-mean cluster analysis model were created in R using standard-fed and wild-fed by host feeding guild and species (Appendix B, 1). The k-means cluster analysis assigned observations into clusters of the nearest mean with 95% probability using the standard algorithm (Lloyd's algorithm) to assign centroids as new means until convergence was reached for each feeding guild and species (see Kanungo et al., 2000).

Table 2. Prepared samples and their applications. Diets: neutral (standard, N), corn (C), wild (W), insect (I), meat (M), corn pulse (CP), insect pulse (IP), and meat pulse (MP).

Year	Host: Common name	Host: Scientific name	Host: Abb.	Host: Feeding guild	Diet	Individuals	*n* =	Species model	Guild model	Diet significance tests	Host + corn (Fig. 2)	Comments
2002	North American opossum	*Didelphis virginiana*	DIVI	Omni.	N	3	7	x	x			
	Striped skunk	*Mephitis mephitis*	MEME	Omni.	N	2	6	x	x			
	Raccoon	*Procyon lotor*	PRLO	Omni.	N	3	9	x	x			
	Eastern gray squirrel	*Sciurus carolinensis*	SCCA	Herb.	N	2	5	x	x			
	Red squirrel	*Tamiasciurus hudsonicus*	TAHU	Fungi.	N	1	3	x	x			
2015	Eastern chipmunk	*Tamias striatus*	TAST	Herb.	C		22					
					N		11	x	x			
	White-footed mouse	*Peromyscus leucopus*	PELE	Herb.	C		19					
					N		18	x	x			
2017	Eastern chipmunk	*Tamias striatus*	TAST	Herb.	W	8	15	x	x			Only ticks from wild-fed hosts were used to create models. Captured hosts were heavily infested with engorged nymphs. All diet treatments were considered to be pulses because dropped ticks could not be identified as wild-fed or treatment-fed. Signatures of tick considered to be of a treatment diet are thus unreliable data.
					N	4	6					
					C	3	4					
					I	2	6					
					M	2	3					
2019	White-footed mouse	*Peromyscus leucopus*	PELE	Herb.	W	4	4	x	x	x	x	
					N	4	5	x	x	x	x	
					C	5	5			x	x	Only data from 2019 were used to analyze diet enrichment.
					CP	2	2					
					I	5	5			x		
					IP	2	2					
					M	3	3			x		
					MP	1	1					

Results

2019 Trapping Success

Forty-six white-footed mice (*Peromyscus leucopus*) (40 male, 6 female, with 2 male and 1 female likely recaptured) were captured in Sherman traps baited with oats from 29 July 2019 through 27 August 2019 in the Reist Sanctuary (Schenectady County, NY, USA) for 350 trap nights with a 0.21 catch per unit effort.

Fur Sampling

I collected fur samples from each animal on the dorsal rump for future isotopic analysis of a different turnover time. This separate experiment is ongoing (Appendix B, 1).

Significance of Diets

I confirm the findings of LoGiudice et al. (2018) that the carbon isotopic signature of molted tick blood meal is reflective of the prior host's diet on an immediate time scale of as little as 4 days. Using our data from 2019, an ANOVA shows significant differences in $\delta^{13}C$ between the corn-fed, insect-fed, meat-fed, standard-fed, and wild-fed ticks (ANOVA, $p = 0.002$) (Table 3). Pairwise comparisons (Tukey HSD) reveal that corn-fed ticks have significantly higher $\delta^{13}C$ than meat-fed ticks ($p = 0.002$), standard-fed ticks ($p = 0.01$), and wild-fed ticks ($p = 0.01$), with no differences between the other pairs. This corroborates the findings of LoGiudice et al. (2018) that enrichment of ^{13}C occurs in ticks that fed on corn-fed hosts over a feeding period of 4 days or less. The 2019 data suggest that $\delta^{15}N$ is not significantly different across diets (ANOVA, $p > 0.1$). There is a discernible distinction in both $\delta^{13}C$ and $\delta^{15}N$ between species fed on corn and those that were not (Fig. 2).

Table 3. Connecting levels report for diets in $\delta^{13}C$ (Tukey HSD). Diets: corn (C), insect (I), wild (W), neutral (standard, N), and meat (M).

Level			Least Squares Mean
Corn	A		-22.15
Insect	A	B	-24.32
Wild		B	-24.92
Neutral		B	-24.94
Meat		B	-26.11

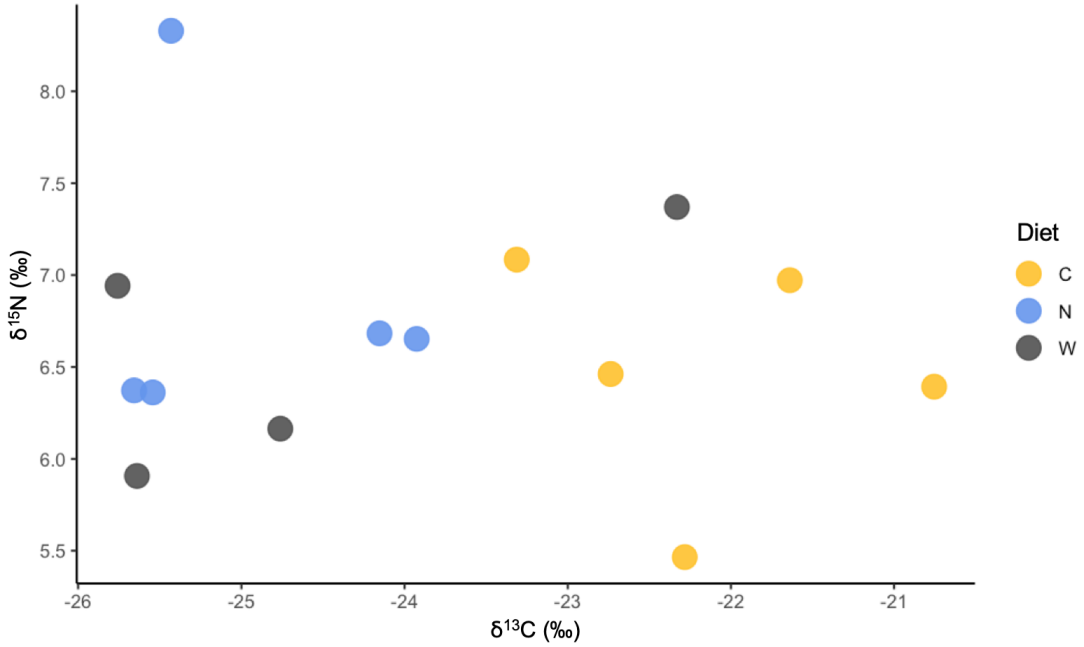

Figure 2. $\delta^{13}C$ and $\delta^{15}N$ of ticks fed on known hosts (white-footed mouse) from the 2019 data. Diets: corn (C), neutral (standard, N), and wild (W).

Applying the Model

The ultimate aim of this project is to determine whether stable isotope analysis can be used to discern the identity of the previous host of field collected ticks. Using the historical data, I created a five-cluster analysis model in R, using means and a confusion matrix (Appendix B, 2 and 3). Only ticks fed on hosts on standard and wild diets were used to create the model. The clusters were created with 95% confidence ellipses (see Syväranta et al., 2013). A five-cluster analysis was

chosen for analyzing host feeding guilds and species due to the accuracy of assignment and best fit for the data.

Identification of Prior Host Feeding Guild and Species

The model successfully assigned fungivores and omnivores to their respective clusters 100% of the time. Other herbivores were split into three clusters, assigned successfully 42%, 19%, and 37% of the time, with 2% being assigned to the fungivore cluster (Fig. 3).

The model successfully assigned red squirrel ticks to the same cluster 100% of the time. It assigned the North American opossum, striped skunk, and raccoon-fed ticks to a cluster with 100% accuracy. It assigned ticks fed on the Eastern gray squirrel to a cluster 100% of the time, shared with the Eastern chipmunk (30% accuracy) and the white-footed mouse (30% accuracy). Two other clusters were composed of the white-footed mouse-fed ticks (33% accuracy) and the Eastern chipmunk-fed ticks (65% accuracy), and the white-footed mouse-fed ticks (33% accuracy) and the Eastern chipmunk-fed ticks (5% accuracy) (Fig. 3).

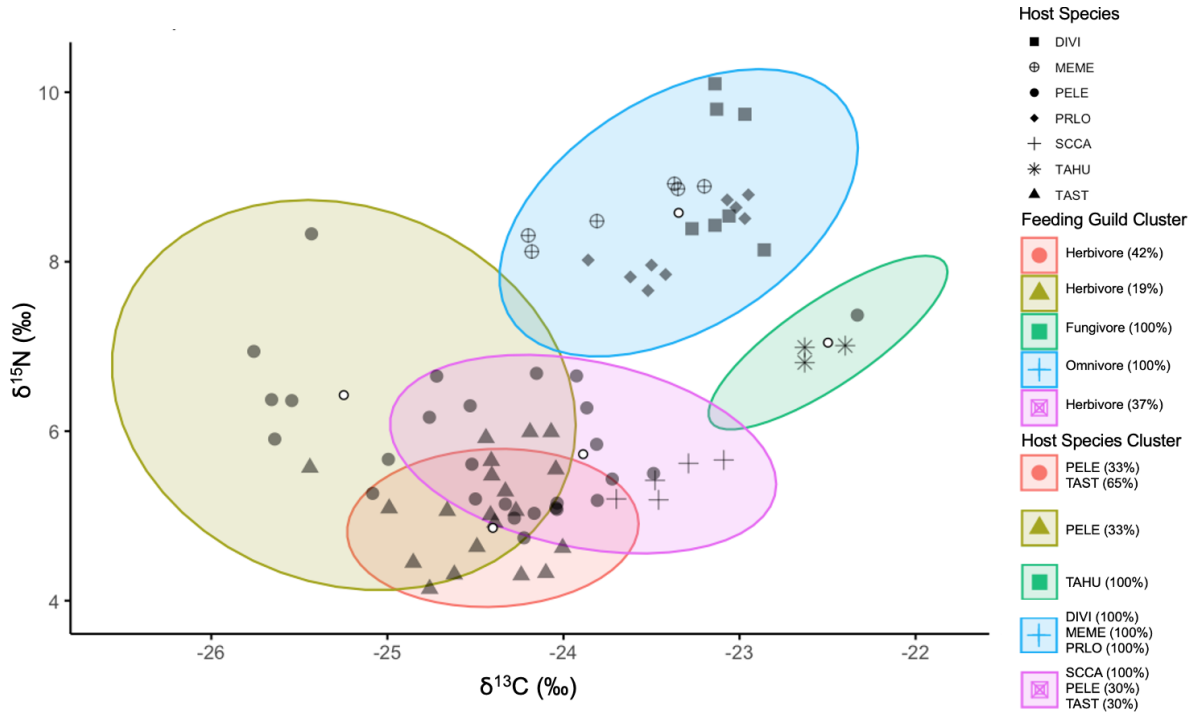

Figure 3. Five-cluster analysis of $\delta^{13}C$ and $\delta^{15}N$ of ticks fed on hosts consuming a standard or wild diet, organized by feeding guild and species. Multivariate normal distribution assumed: all guilds except fungivore/red squirrel (TAHU) (n =3) fit a normal distribution. Black points indicate samples, white points indicate means.

Distribution of Samples

The model distributed 77 samples across the five clusters (Fig. 3, Table 4). The most dense (non-omnivore) area of the model was the three-way overlap of the three herbivore clusters (19%, 37%, and 42%), accounting for 23% of herbivore samples. This area corresponds to the three-way overlap between white-footed mouse (33%)/Eastern gray squirrel (100%), white-footed mouse (30%)/Eastern chipmunk (30%), white-footed mouse (33%), and Eastern chipmunk (65%) clusters, encompassing 35% of Eastern chipmunk and 19% of white-footed mouse samples. This area accounted for 16% of all known data. The second most dense area was the herbivore (37%) cluster, accounting for 17% of herbivore samples. This area corresponds with the Eastern gray squirrel (100%), white-footed mouse (30%), and Eastern chipmunk (30%) cluster, encompassing 19% of white-footed mouse and 80% of Eastern gray squirrel samples. This area accounts for 12% of all known samples.

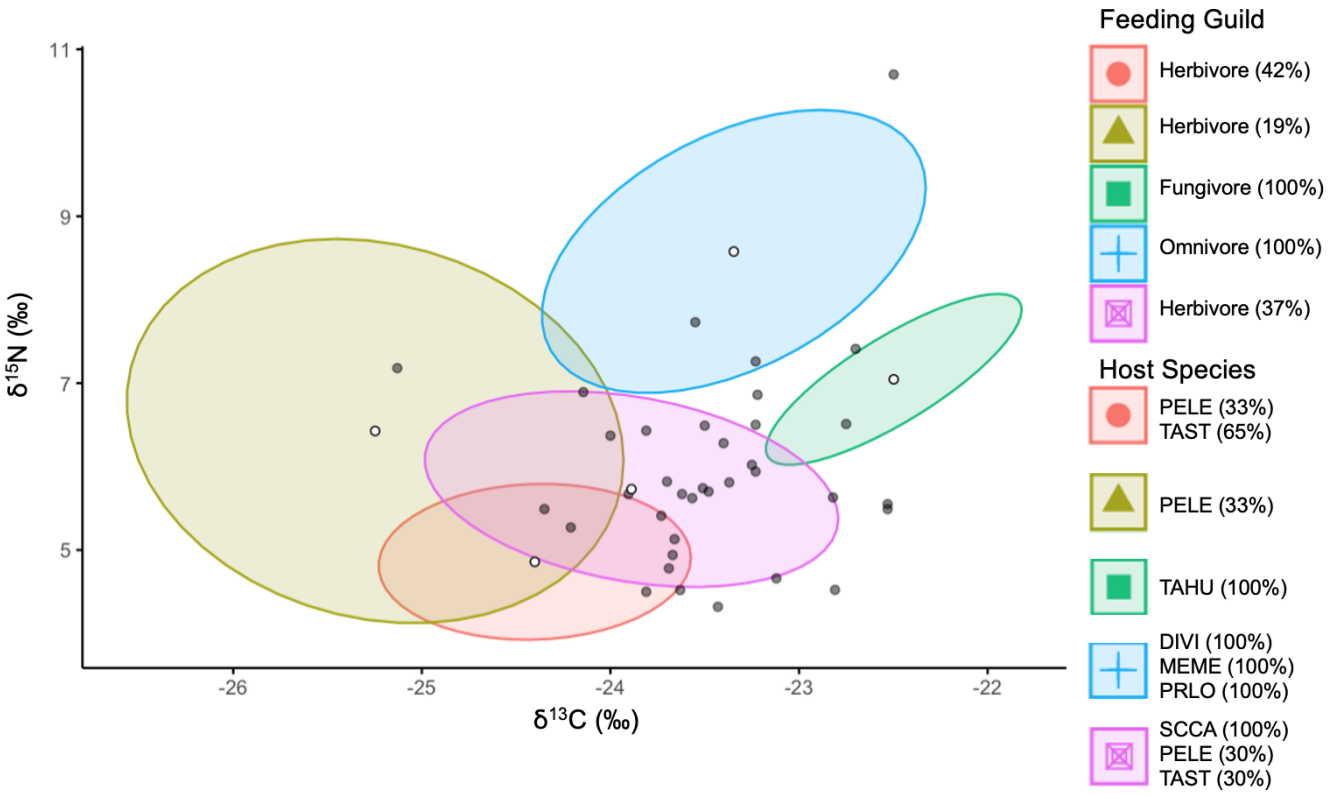

Figure 4. Five-cluster analysis of $\delta^{13}C$ and $\delta^{15}N$ of ticks fed on standard-fed or wild-fed hosts by feeding guild and species, overlaid by signatures of field-collected ticks fed on an unknown host. White points indicate cluster means.

Isotopic signatures of field-collected ticks fed on unknown hosts were overlaid on the cluster model (Fig. 4). Seventy-two percent of unknown ticks fell into a feeding guild or species cluster, while 28% off samples did not fall into a cluster (Table 4). However, when using only convex hulls, 36% of ticks fed on an unknown host fell into a cluster (see Syväranta et al., 2013, Appendix A, Fig. A). The majority field-collected ticks fell into the herbivore (37%) cluster for guild, and the Eastern gray squirrel (100%), white-footed mouse (30%), and Eastern chipmunk (30%) cluster (Fig. 3).

Table 4. Distribution of unknown ticks using the four-cluster analysis model.

Color	Guild cluster (accuracy*)	n in guild	Percent of host guild	Species cluster (accuracy*)	n knowns assigned	Percent of host species	Percent of all knowns	n unknowns assigned	Percent unknowns assigned
Olive	Herbivore (19%)	7 herbivore	13% of herbivore	PELE (33%)	6 PELE 1 TAST	22% of PELE 5% of TAST	9%	1	3%
Blue	Omnivore (100%)	22 omnivore	100% of omnivore	DIVI (100%) MEME (100%) PRLO (100%)	6 MEME 9 PRLO 7 DIVI	100% of MEME 100% of PRLO 100% of DIVI	29%	2	6%
Purple	Herbivore (37%)	9 herbivore	17% of herbivore	SCCA (100%) PELE (30%) TAST (30%)	5 PELE 4 SCCA	19% of PELE 80% of SCCA	12%	13	36%
Red	Herbivore (42%)	4 herbivore	8% of herbivore	PELE (33%) TAST (65%)	4 TAST	20% of TAST	5%	1	3%
Green	Fungivore (100%)	3 fungivore 1 herbivore	100% of fungivore 2% of herbivore	TAHU (100%)	3 TAHU 1 PELE	100% of TAHU 4% of PELE	5%	1	3%
Olive + purple	Herbivore (19%) + Herbivore (37%)	7 herbivore	13% of herbivore	PELE (33%) + SCCA (100%) PELE (30%) TAST (30%)	4 PELE 3 TAST	15% of PELE 15% of TAST	9%	2	6%
Olive + red	Herbivore (19%) + Herbivore (42%)	7 herbivore	13% of herbivore	PELE (33%) + PELE (33%) TAST (65%)	5 TAST 2 PELE	25% of TAST 7% of PELE	9%	0	0%
Olive + purple + red	Herbivore (19%) + Herbivore (37%) + Herbivore (42%)	12 herbivore	23% of herbivore	PELE (33%) + SCCA (100%) PELE (30%) TAST (30%) + PELE (33%) TAST (65%)	7 TAST 5 PELE	35% of TAST 19% of PELE	16%	2	6%
Purple + red	Herbivore (37%) + Herbivore (42%)	5 herbivore	10% of herbivore	SCCA (100%) PELE (30%) TAST (30%) + PELE (33%) TAST (65%)	4 PELE 1 SCCA	15% of PELE 20% of SCCA	6%	4	11%

* The accuracy with which the model assigned the data points to their actual guild or species.

Discussion

Model Success

It is apparent that the model consists of two defined clusters: ticks that fed on omnivorous mesopredators with medium-high $\delta^{13}C$ and $\delta^{15}N$, and those that fed on herbivorous rodents with low-medium $\delta^{13}C$ and $\delta^{15}N$. The mesopredators were assigned to their cluster with 100% accuracy, while the herbivores were spread across three clusters with varying degrees of accuracy, supporting the claim that the two distinct clusters exist. For the rodent samples, the chipmunk and white-footed mice samples are not distinguishable from each other isotopically (LoGiudice et al., 2018). The chipmunk and white-footed mouse ticks fell into the three clusters, while the gray squirrel ticks fell into only one of the three. Additionally, the chipmunk and white-footed mouse samples overlap significantly, while the gray squirrel samples have higher $\delta^{13}C$ on the x-axis. However, with the $\delta^{15}N$ data constructing the y-axis, the two main clusters are distinguishable when organized by feeding guild instead of species.

Distribution of Field-Collected Ticks Across Clusters

Using the clusters constructed in the model, I estimate that up to 67% of the field-collected adult ticks fed on herbivores or rodents (Table 3). Additionally, I estimate that only 6% of field-collected ticks fed on omnivores, supporting the claim that nymphal ticks primarily feed on small mammals. Only one sample with increased enrichment of both ^{13}C and ^{15}N fell outside of the omnivore cluster, likely having fed on a carnivore. These findings are in agreement with those of LoGiudice et al., 2003, which estimated that between 26% and 63% of ticks fed on herbivores and between 6% and 3% of ticks fed on omnivores as nymphs, depending on the host community composition (Appendix A, Table C). This confirms the acknowledged importance of rodents as dynamic hosts (Ostfeld, 1997; Magnarelli, 2011).

Model Limitations

Although the model was successful, it is not without significant limitations. While no herbivore cluster overlaps with the omnivore cluster and vice-versa, the distribution of the rodents is variable across the three herbivore clusters (Fig. 4). The separation of clusters by feeding guild and overlap by species is suggestive of a successful construction of clusters by feeding guild and inconclusive construction of clusters by species. Further, the ability to conclude an adult tick's prior host using stable isotope analysis is limited to feeding guild using this current model.

The sample sizes used to create the model and the applied field-collected samples are both relatively small, causing more limitations. Because of this, it is difficult to discriminate between true sample similarity and a limited range of signatures. Pseudoreplication also contributes to false accuracy. By using multiple ticks fed on the same individual, natural variation between individuals is limited. Because of this, clusters may appear to be overly accurate. Pseudoreplicates are included in the model due to limited data and may falsely strengthen the model's accuracy. Further, the omnivore samples came from three or two individuals while the fungivore samples came from a single individual. Consequently, the fungivore/red squirrel cluster must be interpreted with caution and reflects the individual, not the species or guild in this model. However, species-specific differences in isotopic signature appear to be influencing the accuracy and function of the model as well.

When the field-collected ticks are applied to the model, 72% of field-collected ticks fall within the bounds of a cluster, 30% of those inside clusters lay near or on an edge. This variation suggests weaknesses in the model due to its construction using small sample sizes and lack of host diversity. A final caveat is that the model was created using both nymphal and adult ticks, which may be a confounding variable. However, the model did not create significantly different clusters between the two instars and does not indicate a difference between isotopic signatures.

Effect of Diet on Model Construction

While the wild and standard diet ticks are not statistically different in both $\delta^{13}C$ and $\delta^{15}N$, both treatments were used to construct the model (Appendix A, Table A, Table B). The field-collected ticks fed exclusively on animals on wild diets. This natural diet of small mammals in the wild is opportunistic and prone to disturbance by enriched foodstuff. As a brief yet broad literature review of each species' diet, mesopredators consume mammals and amphibians, insects, and birds and their eggs while rodents consume acorns, seeds, arthropods and insects, and fungi (Appendix A, Table D). The rodents in this study are generally more opportunistic than the mesopredators, acting opportunistically omnivorous (Rose et al., 2014). This behavior in rodents makes them prone to interference in isotopic enrichment, particularly in $\delta^{15}N$ due to protein in animal tissue. Consequently, field-collected ticks that fed on these small mammals would also be enriched.

Recognizing the significant enrichment of $\delta^{13}C$ in the signatures of ticks fed on hosts on a corn diet after three days or more, the opportunistic granivore/herbivores of Rodentia most likely occupy a larger range of $\delta^{13}C$ than mesopredator omnivores due to inclinations to consume C4 plants. C3 plants commonly occupy a $\delta^{13}C$ range of -25‰ to -29‰, while C4 plants span -11‰ to -16‰ (O'Leary, 1988; unpublished data). The consumption of both endemic and exotic C3 and C4 plants would alter the $\delta^{13}C$ signature of respective ticks to the degree with which the prior host ate the plant. There are few native C4 plants in New York state; it is estimated only 0.5% of flora are (Still and Berry, 2003). However, invasives, agriculture, and human activity may expose small mammals to C4 foodstuff, increasing the respective $\delta^{13}C$ isotopic signature. The relatively larger sample size of herbivore samples in the model's creation also widens this range.

Host Specificity and Further Remarks

The model produced does not account for the isotopic signatures of other important hosts, such as short-tailed shrews (*Blarina brevicauda*) and white-tailed deer (*Odocoileus virginianus*), which are hypothesized to be important yet elusive reservoir hosts, feeding, and/or transport hosts

for larval ticks (Brisson et al., 2008; Telford et al., 1990; Kugeler et al., 2015). In the case of the white-tailed deer, it has been observed that the red deer (*Cervus elaphus*) functions as a transport host for the enhanced dispersal of nymphal castor bean tick (*Ixodes ricinus*) in addition to being known adult hosts (Qviller et al., 2016). This commensalism may also exist between the nymphal blacklegged tick and the white-tailed deer. Additionally, it has been suggested that up to 29% of larvae can feed on white-tailed deer, in comparison to 44% fed on white-footed mice at this life stage (Huang et al., 2019). White-tailed deer may dilute infection of nymphs in this role, but may foster population growth of ticks as their adult host (Huang et al., 2019).

Shrews have been suggested as even more significant hosts than white-footed mice and may feed 35% of ticks and 55% of ticks infected with *B. burgdorferi*, at least when other rodent host populations crash (Brisson et al., 2008). LoGiudice et al., 2003 estimated that between 66% and 33% of nymphs fed on shrews, depending on low and high mouse/chipmunk densities (Appendix A, Table C). During population crashes of these hosts, shrews function as "rescue hosts" and may contribute to persisting tick populations and perpetuate infection by feeding multiple life stages of ticks (LoGiudice et al., 2003). Knowing that shrews are insectivorous, consuming primarily earthworms and grains, their isotopic signature would likely be enriched in both $\delta^{13}C$ and $\delta^{15}N$ (Babcock, 1914). Adding this data to the model would disrupt the omnivorous mesopredator cluster, potentially creating a new insectivorous cluster.

Both mice and shrews must be functioning as dual larval and nymphal hosts to perpetuate *B. burgdorferi* in a given ecosystem. Close research must continue to investigate which hosts and in what percent contribute to ongoing infection of ticks. With climate change and human health having the most impact on public policy, efforts in disease ecology must continue to extricate complex relationships, including those of *I. scapularis* and its various dynamic hosts.

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Appendix A

Table A. Connecting levels report for diets and foodstuff in $\delta^{13}C$ (Tukey HSD).

Level									Least squares mean
Dried Corn	A								-10.81812
Frozen Corn	A								-11.54227
Rodent Chow		B	C						-22.05351
Corn Diet		B							-22.14538
Ground Beef		B	C	D					-23.66143
Insect Diet			C	D	E				-24.31741
Wild Diet				D	E	F			-24.91946
Neutral Diet				D	E	F			-24.94205
Meat Diet					E	F	G		-26.10773
Chuck Beef					E	F	G	H	-26.52452
Grasshopper						F	G	H	-27.09667
Sunflower Seeds							G	H	-28.22692
Walnut								H	-28.58250

Table B. Connecting levels report for diets and foodstuff in $\delta^{15}N$ (Tukey HSD).

Level									Least squares mean
Meat Diet	A								7.845989
Insect Diet	A								7.642376
Neutral Diet	A	B							6.879528
Wild Diet	A	B							6.706045
Corn Diet	A	B	C						6.475058
Ground Beef			C	D					4.845714
Chuck Beef		B	C	D	E				4.67567
Dried Corn				D	E	F			3.047977
Sunflower Seeds				D	E	F	G		2.683231
Grasshopper					E	F	G		2.126667
Rodent Chow						F	G	H	0.826891
Walnut							G	H	0.535
Frozen Corn								H	-0.598732

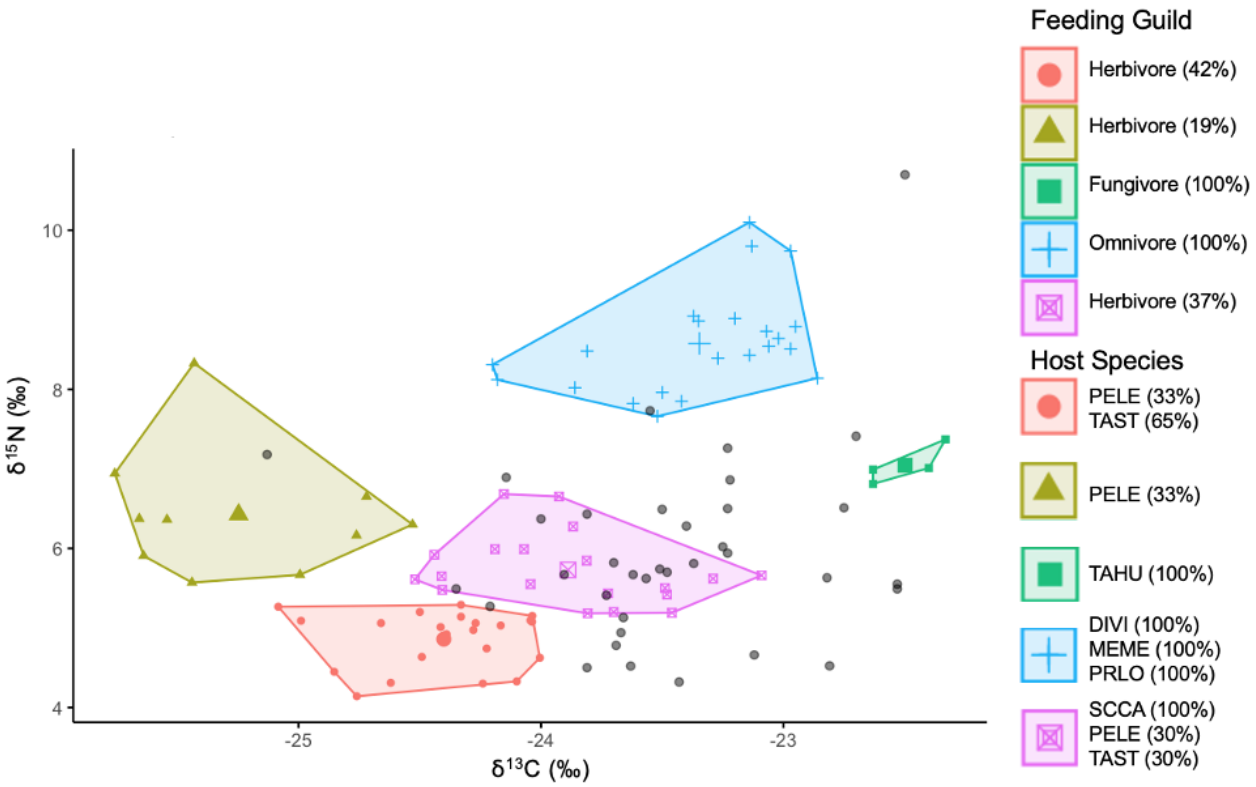

Figure A. Five-cluster analysis of $\delta^{13}C$ and $\delta^{15}N$ of ticks fed on standard-fed or wild-fed hosts by feeding guild, overlaid by signatures of field-collected ticks of an unknown prior host in black. Clusters are created as convex hulls. Smaller points indicate samples, larger points indicate means.

Table C. Effect of mouse and chipmunk density on tick density and distribution across hosts in a community. Adapted from LoGiudice et al., 2003.

	Low mouse and chipmunk density		Average mouse and chipmunk density		High mouse and chipmunk density		Body burden				
Species	Body burden * animal density	Body burden / total ticks per hectare * 100 (%)	Body burden * animal density	Body burden / total ticks per hectare * 100 (%)	Body burden * animal density	Body burden / total ticks per hectare * 100 (%)	Mean	(SE)	N	Density, no. per hectare	Density source
White-footed mouse	0	0	1390	20.45	2780	30.60	27.8	-3.3	28	100	Original data
Eastern chipmunk	0	0	900	13.24	1800	19.81	36	-11	57	50	Original data
White-tailed deer	59.75	1.33	59.75	0.88	59.75	0.66	239	-99	12	0.25	Original data
Raccoon	25.4	0.56	25.4	0.37	25.4	0.28	127	-30	12	0.2	Literature
Virginia opossum	254	5.64	254	3.74	254	2.80	254	-115	21	1	Literature
Striped skunk	3.34	0.07	3.34	0.05	3.34	0.04	66.8	-12.7	4	0.05	Literature
Short-tailed shrew	1572.5	34.89	1572.5	23.14	1572.5	17.31	62.9	-17.3	42	25	Literature
Birds	53.72	1.19	53.72	0.79	53.72	0.59	1.7	-0.4	74	31.6	Original and literature data
Sorex shrews	1387.5	30.79	1387.5	20.42	1387.5	15.27	55.5	-32	8	25	Literature
Red and gray squirrel	1150.2	25.52	1150.2	16.92	1150.2	12.66	142	-58	10	8.1	Literature
Total ticks in hectare	4506		6796		9086						
% ticks from herbivores	25.52		50.62		63.06						
% ticks from omnivores	6.27		4.16		3.11						
% ticks from shrews	65.68		43.55		32.58						

Table D. Literature review of mesopredator and rodent diets.

Species	Feeding guild	Primary foodstuff	Source
North American opossum	Omni.	Fruit, amphibians, mammals	McRuer and Jones, 2009
Striped skunk	Omni.	Insects, birds and their eggs, mammals	Azevedo et al., 2006
Raccoon	Omni.	Birds and their eggs, wheat seeds, insects	Azevedo et al., 2006
Red squirrel	Fungi.	Pine and conifer seeds, fungi, buds	Krauze-Gryz and Gryz, 2015
Eastern gray squirrel	Herb.	Acorns and pine cones	Spritzer, 2002
Eastern chipmunk	Herb.	Acorns, insects, fungi	Wrazen and Svendsen, 1978
White-footed mouse	Herb.	Fruits, seeds, arthropods	Rose et al., 2014

Appendix B

1. One short-tailed shrew (*Blarina brevicauda*) individual was captured, sampled, and released during trapping, in addition to acquiring fur samples from five frozen short-tailed shrews with a post-mortem interval ranging from 28 September 2017 to 22 October 2019. Fur was cleaned using a 2:1 chloroform-methanol solution. Samples soaked in solution for 10 to 15 minutes, stirred occasionally, and filtered through stainless steel mesh using a vacuum pump. This was repeated two more times with a final rinse using the 2:1 chloroform-methanol solution before drying under the stainless steel mesh for 48 hours.

2. Code for the guild cluster analysis model using R by host guild.

```{r}
library(ClusterR)
x=Known_ticks[7:8]
x=na.omit(x)
summary(x)
y=Known_ticks[3]
dat=center_scale(x, mean_center = T, sd_scale = T)
gmm = GMM(dat, 2, dist_mode = "maha_dist", seed_mode = "random_subset", km_iter = 10, em_iter = 10, verbose = F)

pr = predict_GMM(dat, gmm$centroids, gmm$covariance_matrices, gmm$weights)

cluster4=kmeans(x, 4)
cluster3=kmeans(x, 3)
cluster5=kmeans(x, 5)
cluster6=kmeans(x, 6)
cluster2=kmeans(x, 2)

calculate.confusion2 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$guild, cluster = cluster2$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:3) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:3,1]
 return(pc)
}

calculate.confusion6 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$guild, cluster = cluster6$cluster)
```

```
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:3) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:3,1]
 return(pc)
}
calculate.confusion5 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$guild, cluster = cluster5$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:3) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:3,1]
 return(pc)
}
calculate.confusion4 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$guild, cluster = cluster4$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:3) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:3,1]
 return(pc)
}
calculate.confusion3 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$guild, cluster = cluster3$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:3) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:3,1]
 return(pc)
}
calculate.confusion6(Known_ticks$guild,cluster6$cluster)
calculate.confusion5(Known_ticks$guild,cluster5$cluster)
calculate.confusion4(Known_ticks$guild,cluster4$cluster)
calculate.confusion3(Known_ticks$guild,cluster3$cluster)
calculate.confusion2(Known_ticks$guild,cluster2$cluster)
```

```
d13C           d15N
 Min.   :-25.76   Min.   : 4.140
 1st Qu.:-24.44   1st Qu.: 5.183
 Median :-24.04   Median : 5.920
 Mean   :-23.98   Mean   : 6.453
 3rd Qu.:-23.37   3rd Qu.: 7.960
 Max.   :-22.33   Max.   :10.100
        [,1]     [,2]     [,3]     [,4]     [,5]     [,6]
Fung 0.0000000 0.0000000 0.0000000 0.0000000 0.0000000 1.0000000
Herb 0.1914894 0.1914894 0.4255319 0.1702128 0.0000000 0.0212766
Omni 0.0000000 0.1851852 0.0000000 0.0000000 0.6296296 0.1851852
        [,1]     [,2]     [,3]     [,4]     [,5]
Fung 0.0000000 0.000000 1.0000000 0.0000000 0.0000000
Herb 0.4680851 0.212766 0.0212766 0.0000000 0.2978723
Omni 0.0000000 0.000000 0.0000000 0.8148148 0.1851852
        [,1]     [,2]      [,3]     [,4]
Fung 0.0000000 1.0000000 0.00000000 0.0000000
Herb 0.0212766 0.1489362 0.59574468 0.2340426
Omni 0.8148148 0.1111111 0.07407407 0.0000000
         [,1]      [,2]     [,3]
Fung 0.00000000 0.00000000 1.0000000
Herb 0.04255319 0.74468085 0.2127660
Omni 0.81481481 0.03703704 0.1481481
         [,1]     [,2]
Fung 1.00000000 0.0000000
Herb 0.04255319 0.9574468
Omni 0.81481481 0.1851852
```

3. Code for the host cluster analysis model using R by host species.

```{r}
library(ClusterR)
x=Known_ticks[7:8]
x=na.omit(x)
summary(x)
y=Known_ticks[2]
dat=center_scale(x, mean_center = T, sd_scale = T)
gmm = GMM(dat, 2, dist_mode = "maha_dist", seed_mode = "random_subset", km_iter = 10, em_iter = 10, verbose = F)

pr = predict_GMM(dat, gmm$centroids, gmm$covariance_matrices, gmm$weights)

cluster4=kmeans(x, 4)
cluster3=kmeans(x, 3)
cluster5=kmeans(x, 5)
cluster6=kmeans(x, 6)
cluster7=kmeans(x, 7)


calculate.confusion7 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$host, cluster = cluster7$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:7) # 9 labels
 {
```

```
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:7,1]
 return(pc)
}

calculate.confusion6 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$host, cluster = cluster6$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:7) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:7,1]
 return(pc)
}
calculate.confusion5 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$host, cluster = cluster5$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:7) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:7,1]
 return(pc)
}
calculate.confusion4 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$host, cluster = cluster4$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:7) # 9 labels
 {
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:7,1]
 return(pc)
}
calculate.confusion3 <- function(states, clusters)
{
 # generate a confusion matrix of cols C versus states S
 d <- data.frame(state = Known_ticks$host, cluster = cluster3$cluster)
 td <- as.data.frame(table(d))
 # convert from raw counts to percentage of each label
 pc <- matrix(ncol=max(clusters),nrow=0) # k cols
 for (i in 1:7) # 9 labels
 {
```

```
  total <- sum(td[td$state==td$state[i],3])
  pc <- rbind(pc, td[td$state==td$state[i],3]/total)
 }
 rownames(pc) <- td[1:7,1]
 return(pc)
}

calculate.confusion7(Known_ticks$host,cluster7$cluster)
calculate.confusion6(Known_ticks$host,cluster6$cluster)
calculate.confusion5(Known_ticks$host,cluster5$cluster)
calculate.confusion4(Known_ticks$host,cluster4$cluster)
calculate.confusion3(Known_ticks$host,cluster3$cluster)
```
```
d13C           d15N
 Min.   :-25.76   Min.   : 4.140
 1st Qu.:-24.44   1st Qu.: 5.183
 Median :-24.04   Median : 5.920
 Mean   :-23.98   Mean   : 6.453
 3rd Qu.:-23.37   3rd Qu.: 7.960
 Max.   :-22.33   Max.   :10.100
        [,1]      [,2]      [,3]      [,4]      [,5]      [,6]      [,7]
DIVI 0.8571429 0.0000000 0.14285714 0.00000000 0.0000000 0.0000000 0.0000000
MEME 1.0000000 0.0000000 0.00000000 0.00000000 0.0000000 0.0000000 0.0000000
PELE 0.0000000 0.1481481 0.03703704 0.03703704 0.3703704 0.1851852 0.2222222
PRLO 0.5555556 0.0000000 0.44444444 0.00000000 0.0000000 0.0000000 0.0000000
SCCA 0.0000000 1.0000000 0.00000000 0.00000000 0.0000000 0.0000000 0.0000000
TAHU 0.0000000 0.0000000 1.00000000 0.00000000 0.0000000 0.0000000 0.0000000
TAST 0.0000000 0.0500000 0.00000000 0.35000000 0.4500000 0.0000000 0.1500000
        [,1]      [,2]      [,3]      [,4]      [,5]      [,6]
DIVI 0.0000000 0.0000000 0.0000000 0.00000000 0.8571429 0.14285714
MEME 0.0000000 0.0000000 0.0000000 0.00000000 1.0000000 0.00000000
PELE 0.3333333 0.2222222 0.3703704 0.03703704 0.0000000 0.03703704
PRLO 0.0000000 0.0000000 0.0000000 0.00000000 0.5555556 0.44444444
SCCA 0.0000000 1.0000000 0.0000000 0.00000000 0.0000000 0.00000000
TAHU 0.0000000 0.0000000 0.0000000 0.00000000 0.0000000 1.00000000
TAST 0.0000000 0.1500000 0.5000000 0.35000000 0.0000000 0.00000000
        [,1]      [,2]      [,3] [,4]      [,5]
DIVI 0.0000000 0.0000000 0.00000000    1 0.0000000
MEME 0.0000000 0.0000000 0.00000000    1 0.0000000
PELE 0.3333333 0.3333333 0.03703704    0 0.2962963
PRLO 0.0000000 0.0000000 0.00000000    1 0.0000000
SCCA 0.0000000 0.0000000 0.00000000    0 1.0000000
TAHU 0.0000000 0.0000000 1.00000000    0 0.0000000
TAST 0.6500000 0.0500000 0.00000000    0 0.3000000
         [,1]      [,2]      [,3]      [,4]
DIVI 1.00000000 0.0000000 0.0000000 0.0000000
MEME 1.00000000 0.0000000 0.0000000 0.0000000
PELE 0.03703704 0.1851852 0.4444444 0.3333333
PRLO 1.00000000 0.0000000 0.0000000 0.0000000
SCCA 0.00000000 0.6000000 0.4000000 0.0000000
TAHU 0.00000000 1.0000000 0.0000000 0.0000000
TAST 0.00000000 0.1000000 0.8000000 0.1000000
         [,1]      [,2]      [,3]
DIVI 1.00000000 0.0000000 0.0000000
MEME 1.00000000 0.0000000 0.0000000
PELE 0.07407407 0.6296296 0.2962963
PRLO 1.00000000 0.0000000 0.0000000
SCCA 0.00000000 0.2000000 0.8000000
TAHU 0.00000000 0.0000000 1.0000000
TAST 0.00000000 0.9000000 0.1000000
```