

# Comparison of DNA and Carbon and Nitrogen Stable Isotope-based Techniques for Identification of Prior Vertebrate Hosts of Ticks

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J. Med. Entomol. 52(5): 1043–1049 (2015); DOI: 10.1093/jme/tjv063

**ABSTRACT** Identification of the vertebrate hosts upon which hematophagous arthropods feed provides key information for understanding the ecology and transmission of vector-borne diseases. Bloodmeal analysis of ticks presents unique challenges relative to other vectors, given the long interval between bloodmeal acquisition and host-seeking, during which DNA degradation occurs. This study evaluates DNA-based and stable isotope-based bloodmeal analysis methodologies for the lone star tick, *Amblyomma americanum* (Linnaeus, 1758), in an experimental study with chicken as the known host. We subjected ticks of different ages and environmental rearing conditions to three DNA-based approaches and a stable isotopic analysis, which relies on the natural variation of nitrogen (<sup>15</sup>N/<sup>14</sup>N) and carbon (<sup>13</sup>C/<sup>12</sup>C) isotopes. While all three DNA-based approaches were successful in identifying the bloodmeal host of the engorged nymphs, only the probe-based RT-PCR was able to detect host DNA in aged ticks, the success of which was low and inconsistent across age and rearing treatments. In contrast, the stable isotope analysis showed utility in determining the host across all ages of ticks when isotopic values of ticks were compared with a panel of candidate vertebrate species. There was a positive shift in both δ<sup>13</sup>C and δ<sup>15</sup>N in adult *A. americanum* until 34 wk postnymphal bloodmeal. Through analyzing the isotopic signatures of eight potential vertebrate host species, we determined that the magnitude of this isotopic shift that occurred with tick age was minor compared with the heterogeneity in the δ<sup>15</sup>N and δ<sup>13</sup>C signatures among species. These results suggest that stable isotopes are a useful tool for understanding tick–host interactions.

**KEY WORDS** *Amblyomma americanum*, bloodmeal analysis, stable isotope, tick

A prerequisite for the prevention of vector-borne disease is to understand the ecology of natural cycles of pathogen transmission. One large component of the natural cycle is the identification of key vertebrate hosts that feed vectors, and also serve as pathogen reservoirs. Bloodmeal analysis is a common tool used to identify the hosts of blood-feeding arthropods. Variations of bloodmeal analysis approaches have evolved for more than half of a century, refining the study of vector biology and our understanding of pathogen transmission (Kent 2009). Currently, the most widely used technique for elucidating vector–host interactions is a polymerase chain reaction (PCR)- and DNA sequencing-based bloodmeal analysis, which has been applied to mosquitoes (Hamer et al. 2009, Kent 2009), kissing bugs

(Kjos et al. 2013), fleas (Graham et al. 2012), and several biting flies (Hellgren et al. 2008, Santiago-Alarcon et al. 2013).

Ticks present unique challenges in bloodmeal host identifications because they are typically collected weeks, months, and even years after the bloodmeal was acquired, following substantial DNA degradation through the molting and ageing processes (Schmidt et al. 2011). Nonetheless, DNA-based approaches for bloodmeal identification have also been used in the analysis of tick-borne systems, but with limited success. For example, Garipey et al. (2012) used universal vertebrate barcoding primers, nested PCR, and DNA sequencing to successfully identify the host species from 72% of *Ixodes scapularis* that were tested. However, the analysis was restricted to engorged ticks removed from known small mammal hosts, so the utility of this approach for learning about diverse and unknown hosts of the previous stage of unfed adult ticks was not established. Using PCR followed by reverse line blot with hybridization to a series of probes specific to either individual species or host groups, the success of detecting bloodmeal sources ranged from 50 to 63% for unfed ticks including *Amblyomma americanum*, *I. scapularis*,

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and *Ixodes ricinus* (Pichon et al. 2005, Humair et al. 2007, Allan et al. 2010, Scott et al. 2012). However, problems with human DNA contamination proved intractable in at least one study (Scott et al. 2012), and the technique is not suited to measure tick feeding on humans. Recently, proteome profiling using spectral analysis (Onder et al. 2013) was introduced as non-DNA-based technique for tick bloodmeal identification in *I. ricinus*; this technique presents an exciting tool, if comprehensive blood spectral libraries become available to characterize local wildlife host communities.

Here, we developed an approach to study North American tick–host interactions based on the measurement of nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and carbon ( $^{13}\text{C}/^{12}\text{C}$ ) stable isotopes in host blood that are retained in the tick through the molt. Many of the elements that are important in physiological processes such as carbon, nitrogen, oxygen, and hydrogen have stable isotopes that occur naturally in the environment, are nontoxic and non-radioactive, and incorporate easily into living tissue. As vertebrates consume food resources, the isotopes from the dietary items are digested and assimilated into vertebrate tissues, providing the basis for variation in isotopic signatures across species based on diet. Stable isotopes have previously been used to understand vector-borne disease systems (Gomez-Diaz and Figuerola 2010), including the identification of bloodmeal hosts in mosquitoes with completely-digested bloodmeals (Rasgon 2008) and fleas with more than one host (Stapp and Salkeld 2009). Schmidt et al. (2011) found that the stable isotope ratios of nitrogen and carbon in unfed nymphal *I. ricinus* reflect the ratios of the hosts upon which they fed as larvae, providing proof-of-concept for the exploitation of variation in the natural abundance of stable isotopes as a way to reveal tick feeding patterns. Our objectives were to subject cohorts of colony-reared ticks of varying ages to four different bloodmeal analytical techniques and compare their success in identifying the host. Techniques include three DNA-based approaches and a stable isotope analysis to identify  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  (ratios of  $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$ , respectively) reflective of their bloodmeal host. We also compared the stable isotope values of the chicken-fed *A. americanum* age cohorts to the stable isotope values of whole blood from several potential vertebrate host species to determine if the stable isotope method can be used to identify the vertebrate species utilized by a tick in a previous life stage.

## Materials and Methods

An experimental colony of *A. americanum* originating from field-collected ticks collected in Edwards and Sutton Counties, TX, was propagated by feeding adults on cattle and larvae and nymphs on domestic chickens. The domestic chicken, *Gallus gallus* (L., 1758), and two other gallinaceous birds (quail and wild turkey) are among at least 10 bird species previously documented as hosts for immature stages of *A. americanum* (Koch 1981). For this study, nymphs that were 20 mo of age postlarval bloodmeal were fed on a single chicken acquired from a local commercial poultry operation to provide a single

nutritional source to the cohort of experimental adult ticks. This host was of good plumage, weight and dietary intake, and was conditioned to a pelleted commercially available poultry ration designed for maintenance and without antibiotics. Fed nymphs were held in an incubator maintained at  $23 \pm 2^\circ\text{C}$ , 85% relative humidity (RH), and a photoperiod of 14:10 (L:D) h. Molted adults were separated into two cohorts: an indoor/incubated cohort (unstressed = under conditions of  $23 \pm 2^\circ\text{C}$ , 85% RH, and a photoperiod of 14:10 [L:D] h) and an outdoor/shaded cohort (stressed = exposed to natural variation in temperature and humidity). The following groups of ticks were sampled for bloodmeal analysis, with a sample size of 10 per treatment group unless noted otherwise (including 1:1 sex ratio for adults): unfed nymphs, newly blood fed nymphs, newly molted adults (6 wk postnymphal bloodmeal;  $n = 20$ ), and both stressed and unstressed adults at 17, 34, and 40 wk postmolt. The final group of adult ticks was harvested at 59 wk postmolt, at which time only ticks in the unstressed group were viable. Harvested ticks were frozen at  $-20^\circ\text{C}$  until the time of processing.

**PCR Methodologies.** Five to 10 individual ticks from each group (representing half of the harvested cohort at each time point) were subjected to molecular analysis. Before DNA extraction, surface contaminants were removed by soaking each tick in 50% bleach for 15 seconds, followed by a 15-s soak in phosphate buffered saline (Graham et al. 2012). Total DNA was extracted from individual ticks after the exoskeleton and midgut of each tick was sliced open using a sterile scalpel. After overnight lysis, manufacturer's instructions for the tissue extraction protocol were followed with a final elution of 50  $\mu\text{l}$  (Qiagen DNeasy Blood and Tissue Kit, Valencia, CA). Extracted DNA served as template for a series of three PCRs. In all reactions, DNA extracted from whole chicken blood served as a positive control, and both water-template and no-template wells served as negative controls.

Conventional PCR was conducted to amplify a portion of the vertebrate cytochrome b (*cytb*) gene using the "BM" primers, as previously described (Cupp et al. 2004; Table 1). This particular *cytb* region was amplified using barcoding primers that have previously been used for bloodmeal analysis and was selected for use in the current study because it targets a relative short fragment of DNA (150 bp), which we considered more appropriate for bloodmeal analysis of ticks in which host DNA may be degraded. The Failsafe PCR System (Epicentre Biotechnologies, Madison, WI) was used. Following gel electrophoresis, representative amplicons from each age cohort were purified (ExoSAP-IT, Affymetrix, Santa Clara, CA) and directly sequenced (Eton Bioscience, San Diego, CA). Positive samples were determined as those that generated a band of appropriate size on the gel, and also generated a DNA sequence with significant homology to *Gallus gallus* when checking against a national database (NCBI Blast) in representatives from the same age class. The oldest tick cohort (59 wk postmolt) was not subjected to conventional PCR because all younger age molted cohorts were negative.

**Table 1. Primer and probe sequences for the three PCR protocols used in the current study**

PCR	Gene	Size	Primer and probe sequences	Reference
Conventional	<i>cytB</i>	150 bp	BM-F: 5'-GCH GAY ACH WVH HYH GCH TTY TCH TC-3' BM-R: 5'-CCC CTC AGA ATG ATA TTT GTC CTC A-3'	(Cupp et al. 2004)
Real-time probe	<i>cytB</i>	185 bp	Forward: 5'-CCT CTA CAA GGA AAC CTC AAA CAC-3' Reverse: 5'-GAC TAG CGT GTG TCC AAT GTA GG-3' Probe: 5'-FAM-AAT CCT CCT CCT CAC ACT CAT AGC CAC CG-BHQ-3'	(Woods et al. 2009)
Real-time SYBR	12S rRNA	175 bp	12S_425F: 5'-TGT AAA ACG ACG GCC AGT GGG ATT AGA TAC CCY ACT ATG C-3' 12S_9R: 5'-CAG GAA ACA GCT ATG ACA GAA CAG GCT CCT CTA G -3' M13 tag included in each primer is shown in italics	(Graham et al. 2012)

**Table 2. Bloodmeal host amplification success using three different PCR-based techniques**

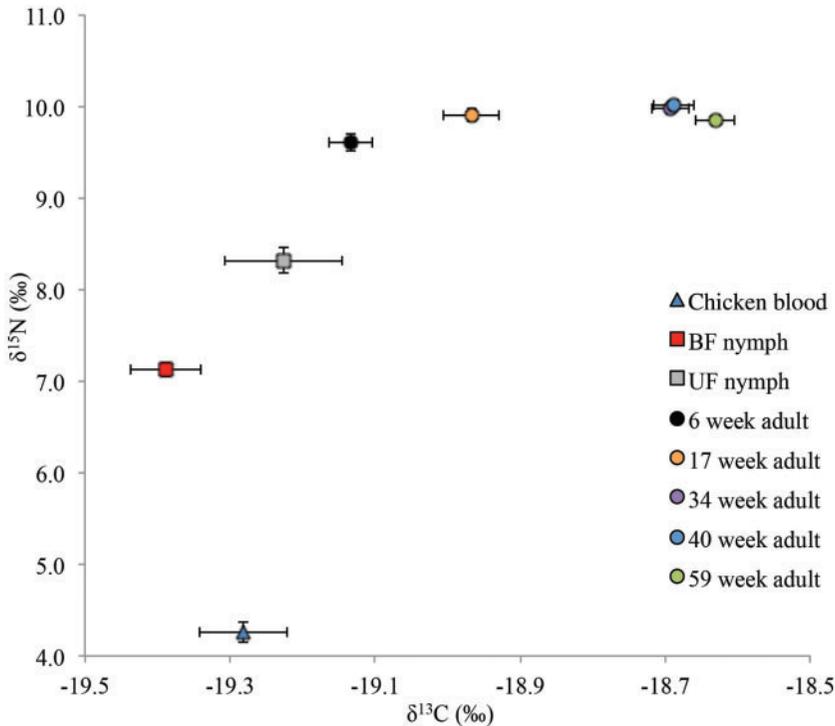
Tick cohort	Rearing condition	Sex	N	Conventional PCR	Probe RT-PCR	SYBR RT-PCR
Unfed nymph	Unstressed		5	0	2 (40%)	0
Blood fed nymph	Unstressed		5	5 (100%)	5 (100%)	5 (100%)
Adults, 6 weeks	Unstressed	Males	5	0	0	0
		Females	5	0	1 (20%)	0
Adults, 17 wk	Total		10	0	1 (20%)	0
		Unstressed		5	0	0
	Stressed	Males	5	0	0	0
		Females	5	0	0	0
Adults, 34 wk	Total		20	0	0	0
		Unstressed		5	0	1 (20%)
	Stressed	Males	5	0	0	0
		Females	5	0	1 (20%)	0
Adults, 40 wk	Total		20	0	2 (10%)	0
		Unstressed		5	0	2 (40%)
	Stressed	Males	5	0	0	0
		Females	5	0	0	0
Adults, 59 wk	Total		20	0	2 (10%)	0
		Unstressed		10	NA	0
	Total	Males	10	NA	0	0
		Females	10	NA	0	0
	Total		20	NA	0	0

Adult age represents weeks postnymphal bloodmeal.

Two different real-time PCRs were carried out on a Stratagene Mx3000 (Agilent Technologies, Santa Clara, CA; Table 2). First, a real-time PCR was performed using chicken-specific primers and probe based on the sequence of the chicken *cytB* gene (Woods et al. 2009) using the iTaq Universal Probes Supermix (BioRad, Hercules, CA). Second, a real-time PCR using SYBR Green was conducted using a universal 12S rRNA primer set with M13 tags to amplify this gene from all vertebrates (Graham et al. 2012). All samples were run in duplicate using the iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA). The final amplification cycle was immediately followed by a melting analysis cycle, during which the PCR software generated a melting peak for each sample. A sample was considered positive when the CT value was <36 and the melting temperature was similar to that of the positive control chicken blood. To aid in interpretation of results, a subset of PCR amplicons from samples with melting temperatures similar to and distinct from the positive control were subjected to direct DNA sequencing, as indicated above.

**Stable Isotope Analysis of Ticks.** Five to 10 individual ticks from each group (representing the

remaining half of the harvested cohort at each time point) were analyzed by elemental analysis isotope ratio mass spectrometry (EA-IRMS) as individual ticks for carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) isotopic values. Each sample was dried, homogenized, weighed into tin capsules, and analyzed on a Carlo Erba NA1500 Series 2 elemental analyzer (EA) coupled to a Finnigan Delta Plus XP (Thermo Fisher Scientific, Waltham, MA) stable IRMS via a Finnigan Conflo III open split interface at the Stable Isotope Geosciences Facility at Texas A&M University. The EA combust the samples at 1,200°C and the resulting  $\text{CO}_2$  and  $\text{N}_2$  gases are separated and analyzed on the IRMS. Results are presented in standard delta ( $\delta$ ) notation:  $\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$ , where R is the ratio of the heavy to light stable isotope in the sample and standard. Results are referenced to the VPDB carbonate standard for  $\delta^{13}\text{C}$  and relative to air for  $\delta^{15}\text{N}$ . Two international standards, USGS 40 and 41, that encompassed the range of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of samples for a two-point calibration and internal laboratory standards every ~12 unknowns are used to measure analytical precision. For most samples, the analytical uncertainty is  $\pm 0.15\%$  for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ,



**Fig. 1.** Stable isotopic results of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  for different life-stages and ages of *A. americanum* fed on a chicken in relation to the values for chicken blood. BF represents blood fed and UF represents unfed nymphal ticks. Weeks represent time postnymphal bloodmeal. X- and Y-axis error bars represent SEs around means. The data points from the 34 and 40 week cohorts are superimposed.

while for some of the unfed nymphs, the small sample volume resulted in an increased analytical uncertainty of  $\pm 0.25$  ‰ for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . Additionally, a sample of the commercial chicken feed pellets provided to the chickens that fed the tick colony was subjected to stable isotope analysis.

**Stable Isotope Analysis of Vertebrate Blood.** We generated a template of stable isotope signatures from a community of vertebrate hosts that co-occur with *A. americanum* populations in nature in the southern United States. These data were generated to facilitate an initial assessment of interspecific versus intraspecific variation in isotopic signatures. Three to 65 whole blood samples from different individuals from the following vertebrate hosts were dried and processed using EA-IRMS methods as described above: white-tailed deer (*Odocoileus virginianus*), white-footed mouse (*Peromyscus leucopus*), American robin (*Turdus migratorius*), broad-headed skink (*Plestiodon laticeps*), feral swine (*Sus scrofa domestica*), and song sparrow (*Melospiza melodia*). Human serum and clot data were obtained from Kraft et al. (2008) for comparative purposes but were not included in the statistical analyses because raw data were not available.

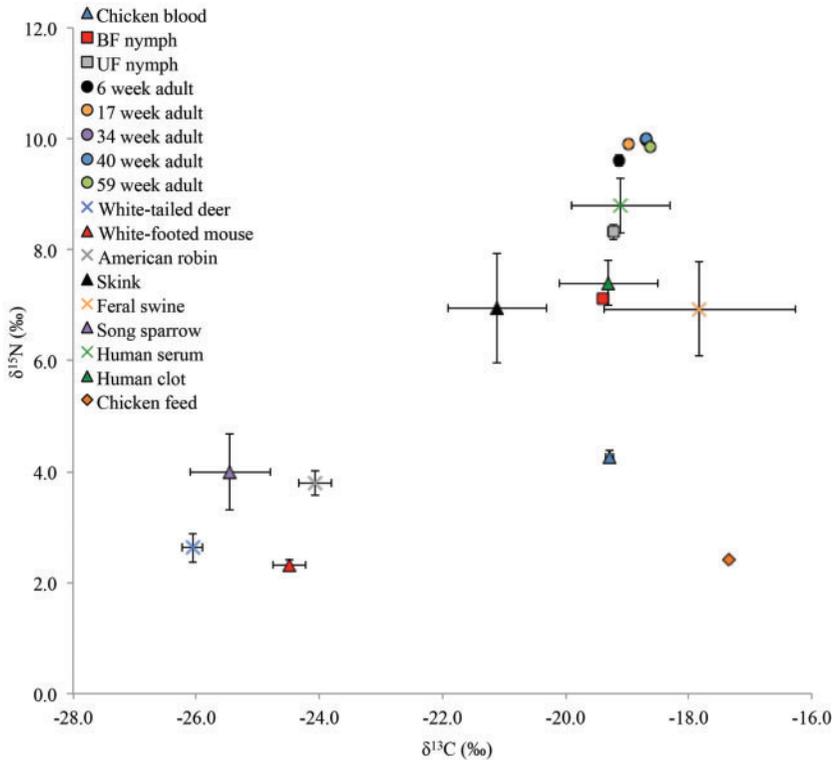
**Statistical Analysis.** We used a multivariate analysis of variance (MANOVA) to compare  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  results for different ages, sexes, and storage environments of the adult *A. americanum* ticks fed on chickens. When MANOVA revealed significant

differences, we used Tukey's honestly significant differences post hoc test to find differences among the groups. Means are presented  $\pm$  SE. All statistical analyses were performed using program R (R development Core Team 2012).

## Results

**DNA-based Bloodmeal Analysis.** Across all three DNA-based approaches, we had the highest success in identifying the bloodmeal host of ticks in the blood fed nymphs group, the only group for which all three methodologies identified chicken as the host in all five ticks (Table 2). The conventional PCR and SYBR RT-PCR failed to detect the host in any of the other tick treatment groups. The probe-based RT-PCR successfully identified the host in 0–40% of ticks in the other age/treatment groups, with no discernable pattern in success related to tick age or treatment (Table 2).

**Stable Isotope-based Bloodmeal Analysis.** There were no differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  between sexes of adult *A. americanum* ticks fed on chickens ( $F=0.39$ ;  $df=2, 99$ ;  $P=0.675$ ) nor between the adult *A. americanum* ticks stored in the stressed and unstressed groups ( $F=1.99$ ;  $df=2, 99$ ;  $P=0.142$ ). There were significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  among different aged adult *A. americanum* ticks ( $F=75.17$ ;  $df=2, 99$ ;  $P<0.001$ ). The post hoc test showed that  $\delta^{13}\text{C}$  was significantly different based on an alpha level of 0.05



**Fig. 2.** The  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  results of *A. americanum* ticks, blood from the chicken upon which they fed, and whole blood from other vertebrate hosts. BF represents blood fed and UF represents unfed nymphal ticks. Weeks represent time post nymphal bloodmeal. Human serum and clot data are from Kraft et al. (2008). X- and Y-axis error bars represent SEs around means. Sample sizes are deer (8) mice (65), robin (3), broad-headed skink (5), feral swine (3), song sparrow (3), and humans (206). The data points from the 34 and 40 week cohorts are superimposed.

for all pair-wise age combinations except those among the three oldest age cohorts (34 to 40 wk, 34 to 59 wk, and 40 to 59 wk; Fig. 1). The post hoc test showed that  $\delta^{15}\text{N}$  was significantly different for all pair-wise age combinations except among the four oldest age cohorts (17 to 34 wk, 17 to 40 wk, 17 to 59 wk, 34 to 40 wk, 34 to 59 wk, and 40 to 59 wk). The pelleted food provided to chickens had a  $\delta^{15}\text{N}$  of 2.4 and a  $\delta^{13}\text{C}$  of  $-17.3$ , which is consistent with corn and cereal grains that have approximate  $\delta^{15}\text{N}$  values of 2 and  $\delta^{13}\text{C}$  values of  $-11$  (Kraft et al. 2008).

There were significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  among the whole blood for seven different vertebrates ( $F = 15.13$ ;  $df = 6, 86$ ;  $P < 0.001$ ; Figure 2). The post hoc test showed that  $\delta^{13}\text{C}$  was significantly different based on an alpha level of 0.05 for all pair-wise species combinations except song sparrow with American robin, white-footed mouse with American robin, feral swine with chicken, white-footed mouse with song sparrow, and white-tailed deer with song sparrow. The post hoc test showed that  $\delta^{15}\text{N}$  was significantly different based on an alpha level of 0.05 for the pair-wise species combinations of white-footed mouse and broad-headed skink, white-tailed deer and broad-headed skink, white-footed mouse and feral swine, and white-tailed deer and feral swine. In summary, for the majority of the pairwise comparisons among vertebrate taxa, the

species differed significantly on the basis of their composition of rare and common forms of carbon and/or nitrogen, likely because of different diets.

## Discussion

Our data suggest that the determination of stable isotope signatures in ticks may facilitate identification of the host upon which ticks have previously fed. This approach is especially promising in analyses of aged ticks, when a series of DNA-based approaches failed. The process of blood feeding and utilization of blood products by the tick is associated with significant changes to the tick midgut that results in challenges to traditional DNA-based approaches for identification of host species. The midgut of ticks serves as a food storage reservoir for hemolyzed whole blood, and where intracellular digestion occurs among a diversity of epithelial cells all designed for slow conservative utilization (Sonenshine and Anderson 2014). The midgut is of endodermal origin and passes through intrastadial metamorphosis to not only provide nutrition in what may be a long wait time until the next host, but may also be a haven for intrastadial transmission of pathogens, symbionts and other microbes. Recent evidence shows that the internal microbiome of *A. americanum* changes over the

course of its transition from engorged nymph, through molt, and between aged adults kept under optimal environmental conditions and those exposed to outdoor environmental oscillations (Menchaca et al. 2013).

Our results show that traditional DNA-based bloodmeal analysis techniques are largely ineffective at providing a result once 6 wk have passed since the ticks' prior bloodmeal. While the conventional PCR and SYBR RT-PCR approaches successfully identified chicken as the host in engorged nymphs, these approaches were unsuccessful in determining the host once ticks molted. The probe RT-PCR has some, albeit low, success at different weeks postbloodmeal, and may, therefore, show some promise for utility in analyses of field-collected ticks of unknown age and unknown host origin. However, although we washed the external surface of ticks with bleach prior to analyses, we are unable to rule out the possibility that chicken DNA could have contaminated the external surface of the ticks, leading to inconsistent amplification of host DNA in a small number of the aged ticks.

We observed an increase in the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  isotopic values with tick over time until 34 and 17 wk post-bloodmeal, respectively, after which the values were statistically indistinguishable (Fig. 1). At different ages postmolt, the values for adult ticks varied more for  $\delta^{13}\text{C}$  than for  $\delta^{15}\text{N}$ , as indicated in the post hoc test and in Fig. 1. This isotopic shift is likely because of the continuous metabolic activity for digestion of the bloodmeal. The results of our study are consistent with the only prior study of tick host identification using stable isotopes; in particular, both studies document an isotopic shift with tick age (Schmidt et al. 2011). Given the consistency between these two studies, which were performed on different tick species from different continents, it is possible that the isotopic shift may be quantified to improve the analytical performance and estimate tick age, as suggested by Schmidt et al. (2011).

The two most significant constraints on survivorship in the off-host phase of tick life cycles are desiccation and efficient utilization of bloodmeals (Balashov 1972). We found that the storage conditions of adult ticks, whether in a climate-controlled unstressed environment or an outdoor stressed environment, did not influence the outcome of the stable isotope results. This characteristic is promising for the stable isotope-based approach, given that ticks in the field experience diverse environmental conditions that appear to have little influence on  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ .

Despite the variation of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  for *A. americanum* feeding on the same vertebrate species in our experiments (chicken), we show that this variation is relatively small in comparison to the large isotopic variation among a diverse group of vertebrate species (Fig. 2). Most pairs of species were significantly different from each other in either or both the nitrogen or carbon signature, thereby demonstrating the potential utility of natural  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  variation in vertebrates to aid in identifying the vertebrate species utilized by a tick in a prior life stage. Future studies should further address the isotopic variation within vertebrate species,

especially focusing on the geographic and seasonal factors that could influence the diets, which would in turn influence stable isotopic values of the vertebrate host species. Additionally, comparing the stable isotopic values for ticks at different life stages that fed upon a series of known hosts will reveal the consistency of results. In our study, we analyzed the isotopic signature of variable numbers of individuals of 8 potential tick hosts, ranging from 3 to 65 individuals per species, to provide a cross-sectional assessment of differences among species. The magnitude of the variation of the host species with the largest number of individuals analyzed (white-footed mouse) was similar to that of the other species in which fewer individuals were analyzed (Fig. 2), supporting our conclusion that the high degree of among species variation in isotopic signature facilitates the utilization of stable isotopes measurements to trace tick host feeding origin. Longitudinal studies to track vertebrate isotope signatures over space and time will afford a more robust understanding of how we must account for variability to determine host feeding patterns of ticks using this method.

The specificity of any vector bloodmeal analysis approach is limited by the completeness of references for comparisons (e.g., DNA sequences, reverse line blot probes, stable isotope signatures). This study and others demonstrate significant isotopic variation among vertebrates; our study shows that this variation can be utilized to identify prior vertebrate hosts. Ultimately, this technique will be useful for answering specific questions within focal geographic regions, in areas of limited host communities (such as islands, caves, urban zones, or forest fragments), or in addressing questions about the vertebrate host species that may be responsible for moving ticks to new areas (e.g., birds versus mammals). The stable isotope technique affords the unique ability to determine host origin of aged ticks, when DNA-based approaches fail.

### Acknowledgments

We appreciate the vertebrate blood provided by Graham Hickling, Isis Kuczaj, Genevieve Pang, and Jean Tsao. Funding was provided in part by the U.S. Department of Agriculture (USDA) Formula Animal Health program Project No. TEX09534, Undergraduate Research Opportunity in Entomology (UROE) at Texas A&M University, and Texas A&M AgriLife Research Project TEX08911. The feeding of ticks on chicken was done in accordance with the Institutional Animal Care and Use Committee-approved Animal Use Protocol No. 2011-213.

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Received 6 October 2014; accepted 8 May 2015.