Temporal Detection Limits of Remnant Larval Bloodmeals in Nymphal *Ixodes scapularis* (Say, Ixodida: Ixodidae) Using Two Next-Generation Sequencing DNA Barcoding Assays

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Abstract

Using next-generation sequencing nymphs, we aimed to determine: 1) if the larval bloodmeal can be detected in *Ixodes scapularis* nymphs and 2) the post-moult temporal window for detection of the larval bloodmeal. Subsets of 30 nymphs fed on a domestic rabbit (*Oryctolagus cuniculus* Linnaeus, Lagomorpha: Leporidae) as larvae were reared and frozen at 11 time points post-moult, up to 150 d. Vertebrate DNA was amplified using novel universal (UP) and species-specific primers (SSP) and sequenced for comparison against cytochrome c oxidase subunit I barcodes to infer host identification. Detectable bloodmeals decreased as time since moult increased for both assays. For the SSP assay, detection of bloodmeals decreased from 96.7% (n = 29/30) in day 0 nymphs to 3.3% (n = 1/30) and 6.7% (n = 2/30) at 4- and 5-mo post-moult, respectively. A shorter temporal detection period was achieved with the UP assay, declining from 16.7% (n = 5/30) in day 0 nymphs to 0/30 in 3-d-old nymphs. Bloodmeal detection was nonexistent for the remaining cohorts, with the exception of 1/30 nymphs at 2-mo post-moult. Host detection was significantly more likely using the SSP assay compared to the UP assay in the first three time cohorts (day 0: χ² = 39.1, P < 0.005; day 2: χ² = 19.2, P < 0.005; day 3: χ² = 23.3, P < 0.005). Regardless of the primer set used, the next-generation sequencing DNA barcoding assay was able to detect host DNA from a larval bloodmeal in the nymphal life stage; however, a short window with a high proportion of detection post-moult was achieved.

Key words: bloodmeal analysis, DNA barcoding, host identification, next-generation sequencing

Worldwide, ticks transmit numerous zoonotic pathogens. In North America, ticks transmit pathogens, such as *Borrelia miyamotoi* (Spirochaetales: Spirochaetaceae), *Anaplasma phagocytophilum* (Rickettsiales: Ehrlichiaeaceae), and *Borrelia burgdorferi* (Spirochaetales: Spirochaetaceae), the causative agents of a relapsing flu-like illness, granulocytic anaplasmosis, and Lyme disease, respectively (Spielman 1976, Ebel 2010, Ogden et al. 2011, Platonov et al. 2011, Rikihisa 2011). These pathogens are maintained in nature through complex and regionally diverse tick-host assemblages that ultimately affect the regional prevalence of the pathogen (Estrada-Peña and de la Fuente 2017). Similar to other ixodid ticks, *Ixodes scapularis* (Say, Ixodida: Ixodidae) has four life stages: egg, larva, nymph, and adult, and requires a bloodmeal from a vertebrate host to moult to the next life stage. During these bloodmeals, pathogens, such as *B. burgdorferi*, can be transmitted to ticks from the vertebrate hosts and vice versa. *Ixodes scapularis* has an extensive host range of at least 125 species in North America alone, resulting in a wide range of species that can potentially impact the transmission of pathogens (Keirans et al. 1996). Understanding these vector-host assemblages is essential for developing effective control and mitigation strategies. Identifying the origin of bloodmeals consumed by vectors through bloodmeal analysis (BMA) can provide valuable insights into vector-host interactions and pathogen transmission (Hopken et al. 2017).

The ideal tick BMA technique should be able to identify a wide range of host species to ensure complete understanding of the entire
range of species including novel or unanticipated species involved in pathogen transmission (Kent 2009, Gómez-Díaz and Figuerola 2010). Due to prerequisites in primers, probes, proteins, or enzyme restriction patterns, previous studies are limited in the range of identifiable host species (Kirstein and Gray 1996; Gray et al. 1999; Pichon et al. 2005, 2006; Humair et al. 2007; Morán Cadenas et al. 2007; Wickramasekara et al. 2008; Léger et al. 2015). Using restriction fragment length polymorphism (RFLP), Wodecka and Skotarczak (2016) demonstrated a tick BMA assay with the ability to identify 60 host species. Although providing the widest range of host identification from BMA in host-seeking ticks, the RFLP assays require a lot of high-quality DNA as well as a library of predefined enzyme restriction patterns for specific species and is currently limited to European species parasitized by *Ixodes ricinus* (Linnaeus, *Ixodida: Ixodidae*) ticks.

DNA barcoding, a method of species identification that uses a region of the cytochrome c oxidase subunit I (COI) mitochondrial gene, a gene unique in almost every vertebrate species (Hebert et al. 2003), was first applied to tick BMA in 2009 (Alcaide et al. 2009). By using universal primers for the COI gene segment, DNA barcoding and its extensive vertebrate barcode libraries, namely the Barcode of Life Database (BOLD), can allow for the identification of a magnitude of host species (Hebert et al. 2003). The ability of DNA barcoding to identify a wide range of host species has been demonstrated through BMA in other vectors, namely mosquitoes (Townzen et al. 2008, Reeves et al. 2018). In engorged *I. scapularis* larvae, Gariepy et al. (2012) demonstrated that DNA barcoding accurately identified the full range of known wild host species. However, for BMA techniques to be useful, they need to detect bloodmeals consumed by the previous tick instar in unengorged ticks post-moult (Gariepy et al. 2012). Although not suitable for all tick species, unengorged host-seeking ticks can often be easily collected in the field. However, the last detectable bloodmeal could have been consumed weeks to months to years post-moult (hereinafter 'post-moult cohorts'): these were days 0, 14, 21, 28, 56, 84, 112, and 150. Thirty nymphs were allocated to each post-moult cohort.

Our objective was to use next-generation sequencing DNA barcoding to 1) identify whether the larval bloodmeal can be detected in *I. scapularis* nymphs, and 2) identify the post-moult temporal window for detection and identification of a larval bloodmeal in *I. scapularis* nymphs fed on known hosts as larvae. The detection limit was assessed using: 1) a species-specific primer (SSP) to ensure the most optimal conditions for detection of remnant host DNA; and 2) universal primers (UP) for the future application in wild host-seeking ticks to capture a wide range of host species potentially involved in *B. burgdorferi* transmission.

**Materials and Methods**

**Tick Rearing and Temporal Post-Moult Cohorts**

*Ixodes scapularis* larvae (*n* = 1,046) were obtained from a pathogen-free colony at the Oklahoma State University tick-rearing facility (Stillwater, OK). The larvae were fed on domestic rabbits (*Oryctolagus cuniculus*) (Linnaeus, Lagomorpha: Leporidae) until fully engorged and then sent to the University of Guelph. Upon arrival, live ticks were washed using dish soap (Dawn ULTRA; Procter & Gamble, Cincinnati, OH), distilled water, and 70% *v/v* ethanol to remove accumulated mold. A subset of engorged larvae (*n* = 30) was frozen at −80°C within 3 d of removal from the host. The subset of engorged larvae were used as a baseline for the capacity of the two assays. The remaining clean engorged larvae were placed inside modified 15-ml screw top vials with a mesh covering with a maximum capacity of 100 ticks per vial to avoid overcrowding (Levin and Schumacher 2016). Engorged larvae were then reared in a humidity chamber held at 23–24°C, with 90–99% relative humidity, and a photoperiod of 16:8 (L:D) h. Humidity was achieved using 1-ml of milli-q water saturated with potassium sulfate (*K*₂*SO*₄) to achieve a minimum relative humidity above 90%. Humidity was monitored using a Zoo Med Hygrotherm (Zoo Med Laboratories Inc., San Luis Obispo, CA). The vials containing engorged larvae were stored in a horizontal position elevated out of K₂SO₄ solution. Due to evaporation, the humidity chamber was checked daily to ensure consistent water levels. See Supp Fig. S1 (online only) for depiction of incubator setup.

All engorged larvae maintained in the incubator were checked daily and dead ticks were removed. If mold was seen on the exterior of ticks, they were gently washed using the previously mentioned three-step washing regime. The incubator was dismantled and cleaned approximately every 3 wk with 70% *v/v* ethanol and K₂SO₄ solution was replaced. During the cleaning of the main incubator, all ticks were stored in a mini-incubator under the same parameters.

At each daily inspection, newly moulted nymphs were recorded with the corresponding date of moult and placed into the modified 15 ml vials at ≤25 nymphs per vial or identically modified 5 ml to keep <10 newly moulted nymphs (Levin and Schumacher 2016). All vials containing nymphs were stored vertically. Nymphs were maintained in the same incubator as engorged larvae and subject to the same three-step washing regime for mold prevention. Nymphs were frozen at −80°C at 11 different time points after the recorded day of moult (hereinafter ‘post-moult cohorts’): these were days 0, 2, 3, 7, 14, 21, 28, 56, 84, 112, and 150. Thirty nymphs were allocated to each post-moult cohort.

**DNA Extraction**

Prior to DNA extraction, all ticks were washed with 70% *v/v* ethanol, followed by DNA-free water (UltraPure DNase/RNase-Free Distilled Water, Invitrogen, Waltham, MA) to eliminate external impurities. The whole body of each tick was transferred into 2-ml screw-cap vials using sterile techniques. To penetrate the chitinous exoskeleton of *I. scapularis* all tubes were filled with 0.75–0.8 g of 2.0 mm yttria stabilized zirconium oxide beads and suspended in 200 µl of Lysis Buffer (700-mM guanidine thiocyanate [Sigma–Aldrich, St. Louis, MO], 30-mM EDTA pH 8.0 [Thermo Fisher Scientific, Waltham, MA], 30-mM Tris–HCl pH 8.0 [Sigma–Aldrich], 0.5% Triton X-100 [Sigma–Aldrich], 5% Tween-20 [Honeywell Fluksa, Charlotte, NC]) and a 1:10 ratio of 20-mg/ml Proteinase K (Promega, Madison, WI). The tubes were then homogenized at 30 Hz using a Tissue Lyser (Qiagen, Basel, Switzerland) for a total of 16 min, rotating the adaptors 180 degrees once after 8 min. The samples were then spun at 6,000 *g* for 30 s in a benchtop microcentrifuge and then incubated at 56°C overnight (18 h) to allow digestion.

Samples were sent to the Canadian Centre for DNA Barcoding (CCDB; Guelph, Ontario, Canada) for further DNA extraction. A manual silica-based membrane protocol (i.e., ‘glass fiber’ [GF], protocol; Ivanova et al. 2006) was utilized to extract DNA from all specimens. Following digestion, lysate was cooled to room temperature and all tubes were spun at 6,000 *g* for 30 s. Next, 150 µl of
lysate was mixed with 300 μl of Binding Mix (3-M guanidine thiocyanate, 10 mM EDTA pH 8.0, 5 mM Tris-HCl pH 6.4, 2% Triton X-100, and 50% ethanol). All samples were then transferred to a 96-well glass fiber plate (PALL AcroPrep 96 1-ml filter plate with 3.0-μm glass fiber media over 0.2-μm bioinert membrane) placed on top of a square-well block. All samples were spun at 5,000 g for 5 min to bind DNA to the glass fiber membrane. For the first DNA washing step, 180 μl of Protein Wash Buffer (1.56-M guanidine thiocyanate, 5.2-mM EDTA pH 8.0, 2.6-mM Tris-HCl pH 6.4, 1.04% Triton X-100, and 70% ethanol) was added to each well followed by centrifugation at 5,000 g for 2 min. Next, 750 μl of Wash Buffer (10-mM Tris-HCl pH 7.4, 50-mM NaCl, 0.5-mM EDTA pH 8.0, and 60% ethanol) was added to each well followed by centrifugation at 5,000 g for 10 min, removing and replacing the plate seal at 5 min. All samples were incubated at 56°C for 30 min to evaporate any remaining ethanol. Placing the GF membrane onto a collection microplate, the DNA was eluted in 50 μl of preheated Elution Buffer (10-mM Tris-HCl, pH 8) to 56°C and incubated at room temperature for 1 min. A final centrifugation at 5,000 g for 5 min was conducted to collect the DNA eluate before storage of samples at −20°C until further polymerase chain reaction (PCR) processing. In total, 12 controls were included at the extraction stage and processed in parallel with samples. Controls consisted of one positive control of O. cuniculus muscle tissue per plate (n = 4) and two negative controls consisting of only Lysis Buffer per plate (n = 8). All samples were randomly distributed across four 96-well plates and all controls were randomly distributed within a single plate.

SSP Design and Optimization

A forward and reverse primer was designed to amplify O. cuniculus templates on the barcode region of the COI gene. Three O. cuniculus COI sequences were downloaded from the BOLD barcode database and aligned using Aliview software (version 1.26, Larsson 2014). Primers were designed to amplify 194 bp in the reverse complement order following the guidelines outlined by the CCDB (Ivanova and Grainger 2007). The SSPs were validated on DNA samples extracted from muscle tissue of O. cuniculus using the same aforementioned GF DNA extraction protocol. Gradient PCR from 30 to 60°C was used to optimize SSP annealing temperature. Primer sequences can be found in Table 1.

Universal Primer Design and Optimization

Two forward and reverse universal primers were designed to amplify templates on the barcode region of the vertebrate COI gene while excluding I. scapularis templates. Full length COI sequences for 96 vertebrate hosts common in Eastern Canada including species belonging to the classes, Aves, Mammalia, and Reptilia were downloaded from the BOLD barcode database (Supp Table S1 [online only]) and aligned using Aliview. A COI sequence for O. cuniculus, a species not native to Eastern Canada, was also downloaded to ensure application of the UP to ticks that experimentally fed on this host. Additionally, a COI sequence for I. scapularis and Homo sapiens (Linnaeus, Primates: Hominoidea) were also downloaded for amplification avoidance. The alignment was used to identify 20–25 bp sequences that were well-conserved across all vertebrate taxa but included nucleotide mismatch positions between the tick and vertebrates. Guidelines used for primer design are outlined by the CCDB (Ivanova and Grainger 2007). A nucleotide mismatch position to exclude human DNA from target vertebrate DNA was not identified. Two primer sites were identified: one well-conserved primer sequence for mammals and one well-conserved primer sequence for reptiles and birds. Two oligonucleotide forward primers (BloodmealF3_t1 and BloodmealF4_t1) were designed for these sites with sequence variation among vertebrate host taxa represented by degenerate bases. The two degenerate forward primers were employed at a 1:1 ratio with a modified published mammal reverse primer cocktail (Mod.Mam.Rev_t1) to amplify the last 190-bp region of COI. The modified mammal reverse primer previously demonstrated amplification and sequencing of vertebrate host DNA with minimal amplification of tick DNA (Gariepy et al. 2012). Briefly, the modified mammal reverse cocktail consisted of three primers VR1_t1, VR1d_t1, and VD1i_t1 mixed in a 1:1:3 volumetric and molar ratio (Ivanova et al. 2007). Primer sequences can be found in Table 1. Gradient PCR from 50 to 60°C was used to optimize primer annealing temperature.

Two-Stage PCR Amplification for Next-Generation Sequencing

A two-stage PCR with fusion primers was utilized to prepare the extracted DNA for sequencing on the Ion S5 platform (Thermo Fisher Scientific). A depiction of the methodological workflow is shown in Fig. 1. For the first round of PCR, all samples were amplified using the SSP and the UP separately. Each primer was tagged with an M13 sequence for a fusion primer binding site in the second round of PCR. Two PCR replicates were run for both the SSP assay and UP assay to increase amplification success (Doi et al. 2019). Eight controls were introduced at this stage; four positive and four negative. Positive controls consisted of O. cuniculus tissue and negative controls of PCR mix and 2 μl of Hyclone ultra-pure water (Thermo Fisher Scientific) were utilized.

The PCR replicates consisted of 6.25 μl of 10% D- (+)-trehalose dihydrate (Fluka Analytical), 2 μl of Hyclone ultra-pure water (Thermo Fisher Scientific), 1.25 μl of 10× PlatinumTaq buffer (Invitrogen), 0.625 μl of 50 mM MgCl₂ (Invitrogen), 0.125 μl of each 10 μM

Table 1. Cytochrome c oxidase subunit I primer sequences used for the species-specific and universal barcode assays

<table>
<thead>
<tr>
<th>Barcode assay</th>
<th>Cocktail name</th>
<th>Primer label</th>
<th>Ratio</th>
<th>Sequence (5′–3′)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal</td>
<td>C_BloodmealF2_t1</td>
<td>BloodmealF3_t1</td>
<td>1</td>
<td>TCATTACAACWATTAYAAYATRAA</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BloodmealF4_t1</td>
<td>1</td>
<td>TCATCACAAGGACATAYATRAA</td>
<td>This study</td>
</tr>
<tr>
<td>Mod.Mam.Rev_t1</td>
<td>VR1_t1</td>
<td>1</td>
<td>TTCTCAACCAACCAAGACAYTG</td>
<td>Ivanova et al. (2006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VR1d_t1</td>
<td>1</td>
<td>TTCTCAACCAACCAAGGATYTG</td>
<td>Ivanova et al. (2006)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VD1i_t1</td>
<td>3</td>
<td>TTCTCAACCAACCAAGAATIGG</td>
<td>Ivanova et al. (2006)</td>
<td></td>
</tr>
<tr>
<td>Species-specific</td>
<td>Oryctolagus cuniculus</td>
<td>MRabbit-COI-F1_T1</td>
<td>1</td>
<td>ATTCCTTCTTCTACTAGCC</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MRabbit-COI-R1_t1</td>
<td>1</td>
<td>TAGAAATAAGGGGGGTTGA</td>
<td>This study</td>
</tr>
<tr>
<td>M13 sequences</td>
<td>M13Foward</td>
<td>1</td>
<td>TGAAAACAGCCGCGAGT</td>
<td>Messing (1983)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M13Reverse</td>
<td></td>
<td>CAGGAAAACAGCTATGAC</td>
<td>Messing (1983)</td>
<td></td>
</tr>
</tbody>
</table>

Each forward and reverse primer was tagged with an M13 forward and reverse sequences, respectively.
primer, 0.0625 µl of 10 mM dNTP (KAPA Biosystems), 0.060 µl of 5 U/µl PlatinumTaq DNA Polymerase (Invitrogen), and 2 µl of template DNA for a total reaction volume of 12.5 µl. Thermocycling conditions for SSP were an initial 2-min denaturation step at 94°C, then 60 cycles at 94°C for 40 s, 61°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. For UP assay, a 1:1 ratio of both forward degenerate primers were employed with the reverse degenerate primers. Thermocycling conditions for UP were an initial 2-min denaturation step at 94°C, then 60 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 5 min.

The second round of PCR employed identical PCR regimes and aforementioned PCR mix as the first round of PCR, but for the addition of ‘fusion primers’ (i.e., adapter sequences and unique molecular identifier tags, UMIs; Table 1). The Ion S5 platform requires all PCR amplicons to include added fusion primers consisting of adapter sequences for characterization by the sequencing platform as well as UMI tags to link sequence reads to individual samples (Prosser and Hebert 2017). These fusion primers were incorporated into amplicons for both PCR replicates generated in the first amplification round. Four additional negative controls were included at this stage consisting of PCR mix and 2 µl of Hyclone ultra-pure water (Thermo Fischer Scientific). All PCR 1 and 2 products were visually analyzed using gel electrophoresis (pre-cast E-Gel 2% 96 Agarose Gels; Invitrogen) before sequencing to ensure adequate success of amplification was achieved. All PCR products from each replicate were then pooled and purified separately using a multi-step carboxylate modified magnetic beads (Aline Biosciences, Woburn, MA) protocol in accordance to Moran et al. (2019) to remove amplicons >600 and <100 bp. Pooled products were then normalized to 1 ng/µl following DNA quantification using Qubit 2.0 fluorometer.

**Sequencing and Bioinformatic Workflow**

For both the SSP and UP assays, regardless of whether visible product was observed as gel visualization was not sufficient for determining product, all PCR 2 products from both PCR replicates were sequenced separately on the Ion S5 platform. To prepare the samples, each sample was diluted to 26 pM:5 µl (1 ng/µl) and 1,010-µl water. Next, the Ion Chef System was utilized for library preparation and chip loading followed by sequencing on Ion S5 using a 530 v.1 chip according to the manufacturer’s instructions. The Ion S5 software automatically de-multiplexes the data by scoring unique UMIs and assigning the resulting reads to their source samples. The data were processed through a bioinformatic pipeline as outlined in Prosser and Hebert (2017). Briefly, reads less than 100 bp (e.g., primer dimers) or reads with low quality scores (QV < 20) were removed. Next, primers and adapter sequences were removed from
the remaining reads (Prosser and Hebert 2017). Operational taxonomic units (OTUs) were formed by dereplicating and clustering the reads with 98% identity (i.e., how similar the compared sequences are when fully aligned). To de-noise the data (i.e., remove low abundance artifactual reads and chimeric reads), remaining OTUs composed of <10 reads were removed (Clarke et al. 2017, Alberdi et al. 2018). To assign taxonomy to the OTUs, a basic local alignment search tool (BLAST) using all BINs (Barcode Index Number) from the Barcode of Life Data System v4 (BOLD; http://www.boldsystems.org/index.php) was conducted. Sequence taxonomic assignments were only accepted as genuine after meeting the following confidence thresholds for identification: at least 95% sequence similarity (identity), a minimum overlap of query sequence with a reference sequence ≥100 nucleotides, and a depth of coverage of at least 100 reads (Prosser and Hebert 2017). Briefly, a custom R script was used to filter the BLAST data to remove query sequences with less than 95% identity to a reference sequence across at least 100 bp (Moran et al. 2019). The data associated with each sample was then consolidated by combining all OTUs matching to a common reference sequence and summing their component reads providing a total read count.

Data Analysis

All samples that yielded a sequence that met the confidence thresholds from one or both of the PCR replicates were considered as a host identification. A two-sided Pearson’s χ²-test of independence was performed to examine the statistical difference between the two barcode assays for each of the nymphal post-moult time cohorts. A Bonferroni adjustment was conducted to account for potential false-positive statistical associations; therefore, an alpha level of 0.0045 was used for all statistical tests. Computations were performed using SPSS Statistics (Version 26, International Business Machines Corp., Armonk, NY).

Results

Species-Specific Barcode Assay

Using the species-specific barcode assay, host DNA was detected from all engorged larvae (n = 30). The percentage of detectable O. cuniculus DNA for the combined PCR replicates for each temporal tick cohort is depicted in Fig. 2. Oryctolagus cuniculus DNA was detected in all 11 post-moult nymph cohorts and a total detection of 25.2% (83/330) of remnant bloodmeals was achieved across all cohorts. Detectable bloodmeals from the post-moult cohorts decreased as time since moult increased with 96.7% (n = 29/30) of bloodmeals detected in nymphs 0-d post-moult with a decline in detection until the day 14 nymph cohort where the average bloodmeal detection plateaued at approximately 5%. The combination of PCR replicates increased the percent of detectable bloodmeals for all cohorts except the post-moult cohorts D14, D28, D54, D84, and D112 (Supp Fig. S2 [online only]). All obtained query sequences that surpassed confidence thresholds matched with the intended O. cuniculus reference sequence. The sequencing performance metrics for both replicates depicted good overall sequencing of target template shown in the read length histogram with little variation in sequence product length (Supp Fig. S3 [online only]).

Controls

Oryctolagus cuniculus DNA exceeding the minimum confidence thresholds was detected in 1 of the 16 (0.06%) negative controls from the species-specific barcode assay. All positive controls consisting of O. cuniculus tissue (n = 4) yielded O. cuniculus identification that met the sequence confidence thresholds in both PCR replicates. Sequencing results from all negative and positive controls are found in Supp Tables S2 and S3 (online only).

**Fig. 2.** Percentage of nymphs in which rabbit DNA was detected with 95% CIs from the combined success of PCR replicates for the species-specific and universal barcode assay. A detectable bloodmeal is defined as a specimen that yielded Oryctolagus cuniculus sequence that met the imposed sequence confidence thresholds (≥95% identity, a minimum overlap of query sequence to ≥100 nucleotides, and a depth of coverage ≥100 reads) for each tick cohort. Temporal tick cohorts include engorged larvae (EL) (n = 30) and the post-moult nymph cohorts ranging from day 0 (D0) to day 150 (D150) with 30 nymphs allocated to each cohort (n = 330). χ² significance at D0 (χ² = 39.095, n = 30, P = 0.000), D2 (χ² = 19.200, n = 30, P = 0.000), and D3 (χ² = 23.254, n = 30, P = 0.000).
Universal Barcode Assay

Using the universal barcode assay, host DNA was detected from all engorged larvae (n = 30). The percentage of detectable O. cuniculus DNA for the combined PCR replicates for each temporal tick cohort is depicted in Fig. 2. Oryctolagus cuniculus DNA was detected in the four post-moult nymph cohorts (D0, D2, D3, and D56) and a total detection of 3.03% (n = 10/330) of remnant bloodmeals was achieved across all 11 post-moult nymph cohorts. Detectable O. cuniculus DNA from the post-moult nymph cohorts decreased as time since moult increased with 16.7% (n = 5/30) of bloodmeals detected in the day 0 cohort declining until the day 3 cohort, where bloodmeal detection was nonexistent for the remaining cohorts with the exception of 3.33% (n = 1/30) detected in the day 56 cohort. The combination of PCR replicates increased the proportion of detectable O. cuniculus DNA for day 0 and day 2 post-moult cohorts (Supp Fig. S4 [online only]).

Eleven species other than O. cuniculus (Linnaeus, Artiodactyla: Suidae) were identified with two species identifications (Homo sapiens and Sus scrofa) meeting the minimum sequence confidence thresholds (Fig. 3), because all ticks were fed on the known host O. cuniculus, H. sapiens, and S. scrofa were deemed as external contaminants. The remaining eight identifications including springtails (Tullbergiida Gisin, Collembola: Onychiuridae), crustaceans (Phoxocephalidae Sars, Amphipoda: Gammaridea and Centromedon pumilus Lilljeborg, Amphipoda: Uristidae), pindi moth (Abantiades latipennis Tindale, Lepidoptera: Hepialidae), isopods (Phreatomerus latipes Chilton, Isopoda: Amphisopodidae), frogs (Xenorhina oxycephala Schlegal, Anura: Microhylidae), sika deer (Cervus nippon Temminch, Artiodactyla: Cervidae), and gorillas (Gorilla gorilla Savage, Primates: Hominidae) did not meet the minimum sequence thresholds and many were biologically implausible contaminants, so they were deemed spurious identifications. The sequencing performance metrics for both replications are shown in Supp Fig. S5 (online only). The sequencing performance metrics for both replications depicted overall suboptimal sequencing of target template shown in the read length histogram with some variation in sequence product length instead of a single peak (Supp Fig. S5 [online only]).

Controls

Oryctolagus cuniculus DNA exceeding the minimum sequence confidence thresholds was detected in 1 of the 16 (0.06%) negative controls from the universal barcode assay. Unidentified DNA (i.e., no BLAST Hit) was detected in two other negative controls.

All positive controls consisting of O. cuniculus tissue (n = 4) yielded O. cuniculus identification that met the sequencing confidence thresholds in both PCR replicates. Sequencing results from all controls are found in Supp Tables S4 and S5 (online only).

Chi-Square Test of Independence

The χ²-test of independence showed that there was a statistical difference between the two barcode assays for nymphs in the first three time cohorts (day 0, 2, and 3; Table 2). Successful host identifications were observed significantly more in the first three time cohorts using the SSP assay compared with the UP assay. No statistical difference of successful bloodmeal identifications between the two assays was observed for the remaining eight time cohorts.

Discussion

Compared with BMA in other hematophagous vectors, BMA in tick vectors is particularly challenging because ticks feed on a wide range of host species, and have prolonged, multistage life cycles. Bloodmeal analysis methods for post-moult tick instars, therefore, need to be able to detect highly degraded DNA. Given these difficulties, the aim of the present study was to apply next-generation sequencing DNA
barcoding to determine whether the larval bloodmeals could be detected in the I. scapularis nymphal life stage followed by identifying the temporal limit of detectable larval bloodmeals post-moult using two different primer assays.

The next-generation sequencing DNA barcode assay was initially coupled with an SSP to provide the optimal conditions for host DNA detection. Bloodmeal detection ranged from 97.67% in day 0 nymphs to 3.33 and 6.67% in nymphs 112- and 150-d old (approximately 4- and 5-mo), respectively. Host DNA can remain in the gut of ticks for long periods of time after consumption (Kirstein and Gray 1996), yet the overall decline in host DNA detection demonstrates the extensive physiological degradation of the host DNA as time since moult increased. The decline in detection is consistent with results from other experimental studies on the temporal detection of remnant bloodmeals in unengorged nymphal ticks (Kirstein and Gray 1996, Pichon et al. 2003, Léger et al. 2015); however, the detection of host DNA using the SSP barcode assay in the present study declined at a faster rate. Kirstein and Gray (1996) documented a decline in host DNA detection after 5-mo post-moult, whereas Pichon et al. (2003) documented a decline in host DNA detection after 7-mo post-moult. It is difficult to compare host DNA detection results among experimental temporal detection studies as different tick species, host species, rearing conditions, DNA extraction protocols, and molecular targets were utilized.

Despite the inability to directly compare results between studies, there are several factors that may contribute to the lower duration and proportion of detection of host DNA in the present study. First, extraction of host DNA from the tick is one of the critical steps in tick BMA and the DNA extraction protocol used can affect the downstream success of any DNA-based BMA technique. Many different extraction protocols have been used in tick BMA with the most common being ammonium hydroxide (Kirstein and Gray 1996, Gray et al. 1999, Pichon et al. 2003, Humair et al. 2007, Allan et al. 2010) and commercial extraction kits (Scott et al. 2012, Che Lah et al. 2015, Léger et al. 2015). The phenol–chloroform protocol is reported to be the most effective for host DNA detection (Wodecka and Skotarczak 2016), but we had greater success using the silica-membrane-based GF protocol on engorged larvae when preliminarily compared with a phenol–chloroform protocol and DNeasy Blood & Tissue extraction kit (Qiagen; Supp Table S6 [online only]). Additionally, the phenol–chloroform technique is highly time consuming and better suited for a low volume of samples, which would not be ideal for field studies that require hundreds of tick samples. It is unknown which extraction protocol is most effective for detecting the last bloodmeal in unengorged ticks and studies directly comparing the yield and amplification success of host DNA after isolation are required.

In addition to DNA extraction, the host species that provides a bloodmeal can affect the quality of blood for DNA-based detection resulting in a dominant template bias. The detection of blood can be impacted by host immune responses, the level of residual heme molecules, which are a known PCR inhibitor, and whether host red blood cells are nucleated (e.g., reptiles and birds) or non-nucleated (e.g., mammals; Sonenshine 1991, Allan et al. 2010, Scott et al. 2012, Léger et al. 2015). Host bias was demonstrated by Léger et al. (2015) in unengorged nymphs 2-mo after moult using two different BMA techniques, qPCR and reverse line blot hybridization (RLBH), where amplified PCR products are hybridized to the group- or species-specific oligonucleotide probes to identify the source of the bloodmeal. Using the two techniques, a greater proportion of remnant bloodmeals were detected from the avian host, which has nucleated red blood cells, compared with the mammal host which does not. It is possible that rabbit bloodmeals may contribute to the low detection threshold in the present study; however, the exact mechanism of inhibition is unknown. Preferential amplification of host bloodmeals over others could introduce biases when detecting multiple unknown host species in wild tick populations. To further test potential dominant template bias, the current next-generation sequencing DNA barcode assay should be tested on unengorged nymphs that have fed as larva on a range of known wild host species groups, including birds, mammals, and reptiles.

Tick rearing conditions may impact tick digestion and need to be considered when analyzing the temporal detection limits of remnant tick bloodmeals. For example, high temperatures and extended light to dark exposure ratios can induce faster digestion of the bloodmeal and therefore increase the degradation of host DNA (Sonenshine 1993). The longest achieved experimental detection of larval bloodmeals was 9-mo in field-maintained nymphs under natural temperature and light conditions (Pichon et al. 2003). This 9-mo temporal detection window would likely satisfy the duration of the last consumed bloodmeals in field collected ticks; however, the technique was restricted to order/family host identification limiting the specificity of detecting hosts that feed wild tick populations. Ticks in the present study were maintained with a long photoperiod of 16:8 (L:D) h and at 24°C, a temperature which exceeded temperatures used in other experimental studies (Kirstein and Gray 1996, Léger et al. 2015), potentially contributing to the comparative low rates of host DNA detection in nymphs >3-d. The photoperiod in the present study was used to ensure moulting within ~1-mo (Levin and Schumacher 2016) and temperature was held at 24°C due to constraints from ambient air temperature.

To capture the wide host range of I. scapularis, the next-generation sequencing DNA barcode assay was coupled with a UP set targeting COI for vertebrates. The temporal detection achieved by the UP barcoding assay was less successful than the SSP barcoding assay. This difference was expected as increasing the degenerate bases in a barcoding assay was less successful than the SSP barcoding assay. COI for vertebrates. The temporal detection achieved by the UP barcoding assay was less successful than the SSP barcoding assay. COI for vertebrates. The temporal detection achieved by the UP barcoding assay was less successful than the SSP barcoding assay. COI for vertebrates. The temporal detection achieved by the UP barcoding assay was less successful than the SSP barcoding assay.

### Table 2. Pearson’s χ²-tests depicting positive adjusted residuals, Pearson χ²-value and P-value for both next-generation DNA barcode assays for each of the nymphal post-moult time cohorts

<table>
<thead>
<tr>
<th>Time cohort</th>
<th>SSP assay</th>
<th>UP assay</th>
<th>Pearson χ²-value (χ²)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>D0</td>
<td>29</td>
<td>5</td>
<td>39.095</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D2</td>
<td>18</td>
<td>2</td>
<td>19.200</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D3</td>
<td>20</td>
<td>2</td>
<td>23.254</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>D7</td>
<td>5</td>
<td>0</td>
<td>5.455</td>
<td>0.020</td>
</tr>
<tr>
<td>D14</td>
<td>1</td>
<td>0</td>
<td>1.017</td>
<td>0.313</td>
</tr>
<tr>
<td>D21</td>
<td>3</td>
<td>0</td>
<td>3.158</td>
<td>0.076</td>
</tr>
<tr>
<td>D28</td>
<td>1</td>
<td>0</td>
<td>1.017</td>
<td>0.313</td>
</tr>
<tr>
<td>D56</td>
<td>1</td>
<td>1</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>D84</td>
<td>2</td>
<td>0</td>
<td>2.069</td>
<td>0.150</td>
</tr>
<tr>
<td>D112</td>
<td>1</td>
<td>0</td>
<td>1.017</td>
<td>0.313</td>
</tr>
<tr>
<td>D150</td>
<td>2</td>
<td>0</td>
<td>2.069</td>
<td>0.150</td>
</tr>
</tbody>
</table>

All P-values are adjusted to 0.0045 to account for multiple comparisons (Bonferroni adjustment) and no statistics are reported for engorged larvae cohort as it is a constant. Statistical significance is denoted in bold.

**Note:** The next-generation sequencing DNA barcode assay was initially coupled with an SSP to provide the optimal conditions for host DNA detection. Bloodmeal detection ranged from 97.67% in day 0 nymphs to 3.33 and 6.67% in nymphs 112- and 150-d old (approximately 4- and 5-mo), respectively.
nymph samples (Fig. 3). These findings indicate that this assay as it was carried out herein may not be suitable for application to field-collected unengorged nymphs.

However, the UP barcoding assay may be improved with the application in wild tick populations due to tick dormancy and seasonality of bloodmeal consumption and moulting. Host-seeking Spring/Summer nymphs experience a period of dormancy over the winter months slowing down the digestion of the tick bloodmeal (Sonenshine 1991) potentially increasing the rate of host detection. Additionally, seasonally targeting the collection of ticks may improve the utility of the assay in the field by reducing the amount of degradation to the last consumed bloodmeal. Variation in host detection was illustrated by the seasonal collection of ticks with higher detection of host DNA achieved in host-seeking nymphs collected in the Fall compared with nymphs collected in the Spring/Summer (Pichon et al. 2003, Morán Cadenas et al. 2007). Although it is unknown when wild-caught nymphs consumed their larval bloodmeal, nymphs collected in the Fall likely consumed their larval bloodmeals more recently than nymphs collected in the Spring/Summer months (Ogden et al. 2004). Tick dormancy and its effects on digestion, as well as seasonally targeting the collection of host-seeking nymphs, may improve the UP barcoding assay detection of the remnant bloodmeals in the field; however, further validation of the UP primer set on wild host species would need to be conducted before use on wild-caught ticks.

In the present study, the proportion of host detection was limited particularly in the later post-moulting cohorts, yet the application of the UP barcode assay in wild tick populations may prove useful for high-density regions of *I. scapularis*. Due to the low proportion of host DNA detection, a large number of ticks are required in order to obtain a representative sample size of host species providing bloodmeals. Thus, the assay is likely not useful in regions with low tick densities. Therefore, seasonally targeting the collection of host-seeking *I. scapularis* in regions with high *I. scapularis* densities may positively affect the utility of the UP barcode assay. In the future, DNA metabarcoding may be a more efficient technique for large tick numbers as it is able to identify multiple host species at once from pooled tick samples alleviating the labor and resource intensive processing of large numbers of individual ticks. Information regarding the proportional contribution of a host species to *B. burgdorferi* transmission will be lost with pooled tick samples; however, valuable insights into tick-host feeding patterns can still be gained. The same concept of seasonally targeting the collection of wild ticks in large numbers also applies to the SSP barcode assay, but this assay would only be useful for investigations targeted at particular host species. Contamination of rabbit DNA was evident in one of the negative extraction controls and is likely the result of technical error (e.g., pipetting error) at the DNA extraction stage. This is evident by the adjacent location of the negative control to a positive rabbit extraction control and is likely the result of technical error that may be improved with the application in wild tick populations due to tick dormancy and seasonality of bloodmeal consumption and moulting. Host-seeking Spring/Summer nymphs experience a period of dormancy over the winter months slowing down the digestion of the tick bloodmeal (Sonenshine 1991) potentially increasing the rate of host detection. Additionally, seasonally targeting the collection of ticks may improve the utility of the assay in the field by reducing the amount of degradation to the last consumed bloodmeal. Variation in host detection was illustrated by the seasonal collection of ticks with higher detection of host DNA achieved in host-seeking nymphs collected in the Fall compared with nymphs collected in the Spring/Summer (Pichon et al. 2003, Morán Cadenas et al. 2007). Although it is unknown when wild-caught nymphs consumed their larval bloodmeal, nymphs collected in the Fall likely consumed their larval bloodmeals more recently than nymphs collected in the Spring/Summer months (Ogden et al. 2004). Tick dormancy and its effects on digestion, as well as seasonally targeting the collection of host-seeking nymphs, may improve the UP barcoding assay detection of the remnant bloodmeals in the field; however, further validation of the UP primer set on wild host species would need to be conducted before use on wild-caught ticks.

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Conclusion
In this study, regardless of the primer set used, the next-generation sequencing DNA barcoding assay was able to detect host DNA from the larval bloodmeal in the nymphal life stage. However, the proportion and duration of bloodmeal detection in unengorged *I. scapularis* nymphs were lower compared with other tick BMA studies. Although higher detection thresholds in host-seeking nymphs were demonstrated using other BMA techniques, the majority of these techniques have limited specificity of host identification and/or a pre-defined range of detectable host species (Kirstein and Gray 1996, Pichon et al. 2003, Morán Cadenas et al. 2007, Allan et al. 2010, Wodecka and Skotarczak 2016). Multiple avenues can be explored to improve detection in host-seeking nymphs using next-generation sequencing DNA barcoding such as, 1) increasing PCR replicates as re-amplification from apparently negative reactions can recover results from samples with low template concentration (Kent and Norris 2005), 2) primers/annealing temperatures with better sensitivity and discrimination, and 3) use group-specific primers that are less degenerate. Lastly, trials with nymphs held under natural environmental conditions should be employed in tandem with all of the aforementioned ways to improve the UP primer assay. Despite the low success in host DNA detection in unengorged nymphs in the present study, the utility of the presented next-generation sequencing DNA barcoding assay in wild host-seeking tick populations is unknown, and field studies are required in tandem with the aforementioned suggested improvements.

**Supplementary Data**
Supplementary data are available at *Journal of Medical Entomology* online.

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