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Survival of Host Blood Proteins in *Ixodes scapularis* (Acari: Ixodidae) Ticks: A Time Course Study

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ABSTRACT *Ixodes scapularis* Say, 1821 larvae were fed on mice and allowed to molt under laboratory conditions. A liquid chromatography–tandem mass spectrometry-based proteomic study was conducted to identify the type of mammalian proteins present in the derived nymphal ticks at different time intervals after molting. Albumin was present for 85 d; transferrin was present for 29 d; and, more importantly, hemoglobin remained detectable for up to 309 d postmolting. Peptides of actin, keratin, and tubulin are highly similar between mouse and tick, and therefore, unambiguous assignment of these proteins to different species was not possible. Establishing a time line for the persistence of hemoglobin, one of the most abundant blood proteins, at detectable levels in ticks after the bloodmeal and molting advances our efforts to use this protein to identify the host species.

KEY WORDS LC-MS/MS, mouse, hemoglobin, vector-borne disease, reservoir

The most common arthropod-borne disease in North America and much of Europe is Lyme borreliosis (LB), also known as Lyme disease. In the United States, LB is caused by *Borrelia burgdorferi*, which infects a variety of mammalian species. *B. burgdorferi* is transmitted between vertebrate reservoir hosts and hard ticks of the species *Ixodes scapularis* Say, 1821 in the central and eastern United States and *Ixodes pacificus* Cooley & Kohls, 1943 in the far-western United States (Caimano 2006).

Establishing the reservoir host species for vector-borne zoonoses in a given geographic area is important, as host–vector interactions provide insight into the ecological factors influencing the incidence of vector-borne diseases. It has been determined that the number of different potential host species living in a given area is in direct correlation with the infection prevalence of nymphal *I. scapularis* (LoGiudice et al. 2003), but identification of which hosts are infected has proven to be a challenge. Identifying reservoir species provides a better understanding of the transmission dynamics of a pathogen between its hosts and vectors, which can also assist with effective population control strategy development, targeting infected wildlife in an endemic region (Pepin et al. 2012).

Early arthropod bloodmeal identification methods relied on immunological tests. Serological tools, such as the precipitin test and hemagglutination inhibition, used by Tempelis (1975) on mosquitoes, were able to identify host bloodmeal sources to the order and family level. More sensitive and rapid polymerase chain reaction (PCR) based methods were later assessed for tick and insect host bloodmeal identification, with one of the first targets being the mitochondrial cytochrome b gene in tick vectors (Kirstein and Gray 1996). This study showed that the DNA for the gene can be detected for up to 165 d postmolt in European *Ixodes ricinus* (L.) nymphs. Humair et al. (2007) subsequently studied the residual DNA from bloodmeals of *I. ricinus* ticks using the mitochondrial 12S rDNA and reported that they could distinguish between 60 known mammalian, bird, and lizard species. Pichon et al. (2006) used the small-subunit ribosomal RNA gene sequences to identify the host species in about half of the *I. ricinus* nymphs collected and, by also detecting the pathogens present, observed that most pathogens were restricted to the ranges of their hosts. This technique was able to identify the host species in about half of the ticks collected. In laboratory conditions, the host species was identifiable for up to 9 mo after molting (Pichon et al. 2003). More recently, the use of multiple 12S rDNA oligonucleotide probes was proposed to distinguish between closely related species of squirrels (Sciuridae) (Goessling et al. 2012).

PCR-based methods offer the possibility to identify genetic material even if the species studied has fed on more than one host (Cadenas et al. 2007). However, there are also several disadvantages of nucleic acid-

Article summary line: A time course study is presented, where the authors use tandem mass spectrometry to follow the degradation of host proteins in the nymphal stage of *I. scapularis* ticks.

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based host identification. These include the comparatively rapid degradation of the target nucleic acids, dependence on DNA primers that may vary in their specificities, and the susceptibility of PCR to contaminating DNA from previous amplifications from different species in the laboratory. These shortcomings arise even when using the more advanced methods, such as DNA barcoding for host identification (Gariepy et al. 2012).

An alternative to these approaches is mass spectrometry (MS)-based host protein identification. The advantage of using mass spectrometers for characterization of proteins lies in the inherent sensitivity and speed of the analysis that can be applied to a multitude of proteins in a sample, even when residual nucleic acids from the host are not present. State-of-the-art mass spectrometers have attomole (10^{-18} mol) detection limits and a detection speed of ≈0.7 s per peptide (Wojcik et al. 2012).

MS has been widely used for the past decade in the study of a variety of biological systems; however, it has yet to prove its capabilities in the field of acarology. One type of commonly used instrument is the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. For the analysis, the protein sample is digested with a protease, such as trypsin, to obtain shorter, more easily detectable peptides. The peptides are mixed with a matrix compound, placed on a stainless steel plate, dried, and the molecules are ionized using short laser bursts. The ions are analyzed simultaneously, offering molecular weight information of hundreds of components (Gobom et al. 2001).

Madden et al. (2002) used this approach to identify salivary secretion proteins from *Amblyomma americanum* (L.) ticks. They used the peptide mass fingerprint profile to distinguish between host and tick proteins and concluded that the host proteome overwhelms the tick proteins present. Because the signal of a peptide is in correlation with its concentration, identification of the low abundance secreted proteins was challenging, but this result is auspicious if host protein identification is desired. In a different study, Stopforth et al. (2010) assessed the hemolymph proteins responsible for the defense response of *Ornithodoros savigny* Audouin, 1827, using a similar MALDI-TOF experiment. However, this study similarly failed in detecting low-level tick proteins.

Because the relative quantities of host proteins are expected to decrease gradually as digestion of the bloodmeal progresses, it is expected that, at a certain point in the tick lifetime, the tick proteins will become more abundant than the host proteins. Therefore, it is apparent that a different, more sensitive mass spectrometer must be used for the comprehensive host proteome time course study. Liquid chromatography (LC) can be used to separate the peptides present in the sample before electrospray ionization (ESI), which increases the analysis time to 1–2 h per sample. Nonetheless, in the absence of the shielding effect of the high-abundance peptides, low-level components can be detected, when the instrument is optimally operating. LC coupled with tandem mass spectrometry (LC-MS/MS) has successfully been used to survey the type of host proteins present in *I. scapularis* and *A. americanum* ticks fed on sheep, rabbits, and mice (Wickramasekara et al. 2008). With the LC-MS/MS approach, host proteins such as actin, histones, tubulin, serum albumin, keratin, and hemoglobin were identified at the nymphal stage.

For the current study, a time course for the persistence of host blood proteins was sought to be established in nymphal *I. scapularis* after they had fed as larvae on the laboratory mouse, *Mus musculus*, and then molted. The stability over time of hemoglobin, a tetrameric protein complex with α and β chains of 141 and 146 amino acids, respectively, were of particular interest. This protein is highly conserved across species, but it also has regions of sequence divergence that can be exploited for host identification down to the species level. With the availability of whole genome sequence and a database of cDNA sequences for *I. scapularis*, the annotated tick proteins were also included in the searchable database for the data analysis. As a result, in addition to identifying host proteins, the LC-MS/MS approach provided information on tick proteins present at each time point.

Materials and Methods

Feeding of *I. scapularis* Larvae. Laboratory-reared larval *I. scapularis* were obtained from Oklahoma State University (Stillwater, OK) and fed on CD-1 outbred mice at the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Diseases (DVBD) in Fort Collins, CO, as described by Piesman (1993) and according to protocol 09–002, approved by the CDC, DVBD Institutional Animal Care and Use Committee. Mice (*M. musculus*) were anesthetized with a ketamine (67 mg/kg) and xylazine (6.7 mg/kg) mixture, and unfed larvae were placed on mice dorsally between the scapulae. Ticks were allowed to attach ad libitum and fed to repletion (3–5 d).

Sampling of *I. scapularis* Nymphs. Larvae were maintained at 24°C, >95% relative humidity, and a photoperiod of 16:8 (L:D) h at the University of Arizona. Molting of the tick population began ≈4 wk after feeding. Nymphs were sampled weekly, starting from the day after molt until no surviving ticks remained. The protein content extracted from a single *I. scapularis* nymph is 50–70 μg (Wickramasekara et al. 2008), corresponding to ≈1 nmol sample. This amount is sufficient for hundreds of analyses using the instrument and workflow described herein; therefore, the method is suitable for analysis of a single field-collected nymph. Because the goal of the current study was to explore the type of mammalian proteins detectable throughout the lifetime of the tick nymphs, at each time point five nymphs were sampled. Sample pooling was necessary to minimize the effects of differential protein profiles because of biological variability between individual ticks. Taking into account the mortality rate of larvae and nymphs and the length of the study, sampling a larger number of ticks was not

feasible in our laboratory. For each measurement, the five ticks were pooled and placed in a 1.5-ml Eppendorf tube in 50 μl ultra-pure water and were mechanically disintegrated and homogenized. Samples were stored at −80°C until further use.

Proteolytic Digestion and MS. In preparation for analysis, pooled samples were thawed and subjected to protein precipitation with 950 μl ethanol. The resultant protein pellets were then dissolved in 8 M urea, reduced with dithiothreitol, alkylated using iodoacetamide, and digested with trypsin. All reagents were obtained from Sigma Aldrich (St. Louis, MO). The peptides generated by tryptic digestion were separated using a Proxeon EASY-nLC system (Thermo Scientific, Odense, Denmark) with a 125-min H_2O: acetonitrile (0.1% formic acid) 5–95% organic gradient. Separation was performed on a Proxeon 10 cm, 75 μm ID C-18 analytical column, preceded by a 2 cm, 100 μm ID C18 precolumn. The eluent was nano-sprayed using an Advion TriVersa NanoMate nanospray system (Advion, Ithaca, NY) at a flow rate of 300 nL/min and spray voltage of 1.77 kV, and analyzed on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany). The LTQ Orbitrap Velos uses the ion trap and Orbitrap analyzers in tandem. In the first scan of each scan series, the peptides eluting at a particular time point from the LC column were surveyed using a high resolution (R = 30,000) precursor mass scan in the Orbitrap (MS). In the next step, the 14 most abundant precursor ions were selected from the MS scan and fragmented using collision-induced dissociation (CID), yielding an MS/MS scan. These MS/MS spectra were collected in the linear ion trap. After a mass was selected for fragmentation, it was included on a dynamic exclusion list for 45 s, ensuring that low abundance precursor ions co-eluting with highly abundant species were promptly fragmented. Sensitivity was improved by using the monoisotopic precursor selection (MIPS) set to 10 ppm mass tolerance. This ensured identification of peptide precursor isotope patterns, and set a narrow dynamic exclusion window to avoid excluding new peptides with mass to charge ratios (m/z values) close to m/z values already analyzed. The LC-MS/MS experiments were performed in triplicate. To avoid sample carryover, after each sample, the chromatographic column was flushed with high organic content solvent for 30 min, followed by a blank injection and a 40-min LC gradient. Data analysis was performed with Sequest, using the Thermo Proteome Discoverer v.1.3 algorithm (Thermo Scientific, Bremen, Germany).

Interpretation of the MS/MS Spectra. The LC-MS/MS output is in the form of an XCalibur.raw file that contains thousands of tandem mass spectra. The charge of the ions is the result of the addition of an H^+ in the acidic environment of the eluents used; the number of charges is loosely correlated with the number of basic groups in each individual ion. These spectra contain information about the mass and charge of the precursor ion and of fragment ions resulting from the cleavage of the peptide amide bonds. In CID experiments, each peptide ion selected for fragmentation collides with a neutral bath gas. This causes the breaking of the weakest bonds in the peptide, yielding b and y fragment ions. The MS/MS spectra were subjected to a database search algorithm. A database containing *I. scapularis* and *M. musculus* proteins present in the SwissProt knowledgebase was compiled *a priori.* Database search algorithms match the experimental spectra to predicted theoretical spectra or peak lists that are generated from candidate peptide sequences present in the database. The confidence of each hit was scored by Thermo Proteome Discoverer, which used the high resolution m/z information of the precursor ion measured in the Orbitrap and low resolution m/z fragment ions generated from the LTQ. After the most likely peptide sequence for each tandem mass spectrum was identified, the algorithm grouped the peptide sequences that passed the set filtering threshold and reported the proteins present. Score filtering was used to obtain high confidence identifications, and the filtering thresholds (Xcorr scores) were set to 1.8 for singly charged, 2.5 for doubly charged, and 3.5 for triply and higher charged peptides. Proteins identified with at least two peptides that passed the Xcorr threshold were considered confident. False discovery rate was determined using a reverse database of the tick and mouse databases and was found to be <0.5%.

Results and Discussion

Quality of MS Data for Host Proteins in Ticks. Under the laboratory conditions used, *I. scapularis* nymphs survived for up to 309 d postmolting or ≈345 d postfeeding. Over this time period, the nymphs were sampled periodically for analysis. The seasonal activity of questing *I. scapularis* immatures in the eastern United States involves larvae feeding in August–September, and molting by October. The next peak in nymphal activity is in the next May–July. Therefore, the survival of nymphs in nature appears to have a similar timeline of ≈10 mo (Gatewood et al. 2009) to that recreated in the laboratory conditions used in this study.

To illustrate the quality of the data and exemplify the information extracted, two fragmentation spectra are detailed in Fig. 1, showing the experimental results of two peptides present day 1 after molt (≈1 mo after feeding) sample. The XCorr scores of these peptides were greater than the threshold, and were identified as component sequences of β-hemoglobin of *M. musculus.* The spectra show the most abundant b^+ and y^+ fragment ions of the isolated precursor ion. With the exception of the first peptide bond cleavage, all fragment ions were present in the spectrum, showing extensive sequence coverage and reliable identification. The measurement error of the precursor m/z was within 7 ppm (part per million), a typical value for accurate peptide identification.

Figure 1a shows the MS/MS spectrum of the doubly charged YFDSFGDLSSASAIMGNAK peptide, part of hemoglobin β chain positions 43–60. The absence of the b_1^+ ion is a common feature of most fragmentation

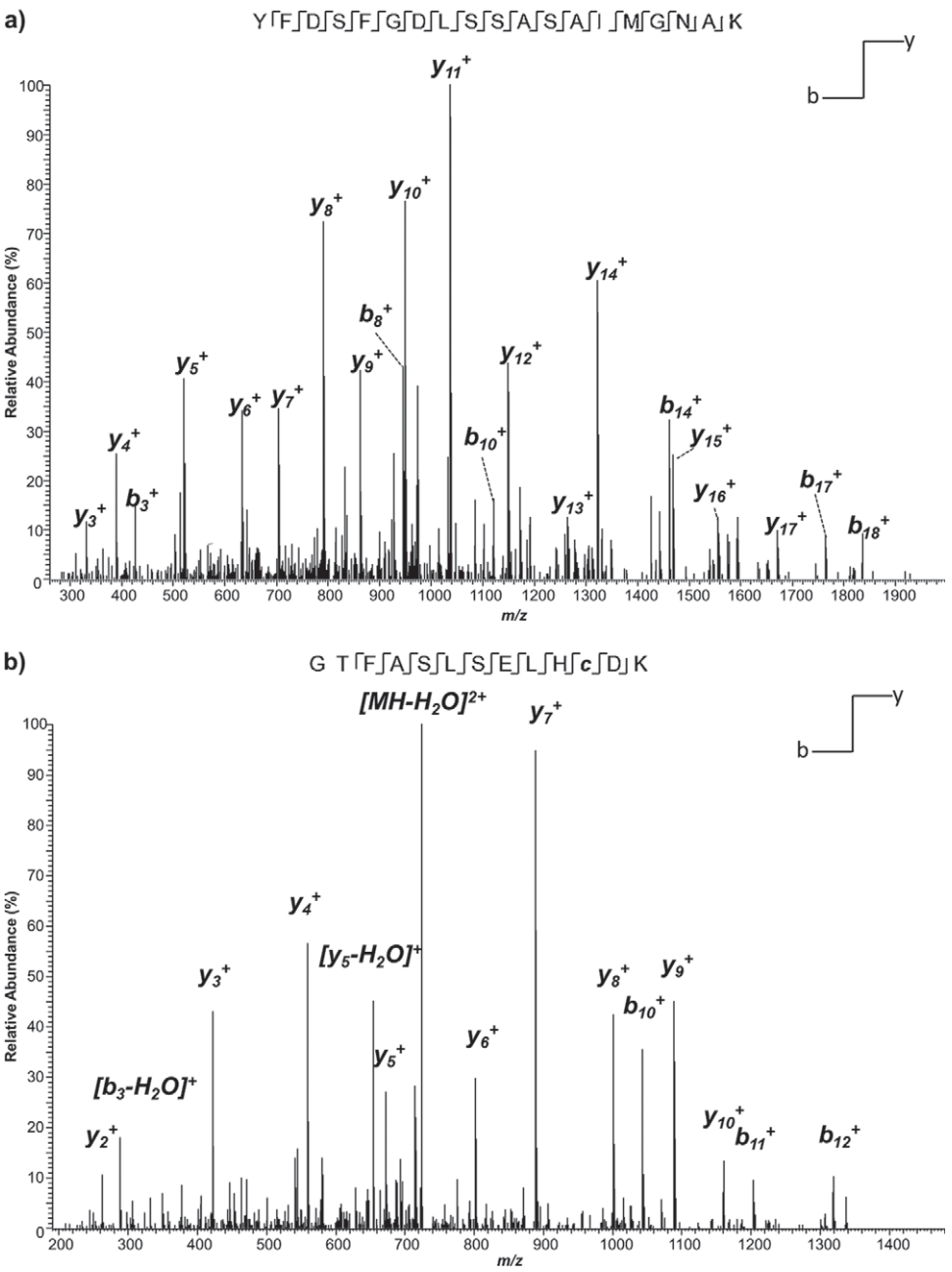


Fig. 1. CID spectra of (a) doubly charged YFDSFGDLSSASAIMGNAK and (b) doubly charged GTFASLSELHcDK peptides, belonging to *M. musculus* hemoglobin β chain. The most abundant b^+ and y^+ ions are labeled on each mass spectrum. The identified amino acid sequences and the fragmentation sites are indicated above the spectra. The mass error of the precursor ion was <7 ppm for both peptides.

spectra when CID is used, and is because of the instability of the gas phase structure of these ions (O'Hair and Reid 2000). This peptide was also detected with a mass increase of 15.99 Da, corresponding to the oxidized methionine form. Figure 1b shows the MS/MS spectrum of the GTFASLSELH*c*DK peptide, which has been identified to be part of the hemoglobin β chain, residues 84–96. The cysteine residue is alkylated and carries an additional carbamidomethyl group that adds 57.03 Da to the mass of the peptide.

The current detected for each ion is dependent on the amount of total sample injected, as well as on the concentration and ionizability of each molecule in part. The total ion current (TIC) is the sum of the current for all ions present in each spectrum, and can be controlled by varying the ion injection time, that is,

Table 1. Important *M. musculus* proteins found in the *I. scapularis* tick population

Sampling day	Total no. of mouse protein groups	Albumin coverage (%)/ (unique peptides)	Transferrin coverage (%)/ (unique peptides)	Hemoglobin coverage (%)/ (unique peptides)	
				α	β
1	47	46.71/ (24)	35.44/ (19)	83.8/ (8)	92.51 (11)
8	20	15.95/ (7)	0	36.62/ (4)	68.03 (8)
15	20	33.06/ (15)	7.03/ (4)	62.68/ (6)	93.87 (11)
22	20	22.70/ (10)	6.31/ (3)	41.55/ (5)	76.87 (9)
29	13	21.05/ (10)	4.02/ (2)	62.68/ (6)	68.02 (8)
36	17	10.53/ (5)	0	50.0/ (4)	69.38 (8)
43	25	13.16/ (6)	0	50.0/ (4)	70.74 (8)
50	17	2.3/ (1)	0	36.62/ (4)	70.74 (8)
57	19	4.44/ (2)	0	36.62/ (4)	72.60 (8)
64	23	0	0	36.62/ (4)	55.78 (6)
78	24	23.36/ (10)	4.02/ (2)	62.68/ (6)	68.71 (8)
85	19	4.44/ (2)	0	56.34/ (5)	68.70 (9)
92	20	0	0	41.55/ (3)	62.85 (7)
99	14	0	0	7.38/ (1)	36.73 (4)
106	19	10.86/ (5)	0	36.62/ (4)	61.90 (7)
113	9	0	0	0	25.85 (3)
120	13	10.69/ (4)	0	42.96/ (5)	63.94 (8)
127	20	0	0	19.01/ (3)	36.73 (4)
134	22	0	0	0	0
155	17	0	0	0	27.89 (3)
162	13	0	0	21.83/ (3)	55.78 (6)
169	13	0	0	0	46.94 (5)
176	15	0	2.05/ (1)	21.83/ (2)	55.78 (6)
183	23	0	0	19.01/ (2)	27.89 (3)
190	27	0	0	0	0
197	20	0	0	0	0
204	24	0	0	0	0
211	24	0	1.72/ (1)	5.97/ (1)	21.92 (3)
218	14	0	0	0	8.16 (1)
232	16	0	0	0	15.65 (2)
253	6	0	0	0	15.65 (2)
260	13	0	0	0	6.8 (1)
274	19	0	0	0	0
281	20	0	0	0	0
288	14	0	0	0	28.57 (3)
295	19	0	0	0	19.72 (2)
309	14	0	0	0	15.65 (2)

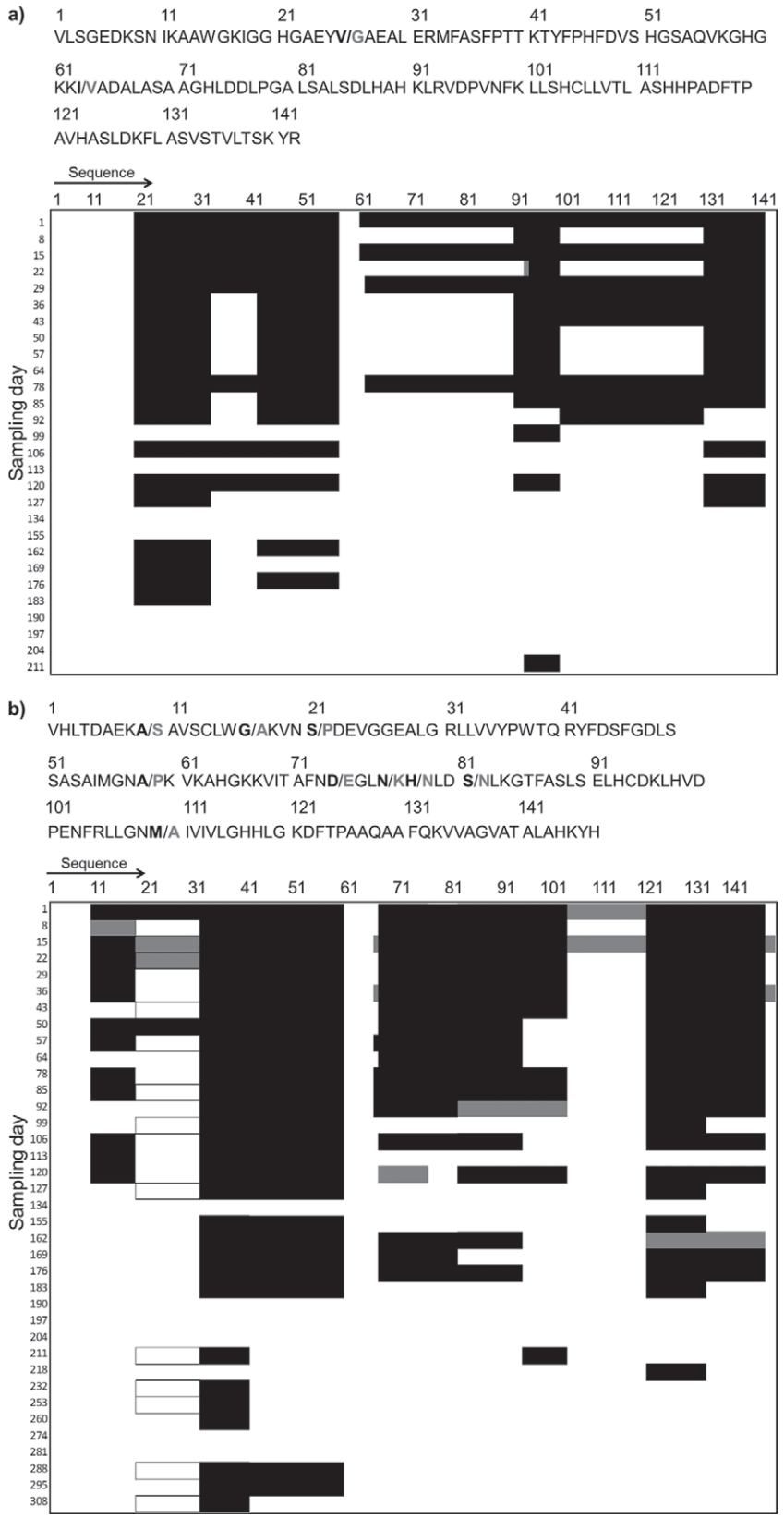
The first column indicates the sampling day after molting; the second column shows the total number of mouse proteins that were found with at least two peptides per protein. The percentage values represent the nominal sequence coverage of the protein for each sample, and the values in parentheses show the number of unique peptides detected.

the time allotted for ion accumulation. Thus, low abundance ions were accumulated for a longer time (e.g., 200 ms), whereas more abundant species required much shorter (<1 ms) injection times. The largest ion current recorded for the LC-MS/MS experiment exemplified here was 5.4×10^8, whereas the ion current of the two peptides was three orders of magnitude lower, 9.9×10^4 and 7.2×10^5, respectively. Nonetheless, by using an LC separation before mass analysis combined with the dynamic exclusion list ensured extensive spectral information even for the less abundant peptides.

Host Proteins Present in the Sampled Nymphs Over the Sampling Period. Table 1 lists by day of collection after molting the unambiguously assigned host proteins that were repeatedly detected in the tick samples of this study. The table includes the total number of mouse proteins that were detected with at least two confirmed peptides for each protein. According to Elias and Gygi (2007), confidence in protein identification increases with the number of constituent peptides assigned. Table 1 also lists the percentage values of the identified sequence coverage of albumin, transferrin, and hemoglobin α and β chains; in parentheses, we indicated the number of peptides detected by MS/MS.

Structural Proteins. Several mammalian proteins were detected consistently in the analyzed tick samples throughout the time course. In samples from ticks that fed on *M. musculus*, there were multiple peptides each of actin, keratin, tubulin, and histone chains 2A, 3, and 4. Although these proteins were found in all

Fig. 2. Mouse hemoglobin (a) α and (b) β chain sequence coverage at different sampling times in the study of mammalian host proteins in the bloodmeal of *I. scapularis* ticks. The two different sequence variants of hemoglobin β and α are indicated with bolded black and gray letters. Sequence coverage of the two variants were overlapped; black regions represent peptides with sequences containing bolded black letters; gray regions were identified as the variant peptides. White boxes in the β coverage map denote peptide regions identified as hemoglobin ε.


a)
1 11 21 31 41 51
VLSGEDKSN IKAAWGKIGG HGAEYV/GAEAL ERMFASFPTT KTYFPHFDVS HGSAQVKGHG
61 71 81 91 101 111
KKI/VADALASA AGHLDDLPGA LSALSDLHAH KLRVDPVNFK LLSHCLLVTL ASHHPADFTP
121 131 141
AVHASLDKFL ASVSTVLTSK YR
Sequence
Sampling day
b)
1 11 21 31 41
VHLTDAEKA/S AVSCLWG/AKVN S/PDEVGGEALG RLLVVYPWTQ RYFDSFGDLS
51 61 71 81 91
SASAIMGNA/PK VKAHGKKVIT AFND/EGLN/KH/NLD S/NLKGTFASLS ELHCDKLHVD
101 111 121 131 141
PENFRLLGNM/A IVIVLGHHLG KDFTPAAQAA FQKVVAGVAT ALAHKYH
Sequence
Sampling day

replicates at all sampling times, examination of the aligned sequences for these sets of proteins revealed that mouse and tick structural proteins were highly similar in sequence. The tubulin peptides were ≈96% identical in sequence between these species, and some of the histone peptides of the different species had complete sequence homology. Therefore, the database search engine could not decisively distinguish between these mouse and tick proteins. A BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the peptides identified belonging to these highly conserved proteins revealed that, with few exceptions, they could not be unambiguously assigned to either species. Mass spectrometry is capable of offering more detailed information on protein structure beyond the primary amino acid sequence, such as the presence of post-translational modifications (such as phosphorylation, methylation, glycosylation, and so forth). However, with the absence of a large scale species-specific library of these modifications, this information cannot be readily used for species identification. On the basis of these findings, we concluded that actin, keratin, tubulin, and the various histones would not be informative for identification of the host species of the last bloodmeal.

Immunoglobulins. It was previously shown that host IgG is present in the tick hemolymph of soft ticks, *Ornithodoros moubata* (Murray) (Minoura et al. 1985). The uptake of immunoglobulin into the hard tick *A. americanum* was also demonstrated by capillary feeding of labeled serum proteins (Jasinskas et al. 2000). This phenomenon of immunoglobin uptake into the hemolymph was the basis of proposals for antitick vaccine (Minoura et al. 1985). However, we found that, by the criterion of ≥2 peptides, the presence of immunoglobulins was observed in the day 1 samples but not thereafter. Previous work suggest that certain ixodid species may have evolved the ability to evade the host immune response by removing immunoglobulins, in particular through the secretion of IgGs from the midgut into the tick saliva via the salivary glands during feeding, thus explaining their limited presence in our samples (Wang and Nuttall 1999).

Transferrin and Albumin. These two serum proteins were detected in samples from day 1, with 46.7 and 35.4% protein sequence identified, respectively (Table 1). Transferrin apparently degraded rapidly; by day 29, only 4% of the protein was detected. Degradation of albumin was slower; this protein was present in the analyzed samples until day 85. However, certain sampling points in the course of the experiment, such as those at days 78, 106, 120, 176, and 211 showed protein identifications that were exceptions to an apparent trend of gradual protein degradation. The presence of transferrin and serum albumin at these time points may be accounted for by differences between individual ticks in a given pool in the rate of protein degradation. Owing to the very high sensitivity of the mass spectrometer used (≈200 ng total protein loaded per experiment), subtle differences in the process of protein digestion or degradation by a single tick may result in a difference in the number of peptides and types of proteins identified in the pool as whole.

Hemoglobin. Throughout the time course experiment, the database search software assigned two β and two α chains of mouse hemoglobin, albeit with various UniProt accession numbers. β-1 chain was identified under protein accession numbers P0288, D0U269, and D4N6R6, and β-2 chain was reported either as Q54AH9 or A8DUM2. α-1 was identified as A8DUV1, and α-2 as P1942. In addition, the ε chain was occasionally identified with accession number P02104. α chain peptides were detected in the majority of pooled tick samples for up to 183 d by the criterion of the presence ≥2 peptides, and for up to 211 d by the criterion of presence of single peptide identification. β-chain peptides were detected in some tick pools up until the end of sampling on day 309. Figure 2 shows hemoglobin survival and indicates the position of identified peptides for the hemoglobin α and β chain variants throughout the time course. Mouse hemoglobin in the day 309 sample was represented by two peptides: VNVEEVGGEALGR and LLVVYPWTQR. The LLVVYPWTQR peptide is a shared peptide between the hemoglobin ε and β chains (positions 31–40). The VNVEEVGGEALGR peptide (hemoglobin ε 18–30) was detected in 10 samples, including the last sample collected on day 308. These peptides are shown in Fig. 2 as white boxes.

One discrepancy in the trend of seemingly diminishing hemoglobin β was the large sequence coverage in the samples collected for days 288–309. These sampling points indicate the host protein content of the last surviving ticks in our study. Therefore, we hypothesize that hemoglobin may be crucial for tick survival and that only ticks with high hemoglobin content (those that either had a larger initial bloodmeal volume or a slower metabolic rate) were able to survive for this long. Future studies that might verify this hypothesis would involve analyzing ticks on the day of their natural death.

Hemoglobin is a major blood component and plausibly could be used for host identification. LC-MS/MS or MALDI-MS could be used for host species identification from field-collected nymphs based on the sequence of the hemoglobin fragment identified or the m/z of the intact α and β chain. The prerequisite of the LC-MS/MS approach, however, is the construction of a comprehensive database composed of hemoglobin sequences of all known tick hosts. We previously carried out de novo sequencing of α and β chains of hemoglobin of several small mammals that are documented or potential reservoirs for Lyme disease and other zoonotic infections (Laskay et al. 2012).

The β-hemoglobin peptide that was identified at 288 and 295 d postmolt in at least some of the ticks was LVVYPWTQRYFDSFGDLSSASALMGNAK. This was 96% identical to the *M. musculus* sequence, differing only by a leucine in place of the isoleucine at position 23 in the reference sequence. Having identical masses, the mass spectrometer used herein was not able to distinguish these two amino acids. The aligned *Rattus norvegicus* sequence differed by two

amino acids, at positions 23 and 27. The corresponding sequence of the β chain *Peromyscus leucopus*, a common reservoir for *B. burgdorferi* and host for *I. scapularis*, and a related species, *Peromyscus maniculatus*, differed at three residues. With mammals in more distant clades, there was more diversity. For instance, the aligned β chain sequence for the chipmunk, *Tamias merriami*, differed at 4 of 28 residues (Laskay et al. 2012).

As discussed, keratin, actin, histone, and tubulin chains are not considered good targets for host species identification, because even though they were commonly represented in the samples, they are too conserved in sequence across the animal kingdom to be informative. It is possible, but not probable, that the mammalian species of interest, like Tamias spp., could have unique regions of these structural proteins. However, mass spectrometry-based de novo sequencing for these proteins from a variety of species would be technically difficult, considering the size of these structural proteins, some of which are >500 amino acid residues in length. The presence of different chain types, isoforms and posttranslational modifications, such as extensive methylation of histones, would further complicate the analysis. These considerations lead us to conclude that hemoglobin is the most promising candidate for host species identification, especially when flat ticks are examined months after a molt.

Another justification for a focus on the α and β globin chains are the reports by Fogaca et al. (1999) and Sforça et al. (2005) that the bovine hemoglobin α chain region from residues 33–61 (FLSFPTTKTYF PHFDLSHGSAQVKGHGAK) shows antimicrobial activity and have proposed that it plays an important role in the defense mechanism of the metastriate tick *Rhipicephalus microplus* against different microorganisms. Part of this region, TYFPHFDVSHGSAQVK, was identified in nymphs in this study, up to 176 d postmolt, and could indicate that this and related peptides may play a similar role in the prostriate tick *I. scapularis*.

Other Mammalian Proteins. Mouse carbonic anhydrase, hemopexin, and apolipoprotein A were detected in the day 1 sample, and serine protease inhibitor was present in the first three samples collected. None of these proteins was detected in any of the later sampling points, indicating a rapid degradation of these during tick metabolism.

One of the main advantages of using mass spectrometry for the large-scale study of the proteome of an entire organism is the quality and quantity of information provided by a single experiment. Because no a priori knowledge is required about the proteins of interest, a vast amount of unbiased information can be obtained regarding the type of proteins present in the sample. The advantage of this feature was used in this study to survey the tick proteins present at each time point, in addition to those within the bloodmeal.

Although not the original intent for the study, we also identified *I. scapularis* proteins in the samples. These include tick versions of actin, keratin, myosin, and tubulin, as well as tick macroglobulins, heme lipoproteins, carrier proteins, several secreted salivary gland proteins, and several tick proteases. Among the identified proteases were cathepsin B and C, which were present in the samples for up to 3 mo after molting. Horn et al. (2009) have recently shown that in *I. ricinus*, hemoglobin digestion occurs intracellularly in the gut tissue with the help of endopeptidases from the cathepsin family. Our results indicate that this process continues for several weeks in *Ixodes* species ticks. Another tick protein of more general interest that was identified is Serpin 4 (Swiss Prot accession #B7QL36), which has been proposed as a target for an antitick vaccine (Imamura et al. 2005). Serpin 4 was present in samples throughout the sampling period.

Based on our current findings, we suggest the use of a MS-based proteomic approach for the detection of hemoglobin fragments for host species identification. A prerequisite of this work is an expansion of a comprehensive hemoglobin database comprising all known *I. scapularis* host species found in its geographic range. Once this database is established, the efficacy of testing field-derived *I. scapularis* collected in Lyme disease endemic regions could be established.

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