Title: Time-resolved proteomic profile of *Amblyomma americanum* tick saliva during feeding

Running Title: Proteins in tick saliva every 24 h during feeding

Tae Kwon Kim[a*]; Lucas Tirloni[a,b*]; Antônio F. M. Pinto[c,d]; Jolene K. Diedrich[d,f]; James J. Moresco[d,f]; John R. Yates III[d]; Itabajara da Silva Vaz Jr[b,e]; and Albert Mulenga[a,#]

[a] Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX, USA.

[b] Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[c] Foundation Peptide Biology Lab, Salk Institute for Biological Studies, La Jolla, CA, USA

[d] Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA.

[e] Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[f] Mass Spectrometry Core, Salk Institute for Biological Studies, La Jolla, CA, USA.

*TKK and LT are co-first authors

Correspondence author
Phone: 979 458 4300
Email: amulenga@cvm.tamu.edu

Abstract

Amblyomma americanum ticks transmit more than a third of human tick-borne disease (TBD) agents in the United States. Tick saliva proteins are critical to success of ticks as vectors of TBD agents, and thus might serve as targets in tick antigen-based vaccines to prevent TBD infections. We describe a systems biology approach to identify, by LC-MS/MS, saliva proteins (tick=1182, rabbit=335) that *A. americanum* ticks likely inject into the host every 24 h during the first 8 days of feeding, and towards the end of feeding using two different sample preparation approaches (in-gel and in-solution). The in-gel approach determined molecular identification of predominant protein bands in tick saliva, and the in-solution added depth to discovery of proteins. Searching against entries in GenBank grouped tick and rabbit proteins in this study into 27 and 25 functional categories. Aside from housekeeping-like proteins, majority of tick saliva proteins belong to the tick-specific (no homology to non-tick organisms: 32%), protease inhibitors (13%), proteases (8%), glycine-rich proteins (6%) and lipocalins (4%) categories. Global secretion dynamics analysis suggests that majority (74%) of proteins in this study are associated with regulating initial tick feeding functions and transmission of pathogens as they are secreted within 24-48 h of tick attachment. Comparative analysis of the *A. americanum* tick saliva proteome to five other tick saliva proteomes identified 284 conserved tick saliva proteins: we speculate that these regulate critical tick feeding functions and might serve as tick vaccine antigens. We discuss our findings in the context of understanding *A. americanum* tick feeding physiology as a means through which we can find effective targets for a vaccine against tick feeding.

Keywords: ticks, saliva, proteome, tick-feeding

Author Summary

The lone star tick, *Amblyomma americanum*, is a medically important species in US that transmits 5 of the 16 reported tick-borne disease agents. Most recently, bites of this tick were associated with red meat allergies in humans. Vaccination of animals against tick feeding has been shown to be a sustainable and effective alternative to current acaricide based tick control method which has several limitations. The pre-requisite to tick vaccine development is to understand the molecular basis of tick feeding physiology. Toward this goal, this study has identified proteins that *A. americanum* ticks inject into the host at different phases of its feeding cycle. This data set has identified proteins that *A. americanum* inject into the host within 24-48 h of feeding before it starts to transmit pathogens. Of high importance, we identified 284 proteins that are present in saliva of other tick species, which we suspect regulate important role(s) in tick feeding success and might represent rich source target antigens for a tick vaccine. Overall, this study provides a foundation to understand the molecular mechanisms regulating tick feeding physiology.

Introduction

Ticks and tick-borne diseases (TBDs) have been on the rise and have greatly impacted human and veterinary medicine. Ticks have gained the attention in public health policy with a recent publication that advocated for One Health solutions listing 17 human TBDs among sources of human health concerns (1). Moreover, the dramatic rise related to ticks and TBDs have caught the attention of United States (US) lawmakers, as shown in the 21st Century Cures Act of 2016, which created the TBD Working Group. Under the Cures Act, the TBD Working Group was tasked with evaluating the impact of TBDs and required research to find solutions (https://www.hhs.gov/ash/advisory-committees/tickbornedisease/index.html). Likewise, six of the 23 human vector-borne diseases that are listed by the World Health Organization are tick borne that include Crimean-Congo haemorrhagic fever, Lyme disease, relapsing fever, rickettsial diseases (spotted fever and Q fever), tick-borne encephalitis, and tularemia (http://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases). In the US, *Amblyomma americanum,* the lone star tick is among one of the tick species of medical and veterinary health significance.

A. americanum is a geographically expanding tick species (2) that is involved in transmission of multiple human and animal disease agents. In public health, *A. americanum* is the principal vector for *Ehrlichia chaffensis*, the causative agent of human monocytic ehrlichiosis (3), and *E. ewingii,* which also causes ehrlichiosis, referred to as human granulocytic ehrlichiosis (4-6). This tick also transmits *Francisella tularensis,* the causative agent for tularemia (7, 8), a yet to be described disease agent, suspected as *Borrelia lonestari,* which causes Lyme disease-like symptoms referred to as southern tick-associated rash illness (STARI) (9, 10) and also an *E. ruminantium*-like organism referred to as the Panola Mountain *Ehrlichia* (PME) (11). There is

also evidence that *A. americanum* may transmit *Rickettsia amblyommii*, *R. rickettsia, and R. parkeri,* the causative agents to rickettsiosis to humans (12, 13). This tick has also been reported to transmit the Heartland and Bourbon viruses to humans (14, 15). Most recently, this tick has been shown to be responsible for causing an α-gal allergy or mammalian meat allergy (MMA) in humans upon tick bite (16). In veterinary health, *A. americanum* transmits *Theileria cervi* to deer (17), and *E. ewingii* to dogs (18). There are reports of mortality in deer fawns that were attributed to a combination of heavy *A. americanum* infestation and *T. cervi* infections (19). In livestock production, heavy infestations were thought to cause low productivity in cattle (20, 21). In the Southern US, *A. americanum* appears to be the most dominant tick species that bite humans, which has been reported to be responsible for 83% of human tick infestations (22).

The success of ticks as pests and vectors of TBD agents is facilitated by secreted tick salivary proteins that are injected into the host to regulate the tick's evasion of host defense (23). There is evidence that repeatedly infested animals develop immunity against tick saliva proteins and are protected against TBD transmissions such as *Francisella tularensis* (24), *B. burgdorferi* (25-27), *Babesia* spp. (28), Thogoto virus (29), tick-borne encephalitis virus (30) and *T. parva bovis* (31). Therefore, identification of tick saliva proteins that ticks inject into the host during feeding might lead to development of tick saliva protein-based vaccines to prevent TBD infections.

The goal of this study was to utilize systems biology approach to identify proteins that *A. americanum* ticks injects every 24 h during feeding. This study builds upon our recent findings that identified *Ixodes scapularis* tick saliva proteins that are secreted every 24 h during first five days of feeding (32), partial and replete fed *Rhipicephalus microplus* (33), and replete fed adult and nymph *Haemaphysalis longicornis* (34). Others have reported proteins in saliva of replete

fed *R. sanguineus* (35) and three and five day fed *Dermacentor andersoni* (36). A related study reported proteins in *Ornithodoros moubata* (soft tick) identified from saliva collected after four months from feeding (37). Most recently, the saliva proteomes of unfed *I. scapularis* and *A. americanum* exposed to different hosts have been identified (38). In this study, we report proteins that *A. americanum* ticks sequentially inject into the host every 24 h during feeding. Comparison of the *A. americanum* tick saliva proteome in this study with other saliva proteomes of other tick species allowed us to identify tick saliva proteins that are likely utilized by multiple tick species to regulate feeding, and these might represent potential antigens for anti-tick vaccine development.

Materials and Methods

Ethics statement

All experiments were done according to the animal use protocol approved by Texas A&M University Institutional Animal Care and Use Committee (IACUC) (AUP 2011-207 and 2011-189) that meets all federal requirements, as defined in the Animal Welfare Act (AWA), the Public Health Service Policy (PHS), and the Humane Care and Use of Laboratory Animals.

A. americanum tick saliva collection

A. americanum ticks were purchased from the tick rearing facility at Oklahoma State University (Stillwater, OK, USA). Routinely, ticks were fed on rabbits as previously described (39, 40). Ticks were restricted to feed on the outer part of the ear of New Zealand rabbits with orthopedic stockinet's glued with Kamar adhesive (Kamar Products Inc., Zionsville, IN, USA). To stimulate female *A. americanum* ticks to attach onto the host to start feeding and to be

130 inseminated to complete the feeding process, male ticks (15 per ear) were pre-fed for three days
131 prior to placing female ticks onto rabbit ears to feed. A total of 50 female *A. americanum* ticks
132 (25 per ear) were placed into tick containment apparatus on each of the three rabbits and allowed
133 to attach.

134 Saliva of female *A. americanum* tick was collected as previously described (32, 33).
135 Saliva was collected from 15 ticks fed for 24, 48, 72, 96, 120, 144, 168, and 192 h, respectively,
136 ten ticks fully fed but not detached from the host (BD) and six ticks spontaneously detached from
137 the host (SD). Briefly, tick saliva was collected every 15 – 30 min intervals for a period of
138 approximately 4 h at room temperature from ticks that were previously injected with 1-3 µL of
139 2% pilocarpine hydrochloride in phosphate buffered saline (PBS, pH 7.4) as published by our
140 group (32, 38).

141

142 *Identification of A. americanum tick saliva proteins by LC-MS/MS*

143 Identification of tick saliva proteins using LC-MS/MS was done in two methods: "in-gel"
144 digestion (GeLCMS) and "in-solution" digestion (shotgun proteomics) of tick saliva protein
145 peptides as described (32-34). For the in-gel preparation approach, saliva from a pool of 30 ticks
146 collected from 24, 72, 120, and 168 h fed were resolved on a Novex 4-20% Tris-Glycine SDS-
147 PAGE gradient (Thermo, Waltham, MA, USA), stained with Coomassie Brilliant Blue, visible
148 protein bands excised and submitted for LC-MS/MS as described (33, 41). For the in-solution
149 digestion method, ~4.5 µg of total tick saliva proteins (in triplicate runs using ~1.5 µg per run)
150 per feeding time point (24, 36, 48, 72, 96, 120, 144, 168, 192, BD, and SD) were processed for
151 LC-MS/MS as published by our group (32-34).

152

153 *Database searching of tandem mass spectra*

154 Proteins in *A. americanum* tick saliva were identified according to the previously
155 described pipeline (32-34). To prepare the protein database used for protein identification, we
156 extracted the coding sequences (CDS) from *A. americanum* transcriptomes that were assembled
157 from Illumina sequence reads (BioProject accession # PRJNA226980) (42) using an automated
158 pipeline in Visual Basic (Microsoft, Redmond, Washington, USA) provided Dr. Jose M. Ribeiro
159 (NIH), based on similarities to known proteins (43). Contigs from the assembled *A. americanum*
160 transcriptome were used to identify open reading frames (ORFs) that were larger than 50 amino
161 acids in all six frames. The identified ORFs were subjected to blastp using several amino acid
162 sequence databases downloaded from NCBI (non-redundant [nr] Acari and refseq-invertebrate),
163 Uniprot (nr-Acari), MEROPS database (44), the GeneOntology (GO) FASTA subset (45) and the
164 conserved domains database (CDD) of NCBI (46) containing the COG (47), PFAM (48), and
165 SMART motifs (49). As a false-discovery approach to identify transcripts related to hosts, we
166 searched the ORFs against the nr-databases from NCBI for rabbit, mouse, rat, goat, sheep, cow,
167 monkey, and humans. CDS were extracted from blastp searches that matched with 70% identity
168 and e-value of $1e^{-40}$. To remove redundancies, CD-HIT (50) was used to remove sequences at
169 98% identity. The extracted CDS (n=110,587) were concatenated with *Oryctolagus cuniculus*
170 from Uniprot (www.uniprot.org) (n=21,148) and reverse sequences of all entries were used to
171 identify peptides from tandem mass spectra.

172 For the in-gel method, proteins were identified by searching MS/MS spectra against the
173 protein database (described above) using the MASCOT software version 2.2 (Matrix Science,
174 London, UK) with the following parameters: tryptic specificity, one missed cleavage and a mass
175 tolerance of 0.2 Da in the MS mode and 0.2 Da for MS/MS ions. Carbamidomethylation of

cysteine was set as a fixed modification, and methionine oxidation was set as variable modifications. Mascot peptide identifications required ion scores higher than the associated identity scores of 20 and 35 for doubly and triply charged peptides, respectively. Protein identifications were accepted if they contained at least 2 identified peptides. To be included in this analysis, all peptide sequences had to have 100% identity with assigned proteins.

For the in-solution approach, proteins were identified by first extracting the tandem mass spectra from Thermo RAW files using RawExtract 1.9.9.2 (51) and then searching against the protein database (described above) using ProLuCID in the Integrated Proteomics Pipeline Ver.5.0.1 (52). At least two peptide matches were required to be considered a protein hit. A cutoff score was established to accept a protein false discovery rate (FDR) of 1% based on the number of decoys. Additionally, a minimum sequence length of six residues per peptide was required. Results were post processed to only accept PSMs with <10ppm precursor mass error. Finally, the protein matches from each sampled time points were concatenated into one file using Identification Compare (IDcompare) program on IP2- Integrated Proteomics Pipeline Ver.5.0.1 (52).

For functional annotation, both tick and rabbit proteins were searched against the following databases: non-redundant (NR), Acari and refseq-invertebrate from NCBI, Acari from Uniprot, MEROPS database (44), the GeneOntology (GO) FASTA subset (45), and the conserved domains database of NCBI (46) containing the COG (47), PFAM (48), and SMART motifs (49). Outputs from the blast searches were used in the classifier program in Dr. Ribeiro's visual basic program (43) to functionally categorize the identified proteins based on the best match from among all the blast screens. The functionally annotated proteins were manually validated.

Relative abundance and graphical visualization of secretion dynamics of A. americanum tick saliva proteins.

Relative abundance and secretion dynamics were determined as described (32) using normalized spectral abundance factors (NSAF) that were validated as reliable in a label-free relative quantification approach (53-55). For each functional category or individual protein, NSAF was expressed as a percent (%) of total NSAF for that time point. Percent NSAF values were normalized using Z-score statistics using the formula $Z = \frac{X - \mu}{\sigma}$, where Z *i*s the Z-score, X is the NSAF for each protein per time point, μ is the mean throughout time points, σ is the standard deviation throughout time points. Normalized percent NSAF values were used to generate heat maps using the heatmap2 function from the gplots library in R (56).

Identification of A. americanum saliva proteins found in saliva of other tick species

A. americanum tick saliva proteins in this study were searched against published tick saliva proteomes of *R. microplus* (32), *I. scapularis* (33), *H. longicornis* (34), *R. sanguineus* (35), *D. andersoni* (36), and *O. moubata* (37) using local BLASTp analysis. Databases of protein sequences reported for each tick saliva proteome were extracted from NCBI or Uniprot and screened by BLASTp using the *A. americanum* saliva proteome (from this study) as the query. Protein matches ≥70% identity was reported.

Results and Discussion

Protein profile and abundance changes every 24 h during A. americanum tick feeding

Previous studies have demonstrated that the protein profile and abundance in salivary glands of female *A. americanum* is dynamic and changes during the course of tick feeding (57).

However, a limitation to the previous study was that it did not inform which salivary gland proteins were secreted during feeding. To attempt at capturing changes in tick saliva protein profiles, we successfully used pilocarpine to induce and collect saliva from *A. americanum* ticks every 24 h during the first eight days of tick feeding as we all as from ticks that had engorged but had not detached, and replete fed ticks as described (32, 58). In early feeding stages (24-72 h), *A. americanum* tick saliva was observed as a white flake that accumulated on the mouthparts over time and was collected every 15 - 30 min for 4 h by washing the mouthparts with sterile phosphate buffered saline. Tick saliva was more evident after 72 h of feeding, observed as droplets of liquid forming at the mouthparts. Proteins in tick saliva were identified by LC-MS/MS sequencing in two approaches: "in-gel" digestion (GeLCMS) and "in-solution" digestion (shotgun proteomics) of tick saliva protein peptides.

For the "in-gel" digestion approach, saliva that was collected from 24, 72, 120, and 168 h fed *A. americamnum* ticks was electrophoresed on a 4-20% SDS-PAGE and Coomassie blue staining. Subsequently visible protein bands (n=157) (Fig. 1) were individually excised, processed for in-gel trypsinization and LC-MS/MS analysis. The peptide MS/MS spectra were searched using MASCOT software version 2.2 (Matrix Science, London, UK) against a combined protein database (tick, rabbit, and human contaminants [i.e keratin]) that was translated from coding domains (n=110,587) that were assembled from Bioproject # PRJNA226980 (42). This analysis identified a total of 76 proteins (294 peptides) in tick saliva of which 55 (229 peptides) and 21 (64 peptides) belonged to tick and rabbit, respectively (Supplemental table 1). Of the total 55 tick saliva proteins 23, 16, 41, and 19 were identified in saliva of 24, 72, 120 and 168 h fed, respectively (Tables 1A-C). Likewise, we identified 1, 19, 8, and 4 rabbit proteins in 24, 72, 120 and 168 h fed tick saliva, respectively (Tables 1A-C).

Table 1A. *Amblyomma america*num saliva proteins (250-100 kDa) identified from in gel digestion and LC-MS/MS during feeding

	Feeding Time Points (h)											
	24 h			72 h			120 h			168 h		
Molecular weight	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description
≥ 250 kDa	C1-C4	N/A	N/A	D1	N/A	N/A	E1-E4	N/A	N/A	F1-F2	N/A	N/A
				D2	XP_002711050.1	keratin 6A-like	E5	Aam-426945	TIL domain containing protein, partial	F3	Aam-28660	putative neutral endopeptidase-like protein, partial
				D3-D8	N/A	N/A		Aam-59478	TIL domain containing protein, partial	F4	Aam-28660	putative neutral endopeptidase-like protein, partial
	C5	Aam-3564	heme lipoprotein precursor	D9	Aam-3564	heme lipoprotein precursor		Aam-587352	TIL domain containing protein, partial	F5-F10	N/A	N/A
							E6-E7	N/A	N/A	F11	Aam-54072	Vitellogenin B
				D10	XP_002719410.1	keratin 10	E8	Aam-3564	heme lipoprotein precursor		Aam-3564	heme lipoprotein precursor
				D11-D12	N/A	N/A	E9-E11	N/A	N/A			
250 - 150 kDa	C6-C7	N/A	N/A	D13	N/A	N/A	E12	Aam-3564	heme lipoprotein precursor	F12	N/A	N/A
	C8	Aam-3564	heme lipoprotein precursor	D14	XP_017197646.1	keratin 6A-like		XP_002711050.1	keratin 6A-like			
							E13	Aam-54072	Vitellogenin B	F13	N/A	N/A
	C9	Aam-3564	heme lipoprotein precursor	D15	XP_017197646.1	keratin 6A-like	E14	N/A	N/A			
		Aam-220360	putative vitellogenin-1, partial				E15	Aam-3564	heme lipoprotein precursor			
150 - 100 kDa	C10	Aam-3564	heme lipoprotein precursor	D16	Aam-220360	putative vitellogenin-1, partial	E17	Aam-3564	heme lipoprotein precursor	F14	Aam-392504	putative alpha-2-macroglobulin-like protein
		Aam-220360	putative vitellogenin-1, partial		Aam-62355	vitellogenin 4, partial		Aam-186025	heme lipoprotein precursor			
								Aam-954297	glucose dehydrogenase, putative			
								Aam-144361	heme lipoprotein precursor			
		Aam-392504	putative alpha-2-macroglobulin-like protein		Aam-62352	putative vitellogenin-1, partial	E18	Aam-3564	heme lipoprotein precursor		Aam-220360	putative vitellogenin-1, partial
		Aam-62355	vitellogenin 4, partial					Aam-186025	heme lipoprotein precursor			
	C11	Aam-3564	heme lipoprotein precursor	D17	Aam-3564	heme lipoprotein precursor		Aam-50363	hypothetical protein		Aam-3564	heme lipoprotein precursor
		Aam-186025	heme lipoprotein precursor					Aam-220360	putative vitellogenin-1, partial			
		Aam-392513	putative alpha-2-macroglobulin-like protein, partial	D18	Aam-3564	heme lipoprotein precursor		Aam-954297	glucose dehydrogenase, putative			
	C12	Aam-220360	putative vitellogenin-1, partial				E19	Aam-3564	heme lipoprotein precursor	F15	Aam-3564	heme lipoprotein precursor
					Aam-186025	heme lipoprotein precursor		Aam-54072	Vitellogenin B			
		Aam-3564	heme lipoprotein precursor					Aam-252303	glucose dehydrogenase, putative	F16	Aam-3564	heme lipoprotein precursor
				D19	Aam-3564	heme lipoprotein precursor		Aam-279098	glucose dehydrogenase, putative			
		Aam-186025	heme lipoprotein precursor					Aam-80557	glucose dehydrogenase, putative		Aam-186025	heme lipoprotein precursor
		Aam-50363	hypothetical protein					Aam-220360	putative vitellogenin-1, partial			

Table 1B. *Amblyomma america*num saliva proteins (100-37 kDa) identified from in gel digestion and LC-MS/MS during feeding

Molecular weight	Feeding Time Points (h)											
	24 h			72 h			120 h			168 h		
	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description
100 - 75 kDa	C13	Aam-3564	heme lipoprotein precursor	D20	N/A		E20	Aam-75534	metabotropic glutamate receptor 2/3	F17	Aam-3564	heme lipoprotein precursor
				D21	CAA41424.1	liver transferrin		Aam-3564	heme lipoprotein precursor		Aam-54072	Vitellogenin B
	C14	AAB58347.2	serum albumin precursor					XP_002711050.1	keratin 6A-like	F18	Aam-3564	heme lipoprotein precursor
				D22	NP_001075813.1	serum albumin precursor	E21	4F5V_A	Chain A, Crystal Structure Of Leporine Serum Albumin		Aam-75534	metabotropic glutamate receptor 2/3
		Aam-3564	heme lipoprotein precursor					Aam-3564	heme lipoprotein precursor	F19	4F5V_A	Chain A, Crystal Structure Of Leporine Serum Albumin
	C15	N/A	N/A		XP_017197646.1	keratin 6A-like		Aam-949183	Basic tail secreted protein		Aam-3564	heme lipoprotein precursor
											XP_002711050.1	keratin 6A-like
75 - 50 kDa	C16	Aam-28434	Serine protease inhibitor	D23	4F5V_A	Chain A, Crystal Structure Of Leporine Serum Albumin	E22	Aam-3564	heme lipoprotein precursor	F20	Aam-3564	heme lipoprotein precursor
							E23	XP_002719410.1	keratin 10			
		Aam-24638	Putative lysosomal & prostatic acid phosphatase	D24	4F5V_A	Chain A, Crystal Structure Of Leporine Serum Albumin		Aam-3564	heme lipoprotein precursor	F21	N/A	N/A
								Aam-271199	putative lysosomal & prostatic acid phosphatase			
		Aam-271199	putative lysosomal & prostatic acid phosphatase	D25	XP_002719410.1	keratin 10	E25	Aam-24638	Putative lysosomal & prostatic acid phosphatase	F22	Aam-3564	heme lipoprotein precursor
								Aam-28434	Serine protease inhibitor			
	C17	Aam-28434	Serine protease inhibitor					XP_002711050.1	keratin 6A-like	F23	Aam-28434	Serine protease inhibitor
								Aam-28434	Serine protease inhibitor			
		Aam-2651	Serine protease inhibitor		AAB58347.2	serum albumin precursor	E26	Aam-488146	serine protease inhibitor		Aam-488146	serine protease inhibitor
								Aam-271199	putative lysosomal & prostatic acid phosphatase			
		Aam-20799	actin		XP_002711050.1	keratin 6A-like		Aam-24638	Putative lysosomal & prostatic acid phosphatase	F24	Aam-20799	actin
								Aam-20799	actin			
		Aam-973854	serine protease inhibitor				E27	XP_017200220.1	beta actin		CAA43139.1	alpha-smooth muscle actin
								Aam-2651	Serine protease inhibitor			
	C18	N/A	N/A	D26	AAA31288.1	Ig gamma heavy chain constant region, partial	E28	Aam-1027384	putative secreted protein, partial		XP_017200220.1	beta actin
								AAB58347.2	serum albumin precursor			
50 - 37 kDa	C19	Aam-30101	AV422	D27	Aam-28434	Serine protease inhibitor	E29	Aam-12781	putative toll-like receptor 5	F25	Aam-1027384	putative secreted protein, partial
								Aam-1027384	putative secreted protein, partial			
		Aam-16505	putative inducible metalloproteinase	D28	N/A	N/A		AAC78495.1	annexin I		XP_002711050.1	keratin 6A-like
					Aam-20799	actin		Aam-20216	putative cement protein RIM36, partial			
		Aam-235530	putative tick til 20	D29	XP_017200220.1	beta actin	E30	Aam-3564	heme lipoprotein precursor	F26	Aam-12781	putative toll-like receptor 5
					Aam-2651	Serine protease inhibitor		Aam-34094	putative glyceraldehyde 3-phosphate dehydrogenase			
		Aam-328185	putative secreted protein precursor	D30	XP_002716936.1	fibrinogen, beta chain		Aam-17458	putative glycine-rich cell wall structural protein, partial	F27	N/A	N/A
					NP_001075579.1	haptoglobin	E31	N/A	N/A			

Table 1C. *Amblyomma america*num saliva proteins (37-10 kDa) identified from in gel digestion and LC-MS/MS during feeding

Molecular weight	Feeding Time Points (h)											
	24 h			72 h			120 h			168 h		
	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description	Gel Band	Contig	Description
37 - 25 kDa	C20	Aam-30101	AV422	D34	Aam-68822	putative secreted protein	E32	Aam-16628	putative secreted protein precursor	F28	Aam-16628	putative secreted protein precursor
		Aam-64998; Aam-19567	GGY domain-containing protein				E33	Aam-68822	putative secreted protein			
		Aam-216130	Histamine binding protein (HBP)/ Lipocalin				E34	Aam-15540	putative tpa exp: secreted protein	F29	N/A	N/A
	C21	Aam-328185	putative secreted protein precursor	D35	P01840.1	Ig kappa-b4 chain C region	E35	Aam-30101	AV422	F30	Aam-15540	putative tpa exp: secreted protein
		Aam-68822	putative secreted protein					Aam-957531	putative low-density lipoprotein receptor domain class a			
		Aam-216130	Histamine binding protein (HBP)/ Lipocalin				E36	Aam-30101	AV422	F31	Aam-64998; Aam-19567	GGY domain-containing protein
				D36	N/A	N/A		Aam-64998; Aam-19567	GGY domain-containing protein			
	C22	N/A	N/A					Aam-70356	putative major epididymal secretory protein he1, partial	F32	N/A	N/A
25 - 20 kDa	C23	N/A	N/A	D37	Aam-16505	putative inducible metalloproteinase	E37	Aam-64998; Aam-19567	GGY domain-containing protein	F33	Aam-16505	putative inducible metalloproteinase
								Aam-30101	AV422			
	C24	Aam-1038189	putative secreted protein precursor		Aam-244823	putative tick til 20		Aam-70356	putative major epididymal secretory protein he1, partial			
								Aam-21134	putative salivary lipid	F34	XP_002711050.1	keratin 6A-like
							E38	Aam-16505	putative inducible metalloproteinase			
				D38	N/A	N/A		Aam-235530	putative tick til 20		Aam-16505	putative inducible metalloproteinase
							E39	Aam-16505	putative inducible metalloproteinase			
20 - 15 kDa	C25	N/A	N/A	D39	AAC61771.1	calgranulin B	E40	Aam-235530	putative tick til 20	F35-F38	N/A	N/A
					P25230.1	Antimicrobial protein CAP18						
				D40	Aam-851110	histidine-rich glycoprotein-like		Aam-16505	putative inducible metalloproteinase			
					CAA24247.1	beta-globin						
	C26	Aam-23118	putative tick cistatins 1		XP_002712698.1	histone cluster 1, H2ag-like	E41	Aam-851110	histidine-rich glycoprotein-like			
					CAA24247.1	beta-globin						
				D41	CAA28447.1	alpha-1-globin	E43	Aam-11351	putative secreted salivary gland peptide			
					Aam-23118	putative tick cistatins 1						
	C27-C28	N/A	N/A		Aam-292762	globin 1, partial		CAA24247.1	beta-globin			
					Aam-11351	putative secreted salivary gland peptide		Aam-88953	putative secreted protein			
					AAA31269.1	beta-hemoglobin, partial		Aam-23118	putative tick cistatins 1			
					P14461.1	Fibrinogen alpha chain		CAA28447.1	alpha-1-globin			
15 - 10 kDa	C29	N/A	N/A	D42	CAA28447.1	alpha-1-globin	E44-E46	N/A	N/A	F39	Aam-20302	putative secreted protein
				D43	Aam-861740	neurocalcin-like protein					Aam-20302	putative secreted protein
										F40	Aam-24452	putative chymotrypsin-elastase inhibitor ixodidin

For in the "in-solution" digestion approach, saliva collected from ticks that had fed for 24, 48, 72, 96, 120, 144, 168, and 192 h as well as ticks that were apparently engorged but were not detached from the host (BD) and replete fed (SD) was subjected to LC-MS/MS analysis. Peptide mass spectra were searched against the combined database (described above) using the

ProLuCID search engine (52). This analysis identified a total of 1612 proteins of which 1182, 335, 30, and 65 were considered as respective tick, rabbit, and contaminants or reversed proteins (Supplemental table 2). We respectively identified 450, 540, 419, 441, 332, 529, 478, 536, 312, and 325 tick proteins in the 10 different saliva samples. Similarly, we respectively identified 127, 130, 115, 147, 112, 140, 199, 198, 78, and 282 as rabbit proteins (Supplemental table 2). The identification of 1182 tick and 335 rabbit unique proteins in tick saliva demonstrates the complexity of tick and host interactions.

Tick and rabbit proteins in A. americanum tick saliva are annotated in multiple functional categories

For putative functional annotation, identified proteins were searched against entries in public databases, NCBI, Uniprot, and MEROPS. This analysis categorized the 55 tick and 21 rabbit proteins that were identified in the "in-gel" digestion method into 12 and 9 functional protein categories, respectively (Tables 1A, 1B and 1C). Proteins that were identified in the "in-solution" digestion approach (1182 tick and 335 rabbit proteins) were categorized into a respective 27 (Tables 2A and 2B) and 25 (Tables 3A and 3B) functional categories. It is interesting but not surprising to note that all proteins that were identified in the "in-gel" digestion approach were among those that were identified in the "in-solution" digestion approach. We are aware of the fact that, utilizing both methods might be perceived as redundant, however the advantage of the "in-gel" digestion method was that, we determined the molecular identities of the predominant protein bands on Coomassie blue-stained *A. americanum* tick saliva SDS-PAGE (Fig. 1, Tables 1A-C). For instance, the predominant protein band between 100-150 kDa (Fig. 1) consists of heme-binding storage proteins called hemelipoproteins and vitellogenins.

Table 2A. Numbers and cumulative relative abundance of tick protein classes in *Amblyomma americanum* saliva during 24-120 h of feeding

	Feeding Time Point									
	24 h		48 h		72 h		96 h		120 h	
Classification	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)
antimicrobial	6	4.20	6	3.30	5	6.01	5	6.15	5	6.73
cytoskeletal	42	4.77	16	1.32	25	2.13	14	1.86	6	0.71
detoxification	19	2.16	15	0.71	12	0.72	15	0.89	3	0.18
evasin	0	0.00	6	0.74	2	0.24	6	0.68	7	1.67
extracellular matrix	20	1.81	11	1.20	17	1.48	11	0.64	6	0.37
glycine rich	23	2.41	43	5.81	44	9.82	41	4.86	27	6.83
heme/iron binding	16	20.20	16	7.48	15	15.27	16	12.35	9	5.81
immune-related	11	2.96	9	2.28	8	4.12	9	4.47	7	3.54
ixodegrin	1	0.46	5	0.82	1	0.84	2	0.47	2	0.62
lipocalin	8	0.74	17	2.90	2	0.55	6	1.01	3	4.39
metabolism, amino acid	3	0.08	0	0.00	1	0.02	0	0.00	0	0.00
metabolism, carbohydrate	11	0.57	7	0.22	8	0.36	9	0.77	3	0.06
metabolism, energy	18	1.22	17	1.54	13	1.48	10	0.94	2	0.04
metabolism, lipid	14	1.07	19	1.14	18	1.19	14	1.33	8	0.57
metabolism, nucleic acid	7	2.48	18	1.99	9	0.97	9	2.19	13	1.22
mucin	3	0.21	5	0.53	4	0.54	6	0.96	4	0.27
nuclear regulation	7	0.39	7	0.45	8	1.04	7	1.24	7	1.00
protease	17	0.72	25	1.07	20	0.97	25	1.46	27	4.08
protease inhibitor	55	22.28	76	21.14	52	11.10	64	15.33	66	20.32
proteasome machinery	8	1.57	6	0.50	6	1.29	6	1.50	6	1.18
protein modification	16	0.59	6	0.14	16	0.84	7	0.22	5	0.13
protein synthesis	6	0.16	2	0.05	4	0.14	2	0.06	0	0.00
signal transduction	14	3.57	11	4.74	3	0.39	7	2.01	8	1.80
tick specific proteins	110	23.99	188	39.44	118	37.88	140	37.87	105	38.28
transcription machinery	4	0.24	1	0.03	3	0.18	3	0.11	0	0.00
transporter/ receptor	10	1.12	6	0.42	4	0.37	7	0.62	3	0.19
transposon element	1	0.02	2	0.03	1	0.05	0	0.00	0	0.00
Total	450	100.00	540	100.00	419	100.00	441	100.00	332	100.00

Table 2B. Numbers and cumulative relative abundance of tick protein classes in *Amblyomma americanum* saliva during 144 to completion of feeding

	Feeding Time Point									
	144 h		168 h		192 h		BD		SD	
Classification	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)
antimicrobial	4	4.31	4	2.87	3	2.98	3	5.04	3	1.49
cytoskeletal	38	3.43	11	0.98	16	1.23	3	0.39	14	1.42
detoxification	21	1.71	11	0.67	18	1.13	7	0.79	12	0.55
evasin	5	1.54	7	3.40	6	3.39	1	0.83	5	2.86
extracellular matrix	18	1.61	7	0.38	19	0.85	14	1.75	0	0.00
glycine rich	14	1.03	19	2.00	31	2.79	9	0.47	8	0.56
heme/iron binding	17	22.96	14	7.31	16	12.38	17	26.07	10	7.23
immune-related	14	3.76	9	2.37	14	3.09	12	4.65	7	1.66
ixodegrin	1	0.58	1	0.20	1	0.62	2	0.78	0	0.00
lipocalin	8	1.11	24	6.57	20	5.69	20	4.07	26	14.76
metabolism, amino acid	4	0.07	2	0.07	1	0.04	0	0.00	0	0.00
metabolism, carbohydrate	22	1.30	4	0.12	14	0.90	14	2.56	5	0.17
metabolism, energy	13	0.81	9	0.34	10	0.50	2	0.30	6	0.15
metabolism, lipid	16	0.95	5	0.39	16	1.01	12	1.29	3	0.39
metabolism, nucleic acid	14	1.80	10	0.93	16	1.72	3	1.11	8	1.21
mucin	7	0.33	3	0.13	9	0.33	3	0.56	0	0.00
nuclear regulation	10	0.74	8	1.49	8	1.02	1	0.26	11	2.15
protease	40	3.37	38	4.57	43	4.21	36	2.93	36	7.70
protease inhibitor	83	17.41	81	15.82	78	14.79	59	19.17	39	17.08
proteasome machinery	7	0.87	6	1.09	6	0.87	4	0.11	6	1.68
protein modification	18	0.58	10	0.26	13	0.38	0	0.00	11	0.44
protein synthesis	2	0.03	2	0.06	5	0.15	0	0.00	2	0.08
signal transduction	14	3.11	10	1.49	9	2.43	7	1.89	5	0.53
tick specific proteins	127	25.15	175	46.18	157	36.86	77	23.84	103	37.75
transcription machinery	2	0.04	1	0.02	1	0.01	0	0.00	1	0.02
transporter/ receptor	10	1.38	6	0.26	6	0.62	6	1.14	3	0.07
transposon element	0	0.00	1	0.04	0	0.00	0	0.00	1	0.02
Total	529	100.00	478	100.00	536	100.00	312	100.00	325	100.00

289 Table 3A. Numbers and cumulative relative abundance of rabbit protein classes in *Amblyomma*
290 *americanum* saliva during 24-120 h of feeding

	Feeding Time Point									
	24 h		48 h		72 h		96 h		120 h	
Classification	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)
Antimicrobial	3	0.00134357	4	0.00192864	3	0.00171397	5	0.0086771	6	0.00763598
Antioxidant	2	0.00042155	1	7.576E-05	1	0.00035322	0	0	0	0
Cytoskeletal	19	0.01798265	14	0.01629188	11	0.00953933	12	0.01698913	7	0.01026072
Extracellular matrix	3	0.00049492	1	0.000267	1	0.0000291	2	0.0001466	2	0.0003605
Fibrinogen	3	0.00022576	4	0.00017475	0	0	1	6.7612E-05	2	0.00022745
Globin/ RBC	20	0.08991802	18	0.02390767	12	0.01107268	15	0.02658361	15	0.02722383
Heme/Iron binding	5	0.01948816	7	0.01314713	2	0.00443612	7	0.00916951	4	0.00596773
Immunity related	9	0.00268497	7	0.0023492	5	0.00211006	8	0.00712898	15	0.01097912
Keratin	17	0.00337019	32	0.01031924	35	0.02503904	41	0.04692297	27	0.03080678
Lipocalin	0	0	1	0.00051272	1	0.00052663	1	0.00283525	1	0.00167895
Metabolism, amino acid	0	0	0	0	0	0	0	0	0	0
Metabolism, carbohydrates	5	0.00133936	4	0.00037672	5	0.00064097	5	0.00074812	3	0.00033307
Metabolism, energy	1	2.8207E-05	0	0	0	0	3	0.00071594	1	0.00018482
Metabolism, lipid	0	0	0	0	0	0	2	0.00093604	2	0.00138574
Metabolism, nucleic acids	0	0	0	0	0	0	0	0	0	0
Nuclear regulation	8	0.00325694	16	0.00871704	15	0.01681692	18	0.01938734	15	0.01609353
Protease	0	0	1	0.00013596	0	0	2	0.00146001	1	4.2416E-05
Protease Inhibitors	8	0.0025413	5	0.00116042	1	0.0001394	5	0.00178596	1	0.00071414
Protein export	0	0	2	0.00017604	2	0.00115523	3	0.00209215	3	0.00247782
Protein modification	7	0.00102993	3	0.0003923	7	0.00221869	6	0.00095378	4	0.00051132
Protein synthesis	4	0.00135166	2	0.0001756	4	0.00122153	4	0.0006344	0	0
Proteasome machinery	2	0.00600273	2	0.0021349	2	0.00578655	2	0.0056957	2	0.00490593
Signal transduction	9	0.00264391	1	0.00047693	7	0.00264375	1	0.00089234	1	0.00035228
Transporter/ Receptor	2	0.00013431	5	0.00049163	1	3.5648E-05	4	0.00061835	0	0
Transcription machinery	0	0	0	0	0	0	0	0	0	0
Total	127	0.15425811	130	0.08321153	115	0.08547884	147	0.15444091	112	0.12214214

291

Table 3B. Numbers and cumulative relative abundance of rabbit protein classes in *Amblyomma americanum* saliva during 144 to completion of feeding

	Feeding Time Point									
	144 h		168 h		192 h		BD		SD	
Classification	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)	Protein Count	NSAF (%)
Antimicrobial	8	0.007565	9	0.018337	9	0.015212	6	0.004394	8	0.015088
Antioxidant	3	0.000677	2	0.00071	3	0.000384	0	0	4	0.000927
Cytoskeletal	20	0.019936	29	0.014844	26	0.019163	6	0.011922	55	0.031601
Extracellular matrix	1	3.63E-05	0	0	1	2.58E-05	1	8.41E-05	3	0.000973
Fibrinogen	1	0.00011	5	0.002512	5	0.001638	0	0	5	0.001423
Globin/ RBC	18	0.105112	19	0.100601	18	0.067832	16	0.052725	21	0.109247
Heme/Iron binding	3	0.005258	9	0.011255	7	0.011786	3	0.005854	9	0.015765
Immunity related	15	0.007953	23	0.02019	17	0.014264	5	0.002246	23	0.025243
Keratin	20	0.010315	34	0.012893	37	0.021782	21	0.010099	40	0.045599
Lipocalin	1	0.002122	1	0.004206	1	0.00311	1	0.001514	1	0.00534
Metabolism, amino acid	0	0	1	0.000201	0	0	0	0	1	3.83E-05
Metabolism, carbohydrates	5	0.001337	9	0.001486	7	0.001096	2	0.000114	7	0.001634
Metabolism, energy	2	0.000319	2	0.000144	2	0.000364	0	0	6	0.000535
Metabolism, lipid	0	0	0	0	0	0	0	0	2	0.000229
Metabolism, nucleic acids	1	0.000108	4	0.000989	0	0	0	0	0	0
Nuclear regulation	19	0.013472	20	0.028935	24	0.022492	10	0.008565	26	0.032846
Protease	0	0	4	0.000876	1	9.16E-05	0	0	6	0.00066
Protease Inhibitors	1	0.000202	3	0.000967	2	0.000151	0	0	7	0.00203
Protein export	3	0.001588	4	0.004882	5	0.003771	2	0.00077	4	0.006886
Protein modification	6	0.000988	7	0.000754	12	0.001567	0	0	16	0.002522
Protein synthesis	0	0	4	0.00057	4	0.00084	0	0	4	0.000866
Proteasome machinery	2	0.003209	2	0.003649	2	0.003418	2	0.000584	2	0.005294
Signal transduction	8	0.003392	5	0.001237	9	0.001945	0	0	12	0.003857
Transporter/ Receptor	3	0.000381	1	6.81E-05	4	0.000521	3	0.000589	13	0.00182
Transcription machinery	0	0	2	6.16E-05	2	6.68E-05	0	0	7	0.000636
Total	140	0.184079	199	0.230369	198	0.191521	78	0.099459	282	0.311061

Along with findings from electrophoretic profile, the "in-solution" approach confirmed the complexity of tick saliva. With redundancy removed at 98% amino acid identity levels, the majority of the identified proteins are tick specific proteins (did not match to proteins in non-tick organisms) of unknown function (32%), followed by protease inhibitors (PI) (13%), proteases (8%), and glycine-rich proteins (6%). Notable protein categories that were ≤ 5% include cytoskeletal, lipocalin, antioxidant/detoxification, extracellular matrix, immune related, heme/iron-binding, mucins, evasins, antimicrobials, and ixodegrins (Tables 2A and 2B,

Supplemental table 2). For rabbit proteins, the majority are categorized as cytoskeletal (19%), followed by keratin (13%), nuclear regulation (8%), immunity-related (8%), globin/RBC degradation (6%), and protein categories that were ≤ 5% include antimicrobials, heme/iron-binding, protease inhibitors, proteases, extracellular matrix, antioxidant/detoxification, fibrinogen and lipocalin (Tables 3A and 3B, Supplemental table 2).

The most abundant category of A. americanum tick saliva proteins is tick-specific

Figures 2A and 2B summarizes daily relative abundance of tick saliva proteins during *A. americanum* tick feeding as determined by normalized spectral abundance factor (NSAF), the index for relative protein abundance (53-55). Fig. 2A shows that three protein categories, tick-specific saliva proteins of unknown function (TSP), protease inhibitors (PI), and heme/iron binding proteins were the most abundant ranging from a respective 24-46%, 11-22%, and 6-26% during feeding (24-192h). Other protein categories at ≤ 10% in abundance include glycine-rich proteins, antimicrobial peptides, evasins, and proteases. For rabbit proteins in *A. americanum* tick saliva, the most predominant functional category was hemoglobin/red blood cell products (RBC) (13-58%) followed by cytoskeletal (6-20%), heme/iron binding (~5-16%), keratin (2-30%), and nuclear regulation (2-20%) (Fig. 2B). It is notable that rabbit functional categories related to immunity, antimicrobial peptides, protease inhibitors and proteases were abundant at ≤8% throughout feeding.

The finding that the majority of proteins identified in this study are of unknown function is not unique to *A. americanum* tick saliva, it is consistent with findings in saliva of *I. scapularis* (32) and tick salivary gland transcriptomics (59-61). This is potentially a reflection of how little information exists on the molecular basis of tick feeding physiology.

Majority of A. americanum tick saliva proteins are associated with early stage tick-feeding processes

To gain insight into broad relationships of secretion dynamics of both tick and rabbit proteins with the tick feeding processes, Z-score statistics normalized NSAF (relative abundance) values were visualized on heat maps (Figs. 3A and 3B). The clustering patterns are influenced by cumulative relative abundance of protein category. The blue to red transition denotes low to high abundance. As shown in figure 3A, the 27 TSP functional categories clustered into four broad secretion patterns (clusters A-D). Broadly, 74% (20/27) of tick protein categories are secreted at high abundance within the first 48 h of feeding (Fig. 3A, clusters A, B, and C) with exception of four categories (evasins, proteases, lipocalins, and nuclear regulation proteins) in cluster D, which are injected into the host at high abundance starting from day five of feeding. These proteins could be important proteins regulating early stages of tick feeding activities such as initiating tick feeding by creating feeding lesion and attaching, while suppressing host tissue repair defenses and also contribute to transmission of TBD agents. Protein categories that were identified in abundance starting from the 192 h feeding time point might be associated regulating the end of the tick-feeding process when the tick detaches from the host skin with minimal damage.

Similarly, the majority of rabbit proteins functional categories (21 of the 25) were detected at high abundance in saliva of *A. americanum* ticks during feeding (Fig. 3B). The 25 rabbit protein categories in saliva of *A. americanum* ticks segregated into four clusters, A-D (Fig. 3B). Rabbit proteins that were secreted at high abundance starting from 24-72 h of feeding are part of clusters A and B. Five of the seven proteins in cluster A are highly abundant at 24 and 48

h feeding time points, while those in clusters C and D were less abundant in the first 48 h of feeding and showed varied abundance levels starting from 72 h of feeding.

Secretion dynamics of non-housekeeping-like A. americanum tick saliva proteins

Supplemental table 2 lists individual proteins that were identified in *A. americanum* tick saliva. Thirteen functional categories not considered as housekeeping-like (antimicrobial, detoxification extracellular matrix/cell adhesion, evasin, glycine-rich, heme/iron binding, immunity-related, ixodegrin, lipocalin, mucin, protease inhibitors, proteases, and TSPs of unknown function) (Tables 2A and 2B) accounted for 76% of total number of proteins and represented more than 82% in relative abundance throughout feeding time points. In the subsequent sections, we have discussed non-housekeeping-like tick proteins individually per category (Figs. 4A-S) and have highlighted housekeeping-like tick proteins and rabbit proteins as a group below. Our lab is interested and is working to understand functions of proteases and protease inhibitors, and our subsequent discussion below is biased toward these two categories.

a) A. americanum tick saliva contains a large diversity of protease inhibitors in nine families

We previously documented at least 18 of the 99 Merops database protease inhibitor (PI) that might be expressed by *A. americanum* and other tick species (62). Here we show that adult *A. americanum* ticks secreted at least 155 PIs belonging into eight PI families. These include Kunitz-type inhibitors (I2, n=68), serine protease inhibitors (serpins, I4, n=21), trypsin inhibitor-like (TIL, I8, n=36), alpha-2-macroglobulins (α2M, I39, n=12), cysteine inhibitors (cystatin, I25, n=12), thyropins (I31, n=3), phosphatidylethanolamine-binding proteins (I51, n=2), and a tick carboxypeptidase inhibitor (TCI, n=1). Of significant interest, nearly 75% of PIs (115/155) in

this study were secreted in saliva within the first 120 h post-attached fed ticks (Supplemental table 2). This strongly suggests that functions of tick saliva PIs are associated with regulating early stages of the tick feeding stages such as tick creation of its feeding site and transmission of TBD agents, which are critical to the success of ticks as pests and vectors of TBD agents.

Of the PI families in this study, serpins are the most studied (42, 62-67), presumably because functional roles of this protein category are relatable to tick feeding physiology. To successfully feed and transmit TBD agents, ticks have to overcome serine protease-mediated host defense pathways that are tightly controlled by inhibitors, including serpins. On this basis, it was proposed that ticks might utilize serpins to evade host defenses to successfully feed (68). From this perspective, it is notable that 90% (19/21) of serpins were identified in saliva of ticks that fed for 24-48 h (Fig. 4A), suggesting these serpins are injected into host and might be involved with regulating tick feeding within hours of the tick starting to feed. It is interesting to note that, *A. americanum* serpin 6 and 19, which were previously validated as inhibitors of host defense system proteases (69, 70) were also found in tick saliva within the first 24 h of feeding this study. It is notable that 20-50% of PIs were identified at a single time point for all PI families except serpins, where only 5% (1/21) was found (Supplemental table 2). This might suggest that the functions of tick saliva serpins are important throughout the tick feeding process, most likely in evading host defenses.

Although not much has been reported on the functional analysis of *A. americanum* tick cystatins, a lone study has reported that RNAi silencing of a cystatin transcript reduced the ability of ticks to feed successfully (71). Several researchers have reported cystatins in other tick species indicated that they play important roles in tick feeding physiology (72). In the soft tick *Ornithodoros moubata,* a cystatin was internalized by host dendritic cells and targeted cathepsin

394 S and cathepsin C, affecting their maturation (73). Cystatins from other tick species also have
395 immunosuppressive functions (74-76). Of the 12 cystatins identified in tick saliva from this study
396 (family I25, Fig. 4B), seven were secreted starting from 72 h of feeding, indicating that majority
397 of cystatins might be involved in regulating tick feeding functions after the tick has initiated
398 feeding. On the contrary, four cystatins were secreted within the first 24 h of feeding and were
399 predominantly secreted throughout feeding.

400 Similar to cystatins, figure 4C shows the secretion dynamics of alpha-2-macroglobulins
401 (α2M), where majority were injected into the host at high abundance toward the end of feeding
402 (BD). This might suggest that α2M could be involved in regulating tick feeding functions toward
403 the end of tick feeding. There are very few studies on α2M in tick feeding physiology. Two
404 studies have reported the functional roles of α2M in soft tick immune defense (77) and anti-
405 microbial activity in *I. ricinus* (78).

406 Kunitz-type inhibitors and trypsin inhibitor-like (TIL) were the two most, in number of
407 proteins, identified in all PI families from this study, comprising of a respective 44 and 23% of
408 total PI proteins in tick saliva during feeding. The secretion dynamics of Kunitz-type inhibitors
409 (Fig. 4D) and TIL (Fig. 4E) is comparable and notable: the tick appears to secrete a different set
410 of these inhibitors every 24 h starting from the first day of tick feeding. This might suggest that
411 functions of these inhibitors are required throughout the tick feeding process. It is also notable
412 that a total 35% of Kunitz-type inhibitors and 22% of TILs were detected in saliva of replete fed
413 ticks (SD), unlike the other PIs in this study.

414

415 *b) Majority of proteases in A. americanum saliva are metalloproteases*

At the time of this study, protease families that were encoded by *A. americanum* were not enumerated, presumably because its genome had not been sequenced. However, analysis of annotated sequences from *I. scapularis* showed that the tick might encode for all protease categories: aspartic, cysteine, serine, metallo-, and threonine proteases (79). Here, we found that *A. americanum* secretes at least 94 proteases in saliva during feeding. These 94 proteases belong in four categories grouped into 15 families: aspartic (family A1, n=4), cysteine (C1, C2, and C13, n=12), metallo- (M12, M13, M14, M15, M17, M20, M28, and M49, n=56), and serine (S1, S10, and S28, n=22) proteases (Supplemental table 2, Figs. 4F, 4G, and 4H). Please note that the heatmap for aspartic proteases was not developed due to low numbers (the secretion dynamics is presented in Supplemental table 2). The heatmaps (Figs. 4F, 4G, and 4H and Supplemental table 2) show that more than 60% (60/94) of proteases are injected into the host various time points during the first five days of feeding, demonstrating that some of the proteases in this study are associated with tick feeding regulation.

The observation that metalloproteases are the majority of proteases in saliva of *A. americanum* is consistent with our previous findings in the *I. scapularis* proteome (32). It is notable that similar to the *I. scapularis* proteome, metalloproteases that were secreted at high abundance during the first 72 h feeding time points are in families M12 and M13 (Fig. 4G), indicating that these proteases regulate initial tick feeding functions that are important to both tick species. Indirect evidence on snake venom M12 proteases that have anti-coagulant activity (80, 81) suggest that secretion of these proteases at high abundance when the tick is initiating feeding might be beneficial to tick feeding to prevent blood from clotting, which might otherwise prevent blood meal feeding. There is also evidence that RNAi silencing of M12 proteases significantly affected tick-feeding efficiency (82). It has been reported in *I. scapularis* saliva a

439 metalloprotease similar to hemorrhagic proteases of snake venom that act towards gelatin,
440 fibrin(ogen), and fibronectin (83). Likewise, indirect evidence suggests that ticks might utilize
441 M13 proteases to regulate host immunity. In mammals, M13 proteases were among other
442 functions involved in modulating neurotransmitter levels, control blood pressure, involved in
443 reproduction and cancer progression (84).

444 Another notable similarity between *A. americanum* and *I. scapularis* proteomes is that
445 both tick species secreted a small number of S1 serine proteases, six and three respectively (Fig.
446 4G and Supplemental table 2). We are interested in S1 serine proteases due to their functional
447 roles in signal transduction as activators of protease-activated receptors (85, 86); could the tick
448 utilize these proteases to interfere with host defense signaling at the tick-feeding site?

449 The observation that *A. americanum* injected cysteine proteases at the beginning of
450 feeding indicate they might be playing some role(s) in the early stages of tick feeding. Several
451 studies have documented potential functional roles of cysteine proteases in tick physiology (87-
452 89). In a lone study, a cysteine protease from *H. longicornis* when silenced by RNAi, showed to
453 be involved with digestion of a blood meal and increased the number of *Babesia* parasites (90).
454 Recently, a cathepsin L from the tick, *R. microplus* (BmCL1), was shown to interact with
455 thrombin at pH 7.5 and impair thrombin-induced fibrinogen clotting via a fibrinogenolytic
456 activity (91). In helminths, cysteine proteases are the most abundant category of proteins
457 identified into excretion/secretion products (92) and have been shown to be involved with host
458 immune evasion (93) and extracellular matrix degradation (94).

459 Majority of studies on tick aspartic proteases are mainly characterized as blood digestion
460 proteins in the midgut, similar to the mammalian lysosome acidic protease, cathepsin D (95). In
461 *H. longicornis* adult ticks, the potential role of these proteins in proteolysis of erythrocyte

462 hemoglobin has been reported (96). Other studies have shown the importance of this protease in
463 embryogenesis, playing roles in vitellin degradation (97) and heme-binding properties (98).
464 Although only four aspartic proteases were identified in *A. americanum* saliva during feeding,
465 three of these proteases were present within the first 96 h of feeding, which may implicate roles
466 in the early stages of tick feeding success (Supplemental table 2).
467
468 *c) Lipocalins/histamine-binding proteins are alternately secreted during tick feeding*
469 Inflammation response is among host defense pathways that ticks must evade to complete
470 feeding. Histamine is one of the key mediators of inflammation in tissue damage that is expected
471 to occur in response to tick feeding (99). From this perspective, lipocalins/tick histamine-binding
472 proteins in tick saliva are suspected to be part of the tick machinery to evade the host's
473 inflammation defense response through sequestration of histamine that is released at the tick-
474 feeding site. In this study, we found 46 lipocalins/tick histamine-binding proteins that show two
475 broad secretion patterns: secreted at multiple feeding time points and those that were alternately
476 secreted at single time points (Fig. 4I). It is interesting to note that of the total 46 lipocalins
477 identified in tick saliva during feeding, 22% (10/46) were present within the first 48 h of feeding,
478 while 35% (16/46) were present after 96 h of feeding, and 43% (20/46) were identified in a
479 single time point (Fig 4I, Supplemental table 2). Given that in addition to regulating
480 inflammation, lipocalins/histamine-binding proteins have other diverse functions such as
481 antimicrobials (100, 101), glucose metabolism (102) and binding several ligands including
482 serotonin and fatty acids (103, 104), it is most likely that these proteins might be involved in
483 regulating several other tick feeding functions besides mediating the tick's anti-inflammation
484 function.

d) Heme binding proteins are secreted at high abundance throughout feeding

Like other animals, ticks require iron and heme (the iron-containing part of hemoglobin) for normal physiological functions (105). However, ticks do not have a heme biosynthesis pathway, therefore they must obtain it from host blood (106). Female ticks that were artificially fed a diet not containing hemoglobin laid sterile eggs (107) demonstrating the importance of heme in tick biology. However, in high abundance heme can be toxic for the tick (108), therefore it is postulated that hemelipoproteins and vitellogenenins could serve as heme binding proteins to remove the excess heme from the tick system. Supplemental table 2 lists a total of 17 heme/iron-binding proteins consisted of hemelipoproteins, vitellogenins, and a ferritin that collectively accounted for the third most abundant protein category in tick saliva throughout feeding (Fig. 2). High abundance of hemelipoproteins here is in consistent with other tick saliva proteomes (32-34). The secretion dynamics summarized in figure 4J revealed two broad secretion patterns, those that are injected into the host from 24 h through 120 h of feeding (HCB and HCC) and those that injected into the host starting from 144 h of feeding through the end of tick feeding (HCD and HCA). Ticks acquire both iron and heme from host blood (106, 109), and thus iron and/heme-binding proteins are important to normal tick physiology. It has been shown that *R. microplus* hemelipoprotein (HeLp) could bind eight heme molecules (110). Given that hemelipoprotein is the most abundant protein in tick hemolymph (111), it could be secreted in saliva as a result of this protein being transferred into the salivary glands when exposed to the hemolymph. However, transcriptional profile and protein localization of these hemelipoproteins in salivary glands of unfed and fed adult ticks suggest that they could act in different pathways during blood-feeding (112). Among other functions it is known that free heme has pro-inflammatory properties (113). Thus, the presence of hemelipoproteins could lower free heme

concentration at the feeding site, reducing inflammation. Other roles of tick hemelipoproteins such as an antioxidant in transporting other compounds such as cholesterol, phospholipids, and free fatty acids have been previously reported (114). It is interesting to note that reduction of vitellogenin receptor (VgR) expression by RNAi resulted in reduced fertility (115) and *Babesia bovis* transmission and oocyte maturation (116).

e) Ticks inject multiple antioxidant proteins into the feeding site

Feeding and digestion of large amounts of host blood exposes ticks to hydroxyl radicals and reactive oxygen species (ROS), which if left uncontrolled could damage tick tissue (117, 118). Expression of antioxidant proteins protect the tick during feeding and digestion of the blood meal. Studies have shown that RNAi silencing of tick antioxidants caused deleterious effects to the tick and prevented them to obtain a full blood meal (119, 120). Previous studies by others and from our lab have documented presence of antioxidants in tick salivary glands (121, 122) and saliva (32-34, 123, 124). In this study we identified 41 putative antioxidant enzymes. These enzymes include glutathione-S-transferase, thioredoxin, superoxide dismutase, catalase, peroxinectin, arylsulfatase, aldehyde dehydrogenase, epoxide hydrolase, sulfotransferase, sulfhydryl oxidase and glycolate oxidase. Figure 4K reveals two broad secretion patterns of tick saliva antioxidant proteins based on NSAF values as an index for abundance: (i) proteins injected into the host in high abundance once at various feeding time points (ACA-ACI) and (ii) proteins that are consecutively injected into the host in high abundance from 24-96 h of feeding (ACF). Like heme/iron binding proteins, tick antioxidants are presumed to function inside the tick; the question is why do ticks inject these into the feeding site? Host tissue injury caused during the creation of the tick-feeding site could trigger release of oxidants such as ROS; could tick saliva

antioxidants function to cleanse the blood meal before the tick ingests it or potentially to protect the host from damage to keep the feeding site balanced?

f) Glycine-rich and extracellular matrix/cell adhesion proteins are secreted early during tick feeding

Within 5-30 min of attachment, the tick secretes an adhesive substance called cement, which anchors ticks onto host skin during its protracted feeding period (125). Tick cement is also suggested to protect the tick from host immune factors (126, 127) and might function as antimicrobials at the feeding site (128). Glycine-rich proteins are among categories of tick proteins that are thought to play key roles in formation of tick cement (125). From this perspective, glycine-rich proteins are among tick proteins that have received significant research attention (129-132). In this study we found a total of 67 glycine-rich proteins, which represented the fifth largest category of proteins identified in tick saliva during feeding (Fig. 2). Nearly 90% (60/67) of the glycine-rich proteins were secreted in abundance within the first four days of feeding (Fig. 4L; GCB, GCD, GCE, GCF, and GCG). Tick cement deposition is completed during the first 96 h of tick feeding (125), and thus it is conceivable that some of the glycine-rich proteins in this study might be involved with tick cement formation. It is interesting to note that some of the glycine-rich proteins that were identified from tick cement in our lab (131) and others (130) were also found in this study (Supplemental table 3). Some of the glycine-rich proteins were secreted from the 144-h time point, long after tick cement formation; these might regulate other tick feeding functions. Although glycine-rich proteins are mostly known for their potential role in tick cement formation, indirect evidence in other organisms indicate that these

553 proteins might be involved in other functions such as host defense and stress response as in
554 plants (133).

555 Figure 4M summarize the secretion dynamics of 37 extracellular matrix proteins that
556 were found in this study. Similar to glycine-rich proteins, majority (27/37) of the extracellular
557 proteins were secreted within the first five days of feeding demonstrating their role in early stage
558 tick feeding regulation. Our speculation is that some of these proteins will play roles in formation
559 of tick cement. In a previous study, RNAi silencing of chitinase, also identified in this study,
560 weakened the tick cement cone to the extent that host blood was leaking out around the
561 mouthparts of attached ticks (40).

562

563 *g) Antimicrobials, mucins, and immune related proteins are secreted throughout the feeding*
564 *process*

565 Once the tick has anchored itself onto the host skin and created its feeding lesion, it faces
566 a difficult task of overcoming host humoral and cellular immunity, and also preventing microbes
567 in the host skin from colonizing the tick-feeding site. Here we show that *A. americanum* secretes
568 immunomodulatory and antimicrobial peptides starting within the early stages of the tick feeding
569 process (Fig. 4N-R). We identified nine antimicrobials consisting of microplusins, lysozymes,
570 and defensins (Fig. 4N). Previous studies showed that microplusin has dual effects against
571 fungus and gram-positive bacteria, lysozyme against gram-positive bacteria, and defensin
572 effective against both gram-positive and -negative bacteria (134-136). The heat map in figure 4N
573 shows that antimicrobials were injected into the host starting at 24 and 48 h (AMCA), from 72 h
574 (AMCC), and from 120 h (AMCB). This secretion pattern suggests that the functions of
575 antimicrobial peptides are needed throughout feeding.

576 Similar to antimicrobials, we identified 12 mucins (Fig. 4O), with ~60% of these proteins
577 (7/12) being secreted at high abundance within 24-48 h of feeding. Functional roles of mucins in
578 ticks have not been studied. However, indirect evidence in mammals suggest that mucins might
579 be involved in antimicrobial activity in that human mucins were shown to encapsulate microbes
580 (137).

581 Among putative immunomodulatory proteins, we identified evasins (Fig. 4P) and
582 ixodegrins (Fig. 4Q). Evasins (n=12, Fig. 4P) were shown to bind to chemokines (138, 139) to
583 reduce leukocytes recruitment to the tick feeding site and therefore contribute to tick evasion of
584 the host's inflammatory defense. It is interesting to note that the 12 evasins identified in tick
585 saliva were present after 24 h of feeding and continued to be secreted throughout feeding at
586 variable levels. This might suggest that evasins might not be involved in regulating tick feeding
587 functions during the first 24 h of tick feeding.

588 Figure 4Q summarizes the secretion pattern of the six ixodegrin-like proteins found in
589 tick saliva during feeding in this study. It is interesting to note, 83% (5/6) of these proteins were
590 identified within the first 48 h of feeding (Supplemental table 2.) These proteins were first
591 described in *I. scapularis* as inhibitors of platelet aggregation (140). Platelet aggregation is the
592 first step in the blood clotting system (141), which ticks must overcome to successfully feed.
593 Thus, the presence of ixodegrins in saliva of *A. americanum* at the start of feeding is beneficial to
594 tick feeding success. Finally, we also found proteins that show similarity to previously
595 characterized immunomodulatory proteins (Fig. 4R), which have been validated in other tick
596 species including p36, which inhibits cell proliferation and cytokine expression (142). These
597 proteins might play roles in mediating the tick's evasion of host immunity.
598

599 *h) Tick-specific secreted saliva proteins (TSP) of unknown function are alternately secreted.*

600 Over one-third of Ixodidae protein sequences deposited into GenBank are annotated as
601 hypothetical, secreted, conserved and unknown proteins. However, some are annotated based on
602 sequence identities and conserved signature motifs, which include basic tail/tailless proteins, 8.9
603 kDa protein family, leucine-rich proteins, AV422 (a tick saliva protein that is high upregulated
604 when ticks are stimulated to start feeding [39, 143]), proteins containing RGD motifs, which
605 might play roles in inhibition of platelet aggregation (140, 144). In this study we have identified
606 a total of 377 (Fig. 4S) tick saliva proteins that fit the above description that we refer here to as
607 tick-specific saliva proteins of unknown functions (TSPs). More than 95% (357/377) of the total
608 TSPs were identified within the first eight days of feeding in tick saliva indicating their potential
609 roles in regulating the tick feeding process. It is interesting to note that the secretion pattern for
610 over a third (128/377) of the total TSPs identified in tick saliva during feeding were alternately
611 injected once during feeding (Supplemental table 2). From the perspective of finding target
612 antigens for tick vaccine development, TSPs represent a unique opportunity in that they do not
613 share any homology to host proteins and might not cross-react with the host.

614

615 *A. americanum secretes multiple housekeeping-like proteins in saliva throughout the feeding*
616 *process*

617 Supplemental table 2 lists 288 housekeeping-like proteins that were identified in this
618 study. Presence of these proteins in *A. americanum* saliva is not unexpected, as similar findings
619 have been previously reported in tick saliva (32-34). The 288 housekeeping-like proteins were
620 classified into 14 categories including those associated with metabolism of amino acids (n=7),
621 carbohydrates (n=25), energy (n=31), lipids (n=31), and nucleic acids (n=33). Other protein

622 categories include those involved in cytoskeletal (n=53), nuclear regulation (n=16), protein
623 modification (n=21), proteasome machinery (n= 8), protein synthesis (n=10), signal transduction
624 (n=24), transposable element (n=3), transcription machinery (n=7), and transporter/receptors
625 (n=17). It is interesting to note that, within the first 24 h of feeding 12 of the 14 categories were
626 identified at high abundance (Fig. 3A).

627 One feature of housekeeping-like tick proteins is that they have high sequence identity
628 with mammalian housekeeping proteins, and for this reason they are discounted as potential
629 target antigens for tick vaccine development. However, based on literature showing that several
630 roles of these proteins in host defense, we think that these proteins play an important role in tick
631 feeding physiology. Housekeeping-like proteins identified here mostly function intracellularly,
632 and they serve as alarm signals to alert the host defense system to injury when secreted outside
633 of the cell (145). There is evidence that in the extracellular space, some of the housekeeping
634 proteins such as heat shock proteins, have anti-inflammatory functions (146), while histone
635 proteins have antimicrobial activity (147). Given high sequence similarity to host housekeeping
636 proteins, could it be that some of the tick housekeeping-like proteins play roles in promoting tick
637 feeding through anti-inflammatory and anti-microbial activity?

638 Another important aspect of tick feeding physiology that has not received much attention
639 is the fact that host blood meal also contains carbohydrates, lipids and other molecules besides
640 host proteins. It is notable that some of the tick housekeeping-like proteins in tick saliva have
641 high similarity to enzymes in the carbohydrate and lipid metabolism pathways. Is the tick
642 pumping these proteins into the feeding site to process these molecules before the tick takes its
643 blood meal?
644

Secretion of rabbit host proteins in A. americanum tick saliva is not random

In this study, we identified 335 rabbit host proteins belonging into 25 different categories that include cytoskeletal (19%), keratin (13%), nuclear regulation (8%), immune-related (8%), hemoglobin/RBC degradation (6%), transporters/receptors (5%), protein modification (5%), and protein categories below 4% included antimicrobials, extracellular matrix, heme/iron binding, detoxification/ antioxidants, metabolism (energy, carbohydrates, lipid, amino acid, and nucleic acids), protein export, protein synthesis, fibrinogen, protease inhibitors, proteases, signal transduction, transcription machinery, proteasome machinery, and lipocalin (Tables 3A and 3B, Supplemental table 2). Relative abundance as determined by NSAF indicated that the most abundant protein categories consisted of hemoglobin/RBC degradation products (58-13%), followed by heme/ iron binding host proteins (13-16%), and cytoskeletal (6-20%) (Fig. 3).

At a glance, presence of rabbit host proteins in *A. americanum* tick saliva could be dismissed as host protein contamination. This observation might be strengthened by the fact that some rabbit host proteins in tick saliva such as keratin, nuclear regulation proteins, and host antimicrobial peptides increased in abundance as feeding progressed. This suggested that secretion of host proteins into tick saliva was a consequence of ticks ingesting an increased amount of host blood, and that some of these host proteins might leak or be regurgitated back into the host via saliva or esophagus. However, our data here suggests that the tick might systematically be utilizing host proteins to regulate its tick-feeding site. For instance, mammals are likely to encode for more than 500 proteases and 150 protease inhibitors (based on rat, mice, and humans [148]), however we found 9 proteases and 8 protease inhibitors from host origin in *A. americanum* tick saliva (Supplemental table 2). We are of the view that if secretion of host proteins was a random process, we could have identified more rabbit host proteases and protease

668 inhibitors. There are reports that human α1-antitrypsin and α2-macroglobulin are secreted
669 following injury as occurs during tick feeding, and if left uncontrolled could lead to delayed
670 wound healing (149), which is beneficial to tick feeding. On this basis, it is highly likely that
671 ticks inject host α1-antitrypsin and α2-macroglobulin into the feeding site as a strategy of
672 evading the host's tissue repair defense response. It is also notable that fibrinogen and neutrophil
673 gelantinase-associated lipocalin, which among other functions plays important roles in wound
674 healing, were identified towards the end of feeding (150-153). This is interesting in that the tick-
675 feeding lesion is completely sealed, preventing leakage of blood, when a replete fed tick detaches
676 from its feeding site. It has been reported in *Opisthorchis viverrini*, the human liver fluke, that
677 they secrete proteins in the granulin family that accelerate wound healing (154). Could it be that
678 the increased abundance of host proteins involved in wound healing are secreted by the tick into
679 the feeding site towards the end of tick feeding is the tick's way to help its host heal?
680
681 *Different tick species might utilize similar proteins to regulate feeding*
682 At the time of preparing data in this study for publication, several other tick saliva
683 proteomes had been published. We took advantage of the availability of these data to test the
684 hypothesis that key proteins that are important to tick feeding might be conserved across tick
685 taxa. Thus, we compared data in this study to saliva proteomes of *I. scapularis* (32), *R. microplus*
686 (33), *H. longicornis* (34), *R. sanguineus* (35), *D. andersoni* (36), and *O. moubata* (37). This
687 analysis revealed that more than 24% (284/1182) of the *A. americanum* tick saliva proteins in
688 this study have homologs in saliva proteomes of other tick species (Supplemental table 4). Table
689 4 highlights the 163, 138, 137, 92, 22, and 11 *A. americanum* tick saliva proteins in 22 categories
690 that were >70% identical to proteins in saliva of *I. scapularis* (32), *H. longicornis* (34), *D.*

691 Table 4. *Amblyomma americanum* tick saliva protein categories that are conserved in other tick
692 saliva proteomes at 70% identity

Classification	*I. scapularis*	*H. longicornis*	*D. andersoni*	*R. microplus*	*O. moubata*	*R. sanguinues*
Cytoskeletal	36	27	19	12	3	0
Detoxification	13	9	9	5	0	2
Extracellular matrix	3	6	9	3	0	1
Glycine rich	5	4	4	4	0	0
Immune related	4	3	3	4	1	1
Metabolism, amino acids	4	1	0	0	0	0
Metaolism, carbohydrates	4	3	6	1	1	0
Metabolim, energy	20	11	12	0	4	0
Metabolism, lipids	2	1	3	4	0	0
Metabolism, nucleic acids	11	9	1	0	2	0
Nuclear regulation	6	5	6	4	0	0
Protein modification	16	14	8	9	7	0
Protease	6	6	8	3	0	0
Proteosome machinery	7	6	5	0	0	6
Protein synthesis	4	2	3	2	0	0
Secreted saliva proteins	5	6	22	14	0	0
Lipocalin	0	0	0	4	0	0
Protease Inhibitors	5	14	10	11	1	1
Signal transduction	6	3	6	1	1	0
Heme/Iron binding	1	7	2	9	2	0
Transcription machinery	2	0	1	1	0	0
Transporters/ receptors	3	1	0	1	0	0
Total Protein Matches	**163**	**138**	**137**	**92**	**22**	**11**

693
694

695 *andersoni* (36), *R. microplus* (33), *O. moubata* (37) and *R. sanguineus* (35), respectively. Of the
696 22 categories of proteins, immune-related proteins were present in all tick saliva proteomes.
697 Likewise, proteins from nine other categories (antioxidant/detoxification, carbohydrate
698 metabolism, cytoskeletal, extracellular matrix, heme/iron binding protease, protease inhibitor,
699 protein modification, and signal transduction) from *A. americanum* saliva were present in five
700 other tick saliva proteomes. It is interesting to note when comparing saliva proteins in the *A.*
701 *americanum* tick-specific saliva secreted protein category with other hard ticks, with the
702 exception of *R. sanguineus*, five proteins were shared with Prostriata *I. scapularis*, and 6, 22, and
703 14 from Metastriata *H. longicornis*, *D. andersoni*, and *R. microplus*, respectively.

We would like the reader to note that with the exception of *I. scapularis* tick saliva proteome for which proteins were identified every 24 h during feeding (32) the other tick saliva proteomes were limited to a narrow range of tick feeding time points and/or fully engorged ticks. This might be the reason that higher numbers of *A. americanum* tick saliva proteins were found compared to *I. scapularis* tick saliva proteome. It is interesting to note that, *A. americanum* and *I. scapularis* are biologically different as they belong to different tick lineages, Prostriata and Metastriata (155, 156). Thus, tick saliva proteins that are shared between these two tick species could regulate evolutionarily conserved proteins that regulate essential tick feeding physiology functions. On this basis, such proteins could be targeted for tick vaccine development. We have previously shown that RNAi silencing of *A. americanum* tick saliva serpin 19, an anti-coagulant (69), which is also conserved in *I. scapularis* ticks (42, 62), caused significant mortality demonstrating the importance of this protein in tick physiology (32).

Conclusion and Future perspective

This study has made a unique contribution toward understanding the molecular basis of *A. americanum* tick feeding physiology. We believe that this study provides a good starting point toward discovery of effective targets for anti-tick vaccine development. Our strategy to identify tick saliva proteins every 24 h during feeding has allowed us to map tick saliva proteins to different phases of the tick feeding process. This is significant as it provides for the opportunity to focus on tick saliva proteins that regulate the tick feeding process that precede critical events such as TBD agent transmission. Majority of TBD agents are transmitted after 48 h of tick attachment (157, 158), and therefore proteins that are secreted from 24 and 48 h of tick feeding time points are prime candidates for tick vaccine research. It is important to acknowledge the fact

727 that, during the course of feeding, *A. americanum* ticks secretion of more than 1500 tick and
728 rabbit host proteins might indicate that the tick has inbuilt systems to evade host immunity, and
729 that it is going to be a challenge to actually find effective targets for anti-tick vaccine
730 development. However, the findings that nearly 300 *A. americanum* tick saliva proteins were
731 also secreted by other tick species is very encouraging as these proteins might provide insight
732 into conserved mechanisms that are utilized by all ticks to successfully feed, and could serve as
733 potential targets for anti-tick vaccine development.

734 We have recently described proteins (n=340) in saliva of unfed *A. americanum* ticks that
735 were stimulated to start feeding on three different hosts: rabbits, dogs, and humans (38). It is
736 notable that 70% (231/340) of proteins in saliva of unfed *A. americanum* ticks were found in the
737 tick saliva proteome described here (Supplemental table 5). The significance of these data is that
738 the 231 tick saliva proteins present in saliva of both unfed and fed ticks represent proteins that
739 are potentially injected into the host within minutes of the tick attaching onto host skin and are
740 likely associated with regulating initial tick feeding events. Immunologically blocking functions
741 of these proteins might significantly disrupt tick feeding and prevent transmission of TBD
742 agents. In summary, this study has set the foundation for in-depth studies to understand *A.*
743 *americanum* tick feeding physiology and find effective targets for development of tick-antigen
744 based vaccines to prevent TBD infections.

745

746 **Acknowledgements:**

747 This research was supported by National Institutes of Health grants (AI081093, AI093858,
748 AI074789, AI074789-01A1S1) to AM and National Center for Research Resources
749 (5P41RR011823) and National Institute of General Medical Sciences (8P41GM103533) to JRY.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (159) partner repository with the dataset identifier PXD014844.

References

1. Dantas-Torres, F., Chomel, B.B., Otranto, D., 2012. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol. 28, 437-446.
2. Raghavan, R.K., Peterson, A.T., Cobos, M.E., Ganta, R., Foley, D., 2019. Current and Future Distribution of the Lone Star Tick, *Amblyomma americanum* (L.) (Acari: Ixodidae) in North America. PLoS One 14, e0209082.
3. Anderson, B.E., Sims, K.G., Olson, J.G., Childs, J.E., Piesman, J.F., Happ, C.M., Maupin, G.O., Johnson, B.J., 1993. *Amblyomma americanum*: a potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. 49, 239-244.
4. Buller, R.S., Arens, M., Hmiel, S.P., Paddock, C.D., Sumner, J.W., Rikhisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F.A., Liddell, A.M., Schmulewitz, N., Storch, G.A., 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. 341, 148-155.
5. Murphy, G.L., Ewing, S.A., Whitworth, L.C., Fox, J.C., Kocan, A.A., 1998. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet. Parasitol. 79, 325-339.
6. Wolf, L., McPherson, T., Harrison, B., Engber, B., Anderson, A., Whitt, P., 2000. Prevalence of *Ehrlichia ewingii* in *Amblyomma americanum* in North Carolina. J. Clin. Microbiol. 38, 2795.
7. Hopla CE, D.C., 1953. The isolation of *Bacterium tularense* from the tick *Amblyomma americanum.* journal of kansas entomological society 26, 71-72.
8. Taylor, J.P., Istre, G.R., McChesney, T.C., Satalowich, F.T., Parker, R.L., McFarland, L.M., 1991. Epidemiologic characteristics of human tularemia in the southwest-central states, 1981-1987. Am. J. Epidemiol. 133, 1032-1038.
9. Armstrong, P.M., Brunet, L.R., Spielman, A., Telford, S.R.,3rd, 2001. Risk of Lyme disease: perceptions of residents of a Lone Star tick-infested community. Bull. World Health Organ. 79, 916-925.
10. James, A.M., Liveris, D., Wormser, G.P., Schwartz, I., Montecalvo, M.A., Johnson, B.J., 2001. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J. Infect. Dis. 183, 1810-1814.
11. Reeves, W.K., Loftis, A.D., Nicholson, W.L., Czarkowski, A.G., 2008. The first report of human illness associated with the Panola Mountain *Ehrlichia* species: a case report. J. Med. Case Rep. 2, 139.
12. Apperson, C.S., Engber, B., Nicholson, W.L., Mead, D.G., Engel, J., Yabsley, M.J., Dail, K., Johnson, J., Watson, D.W., 2008. Tick-borne diseases in North Carolina: is "*Rickettsia amblyommii*" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector Borne Zoonotic Dis. 8, 597-606.
13. Breitschwerdt, E.B., Hegarty, B.C., Maggi, R.G., Lantos, P.M., Aslett, D.M., Bradley, J.M., 2011. *Rickettsia rickettsii* transmission by a lone star tick, North Carolina. Emerg. Infect. Dis. 17, 873-875.
14. McMullan, L.K., Folk, S.M., Kelly, A.J., MacNeil, A., Goldsmith, C.S., Metcalfe, M.G., Batten, B.C., Albarino, C.G., Zaki, S.R., Rollin, P.E., Nicholson, W.L., Nichol, S.T., 2012. A new phlebovirus associated with severe febrile illness in Missouri. N. Engl. J. Med. 367, 834-841.

15. Savage, H.M., Godsey, M.S.,Jr, Lambert, A., Panella, N.A., Burkhalter, K.L., Harmon, J.R., Lash, R.R., Ashley, D.C., Nicholson, W.L., 2013. First detection of heartland virus (Bunyaviridae: Phlebovirus) from field collected arthropods. Am. J. Trop. Med. Hyg. 89, 445-452.
16. Steinke, J.W., Platts-Mills, T.A., Commins, S.P., 2015. The alpha-gal story: lessons learned from connecting the dots. J. Allergy Clin. Immunol. 135, 96; quiz 597.
17. Laird, J.S., Kocan, A.A., Kocan, K.M., Presley, S.M., Hair, J.A., 1988. Susceptibility of *Amblyomma americanum* to natural and experimental infections with *Theileria cervi*. J. Wildl. Dis. 24, 679-683.
18. Little, S.E., O'Connor, T.P., Hempstead, J., Saucier, J., Reichard, M.V., Meinkoth, K., Meinkoth, J.H., Andrews, B., Ullom, S., Ewing, S.A., Chandrashekar, R., 2010. *Ehrlichia ewingii* infection and exposure rates in dogs from the southcentral United States. Vet. Parasitol. 172, 355-360.
19. Yabsley, M.J., Quick, T.C., Little, S.E., 2005. Theileriosis in a white-tailed deer (*Odocoileus virginianus*) fawn. J. Wildl. Dis. 41, 806-809.
20. Barnard, D.R., Ervin, R.T., Epplin, F.M., 1992. Bio-Economic Impact of *Amblyomma americanum* in Beef Cattle Production Systems. Tick Vector Biology, 55-69.
21. Tolleson, D.R., Teel, P.D., Stuth, J.W., Strey, O.F., Welsh, T.H.,Jr, Carstens, G.E., Longnecker, M.T., Banik, K.K., Prince, S.D., 2010. Effects of a lone star tick (*Amblyomma americanum*) burden on performance and metabolic indicators in growing beef steers. Vet. Parasitol. 173, 99-106.
22. Felz, M.W., Durden, L.A., Oliver, J.H.J., 1996. Ticks parasitizing humans in Georgia and South Carolina. The Journal of parasitology 82,3, 505-508.
23. Nuttall, P.A., Labuda, M., 2004. Tick-host interactions: saliva-activated transmission. Parasitology 129 Suppl, 177.
24. Bell, J.F., Stewart, S.J., Wikel, S.K., 1979. Resistance to tick-borne *Francisella tularensis* by tick-sensitized rabbits: allergic klendusity. Am. J. Trop. Med. Hyg. 28, 876-880.
25. Burke, G., Wikel, S.K., Spielman, A., Telford, S.R., McKay, K., Krause, P.J., Tick-borne Infection Study Group, 2005. Hypersensitivity to ticks and Lyme disease risk. Emerg. Infect. Dis. 11, 36-41.
26. Nazario, S., Das, S., de Silva, A.M., Deponte, K., Marcantonio, N., Anderson, J.F., Fish, D., Fikrig, E., Kantor, F.S., 1998. Prevention of *Borrelia burgdorferi* transmission in guinea pigs by tick immunity. Am. J. Trop. Med. Hyg. 58, 780-785.
27. Wikel, S.K., Ramachandra, R.N., Bergman, D.K., Burkot, T.R., Piesman, J., 1997. Infestation with pathogen-free nymphs of the tick *Ixodes scapularis* induces host resistance to transmission of *Borrelia burgdorferi* by ticks. Infect. Immun. 65, 335-338.
28. Francis, J., Little, D.A., 1964. Resistance of Droughtmaster cattle to tick infestation and babesiosis. Aust. Vet. J. 40, 247-253.
29. Jones, L.D., Nuttall, P.A., 1990. The effect of host resistance to tick infestation on the transmission of Thogoto virus by ticks. J. Gen. Virol. 71 (Pt 5), 1039-1043.
30. Votiakov, V., P Mishaeva, N., 1980. Possibility of Protecting Vertebrates Against Transmissive Infection with Tick-Borne Encephalitis Virus. Vopr Virusol. 2,170-173.
31. Fivaz, B.H., Norval, R.A., Lawrence, J.A., 1989. Transmission of *Theileria parva bovis* (Boleni strain) to cattle resistant to the brown ear tick *Rhipicephalus appendiculatus* (Neumann). Trop. Anim. Health Prod. 21, 129-134.

32. Kim, T.K., Tirloni, L., Pinto, A.F., Moresco, J., Yates, J.R.,3rd, da Silva Vaz, I.,Jr, Mulenga, A., 2016. *Ixodes scapularis* Tick Saliva Proteins Sequentially Secreted Every 24 h during Blood Feeding. PLoS Negl Trop. Dis. 10, e0004323.
33. Tirloni, L., Reck, J., Terra, R.M., Martins, J.R., Mulenga, A., Sherman, N.E., Fox, J.W., Yates, J.R.,3rd, Termignoni, C., Pinto, A.F., da Silva Vaz, I.,Jr, 2014. Proteomic Analysis of Cattle Tick *Rhipicephalus* (*Boophilus*) *microplus* Saliva: A Comparison between Partially and Fully Engorged Females. PLoS One 9, e94831.
34. Tirloni, L., Islam, M.S., Kim, T.K., Diedrich, J.K., Yates, J.R.,3rd, Pinto, A.F., Mulenga, A., You, M.J., Da Silva Vaz, I.,Jr, 2015. Saliva from nymph and adult females of *Haemaphysalis longicornis*: a proteomic study. Parasit. Vectors 8, y.
35. Oliveira, C.J., Anatriello, E., de Miranda-Santos, I.K., Francischetti, I.M., Sa-Nunes, A., Ferreira, B.R., Ribeiro, J.M., 2013. Proteome of *Rhipicephalus sanguineus* tick saliva induced by the secretagogues pilocarpine and dopamine. Ticks Tick Borne Dis. 4, 469-477.
36. Mudenda, L., Pierle, S.A., Turse, J.E., Scoles, G.A., Purvine, S.O., Nicora, C.D., Clauss, T.R., Ueti, M.W., Brown, W.C., Brayton, K.A., 2014. Proteomics informed by transcriptomics identifies novel secreted proteins in *Dermacentor andersoni* saliva. Int. J. Parasitol. 44, 1029-1037.
37. Diaz-Martin, V., Manzano-Roman, R., Oleaga, A., Encinas-Grandes, A., Perez-Sanchez, R., 2013. Cloning and characterization of a plasminogen-binding enolase from the saliva of the argasid tick *Ornithodoros moubata*. Vet. Parasitol. 191, 301-314.
38. Tirloni, L., Kim, T.K., Pinto, A.F.M., Yates, J.R.,3rd, da Silva Vaz, I.,Jr, Mulenga, A., 2017. Tick-Host Range Adaptation: Changes in Protein Profiles in Unfed Adult *Ixodes scapularis* and *Amblyomma americanum* Saliva Stimulated to Feed on Different Hosts. Front. Cell. Infect. Microbiol. 7, 517.
39. Mulenga, A., Kim, T.K., Ibelli, A.M., 2013a. Deorphanization and target validation of cross-tick species conserved novel *Amblyomma americanum* tick saliva protein. Int. J. Parasitol. 43, 439-451.
40. Kim, T.K., Curran, J., Mulenga, A., 2014. Dual silencing of long and short *Amblyomma americanum* acidic chitinase forms weakens the tick cement cone stability. J. Exp. Biol. 217, 3493-3503.
41. Shevchenko, A., Wilm, M., Vorm, O., Mann, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. 68, 850-858.
42. Porter, L., Radulovic, Z., Kim, T., Braz, G.R., Da Silva Vaz, I.,Jr, Mulenga, A., 2015. Bioinformatic analyses of male and female *Amblyomma americanum* tick expressed serine protease inhibitors (serpins). Ticks Tick Borne Dis.
43. Karim, S., Singh, P., Ribeiro, J.M., 2011. A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS One 6, e28525.
44. Rawlings, N.D., Barrett, A.J., Bateman, A., 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 40, 343.
45. Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M., Sherlock, G., 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25-29.

46. Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res. 43, 222.
47. Tatusov, R.L., Fedorova, N.D., Jackson, J.D., Jacobs, A.R., Kiryutin, B., Koonin, E.V., Krylov, D.M., Mazumder, R., Mekhedov, S.L., Nikolskaya, A.N., Rao, B.S., Smirnov, S., Sverdlov, A.V., Vasudevan, S., Wolf, Y.I., Yin, J.J., Natale, D.A., 2003. The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4, 41.
48. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S.R., Griffiths-Jones, S., Howe, K.L., Marshall, M., Sonnhammer, E.L., 2002. The Pfam protein families database. Nucleic Acids Res. 30, 276-280.
49. Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P., Bork, P., 2000. SMART: a web-based tool for the study of genetically mobile domains. Nucleic Acids Res. 28, 231-234.
50. Fu, L., Niu, B., Zhu, Z., Wu, S., Li, W., 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics 28, 3150-3152.
51. McDonald, W.H., Tabb, D.L., Sadygov, R.G., MacCoss, M.J., Venable, J., Graumann, J., Johnson, J.R., Cociorva, D., Yates, J.R.,3rd, 2004. MS1, MS2, and SQT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. Rapid Commun. Mass Spectrom. 18, 2162-2168.
52. Xu, T., Park, S.K., Venable, J.D., Wohlschlegel, J.A., Diedrich, J.K., Cociorva, D., Lu, B., Liao, L., Hewel, J., Han, X., Wong, C.C., Fonslow, B., Delahunty, C., Gao, Y., Shah, H., Yates, J.R.,3rd, 2015. ProLuCID: An improved SEQUEST-like algorithm with enhanced sensitivity and specificity. J. Proteomics .
53. Florens, L., Carozza, M.J., Swanson, S.K., Fournier, M., Coleman, M.K., Workman, J.L., Washburn, M.P., 2006. Analyzing chromatin remodeling complexes using shotgun proteomics and normalized spectral abundance factors. Methods 40, 303-311.
54. Paoletti, A.C., Parmely, T.J., Tomomori-Sato, C., Sato, S., Zhu, D., Conaway, R.C., Conaway, J.W., Florens, L., Washburn, M.P., 2006. Quantitative proteomic analysis of distinct mammalian Mediator complexes using normalized spectral abundance factors. Proc. Natl. Acad. Sci. U. S. A. 103, 18928-18933.
55. Zhu, W., Smith, J.W., Huang, C.M., 2010. Mass spectrometry-based label-free quantitative proteomics. J. Biomed. Biotechnol. 2010, 840518.
56. Warnes, G.R., Bolker, B., Bonebakker, L., Gentleman, R., Huber, W., Liaw, A., Lumley, T., Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., Venables, B., 2013. Various R programming tools for plotting data. The Comprehensive R Archive Network.
57. McSwain, J.L., Essenberg, R.C., Sauer, J.R., 1982. Protein changes in the salivary glands of the female lone star tick, *Amblyomma americanum*, during feeding. J. Parasitol. 68, 100-106.
58. Tatchell, R.J., 1967. A modified method for obtaining tick oral secretion. J. Parasitol. 53, 1106-1107.
59. Karim, S., Ribeiro, J.M., 2015. An Insight into the Sialome of the Lone Star Tick, *Amblyomma americanum*, with a Glimpse on Its Time Dependent Gene Expression. PLoS One 10, e0131292.
60. Ribeiro, J.M., Alarcon-Chaidez, F., Francischetti, I.M., Mans, B.J., Mather, T.N., Valenzuela, J.G., Wikel, S.K., 2006. An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks. Insect Biochem. Mol. Biol. 36, 111-129.

61. Valenzuela, J.G., Francischetti, I.M., Pham, V.M., Garfield, M.K., Mather, T.N., Ribeiro, J.M., 2002. Exploring the sialome of the tick *Ixodes scapularis*. J. Exp. Biol. 205, 2843-2864.
62. Porter, L.M., Radulovic, Z.M., Mulenga, A., 2017. A repertoire of protease inhibitor families in *Amblyomma americanum* and other tick species: inter-species comparative analyses. Parasit. Vectors 10, 1.
63. Blisnick, A.A., Foulon, T., Bonnet, S.I., 2017. Serine Protease Inhibitors in Ticks: An Overview of Their Role in Tick Biology and Tick-Borne Pathogen Transmission. Front. Cell. Infect. Microbiol. 7, 199.
64. Chmelar, J., Kotal, J., Langhansova, H., Kotsyfakis, M., 2017. Protease Inhibitors in Tick Saliva: The Role of Serpins and Cystatins in Tick-host-Pathogen Interaction. Front. Cell. Infect. Microbiol. 7, 216.
65. Law, R.H., Zhang, Q., McGowan, S., Buckle, A.M., Silverman, G.A., Wong, W., Rosado, C.J., Langendorf, C.G., Pike, R.N., Bird, P.I., Whisstock, J.C., 2006. An overview of the serpin superfamily. Genome Biol. 7, 216. Epub 2006 May 30.
66. Mulenga, A., Khumthong, R., Blandon, M.A., 2007a. Molecular and expression analysis of a family of the *Amblyomma americanum* tick Lospins. J. Exp. Biol. 210, 3188-3198.
67. Mulenga, A., Khumthong, R., Chalaire, K.C., 2009. *Ixodes scapularis* tick serine proteinase inhibitor (serpin) gene family; annotation and transcriptional analysis. BMC Genomics 10, 217.
68. Mulenga, A., Sugino, M., Nakajim, M., Sugimoto, C., Onuma, M., 2001. Tick-Encoded serine proteinase inhibitors (serpins); potential target antigens for tick vaccine development. J. Vet. Med. Sci. 63, 1063-1069.
69. Kim, T.K., Tirloni, L., Radulovic, Z., Lewis, L., Bakshi, M., Hill, C., da Silva Vaz, I.,Jr, Logullo, C., Termignoni, C., Mulenga, A., 2015. Conserved *Amblyomma americanum* tick Serpin19, an inhibitor of blood clotting factors Xa and XIa, trypsin and plasmin, has anti-haemostatic functions. Int. J. Parasitol. 45, 613-627.
70. Mulenga, A., Kim, T., Ibelli, A.M., 2013a. *Amblyomma americanum* tick saliva serine protease inhibitor 6 is a cross-class inhibitor of serine proteases and papain-like cysteine proteases that delays plasma clotting and inhibits platelet aggregation. Insect Mol. Biol. 22, 306-319.
71. Karim, S., Miller, N.J., Valenzuela, J., Sauer, J.R., Mather, T.N., 2005. RNAi-mediated gene silencing to assess the role of synaptobrevin and cystatin in tick blood feeding. Biochem. Biophys. Res. Commun. 334, 1336-1342.
72. Wang, X., Shaw, D.K., Sakhon, O.S., Snyder, G.A., Sundberg, E.J., Santambrogio, L., Sutterwala, F.S., Dumler, J.S., Shirey, K.A., Perkins, D.J., Richard, K., Chagas, A.C., Calvo, E., Kopecky, J., Kotsyfakis, M., Pedra, J.H.F., 2016. The Tick Protein Sialostatin L2 Binds to Annexin A2 and Inhibits NLRC4-Mediated Inflammasome Activation. Infect. Immun. 84, 1796-1805.
73. Zavasnik-Bergant, T., Vidmar, R., Sekirnik, A., Fonovic, M., Salat, J., Grunclova, L., Kopacek, P., Turk, B., 2017. Salivary Tick Cystatin OmC2 Targets Lysosomal Cathepsins S and C in Human Dendritic Cells. Front. Cell. Infect. Microbiol. 7, 288.
74. Sa-Nunes, A., Bafica, A., Antonelli, L.R., Choi, E.Y., Francischetti, I.M., Andersen, J.F., Shi, G.P., Chavakis, T., Ribeiro, J.M., Kotsyfakis, M., 2009. The immunomodulatory action of sialostatin L on dendritic cells reveals its potential to interfere with autoimmunity. J. Immunol. 182, 7422-7429.

75. Wang, Y., Zhou, Y., Gong, H., Cao, J., Zhang, H., Li, X., Zhou, J., 2015. Functional characterization of a cystatin from the tick *Rhipicephalus haemaphysaloides*. Parasit. Vectors 8, 5.
76. Wei, N., Lin, Z., Xu, Z., Gong, H., Zhang, H., Zhou, Y., Cao, J., Li, G., Zhou, J., 2019. Immunosuppressive effects of tick protein RHcyst-1 on murine bone marrow-derived dendritic cells. Parasit. Vectors 12, 1.
77. Kopacek, P., Weise, C., Saravanan, T., Vitova, K., Grubhoffer, L., 2000. Characterization of an alpha-macroglobulin-like glycoprotein isolated from the plasma of the soft tick *Ornithodoros moubata*. Eur. J. Biochem. 267, 465-475.
78. Buresova, V., Hajdusek, O., Franta, Z., Sojka, D., Kopacek, P., 2009. IrAM-An alpha2-macroglobulin from the hard tick Ixodes ricinus: characterization and function in phagocytosis of a potential pathogen *Chryseobacterium indologenes*. Dev. Comp. Immunol. 33, 489-498.
79. Mulenga, A., Erikson, K., 2011. A snapshot of the *Ixodes scapularis* degradome. Gene 482, 78-93.
80. Fox, J.W., Serrano, S.M., 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. Toxicon 45, 969-985.
81. Kini, R.M., 2006. Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem. J. 397, 377-387.
82. Decrem, Y., Mariller, M., Lahaye, K., Blasioli, V., Beaufays, J., Zouaoui Boudjeltia, K., Vanhaeverbeek, M., Cerutti, M., Brossard, M., Vanhamme, L., Godfroid, E., 2008. The impact of gene knock-down and vaccination against salivary metalloproteases on blood feeding and egg laying by *Ixodes ricinus*. Int. J. Parasitol. 38, 549-560.
83. Francischetti, I.M., Mather, T.N., Ribeiro, J.M., 2003. Cloning of a salivary gland metalloprotease and characterization of gelatinase and fibrin(ogen)lytic activities in the saliva of the Lyme disease tick vector Ixodes scapularis. Biochem. Biophys. Res. Commun. 305, 869-875.
84. Turner, A.J.,2004. Handbook of Proteolytic Enzymes1.
85. Ewen, D., Clarke, S.L., Smith, J.R., Berger, C., Salmon, G., Trevethick, M., Shute, J.K., 2010. The role of protease-activated receptors PAR-1 and PAR-2 in the repair of 16HBE 14o(-) epithelial cell monolayers in vitro. Clin. Exp. Allergy 40, 435-449.
86. Cattaruzza, F., Amadesi, S., Carlsson, J.F., Murphy, J.E., Lyo, V., Kirkwood, K., Cottrell, G.S., Bogyo, M., Knecht, W., Bunnett, N.W., 2014. Serine proteases and protease-activated receptor 2 mediate the proinflammatory and algesic actions of diverse stimulants. Br. J. Pharmacol. 171, 3814-3826.
87. Sojka, D., Francischetti, I.M., Calvo, E., Kotsyfakis, M., 2011. Cysteine proteases from bloodfeeding arthropod ectoparasites. Adv. Exp. Med. Biol. 712, 177-191.
88. Sun, Y., He, L., Yu, L., Guo, J., Nie, Z., Liu, Q., Zhao, J., 2019. Cathepsin L-a novel cysteine protease from *Haemaphysalis flava* Neumann, 1897. Parasitol. Res.
89. Zhang, T.T., Qiu, Z.X., Li, Y., Wang, W.Y., Li, M.M., Guo, P., Liu, J.Z., 2019. The mRNA expression and enzymatic activity of three enzymes during embryonic development of the hard tick *Haemaphysalis longicornis*. Parasit. Vectors 12, 8.
90. Tsuji, N., Miyoshi, T., Battsetseg, B., Matsuo, T., Xuan, X., Fujisaki, K., 2008. A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. PLoS Pathog. 4, e1000062.

91. Xavier, M.A., Tirloni, L., Torquato, R., Tanaka, A., Pinto, A.F.M., Diedrich, J.K., Yates, J.R.,3rd, da Silva Vaz, I.,Jr, Seixas, A., Termignoni, C., 2019. Blood anticlotting activity of a *Rhipicephalus microplus* cathepsin L-like enzyme. Biochimie 163, 12-20.
92. Cwiklinski, K., de la Torre-Escudero, E., Trelis, M., Bernal, D., Dufresne, P.J., Brennan, G.P., O'Neill, S., Tort, J., Paterson, S., Marcilla, A., Dalton, J.P., Robinson, M.W., 2015. The Extracellular Vesicles of the Helminth Pathogen, *Fasciola hepatica*: Biogenesis Pathways and Cargo Molecules Involved in Parasite Pathogenesis. Mol. Cell. Proteomics 14, 3258-3273.
93. Berasain, P., Carmona, C., Frangione, B., Dalton, J.P., Goñi, F., 2000. *Fasciola hepatica*: Parasite-Secreted Proteinases Degrade All Human IgG Subclasses: Determination of the Specific Cleavage Sites and Identification of the Immunoglobulin Fragments Produced. Exp Parasitol. 94, 99-110.
94. Robinson, M.W., Corvo, I., Jones, P.M., George, A.M., Padula, M.P., To, J., Cancela, M., Rinaldi, G., Tort, J.F., Roche, L., Dalton, J.P., 2011. Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop. Dis. 5, e1012.
95. Akov, S., 1982. Blood Digestion in Ticks. pp 197-212. Obenchain, F.D and Galun R., Physiology of Ticks.
96. Boldbaatar, D., Sikalizyo Sikasunge, C., Battsetseg, B., Xuan, X., Fujisaki, K., 2006. Molecular cloning and functional characterization of an aspartic protease from the hard tick *Haemaphysalis longicornis*. Insect Biochem. Mol. Biol. 36, 25-36.
97. Logullo, C., Vaz Ida, S., Sorgine, M.H., Paiva-Silva, G.O., Faria, F.S., Zingali, R.B., De Lima, M.F., Abreu, L., Oliveira, E.F., Alves, E.W., Masuda, H., Gonzales, J.C., Masuda, A., Oliveira, P.L., 1998. Isolation of an aspartic proteinase precursor from the egg of a hard tick, *Boophilus microplus*. Parasitology 116 (Pt 6), 525-532.
98. Sorgine, M.H., Logullo, C., Zingali, R.B., Paiva-Silva, G.O., Juliano, L., Oliveira, P.L., 2000. A heme-binding aspartic proteinase from the eggs of the hard tick *Boophilus microplus*. J. Biol. Chem. 275, 28659-28665.
99. Paesen, G.C., Adams, P.L., Harlos, K., Nuttall, P.A., Stuart, D.I., 1999. Tick histamine-binding proteins: isolation, cloning, and three-dimensional structure. Mol. Cell 3, 661-671.
100. Fluckinger, M., Haas, H., Merschak, P., Glasgow, B.J., Redl, B., 2004. Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores. Antimicrob. Agents Chemother. 48, 3367-3372.
101. Chan, Y.R., Liu, J.S., Pociask, D.A., Zheng, M., Mietzner, T.A., Berger, T., Mak, T.W., Clifton, M.C., Strong, R.K., Ray, P., Kolls, J.K., 2009. Lipocalin 2 is required for pulmonary host defense against *Klebsiella* infection. J. Immunol. 182, 4947-4956.
102. Cho, K.W., Zhou, Y., Sheng, L., Rui, L., 2011. Lipocalin-13 regulates glucose metabolism by both insulin-dependent and insulin-independent mechanisms. Mol. Cell. Biol. 31, 450-457.
103. Ganfornina, M.D., Kayser, H., Sanchez, D., 2000. Lipocalins in Arthropoda: Diversification and Functional Explorations. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience .
104. Smathers, R.L., Petersen, D.R., 2011. The human fatty acid-binding protein family: evolutionary divergences and functions. Hum. Genomics 5, 170-191.

105. Koreny, L., Obornik, M., Lukes, J., 2013. Make it, take it, or leave it: heme metabolism of parasites. PLoS Pathog. 9, e1003088.
106. Braz, G.R., Coelho, H.S., Masuda, H., Oliveira, P.L., 1999. A missing metabolic pathway in the cattle tick *Boophilus microplus*. Curr. Biol. 9, 703-706.
107. Perner, J., Sobotka, R., Sima, R., Konvickova, J., Sojka, D., Oliveira, P.L., Hajdusek, O., Kopacek, P., 2016. Acquisition of exogenous haem is essential for tick reproduction. Elife 5, 10.7554/eLife.12318.
108. Toh, S.Q., Glanfield, A., Gobert, G.N., Jones, M.K., 2010. Heme and blood-feeding parasites: friends or foes? Parasit. Vectors 3, 108.
109. Lara, F.A., Lins, U., Bechara, G.H., Oliveira, P.L., 2005. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. J. Exp. Biol. 208, 3093-3101.
110. Maya-Monteiro, C.M., Daffre, S., Logullo, C., Lara, F.A., Alves, E.W., Capurro, M.L., Zingali, R., Almeida, I.C., Oliveira, P.L., 2000. HeLp, a heme lipoprotein from the hemolymph of the cattle tick, *Boophilus microplus*. J. Biol. Chem. 275, 36584-36589.
111. Gudderra, N.P., Sonenshine, D.E., Apperson, C.S., Roe, R.M., 2002a. Hemolymph proteins in ticks. Journal of Insect Physiology 48, 269-278.
112. Gudderra, N.P., Sonenshine, D.E., Apperson, C.S., Roe, R.M., 2002b. Tissue distribution and characterization of predominant hemolymph carrier proteins from *Dermacentor variabilis* and *Ornithodoros parkeri*. J. Insect Physiol. 48, 161-170.
113. Dutra, F.F., Bozza, M.T., 2014. Heme on innate immunity and inflammation. Front. Pharmacol. 5, 115.
114. Maya-Monteiro, C.M., Alves, L.R., Pinhal, N., Abdalla, D.S., Oliveira, P.L., 2004. HeLp, a heme-transporting lipoprotein with an antioxidant role. Insect Biochem. Mol. Biol. 34, 81-88.
115. Seixas, A., Alzugaray, M.F., Tirloni, L., Parizi, L.F., Pinto, A.F.M., Githaka, N.W., Konnai, S., Ohashi, K., Yates Iii, J.R., Termignoni, C., da Silva Vaz, I.,Jr, 2018. Expression profile of *Rhipicephalus microplus* vitellogenin receptor during oogenesis. Ticks Tick Borne Dis. 9, 72-81.
116. Hussein, H.E., Johnson, W.C., Taus, N.S., Suarez, C.E., Scoles, G.A., Ueti, M.W., 2019. Silencing expression of the *Rhipicephalus microplus* vitellogenin receptor gene blocks *Babesia bovis* transmission and interferes with oocyte maturation. Parasit. Vectors 12, 1.
117. Narasimhan, S., Deponte, K., Marcantonio, N., Liang, X., Royce, T.E., Nelson, K.F., Booth, C.J., Koski, B., Anderson, J.F., Kantor, F., Fikrig, E., 2007. Immunity against *Ixodes scapularis* salivary proteins expressed within 24 hours of attachment thwarts tick feeding and impairs *Borrelia* transmission. PLoS One 2, e451.
118. Rojkind, M., Dominguez-Rosales, J.A., Nieto, N., Greenwel, P., 2002. Role of hydrogen peroxide and oxidative stress in healing responses. Cell Mol. Life Sci. 59, 1872-1891.
119. Adamson, S., Browning, R., Singh, P., Nobles, S., Villarreal, A., Karim, S., 2014. Transcriptional activation of antioxidants may compensate for selenoprotein deficiencies in *Amblyomma maculatum* (Acari: Ixodidae) injected with selK- or selM-dsRNA. Insect Mol. Biol. 23, 497-510.
120. Kumar, D., Budachetri, K., Meyers, V.C., Karim, S., 2016. Assessment of tick antioxidant responses to exogenous oxidative stressors and insight into the role of

catalase in the reproductive fitness of the Gulf Coast tick, *Amblyomma maculatum*. Insect Mol. Biol. 25, 283-294.

121. Das, S., Banerjee, G., DePonte, K., Marcantonio, N., Kantor, F.S., Fikrig, E., 2001. Salp25D, an *Ixodes scapularis* antioxidant, is 1 of 14 immunodominant antigens in engorged tick salivary glands. J. Infect. Dis. 184, 1056-1064.

122. Wu, J., Wang, Y., Liu, H., Yang, H., Ma, D., Li, J., Li, D., Lai, R., Yu, H., 2010. Two immunoregulatory peptides with antioxidant activity from tick salivary glands. J. Biol. Chem. 285, 16606-16613.

123. Lewis, L.A., Radulovic, Z.M., Kim, T.K., Porter, L.M., Mulenga, A., 2015. Identification of 24h *Ixodes scapularis* immunogenic tick saliva proteins. Ticks Tick Borne Dis. 6, 424-434.

124. Radulovic, Z.M., Kim, T.K., Porter, L.M., Sze, S.H., Lewis, L., Mulenga, A., 2014. A 24-48 h fed *Amblyomma americanum* tick saliva immuno-proteome. BMC Genomics 15, 518.

125. Kemp, D.H., Stone, B.F., Binnington, K.C., 1982. Tick Attachment and Feeding: Role of the Mouthparts, Feeding Apparatus, Salivary Gland Secretions and the Host Response, in Obenchain, F., Galun, R. (Eds.), Physiology of Ticks. Pergamon Press Ltd., Oxford, UK, pp. 138-139.

126. Binnington, K.C., Kemp, D.H., 1980. Role of tick salivary glands in feeding and disease transmission. Adv. Parasitol. 18, 315-339.

127. Sonenshine, D.E., 1993. Biology of Ticks. Oxford University Press, Oxford.

128. Alekseev, A.N., Burenkova, L.A., Podboronov, V.M., Chunikhin, S.P., 1995. Bacteriocidal qualities of ixodid tick (Acarina: Ixodidae) salivary cement plugs and their changes under the influence of a viral tick-borne pathogen. J. Med. Entomol. 32, 578-582.

129. Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., Morzaria, S., Musoke, A., Nene, V., 2002. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833-842.

130. Bullard, R., Allen, P., Chao, C.C., Douglas, J., Das, P., Morgan, S.E., Ching, W.M., Karim, S., 2016. Structural characterization of tick cement cones collected from in vivo and artificial membrane blood-fed Lone Star ticks (*Amblyomma americanum*). Ticks Tick Borne Dis. 7, 880-892.

131. Hollmann, T., Kim, T.K., Tirloni, L., Radulovic, Z.M., Pinto, A.F.M., Diedrich, J.K., Yates, J.R.,3rd, da Silva Vaz, I.,Jr, Mulenga, A., 2017. Identification and characterization of proteins in the *Amblyomma americanum* tick cement cone. Int. J. Parasitol.

132. Mulenga, A., Sugimoto, C., Sako, Y., Ohashi, K., Musoke, A., Shubash, M., Onuma, M., 1999. Molecular characterization of a *Haemaphysalis longicornis* tick salivary gland-associated 29-kilodalton protein and its effect as a vaccine against tick infestation in rabbits. Infect. Immun. 67, 1652-1658.

133. Czolpinska, M., Rurek, M., 2018. Plant Glycine-Rich Proteins in Stress Response: An Emerging, Still Prospective Story. Front. Plant. Sci. 9, 302.

134. Rossi, D.C.P., Silva, F.D., Daffre, S., Martinez, L.R., Nosanchuk, J.D., Frases, S., Fonseca, F.L., Rodrigues, M.L., Campos, C.B.L., 2011. Effects of microplusin, a copper-chelating antimicrobial peptide, against *Cryptococcus neoformans*. femsle 324, 64-72.

135. Silva, F.D., Rezende, C.A., Rossi, D.C., Esteves, E., Dyszy, F.H., Schreier, S., Gueiros-Filho, F., Campos, C.B., Pires, J.R., Daffre, S., 2009. Structure and mode of action of microplusin, a copper II-chelating antimicrobial peptide from the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*. J. Biol. Chem. 284, 34735-34746.

136. Nakajima, Y., van der Goes van Naters-Yasui, A, Taylor, D., Yamakawa, M., 2003. Antibacterial peptide defensin is involved in midgut immunity of the soft tick, *Ornithodoros moubata*. Insect Mol. Biol. 11, 611-618.

137. Petrou, G., Crouzier, T., 2018. Mucins as multifunctional building blocks of biomaterials. Biomater. Sci. 6, 2282-2297.

138. Eaton, J.R.O., Alenazi, Y., Singh, K., Davies, G., Geis-Asteggiante, L., Kessler, B., Robinson, C.V., Kawamura, A., Bhattacharya, S., 2018. The N-terminal domain of a tick evasin is critical for chemokine binding and neutralization and confers specific binding activity to other evasins. J. Biol. Chem. 293, 6134-6146.

139. Hayward, J., Sanchez, J., Perry, A., Huang, C., Rodriguez Valle, M., Canals, M., Payne, R.J., Stone, M.J., 2017. Ticks from diverse genera encode chemokine-inhibitory evasin proteins. J. Biol. Chem. 292, 15670-15680.

140. Francischetti, I.M., My Pham, V., Mans, B.J., Andersen, J.F., Mather, T.N., Lane, R.S., Ribeiro, J.M., 2005. The transcriptome of the salivary glands of the female western black-legged tick *Ixodes pacificus* (Acari: Ixodidae). Insect Biochem. Mol. Biol. 35, 1142-1161.

141. Smith, S.A., Travers, R.J., Morrissey, J.H., 2015. How it all starts: Initiation of the clotting cascade. Crit. Rev. Biochem. Mol. Biol. 50, 326-336.

142. Konnai, S., Nakajima, C., Imamura, S., Yamada, S., Nishikado, H., Kodama, M., Onuma, M., Ohashi, K., 2009. Suppression of cell proliferation and cytokine expression by HL-p36, a tick salivary gland-derived protein of *Haemaphysalis longicornis*. Immunology 126, 209-219.

143. Mulenga, A., Blandon, M., Khumthong, R., 2007b. The molecular basis of the *Amblyomma americanum* tick attachment phase. Exp. Appl. Acarol. 41, 267-287.

144. Francischetti, I.M., Sa-Nunes, A., Mans, B.J., Santos, I.M., Ribeiro, J.M., 2009. The role of saliva in tick feeding. Front. Biosci. (Landmark Ed) 14, 2051-2088.

145. Rock, K.L., Kono, H., 2008. The inflammatory response to cell death. Annu. Rev. Pathol. 3, 99-126.

146. Zininga, T., Ramatsui, L., Shonhai, A., 2018. Heat Shock Proteins as Immunomodulants. Molecules 23, 10.3390/molecules23112846.

147. Poirier, A.C., Schmitt, P., Rosa, R.D., Vanhove, A.S., Kieffer-Jaquinod, S., Rubio, T.P., Charriere, G.M., Destoumieux-Garzon, D., 2014. Antimicrobial histones and DNA traps in invertebrate immunity: evidences in Crassostrea gigas. J. Biol. Chem. 289, 24821-24831.

148. Puente, X.S., Lopez-Otin, C., 2004. A genomic analysis of rat proteases and protease inhibitors. Genome Res. 14, 609-622.

149. Shah, D., Mital, K., 2018. The Role of Trypsin:Chymotrypsin in Tissue Repair. Adv. Ther. 35, 31-42.

150. Mosesson, M.W., 2005. Fibrinogen and fibrin structure and functions. J. Thromb. Haemost. 3, 1894-1904.

151. Abdollahi, M., Ng, T.S., Rezaeizadeh, A., Aamidor, S., Twigg, S.M., Min, D., McLennan, S.V., 2017. Insulin treatment prevents wounding associated changes in tissue and circulating neutrophil MMP-9 and NGAL in diabetic rats. PLoS One 12, e0170951.

152. Laurens, N., Koolwijk, P., de Maat, M.P., 2006. Fibrin structure and wound healing. J. Thromb. Haemost. 4, 932-939.

153. Thorsvik, S., van Beelen Granlund, A., Svendsen, T.D., Bakke, I., Royset, E.S., Flo, T.H., Damas, J.K., Ostvik, A.E., Bruland, T., Sandvik, A.K., 2019. Ulcer-associated cell lineage expresses genes involved in regeneration and is hallmarked by high neutrophil gelatinase-associated lipocalin (NGAL) levels. J. Pathol.

154. Smout, M.J., Sotillo, J., Laha, T., Papatpremsiri, A., Rinaldi, G., Pimenta, R.N., Chan, L.Y., Johnson, M.S., Turnbull, L., Whitchurch, C.B., Giacomin, P.R., Moran, C.S., Golledge, J., Daly, N., Sripa, B., Mulvenna, J.P., Brindley, P.J., Loukas, A., 2015. Carcinogenic parasite secretes growth factor that accelerates wound healing and potentially promotes neoplasia. PLoS Pathog. 11, e1005209.

155. Black, W.C.,4th, Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc. Natl. Acad. Sci. U. S. A. 91, 10034-10038.

156. Hoogstraal H., Aeschilimann, A., 1982. Tick-host specificity. Mitt. Schweiz. Entomol. Ges.

157. des Vignes, F., Piesman, J., Heffernan, R., Schulze, T.L., Stafford, K.C.,3rd, Fish, D., 2001. Effect of tick removal on transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* nymphs. J. Infect. Dis. 183, 773-778.

158. Ebel, G.D., Kramer, L.D., 2004. Short report: duration of tick attachment required for transmission of powassan virus by deer ticks. Am. J. Trop. Med. Hyg. 71, 268-271.

159. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, Inuganti A, Griss J, Mayer G, Eisenacher M, Pérez E, Uszkoreit J, Pfeuffer J, Sachsenberg T, Yilmaz S, Tiwary S, Cox J, Audain E, Walzer M, Jarnuczak AF, Ternent T, Brazma A, Vizcaíno JA (2019). The PRIDE database and related tools and resources in 2019: improving support for quantification data. Nucleic Acids Res 47(D1):D442-D450.

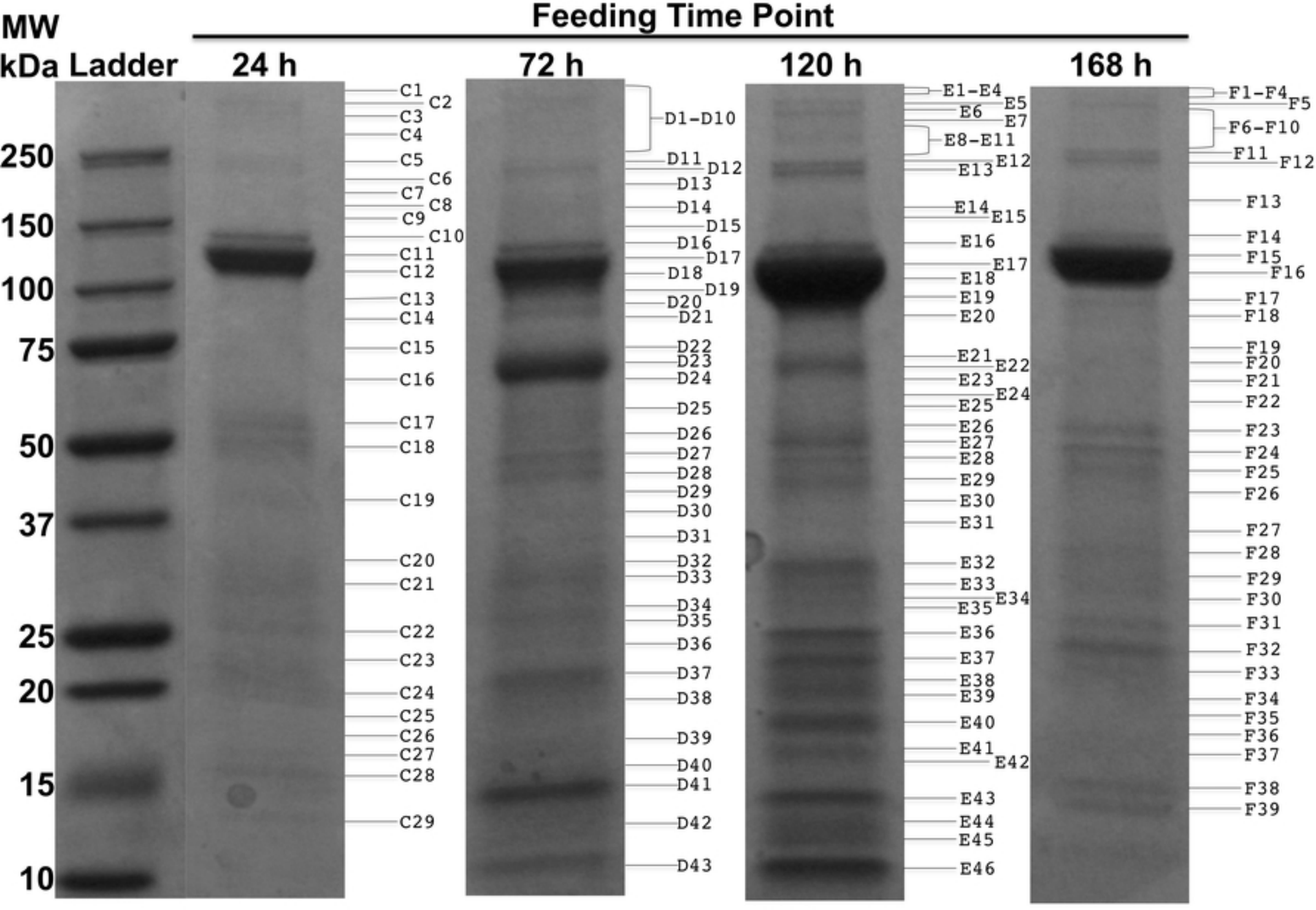
MW
kDa Ladder
Feeding Time Point
24 h
72 h
120 h
168 h
250
150
100
75
50
37
25
20
15
10
C1
C2
C3
C4
C5
C6
C7
C8
C9
C10
C11
C12
C13
C14
C15
C16
C17
C18
C19
C20
C21
C22
C23
C24
C25
C26
C27
C28
C29
D1-D10
D11
D12
D13
D14
D15
D16
D17
D18
D19
D20
D21
D22
D23
D24
D25
D26
D27
D28
D29
D30
D31
D32
D33
D34
D35
D36
D37
D38
D39
D40
D41
D42
D43
E1-E4
E5
E6
E7
E8-E11
E12
E13
E14
E15
E16
E17
E18
E19
E20
E21
E22
E23
E24
E25
E26
E27
E28
E29
E30
E31
E32
E33
E34
E35
E36
E37
E38
E39
E40
E41
E42
E43
E44
E45
E46
F1-F4
F5
F6-F10
F11
F12
F13
F14
F15
F16
F17
F18
F19
F20
F21
F22
F23
F24
F25
F26
F27
F28
F29
F30
F31
F32
F33
F34
F35
F36
F37
F38
F39

Figure 1

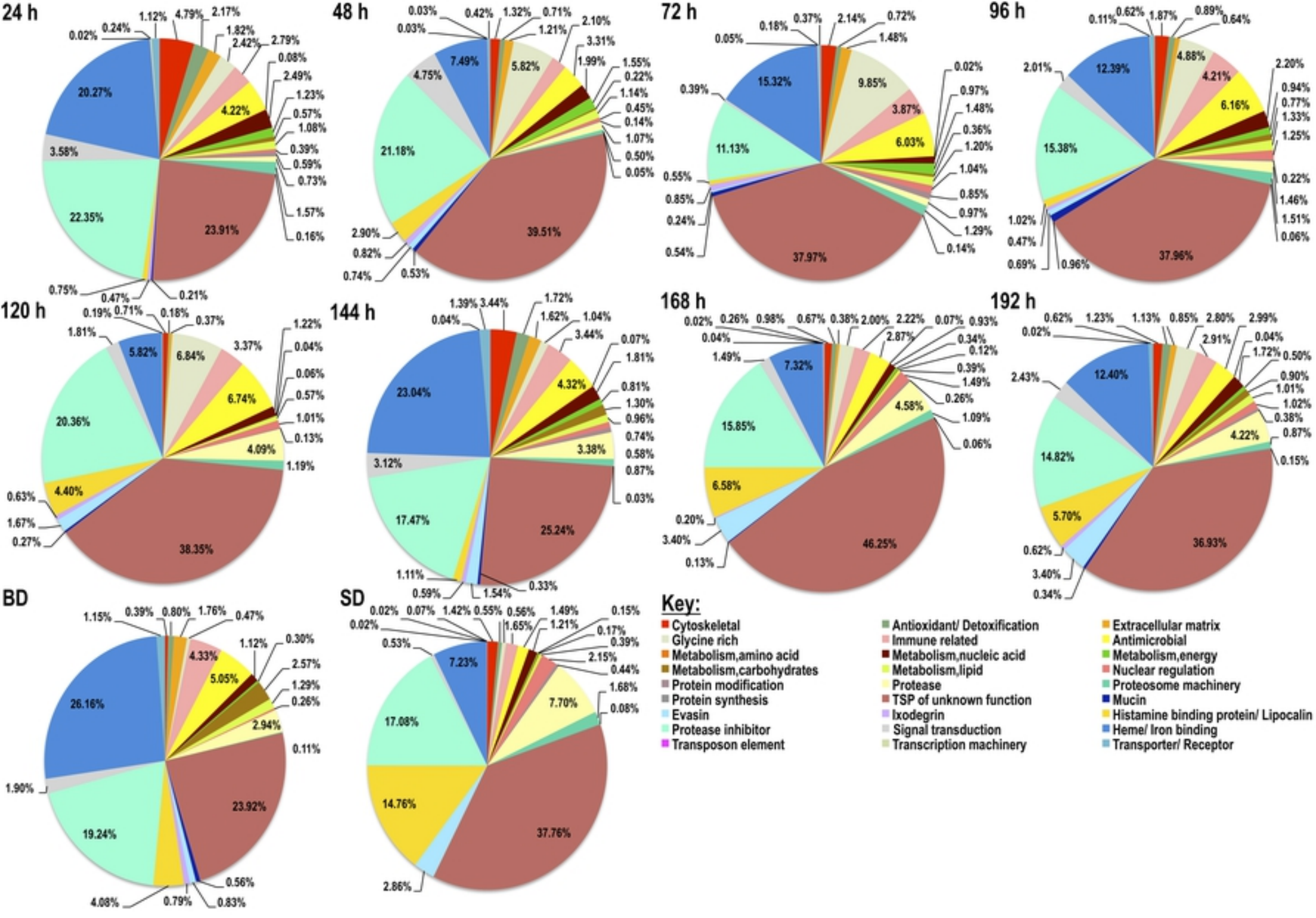
24 h
1.12%
4.79%
2.17%
0.02%
0.24%
1.82%
2.42%
2.79%
0.08%
2.49%
20.27%
1.23%
4.22%
0.57%
1.08%
0.39%
3.58%
0.59%
0.73%
1.57%
22.35%
23.91%
0.16%
0.75%
0.47%
0.21%
48 h
0.03%
0.42%
1.32%
0.71%
0.03%
1.21%
2.10%
3.31%
7.49%
5.82%
1.99%
1.55%
0.22%
4.75%
1.14%
0.45%
0.14%
21.18%
1.07%
0.50%
0.05%
2.90%
0.82%
39.51%
0.74%
0.53%
72 h
0.18%
0.37%
2.14%
0.72%
0.05%
1.48%
15.32%
9.85%
0.02%
0.39%
0.97%
3.87%
1.48%
0.36%
6.03%
11.13%
1.20%
1.04%
0.55%
0.85%
0.97%
0.85%
0.24%
1.29%
0.54%
37.97%
0.14%
96 h
0.11%
0.62%
1.87%
0.89%
0.64%
2.01%
12.39%
4.88%
2.20%
4.21%
0.94%
6.16%
0.77%
1.33%
1.25%
15.38%
0.22%
1.46%
1.02%
1.51%
0.47%
0.06%
0.69%
0.96%
37.96%
120 h
0.19%
0.71%
0.18%
0.37%
1.81%
1.22%
5.82%
6.84%
3.37%
0.04%
0.06%
6.74%
0.57%
20.36%
1.01%
0.13%
4.09%
1.19%
4.40%
0.63%
1.67%
0.27%
38.35%
144 h
1.39%
3.44%
1.72%
0.04%
1.62%
1.04%
3.44%
0.07%
23.04%
1.81%
4.32%
0.81%
1.30%
0.96%
3.38%
0.74%
0.58%
3.12%
0.87%
0.03%
17.47%
25.24%
1.11%
0.59%
1.54%
0.33%
168 h
0.02%
0.26%
0.98%
0.67%
0.38%
2.00%
2.22%
0.07%
0.93%
0.04%
2.87%
0.34%
1.49%
7.32%
0.12%
0.39%
1.49%
0.26%
4.58%
1.09%
15.85%
0.06%
6.58%
0.20%
3.40%
46.25%
0.13%
192 h
0.62%
1.23%
1.13%
0.85%
2.80%
2.99%
0.02%
2.91%
0.04%
1.72%
0.50%
12.40%
0.90%
2.43%
1.01%
1.02%
0.38%
4.22%
0.87%
14.82%
0.15%
5.70%
0.62%
36.93%
3.40%
0.34%
BD
1.15%
0.39%
0.80%
1.76%
0.47%
1.12%
0.30%
4.33%
5.05%
2.57%
1.29%
0.26%
26.16%
2.94%
0.11%
1.90%
23.92%
19.24%
0.56%
4.08%
0.79%
0.83%
SD
0.02%
0.07%
1.42%
0.55%
0.56%
1.49%
0.15%
0.02%
1.65%
1.21%
0.17%
0.53%
0.39%
7.23%
2.15%
0.44%
1.68%
7.70%
0.08%
17.08%
14.76%
37.76%
2.86%
Key:
Cytoskeletal
Glycine rich
Metabolism,amino acid
Metabolism,carbohydrates
Protein modification
Protein synthesis
Evasin
Protease inhibitor
Transposon element
Antioxidant/ Detoxification
Immune related
Metabolism,nucleic acid
Metabolism,lipid
Protease
TSP of unknown function
Ixodegrin
Signal transduction
Transcription machinery
Extracellular matrix
Antimicrobial
Metabolism,energy
Nuclear regulation
Proteosome machinery
Mucin
Histamine binding protein/ Lipocalin
Heme/ Iron binding
Transporter/ Receptor

Figure 2A

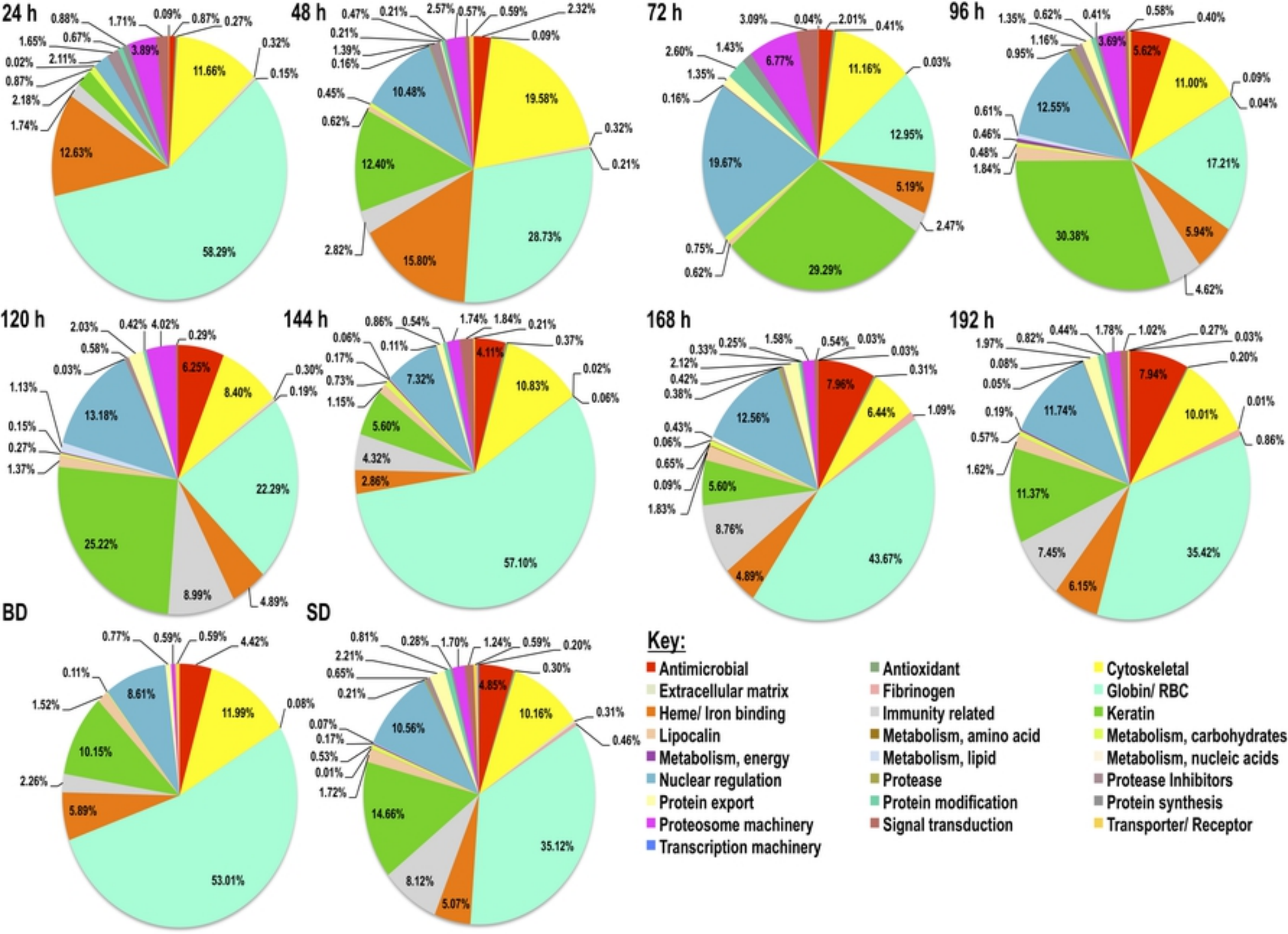
24 h
0.88%
1.71%
0.09%
0.87%
0.27%
1.65%
0.67%
3.89%
0.32%
0.02%
2.11%
11.66%
0.15%
0.87%
2.18%
1.74%
12.63%
58.29%
48 h
0.47%
0.21%
2.57%
0.57%
0.59%
2.32%
0.21%
0.09%
1.39%
0.16%
0.45%
10.48%
19.58%
0.62%
0.32%
12.40%
0.21%
2.82%
15.80%
28.73%
72 h
3.09%
0.04%
2.01%
0.41%
2.60%
1.43%
6.77%
11.16%
0.03%
1.35%
0.16%
12.95%
19.67%
5.19%
2.47%
0.75%
0.62%
29.29%
96 h
1.35%
0.62%
0.41%
0.58%
0.40%
1.16%
3.69%
5.62%
0.95%
11.00%
0.09%
0.04%
0.61%
12.55%
0.46%
17.21%
0.48%
1.84%
30.38%
5.94%
4.62%
120 h
2.03%
0.42%
4.02%
0.29%
0.58%
6.25%
0.03%
8.40%
0.30%
0.19%
1.13%
13.18%
0.15%
0.27%
22.29%
1.37%
25.22%
8.99%
4.89%
144 h
0.86%
0.54%
1.74%
1.84%
0.21%
0.06%
0.11%
4.11%
0.37%
0.17%
7.32%
10.83%
0.73%
0.02%
1.15%
0.06%
5.60%
4.32%
2.86%
57.10%
168 h
0.33%
0.25%
1.58%
0.54%
0.03%
0.03%
2.12%
7.96%
0.31%
0.42%
0.38%
6.44%
12.56%
1.09%
0.43%
0.06%
0.65%
0.09%
5.60%
1.83%
8.76%
4.89%
43.67%
192 h
1.97%
0.82%
0.44%
1.78%
1.02%
0.27%
0.03%
0.08%
7.94%
0.20%
0.05%
0.19%
11.74%
10.01%
0.01%
0.57%
0.86%
1.62%
11.37%
7.45%
6.15%
35.42%
BD
0.77%
0.59%
0.59%
4.42%
0.11%
8.61%
1.52%
11.99%
0.08%
10.15%
2.26%
5.89%
53.01%
SD
0.81%
0.28%
1.70%
1.24%
0.59%
0.20%
2.21%
0.30%
0.65%
4.85%
0.21%
10.16%
0.31%
10.56%
0.46%
0.07%
0.17%
0.53%
0.01%
1.72%
14.66%
35.12%
8.12%
5.07%
Key:
Antimicrobial
Extracellular matrix
Heme/ Iron binding
Lipocalin
Metabolism, energy
Nuclear regulation
Protein export
Proteosome machinery
Transcription machinery
Antioxidant
Fibrinogen
Immunity related
Metabolism, amino acid
Metabolism, lipid
Protease
Protein modification
Signal transduction
Cytoskeletal
Globin/ RBC
Keratin
Metabolism, carbohydrates
Metabolism, nucleic acids
Protease Inhibitors
Protein synthesis
Transporter/ Receptor

Figure 2B

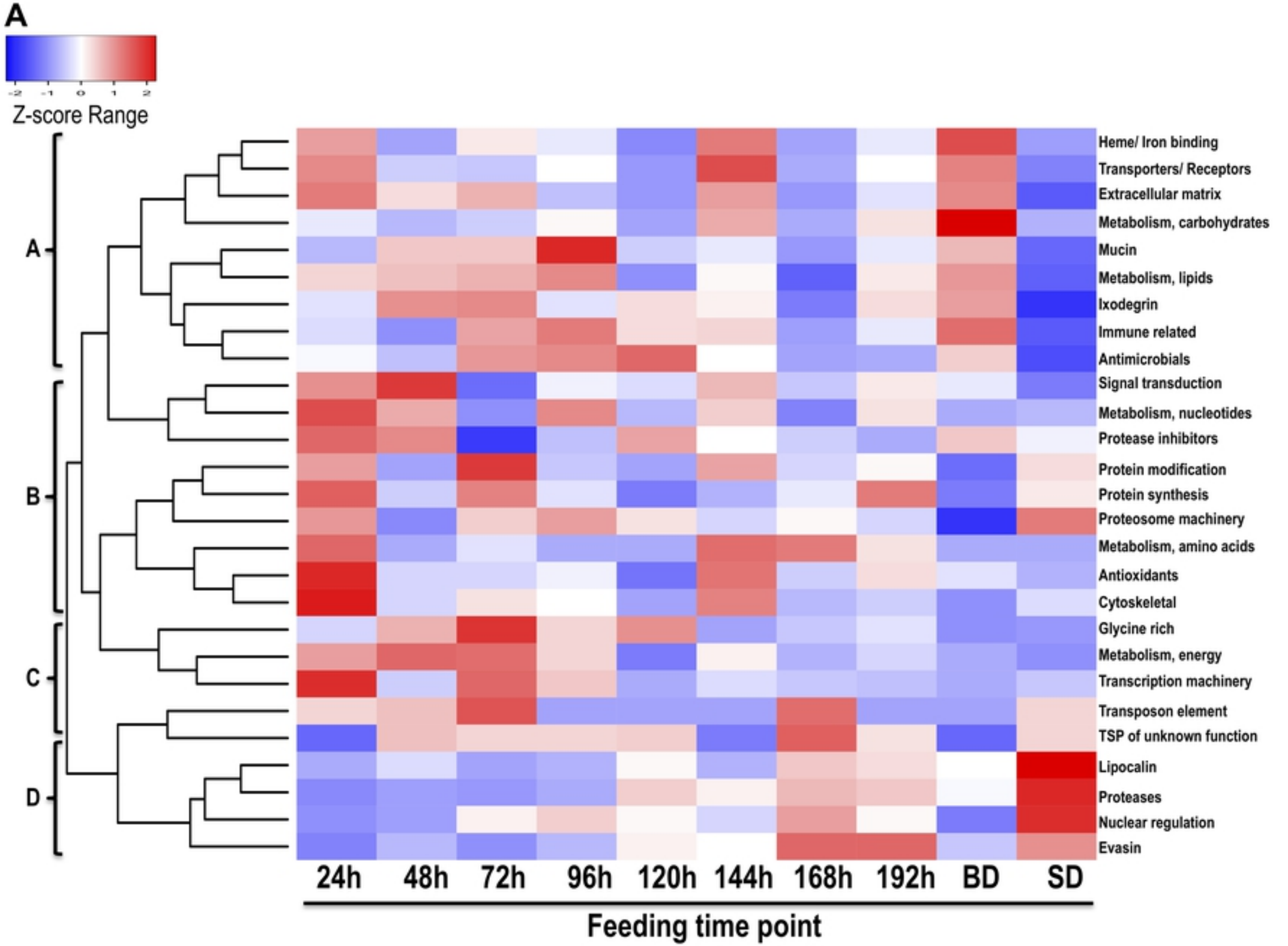
A
-2
-1
0
1
2
Z-score Range
A
B
C
D
Heme/ Iron binding
Transporters/ Receptors
Extracellular matrix
Metabolism, carbohydrates
Mucin
Metabolism, lipids
Ixodegrin
Immune related
Antimicrobials
Signal transduction
Metabolism, nucleotides
Protease inhibitors
Protein modification
Protein synthesis
Proteosome machinery
Metabolism, amino acids
Antioxidants
Cytoskeletal
Glycine rich
Metabolism, energy
Transcription machinery
Transposon element
TSP of unknown function
Lipocalin
Proteases
Nuclear regulation
Evasin
24h
48h
72h
96h
120h
144h
168h
192h
BD
SD
Feeding time point

Figure 3A

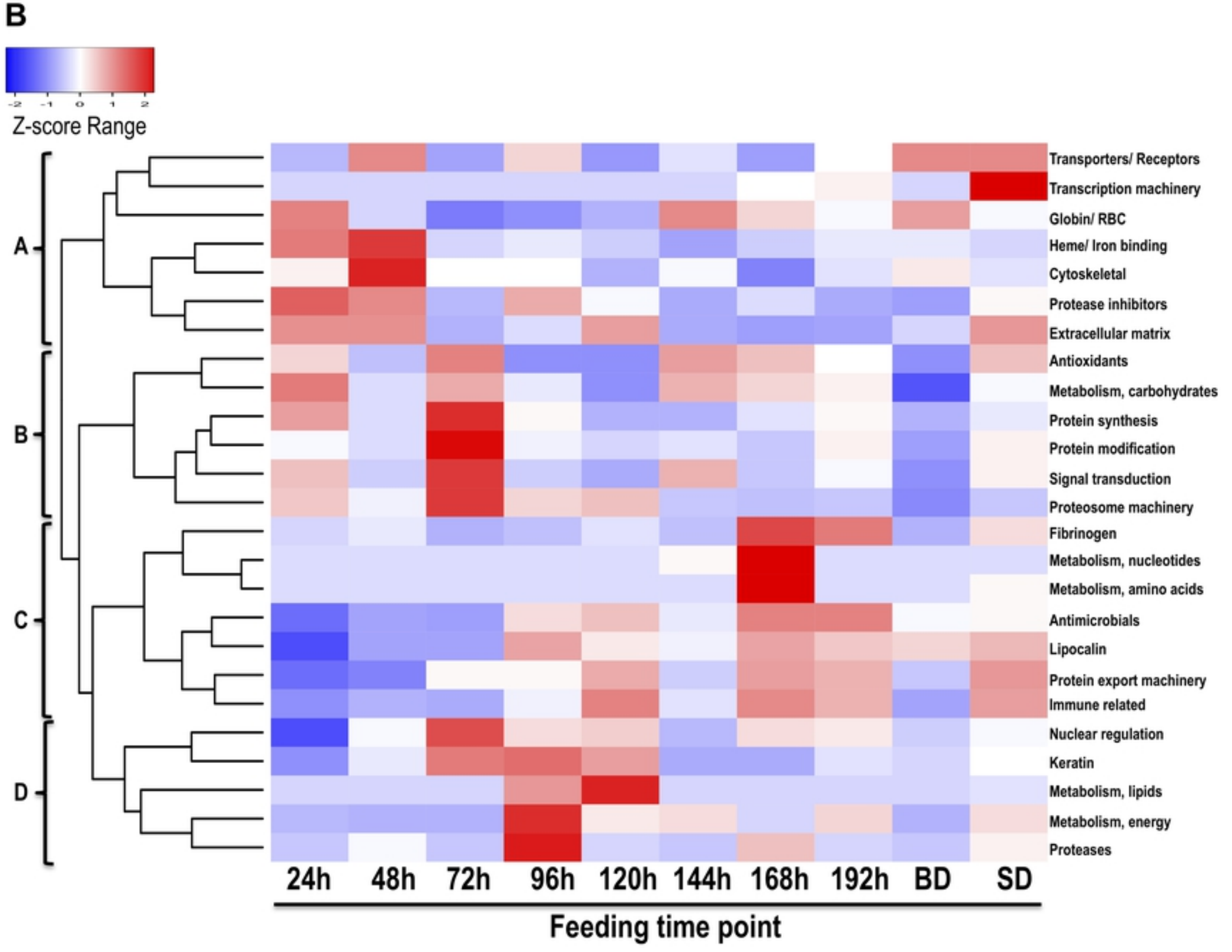
B
-2
-1
0
1
2
Z-score Range
A
B
C
D
Transporters/ Receptors
Transcription machinery
Globin/ RBC
Heme/ Iron binding
Cytoskeletal
Protease inhibitors
Extracellular matrix
Antioxidants
Metabolism, carbohydrates
Protein synthesis
Protein modification
Signal transduction
Proteosome machinery
Fibrinogen
Metabolism, nucleotides
Metabolism, amino acids
Antimicrobials
Lipocalin
Protein export machinery
Immune related
Nuclear regulation
Keratin
Metabolism, lipids
Metabolism, energy
Proteases
24h
48h
72h
96h
120h
144h
168h
192h
BD
SD
Feeding time point

Figure 3B

Kim et al., Figure 4

Figure 4