JOURNAL OF PROTEOMICS 105 (2014) 204–216

Available online at www.sciencedirect.com

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Comparative proteomics for the characterization of the most relevant *Amblyomma* tick species as vectors of zoonotic pathogens worldwide☆

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ARTICLE INFO

Available online 29 December 2014

Keywords:
Tick
Proteomics
Vector–host interactions
Amblyomma
Phyloproteomics

ABSTRACT

Ticks transmit zoonotic pathogens worldwide. Nevertheless, very little information is available on their genome, transcriptome and proteome. Herein, we characterized the proteome of *Amblyomma americanum* adults and nymphs because of their role in pathogen transmission and compared the proteome of *A. americanum*, *A. cajennense* and *A. variegatum* adult ticks. We also used de novo sequencing proteomics data for the analysis of the phylogenetic relationships between the three *Amblyomma* spp. in a proof of concept for phyloproteomics. The results showed that host and tick proteins involved in blood digestion, heme detoxification, development and innate immunity were differentially represented between adults and nymphs. Although these ticks were unfed, over-represented host proteins may supply nutrients during off-host periods. Tick proteins involved in tick attachment, feeding, heat shock response, protease inhibition and heme detoxification were differentially represented between *Amblyomma* spp., suggesting adaptation processes to biotic and abiotic factors. These results suggested that phyloproteomics might be a useful tool for the phylogenetic analysis of tick species in which sequence data is a limiting factor and demonstrate the possibilities of proteomics studies for the characterization of relevant tick vector species and provide new relevant information to understand the physiology, development and evolution of these tick species.

Biological significance
This is the first report on the proteome of the most important *Amblyomma* tick species for their relevance as vectors of zoonotic pathogens worldwide. Nevertheless, very little information is available on the genome, transcriptome and proteome of these vector ectoparasites. The results reported herein provide new relevant information to understand the physiology, development and evolution of these tick species. Phyloproteomics using de novo protein sequencing was assayed as a new approach for the phylogenetic analysis of tick species in which sequence data is a limiting factor.
This article is part of a Special Issue entitled: Proteomics of non-model organisms.

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http://dx.doi.org/10.1016/j.jprot.2013.12.016

1. Introduction

The arthropod subphylum Chelicerata includes Acari that contains obligate blood-feeding ectoparasites such as *Amblyomma* spp. ticks that vector emerging or re-emerging zoonotic pathogens including those causing typhus, Human and canine ehrlichiosis, Lyme disease, Rocky Mountain Spotted Fever, African Tick-Bite Fever, Flinders Island Spotter Fever, Heartwater, theileriosis, babesiosis, Crimean-Congo Hemorrhagic Fever [1]. According to the latest revision of the tick classification and nomenclature [2], the genus *Amblyomma* contains 130 species with a worldwide distribution, but three of them are the most widely distributed *Amblyomma* spp. vectors of zoonotic pathogens. *Amblyomma americanum* (lone star tick) is the most abundant tick specie in the southeastern regions of North America and the main vector of agents causing of ehrlichiosis, Southern Tick-Associated Rash Illness and tularemia [3]. *Amblyomma cajennense*, found throughout the Americas from the southern USA to northern Argentina [4], is considered to be a tick of great public health importance in these regions because of its major role in transmitting *Rickettsia rickettsii*, the causal agent of Rocky Mountain spotted fever [5], high infestation rates in cattle farms and resistance to several acaricides [6]. And finally, *Amblyomma variegatum*, found in the tropics and subtropics, is endemic in savannas in many countries in sub-Saharan Africa as well as in southern Arabia, the Caribbean, and some islands in the Atlantic and Indian Oceans [7]. The long mouthparts of *A. variegatum* cause severe and painful bites, leaving large wounds on the skin, thereby producing substantial economic losses in domestic ruminants by exsanguination or by physical injury. In addition, *A. variegatum* is host of a number of microbial pathogens including *Ehrlichia ruminantium*, the agent of heartwater, and *Rickettsia africae*, the agent of African tick-bite fever, which is an emerging zoonosis in rural sub-Saharan Africa and the Caribbean [8].

However, these tick species are largely understudied. The only tick genome currently close to completion is that of the black-legged deer tick, *Ixodes scapularis* [9,10] and there are only a small number of proteomics studies performed on these ticks, mainly focused on specific tissues like salivary glands and midgut proteins that are determined to contain proteins holding a key role in tick physiology and pathogen transmission [11,12].

More comprehensive genomics, transcriptomics and proteomics analyses of the Acari and particularly for tick species that are vector of human and animal pathogens are urgently needed for evolutionary studies and the identification of pathways involved in host–vector–pathogen interactions with implications for disease control.

In order to expand our knowledge of the *Amblyomma* spp., in this work we characterized the proteomes of *A. americanum* adults and nymphs because of their role in the transmission of an increased number of disease causing pathogens. Moreover, we carried out a proteomics study of *A. americanum*, *A. cajennense* and *A. variegatum* adult ticks to increase the information available for the characterization of these important *Amblyomma* spp. We applied a novel method by using *de novo* sequencing proteomics data for the analysis of the phylogenetic relationships between the three *Amblyomma* spp. in a proof of concept for phyloproteomics.

2. Materials and methods

2.1. Tick collection

A. americanum unfed ticks were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Larvae and nymphs were fed on rabbits and adult ticks were fed on sheep. Off-host ticks were maintained in a 12 h light:12 h dark photoperiod at 22–25 °C and 95% relative humidity. Animals were housed at the Tick Rearing Laboratory with the approval and supervision of the OSU Institutional Animal Care and Use Committee.

A. cajennense unfed ticks were obtained from a laboratory colony maintained at the University of Tamaulipas, Mexico. Originally, this tick strain was established at CENAPA, Cuernavaca, Mexico. Ticks were fed on cattle and collected after repletion to allow for oviposition and hatching in humidity chambers at 12 h light:12 h dark photoperiod, 22–25 °C and 95% relative humidity. Ticks were maintained in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (http://www.nal.usda.gov/awic/pubs/noawicpubs/careuse.htm). The protocol was approved by the Committee on the Ethics of Animal Experiments of FOMIX (TAMPS-2007-C13-73622) and the University of Tamaulipas (0073622).

A. variegatum unfed ticks were collected from cattle in three areas of Plateau State, Nigeria [13]. These ticks were brought to the Parasitology Division, National Veterinary Research Institute, Vom Plateau State, Nigeria, where they were morphologically identified using the guidelines for ticks identification [14] and independently corroborated by A. Estrada-Peña (University of Zaragoza, Spain).

All ticks were stored in 70% ethanol at −20 °C until used for protein extraction.

2.2. Protein extraction

A total of sixty ticks from each group (*A. americanum*, *A. cajennese*, and *A. variegatum* adult males and females) and one hundred and fifty *A. americanum* nymphs were equally divided into four different biological samples for each group which were pulverized in liquid nitrogen and homogenized with a glass homogenizer (10 strokes) in 1 ml lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM Tris–HCl, pH 8.0). Samples were sonicated for 1 min in an ultrasonic cooled bath followed by 10 s of vortex. After 3 cycles of sonication-vortex, the homogenates were centrifuged at 200 ×*g* for 5 min at 4 °C to remove cellular debris. The supernatants were collected and protein concentration was determined using the BCA Protein Assay (Thermo Scientific, San Jose, CA, USA) using BSA as standard.

2.3. 2D-DIGE of A. americanum developmental stages

Two hundred micrograms of protein from each *A. americanum* females, males and nymphs groups were purified with a 2-D Clean-Up Kit (GE Healthcare, Madrid, Spain) according to the

manufacturer's instructions and protein concentration was determined using the 2D-Quant Kit (GE Healthcare). CyDye minimal labeling was performed following the manufacturer's protocols (GE Healthcare). Briefly, 50 μg of protein of each sample was labeled with 400 pmol of Cy3 or Cy5 fluorochromes dissolved in anhydrous DMF (Sigma, St. Louis, MO, USA) for 30 min on ice, in the dark and following the random labeling scheme shown in Table 1. Reactions were quenched by adding 1 μL of 10 mM lysine followed by incubation for 10 min on ice, in the dark. For internal standardization, a pool of equal amounts of all samples (25 μg per sample) was created and labeled with Cy2 dye with the same procedure but scaling adjusting the quantities of reagents according to the amount of protein (300 μg). The twelve individual labeled samples corresponding to four biological replicates from males, females and nymphs ticks were distributed randomly across six DIGE gels with the internal standard pooled sample also present in each separation (Table 1). After sample combination, an equal volume of 2× sample buffer was added (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% w/v DTT and 2% v/v IPG buffer, pH 3–11). The two-dimensional electrophoresis was carried out using reagents and equipment from GE Healthcare. For the first dimension, 24-cm 3–11 NL pH range IPG strips were rehydrated overnight in 450 μL of DeStreak Rehydration Solution supplemented with 0.5% IPG buffer pH 3–11 using a reswelling tray. IEF was performed at 20 °C using an Ettan IPGphor 3. Samples were applied using anodic cup loading and the isoeletrofocusing was carried out using the following conditions: 300 V for 3 h, 300–1000 V for 6 h, 1000–10,000 V for 3 h, 10,000 V for 3 h and 500 V for 3 h. Prior second dimension, proteins present in focused IPG strips were reduced and alkylated by successive incubations in two different equilibration buffer solutions (50 mM Tris–HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.2% bromophenol blue, supplemented with either 0.5% w/v DTT for the first incubation or 4.5% w/v iodoacetamide for the second incubation) for 15 min each with gentle rocking. Equilibrated IPG strips were placed onto 12% homogeneous SDS-polyacrylamide gels casted in low fluorescence glass plates using an Ettan-DALT Six System. Electrophoresis was carried out at 20 °C and 0.5 W/gel for 30 min followed by a second step at 15 W/gel for 4 h.

Table 1 – Experimental design for DIGE analysis of *Amblyomma americanum* development stages (indicating CyDye labeling, the four biological replicates per each sample and the combination of samples for each gel).

Gel number	Cy3 labeling	Cy5 labeling	Cy2 labeling
Gel 1	M 2	F 1	Internal standard
Gel 2	NY 1	M 3	Internal standard
Gel 3	F 3	NY 4	Internal standard
Gel 4	NY 3	F 2	Internal standard
Gel 5	M 4	NY 2	Internal standard
Gel 6	F 4	MA1	Internal standard

Abbreviations: M, Males; F, Females; NY, Nymphs.

2.3.1. Image acquisition and data analysis

Proteins were visualized using an Ettan DIGE Imager (GE Healthcare) following the manufacturer's instructions. Image analysis was performed with DeCyder 2D Software, version 7.0 (GE Healthcare). Eighteen images were considered for the analysis, 12 corresponded to the different samples labeled with Cy3 and Cy5 and 6 corresponded to sample pool labeled with Cy2 and acquired individually with each gel. Spot co-detection, normalization of each spot against the corresponding value of the internal pool and volume ratios calculation were carried out using Differential In-Gel Analysis (DIA) module. In the Biological Variation Analysis (BVA) module, the 18 spot maps were distributed in 4 groups, that is, standards (6 images), and the 3 different groups of samples (4 males, 4 females and 4 nymphs). The most representative standard image with average quality was assigned as master. Paired comparisons were carried out between adult females and males and nymphs and the proteins with differences in their abundance patterns were compared by Student's *t*-test ($p = 0.05$) and only significant average ratios of ±2.5-fold difference were considered for mass spectrometry analysis. All proteins of interest were manually checked to avoid false positives.

For preparative gel, 2-D electrophoresis of 250 μg unlabeled pool proteins was carried out in the same conditions as described above for CyDye labeled samples, but in this case, after second dimension the gel was stained with Sypro Ruby (Molecular Probes, Invitrogen, Eugene, OR, USA) following the protocol recommended by the manufacturer. Gel image was matched automatically in the BVA module of DeCyder software with the DIGE images and the protein spots of interest were manually excised from the gels, dehydrated with acetonitrile and vacuum-dried in a Speed Vac.

2.3.2. Trypsin digestion and MS analysis

After drying, spots were re-hydrated and digested overnight at 37 °C with 12.5 ng/μl trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate, pH 8.8 [15]. Trifluoroacetic acid was added to a final concentration of 1% and the peptides were finally desalted onto OMIX Pipette tips C_{18} (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at 20 °C until mass spectrometry analysis.

The desalted protein digests were resuspended in 0.1% formic acid and analyzed by RP-LC-MS/MS using an Easy-nLC II system coupled to an ion trap LCQ Fleet mass spectrometer (Thermo Scientific, San Jose, CA, USA). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Thermo Scientific), and then separated using a 0.075 mm × 100 mm C18 RP column (Thermo Scientific) operating at 0.3 μl/min. Peptides were eluted using a 40-min gradient from 5 to 35% solvent B (Solvent A: 0.1% formic acid in water, solvent B: 0,1% formic acid in acetonitrile). ESI ionization was done using a Fused-silica PicoTip Emitter ID 10 μm (New Objective, Woburn, MA, USA) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by three data dependent MS/MS scans (Top 3), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35%, and dynamic exclusion applied during 30 s periods.

Protein identification was carried out using the SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Scientific). The MS/MS raw files were searched against the Ixodida (40,849 entries in June 2013) and Ruminantia (66,519 entries in June 2013) Uniprot databases with the following constraints: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. A false discovery rate (FDR) < 0.01 was considered as condition for successful peptide assignments subsequent protein identification.

2.4. Total proteome analysis of Amblyomma spp. by protein one-step in gel digestion, LC-MS/MS and peptide identification

Two hundred micrograms of protein from *A. americanum*, *A. cajennense* and *A. variegatum* ticks obtained following the protocol described in Section 2.2 were precipitated following the methanol/chloroform procedure [16], resuspended in 100 μl Laemmli sample buffer and applied onto 1.2-cm wide wells on a 12% SDS-PAGE gel. The electrophoretic run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by staining with GelCode Blue Stain Reagent (Thermo Scientific), excised, cut into 2 × 2 mm cubes and digested overnight at 37 °C with 60 ng/μl sequencing grade trypsin (Promega, Madison, WI, USA) at 5:1 protein: trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8 containing 10% (v/v) acetonitrile (Shevchenko et al. [15]). The resulting tryptic peptides from each band were extracted by 30 min-incubation in 12 mM ammonium bicarbonate, pH 8.8. Trifluoroacetic acid was added to a final concentration of 1% and the peptides were finally desalted onto OMIX Pipette tips C_{18} (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at −20 °C until mass spectrometry analysis in the CBMSO Proteomics Facility (Madrid, Spain). The experiment was performed by duplicate with similar results.

Briefly, the desalted protein digest was resuspended in 0.1% formic acid and analyzed by RP-LC-MS/MS using an Agilent 1100 LC system (Agilent Technologies) coupled to a linear ion trap LTQ-Velos mass spectrometer (Thermo Scientific). The peptides were separated by reverse phase chromatography using a 0.18 mm × 150 mm Bio-Basic C18 RP column (Thermo Scientific) at 1.8 μl/min. Peptides were eluted using a 120-min gradient from 5 to 40% solvent B in solvent A (Solvent A: 0.1% formic acid in water; solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI ionization was done using a microspray metal needle kit (Thermo Scientific) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35%, and dynamic exclusion applied during 30 s periods. Database searching of MS/MS raw files was performed similar as described in Section 2.3.2, against the Ixodida-Uniprot database with the SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Scientific). A false discovery rate (FDR) < 0.01 was considered as cut-off for positive peptides identification. Differential protein representation for individual proteins between different samples was determined using χ^2 test statistics with Bonferroni correction in the IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/) ($p = 0.05$).

2.5. Phylogenetic analysis

In order to generate *de novo* sequences for the phylogenetic analysis, the raw MS/MS files were further processed using PEAKS Studio v 6.0 software (Bioinformatics Solutions Inc.) with the same general parameters as for the routinely applied software Proteome Discoverer 1.3, (Thermo Scientific) as previously described. Additionally, in PEAKS a special algorithm is used to generate *de novo* sequences of the input spectrum and the SPIDER module is used to identify variations from sequences using a homology match query. The filtering of the scores for all identified peptides is done by assigning a −10lg P value of 30 that was established after manual analyses of the obtained peptide matches to assure quality of the identifications.

Sequences of 30 different peptides belonging to 25 proteins identified in all three *Amblyomma* spp. were generated *de novo* from the *A. americanum*, *A. cajennense* and *A. variegatum* MS/MS raw data and used for multi locus analysis (MLA). An algorithm was developed to check peptide sequences against Arthropoda-Uniprot database to select those with low probability of representing paralogous and not orthologous sequences. The algorithm included (a) Blastp search using the *I. scapularis* reference protein sequence and (b) Blastx search using the *I. scapularis* reference peptide sequence. Sequences that did not produce a positive hit were considered as unlikely to have very closely related paralogs and those included in the analysis. The *I. scapularis* reference sequences were used because this is the only tick genome available in databases and the one used as outgroup in our analysis. Protein sequence alignment was done using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and manually corrected to respect the boundaries of the tryptic peptides. The evolutionary history was inferred using 16S rDNA and protein sequences by the Maximum Likelihood (ML) method based on the General Time Reversible and JTT matrix-based models, respectively [17,18]. Reference *I. scapularis* sequences were used as outgroup. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) and JTT approach, respectively and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated. Stability or accuracy of inferred topologies was assessed via bootstrap analysis of 1000 replications. Evolutionary analyses were conducted in MEGA5 [19].

3. Results and discussion

3.1. 2-D DIGE analysis of A. americanum development stages protein profiles

Two-dimensional DIGE minimal labeling approach in combination with MS was used to characterize differential protein expression between adult and nymphal stages of *A. americanum*

ticks. Protein abundance was compared between males and females adults and nymphs. In order to generate unbiased results, a randomization of samples for labeling before running 2-D gels was performed using four replicates of each biological sample (Table 1). The internal standard, made by pooling equal amounts of all samples of the experiment, was run on every 2-D DIGE gel (Table 1). This means that we had a standard for every spot on the gel, and that all gels within the same experiment were quantitatively linked when gel images were processed with the DeCyder software. In a conventional 2-D electrophoresis a large number of replicates is needed, but in DIGE, because the internal standard virtually eliminates gel-to-gel variation, technical replicates are not necessary performing 2-D DIGE.

Evaluation of protein profiles of 18 spot maps obtained were performed with the DeCyder 7.0 software. An average of 630 spots (S.D. = 45) were automatically detected, of which 457 spots matched with the master gel. The group to group comparisons between males, females and nymphs spot maps were done and the differences obtained were considered significant when the calculated average ratio showed a value lower than −2.5 or higher than +2.5, with Student *t*-test ($p < 0.05$) because our experience in proteomic studies has indicated that 2.5 fold of difference is enough to find relevant changes in proteins. Lower values may be poorly reproducible and a higher cut off would eliminate from the analysis many relevant proteins. With these criteria, no significant differences were found between males and females and 24 spots differed in abundance between adults and nymphs, with 9 spots (37%) more abundant in nymphs (Fig. 1A and B). Due to similar spot patterns shown by males and females, the results and discussion were referred to adults instead of males and females separately.

The differentially expressed spots were excised from a preparative gel, trypsin-digested and analyzed by RP-LC-MS/MS as described in the Materials and methods section. From 24 spots, 22 proteins were identified (Table 2). Many of the identified spots corresponded to different isoforms to the same protein, which resulted in the identification of 17 unique proteins. Of these 17 proteins, 3 corresponded to host proteins (serum albumin and alpha and beta hemoglobins) and the rest were assigned to tick proteins (Table 2).

The most abundant proteins over-represented in *A. americanum* nymphs or adults corresponded to vertebrate host proteins (serum albumin and hemoglobin; 65 peptides), tick structural proteins (paramyosin and actin-binding; 21 peptides) and tick hemoglobin processing proteins (heme lipoprotein, P450, cytochrome b and leucine aminopeptidase; 20 peptides) (Table 2). Although these ticks were unfed, over-represented host proteins have been shown to be present in ticks after molting and may act as protein reserve to supply nutrients during off-host periods [11,20,21]. Interestingly, some of the proteins over-represented in both nymphs and adult ticks are involved in hemoglobin processing and heme binding and transport. Tick carrier proteins that are able to bind, transport, and store host-blood heme are involved in blood digestion, heme detoxification and other functions such as innate immunity [22–24], and represent a unique evolutionary strategy of the Chelicerata to both mitigate heme toxicity and utilize the molecule as a prosthetic group [25]. These proteins may be also involved in metabolizing stored host proteins such as hemoglobin.

Additionally, a G-protein-linked acetylcholine receptor gar-2A (spot 20) and a Wnt protein (spot 23) were over-represented in tick nymphs. These proteins are expressed throughout development, from embryonic to adult stage, and the increases found probably reflected the differences in the developmental process between adults and nymphs [26,27].

3.2. Proteome profiling of *A. americanum* development stages by LC-MS/MS

To complement the 2D-DIGE analysis, the total proteome of each of the four pools prepared from *A. americanum* females, males and nymphs were in-gel concentrated, digested with trypsin and the resulting peptides were separated by RP-LC-MS/MS. After database search against the Ixodida and Ruminantia Uniprot databases as described in Materials and

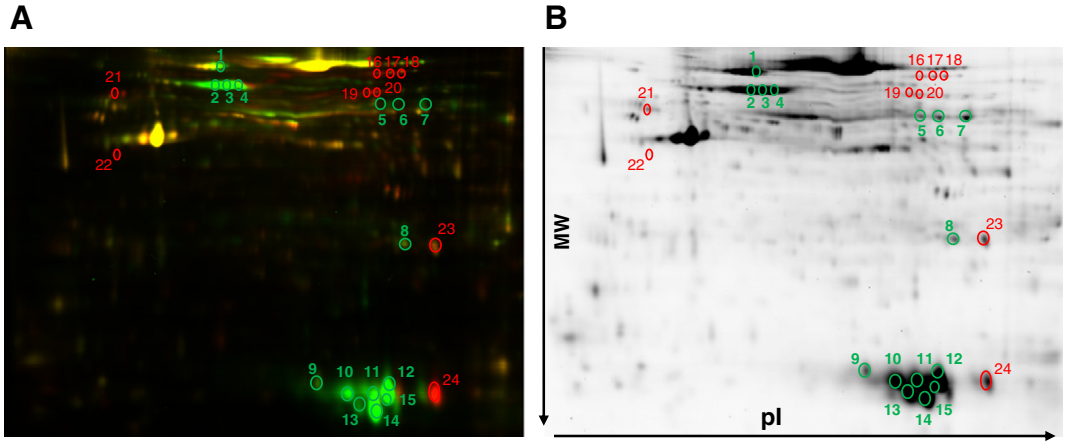

Fig. 1 – 2-D DIGE analysis of *A. americanum* development stages. (A) Representative images of DIGE gels. Overlay image of proteins of Cy3-labeled adults (green) and Cy5-labeled nymphs (red). (B) Representative images of DIGE gels. Pooled internal standard proteome. Proteins that were differentially represented with an average ratio of ± 2.5-fold are circled. Green and red circles indicate proteins that were over-represented in adults or nymphs, respectively.

Table 2 – Differentially represented proteins in *Amblyomma americanum* adults and nymphs (identified by LC-MS/MS after DIGE analysis).

Spot number[a)]	Accession number[b)]	Protein ID[b)]	Mw/pI[c)]	Number of matched peptides	Sequence coverage (%)	Average -fold change	T-Test	Over-represented in:
2	P02769	Serum albumin (*Bos taurus*)	69.2/6.2	8	13.84	7.09	1.20E−07	Adults
3	P02769	Serum albumin (*Bos taurus*)	69.2/6.2	10	16.97	10.56	9.10E−07	Adults
4	P02769	Serum albumin (*Bos taurus*)	69.2/6.2	7	12.35	8.23	1.30E−06	Adults
9	P04346	Hemoglobin subunit beta-A (*Bos javanicus*)	16.0/6.9	10	44.14	17.96	0.00016	Adults
13	P02070	Hemoglobin subunit beta (*Bos taurus*)	15.9/7.6	5	41.38	12.58	2.90E−06	Adults
14	P01967	Hemoglobin subunit alpha-2 (*Bos mutus grunniens*)	15.0/8.4	4	24.82	12.09	4.00E−06	Adults
15	P01968	Hemoglobin subunit alpha-2 (*Bos mutus grunniens*)	15.0/8.9	5	36.17	11.71	0.00079	Adults
1	G3MRF0	Putative uncharacterized protein (*Amblyomma maculatum*)	67.4/7.4	2	5.59	3.64	2.20E−05	Adults
5	Not identified					2.61	0.00067	Adults
6	L7MIU4	Putative actin-binding cytoskeleton protein filamin (*Rhipicephalus pulchellus*)	78.6/8.3	4	6.76	3.19	0.00014	Adults
7	Q9NB96	P450 CYP319A1 (*Boophilus microplus*)	60.9/8.7	6	16.57	3.46	1.40E−05	Adults
8	O99828	Ubiquinol-cytochrome-c reductase complex cytochrome b subunit (*Ripicephalus sanguineus*)	41.1/8.8	2	6.42	5.48	0.00031	Adults
10	G3MPV4	Nucleoside diphosphate kinase (*Amblyomma maculatum*)	17.1/7.4	5	38.82	6.35	7.60E−05	Adults
11	G3MIJ7	Putative uncharacterized protein (*Amblyomma maculatum*)	18.6/7.4	2	16.47	5.74	0.00083	Adults
12	F0J8L0	Leucine aminopeptidase (*Amblyomma variegatum*)	25.6/8.1	4	21.43	2.74	0.00065	Adults
16	A0MVX0	Heme lipoprotein (*Amblyomma americanum*)	176.9/6.8	2	1.39	2.58	1.70E−05	Nymphs
17	A0MVX0	Heme lipoprotein (*Amblyomma americanum*)	176.9/6.8	3	2.31	2.76	5.70E−06	Nymphs
18	B7QH37	Activating signal cointegrator 1 complex subunit 3, helc1, putative (*Ixodes scapularis*)	173.6/8.3	3	4.30	2.80	5.90E−06	Nymphs
19	Not identified					2.55	0.00024	Nymphs
20	B7QLV5	G-protein-linked acetylcholine receptor gar-2A, putative (*Ixodes scapularis*)	90.0/8.7	2	4.12	2.67	0.00036	Nymphs
21	Q86RN8	Paramyosin (*Boophilus microplus*)	101.9/5.7	4	5.04	2.72	0.00013	Nymphs
22	G3MHD3	ATP synthase subunit beta (*Amblyomma maculatum*)	59.1/5.7	8	19.17	2.91	0.00016	Nymphs
23	B7QGH6	Protein Wnt (*Ixodes scapularis*)	35.1/10.0	2	9.43	2.77	1.20E−05	Nymphs
24	Q4PN05	Putative salivary protein (*Ixodes scapularis*)	25.0/8.0	2	12.66	6.63	7.20E−06	Nymphs

a) Spot numbers refer to the 2D gel proteins of interest that were analyzed by MS (Fig. 1).
b) Accession number and protein identity are listed according to the UniProt database for the best match.
c) Abbreviations: Mw, molecular weight (kDa); pI, isoelectrical point.

Methods, a total of 350 unique tick proteins were identified with an FDR < 0.01, of which 218, 222 and 185 were present in *A. americanum* females, males and nymphs, respectively (see Supplementary Information, Table S1A). A total of 93 tick proteins were identified in all samples, but some proteins were identified in one sample only (Fig. 2A).

The most host abundant proteins identified were albumins and hemoglobins, similar to DIGE results (see Supplementary Information, Table S1B). Albumin peptides represented the 26% and 28% of the total host peptides identified in females and males, respectively, whereas peptides from hemoglobins were the 18%, 21% and 17% in females, males and nymphs, respectively, corroborating the storage and transmission of these proteins intrastadially [11,20,21].

Although in all analyzed samples host proteins were detected, the host to tick protein ratio between *A. americanum* stages increased with aging (Fig. 2B). The number of peptides per protein used to identify each protein was higher for host than for tick proteins, reflecting differences between protein databases (Fig. 2C) and also increased in host proteins with the development, probably reflecting the effect of previous feeding of ticks. Additionally, due to the detection of a large number of host proteins, this approach could also be applied not only for the systematic analysis of developmental stages and species relationships, but also for the identification of the host species on which the tick fed [21,41].

The statistical analysis did not show significant differences between males and females, in accordance with DIGE results, and only three proteins, Glutathione S-Transferase and two uncharacterized proteins (Uniprot accession numbers: G3MNX9, E5L874, G3MM43) were over-represented in nymphs with respect to adults. These results showed that DIGE is a powerful technique for the detection of subtle differences between similar samples that may be masked when the analysis is performed at the level of peptides. However, the LC-MS/MS approach allowed the identification of a greater number of differentially represented proteins. Although most of the proteins found differentially represented by DIGE were detected by LC-MS/MS, the application of a labeling quantitative approach before LC-MS/MS will probably detect a higher number of significant differences between samples.

3.3. Proteome analysis of three Amblyomma spp. by LC-MS/MS

Because no differences were found between males and females in the *A. americanum* analysis, we decided to mix the

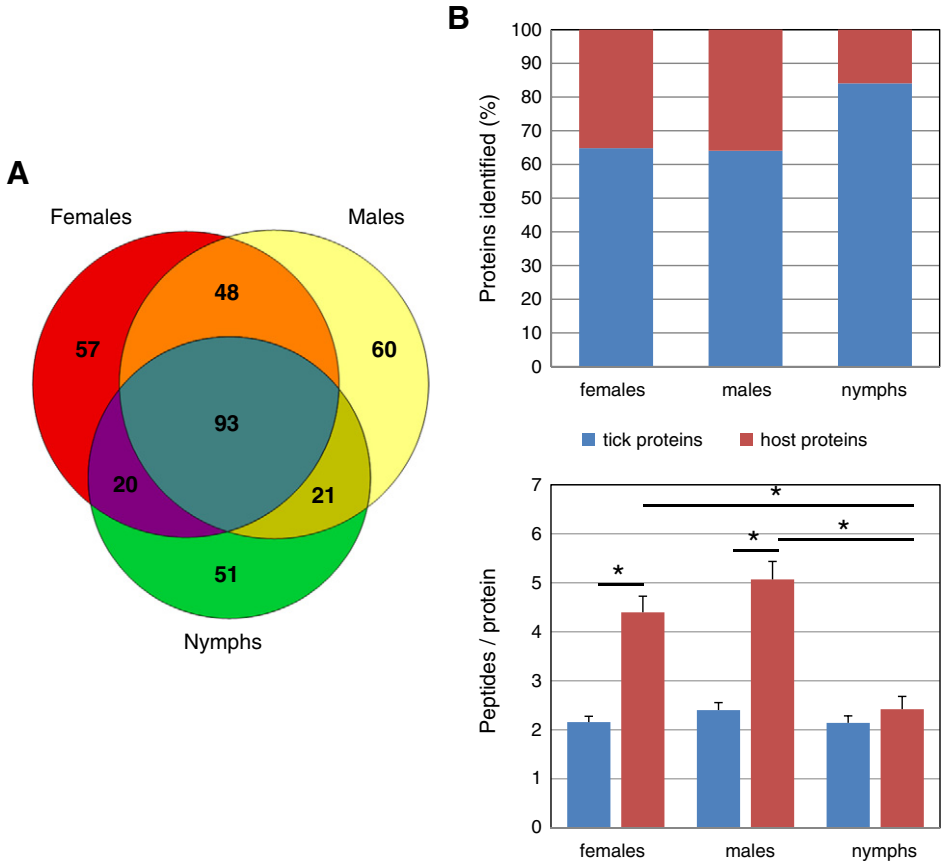

Fig. 2 – Global proteome analysis of *Amblyomma americanum* ticks. Protein extracts from *A. americanum* females, males and nymphs were in gel concentrated, trypsin digested and the resulting peptides were analyzed by RP-LC-MS/MS. Raw data were searched against Ixodida and Ruminantia Uniprot databases as described in the Materials and methods section. (A) Venn diagram of tick proteins identified. (B) Percent distribution of tick and host proteins identified. (C) Number of peptides used for protein identification in the different samples. The number of peptides per protein on each sample was represented as Ave + S.E. and compared between groups by χ^2 test (*p < 0.05).

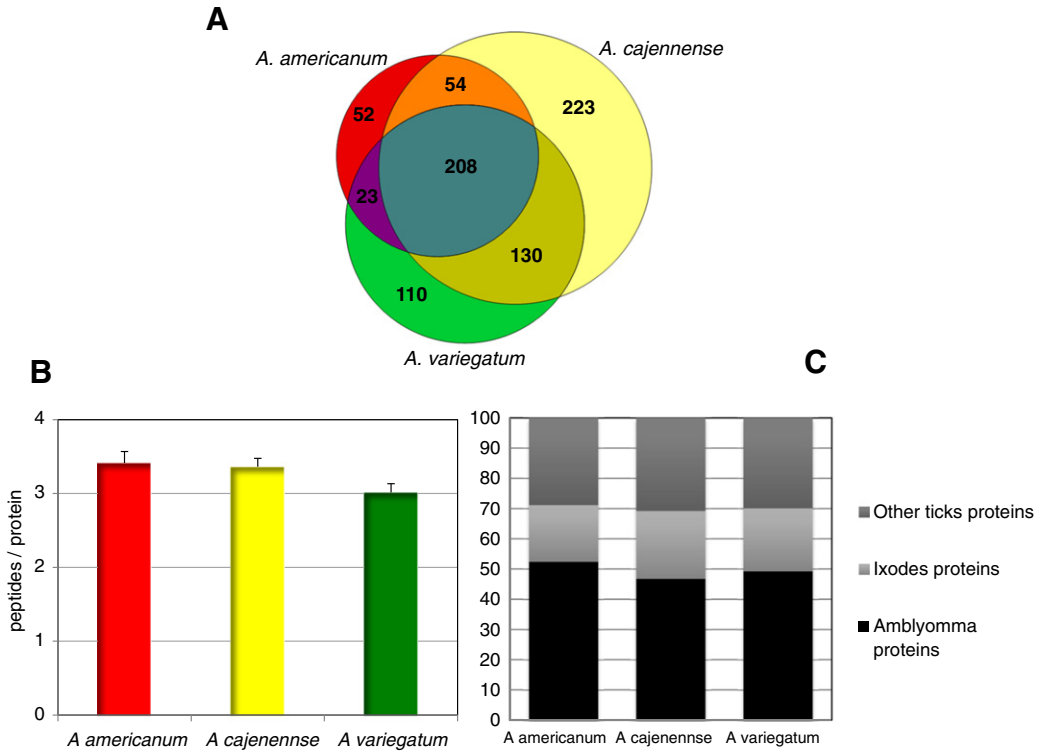

Fig. 3 – Global proteome analysis of *Amblyomma* spp. ticks. Protein extracts from *A. americanum*, *A. cajennense* and *A. variegatum* ticks were in gel concentrated, trypsin digested and the resulting peptides were analyzed by RP-LC-MS/MS. Raw data were searched against the Ixodida-Uniprot database as described in the Materials and methods section. (A) Venn diagram of tick proteins identified. (B) Number of peptides used for protein identification in the different samples. The number of peptides per protein on each sample was represented as Ave + S.E. and compared between groups by χ^2 test ($p > 0.05$). (C) Percent distribution of proteins identified in *Amblyomma* spp., *Ixodes* spp. and other tick spp.

same quantity of proteins from males and females of each *Amblyomma* species. Total proteome from *A. americanum*, *A. cajennense* and *A. variegatum* adult ticks were in-gel concentrated, trypsin-digested and analyzed RP-LC-MS/MS. After Ixodida-Uniprot database search, a total of 800 unique proteins were identified with an FDR < 0.01, of which 337, 615 and 471 were present in *A. americanum*, *A. cajennense* and *A. variegatum* adult ticks respectively (see Supplementary Information, Table S2). A total of 208 proteins were identified in all samples, but some proteins were identified in one sample only (Fig. 3A).

In all *Amblyomma* spp. the mean number of peptides used to identify each protein with FDR < 0.01 was three or more peptides, indicating a good quality of the data obtained (Fig. 3B). Of the proteins identified, around 50% were proteins assigned to *Amblyomma* spp. (52%, 47% and 49% for *A. americanum*, *A. cajennese* and *A. variegatum*, respectively) whereas the rest of the proteins were mainly assigned to *Ixodes* spp. (39%, 42% and 41% for *A. americanum*, *A. cajennese* and *A. variegatum*, respectively) (Fig. 3C). These results are in accordance with the tick proteins available in databases because the only tick genome close to completion is that of the black-legged deer tick, *I. scapularis* [9], with 23,047 proteins currently in the Uniprot database (data from June 2013) whereas only 437, 32 and 3138 protein sequences exist for *A. americanum*, *A. cajennense* and *A. variegatum*, respectively.

Differences were observed between *Amblyomma* spp. for some of the identified proteins (Table 3). For example, the content in cement proteins was greater in *A. variegatum* with respect to *A. cajennense* and *A. americanum* (1.7 and 13.5 fold, respectively) and also in *A. cajennense* with respect to *A. americanum* (8 fold) (Fig. 4A). These results showed a correlation between the abundance of cement proteins and the weight of replete females (approximately 530, 639 and 2950 mg for *A. americanum* [28], *A. cajennese* [29] and *A. variegatum* [30], respectively) (Fig. 4B), suggesting a mechanism to prepare and maintain the attachment to the host. Also, the heat shock response was more represented in *A. cajennense* and *A. variegatum* when compared to *A. americanum* (Fig. 4C). Moreover, the hemelipoproteins involved in blood digestion and heme detoxification were increased in 3 and 2.2 fold in *A. americanum* with respect to *A. cajennense* and *A. variegatum*, respectively (Fig. 4D), and also *A. americanum* showed higher levels of protease inhibitors than *A. cajennense* and *A. variegatum* (Fig. 4E) that may represent an evolutionary advantage to aid in protection against pathogen infection [24,31–34].

The proteomic analysis shown here used whole tick tissues and not individual tissues separately. For comparative proteomics studies this approach is valid to identify differences and similarities between tick species. However, using adult tick tissues may results in better proteome coverage [35,36].

3.4. Phyloproteomics proof of concept

The *Amblyomma* tick species included in this study are among the most abundant vectors of zoonotic pathogens worldwide

Table 3 – Differentially represented proteins in *Amblyomma* spp.

Accession number	Protein ID	*A americanum* vs. *A cajennense*	*A americanum* vs. *A variegatum*	*A cajennese* vs. *A variegatum*
A0MVX0	Heme lipoprotein (*Amblyomma americanum*)	Up	Up	=
A7UI32	Lospin 17 (*Amblyomma americanum*)	Up	Up	=
B5M727	Alpha-2-macroglobulin (*Amblyomma americanum*)	Up	=	=
B7P1Q2	Myosin heavy chain, skeletal muscle or cardiac muscle (*Ixodes scapularis*)	Down	Down	=
B7P5D8	Paramyosin (*Ixodes scapularis*)	=	Up	=
B7PGA6	Kettin (*Ixodes scapularis*)	Down	Down	=
B7PMN6	Cuticular protein (*Ixodes scapularis*)	=	=	Down
E1CAX9	Vitellogenin-1 (*Haemaphysalis longicornis*)	Up	Up	=
E2J6U6	Hypothetical glycine rich secreted cement protein (*Hyalomma marginatum*)	=	=	Down
F0J8B9	Serine protease-like protein (*Amblyomma variegatum*)	=	=	Down
F0J8K3	Salivary protein 313 (*Amblyomma variegatum*)	=	=	Down
F0J8N3	Glycine-rich protein 44 (*Amblyomma variegatum*)	=	Down	=
F0J8R7	Porin/voltage-dependent anion-selective channel protein (*Amblyomma variegatum*)	=	Down	=
F0J8T5	Putative cement protein (*Amblyomma variegatum*)	=	=	Down
F0J8W4	Heme lipoprotein (*Amblyomma variegatum*)	Up	=	Down
F0J965	Succinyl-CoA synthetase small subunit (*Amblyomma variegatum*)	Down	=	Up
F0J9N1	Heme lipoprotein (*Amblyomma variegatum*)	=	=	Down
F0J9N8	Hemelipoglycoprotein (*Amblyomma variegatum*)	=	Down	Down
F0JA65	Putative cement protein (*Amblyomma variegatum*)	=	=	Down
F0JA84	Putative cement protein (*Amblyomma variegatum*)	=	Down	Down
G3MG31	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	Up	=
G3MGI3	Putative uncharacterized protein (*Amblyomma maculatum*)	=	Down	=
G3MHB6	Putative uncharacterized protein (*Amblyomma maculatum*)	=	=	Up
G3MHT9	Putative uncharacterized protein (*Amblyomma maculatum*)	=	=	Up
G3ML01	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	=	=
G3MLU9	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	Up	=
G3MLX4	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	=	=
G3MMF0	Putative uncharacterized protein (*Amblyomma maculatum*)	=	=	Up
G3MPE0	Catalase(*Amblyomma maculatum*)	Up	=	=
G3MQA4	Putative uncharacterized protein (*Amblyomma maculatum*)	=	Up	=
G3MQW1	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	=	=
G3MRB8	Adenosylhomocysteinase (*Amblyomma maculatum*)	=	=	Down
G3MSN9	Putative uncharacterized protein (*Amblyomma maculatum*)	Up	Up	=
J7LVN2	Paramyosin (*Haemaphysalis longicornis*)	Up	Up	=
L7M0V0	Putative heat shock-related protein (*Rhipicephalus pulchellus*)	=	=	Down
L7M2B3	Putative chorion peroxidase-like protein (*Rhipicephalus pulchellus*)	=	Down	=
L7M6R1	Putative calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type calcium pump isoform 1 (*Rhipicephalus pulchellus*)	Down	Down	=
L7M840	Putative multifunctional chaperone 14-3-3 family (*Rhipicephalus pulchellus*)	Down	Down	=
L7M921	Putative lysosomal & prostatic acid phosphatase (*Rhipicephalus pulchellus*)	Up	Up	=
L7MEG0	Putative heat shock protein (*Rhipicephalus pulchellus*)	Down	Down	=
L7MFP6	Uncharacterized protein (*Rhipicephalus pulchellus*)	Up	=	=
L7MJ61		=	Down	=

Table 3 (*continued*)

Accession number	Protein ID	*A americanum* vs. *A cajennense*	*A americanum* vs. *A variegatum*	*A cajennense* vs. *A variegatum*
	Putative heparan sulfate proteoglycan 2 (*Rhipicephalus pulchellus*)			
L7MM43	Putative 60s acidic ribosomal protein (*Rhipicephalus pulchellus*)	Down	=	=
L7MMF9	Putative glycine-rich cell wall structural protein (*Rhipicephalus pulchellus*)	=	=	Down
Q86RN8	Paramyosin (*Boophilus microplus*)	Up	Up	=

Protein levels in *Amblyomma* spp. were compared in pairs between the different species. Abbreviations: Up, Protein over-represented in the first specie with respect to the second specie under comparison. Down, Protein under-represented in the first specie with respect to the second specie under comparison. =, No difference between samples.
Differential protein representation for individual proteins between different samples was determined using χ^2 test statistics with Bonferroni correction in the IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6_form/) ($p = 0.05$).

[1]. However, as of January 2013, only 7,454, 1,769 and 5,421 EST and nucleotide sequences were available on GenBank for *A. americanum*, *A. cajennense* and *A. variegatum*, respectively. Protein database included only 437, 32 and 3138 sequences for *A. americanum*, *A. cajennense* and *A. variegatum*, respectively. Therefore, more genomics, transcriptomics and proteomics data is required for the characterization of these tick species. Despite the limitations of *de novo* protein sequencing, proteomics data is particularly relevant for these studies because this information is not affected by sequencing and assembly

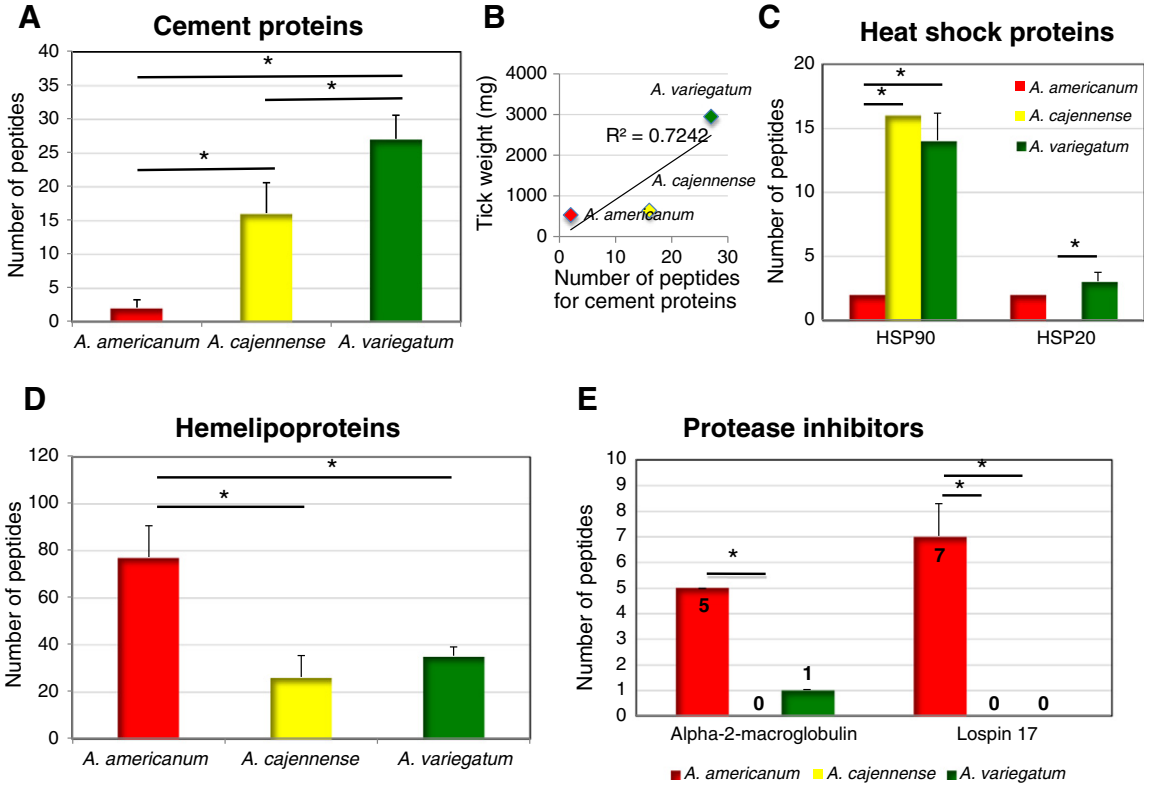

Fig. 4 – Proteins affecting relevant biological processes in *Amblyomma* spp. ticks. (A) Number of peptides for tick Cement proteins involved in attachment to the host identified in *Amblyomma* spp. (B) Correlation between tick weight (mg) and the total number of peptides used to identify cement proteins in *Amblyomma* spp. Correlation coefficient (R^2) is shown. (C) Number of peptides for Heat shock proteins identified in *Amblyomma* spp. (D) Number of peptides for tick Hemelipoproteins involved in blood digestion identified in *Amblyomma* spp. (E) Number of peptides for Protease inhibitor proteins identified in *Amblyomma* spp. The number of peptides per protein on each pathway were represented as Ave + S.D. and compared between groups by χ^2 test (*$p < 0.05$).

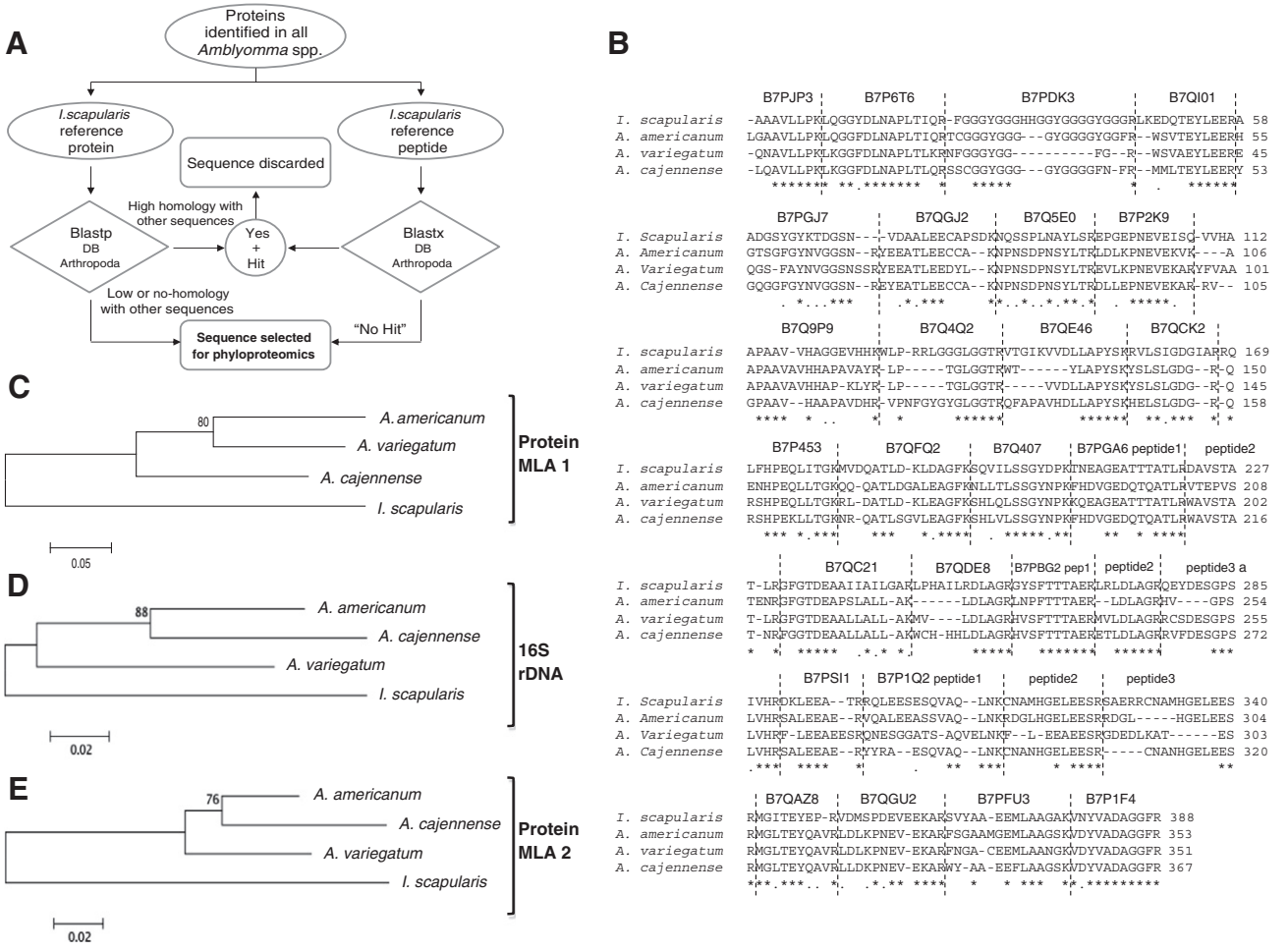

Fig. 5 – Phyloproteomics prove of concept. (A) Algorithm to check peptide sequences against Arthropoda-Uniprot database using (a) Blastp search with the *I. scapularis* reference protein sequence and (b) Blastx search with the *I. scapularis* reference peptide sequence. Sequences that did not produce a positive hit were considered as unlikely to have very closely related paralogs and those included in the analysis. The *I. scapularis* reference sequences were used because this is the only tick genome available in databases and the one used as outgroup in our analysis. (B) Protein sequences were generated de novo in *Amblyomma* spp. (30 peptides belonging to 25 different proteins are shown between discontinuous lines) and aligned with reference *I. scapularis* sequences (UNIPROT accession numbers are listed on top of peptide sequences and referenced in Table S3). Amino acids identical among *Amblyomma* spp. and *I. scapularis* are marked with asterisks (*) while amino acids identical among *Amblyomma* spp. only are marked by dots (.). (C) The evolutionary history was inferred using all protein sequences (60% homology among *Amblyomma* spp.) by ML. (D) The evolutionary history was inferred using 16S rDNA (Genbank accession numbers L34313, L34317, L34312, L43865). (E) The evolutionary history was inferred using the most variable protein sequences (45% homology among *Amblyomma* spp.) by ML. The trees with the highest log likelihood (C; −2984.3305), (D; −1202.1836) and (E; −449.2066) are shown. *I. scapularis* sequences were used as outgroup. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of (C) 492, (D) 450 and (E) 89 positions in the final dataset. Stability or accuracy of inferred topologies were assessed via bootstrap analysis of 1000 replications.

problems associated with genomics and transcriptomics projects [37]. For example, recent evolutionary studies suggest that *Amblyomma* genus contains more species than those actually considered, with some species being polyphyletic [7,38]. However, the limited sequence information available affects the completion of these studies. In this context, we proposed a phyloproteomics approach, by using *de novo* sequencing of peptides for MLA. The "phyloproteomics" term has been used before [39,40], but the basic approach used by these authors differs from the *de novo* sequencing approach of the current study since they employed absence/presence data of MS peaks to cluster and identify organisms to species level but not for MLA.

To prove this concept, peptides sequences generated *de novo* for 30 different peptides belonging to 25 proteins identified in all three *Amblyomma* spp. (*I. scapularis* UNIPROT accession numbers are listed in Table S3 in the Supplementary Information) were selected for analysis after fulfilling the criteria of being with high probability orthologous and not paralogous sequences (Fig. 5A). As expected, these peptide sequences were more homologous

among *Amblyomma* spp. (60% similarity) than when compared to *I. scapularis* (52% similarity) (Fig. 5B). Protein sequences generated *de novo* in all three *Amblyomma* spp. (Fig. 5B) were used for MLA in comparison with established 16S rDNA phylogeny (Fig. 5C). Reference *I. scapularis* sequences were used as outgroup. Protein sequences included in the analysis belonged to 4 different functional categories, muscle development, cytoskeleton, metabolism and other (data not shown). The topology of the trees obtained was similar when MLA was done separately for each category or grouping all proteins together both by ML and Neighbor-Joining (Fig. 5C and data not shown). However, an apparent discrepancy was observed between protein MLA and 16S rDNA analyses in the closer association between *A. variegatum* and neotropical ticks, *A. americanum* and *A. cajennense* using protein sequences (Fig. 5C and D). This discrepancy could reflect the fact that peptide sequences obtained in all three *Amblyomma* spp. likely belong to highly conserved sequences that may not be the most informative for phylogenetic studies. In fact, when only the most variable protein sequences were included in the analysis (45% homology among *Amblyomma* spp.), the topology of the tree was similar to the tree obtained using 16S rDNA sequences (Figs. 5D and E).

These results suggested that phyloproteomics might be a useful tool for the phylogenetic analysis of tick species in which sequence data is a limiting factor but limitations associated with sequence homology should be considered when selecting datasets for analysis.

4. Conclusions

In summary, the results showed no differences between unfed adult female and male *A. americanum* ticks but differentially represented proteins between adults and nymphs underlined important differences between these two developmental stages. Although these ticks were unfed, over-represented host proteins may act as protein reserve to supply nutrients during off-host periods. Tick proteins involved in tick attachment and feeding, heat shock response, protease inhibition, blood digestion and heme detoxification were differentially represented between *Amblyomma* tick spp., suggesting adaptation processes to biotic and abiotic variables in these ticks. The results suggested that phyloproteomics might be a useful tool for the phylogenetic analysis of tick species in which sequence data is a limiting factor but limitations associated with sequence homology should be considered when selecting datasets for analysis. These results demonstrate the possibilities of proteomics studies for the characterization of relevant tick vector species and provide new relevant information to understand the physiology, development and evolution of these tick species.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.12.016.

Acknowledgments

We thank A. Marina and M. del Valle (Proteomics Facility, CBM-SO, Spain) and A. Estrada-Peña (University of Zaragoza, Spain) for technical assistance. We would like to acknowledge K.M. Kocan (Oklahoma State University, USA), C. Almazán (University of Tamaulipas, Mexico) and N.I. Ogo (National Veterinary Research Institute, Nigeria) for providing tick samples. Venn diagrams were drawn with the VennDiagramPlotter software provided by the Proteomics group of the Pacific Northwest National Laboratory (PNNL) in Richland, Washington. This research was supported by grants BFU2011-23896 and the EU FP7 ANTIGONE project number 278976. M. Popara is an Early Stage Researcher supported by the POSTICK ITN (Post-graduate training network for capacity building to control ticks and tick-borne diseases) within the FP7-PEOPLE-ITN programme (EU Grant No. 238511).

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