Expression of Heat Shock and Other Stress Response Proteins in Ticks and Cultured Tick Cells in Response to Anaplasma spp. Infection and Heat Shock

Margarita Villar,1 Nieves Ayllón,1 Ann T. Busby,2 Ruth C. Galindo,1 Edmou F. Blouin,2 Katherine M. Kocan,2 Elena Bonzón-Kulichenko,3 Zorica Zivkovic,4 Consuelo Almazán,5 Alessandra Torina,6 Jesús Vázquez,3 and José de la Fuente1,2

1 Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain
2 Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA
3 Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Cantoblanco, 28049 Madrid, Spain
4 Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, The Netherlands
5 Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Tamaulipas, Km. 5 carretera Victoria-Mante, Ciudad Victoria, 87000 Tamaulipas, Mexico
6 Intituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi no. 3, Palermo, 90129 Sicily, Italy

Correspondence should be addressed to José de la Fuente, jose_delafuente@yahoo.com

1 Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13005 Ciudad Real, Spain
2 Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK 74078, USA
3 Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Cantoblanco, 28049 Madrid, Spain
4 Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584CL Utrecht, The Netherlands
5 Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Tamaulipas, Km. 5 carretera Victoria-Mante, Ciudad Victoria, 87000 Tamaulipas, Mexico
6 Intituto Zooprofilattico Sperimentale della Sicilia, Via G. Marinuzzi no. 3, Palermo, 90129 Sicily, Italy

1. Introduction

Ticks are ectoparasites of wild, domestic animals and humans and are considered to be the most important arthropod vector of pathogens in some regions [1, 2]. The genus Anaplasma includes intraerythrocytic pathogens of ruminants, A. marginale, A. centrale, A. bovis, and A. ovis [2, 3]. Also included in this genus are A. phagocytophilum, which infects granulocytic leukocytes of a wide range of hosts including humans, wild and domestic animals, and A. platys that infects dog platelets [2, 3]. Ticks are biological vectors of Anaplasma spp. but different tick species transmit A. marginale, A. centrale, A. bovis, A. ovis, A. phagocytophilum, and A. platys [1]. Mammalian or tick hosts with persistent infection serve as reservoirs of these pathogens in nature [3].

The ticks and the pathogens that they transmit have coevolved molecular interactions involving genetic traits of both the tick and the pathogen that mediate their development...
studies to characterize protein expression in ticks are di
gies have been made during the last decades, proteomics
and survival, but these mechanisms are not well defined [3–
Furthermore, although advances in proteomics technolo-
5]. Furthermore, although advances in proteomics technolo-
a on to conduct [5–17]. Most of these studies have focused on the
sialome (salivary gland secretory proteome) analysis of ticks
[6–12] and the analysis of host-tick-pathogen interactions
in an attempt to identify potential candidates for vaccine
development against tick-borne diseases [5, 13–17].

The heat shock and other stress responses are a conserved
reaction of cells and organisms to elevated temperatures
and other stress conditions such as toxicity and pathogen
infection [18–21]. The heat-shock proteins (HSPs) and other
stress response proteins (SRPs) protect cells and organisms
from damage, allow resumption of normal cellular and
physiological activities, and overall provide higher levels
tolerance to environmental stress. Crucial to cell survival is
the sensitivity of proteins and enzymes to heat inactivation
and denaturation. Therefore, adaptive mechanisms exist that
protect cells from the proteotoxic effects of heat stress.

At the molecular level, the heat-shock response is a transient
reprogramming of cellular activities mediated by the
synthesis of HSPs [18–21]. In most organisms, the major
groups of HSPs, HSP100, HSP90, HSP70, HSP60, and small
HSPs are represented by a few members of each class [18, 21].
HSPs are functionally linked to the large and diverse group
of molecular chaperones that are defined by their capacity
to recognize and bind substrate proteins that are in an
unstable inactive state [18, 21]. Additionally, extracellular
and membrane bound HSPs such as HSP70 are involved in
binding to antigens and presenting them to the immune
system [18, 20, 21].

The expression of the heat-shock genes encoding the
different HSPs is primarily regulated at the transcriptional
level [20]. The thermoinducibility is attributed to conserved
cis-regulatory promoter elements (HSEs) located in the
TATA-box-proximal 5′-flanking regions of heat-shock genes.
The occurrence of multiple HSEs within a few hundred base
pairs is a signature of most eukaryotic heat-shock genes.
The eukaryotic HSE consensus sequence has been ultimately
defined as alternating units of 5-nGAAn-3 [20]. HSEs are the
binding sites for the transactive heat shock factor (HSF), and
efficient binding requires at least three units, resulting in 5′-
nGAAnnTTCnGAAn-3 [20].

In this paper, the expression of HSPs and other SRPs was
characterized in ticks and cultured tick cells by proteomics
and transcriptomics analyses in response to Anaplasma spp.
infection and heat shock. The transcriptomics analyses of
ticks and tick cells in response to A. marginale and A. phago-
cytophilum infection and the proteomics analysis of tick cells
A. marginale interactions were published previously [4, 5].
The proteomics analysis of tick cells A. phagocytophilum
interactions, the proteomics and transcriptomics analyses of
R. ruranicus-A. ovis interactions, and the characterization of
HSPs mRNAs en tick cells cultured at different temperatures
are unpublished and thus methods were described here in
detail. However, herein we did not present all results from
these analyses, but focused on the analysis of HSPs and other
SRPs expression. These results illustrated the complexity of
the stress response in ticks and suggested a function for the
HSPs and other SRPs during Anaplasma spp. infection in
these organisms.

2. Materials and Methods

2.1. Ticks, Tick Cell Cultures, and Samples Preparation. ISE6
and IDE8 cells, originally derived from Ixodes scapularis
embryos (provided by U.G. Munderloh, University of Minne-
sota, USA), were cultured in L15B medium as described
previously [22], but for ISE6 cells the osmotic pressure
was lowered by the addition of one fourth sterile water by
volume. The ISE6 cells were inoculated with A. phagocy-
tophilum-(NY18 isolate-) infected HL-60 cells as described
previously [23, 24]. The IDE8 tick cells were inoculated with
the Virginia isolate of A. marginale and monitored by stained
smears and with phase contrast microscopy [22]. Uninfected
and infected cultures (N = 3–5 independent cultures
each) were sampled at 13 days postinfection (dpi) (percent
infected cells, 26%–31%) for A. phagocytophilum and at
3 dpi (percent infected cells, 30%–40%) for A. marginale.
The cells were centrifuged at 10,000 X g for 3 min and cell
pellets were frozen in liquid nitrogen until used for RNA and
protein extraction. Approximately 106–107 cells were pooled
from each condition. For proteomics analysis, A. phagocy-
tophilum-infected and uninfected ISE6 cells were lysed in
350 µl lysis buffer (PBS, 1% Triton X-100, 1 mM sodium
vanadate, 1 mM NaF, 1 mM PMSF, 1 µg/ml leupeptin, and
1 µg/ml pepstatin) for 30 min at 4°C. A. marginale-infected
and uninfected IDE8 cells were lysed in 30 mM Tris-
HCl, pH8.8, 7 M Urea, 2 M thiourea, and 4% CHAPS
electrophoresis reagent (Sigma, St. Louis, MO, USA). Total
cel extracts were centrifuged at 200 × g for 5 min to
remove cellular debris. The supernatants were collected and
protein concentration was determined using the Bradford
Protein Assay (Bio-Rad, Hercules, CA, USA) with BSA as
standard.

For analysis of HSP70 and HSP20 mRNA levels in
response to heat shock, IDE6 cells were cultured in L15B
medium as described previously [22] and incubated for
24 h at 4°C, 31°C (normal growth conditions), and 37°C
prior to RNA extraction. Total RNA was extracted from
two independent cultures for each condition as described
previously [4, 5].

Dermacentor variabilis, Dermacentor andersoni, and Rhi-
pecophilus sanguineus male ticks were obtained from labora-
tory colonies maintained at the Oklahoma State University,
Tick Rearing Facility. Offhost ticks were maintained at 95% relative humidity in a 12 hr light: 12 hr dark photoperiod at
22–25°C. In order to obtain infected ticks, male ticks were
fed for one week on a splenectomized calf with ascending A.
marginale parasitemia that was experimentally infected with
the Virginia isolate of A. marginale [5]. The ticks were then
removed and maintained offhost for 4 days and then fed for
an additional week on an uninfected calf to cause infection of
salivary glands and other tick tissues. Uninfected ticks were
fed in a similar way on the uninfected calf. Rhipicephalus (Boophilus) microplus male ticks (Mozambique strain) were
reared in cattle at the Utrecht Centre for Tick-borne Diseases,
Utrecht University, The Netherlands. Ticks were infected by feeding on a calf experimentally infected with a Texas isolate of A. marginale [5]. Rhipicephalus (Boophilus) annulatus larvae were allowed to feed on a calf naturally infected with A. marginale in Tamaulipas, Mexico (approximately 4% rickettsemia during tick feeding) and collected as adults after 21 days of feeding. Uninfected ticks were fed in a similar manner on an uninfected calf. The I. scapularis nymphs uninfected and infected with A. phagocytophilum (Gaillard and Dawson strains) were obtained from a laboratory colony rear at the Centers for Disease Control and Prevention, Atlanta, GA, USA [4]. Tick larvae were fed on infected or uninfected mice, collected after feeding and allowed to molt to the nymphal stage [4]. Animals were housed with the approval and supervision of the Institutional Animal Care and Use Committees. Total RNA was extracted with TriReagent (Sigma) as described previously [4, 5].

Rhipicephalus turanicus adult female ticks feeding on sheep were collected in farms in Sicily, Italy. DNA (for characterization of pathogen infection), RNA (for transcriptomics analysis), and proteins (for proteomics analysis) were extracted with TriReagent (Sigma) following manufacturers recommendations. Anaplasma spp. infection was characterized by PCR and sequence analysis of cloned major surface protein 4 (msp4) amplicons [25, 26]. After analysis, two ticks were positive for A. ovis and negative for other Anaplasma, Ehrlichia, Rickettsia, and Theileria spp. [26] Uninfected ticks were negative for all pathogens analyzed and were used as controls (N = 10) for proteomics and transcriptomics analyses. Interfering components for 2D DIGE experiments were removed from protein samples by using a 2D Clean up Kit (GE Healthcare, Madrid, Spain) according to the manufacturer’s instructions. The protein pellet was resuspended in 25 μl of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM Tris-HCl, and pH 8.0) and protein concentration was determined using the 2D Quant Kit (GE Healthcare). In order to reduce individual-to-individual variation and to obtain enough protein for analysis, protein samples that were pooled resulted in 18.5 μg total protein from the infected ticks.

2.2. Proteomics Analysis of Tick Cells Infected with A. phagocytophilum

2.2.1. Protein One-Step In-Gel Digestion, Peptide iTRAQ Labeling, and IEF Fractionation. Hundred μg of protein extracts from each experimental condition were resuspended in a volume up to 300 μl of sample buffer and applied using a 5-well comb on a conventional SDS-PAGE gel (1.5 mm-thick, 4% stacking, and 10% resolving). The 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 Coomassie Brilliant Blue R-250 staining, excised, cut into cubes (2 × 2 mm), and digested overnight at 37°C with 60 ng/μl 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) ACN, and 0.01% (w/v) 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5) [27, 28]. The resulting tryptic peptides from each proteome were extracted by 1h-incubation in 12 mM ammonium bicarbonate, pH 8.8. TFA was added to a final concentration of 1% and the peptides were finally desalted onto C18 OASIS HLB Extraction cartridges (Waters, Milford, Massachusetts, USA) to remove the amine-containing buffers and dried down. Dried peptides were resuspended in triethylammoniumbicarbonate (TEAB), pH 8.53 and labeled with iTRAQ reagents (Applied Biosystems, Madrid, Spain) for 1 h at room temperature. Samples from uninfected and infected ISE6 cell cultures were labeled with 116 and 117 iTRAQ tags, respectively. The two labeled samples were resuspended in 100 μl 0.1% formic acid and combined into one tube. The mixture was dried down, redisolved in 3.3 ml 5 mM ammonium formiate, pH 3, cleaned up with SCX Oasis cartridges (Waters) using as elution solution 1 M ammonium formiate pH 3, containing 25% ACN, and dried down. The peptide pools were resuspended in 0.5 ml 0.1% TFA, desalted onto C18 Oasis cartridges using as elution solution 50% ACN in 5 mM ammonium formiate, pH 3 and dried down.

The sample was taken up in focusing buffer (5% glycerol and 2% IPG buffer pH 3–10 (GE Healthcare, Madrid, Spain) loaded onto 24 wells over a 24 cm-long Immobiline DryStrip, pH3–10 (GE Healthcare), and separated by IEF on a 3100 OFFgel fractionator (Agilent, Santa Clara, CA, USA), using the standard method for peptides recommended by the manufacturer. The recovered fractions were acidified with 20 μl of 1 M ammonium formiate, pH 3, and the peptides were desalted using OMIX C18 tips (Varian, Palo Alto, CA, USA). After elution with 50% ACN in 5 mM ammonium formiate, pH 3, the peptides were dried down prior to RP-HPLC-LIT analysis.

2.2.2. LC-MS/MS Analysis and Peptide Identification. All samples were analyzed by LC-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA, USA) as described previously [29, 30]. The LTQ was programmed to perform a data-dependent MS/MS scan on the 15 most intense precursors detected in a full scan from 400 to 1600 amu (3 μ scans, 200 ms injection time, and 10 000 ions target). Singly charged ions were excluded from the MS/MS analysis. Dynamic exclusion was enabled using the following parameters: 2 repeat counts, 90 s repeat duration, 500 exclusion size list, 120 s exclusion duration, and 2.1 amu exclusion mass width. PQD parameters were set at 100 ms injection time, 8 microscans per scan, 2 amu isolation width, 28% normalized collision energy, 0.6 activation Q, and 0.3 ms activation time. For PQD spectra generation 10 000 ions were accumulated as target and automatic gain control was used to prevent overfilling of the ion trap.

Protein identification was carried out as described previously [29] using SEQUEST algorithm (Bioworks 3.2 package, Thermo Finnigan), allowing optional (Methionine oxidation) and fixed modifications (Cysteine carboxymidomethylation, Lysine, and N-terminal modification of +144.1020 Da). The MS/MS raw files were searched
against the alphaproteobacteria combined with the arachnida Swissprot database (Uniprot release 15.5, 7 July, 2009) supplemented with porcine trypsin and human keratins. This joint database contains 638408 protein sequences. The same collections of MS/MS spectra were also searched against inverted databases constructed from the same target databases. The alphaproteobacteria Swissprot database was used to identify and discard Anaplasma and possible bacterial symbiotic sequences from further analyses. Statistical analysis and determination of error rates were performed with the Probability Ratio Method [31]. False Discovery Rate (FDR) was used as a measure of statistical significance of peptide identification and was calculated using the refined method proposed by Navarro and Vazquez [32].

2.2.3. Peptide Quantification and Statistics. The intensity of the centroided iTRAQ reporter ion signals was computed by the QuixToT software, correcting for isotope overlap between iTRAQ reporter ions [33]. The sensitivity threshold and mass tolerance for extracting the iTRAQ ratios were set to 0 and ±0.4 Da, respectively. Statistical analysis of the data was done on the basis of a novel random-effects model recently developed and validated in our laboratory that includes four different sources of variance: at the spectrum fitting, scan, peptide, and protein levels [34]. The log₂ ratio of peptide concentration in samples A and B determined by scan s coming from peptide p derived from protein q is expressed as \( x_{qsp} = \log_2(A/B) \). The statistical weight associated to the scan, \( w_{qsp} \), is calculated from the spectrum fitting and the scan variance, \( \sigma^2_q \), as described in [34]. The log₂-ratio value associated to each peptide, \( x_{qp} \), is calculated as a weighted average of the scans used to quantify the peptide, and the value associated to each protein, \( x_{q} \), is similarly the weighted average of its peptides. Besides, a grand mean, \( \bar{x} \), is calculated as a weighted average of the protein values. In turn, the statistical weight associated to each peptide, \( w_{qp} \), is calculated from the corresponding scan weights and the peptide variance, \( \sigma^2_p \), and that of each protein, \( w_{q} \), is calculated from the corresponding peptide weights and the protein variance, \( \sigma^2_p \). In all cases, the statistical weights are the inverses of variances. Outliers at the scan and peptide levels are detected by calculating the probability that the measurements deviate from the expected average according to their respective variances, and controlling for the false discovery rate at each level, FDR$_{qps}$ and FDR$_{qp}$, respectively. Details about the statistical model and the algorithm used to calculate the variances at the scan, peptide, and protein levels can be found in Jorge et al. [34]. Differential protein expression in early versus late infections was statistically analyzed separately for early and late infections for each BP protein ontology category by a Fisher two-tailed test (\( P = .05 \)) using Statistica 6.0 software (StatSoft Inc., 12, OK, USA).

2.3. Proteomics Analysis of Tick Cells Infected with A. Marginale. Proteomics analysis was performed at Applied Biomics (Hayward, CA, USA; http://www.appliedbiomics.com) by two-dimensional difference in gel electrophoresis (2D DIGE) as reported previously [5].

2.4. Proteomics Analysis of Ticks Naturally Infected with A. ovis

2.4.1. Two-Dimensional Difference in Gel Electrophoresis (2D DIGE). CyDye DIGE fluor labeling kit for scarce protein samples (GE Healthcare) was used to label tick proteins according to the manufacturer’s protocol. Briefly, for cysteine reduction before labeling, 5 µg of protein of each sample were incubated with 2 nmol Tris (2carboxyethyl) phosphine hydrochloride (TCEP; Sigma) at 37°C for 1 h in the dark and, for labeling, 4 nmol of Cy5 dye in 2 µl of anhydrous DMF (Sigma) were added and the samples were incubated at 37°C for 30 min in the dark. For internal standardization, a pool of equal amounts of all samples (5 µg per sample) was created and labeled with Cy3 dye with the same procedure but scaling adjusted the quantities of reagents according to the amount of protein (10 µg). The reaction was quenched by adding an equal volume of 2 x sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v IPG buffer pH 3–11, and 0.2% w/v DTT). Before 2D separation, 5 µg of the Cy3-pool was mixed with 5 µg of each sample.

For the first dimension, 24-cm 3–11 NL pH range IPG strips were rehydrated overnight in 450 µl of DeStreak Rehydration Solution (GE Healthcare) supplemented with 0.5% IPG buffer pH 3–11 (GE Healthcare) using a reswelling tray. IEF was performed at 20°C using an Ettan IPGphor 3 (GE Healthcare). Samples were applied using anodic cup loading and the isoelectrofocusing was carried out using the following conditions: 300 V for 3 h, 300–1000 V for 6 h, 1000–10000 V for 3 h, 10000 V for 3 h, and 500 V for 3 h. Prior to second dimension, focused IPG strips were incubated for 10 min equilibration buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.5% w/v DTT, and traces of bromophenol blue. Equilibrated IPG strips were placed onto 12% homogeneous SDS-polyacrylamide gels casted in low-fluorescence glass plates using an Etan-DALT Six System (GE Healthcare). 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 hours.

2.4.2. 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). Four images were considered for the analysis, 2 corresponded to samples labeled with Cy5, and 2 corresponded to the sample pool labeled with Cy3 and acquired individually with each gel. Spot codetection, 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 4 spot maps were distributed in 3 groups, that is, standard, and the 2 different samples (one control and one infected) and the standard image most representative with average quality were assigned as master. After match images, average ratios between groups were calculated. Protein spots with 5-fold as threshold in the average ratio were considered as differentially expressed between samples under comparison.

2.4.3. Selection and Preparation of Protein Samples for Mass Spectrometry. For preparative gel, equal protein amounts of all samples were pooled. 2D electrophoresis was carried out in the same conditions 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. Proteins were visualized by fluorescence using an Etta DIGE Imager (GE Healthcare) selecting 100 μm as pixel size and channel Sypro Ruby 1 with 0.4 of exposure and gel image was acquired. The gel was matched automatically in the BVA module of DeCyder software with the DIGE image in order to select the spots of interest for mass spectrometry analysis. The 2D electrophoresis stained gel was washed twice for 10 min with distilled water. Selected protein spots were visualized with a UV benchtop transilluminator (UVP, Cambridge, UK), manually excised from the gels, dehydrated with acetonitrile, and vacuum dried (Savant miniator (UVP, Cambridge, UK), manually excised from the gels were covered again with 50 mM NH₄HCO₃ and incubated at 37°C for 1 hr in an ice bath. The digestion bufer was removed and the gel was covered again with 50 mM NH₄HCO₃ and incubated at 37°C for 12 hr. Whole supernatants were allowed to dry and then stored at 20°C until mass spectrometry analysis.

2.4.4. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Analysis. Peptide mass fingerprinting was conducted as described previously [37] using an Autoflex (Bruker Daltonics, Bremen, Germany) mass spectrometer in a positive ion reflector mode employing 2, 5-dihydroxybenzoic acid as matrix, and an AnchorChip surface target (Bruker Daltonics). Peak identification and monoisotopic peptide mass assignment were performed automatically using Flexanalysis software, version 2.2 (Bruker Daltonics). Database searches were performed using MASCOT (http://matrixscience.com) [38] against the NCBI nonredundant protein sequence database (http://www.ncbi.nlm.nih.gov). The selected search parameters were as follows: tolerance of two missed cleavages, carbamidomethylation (Cys), and oxidation (Met) as fixed and variable modifications, respectively, and setting peptide tolerance to 100 ppm after close-external calibration. A significant MASCOT probability score \( P < .05 \) was considered as condition for successful protein identification.

2.4.5. Reverse Phase-Liquid Chromatography (RP-LC) MS/MS Analysis. When peptide mass fingerprinting failed to identify a spot, the protein digest was dried, resuspended in 7 μl of 0.1% formic acid, and analyzed by RP-LC-MS/MS in a Surveyor HPLC system coupled to an ion trap Deca XP mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The peptides were separated by reverse phase chromatography using a 0.18 mm × 150 mm BioBasic C18 RP column (Thermo Fisher Scientific), operating at 1.8 μl/min. Peptides were eluted using a 50-min gradient from 5 to 40% solvent B (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 Fisher Scientific) interface. Peptides were detected in survey scans from 400 to 1600 amu (8 μs-scans), followed by three data-dependent MS/MS scans, using an isolation width of 3 amu, normalized collision energy of 30%, and dynamic exclusion, applied during 3-min periods. Peptide identification from raw data was carried out using the SEQUEST algorithm (Bioworks Browser 3.2, Thermo Fisher Scientific) and the PHENYX 2.6 search engine (GENEBIO, Switzerland). Database search was performed against the Apicomplexa, aprotobacteria, and metazoas databases downloaded from the Protein Knowledgeedgebase (UniProtKB) (http://www.uniprot.org). The following constraints were used for the searches: trypic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 2 Da for precursor ions, and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and fixed Cys carbamidomethylation. If the Sequest and Phenyx searches did not yield positive results, high-quality spectra that had not been assigned to any protein identification were selected and a manual de novo interpretation was conducted that was confirmed with PEAKS Studio 4.5 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

2.5. Real-Time RT-PCR. Real-time RT-PCR was performed on RNA samples from IDE8 and ISE6 tick cells and ticks with gene-specific primers ([4, 5] and HSP20F: 5′-GAGGCTTGCTGTTCT-3′ and HSP70R: 5′-CTTGATGTCCA-3′ and HSP20 and HSP70, resp.) using the iScript One-Step RT-PCR Kit with SYBR Green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer’s recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample [39]. The mRNA levels were normalized against tick 16S rRNA [4, 5] using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0) [40]. Data from cells cultured at 31°C (normal growth conditions) were compared with data from cells cultured at 4°C and 37°C and between Anaplasma-infected and uninfected ticks and tick cells using the Student’s t-test \( P = .05 \).
3. Results and Discussion

In this study, proteomics and transcriptomics were used to characterize heat shock and other stress responses in ticks and tick cells in response to *Anaplasma* spp. infection and heat shock (Figure 1). These analyses included both natural (*I. scalparis* ticks and cells *A. phagocytophilum*, *D. variabilis/D. andersoni/R. microplus/R. annulatus/R. sanguineus-A. marginale, and *R. turanicus*-A. ovis) and nonnatural (*I. scalparis* cells *A. marginale*) tick-pathogen interactions (Figure 1). Gene/proteins considered in this paper included HSPs and other SRPs such as glutathione-S transferase (GST), selenoprotein (SEL), metallothionein (MET), and ferritin1 (FER1). These proteins have been shown to be involved in the cellular response to different stress conditions such as heat shock (HSPs; [18, 19, 21]), endogenous and environmental chemicals (GST; [41]), oxidative stress (SEL, MET, FER1; [42–44]), and metals (MET, FER1; [43, 44]). Additionally, these proteins have been reported to be regulated by tick attachment, blood feeding, or pathogen infection [4, 5, 14, 15, 45–51] as well as expressed in unfed and uninfected ticks and tick cells [10, 11, 52, 53].

### 3.1. Analysis of HSPs and Other SRPs in Cultured *I. scalparis* Tick Cells in Response to *A. marginale* Infection

At the mRNA level, HSP70, GST, SEL W2a, and salivary SEL M genes were upregulated while FER1 was downregulated in *A. marginale*-infected IDE8 tick cells [4, 5]. The mRNA levels for HSP20 and HSP70 were further evaluated by real-time RT-PCR in ISE6 tick cells in response to *A. marginale* infection.
Figure 2: 2D DIGE analysis of the total proteome of uninfected and *A. marginale*-infected *I. scapularis* IDE8 tick cells showing GST underexpression in infected cells [5]. (a) 2D representative maps of uninfected (left) and infected (right) IDE8 cells that were labeled with Cy3 and Cy5, respectively. Circled spots correspond to proteins that were differentially expressed after infection. (b, c) Amplification of gel area where GST protein was localized after MS identification in uninfected (green square) and infected (red square) IDE8 cells. (d) 2D DIGE gel image corresponding to the overlapping Cy3 and Cy5 fluorochromes for uninfected versus infected paired samples in the GST region mentioned above.

The results showed that both HSP20 (2.6 ± 2.4 infected to uninfected cells mRNA ratio, Ave ± SD) and HSP70 (2.4 ± 1.2) were upregulated in *A. marginale*-infected tick cells. These results suggested that the stress response was activated in cultured *I. scapularis* tick cells in response to *A. marginale* infection [5]. However, at the protein level, GST was underexpressed in infected IDE8 tick cells [5], probably reflecting a posttranscriptional mechanism induced by *A. marginale* to control tick stress response to infection (Figure 2). In fact, functional analyses conducted by RNA interference (RNAi) in IDE8 tick cells demonstrated that GST gene knockdown resulted in lower *A. marginale* infection levels, thus suggesting that while GST gene expression is activated in response to pathogen infection, it is required for *A. marginale* infection, trafficking, and/or multiplication in tick cells [5, 54].

### Table 1: HSP differential expression between *A. phagocytophilum*-infected and control-uninfected *I. scapularis* ISE6 tick cells.

| Protein description | Fold change | UNIPROT accession number | FDR |
|---------------------|-------------|--------------------------|-----|
| HSP70-2             | +1.42       | B4YTT9                   | 0.000 |
| HSP70-1             | +1.30       | B4YTT8                   | 0.002 |
| HSP70               | +1.20       | B7PEN4                   | 0.011 |
| HSP, putative       | −1.45       | B7P1Z8                   | 0.016 |
| HSP20, putative     | −5.81       | B7P7F7                   | 0.004 |

*a* and *−* indicate protein overexpression and underexpression in *A. phagocytophilum*-infected cells, respectively.

False discovery rate (FDR) was used as a measure of statistical significance of peptide identification and was calculated using the refined method proposed by Navarro and Vazquez [32].

3.2. Analysis of HSPs and Other SRPs in Cultured *I. scapularis* Tick Cells in Response to *A. phagocytophilum* Infection.

Proteomics analysis of ISE6 tick cells in response to *A. phagocytophilum* infection demonstrated that while HSP70 was overexpressed in infected cells, other putative HSPs such as HSP20 were underexpressed after infection (Table 1). However, HSPs represented only 10% (3/31) and 4% (2/50) of over and underexpressed proteins, respectively, in *A. phagocytophilum*-infected ISE6 tick cells (unpublished
results). At the mRNA level, FER1, SEL W2a, SEL M, and GST expression did not change significantly between A. phagocytophilum-infected and uninfected ISE6 tick cells [4]. The mRNA levels for HSP20 and HSP70 were evaluated by real-time RT-PCR in ISE6 tick cells in response to A. phagocytophilum infection. As with other SRPs, the results showed that both HSP20 (8 ± 8 infected to uninfected cells mRNA ratio, Ave ± SD) and HSP70 (0.5 ± 0.4) mRNA levels did not change significantly in A. phagocytophilum-infected ISE6 tick cells when compared to uninfected cells. These results demonstrated differences in the response of ISE6 tick cells to A. marginale and A. phagocytophilum infections [4]. As discussed previously for GST protein expression in A. marginale-infected IDE8 tick cells, differences in HSP expression between proteomics and transcriptomics analyses probably reflected a posttranscriptional mechanism induced by A. phagocytophilum to control tick response to infection.

3.3. Analysis of HSPs and Other SRPs in Anaplasma-Infected Ticks. In A. marginale-infected D. variabilis male ticks, FER1 mRNA levels were lower in the guts and did not change in the salivary glands of infected ticks [5]. For GST, mRNA levels did not change in both guts and salivary glands after infection [5]. In A. marginale-infected D. andersoni male ticks, FER1 mRNA levels did not change in guts and salivary glands, and GST mRNA levels were similar and lower in guts and salivary glands, respectively, when compared to uninfected controls. While SEL M and FER1 were not differentially expressed in R. microplus salivary glands, GST mRNA levels were significantly higher in infected ticks. In R. annulatus and R. sanguineus, A. marginale infection did not change GST and FER1 mRNA levels in guts and salivary glands. These results demonstrated differences between tick species in the stress response to A. marginale infection.

In A. phagocytophilum-infected I. scapularis nymphs, the expressions of FER1 and GST were significantly downregulated at the mRNA level [4]. The mRNA levels were similar in I. scapularis nymphs infected with different strains of A. phagocytophilum [4]. However, as shown before in ISE6 tick cells, these results were different from those obtained in response to A. marginale infection and may reflect pathogen-specific and/or tick species-specific differences in the effect of Anaplasma spp. infection on gene expression.

Proteomics analysis of R. turanicus ticks infected with A. ovis was conducted in comparison with their respective uninfected controls and the proteins that were differentially expressed with an average ratio of $\pm 5$-fold after DeCyder software analysis of DIGE gels was considered. Two experiments were conducted with similar results. Of the 50 identified differentially expressed proteins (30 overexpressed and 20 underexpressed in infected ticks), none corresponded to HSPs or other SRPs. At the mRNA level, GST was downregulated (0.008 ± 0.007 infected to uninfected cells mRNA ratio, Ave ± SD) while FER1 expression (0.02 ± 0.02) did not change in infected ticks.

R. turanicus is a natural vector of A. ovis [55]. D. variabilis, D. andersoni, R. microplus, R. annulatus, and R. sanguineus are natural vectors of A. marginale, and I. scapularis is a natural vector of A. phagocytophilum in different regions of the world [1–3]. However, I. scapularis does not vector A. marginale [1–3]. The results of HSPs and other SRPs expression suggested that, at least when ticks are the pathogen’s natural vector, heat shock and other stress responses are not strongly activated, probably reflecting tick-pathogen coevolution [3–5]. This fact was demonstrated in A. marginale-infected D. variabilis, D. andersoni, R. microplus, R. annulatus, and R. sanguineus, in A. phagocytophilum-infected I. scapularis ticks and ISE6 tick cells, and in A. ovis-infected R. turanicus, but not in A. marginale-infected IDE8-cultured tick cells, which were not derived from a natural vector species [1–3] (Figure 3). In fact, except for FER1 expression that was consistently downregulated or did not change in response to Anaplasma infection, the expression of the other stress response genes was upregulated in A. marginale-infected I. scapularis IDE8 cells while did not change or were downregulated in other tick-Anaplasma interactions (Figure 3). These results suggested that while cultured tick cells are a useful tool for the study of tick-Anaplasma interactions, experiments should be conducted with the natural tick vector and pathogen.

3.4. Expression of HSP20 and HSP70 Genes in I. scapularis IDE6 Tick Cells in Response to Heat Shock. To further characterize the expression of HSP20 and HSP70 genes in response to heat shock, the mRNA levels for HSP20 and HSP70 were evaluated by real-time RT-PCR in IDE6 tick cells incubated at different temperatures. The results demonstrated that both HSP20 and HSP70 were upregulated after heat shock at 37°C but not at 4°C when compared to control cells grown at 31°C (Figure 4). Although the mRNA levels for HSP70 were 72-fold higher than those for HSP20 at

Figure 3: Differential expression of heat shock and other stress response genes in different tick-Anaplasma interactions. The mRNA levels of HSP20, HSP70, GST, SEL, and FER1 were characterized by real-time RT-PCR in uninfected and Anaplasma-infected ticks and tick cells. Arbitrarily, +10 and −10 values were used to represent gene upregulation and downregulation, respectively. When the mRNA levels did not change after pathogen infection, a zero value was used. A ±5 value was used when pathogen infection was characterized in the same species ticks and tick cells or in tick guts and salivary glands. Abbreviations: A. m., A. marginale; A. p., A. phagocytophilum; A. v., A. ovis; I. s., I. scapularis; D. v., D. variabilis; D. a., D. andersoni; R. s., R. sanguineus; R. a., R. annulatus; R. m., R. microplus; R. t., R. turanicus.
31°C, a 5- and 3-fold increase in HSP20 and HSP70 mRNA levels was obtained after heat shock at 37°C, respectively, (Figure 4). These results showed that, as in other organisms, HSPs are upregulated in tick cells in response to heat shock.

4. Conclusions

The results of these studies demonstrated that the stress response was activated in ticks and cultured tick cells after *Anaplasma* spp. infection and heat shock. However, under natural vector-pathogen relationships, HSPs and other SRPs were not strongly activated, probably reflecting tick-pathogen coevolution. Nevertheless, at least as shown by proteomics analysis of ISE6 tick cells in response to *A. phagocytophilum* infection, some HSPs such as the HSP70 family were overexpressed while other putative HSPs such as HSP20 were underexpressed in infected cells. Furthermore, these results demonstrated pathogen-specific and tick species-specific differences in the expression of HSPs and other SRPs in ticks and tick cells infected with *Anaplasma* spp. Additionally, our results suggested the existence of posttranscriptional mechanisms induced by *Anaplasma* spp. to control tick response to infection. In summary, the results presented herein illustrate the complexity of the stress response in ticks and suggest a function for HSPs and other SRPs during *Anaplasma* infection in ticks.

Acknowledgments

This research was supported by the Ministerio de Ciencia e Innovación, Spain (Project no. BFU2008-01244/BMC), the CSIC intramural Project no. PA1002451 to J. Fuente, and the Walter R. Sitlington Endowed Chair for Food Animal Research to K. M. Kocan. *Villar* and R. C. Galindo were funded by the JAE-DOC program (CSIC-FSE) and MEC, Spain, respectively.

References

[1] J. de la Fuente, A. Estrada-Pena, J. M. Venzal, K. M. Kocan, and D. E. Sonenshine, “Overview: ticks as vectors of pathogens that cause disease in humans and animals,” *Frontiers in Bioscience*, vol. 13, pp. 6938–6946, 2008.

[2] J. S. Dumler, A. F. Barbet, C. P. I. Bekker et al., “Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia with Anaplasma, Cowdria with Ehrlichia* and *Ehrlichia* with *NeoRickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and ‘HGE agent’ as subjective synonyms of *Ehrlichia phagocytophila*,” *International Journal of Systematic and Evolutionary Microbiology*, vol. 51, no. 6, pp. 2145–2165, 2001.

[3] K. M. Kocan, J. De La Fuente, E. F. Blouin, and J. C. Garcia-Garcia, “*Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*): recent advances in defining host-pathogen adaptations of a tick-borne *Rickettsia*,” *Parasitology*, vol. 129, supplement, pp. S285–S300, 2004.

[4] Z. Zivkovic, E. F. Blouin, R. Manzano-Roman et al., “*Anaplasma phagocytophilum* and *Anaplasma marginale* different gene expression responses in cultured tick cells,” *Comparative and Functional Genomics*, vol. 2009, Article ID 705034, 2009.

[5] J. de la Fuente, E. F. Blouin, R. Manzano-Roman et al., “Functional genomic studies of tick cells in response to infection with the cattle pathogen, *Anaplasma marginale*,” *Genomics*, vol. 90, no. 6, pp. 712–722, 2007.

[6] R. D. Madden, J. R. Sauer, and J. W. Dillwith, “A proteomics approach to characterizing tick salivary secretions,” *Experimental and Applied Acarology*, vol. 32, no. 1-2, pp. 77–87, 2004.

[7] R. D. Madden, J. R. Sauer, and J. W. Dillwith, “Erratum: a proteomics approach to characterizing tick salivary secretions,” *Experimental and Applied Acarology*, vol. 32, no. 1-2, p. 129, 2004, Corrected and republished in: *Experimental and Applied Acarology*, vol. 32, pp. 77-87, 2004.

[8] J. G. Valenzuela, “High-throughput approaches to study salivary proteins and genes from vectors of disease,” *Insect Biochemistry and Molecular Biology*, vol. 32, no. 10, pp. 1199–1209, 2002.

Figure 4: Heat shock response in *I. scapularis* ISE6 tick cells. ISE6 cells were incubated for 24 h at 4°C, 31°C (normal growth conditions), and 37°C prior to RNA extraction. Total RNA was extracted from two independent cultures for each condition, and HSP20 and HSP70 mRNA levels were analyzed by real-time RT-PCR. The mRNA levels were normalized against tick 16S rRNA and compared between control cells grown at 31°C and cells cultured at 4°C and 37°C using the Student’s t-test (*P < .05).
[9] A. Oleaga, A. Escudero-Población, E. Camafeita, and R. Pérez-Sánchez, "A proteomic approach to the identification of salivary proteins from the argasid ticks Ornithodoros moubata and Ornithodoros erraticus," Insect Biochemistry and Molecular Biology, vol. 37, no. 11, pp. 1149–1159, 2007.

[10] I. M. B. Francischetti, B. J. Mans, Z. Meng et al., "An insight into the salivome of the soft tick, Ornithodoros parkeri," Insect Biochemistry and Molecular Biology, vol. 38, no. 1, pp. 1–21, 2008.

[11] I. M. B. Francischetti, Z. Meng, B. J. Mans et al., "An insight into the salivary transcriptome and proteome of the soft tick and vector of epizootic bovine abortion, Ornithodoros coricicus," Journal of Proteomics, vol. 71, no. 5, pp. 493–512, 2008.

[12] B. J. Mans, J. F. Andersen, I. M. B. Francischetti et al., "Comparative sialomics between hard and soft ticks: implications for the evolution of blood-feeding behavior," Insect Biochemistry and Molecular Biology, vol. 38, no. 1, pp. 42–58, 2008.

[13] F. J. Alarcon-Chaidez and S. K. Wikel, "Comparative aspects of the tick-host relationship: immunobiology, genomics and proteomics," Symposia of the Society for Experimental Biology, no. 55, pp. 185–243, 2004.

[14] A. Rachinsky, F. D. Guerrero, and G. A. Scoles, "Differential protein expression in ovaries of uninfected and Babesia-infected southern cattle ticks, Rhipicephalus (Boophilus) microplus;" Insect Biochemistry and Molecular Biology, vol. 37, no. 12, pp. 1291–1308, 2007.

[15] A. Rachinsky, F. D. Guerrero, and G. A. Scoles, "Proteomic profiling of Rhipicephalus (Boophilus) microplus midgut responses to infection with Babesia bovis," Veterinary Parasitology, vol. 152, no. 3–4, pp. 294–315, 2008.

[16] J. Vennestrøm and P. M. Jensen, "Ixodes ricinus: the potential of two-dimensional gel electrophoresis as a tool for studying host-vector-pathogen interactions," Experimental Parasitology, vol. 115, no. 1, pp. 53–58, 2007.

[17] S. Wickramasekara, J. Bunikis, V. Wysocki, and A. G. Barbour, "Identification of residual blood proteins in ticks by mass spectrometry proteomics," Emerging Infectious Diseases, vol. 14, no. 8, pp. 1273–1275, 2008.

[18] L. Tutar and Y. Tutar, "Heat shock proteins: an overview," Current Pharmaceutical Biotechnology, vol. 11, no. 2, pp. 216–222, 2010.

[19] A.-L. Joly, G. Wettstein, G. Mignot, F. Ghiringhelli, and C. Garrido, "Dual role of heat shock proteins as regulators of apoptosis and innate immunity," Journal of Innate Immunity, vol. 2, no. 3, pp. 238–247, 2010.

[20] C. Wu, "Heat shock transcription factors: structure and regulation," Annual Review of Cell and Developmental Biology, vol. 11, pp. 441–469, 1995.

[21] M. J. Schlesinger, "Heat shock proteins," The Journal of Biological Chemistry, vol. 265, no. 21, pp. 12111–12114, 1990.

[22] E. F. Blouin, J. T. Saliki, J. de la Fuente, J. C. García-García, and K. M. Kocan, "Antibodies to Anaplasma marginale major surface proteins 1a and 1b inhibit infectivity for cultured tick cells," Veterinary Parasitology, vol. 111, no. 2-3, pp. 247–260, 2003.

[23] U. G. Munderloh, S. D. Jauron, V. Fingerle et al., "Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture," Journal of Clinical Microbiology, vol. 37, no. 8, pp. 2518–2524, 1999.

[24] J. de la Fuente, P. Ayoubi, E. F. Blouin, C. Almazán, V. Naranjo, and K. M. Kocan, "Gene expression profiling of human promyelocytic cells in response to infection with Anaplasma phagocytophilum," Cellular Microbiology, vol. 7, no. 4, pp. 549–559, 2005.

[25] J. de la Fuente, M. W. Atkinson, V. Naranjo et al., "Sequence analysis of the msp4 gene of Anaplasma ovis strains," Veterinary Microbiology, vol. 119, no. 2–4, pp. 375–381, 2007.

[26] A. Torina, J. Vicente, A. Alongi et al., "Observed prevalence of tick-borne pathogens in domestic animals in Sicily, Italy during 2003–2005," Zoonoses and Public Health, vol. 54, no. 1, pp. 8–15, 2007.

[27] A. Shevchenko, H. Tomasz, J. Haviš, J. V. Olsen, and M. Mann, "In-gel digestion for mass spectrometric characterization of proteins and proteomes," Nature Protocols, vol. 1, no. 6, pp. 2856–2860, 2007.

[28] H. Katayama, T. Tabata, Y. Ishihama, T. Sato, Y. Oda, and T. Nagasu, "Efficient in-gel digestion procedure using 5-cyclohexyl-1-pentyl-β-D- maltoside as an additive for gel-based membrane proteomics," Rapid Communications in Mass Spectrometry, vol. 18, no. 20, pp. 2388–2394, 2004.

[29] D. López-Ferrer, S. Martínez-Bartolomé, M. Villar, M. Campllos, F. Martín-Maroto, and J. Vázquez, "Statistical model for large-scale peptide identification in databases from tandem mass spectra using SEQUEST," Analytical Chemistry, vol. 76, no. 23, pp. 6833–6860, 2005.

[30] I. Ortega-Pérez, E. Cano, F. Were, M. Villar, J. Vázquez, and J. M. Redondo, "c-Jun N-terminal kinase (JNK) positively regulates NFATc2 transactivation through phosphorylation within the N-terminal regulatory domain," The Journal of Biological Chemistry, vol. 280, no. 21, pp. 20867–20878, 2005.

[31] S. Martínez-Bartolomé, P. Navarro, F. Martín-Maroto et al., "Properties of average score distributions of SEQUEST: the probability ratio method," Molecular and Cellular Proteomics, vol. 7, no. 6, pp. 1135–1145, 2008.

[32] P. Navarro and J. Vazquez, "A refined method to calculate false discovery rates for peptide identification using decoy databases," Journal of Proteome Research, vol. 8, no. 4, pp. 1792–1796, 2009.

[33] I. P. Shadforth, T. P. J. Dunkley, K. S. Lilley, and C. Bessant, "i-Tracker: for quantitative proteomics using iTraqT™," BMC Genomics, vol. 6, article 145, 2005.

[34] I. Jorge, P. Navarro, P. Martinez-Acedo et al., "Statistical model to analyze quantitative proteomics data obtained by 18O/16O labeling and linear ion trap mass spectrometry: application to the study of vascular endothelial growth factor-induced angiogenesis in endothelial cells," Molecular and Cellular Proteomics, vol. 8, no. 5, pp. 1130–1149, 2009.

[35] N. B. Ivanova, J. T. Dimos, C. Schaniel, J. A. Hackney, K. A. Moore, and I. R. Lemischka, "A stem cell molecular signature," Science, vol. 298, no. 5593, pp. 601–604, 2002.

[36] J. P. Shaffer, "Multiple hypothesis testing," Annual Review of Psychology, vol. 46, no. 1, pp. 561–584, 1995.

[37] V. Naranjo, M. Villar, M. Mañes, P. Martín-Hernando et al., "Proteomic and transcriptomic analyses of differential stress/inflammatory responses in mandibular lymph nodes and oropharyngeal tonsils of European wild boars naturally infected with Mycobacterium bovis," Proteomics, vol. 7, no. 2, pp. 220–231, 2007.

[38] D. N. Perkins, D. J. C. Pappin, D. M. Creasy, and J. S. Cottrell, "Probability-based protein identification by searching sequence databases using mass spectrometry data," Electrophoresis, vol. 20, no. 18, pp. 3551–3567, 1999.

[39] K. M. Ririe, R. P. Rasmussen, and C. T. Winder, "Product differentiation by analysis of DNA melting curves during the polymerase chain reaction," Analytical Biochemistry, vol. 245, no. 2, pp. 154–160, 1997.
[40] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method,” *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[41] J. D. Hayes, J. U. Flanagan, and I. R. Jowsey, “Glutathione transferases,” *Annual Review of Pharmacology and Toxicology*, vol. 45, pp. 51–88, 2005.

[42] P. D. Whanger, “Selenoprotein expression and function—Selenoprotein W,” *Biochimica et Biophysica Acta*, vol. 1790, no. 11, pp. 1448–1452, 2009.

[43] A. Formigari, P. Irato, and A. Santon, “Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: biochemical and cytochemical aspects,” *Comparative Biochemistry and Physiology—C*, vol. 146, no. 4, pp. 443–459, 2007.

[44] P. Arosio and S. Levi, “Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage,” *Biochimica et Biophysica Acta*, vol. 1800, no. 8, pp. 783–792, 2010.

[45] N. Rudenko, M. Golovchenko, M. J. Edwards, and L. Grubhoffer, “Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection,” *Journal of Medical Entomology*, vol. 42, no. 1, pp. 36–41, 2005.

[46] J. M. C. Ribeiro, F. Alarcon-Chaidez, I. M. B. Francischetti et al., “An annotated catalog of salivary gland transcripts from *Ixodes scapularis* ticks,” *Insect Biochemistry and Molecular Biology*, vol. 36, no. 2, pp. 111–129, 2006.

[47] K. R. Macaluso, A. Mulenga, J. A. Simser, and A. F. Azad, “Differential expression of genes in uninfected and rickettsia-Infected *Dermacentor variabilis* ticks as assessed by differential-display PCR,” *Infection and Immunity*, vol. 71, no. 11, pp. 6165–6170, 2003.

[48] A. Mulenga, K. R. Macaluso, J. A. Simser, and A. F. Azad, “Dynamics of Rickettsia-tick interactions: identification and characterization of differentially expressed mRNAs in uninfected and infected *Dermacentor variabilis*,” *Insect Molecular Biology*, vol. 12, no. 2, pp. 185–193, 2003.

[49] A. Mulenga, J. A. Simser, K. R. Macaluso, and A. F. Azad, “Stress and transcriptional regulation of tick ferritin HC,” *Insect Molecular Biology*, vol. 13, no. 4, pp. 423–433, 2004.

[50] A. Mulenga, M. Blandon, and R. Khumthong, “The molecular basis of the Amblyomma americanum tick attachment phase,” *Experimental and Applied Acarology*, vol. 41, no. 4, pp. 267–287, 2007.

[51] A.E. Lew-Tabor, P. M. Moolhuijzen, M. E. Vance et al., “Suppressive subtractive hybridization analysis of *Rhipicephalus* (Boophilus) microplus larval and adult transcript expression during attachment and feeding,” *Veterinary Parasitology*, vol. 167, no. 2–4, pp. 304–320, 2010.

[52] E. Esteves, F. A. Lara, D. M. Lorenzini et al., “Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus (Boophilus) microplus*,” *Insect Biochemistry and Molecular Biology*, vol. 38, no. 5, pp. 568–580, 2008.

[53] P. M. Untalan, F. D. Guerrero, L. R. Haines, and T. W. Pearson, “Proteome analysis of abundantly expressed proteins from unfed larvae of the cattle tick, *Boophilus microplus*,” *Insect Biochemistry and Molecular Biology*, vol. 35, no. 2, pp. 141–151, 2005.

[54] K. M. Kocan, Z. Zivkovic, E. F. Blouin et al., “Silencing of genes involved in *Anaplasma marginale*-tick interactions affects the pathogen developmental cycle in *Dermacentor variabilis*,” *BMC Developmental Biology*, vol. 9, no. 1, article 42, 2009.

[55] A. Torina, A. Alongi, V. Naranjo et al., “Prevalence and genotypes of *Anaplasma* species and habitat suitability for ticks in a Mediterranean ecosystem,” *Applied and Environmental Microbiology*, vol. 74, no. 24, pp. 7578–7584, 2008.
Submit your manuscripts at http://www.hindawi.com