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The sialotranscriptome of *Antricola delacruzi* female ticks is compatible with non-hematophagous behavior and an alternative source of food

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Abstract

The hosts for *Antricola delacruzi* ticks are insectivorous, cave-dwelling bats on which only larvae are found. The mouthparts of nymphal and adult *A. delacruzi* are compatible with scavenging feeding because the hypostome is small and toothless. How a single blood meal of a larva provides energy for several molts as well as for oviposition by females is not known. Adults of *A. delacruzi* possibly feed upon an unknown food source in bat guano, a substrate on which nymphal and adult stages are always found. Guano produced by insectivorous bats contains twice the amount of protein and 60 times the amount of iron as beef. In addition, bacteria and chitin-rich fungi proliferate on guano. Comparative data on the transcriptome of the salivary glands of *A. delacruzi* is nonexistent and would help to understand the physiological adaptations of salivary glands that accompany different sources of food as well as the steps taken by the Acari towards haematophagy, believed to have evolved from scavenging dead animals. Annotation of the transcriptome of salivary glands from female instars of *A. delacruzi* collected on guano categorized 5.7% of the clusters of expressed genes as putative secreted proteins. They included abundantly expressed TIL domain-containing proteins (possible anti-microbials), an abundantly

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Authors' contributions

IKFMS conceived the study; IKFMS and JMCR acquired data, analyzed the results, performed the statistical analyses and wrote the manuscript; MBL and BJM analyzed the results and wrote the manuscript and revised it critically for important intellectual content; MBL collected and identified the ticks; SCR and IMBF generated the molecular studies and acquired data; GCB analyzed the results and performed the hierarchical clustering. All authors read and approved the final manuscript and none of them present any conflicts of interest with the work reported herein.

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expressed protein similar to a serum amyloid found in the sialotranscriptomes of *Ornithodoros* spp., a savignygrin, a family of mucin/peritrophin/cuticle-like proteins, antimicrobials and an HIV envelope-like glycoprotein also found in soft ticks. When comparing the transcriptome of *A. delacruzi* with those of blood-feeding female soft and hard ticks some notable differences were observed; they consisted of the following transcripts over- or under-represented or absent in the sialotranscriptome of *A. delacruzi* that may reflect its source of food: ferritin, mucins with chitin-binding domains and TIL domain-containing proteins versus lipocalins, basic tail proteins, metalloproteases, glycine-rich proteins and Kunitz protease inhibitors, respectively.

Keywords

Antricola delacruzi; Hematophagy; Scavenging; Transcriptome; Salivary glands; Bat guano

1. INTRODUCTION

Ticks are specialized mites in the suborder Ixodida of the order Parasitiformes. After hatching and hardening their cuticles tick larvae seek and attach to a preferred terrestrial vertebrate host. This developmental stage is then followed by nymphs and adults, stages which generally are thought to be all obligatorily histiophagous and/or hematophagous. Salivary glands are essential for ticks to acquire food and also for osmoregulation (McMullen et al., 1976; Rudolph and Knulle, 1974). In order to acquire blood from their hosts, ticks have evolved a complex cocktail of salivary components that help them overcome host defenses against blood loss and the inflammatory reactions that develop at the feeding site. These may disrupt blood flow or trigger host-defensive behavior by the sensation of pain or itching. Accordingly, saliva of all blood-sucking arthropods contains anti-clotting, anti-platelet, vasodilatory, anti-inflammatory and immunomodulatory components (Francischetti et al., 2009). To date transcriptomes of salivary glands (sialotranscriptomes) of several species of hard (Ixodidae) and soft (Argasidae) ticks have been characterized and the corresponding databases indicate a large expansion of genes coding for metalloproteases, cysteine-rich proteins similar to metalloprotease domains (the ixostatins and ixodegrins), lipocalins, Kunitz-domain containing proteins, RGD-containing peptides, defensins, and many novel protein families that may include anti-microbial proteins (Francischetti et al., 2009) besides a large diversity of abundantly expressed glycine-rich proteins (Maruyama et al., 2010).

Antricola delacruzi is a species of cave-dwelling tick and its hosts are bats of the genus *Pteronotus* (Labruna et al., 2011), which belong to the Mormoopidae family of ghost-faced, moustached, and naked-backed insectivorous bats that feed on insects close to water (Kunz et al., 2011). Interestingly, haematophagy seems not to be present in all of the post-larval developmental stages of *A. delacruzi* because nymphs and adults are never found on hosts, only on bat guano (feces) on the floor of caves, and to a lesser extent, on the cave walls (Labruna et al., 2008). Oliver (Oliver, 1989) reported that species of *Antricola* are truly obligatory autogenous ticks, i.e., they oviposit without having fed on blood at the adult stage. It has been suggested that *A. delacruzi* may form a cluster of tick species that are basal to the Ixodida as a first evolutionary step from the Holothyrida, a group of free-living scavenging mites that live on dead arthropods (Estrada-Pena et al., 2008), behaviour from which hematophagy is believed to have evolved (Mans et al., 2002a; Mans and Neitz, 2004; Walter and Proctor, 1998). However, recent phylogenetic analyses indicate that the genus *Antricola* is not basal to the Ixodida (Dantas-Torres et al., 2011; Labruna et al., 2008), and might represent a more recent event in tick evolution. *A. delacruzi* is a troglolithic species in that its entire life cycle is completed in caves. In accordance with the characteristics of most

troglobites, this species has a semi-transparent cuticle, it has no eyes and its eggs are white (M.B. Labruna, unpublished data).

The source of food for adult instars of *A. delacruzi* remains an open matter and raises the question of how the small amount of host tissues ingested by the histiophagous and/or hematophagous larval stage supports production of eggs by the female. Guano deposits support a great variety of troglobytic biota, which rely on it as their sole source of nutrients (Harris, 1970; Howarth, 1972). De la Cruz and Estrada-Peña (DelaCruz and EstradaPena, 1995) raised the hypothesis that guano of insectivorous bats is the food source for *Antricola* females. If so, their salivary glands should be adapted accordingly. Comparative data on the sialome of *A. delacruzi* is nonexistent and would be of interest to better understand the physiological adaptations of salivary glands that accompany different sources of food and ecosystems. Another reason for examining the salivary gland transcriptome of *A. delacruzi* is to understand the steps taken by the Argasidae, one of the families to which ticks belong, towards blood-feeding, and to test competing hypotheses for evolution of ticks, whether it occurs through co-speciation of host and tick (Hoogstraal H, 1982) or through ecological specificity of the tick (Klompen et al., 1996).

The present work addresses the following two questions: are salivary glands of *A. delacruzi* females equipped for hematophagy and/or are they equipped for an alternative source of food such as guano of insectivorous bats? In order to begin to address these questions we generated and annotated a sialotranscriptome for adult females of *A. delacruzi* collected on bat guano generated from non-normalized cDNA libraries. We compared this sialotranscriptome to similarly constructed sialotranscriptomes from salivary glands of feeding hematophagous females of various species of soft and hard ticks. This exercise revealed that *A. delacruzi* females are not equipped for hematophagy and that they abundantly express transcripts encoding proteins compatible with functions of guanophagy and defense against fungi and microorganisms.

2. MATERIALS AND METHODS

2.1. Ticks

Adult ticks were collected in April 2007 in a cave within the Porto Velho Municipality (08°40' 43" S, 63°51' 05" W), state of Rondônia, western Amazon, northern Brazil. The ticks were crawling on guano when they were collected. The cave structure is such that it creates a specific hot and humid ecosystem, which is constant throughout the year, with internal air temperature and relative humidity ranging from 33°C to 38 °C and 91% to 93%, respectively, while the midday temperature outside the cave usually varies from 25°C to 27 °C. In addition, the atmosphere in the cave was rich in nitrogen compounds. The guano inside the cave was abundant (approximately 10–30 cm in depth), moist, and sticky. A description of the cave has been made by Labruna and colleagues (Labruna et al., 2011).

2.2. Salivary gland isolation and library construction

Tick salivary gland extracts were prepared by collecting glands from *A. delacruzi* female adult ticks. Glands were dissected by first bisecting the tick and then teasing the salivary glands away from the other internal organs and the tick exoskeleton. Glands were rinsed by immersion in PBS and added to RNA Later and stored frozen at –75°C until further analysis. Ticks were collected from guano in the same manner as that employed for a study of the morphology of salivary glands, which demonstrated that they are granular and thus not degenerated and in a state compatible with feeding (Estrada-Peña et al., 2008). *A. delacruzi* salivary gland mRNA from 50 pairs of glands was isolated using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA). The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit

(Clontech, Palo Alto, CA). This system utilizes oligoribonucleotide (SMART IV) to attach an identical sequence at the 5' end of each reverse-transcribed cDNA strand. This sequence is then utilized in subsequent PCR reactions and restriction digests.

First-strand synthesis was carried out using PowerScript reverse transcriptase at 42 °C for 1 h in the presence of the SMART IV and CDS III primers. Second-strand synthesis was performed by a long distance (LD) PCR based protocol, using Advantage™ Taq Polymerase (Clontech) mix in the presence of the 50 PCR primer and the CDS III primer. The cDNA synthesis procedure resulted in the creation of SfiI A & B restriction enzyme sites at the ends of the PCR products that are used for cloning into the phage vector. The PCR conditions were: 95 °C for 20 s; 24 cycles of 95 °C for 5 s, 68 °C for 6 min. A small portion of the cDNA obtained by PCR was analyzed on a 1.1% agarose gel to check for the quality and range of cDNA synthesized. Double-stranded cDNA was immediately treated with proteinase K (0.8 mg/ml) at 45 °C for 20 min and the enzyme was removed by ultrafiltration through a Microcon (Amicon) YM-100 centrifugal filter device. The cleaned, double-stranded cDNA was then digested with SfiI at 50 °C for 2 h, followed by size fractionation on a ChromaSpin-400 column (Clontech, Palo Alto, CA). The profile of the fractions was checked on a 1.1% agarose gel and fractions containing cDNAs of more than 400 bp were pooled and concentrated using a Microcon YM-100. The cDNA mixture was ligated into the TriplEx2 vector (Clontech, Palo Alto, CA) and the resulting ligation mixture was packaged using the GigaPacks III Plus packaging extract (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The packaged library was plated by infecting log phase XL1-Blue *E. coli* cells (Clontech, Palo Alto, CA). The percentage of recombinant clones was determined by performing a blue–white selection screening on LB/MgSO₄ plates containing X-gal/IPTG. Recombinants were also determined by PCR, using vector primers (50 µl TriplEx2 Sequencing Primer and 30 µl TriplEx2 Sequencing) flanking the inserted cDNA and visualizing the products on a 1.1% agarose/EtBr gel.

2.3. Sequencing of the *A. delacruzi* cDNA library

The *A. delacruzi* salivary gland cDNA library was plated on LB/MgSO₄ plates containing X- gal/IPTG, to an average of 250 plaques per 150mm Petri plate. Recombinant (white) plaques were randomly selected and transferred to 96-well MICROTTEST™ U Bottom plates (BD BioSciences, Franklin Lakes, NJ), containing 100 µl of SM buffer [0.1M NaCl; 0.01M MgSO₄; 7H₂O; 0.035M Tris–HCl (pH 7.5); 0.01% gelatin] per well. The plates were covered and placed on a gyrating shaker for 30 min at room temperature. The phage suspension was either immediately used for PCR or stored at 4°C for future use. To amplify the cDNA using a PCR reaction, 4 µl of the phage sample was used as a template. The primers were sequences from the TriplEx2 vector and named pTEx2 5seq (50-TCC GAG ATC TGG ACG AGC-30) and pTEx2 3LD (50- ATA CGA CTC ACT ATA GGG CGA ATT GGC-30), positioned at the 5' and the 3' end of the cDNA insert, respectively. The reaction was carried out in 96-well flexible PCR plates (Fisher Scientific, Pittsburgh, PA) using the TaKaRa EX Taq polymerase (TAKARA Mirus Bio, Madison, WI), on a Perkin Elmer GeneAmps PCR system 9700 (Perkin Elmer Corp., Foster City, CA). The PCR conditions were: one hold of 95°C for 3 min; 25 cycles of 95°C for 1 min, 61°C for 30 s; 72°C for 2 min. The amplified products were analyzed on a 1.5% Agarose/EtBr gel. Approximately 200–250 ng of each PCR product was transferred to Thermo-Fast 96-well PCR plates (ABgene Corp., Epsom, Surrey, UK) and frozen at –20°C, before cycle sequencing using an ABI3730XL machine.

2.5. Bioinformatic tools and procedures used

Expressed sequence tags (ESTs) were trimmed of primer and vector sequences, clustered, and compared with other databases. The BLAST tool (Altschul et al., 1990), CAP3

assembler (Huang and Madan, 1999), ClustalX (Thompson et al., 1997), and Treeview software (Page, 1996) were used to compare, assemble, and align sequences and to visualize alignments. For functional annotation of the transcripts we used the tool BlastX (Altschul et al., 1997) to compare the nucleotide sequences to the NR protein database of the National Center for Biotechnology Information (NCBI) and to the Gene Ontology (GO) database (Ashburner et al., 2000). The tool, Reverse Position Specific Blast (RPSBLAST) (Altschul et al., 1997) was used to search for conserved protein domains in the Pfam (Bateman et al., 2000), SMART (Letunic et al., 2002), Kog (Tatusov et al., 2003), SwissProt and Conserved Domains Databases (CDD) (Marchler-Bauer et al., 2002). We have also compared the transcripts with other subsets of mitochondrial and rRNA nucleotide sequences downloaded from NCBI. Segments of the three-frame translations of the EST (we did not use six-frame translations because the libraries are unidirectional) that start with a methionine within the first 300 predicted amino acids (AAs) and that have a minimum length of 40 AAs were submitted to the SignalP server (Nielsen et al., 1997) to help identify translation products that could be secreted. Functional annotation of the transcripts was based on all the comparisons above.

Following inspection of all these results, transcripts were classified as either Secretory (S), Housekeeping (H) or of Unknown (U) function, with further subdivisions based on function and/or protein families. Phylogenetic analysis and statistical neighbor joining (NJ) bootstrap tests of the phylogenies were done with the Mega package (Kumar et al., 2004). Comparisons of groups of ESTs to verify if there are significant differences in their distribution among species of ticks was performed with the χ^2 test followed by the Bonferroni correction procedure for multiple comparisons. A heat map of the levels of expression was constructed using Multi Experiment Viewer software and the number of similar ESTs expressed in each species. Data used in Supplemental Table 3 and in the heat map is derived from non-normalized cDNA libraries constructed by some of the authors with the same methodology employed for the *A. delacruzi* library. The EST sequences of this work have been submitted to DBEST (GenBank accessions JK816457–JK817576) and coding sequence to the TSA database (GenBank accessions JU962543–JU962580).

3. RESULTS AND DISCUSSION

3.1. Characteristics of the cDNA library

A total of 1147 clones were sequenced and used to assemble a database (see Supplemental Table S1 at <http://exon.niaid.nih.gov/transcriptome.html>) that yielded 923 clusters of related sequences, of which 866 contained only one EST. The consensus sequence of each cluster is named either a contig (deriving from two or more sequences) or a singleton (deriving from a single sequence). For simplicity's sake, this paper uses 'cluster' to denote sequences deriving both from consensus sequences and from singletons. The 923 clusters were compared using the program BlastX, BlastN, or RPSBLAST (Altschul et al., 1997) to the nonredundant (NR) protein database of the National Center of Biological Information (NCBI), to a GO database (Ashburner et al., 2000), to the CDD of the NCBI (Marchler-Bauer et al., 2002), and to a custom- prepared subset of the NCBI nucleotide database containing either mitochondrial or rRNA sequences.

Because the libraries used are unidirectional, the three- frame translations of the data set were also derived, and open reading frames (ORF) starting with a methionine and longer than 40 AA residues were submitted to SignalP server (Nielsen et al., 1997) to help identify putative- secreted proteins. The EST assembly, BLAST, and signal peptide results were loaded into an Excel spreadsheet for manual annotation, which can be browsed as the Supplemental Table S1.

Four categories of expressed genes derived from the manual annotation of the contigs (Table 1). The putatively secreted (S) category contained 5.7% of the clusters and 10.9% of the sequences, with an average number of 2.38 sequences per cluster. The housekeeping (H) category had 42.9% and 45.0% of the clusters and sequences, respectively, and an average of 1.31 sequences per cluster. Transcripts of transposable elements were represented by two sequences contained in two clusters. 51.3 percent of the clusters, containing 43.9% of all sequences, were classified as unknown (U), because no functional assignment could be made. This category had an average of 1.06 sequences per cluster.

It is noteworthy that the abundance of transcripts encoding putative secreted proteins in females of *A. delacruzi* is relatively smaller than that found in hematophagous females of *Rhipicephalus sanguineus* (21% of clusters and 26% of sequences; (Anatriello et al., 2010b), of *Rhipicephalus microplus* (11% of clusters and 10% of sequences; Maruyama et al., unpublished work), of *Amblyomma variegatum* (32% of clusters and 49% of sequences; (Ribeiro et al., 2011b), of *Ixodes scapularis* (28% of clusters and 29% of sequences; (Ribeiro et al., 2006), and of *Ornithodoros parkeri* (10% of clusters and 16% of sequences; (Francischetti et al., 2008a), being closer to the abundance of transcripts for putative secreted proteins of the bird-feeding soft tick *Argas monolakensis* (6% of clusters and 13% of sequences; (Mans et al., 2008).

3.2. Housekeeping (H) genes

The 397 clusters (comprising 520 EST) attributed to H genes expressed in the salivary glands of *A. delacruzi* were further characterized into 21 subgroups according to function (Table 2). In accordance with findings in other sialotranscriptomes of hematophagous arthropods (Francischetti et al., 2002; Ribeiro et al., 2004a; Ribeiro et al., 2004b) and as expected for an organ specialized in secreting polypeptides, among the larger sets of clusters two were associated with protein synthesis machinery (139 EST in 86 clusters) and energy metabolism (29 clusters containing 36 EST). Also among the larger sets was a group of 86 EST that grouped into 85 clusters, representing highly conserved proteins of unknown function, presumably associated with cellular function, but still uncharacterized. In *A. delacruzi* there is a greater proportion of sets in this category (21% of the clusters classified as housekeeping) than in other hard and soft ticks (*Argas monolakensis*, *Ornithodoros coriaceus*, *O. parkeri*, *Rhipicephalus sanguineus*, and *Ixodes scapularis* and *I. pacificus* for which transcriptomes are available (see <http://exon.niaid.nih.gov/transcriptome.html>). In these ticks the proportion of this set is 11.1%, 15.6%, 4.9%, 8.1%, 14.6% and 7.2%, respectively. They are named conserved proteins of unknown function in Supplemental Table S1, immediately preceding the clusters of the Unknown class. The complete list of all 397 gene clusters, along with further information about each, is given in Supplemental Table S1.

3.3. Possibly secreted (S) class of expressed genes

Inspection of Supplemental Table S1 indicates that several expressed genes contain a signal peptide and code for proteins with TIL-domains, a cystatin, a serpin, a lipocalin, a serum amyloid, a metalloprotease, a savignygrin-like protein, mucin, peritrophin and cuticle-like proteins, glycine-rich proteins, antimicrobials, a dioxygenase involved in catabolism of lysine, and a protein similar to a fragment of an HIV envelope glycoprotein found in *I. scapularis* (Ribeiro et al., 2006). There were also several contigs encoding proteins of unknown function. The contigs and the distribution of ESTs within are listed in Table 3.

3.4. Analysis of the *A. delacruzi* sialotranscriptome

Several clusters of sequences coding for housekeeping and putative secreted polypeptides indicated in Supplemental file S1 are abundant and complete enough to extract consensus

sequences of novel sequences. A total of 196 novel sequences, 22 of which code for putative secreted proteins, are grouped together in Supplemental file S2, available at <http://exon.niaid.nih.gov/transcriptome.html>.

3.5. Comparison of the sialotranscriptomes of hard and soft, adult female ticks

The differences in food sources between *A. delacruzi* females and hematophagous females of other species of soft and hard ticks could be reflected in their sialotranscriptomes. Therefore we examined the proportions of sets of ESTs encoding different proteins related to salivary gland function that are present in the sialotranscriptome of *A. delacruzi* females and in the published sialotranscriptomes of blood feeding females of *Ornithodoros parkeri*, *O. coriaceus*, *Argas monolakensis*, *R. sanguineus*, *Ixodes scapularis* and *I. pacificus*. The sialotranscriptome of *R. sanguineus* presents ESTs differentially distributed in the early and late phases of hematophagy that occur without and with the assistance of the male, respectively. The details of construction of each library are identical and can be found in the authors' publications (Anatriello et al., 2010a; Francischetti et al., 2005; Francischetti et al., 2009; Francischetti et al., 2008a; Francischetti et al., 2008b; Mans et al., 2008; Ribeiro et al., 2006; Ribeiro et al., 2011). An unpublished sialotranscriptome of *R. microplus* females, constructed with an identical strategy, was also used. The comparisons between species indicated that indeed some groups of sequences encoding similar putative biological functions were more or less abundant than expected from a random distribution, as evaluated by the χ^2 test (Supplemental Table 3). We found significant differential expression of genes between females of *A. delacruzi* and of blood feeders for putative proteins related to defense, to iron transport, to mucins and chitinases and to inhibitors of the homeostasis of vertebrate hosts as follows. A heat map of the distribution of similar ESTs within the selected transcriptomes is shown in Figure 1 and emphasizes their differential distribution according to the biology of the females, whether hematophagous or non-hematophagous; noteworthy are the reciprocal expression profiles of trypsin inhibitor-like, cysteine-rich domain family (TIL) proteins, serum amyloids, ferritins, mucins and chitinases, and a conserved protein similar to HIV env in *A. delacruzi* and inhibitors of host homeostatic proteins and glycine-rich proteins of hematophagous females.

The sialotranscriptome of *A. delacruzi* presented two contigs encoding a putative trypsin inhibitor-like, cysteine-rich domain family (TIL) protein, one of which contained 35 ESTs and the other, one EST. These ESTs were significantly more abundant in *A. delacruzi* than in the sialotranscriptomes of any of the other tick species examined in this study (Figure 1 and Supplemental Table 3). However, TIL-domain containing proteins and/or their encoding genes have been described in *O. coriaceus* (Francischetti et al., 2008b), *R. sanguineus* (Anatriello et al., 2010b), *R. microplus* [Maruyama et al, unpublished results; (Sasaki et al., 2008)] and *Ixodes scapularis* (Ribeiro et al., 2006) female ticks, among other species. The functions of TIL domain proteins in a non-hematophagous female tick such as *A. delacruzi* may be at least two. One possible role is suggested by data on BmSI-7, one of the TIL inhibitors described in *R. microplus*. This proteins inhibits subtilisin A and Pr1 proteases of entomopathogenic fungi (Cruz et al., 2010), with which *A. delacruzi* females have ample opportunities to become infected because fungi proliferate on bat guano (Lyon et al., 2004). Secondly, the fungi that proliferate on bat guano may be a source of nutrients and, indeed, many Acari are mycophagous, including some that eat fungi growing on bat guano (Estrada-Barcenas et al., 2010). Tick salivary TIL domain proteins may indirectly assist mycophagy by neutralizing harmful fungal enzymes.

The *A. delacruzi* TIL-domain containing polypeptide was aligned with other similar tick proteins obtained from work by Francischetti and colleagues (Francischetti et al., 2009) to produce the phylogram shown in Figure 2. It shows that the TIL-domain-containing protein of *A. delacruzi* has a close homology to a similar protein of a soft tick, and presents more

distant relationships with similar proteins of hard metastriate ticks; a TIL-domain-containing protein of *I. scapularis* is an outlier of the phylogram. The phylogram confirms that Antricola is phylogenetically closer to other Agasidae ticks than to Ixodidae ticks, especially prostriate Ixodidae. However, all proteins contained a framework motif of thirteen cysteines as well as a conserved and adjacent proline and glycine; soft and metastriate hard ticks present two additional conserved cysteines as well as a few other conserved amino acid residues.

ESTs encoding putative chitin-binding proteins or mucins (12 ESTs distributed in 6 contigs) were also present in the sialotranscriptome of *A. delacruzi* and were significantly more abundant than in the other ticks examined in this study with the exception of *O. parkeri* and females of *R. sanguineus* (Supplemental Table 3). Chitin is abundant in guano from insectivorous bats (Emerson and Roark, 2007) and it derives from bats' immense consumption of insects and/or from the fungi growing on their guano. Chitin-binding proteins may assist *A. delacruzi* as defensive proteins against chitin-containing pathogens such as fungi. Indeed, the expression of genes encoding chitin-binding proteins increases in several tissues of *Anopheles gambiae* when they become infected with *Plasmodium berghei*, including in their salivary glands (Dimopoulos et al., 1998). Mucins may also form a protective barrier to filter out the pathogens (Lehane, 1997) present in bat guano.

The sialotranscriptome of *A. delacruzi* also presented a contig containing 15 ESTs encoding a putative serum amyloid-like protein. Serum amyloid A (SAA) proteins are highly conserved among animals; they are found in chordates (Uhlir et al., 1994) and transcripts or proteins have been described to date in few invertebrates such as the water flea, *Daphnia pulex* (Colbourne et al., 2011), the sea cucumber *Holothuria glaberrima* (Santiago-Cardona et al., 2003), and in tissues of various species of ticks, including salivary glands of feeding females of *Dermacentor andersoni*, *O. coriaceus* and *O. parkeri* (Francischetti et al., 2009). The contig from *A. delacruzi* encoding a putative SAA is similar to one found in *O. parkeri* and another in *O. coriaceus*, however there are significantly less transcripts in the transcriptomes of these latter species (Supplemental Table 3). SAA is an apolipoprotein and it contains amphipathic helices similar to other apolipoproteins, motifs that may be responsible for their lipid-binding properties (Ohta et al., 2009). In vertebrates SAA proteins bind to serum lipids and are components of the acute phase response (Uhlir et al., 1994) and opsonins for Gram-negative bacteria (Shah et al., 2006). In invertebrates their function is unknown, however levels of SAA participate in the acute phase response of sea cucumbers injected with lipopolysaccharides. These features suggest a role for a SAA in innate immunity and in scavenging of lipid nutrients for *A. delacruzi*. The proportion of total lipids in dry matter and organic matter of guano from insectivorous bats is 20% and 24%, respectively (Emerson and Roark, 2007), a significant amount of this nutrient. The *A. delacruzi* serum amyloid-like protein was aligned with similar proteins from other ticks obtained from work by Francischetti and colleagues (Francischetti et al., 2009); Figure 3A) to produce the phylogram shown in Figure 3B. It shows that, again, *A. delacruzi* serum amyloid has a close homolog to a similar protein of a soft tick, but also that it presents relationships with similar proteins of both metastriate and prostriate hard ticks; another serum amyloid protein of a prostriate tick is an outlier of the phylogram. Again, *A. delacruzi* is phylogenetically closer to other Agasidae ticks than to Ixodidae ticks, especially prostriate Ixodidae. All proteins contained a framework motif of eight cysteines as well as additional conserved amino acids; the amyloid proteins of *A. delacruzi* and hard and soft ticks contained two conserved methionines and a conserved histidine and asparagine.

ESTs encoding proteins related to ferritin were represented by two clusters and one was abundantly expressed and contained 17 ESTs. Transcripts for ferritin are quite scarce or absent in the sialotranscriptomes obtained to date for female hard ticks, but are relatively

common in those of soft ticks. If *A. delacruzi* females indeed ingest guano, the abundance of transcripts for ferritin could be a consequence of the high amounts of iron (Fenolio et al., 2006) and of pathogens present in bat guano. Excess amounts of iron are toxic for the host, furthermore, iron is an essential element for pathogens (Schaible and Kaufmann, 2004). In vertebrates ferritins are part of the acute phase response in order to sequester iron from invading pathogens and to detoxify excess amounts (Harrison and Arosio, 1996). Consequently, the synthesis of ferritin is inversely proportional to levels of seric iron and secreted ferritin exerts the iron-withholding strategy of innate immunity. Acute phase response proteins are highly conserved and are also described in arthropods. For example, expression of ferritin increases in echinoderm coelomocytes activated with interleukin-1 α or LPS suggesting that in this species ferritin is involved with changes in iron concentrations (Beck et al., 2002). Chinese mitten crabs also increase the transcription of ferritins in their hemocytes when challenged with *Listonella anguillarum* bacteria (Kong et al., 2010).

A. delacruzi also expressed seven transcripts encoding a protein conserved in ticks that is similar to a secreted protein previously described in *O. parkeri* and in *I. scapularis*. As noted by Francischetti and colleagues (Francischetti et al., 2008a), it is significantly similar to a segment of the envelope protein of human and simian immunodeficiency virus associated with viral cell entry and apoptotic-inducing activities. The role of these proteins in tick saliva remains to be determined. Figure 4 shows the clustal alignment and bootstrapped phylogram of this conserved tick salivary secreted protein family.

Noteworthy absences or underrepresented transcripts in the transcriptome of *A. delacruzi* were transcripts encoding Kunitz-containing protease inhibitors, which are abundant in salivary glands of all hematophagous females (Supplemental Table 3 and Figure 1), as well as the absence of basic tail protein family members, abundant in soft ticks and prostriates. Members of this latter family in *I. scapularis* have been shown to inhibit clotting by their anti-trombin activity, and inhibition of their expression by RNA interference impaired blood feeding (Narasimhan et al., 2002; Narasimhan et al., 2004). Transcripts for lipocalins, metalloproteases and glycine-rich proteins, among other inhibitors of host homeostatic proteins, were significantly less abundant in salivary glands of female *A. delacruzi* when compared to the number of similar transcripts found in salivary glands of blood-feeding females. The *A. delacruzi* transcriptome contained one transcript encoding a protein similar to a savignygrin first found in *O. savignyi* (Mans et al., 2002b) and also abundantly expressed in *O. parkeri* (Francischetti et al., 2008a) and *A. monolakensis* (Mans et al., 2008). Savignygrin from these species of ticks contains an RGD disintegrin motif, but the sequence from *A. delacruzi* does not. All of these findings indicate that females of *A. delacruzi* are not equipped for hematophagy. Although being *bona fide* hematophagous instars, the data shown in Figure 1 and Supplemental Table 3 indicates that *R. microplus* females, nevertheless, express significantly less transcripts for Kunitz-containing putative protease inhibitors and lipocalins than females from other species of hematophagous ticks. This is because in this monoxene species the male is covering the needs for inhibition of hemostasis and inflammation during the female's blood meal (unpublished data).

3.6. Adaptation of ticks to alternative sources of food

The more than 1,100 species of bats represent approximately 20% of all mammalian species, and are among the most diverse, abundant, and widely distributed group of mammals (Simmons, 2005a; Tudge, 2000). Bats are hosts to at least 18 families and over 1,000 species of Acari, a diversity which surpasses that of all other mammalian Acari (2010). Several of these ectoparasites are restricted to bats, the case of the genus *Antricola*. Incidental to specialization of *Antricola* spp. for bats is the nature of their food source. Similarly to trombiculid mites (chiggers), only the first developmental stages of *Antricola* spp. are known to ingest blood under natural conditions (Labruna et al., 2011; Labruna et al., 2008).

If blood were the only source of nutrients for *Antricola* one would have to explain the feat of a single blood meal of a larva providing energy for several molts and for oviposition by females.

Adult *Antricola* spp. have non-functional mouthparts resembling primitive mesostigmatid-type chelicera and most likely do not feed on vertebrate hosts (Klomp et al., 1996). The mouthparts are compatible with scavenging because the hypostome is very small and without teeth. In the present work we have shown that the repertoire of salivary molecules of *A. delacruzi* is similar to what has been proposed to compose the sialome of primitive (female) ticks: it contains transcripts for proteases, antimicrobials and protease inhibitors (Mans, 2011), although transcripts for proteases are conspicuously scarce. This raises the issues of what *A. delacruzi* females scavenge and what this behavior represents, a primitive or a regressed feeding strategy. Herein, we make a case for guanophagy and for *A. delacruzi*'s place in the evolutionary history of ticks.

3.6.1. Bat guano and feeding behaviors—Feces of mammals are usually poor in nutrients, but because bats eat up to one-half of their body weight per day [(Kunz et al., 2011); Allen, 1939, cited in (Klomp, 1965)] and defecate an average of 60 times per day (Klomp, 1965) and because insectivorous bats have very short digestive tracts [Allen, 1939, cited in (Klomp, 1965)], rapid intestinal transit times (Buchler, 1975; Klomp, 1965; Mitzutani H, 1992) and a digestive efficiency of approximately 80–90% for lipids and protein and approximately 60% for chitin (Stalinski, 1994; Webb PI, 2009), their guano is very rich in nutrients. Microbial biofilms that form on guano might also supply nutrients (Fenolio et al., 2006). A few studies on the composition of guano produced by insectivorous bats have been made. One such study shows that it contains twice the amount of protein, 27 times the amount of calcium and 60 times the amount of iron as a commercial hamburger sandwich (Fenolio et al., 2006) and another that the fecal dry and organic matter contain 20% and 24% of lipid, 44% and 53% of carbon, 8% and 9% of nitrogen and 8% and 4% of phosphorous, respectively (Emerson and Roark, 2007). Chitin is, obviously, abundantly ingested by insectivorous bats and this may explain the significantly higher amount of nitrogen in guano produced by this group of bats than by fruit bats (Emerson and Roark, 2007). By means of the action of chitinases produced by intestinal bacteria present in insectivorous bats, chitin can be a source of nutrients to bats (Whitaker et al., 2004), but also to guano-feeders. Bat guano might also contain abundant amounts of taurine, a component of the bile salts excreted in feces. Indeed, the sialotranscriptome of *A. delacruzi* contains a contig (contig 738 with one transcript, Supplemental Table 1) significantly similar to taurine catabolism dioxygenase (tauD) required for the utilization of taurine (2-aminoethanesulphonic acid) as a source of sulphur (vanderPloeg et al., 1996). The nutritional value of bat guano is attested to by the fact that deposits of it support a great variety of cave-adapted troglodytic biota (Harris, 1970; Poulson TL, 2000) including fungi (Kajihiro, 1965) and mites (Radovsky and Krantz, 2003). Thus, in cave ecosystems guano may be an alternative source of energy to blood and it has been shown to indeed influence physiological specialization (Gnaspini P, 2000; Harris, 1970; Howarth, 1983; Poulson TL, 2000). Fenolio and colleagues (Fenolio et al., 2006) reported that cave-adapted salamanders eat bat guano and performed isotope assays to determine that guano is assimilated into salamander muscles. Invertebrate communities associated with bat guano increase in density after the bats appear and deposit fresh guano (Poulson TL, 2000).

3.6.2. Evolutionary considerations on regression of feeding behaviors—De la Cruz and Estrada-Peña (De la Cruz and Estrada-Peña, 1995) speculated that nymphal and adult stages of *Antricola* spp. could feed upon an unknown food source in bat guano, a substrate on which its nymphal and adult stages are always found in caves (Labruna et al., 2011; Labruna et al., 2008). The nutritional value of guano presented above and our data

sustain this possibility. Our findings must now be discussed in the light of knowledge on the evolution of Acari and ticks. Estrada-Peña and colleagues (Estrada-Peña et al., 2008) suggested that *A. delacruzi* may form a cluster of tick species that are basal to the Ixodida as a first evolutionary step from the Holothyrida, a group of free-living scavenging mites that live on dead arthropods. However, genetic data and phylogenetic analysis do not support the conjecture that *Antricola* is primitive to all other ticks. In fact, recent data indicate that *Antricola* is a genus derived within the Ornithodorinae (Dantas-Torres et al., 2011; Mans et al., 2011). The hereby reported sialome of *A. delacruzi* is compatible with a phylogram of Argasidae (Dantas-Torres et al., 2011), made by comparison of mitochondrial 16S rRNA genes. It suggests, albeit with a bootstrap support of 50%, that *Antricola* spp and *Nothoaspis amazoniensis* (Nava et al., 2010)[82], are monophyletic from a common ancestor with a hematophagous adult. Since the females of *Antricola* spp and *N. amazonensis* are all non-hematophagous it is possible that they are indeed monophyletic and that non-hematophagous adults evolved from their ancestral species due to alternative food sources in the ecosystems. The ancestral blood feeding ticks had to adapt to the different homeostatic systems of diverse vertebrates and this occurred independently in the soft and hard tick families through lineage specific expansions of gene duplications (Mans et al., 2008). In the same manner that ticks adapted to different host homeostatic systems they possibly adapted to sources of food other than blood.

Given what is known about evolution of ticks, guanophagy represents a regression from hematophagy to scavenging since the female stage of >95% of nearly 900 known tick species are bona fide blood feeders, a plesiomorphic or ancestral feature relative to *Antricola* spp. The non-bloodfeeding habits of *Antricola* sp. are apomorphic, or recent, and distinguish it from the blood feeders that share their ancestors. Other examples of regression of hematophagy in parasites exist such as in the entirely predaceous mite *Mitonyssoides stercoralis* (Howarth, 1983), the more advanced developmental stages of which are also always found on bat guano and to date the sole example of a non-parasitic Macronyssidae mite. Grimaldi and Engel (Grimaldi D, 2005) have proposed that all the Culicomorpha, which includes all mosquitoes but also the non blood feeding Chironomidae, have evolved from hematophagous ancestors. *Toxorhynchites amboinensis* is an autogenous mosquito, the larvae of which are predatory, feeding on larvae of other mosquitoes. Similarly to *A. delacruzi*, its sialotranscriptome reflects this (Calvo et al., 2008).

3.6.3. Evolutionary forces for speciation of ticks—The data generated herein may also help resolve the questions about whether ticks evolved by co-speciating with their hosts as proposed by Hoogstraal and Aeschlimann (Hoogstraal H, 1982) or whether ecological specificity determined divergence of species as proposed by Klompen and colleagues (Klompen et al., 1996). This work argues that both host and ecosystem were associated forces: nutrient-rich bat guano is just another (processed) host tissue for *A. delacruzi* and bats shape the tick's ecosystem through their digestive physiology and guano. The question remains about why molted *A. delacruzi* nymphs did not return to their bat hosts, but remained on guano while other tick species that feed on bats complete the traditional life cycle of ticks. Bats are heterothermic and body temperatures change according to the level of activity, but since the roosting caves for hosts of *A. delacruzi* maintain high temperatures (37° C) it is unlikely that host body temperature plays a role in this regression of parasitism. Furthermore, there are many species of hematophagous ticks that feed on poikilothermic hosts. A study of bat behavior (could they prey on larger nymphal and adult ticks, but not on the minute larvae?) may explain the switch in feeding habits of this species of tick. Another question remains, that of whether *A. delacruzi* underwent allopatric speciation due to a refuge effect of caves (Vandel, 1965) on a tick already adapted to guano or underwent parapatric speciation due to the adaptative shift (Howarth, 1972) of a hematophagous species at all development stages to a new, guano-rich habitat.

Antricola adults and nymphs do not feed on blood, but larvae do. Therefore the ability to feed on blood has been retained to a certain extent in this genus. The main question now raised is whether the sialome of larval ticks have a similar composition to those of nymphs or hard ticks. If all the blood-feeding related families and proteins involved in host modulation by argasids have been retained in larvae that still feed successfully on bats this would then mean that the proteins/families are still present in the genomes of these ticks, but that their expression is differentially regulated in various lifestyles. Does the sialome of females of *A. delacruzi* indeed reflect a return to the ancestral primitive non-hematophagous state? This conjecture can be made, but will depend on an assumption that guano feeding and ancestral scavenging are related. Given that bats originated 60MYA (Simmons, 2005b) and ticks somewhere over 100MYA it would suggest that the ancestral scavenging mode was not related to guano-like feeding. The relative limited number of species involved in the lifestyle of *Antricola*, most derived from the New World and their terminal place on the tick tree, would suggest that guano-feeding is a derived trait and that any special adaptations to this lifestyle might be unique to this genus. Furthermore, since the phylogram generated by Dantas-Torres and colleagues (Dantas-Torres et al., 2011) places hematophagous species as basal to *A. delacruzi*, and since members of *Antricola* are exclusive to bats, which evolved less than 60 MYA, it will be interesting to examine if the evolutionary history of these taxa coincides.

3.7. Final considerations

In this work we describe the sialotranscriptome of *A. delacruzi* females collected on bat guano and compare it with similarly constructed transcriptomes from salivary glands of soft and hard female ticks collected when they are taking a blood meal. The transcriptional profiles of the different sialotranscriptomes indicate that indeed *A. delacruzi* females do not feed on blood because their salivary glands do not express or express very few transcripts coding for several key anti-homeostatic proteins. Conversely, the sialotranscriptome of *A. delacruzi* females contains signature transcripts that are compatible with a food source consisting of iron- and chitin-rich bat guano and with defense against the biota that grows on it. Interestingly, the soft tick *O. amblus* is found on sea bird guano, but all developmental stages are hematophagous (Clifford CM, 1980). In conclusion, in terms of nourishment/energy for oviposition, bat guano is possibly nutritionally equivalent to blood and it seems to have influenced the physiological specialization of tick saliva. It will be interesting to examine the sialotranscriptomes of species of ticks with different biologies such as females of *Nothoaspis amazoniensis* and ticks that feed on amphibians and reptiles in order to further test the hypotheses put forth here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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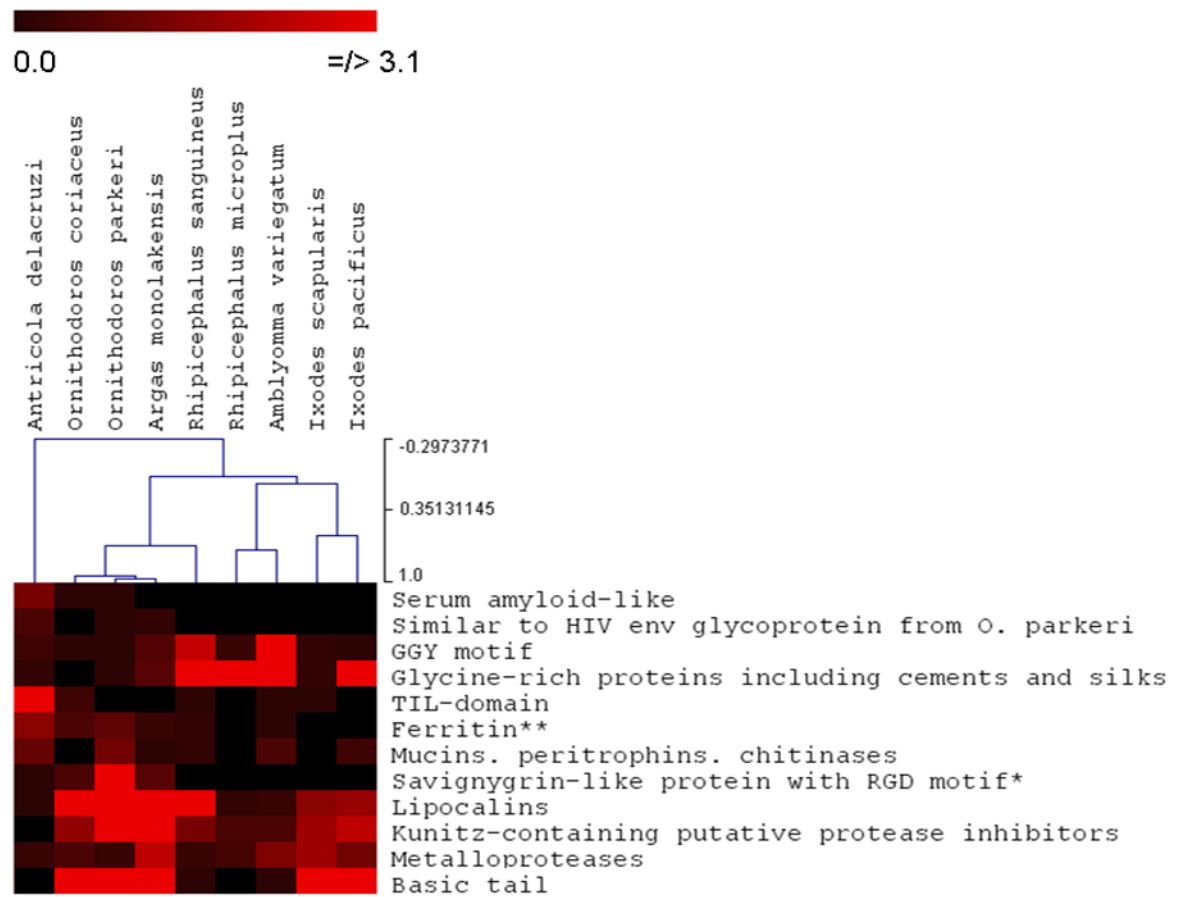
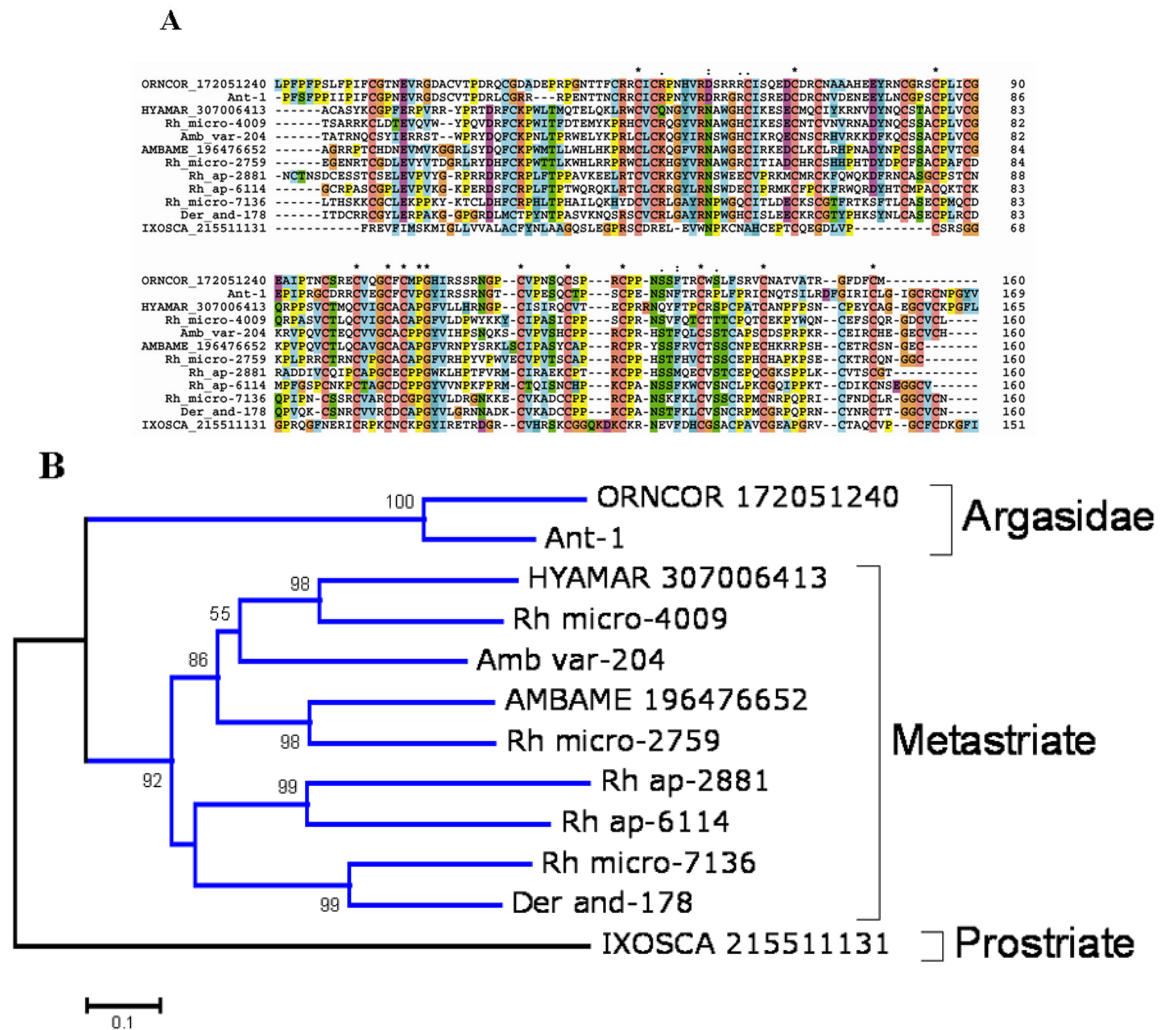
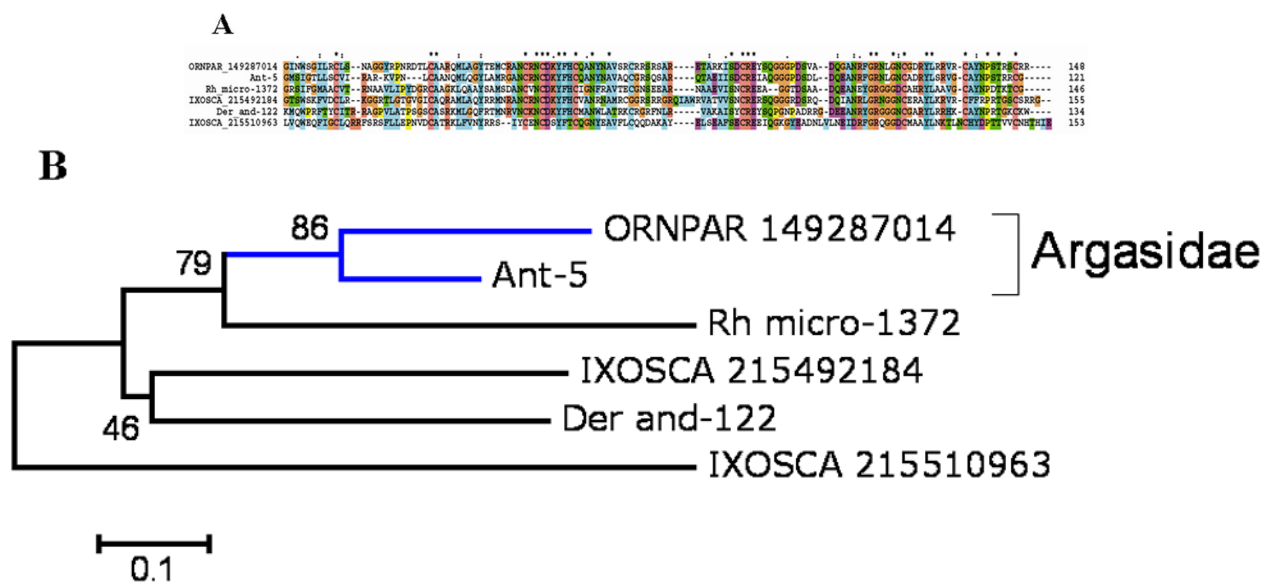


Figure 1.

A heat map of the most abundantly expressed transcripts in *A. delacruzi* females and of the most abundantly expressed transcripts in hematophagous female ticks of genes encoding putative inhibitors of proteins involved in host homeostasis. Data is derived from non-normalized cDNA libraries constructed with the same methodology employed for the *A. delacruzi* library.

**Figure 2.**

Tick TIL domain-containing proteins. (A) Clustal alignment. Symbols over the figure indicate (*) Amino acid identity, (:) similarity and (.) less similarity. (B) bootstrapped phylogram (10,000 iterations) of the alignment in (A). Sequences deposited at NCBI are represented by six capital letters deriving from the genus and species name followed by their GenBank accession number. Remaining sequences were derived from analysis of publicly available EST's and described in a previous review (Francischetti et al. 2009). Values near nodes indicate bootstrap support above 50%. Smaller values are not represented. The bar at the bottom indicates 10% amino acid divergence. Species related to acronyms for naming sequences used to construct the alignment and phylogram are: ORNCOR, *O. coriaceus*; Ant: *A. delacruzi*; HYAMAR: *Hyalomma marginatum*; Rh micro: *R. microplus*; Amb var: *Amblyomma variegatum*; Rh ap: *R. appendiculatus*; and Der: *Dermacentor andersoni*; IXOSCA: *I. scapularis*.

**Figure 3.**

Tick amyloid salivary secreted protein family, with 15 ESTs on *Anticolas delacruzi*. (A) Clustal alignment. (B) Bootstrapped phylogram. For other information, see Figure 1. Species related to acronyms for naming sequences used to construct phylogram are: ORNPARG, *O. parkeri*; Ant: *A. delacruzi*; Rh micro: *R. microplus*; IXOSCA: *I. scapularis*; and Der: *Dermacentor andersoni*.

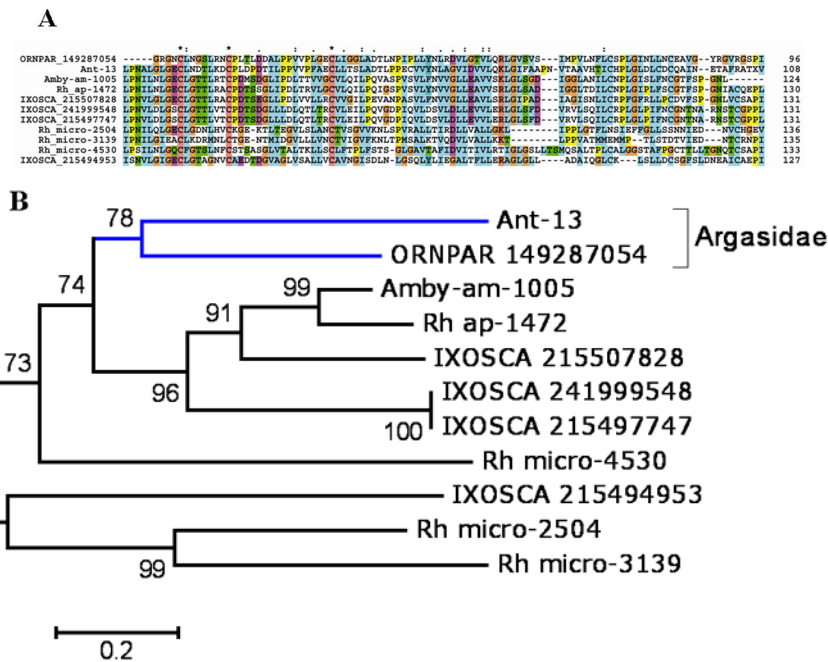


Figure 4.

Conserved tick salivary secreted protein family, similar to HIV env glycoprotein, with 7 ESTs in *Antricola delacruzi*. (A) Clustal alignment. (B) Bootstrapped phylogram. For other information, see Figure 1. Species related to acronyms for naming sequences used to construct phylogram are: Ant: *A. delacruzi*; ORNP_149287054, *O. parkeri*; Amby am: *Amblyomma americanum*; Rh ap: *R. appendiculatus*; Rh micro: *R. microplus*; IXOSCA: *I. scapularis*.

Table 1

Abundance of transcripts according to functional class

Class	Clusters ^a	Sequences	Sequences/cluster
Secreted	50 (5.7)	119 (10.9)	2.38
Housekeeping	397 (42.9)	520 (45.0)	1.31
Transposable element	2 (<0.01)	2 (0.2)	1.00
Unknown	474 (51.3)	507 (43.9)	1.06
Total	925	1155	1.25

^aNumber (percent of total).

Table 2

Functional classification of housekeeping transcripts

Function	Clusters	Sequences	Sequences/cluster
Protein synthesis	86	139	1.6
Unknown conserved	83	86	1.0
Signal transduction	33	41	1.2
Energy metabolism	29	36	1.2
Protein modification	27	29	1.1
Nuclear regulation	20	20	1.0
Transcription machinery	19	19	1.0
Cytoskeletal	18	48	2.7
Transcription factor	12	14	1.2
Protein export	10	10	1.0
Carbohydrate metabolism	10	10	1.0
Lipid metabolism	8	8	1.0
Transporters/Storage	8	27	3.4
Immunity	8	8	1.0
Amino acid metabolism	6	6	1.0
Detoxification	5	5	1.0
Extracellular matrix and adhesion	5	5	1.0
Proteasome machinery	4	4	1.0
Metabolism, nucleotide	4	4	1.0
Metabolism, intermediate	1	1	1.0
Nuclear export	1	1	1.0
Total	397 (42.9)	520 (45.0)	

Table 3

Functional classification of transcripts coding for secreted proteins

Family	Clusters	Sequences	Sequences/cluster
TIL domain-containing	2	36	18
Cystatin *	1	1	1
Serpin *	1	1	1
Similar to amyloid protein found in <i>Ornithodoros sialome</i>	1	15	15
Salivary lipocalin	1	1	1
Similar to savignygrin, but without RGD motif	1	1	1
Mucin/peritrophin/cuticle-like	6	12	2
Enzymes possibly associated with salivary function	2	4	2
Glycine-rich proteins	3	3	1
Possible antimicrobials	5	9	
Similar to <i>Ixodes scapularis</i> secreted protein and to HIV envelope glycoprotein	1	7	7
Other possible secreted peptides	26	29	1.1
TOTAL	50	119	

* may be housekeeping