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A deep insight into the whole transcriptome of midguts, ovaries and salivary glands of the *Amblyomma sculptum* tick

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ABSTRACT

Ticks stand out as the second most important vector of human pathogens and their control is a key issue for public health strategies. The success of these arthropods to parasitize a large range of vertebrate hosts and their importance as a vector of rickettsiosis are intimately related with the physiology of its internal organs. The midguts, ovaries and salivary glands all participate as key sites for rickettsial entry into tick hemolymph, ovarian amplification and transmission to vertebrate hosts, respectively. Although transcriptomic efforts have documented several tick sialomes aspects, very little is known about the repertoire of genes that orchestrate the physiology of midguts and ovaries. The aim of this study was to construct a deep catalogue from the repertoire of genes and putative proteins expressed in the selected internal organs of *Amblyomma scultpum*, a key vector of spotted fever rickettsiosis, through the employment of a RNAseq strategy. A total of 200 million reads derived from midguts, ovaries and salivary glands and sequenced by Illumina HiSeq, Ion Torrent PGM and 454 pyrosequencing, were assembled into contigs. The mapping of the contigs allowed for the identification of 25,569 CDS expressed in midguts, 21,230 in the ovaries and 10,697 in salivary glands. In depth analysis of the transcriptomes revealed several organ specific metabolic processes that expand the knowledge base of tick biology and its role in infectious diseases. The annotation approach used in this work facilitated the discovery of a higher number of novel genes and processes and gives insight into the roles that they play in the *A. scultpum* transcriptome.

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1. Introduction

Ticks are obligate hematophagous arthropods (Order Acari, Class Arachnida), and they are widespread in the most diverse ecosystems of the planet [7,22,56]. With their ability to parasitize a wide variety of vertebrate hosts, these arthropods are the second most important vector of pathogenic agents for humans, second only to mosquitoes [26,84].

Amblyomma sculptum is one of the main species of ticks of medical and veterinary importance. It is the main vector of spotted fever rickettsiosis in a large region of the Americas, from the southern United States to northern Argentina [1,8,9,10,53,59,65]. This specie was previously described as *Amblyomma cajennense* and was recently reclassified taxonomically and renamed to *A. sculptum* [59] due to the morphological differences in tick populations from many regions of the American continent. It presents an aggressive form of parasitism as it naturally infests a wide range of hosts, including humans and wild and domestic animals [47,48,50,67].

Ticks depend on the adaptation of their mouth structures and changes in the gene expression patterns of salivary glands during the blood feeding and engorgement processes [3,44,52,85]. The intestinal tract of ticks also is an important interface with the external environment, since it is the site where proteins and other nutrients from the ingested blood are metabolized [70]. In addition, this organ serves as the main entry pathway for pathogens. The epidemiological implications of these functions have been studied previously in the midgut infection of *Ixodes scapularis* by *Borrelia burgdorferi* [58,62]. The tick's ovary is the most important organ in the interface between the tick and rickettsia as it plays a role in the transovarian transmission and amplification

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of rickettsiae in the environment [69]. Exploring the molecular processes of this organ would constitute an essential step for the epidemiology of *A. sculptum*, and it could support improved strategies for the biological control of this important tick species.

In this context, the field of transcriptomics has emerged in recent years as a promising strategy to discover new genes and peptides from tick tissue [4,16,28,30,79]. It has been particularly useful for the discovery of pharmacological peptides with anti-tumoral activities [18]. Although three previous works have covered *A. sculptum* salivary transcriptomes or sialomes [6,30,52], little information about the transcriptomes of *A. scultpum* ovaries and midguts has been available until this study. Since the transcriptomic repertoire modulating the biological and physiological processes in A. sculptum midguts and ovaries remains understudied, our objective was to construct a reference transcriptome for the midguts (MDG), ovaries (OVA), and salivary glands (SG) of *A. scultpum*. This information is important to elucidate the molecular details about the biology of one of the most important ticks regarding rickettsiology in Central and South America.

2. Materials and methods

2.1. Tick feeding experiments, dissection of internal organs and total RNA isolation

Tick specimens of *A. sculptum* (specific pathogen free) were obtained from the colony of the Ixodology Laboratory of the Federal University of Uberlandia, Brazil. The colony was developed from specimens isolated from the same region of the Brazilian Pantanal region [30]. The specimens were maintained in a BOD incubator, according to previously described protocols [46]. For the midgut transcriptomes, two groups of 75 *A. sculptum* couples (one group previously infected and another uninfected with *R. amblyommii*) were fed on rabbits for a period of 3–4 days for males and 8–10 for females. The uninfected male ticks were utilized solely to fertilize the *A. sculptum* female specimens for both treatments (infected and uninfected). However, the females in both of these groups were allowed to feed until complete engorgement (8–10 days of feeding) in order to evaluate the oogenesis/vitellogenesis processes. The female ticks from both the infected and uninfected groups were dissected and their internal organs collected according to Maruyama et al. [52] in RNAse free conditions. The collected tissues were maintained in RNA Later (Ambion®, USA) at −80 °C. The total RNA extraction of the samples was carried out using the RNAqueous® RNA extraction kit (Ambion®, USA) according to the manufacturer's recommendations. The RNA quality and concentration measurements were carried out with NanoDrop 2000 (ThermoScientificTM, USA) and Bioanalyzer Agilent 2100 (Agilent Technologies, Santa Clara, USA) at the Center of Biomolecules, Federal University of Vicosa (UFV), Brazil. All the procedures in this work were approved by the Animal Experimentation Ethics Committee of the UFV, under protocol number 07/2012, according to the Ethical Principles on Animal Research established by the Brazilian College for Animal Experimentation (COBEA) and current Brazilian legislation.

2.2. cDNA libraries, NGS sequencing and availability of datasets

The cDNA libraries representative of the two ovary samples and two midgut samples from the infected and uninfected groups were constructed using the cDNA Synthesis Kit (Clontech, Carlsbad, USA) according to the manufacturer's instructions. The libraries were sequenced using the Illumina HiSeq 2000 system. For the transcriptomes derived from the midguts, we employed the term “mialome”, which was first used by Anderson et al. [4]. An additional cDNA library constructed from the total RNA from uninfected *A. sculptum* females was sequenced with the Ion Personal Genome Machine® (PGM™, USA) System, according to the manufacturer's protocols.

The ovary and midgut reads generated in this work were deposited in the NCBI under the BioProject accession number PRJNA309641. To improve the transcriptome assembly, an additional, previously published dataset from *A. sculptum* sialomes (SG) [30], was downloaded from the Sequence Read Archive (SRA) database under accession SRX498136 and utilized as a template.

2.3. De novo assembly and gene prediction by Augustus

The sequenced reads were trimmed for quality and filtered using the CLC Genomics Workbench package, version 8.0 (CLC Bio, Denmark). All the libraries were grouped and de novo assembled in contigs using default parameters, including automatic detection of bubble and k-mer sizes. After the de novo assembly, all reads from each one of the 9 libraries were mapped against the generated contigs, with a percentage of mapped reads higher than 90% for all nine libraries, which corroborates the accuracy of the de novo assembly step (data not shown).

The CDS were predicted using Augustus version 2.5.5 (http://augustus.gobics.de) using an intronless gene model and the *Ixodes scapularis* trained dataset. This particular approach is considered to be more accurate in the gene prediction from eukaryotic sequences [75,76,74,83]. The trained data was produced from the latest *Ixodes scapularis* structural genome available at the Ensembl Metazoa database (http://metazoa.ensembl.org/Ixodes_scapularis/Info/Index). To detect the differences between the CDS expressed in each *A. sculptum* organ (MDG, OVA or SG), the reads of each library were mapped for the whole transcriptome using the CLC Genomics Workbench 8.0 package (CLC Bio, Denmark).

2.4. Functional annotation of CDS and mapping of the reads

The CDS were functionally annotated using BLAST searches, including BLASTp, BLASTx, RPSBLAST and BLASTn algorithms [2]. These searches were performed considering an E-value cut-off of $1E^{-04}$, as previously proposed [41]. The following databases were used as reference: (1) the non-redundant protein database (NR) of the National Center for Biology Information (NCBI); (2) UniProt Knowledge base; (3) NCBI-Acari; (4) Conserved Domains Database (CDD); (5) Clusters of Orthologous Groups (COG); (6) EuKaryotic Orthologous Groups (KOG); (7) The Protein Families (PFAM); (8) Simple Modular Architecture Research Tool (SMART) SMART; (9) UNIPROT-Acari; (10) *Bombyx mori*; (11) Chelicerata; (12) rickettsiae; (13) *Amblyomma americanum*; (14) *A. cajennense* (*A. sculptum* complex); (15) *A. maculatum*; (16) *A. parvum*;(17) *A. triste*; (18) Argas; (19) Dermacentor; (20) *I. scapularis*; (21) *I. ricinus*; (22) *Rhipicephalus* and (23) *Haemaphysalis*. In addition, we predicted signal peptides and transmembrane domains. Subcellular localization and related metabolic pathways were addressed by using the SignalP, TMHMM, TargetP and KEGG databases, respectively. For the final annotation, the following parameters were considered: (1) Minor E-value ($<1E^{-04}$); (2) Higher score and (3) key-words from the main blast results (KOG, COG, GO, SwissProt, UniprotKb, Acari, etc). The annotation of all the CDS derived from the whole transcriptome of *A. sculptum*'s internal organs was listed in a spreadsheet, which can be explored on a large scale with the Visual Basic software, as has been done in previous projects [30,33].

For the gene expression comparisons to determine the number of CDS expressed in each organ (MDG, OVA and SG), we mapped the reads derived from each transcriptome against the reference transcriptome (27,308 CDS), using the default settings of the CLC Genomics Workbench 8.0 package. The results of the mapping step were organized in conjunction with the annotation results in an Excel spreadsheet, as established in previous studies [30,43]. The mapped reads from each transcriptome were merged manually according to the organ of origin (MDG, OVA and SG). The normalization of the transcriptomes was performed using the GFOLD software and the RPKM calculus algorithm for each CDS expressed in each transcriptome

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