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Gene expression profiling of adult female tissues in feeding *Rhipicephalus microplus* cattle ticks ☆

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ABSTRACT

The southern cattle tick, *Rhipicephalus microplus*, is an economically important pest, especially for resource-poor countries, both as a highly adaptive invasive species and prominent vector of disease. The increasing prevalence of resistance to chemical acaricides and variable efficacy of current tick vaccine candidates highlight the need for more effective control methods. In the absence of a fully annotated genome, the wealth of available expressed sequence tag sequence data for this species presents a unique opportunity to study the genes that are expressed in tissues involved in blood meal acquisition, digestion and reproduction during feeding. Utilising a custom oligonucleotide microarray designed from available singletons (BmiGI Version 2.1) and expressed sequence tag sequences of *R. microplus*, the expression profiles in feeding adult female midgut, salivary glands and ovarian tissues were compared. From 13,456 assembled transcripts, 588 genes expressed in all three tissues were identified from fed adult females 20 days post infestation. The greatest complement of genes relate to translation and protein turnover. Additionally, a number of unique transcripts were identified for each tissue that relate well to their respective physiological/biological function/role(s). These transcripts include secreted anti-hemostatics and defense proteins from the salivary glands for acquisition of a blood meal, proteases as well as enzymes and transporters for digestion and nutrient acquisition from ingested blood in the midgut, and finally proteins and associated factors involved in DNA replication and cell-cycle control for oogenesis in the ovaries. Comparative analyses of adult female tissues during feeding enabled the identification of a catalogue of transcripts that may be essential for successful feeding and reproduction in the cattle tick, *R. microplus*. Future studies will increase our understanding of basic tick biology, allowing the identification of shared proteins/pathways among different tissues that may offer novel targets for the development of new tick control strategies.

1. Introduction

The southern cattle tick, *Rhipicephalus microplus*, is a major pest of cattle in the tropical and sub-tropical regions of the world including the South Americas, southern Asia, Madagascar and the southern and eastern coasts of Africa (Lynen et al., 2008). This species is regarded as the most economically devastating tick species worldwide due to three main factors (Guerrero et al., 2006). Firstly, *R. microplus* is a highly adaptable species that is spreading rapidly to occupy previously unaffected areas, such as the Ivory Coast in western Africa and the Limpopo province in South Africa, even displacing endemic tick species (Tønnesen et al., 2004). Secondly, *R. microplus* is a well described vector for the causative agents of Asiatic redwater (*Babesia bovis*) and bovine anaplasmosis (*Anaplasma* spp.) (de la Fuente et al., 2007; Madder et al., 2007; Lynen et al., 2008). Thirdly, an increase in resistance to all major classes of acaricides has been reported for this species, as well as the occurrence of a strain resistant to the Bm86-based tick vaccine (de la Fuente et al., 2000; Rajput et al., 2006; Li et al., 2007).

Due to the tremendous impact that this species has on animal health, rationales were provided for whole genome sequencing of *R. microplus* (Guerrero et al., 2006). The genome size of the cattle tick has been estimated at 7.1×10^3 megabases with approximately 60% constituting repetitive sequences (Ullmann et al., 2005; Pagel van Zee et al., 2007) and assembly of the genome is currently in its primary stages (Moolhuijzen et al., 2011; Bellgard et al., 2012). However, the BmiGI (*Boophilus microplus* Gene Index) database of 42,512 expressed sequence tags (ESTs) that was derived from various tissues, life stages and geographical strains as

☆ *Note:* The microarray and sequence data related to this work is available via the NCBI GEO database, GEO accession No. GSE35867.

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well as larvae exposed to various temperatures, host odors and acaricides is available for analysis (Wang et al., 2007).

The BmiGI has been used successfully in high-throughput DNA microarray analyses investigating acaricide-induced gene expression in larvae (Saldivar et al., 2008), organ-specific responses to pathogen infection in male adults (Mercado-Curiel et al., 2011), the transcriptional effects of RNA interference (RNAi)-mediated gene silencing in adult females (Lew-Tabor et al., 2011) as well as responses in feeding larvae and adult females on different cattle species (Rodriguez-Valle et al., 2010). The vast number of genes that are unannotatable, however, limit the full impact of these studies. The latter is largely due to two factors. Firstly, there is considerable evolutionary distance between ticks and other model organisms, limiting homology-based gene predictions, and secondly limited functional protein data of tick protein sequences is available (Hill and Wikel, 2005; Pagel van Zee et al., 2007).

Papers have been published describing the various sialomes of both hard and soft tick species (reviewed by Mans et al., 2008). However, little is known about the gene expression profiles in the various tissues of feeding adult ticks, especially those involved in blood meal acquisition (salivary glands), digestion (midgut) and reproduction (ovaries). A study using EST sequencing showed that a number of genes are uniquely expressed in both salivary glands and ovaries of feeding, female *R. microplus* (de Miranda Santos et al., 2004). More recently, DNA microarray analyses of the transcriptomes of adult male salivary glands and midgut showed a significant response in gene expression during feeding (Mercado-Curiel et al., 2011). This shows that DNA microarrays are a valid high-throughput approach that can be used to elucidate the underlying molecular processes and biochemical pathways that are involved during tick feeding.

To date, transcriptome analysis has been performed using North and South American, as well as Australian, strains of *R. microplus* (Saldivar et al., 2008; Rodriguez-Valle et al., 2010; Mercado-Curiel et al., 2011). However, gene expression profiling of African strains of *R. microplus* on African cattle breeds is lacking. The latter is of vital importance as control of *R. microplus* may vary dramatically in different geographical areas as seen for the Bm86 vaccine (de la Fuente et al., 2000). Therefore, two aims were addressed in this study. Firstly, a custom oligonucleotide microarray platform was designed for the comparison of gene expression in the salivary glands, midgut and ovaries of feeding adult *R. microplus* females ticks from a Mozambique reference strain on a South African *Bos indicus* cattle breed. Global analyses of gene expression in these tissues demonstrated that a total of 588 transcripts were shared between tissues during feeding, while a number of up-regulated transcripts displayed tissue specificity. Secondly, multiple database sequence similarity searches and extensive manual curation were employed to functionally annotate transcripts. This study provides a combined functional genomics overview of tissues involved in feeding and reproduction, offering new insights into the complex gene expression profiles related to tissue function and basic *R. microplus* tick biology.

2. Materials and methods

2.1. Tick rearing and sample collection

Rhipicephalus microplus larvae (Mozambique strain, provided by ClinVet Pty. Ltd., South Africa) were allowed to feed on Holstein–Friesian cattle under controlled conditions at the University of Pretoria Biomedical Research Centre (UPBRC), South Africa. Ethical and relevant Section 20 clearances were obtained from the South African Department of Agriculture, Forestry and Fisheries (ethical clearance number: EC022-10), as well as the University of Pretoria Animal Use and Care Committee. Larvae were allowed to advance through their life stages until mature females dropped off the host animal. Ticks were sampled on days 4, 5, 7, 13, 15 and 20. Adult tissues were collected and processed according to the method of Nijhof et al. (2010). The various tissues were stored in TRI REAGENT® (Molecular Research Center, Inc., USA) at −70 °C.

2.2. Microarray probe design from R. microplus sequences

Using available EST data from GenBank (http://www.ncbi.nlm.nih.gov/nucest/) and the gene index of *R. microplus* (BmiGI release 2.1) from the Harvard gene index project (http://compbio.dfci.harvard.edu/tgi/tgipage.html), a sequence dataset was assembled from some 60,000 ESTs and 13,643 unique sequences to a final sequence database consisting of 13,456 contiguous sequences, using the online bioinformatic tools, cd-hit-est (http://www.bioinformatics.org/cd-hit/) and cap3 (http://pbil.univ-lyon1.fr/cap3.php). Detection and removal of vector sequences from EST data were performed with the VecScreen tool (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen_docs.html), using the UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html). The final sequence dataset was submitted online for array design using the Agilent 8 × 15 k microarray and eArray microarray design platforms (https://earray.chem.agilent.com/earray/). A set of 60 mer probes, incorporating a 3′ bias for possible incomplete cDNA synthesis, was designed for complete representation of all assembled transcripts. Probe quality was assessed from base composition scores and the probes were randomly distributed across the array. Additional quality control probes and housekeeping controls were included. These are elongation factor 1 alpha (GenBank accession No. EW679365), H3 Histone family 3A (GenBank accession No. CV442167), Ribosomal protein L4 (GenBank accession No. CV447629), TATA box binding protein (GenBank accession No. CV453818) and subolesin (GenBank accession No. EU301808). Sequences of assembled transcripts used for custom array design, as well as microarray data related to this work, are available from the NCBI GEO public database (GEO accession No. GSE35867). The customised array slide was manufactured by Agilent Technologies (USA).

2.3. Isolation of total RNA and microarray analyses

Total RNA was isolated according to the manufacturer's guidelines for TRI REAGENT® and purified with the RNeasy kit (QIAGEN, USA). Final RNA concentrations, purity and integrity were assessed with the Nanodrop-1000 (Thermo Fisher Scientific, USA) and the Bioanalyzer 2100 micro-fluidics systems (Agilent Technologies). A reference RNA pool consisting of equivalent amounts of RNA from each life stage and adult tissue was prepared in order to allow the independent analysis of both immature and mature life stages. Test groups for the current study consisted of tissues (salivary gland, midgut and ovary) collected from 15 partially fed females (day 20), from two biological replicates.

High quality DNase I-treated total RNA was used for cDNA synthesis using SuperScript™ III (Invitrogen™ life technologies, USA), an oligo (dT_{25}) primer (5′-$(T)_{25}$VN-3′; N = ATGC; V = AGC), random nanomers and aminoallyl dUTP for Cyanine 3-dCTP/Cyanine 5-dCTP dye coupling. Template was labeled with Cy3 (reference pool) or Cy5 (test sample), purified using the QIAquick PCR purification kit (QIAGEN, USA) and the concentration and coupling efficiency determined using the Nanodrop-1000 system (Thermo Fisher Scientific Inc., USA). Equivalent picomoles of Cy3-labeled cDNA from the common reference pool were hybridised with Cy5-labeled individual test cDNA. Both biological and technical replication were employed for each test sample. Hybridisation was performed at 65 °C for 17 h. Prior to scanning, each slide was washed, rinsed