Comparative microarray analyses of adult female midgut tissues from feeding *Rhipicephalus* species.

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Please note: Supplementary material associated with this article. Microarray data has been submitted to the Gene Expression Omnibus and can be accessed via the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=uzupceegdjqvrwz&acc=GSE55826

Abstract

The cattle tick, *Rhipicephalus microplus*, has a debilitating effect on the livestock industry worldwide, owing to its being a vector of the causative agents of bovine babesiosis and anaplasmosis. In South Africa, co-infestation with *R. microplus* and *R. decoloratus*, a common vector species on local livestock, occurs widely in the northern and eastern parts of the country. An alternative to chemical control methods is sought in the form of a tick vaccine to control these tick species. However, sequence information and transcriptional data for *R. decoloratus* is currently lacking. Therefore, this study aimed at identifying genes that are shared between midgut tissues of feeding adult female *R. microplus* and *R. decoloratus* ticks. In this regard, a custom oligonucleotide microarray comprising of 13,477 *R. microplus* sequences was used for transcriptional profiling and 2,476 genes were found to be shared between these *Rhipicephalus* species. In addition, 136 transcripts were found to be more abundantly expressed in *R. decoloratus* and 1,084 in *R. microplus*. Chi-square analysis revealed that genes involved in lipid transport and metabolism are significantly overrepresented in *R. microplus* and *R. decoloratus*. This study is the first transcriptional profiling of *R. decoloratus* and is an additional resource that can be evaluated further in future studies for possible tick control.

Introduction

The cattle tick, *Rhipicephalus microplus* is one of the most economically important tick species that affect global cattle production to date. This tick affects cattle indirectly by transmitting the protozoan (*Babesia bovis* and *B. bigemina*) and prokaryotic (*Anaplasma marginale*) pathogens, causing babesiosis and anaplasmosis, resulting in losses of milk and beef production (de la Fuente and Kocan, 2006; Wang *et al*., 2007). The cattle tick mostly occurs in tropical and subtropical regions between the 32°S and 40°N longi tudes (Pipano *et al*., 2003), preferring a warm and humid climate. Furthermore, these ticks are best suited to cultivated land and woodlands where they are protected from desiccation. Due to the preference for a warmer climate, it can be speculated that the occurrence of *R. microplus* would spread to non-endemic areas due to climate change, leading to an even more widespread occurrence of disease causing pathogens such as *Babesia bovis*. This has been supported by reports that have indicated that *R. microplus* is displacing endemic *R. decoloratus* in South Africa (Tønnesen *et al*., 2004), spreading into areas that were previously unoccupied (Lynen *et al*., 2008). This is due to the fact that *R. microplus* has a higher reproductive rate than *R. decoloratus* and is less susceptible to host resistance (Lynen *et al*., 2008; Zeman and Lynen, 2010). This is a concern as *R. decoloratus* ticks transmit the less virulent *B. bigemina* strain, whereas *R. microplus* transmits both *B. bigemina* and the more virulent *B. bovis* strain (Homer *et al*., 2000; Jongejan and Uilenberg, 2004). Finally, chemical acaricides are losing their efficacy and a growing resistance to virtually all classes of acaricides is becoming a global problem (Rajput *et al*., 2006). This

highlights the need for an alternative method to chemical control such as tick vaccines.

Immunological control of *R. microplus* was reported in the late 1980s when a low-abundance membrane-bound glycoprotein, Bm86, was used to confer protection against ticks in cattle (Willadsen *et al*., 1989; Willadsen, 2006). Consequently, two Bm86-based vaccines, GAVAC^{TM} and TickGARD^{TM}, were commercialized and used for cattle vaccination (Willadsen *et al*., 1995; Canales *et al*., 1997; de la Fuente *et al*., 1998). Initial reports on the efficacy of Bm86 vaccines were highly promising, indicating that three doses of purified antigen were sufficient to cause a 92% reduction in subsequent larval progeny (Willadsen *et al*., 1989). As part of an integrated pest management program, the vaccine led to a two-third reduction in the number of acaricide treatments necessary to maintain acceptable levels of tick infestation (de la Fuente *et al*., 1998). However, due to the reported variability in its efficacy and other industrial considerations, TickGARD is no longer commercially available. In contrast, GAVAC^{TM} is still marketed in North and South America despite its reported shortcomings and a lack of widespread public acceptance of this vaccine (Guerrero *et al*., 2012). Vaccines do, however, still provide a more environmentally-friendly alternative to acaricides and alleviate the selective pressure for acaricide-resistant ticks (de la Fuente *et al*., 2007). Given the shortcomings of the Bm86 vaccines, novel vaccines need to be discovered. To date, the rate-limiting step in vaccine production has been the identification of effective antigens (Mulenga *et al*., 2000; Willadsen, 2001), but studies involving functional genomics, combined with reverse vaccinology, have led to the identification of numerous potential vaccine candidates (Maritz-Olivier *et al*., 2012).

Microarray technology is typically limited to model organisms, unless a substantial amount of representative sequence data is available (Naidoo *et al*., 2005). For this reason, studying the *R. microplus* transcriptome on a large scale has only recently been made possible due to the availability of a partially assembled genome and large EST databases (Lee et al., 2005; Wang et al., 2007; Bellgard et al., 2012). The BmiGI website is one such database and was launched in 2005 (Guerrero *et al*., 2005). It has subsequently been used for the design of custom microarrays to identify genes that are differentially expressed in larvae as a result of exposure to acaricides (Saldivar *et al*., 2008). DNA microarrays have also been used, among other studies, to identify *R. microplus* genes that are involved with host sensing and feeding (Rodriguez-Valle *et al*., 2010). Unfortunately, studies that focus on the transcriptome of ticks are severely impeded by the fact that most *R. microplus* nucleic acid sequences cannot be annotated (Wang et al., 2007; Bellgard et al., 2012). Up to 60% of the genome lacks similarity with sequences from other organisms (Wang *et al*., 2007), thereby negatively affecting the confidence of results for downstream analyses, such as the prediction of subcellular localization.

Both *R. microplus* and *R. decoloratus* (African blue tick) infest livestock in South Africa (Terkawi *et al*., 2011), making a vaccine targeting both ticks ideal. Compared to *R. microplus*, virtually no sequence data is currently available for *R. decoloratus*. However, based on phylogenetic analyses with mitochondrial 12S and cytochrome c oxidase I sequences, high sequence identity (93% and 88%, respectively) was obtained showing that these two ticks species are presumably closely related (Murrell *et al*., 2001). Previously, cross-species transcriptomic analysis of non-model

organisms, using established array platforms for related species, have been performed successfully for other parasites including *Anopheles stephensi* and *Ancylostoma caninum* (Vontas *et al*., 2007; Cantacessi *et al*., 2009). It was therefore hypothesized that cross-species microarray technology could be employed to identify genes (and their encoded proteins) that are conserved or unique to the midgut tissues of feeding adult females of these two economically important tick species.

In this study, a previously designed custom oligonucleotide microarray, specific for *R. microplus* sequences (Stutzer *et al*., 2013), was used to identify genes that are shared between the midgut of *R. microplus* and *R. decoloratus* adult female ticks. Some 2,476 genes were found to be shared, while 1,084 were more highly expressed in *R. microplus* and 136 in *R. decoloratus*. Furthermore, Chi-square analysis revealed an up-regulation of genes involved in lipid transport and metabolism in both these ticks, perhaps suggesting a potential class of genes to target for tick-control.

Materials and Methods

Tick rearing and sample collection

Pathogen-free *R. decoloratus* and *R. microplus* (Mozambique strain) larvae were obtained from ClinVet International (South Africa). These were stored at 25°C (75–85% humidity) and fed on Holstein-Friesian (*Bos taurus*) cattle at the University of Pretoria Biomedical Research Centre (UPBRC), Onderstepoort veterinary campus (South Africa). Engorged female ticks (3 biological replicates, 10 individuals per replicate) were collected 20 days post-infestation and dissected as per method outlined by Nijhof *et al.* (2010). Only the midgut tissues were used for this study and these were snap-frozen in TRI REAGENT® (Molecular Research Center, Inc.) and stored at −70°C. Ethical clearance was obtained from the South African Dept. of Agriculture, Forestry and Fisheries as well as the University of Pretoria's Animal Use and Care Committee (Project approval number EC022-10).

Isolation of total RNA and cDNA synthesis

Midgut tissues were homogenized using 16G, 18G and 23G needles prior to RNA isolation following the manufacturer's guidelines for TRI REAGENT® (Molecular Research center Inc., USA). This was followed by an additional purification step using the RNeasy Mini kit, followed by on-column DNase digestion using the RNase-free DNase set (Qiagen, USA). Finally, RNA purity and integrity were assessed using the Experion™ RNA StdSens Analysis Kit (Bio-Rad, USA). cDNA synthesis was performed using Superscript™ III (Invitrogen™ Life Technologies, USA), poly-dT (5'-$(T)_{25}$VN-3'; N = ATGC; V = AGC), random nonamer oligonucleotides and aminoallyl dUTP (Fermentas, USA). The cDNA concentration was determined using

the Nanodrop-1000 (Thermo Fisher Scientific, USA). Samples were labeled with Cy3 and Cy5 by incubating the cDNA samples with the respective dye in a desiccator at room temperature for two hours, followed by purification of the labeled cDNA using the QIAquick®PCR Purification Kit (Qiagen, USA).

Microarray

Design of a custom oligonucleotide microarray for *R. microplus* representing 13,477 contigs was reported previously (Maritz-Olivier *et al*., 2012). This database comprised all available EST data from GenBank (http://www.ncbi.nlm.nih.gov/nucest) and release 2.1 of the *R. microplus* gene index (Wang *et al*., 2007), which was then used to create an Agilent 8x15k 60-mer microarray. A balanced block microarray design incorporating a dye-swap was performed using three biological replicates and 20 picomoles of each Cy3- and Cy5-labeled cDNA were hybridized per array (four arrays in total) for 17 h at 65 ℃. Slides were washed and rinsed in the Gene Expression Wash Buffer kit (Agilent Technologies, USA) and scanned using the GenePix™4 000B scanner (Molecular Devices Inc., USA).

Microarray data analysis and functional annotation

Spot finding was performed using the GenePix Pro 6.0 feature extraction software (Molecular Devices Inc., USA) using default parameters, as well as manual inspection of all identified features. Using the *limma* package in the R environment (http://CRAN.R-project.org), within slide normalization was performed using locally weighted scatterplot smoothing (LOWESS), followed by A quantile between slide normalization. Transcripts significantly up- or down regulated between *R. microplus*

and *R. decoloratus* were identified, *P* values were adjusted for multiple comparison false discovery rates and only transcripts with *P* values < 0.05 were considered for further analyses. Transcripts with $-1.7 \leq \log_2$ fold change ≤ 1.7 were considered to be shared between the two tick species in question. Transcripts with $-1.7 > \log_2$ fold change or $\log_2$ fold change > 1.7 were seen as *R. decoloratus*- or *R. microplus*-favored, respectively. All microarray and corresponding nucleotide sequence data can be accessed via the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) accession number: GSE55826.

Functional annotation of identified transcripts

Functional annotation of differentially expressed and shared transcripts was done using the desktop cDNA Annotation System (dCAS, v.1.4.3) (Guo *et al*., 2009). BLAST searches were performed for each transcript against the following databases: GO, KOG, Mit-Pla, NR, Pfam, RRNA, and SMART (http://exon.niaid.nih.gov). Based on the results from KOG, term enrichment was calculated using χ^2 analysis in order to identify biological processes with significant differences between the two tick species. In order to predict the most likely reading frame for each contiguous sequence, Prot4EST was used (Wasmuth and Blaxter, 2004). These reading frames were then further analyzed for the presence of signal peptides and membrane spanning regions, which was predicted using SignalP (v. 4.0) (Bendtsen *et al*., 2004) and TMHMM (v. 2.0) (Sonnhammer *et al*., 1998), respectively. Finally, the possibility of GPI-anchoring was investigated using 4 programs: PredGPI (http://gpcr.biocomp.unibo.it/predgpi/pred.htm), GPI-SOM (http://gpi.unibe.ch/), MemType2L (http://www.csbio.sjtu.edu.cn/bioinf/MemType/), and BigPI (http://mendel.imp.ac.at/gpi/gpiserver.html). For a protein to be considered as a

potentially GPI-anchored protein, at least two of abovementioned programs had to predict a GPI-anchoring signal.

Semi-quantitative real-time PCR (qPCR) validation of array results

Seven transcripts (Contig 1269, Contig 1362, Contig 1514, Contig 3185, CV456291, Contig 5295 and Contig 8515), where the $\log_2$ fold change between the two tick species was very small, were selected for analysis using qPCR and primers were designed (Supplementary Tables S1 and S2). RNA samples used for the microarray hybridizations were used for the qPCR experiments and cDNA was synthesized according to the manufacturer's guidelines for the iScript kit (Bio-Rad, USA). Each primer set was tested with standard PCR techniques prior to use in qPCR analysis. Semi-quantitative PCR was performed in a 10 µl final reaction volume containing 2.5 pmol of each primer set corresponding to the selected gene using the KAPA SYBR®FAST qPCR kit as per manufacturer guidelines (KAPA Biosystems, USA). Previously validated reference genes used for normalization of expression levels included Contig 8723, EF1α and PPIA (Nijhof *et al*., 2009; Stutzer *et al*., 2013). Reactions were performed in triplicate on a LightCycler® 480 (Roche Applied Science, Germany) and included no template negative controls. PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles consisting of template denaturation at 95°C for 3 seconds, primer annealing at 56°C for 7 seconds and primer extension at 72°C for 4 seconds. Post-PCR melting curve analysis was performed to ensure that no nonspecific amplification had taken place. Relative transcript abundance was subsequently determined by comparing the extracted Ct values relative to that of the three selected reference genes, using qBase (Hellemans *et al*., 2007). Finally, fold changes were calculated by dividing the

average normalized relative quantities of each gene in the three *R. microplus* samples with those in *R. decoloratus*.

Results

Identification and annotation of midgut transcripts in R. microplus and *R. decoloratus*

As outlined in Maritz-Olivier *et al*. (2012), 13,477 unique contigs were used for the design of a custom oligonucleotide microarray slide. Of these, 3,696 had *P* values smaller than 0.05. Most of these transcripts (2,476) were shared between *R. microplus* and *R. decoloratus* (Table 1, Figure 1A), whereas 1,084 transcripts were more abundant in *R. microplus* and 136 in *R. decoloratus* (Table 1, Figure 1B and 1C). Potential signal peptides and membrane spanning regions were predicted for the relevant genes and these are summarized in Table 1. Most proteins were found to be intracellular (2,702), followed by membrane proteins (664), secreted proteins (280) and GPI-anchored proteins (50).

Functional classification of transcripts shared between *R. decoloratus* and *R. microplus,* using KOG classification, identified 718 (29%) annotatable and 1,758 (71%) unannotatable genes (Figure 1A). Of the annotatable transcripts, the major functional classes identified related to posttranslational modification, protein turnover and chaperones (13.8%), transcription (10.2%) and signal transduction mechanisms (9.7%) (Figure 1A).

For transcripts up-regulated specifically in *R. microplus*, only 323 (30%) functionally annotatable (with 761 unannotatable) genes were identified (Figure 1B). The top three functional classes identified related to: posttranslational modification, protein

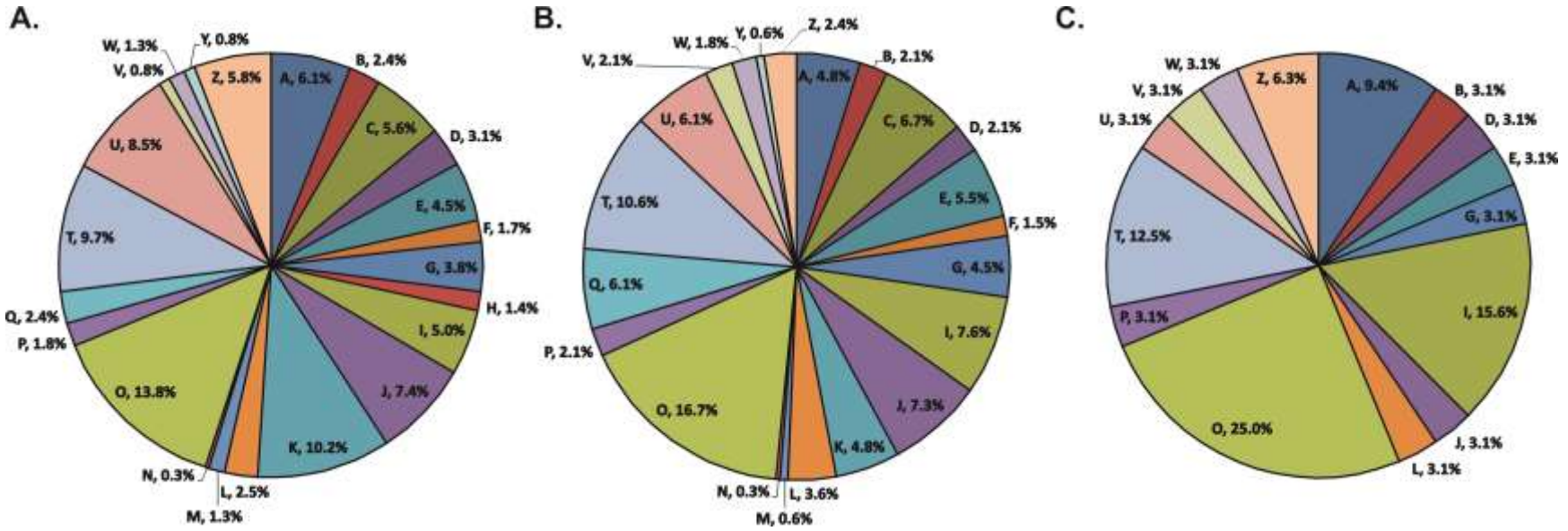

Figure1. Functional classification and distribution of differentially expressed genes from midgut tissues of feeding *Rhipicephalus* ticks (*P* values < 0.05).

Annotation of transcripts achieved using *E*-value cut-offs ≤ 1x10^{-10} and functional classification assigned according to the eukaryotic orthologous functional groups (KOGs) (Tatusov *et al*., 2003). The percentages of unique transcripts that are shared (A) or uniquely up-regulated in *R. microplus* (B) and *R. decoloratus* (C) during feeding are indicated. The functional classifications that are represented include: ARNA processing and modification; B- Chromatin structure and dynamics; C- Energy production and conversion; D- Cell cycle control, cell division, chromosome partitioning; E- Amino acid transport and metabolism; F- Nucleotide transport and metabolism; G- Carbohydrate transport and metabolism; H- Coenzyme transport and metabolism; I- Lipid transport and metabolism; J- Translation, ribosomal structure and biogenesis; K- Transcription; L- Replication, recombination and repair; M- Cell wall/membrane/envelope biogenesis; N- Cell motility; O- Posttranslational modification, protein turnover, chaperones; P- Inorganic ion transport and metabolism; Q- Secondary metabolites biosynthesis, transport and catabolism; TSignal transduction mechanisms; U- Intracellular trafficking, secretion, and vesicular transport; V- Defense mechanisms; W- Extracellular structures; Y- Nuclear structure; Z- Cytoskeleton.

Table1. Membrane topology and cellular localization prediction of abundant genes identified from midgut tissues of *R. microplus* and *R. decoloratus*.

Gene classification	Total	Non-secreted, intracellular	Membrane-associated	Secreted soluble	GPI-anchored
Shared between species	2,476	1,829	436	174	37
Up-regulated in *R. decoloratus*	136	99	26	11	0
Up-regulated in *R. microplus*	1,084	774	202	95	13
Total	3,696	2,702	664	280	50

turnover and chaperones (17.0%), signal transduction mechanisms (10.8%) and lipid transport and metabolism (7.7%) (Figure 1B).

Of the 136 transcripts found to be specifically up-regulated in *R. decoloratus*, only 32 (24%) were annotatable using KOG with 104 (76%) unannotatable transcripts (Figure 1C). The three largest functional classes identified, were similar to *R. microplus* and included: posttranslational modification, protein turnover and chaperones (25.0%), lipid transport and metabolism (15.6%), as well as signal transduction mechanisms (12.5%) (Figure 1C).

Considering the 20 annotatable genes that are most highly expressed in *R. microplus* and in *R. decoloratus* (Supplementary Table S3), it is evident that genes involved in posttranslational modification, protein turnover and chaperones represent a major group of up-regulated genes for both species. Of these transcripts, 6 of the 20 most up-regulated annotatable genes in *R. decoloratus* are involved in lipid transport and metabolism, whereas only 2 such genes were identified in the corresponding list of *R. microplus* genes.

Chi-square analysis

The percentage occurrence (via $\chi 2$ analysis) of each KOG biological term for each gene list was compared with an in-house assembled *R. microplus* database to determine their abundance in *R. microplus* and/or *R. decoloratus* (Maritz-Olivier *et al*., 2012). It was found that genes involved in lipid transport and metabolism were significantly enriched among the abundant transcripts for *R. microplus* and *R. decoloratus,* (*P value* = 0.035 and *P value* = 0.007, respectively) (Table 2). This may

Table 2. Identification of significantly enriched biological terms. Numbers indicate the percentage occurrence of the biological term in the respective gene list. The percentages in brackets indicate the χ2 probability that there is no difference in the occurrence of the biological term in the gene list compared to its occurrence in the in-house assembled *R. microplus* database. Smaller percentages are therefore more significant. Bold values with arrows indicate enriched or depleted biological terms.

Biological process	**% Occurrence in *R. microplus* database**	**% Occurrence in shared genes (χ^2 probability)**	**% Occurrence in *R. microplus* up-regulated genes (χ^2 probability)**	**% Occurrence in *R. decoloratus* up-regulated genes (χ^2 probability)**
Amino acid transport and metabolism	5.6	4.5 (23.50%)	5.6 (98.9%)	3.1 (55.0%)
Carbohydrate transport and metabolism	4.0	3.8 (72.3%)	4.6 (60.3%)	3.1 (79.2%)
Cell cycle control, cell division, chromosome partitioning	2.5	3.1 (39.2%)	2.2 (70.7%)	0 (36.4%)
Cell motility	0.1	0.3 (41.4%)	0.3 (46.7%)	0 (83.1%)
Cell wall/membrane/envelope biogenesis	0.9	1.3 (44.0%)	0.6 (56.2%)	3.1 (20.7%)
Chromatin structure	2.5	2.4 (82.8%)	2.2 (70.7%)	3.1 (82.4%)

Biological process	% Occurrence in *R. microplus* database	% Occurrence in shared genes (χ^2 probability)	% Occurrence in *R. microplus* up-regulated genes (χ^2 probability)	% Occurrence in *R. decoloratus* up-regulated genes (χ^2 probability)
and dynamics				
Coenzyme transport and metabolism	1.2	1.4 (61.5%)	0.9 (70.0%)	N/A (53.9%)
Cytoskeleton	4.3	5.9 (7.60%)	2.5 (11.1%)	6.3 (59.6%)
Defence mechanisms	1.3	0.8 (29.30%)	2.2 (20.7%)	3.1 (37.2%)
Energy production and conversion	5.3	5.6 (74.30%)	6.8 (24.1%)	0 (18.2%)
Extracellular structures	1.0	1.3 (58.70%)	1.9 (16.9%)	3.1 (24.5%)
Inorganic ion transport and metabolism	2.4	1.8 (30.20%)	2.2 (75.2%)	3.1 (80.6%)
Intracellular trafficking, secretion, and vesicular transport	6.3	**8.6 (3.10%)** ↑	6.2 (94.2%)	3.1 (46.1%)
Lipid transport and metabolism	5.0	5.1 (99.9%)	**7.7 (3.5%)** ↑	**15.6 (0.7%)** ↑
Nuclear structure	0.8	0.8 (85.3%)	0.6 (76.6%)	N/A (61.9%)
Nucleotide transport and metabolism	2.2	1.7 (39.9%)	1.5 (46.1%)	N/A (40.0%)
Posttranslational	14.2	13.9 (76.6%)	17.0 (16.8%)	25 (8.30%)

Biological process	% Occurrence in *R. microplus* database	% Occurrence in shared genes (χ^2 probability)	% Occurrence in *R. microplus* up-regulated genes (χ^2 probability)	% Occurrence in *R. decoloratus* up-regulated genes (χ^2 probability)
modification, protein turnover, chaperones				
Replication, recombination and repair	2.8	2.5 (67.1%)	3.7 (34.1%)	3.1 (90.9%)
RNA processing and modification	7.3	6.2 (26.9%)	5.0 (11.7%)	9.4 (65.2%)
Secondary metabolites biosynthesis, transport and catabolism	2.5	2.4 (86.2%)	3.1 (49.8%)	N/A (36.7%)
Signal transduction mechanisms	11.3	9.8 (21.7%)	10.8 (78.6%)	12.5 (83.6%)
Transcription	8.4	10.3 (13.3%)	**5.0 (2.90%)** ↓	N/A (28.1%)
Translation, ribosomal structure and biogenesis	8.0	7.4 (59.10%)	7.4 (72.9%)	3.1 (9.6%)

suggest a possible preference for lipids as a nutrient source during feeding, 20 days post infestation. Of all the biological processes shared between the two species, intracellular trafficking, secretion, and vesicular transport were significantly enriched (P = 0.031) (Table 2). Interestingly, depletion of genes involved in transcription was observed among the genes more abundantly expressed in *R. microplus*. Selected transcripts up-regulated in either *R. microplus* or *R. decoloratus* are shown in Table 3. Of these, around seven transcripts may play a role in β-oxidation.

Microarray validation using semi-quantitative PCR

Validation of transcripts expressed in both tick species was done using *semi-quantitative* PCR. In the absence of *R. decoloratus* sequence data, five putatively shared transcripts were chosen at random and their relative abundance in *R. microplus* midgut was determined. Data was normalised against three reference genes including: previously evaluated cyclophilin and elongation factor 1α (Nijhof *et al*., 2009) and an in-house identified reference gene, Contig8723 (Stutzer *et al*., 2013). The results are summarised in Supplementary Table S2. A high degree of correlation (0.8) between qPCR and microarray data was determined with an overall concordance observed in the direction and magnitude of the fold change values obtained.

Contig number	***E*-value (KOG)**	**Annotation**	**Putative biological role**
Contig6062	8E-56	Putative gamma-butyrobetaine dioxygenase	L-carnitine synthesis and role in β-oxidation
Contig890	5E-28	Putative hydroxysteroid dehydrogenase-like protein	β-oxidation
CV447153	7E-20	Putative hydroxysteroid dehydrogenase-like protein	β-oxidation
Contig8127	7E-11	Putative lipoprotein, Vitellogenin	A major yolk protein used in embryogenesis
Contig3708	3E-21	Putative long chain fatty acid acyl-CoA ligase	Phospholipid biosynthesis
Contig4293	2E-80	Putative long-chain-fatty-acid-CoA ligase	Lipid biosynthesis and β-oxidation
Contig4388	3E-26	Putative long-chain-fatty-acid-CoA ligase	Lipid biosynthesis and β-oxidation
Contig717	1E-13	Putative long-chain-fatty-acid-CoA ligase	Lipid biosynthesis and β-oxidation
TC15677	4E-89	Putative long-chain-fatty-acid-CoA ligase	Lipid biosynthesis and β-oxidation
TC22152	6E-24	Putative phosphatidate phosphatase	Lipid metabolism
Contig7499	1E-17	Putative phosphatidylinositol transfer protein SEC14	Transfer of phosphatidylinositol/ phosphatidylcholine
Contig2302	1E-19	Putative phosphoethanolamine N-methyltransferase	Phospholipid biosynthesis
Contig4499	9E-21	Putative retinaldehyde-binding-like protein, Clavesin	Binds phosphatidylinositol 3,5-bisphosphate, morphology of endosomal and lysosomal compartments
Contig7995	1E-104	Putative sphingomyelin phosphodiesterase	Phospholipid catabolism
Contig8380	7E-89	Putative sterol O-acyltransferase	Anabolism of cholesterol esters/ triglycerides, lipoprotein assembly and cholesterol absorption
Contig6686	1E-41	Putative triacylglycerol lipase	Catabolism of cholesteryl esters and

Contig number	***E*-value (KOG)**	**Annotation**	**Putative biological role**
			triglycerides

Discussion

The frequency of resistance to chemical control is rising within tick populations and the co-infestation of cattle with different tick species is a common occurrence. The efficacy of alternative approaches to tick control (including cross-protection), such as vaccination has been demonstrated previously with Bm86-based vaccines (de Vos *et al*., 2001; Willadsen, 2006; Canales *et al.*, 2009; Rodríguez-Valle *et al.*, 2012). To date, no alternative commercial vaccines have been produced and there are various limitations to the existing Bm86-based vaccines. To test the hypothesis that conserved proteins are present in *R. microplus* and *R. decoloratus*, a DNA microarray-based strategy was followed. Finding genes that are conserved between these two tick species that presents similar epitopes would be advantageous, since the primary limiting step in the identification of possible cross-protective tick vaccines is the identification of suitable vaccine candidates.

Microarray technology allows the simultaneous analysis of expression levels of thousands of genes in a single experiment. In this study, a custom in-house designed oligonucleotide microarray comprising known *R. microplus* sequences was used to identify genes that are shared between the midgut of adult female *R. microplus* and *R. decoloratus* ticks. Global transcriptomic analyses of the fold change in expression between the two ticks revealed that 2,476 genes were expressed at similar levels among the two tick species, whereas 1,084 were found to be more highly expressed in the midgut of *R. microplus* females, compared to 136 genes that were more highly expressed in the midgut of *R. decoloratus* females. Using KOG annotation, it was noted that the three largest clusters of biological processes among the genes expressed in both *R. microplus* and *R. decoloratus*

were signal transduction mechanisms, lipid transport and metabolism and posttranslational modification, protein turnover and chaperones.

Chi-square analysis indicated a significant increase in midgut genes involved in lipid transport and metabolism. The latter suggested an important role of lipids as a nutrient source during feeding for both the tick species investigated. However, it has been reported previously that intracellular absorption of nutrients from the blood meal is primarily directed at hemoglobin (Sonenshine, 1991), a protein. Therefore, it is expected that an up-regulation in genes involved in the breakdown of protein should occur. In this regard, a large subset of genes involved in protein turnover was identified that might be responsible for the breakdown of hemoglobin. Apart from the absorption of hemoglobin, lipid inclusions of varying size are found in digestive cells during the slow feeding phase (Sonenshine, 1991). Considering that the hemoglobin content per red blood cell (by weight) is approximately 100 times higher than that of either lipids or cholesterol (Weed *et al*., 1963), the primary source of energy from the blood meal might in fact be hemoglobin. It must still be borne in mind that the ATP yield from lipid metabolism is more than twice that obtained from proteins (Livesey, 1984). However, the specific contribution of lipids to metabolic flux remains a hypothesis that will have to be tested in future. In a related comparative study of midguts and other tissues of feeding *R. microplus* females, similar transcripts were identified that are involved in lipid metabolism (Stutzer *et al*., 2013). Therefore, up-regulation of components involved in lipid metabolism appears to be a conserved attribute in basic tick biology (i.e. energy metabolism and oogenesis) and represents a novel pathway that can be explored for potential cross-species vaccine design.

An exciting new development in tick vaccine research is the application of reverse vaccinology for the identification of novel vaccine candidates (Rappuoli, 2000). In this study, genes found to be shared between the midgut tissues of *R. microplus* and *R. decoloratus* adult females have been evaluated using our reverse vaccinology pipeline with promising results (Maritz-Olivier *et al*., 2012). With vaccine design in mind, considering genes that are expressed in the midgut of both tick species, 6,730 genes were identified. It has been hypothesized that membrane proteins are ideal vaccine candidates, as they come into direct contact with the host's immune factors (Rappuoli and Bagnoli, 2011). From the 6,730 genes that are actively expressed in the midgut of both *R. microplus* and *R. decoloratus* females, 1,224 are predicted to contain membrane-spanning helices. This is a significant improvement on the problem of identifying suitable tick vaccine candidates (Nuttall *et al*., 2006).

In this study, the first cross-species tick microarray was performed to identify related sequences from two economically important tick species *R. microplus* and *R. decoloratus*. This work further offers a preliminary insight into shared metabolic processes important for basic tick biology, thereby offering potential targets for next generation vaccine and acaricide development. The current lack of sequence data for *R. decoloratus* will be addressed by performing next-generation sequencing technologies, such as RNA-Seq that would not only provide sequence data, but also information regarding expression levels within this species (Nagalakshmi *et al*., 2010). Such sequence data could subsequently be used to design an oligonucleotide microarray specific to *R. decoloratus* for future experiments. Additional proteomic studies will also offer the potential advantage of confirming reading frames of putative genes (and encoded proteins), as well as the relationship between gene

expression- and protein levels. The importance of genes that are involved in lipid transport and metabolism could be further assessed with gene knockdown and inhibition studies. In conjunction with small-scale vaccination trials, novel targets for new generation acaricides, as well as vaccine antigens that impair lipid metabolism in different tick species could be developed.

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References

Bellgard, M.I., Moolhuijzen, P.M., Guerrero, F.D., Schibeci, D., Rodriguez-Valle, M., Peterson, D.G., Dowd, S.E., Barrero, R., Hunter, A., Miller, R.J., Lew-Tabor, A.E. (2012). CattleTickBase: an integrated Internet-based bioinformatics resource for Rhipicephalus (Boophilus) microplus. Int J Parasitol. 42(2): 161-9.

Bendtsen, J.D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783-795.

Canales, M., Almazá n, C., Naranjo, V., Jongejan, F., de la Fuente, J. (2009). Vaccination with recombinant *Boophilus annulatus* Bm86 ortholog protein, Ba86,

protects cattle against *B. annulatus* and *B. microplus* infestations. BMC Biotechnol. 9: 29.

Canales, M., Enríquez, A., Ramos, E., Cabrera, D., Dandie, H., Soto, A., Falcón, V., Rodríguez, M., and de la Fuente, J. (1997). Large-scale production in Pichia pastoris of the recombinant vaccine GavacTM against cattle tick. Vaccine 15, 414-422.

Cantacessi, C., Loukas, A., Campbell, B.E., Mulvenna, J., Ong, E.K., Zhong, W., Sternberg, P.W., Otranto, D., Gasser, R.B. (2009). Exploring transcriptional conservation between *Ancylostoma caninum* and *Haemonchus contortus* by oligonucleotide microarray and bioinformatic analyses. Mol Cell Probes. 23(1):1-9.

de la Fuente, J., Almazán, C., Canales, M., Pérez de la Lastra, J. M., Kocan, K. M., and Willadsen, P. (2007). A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim. Health Res. Rev. 8, 23-28.

de la Fuente, J., and Kocan, K. M. (2006). Strategies for development of vaccines for control of ixodid tick species. Parasite Immunol. 28, 275-283.

de la Fuente, J., Rodríguez, M., Redondo, M., Montero, C., García-García, J. C., Méndez, L., Serrano, E., Valdés, M., Enriquez, A.,Canales, M., Ramos, E., Boué, O., Machado, H., Lleonart, R., de Armas, C. A., Rey, S., Rodríguez, J. L., Artiles, M., and García, L. (1998). Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick *Boophilus microplus*. Vaccine. 16, 366-373.

de Vos, S., Zeinstra, L., Taoufik, O., Willadsen, P., Jongejan, F. (2001). Evidence for the utility of the Bm86 antigen from *Boophilus microplus* in vaccination against other tick species. Exp Appl Acarol. 25(3): 245-61.

Guerrero, F. D., Miller, R. J., and Pérez de León, A. A. (2012). Cattle tick vaccines: Many candidate antigens, but will a commercially viable product emerge? Int. J. Parasitol. 42, 421-427.

Guerrero, F. D., Miller, R. J., Rousseau, M.-E., Sunkara, S., Quackenbush, J., Leed, Y., and Nene, V. (2005). BmiGI: A database of cDNAs expressed in *Boophilus microplus*, the tropical/southern cattle tick. Insect Biochem. Mol. Biol. 35, 585-595.

Guo, Y., Ribeiro, J. M., Anderson, J. M., and Bour, S. (2009). dCAS: a desktop application for cDNA sequence annotation. Bioinformatics 25, 1195-1196.

Hellemans, J., Mortier, G., Paepe, A. D., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, Article R19.

Homer, M. J., Aguilar-Delfin, I., Telford, S. R., Krause, P. J., and Persing, D. (2000). Babesiosis. Clin. Microbiol. Rev. 13, 451-469.

Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. Parasitology. 129, S3-S14.

Lee, Y., Tsai, J., Sunkara, S., Karamycheva, S., Pertea, G., Sultana, R., Antonescu, V., Chan, A., Cheung, F., Quackenbush, J. (2005). The TIGR Gene Indices: clustering and assembling EST and known genes and integration with eukaryotic genomes. Nucleic Acids Res. 33: D71-4.

Livesey, G. (1984). The energy equivalents of ATP and the energy values of food proteins and fats. Br. J. Nutr. 51, 15-28.

Lynen, G., Zeman, P., Bakuname, C., Di Giulio, G., Mtui, P., Sanka, P., and Jongejan, F. (2008). Shifts in the distributional ranges of Boophilus ticks in Tanzania: evidence that a parapatric boundary between *Boophilus microplus* and B. decoloratus follows climate gradients. Exp. Appl. Acarol. 44, 147-164.

Maritz-Olivier, C., van Zyl, W., and Stutzer, C. (2012). A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine

candidates in the cattle tick, *Rhipicephalus microplus*. Ticks Tick Borne Dis. 3, 179-187.

Mulenga, A., Sugimoto, C., and Onuma, M. (2000). Issues in tick vaccine development: identification and characterization of potential candidate vaccine antigens. Microbes Infect. 2, 1353-1361.

Murrell, A., Campbell, N. J. H., and Barker, S. C. (2001). A Total-Evidence Phylogeny of Ticks Provides Insights into the Evolution of Life Cycles and Biogeography. Mol. Phylogenet. Evol. 21, 244-258.

Nagalakshmi, U., Waern, K., and Snyder, M. (2010). RNA-Seq: A Method for Comprehensive Transcriptome Analysis. *Curr. Protoc. Mol. Biol.* **Chapter 4:Unit 4.11,** 1-13.

Naidoo, S., Denby, K. J., and Berger, D. K. (2005). Microarray experiments: considerations for experimental design. S. Afr. J. Sci. 101, 347-354.

Nijhof, A. M., Balk, J. A., Postigo, M., and Jongejan, F. (2009). Selection of reference genes for quantitative RT-PCR studies in *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus appendiculatus* ticks and determination of the expression profile of Bm86. BMC Mol. Biol. 10, 112.

Nijhof, A.M., Balk, J.A., Postigo, M., Rhebergen, A.M., Taoufik, A., Jongejan, F., 2010. Bm86 homologues and novel ATAQ proteins with multiple epidermal growth factor (EGF)-like domains from hard and soft ticks. Int. J. Parasitol. 40(14), 1587-97.

Nuttall, P. A., Trimnell, A. R., Kazimirova, M., Labuda, M., and Telford, S. R. (2006). Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. Parasite Immunol. 28, 155-163.

Pipano, E., Alekceev, E., Galker, F., Fish, L., Samish, M., and Shkap, V. (2003). Immunity against *Boophilus annulatus* induced by the Bm86 (Tick-GARD) vaccine. Exp. Appl. Acarol. 29, 141-149.

Rajput, Z. I., Hu, S.-H., Chen, W.-J., Arijo, A., and Xiao, C.-W. (2006). Importance of ticks and their chemical and immunological control in livestock. J. Zhejiang Univ. Sci. B. 7, 912-921.

Rappuoli, R. (2000). Reverse vaccinology. Curr. Opin. Microbiol. 3, 445-450.

Rappuoli, R., and Bagnoli, F. (2011). "Vaccine design: Innovative approaches and novel strategies." Caister Academic Press, Norfolk, UK.

Rodriguez-Valle, M., Lew-Tabor, A., Gondro, C., Moolhuijzen, P., Vance, M., Guerrero, F. D., Bellgard, M., and Jorgensen, W. (2010). Comparative microarray analysis of *Rhipicephalus (Boophilus) microplus* expression profiles of larvae pre-attachment and feeding adult female stages on *Bos indicus* and *Bos taurus* cattle. BMC Genomics 11, 437-453.

Rodríguez-Valle, M., Taoufik, A., Valdés, M., Montero, C., Ibrahin, H., Hassan, S.M., Jongejan, F., de la Fuente, J. (2012). Efficacy of *Rhipicephalus (Boophilus) microplus* Bm86 against *Hyalomma dromedarii* and *Amblyomma cajennense* tick infestations in camels and cattle. Vaccine. 30(23): 3453-8.

Saldivar, L., Guerrero, F. D., Miller, R. J., Bendele, K. G., Gondro, C., and Brayton, K. A. (2008). Microarray analysis of acaricide-inducible gene expression in the southern cattle tick, *Rhipicephalus (Boophilus) microplus*. Insect Mol. Biol. 17, 597-606.

Sonenshine, D. E. (1991). "Biology of Ticks." Oxford University Press, New York.

Sonnhammer, E. L. L., Heijne, G. v., and Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. In "Proc. of Sixth Int.

Conf. on Intelligent Systems for Molecular Biology", pp. 175-182. AAAI Press, Menlo Park, CA.

Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., Krylov, D. M., Mazumder, R., Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Smirnov, S., Sverdlov, A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J., and Natale, D. A. (2003). The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4, 41.

Terkawi, M. A., Thekisoe, O. M., Katsande, C., Latif, A. A., Mans, B. J., Matthee, O., Mkize, N., Mabogoane, N., Marais, F., Yokoyama, N., Xuan, X., and Igarashi, I. (2011). Serological survey of *Babesia bovis* and *Babesia bigemina* in cattle in South Africa. Vet. Parasitol. 182, 337-342.

Tønnesen, M. H., Penzhorn, B. L., Bryson, N. R., St oltsz, W. H., and Masibigiri, T. (2004). Displacement of Boophilus decoloratus by *Boophilus microplus* in the Soutpansberg region, Limpopo Province, South Africa. Exp. Appl. Acarol. 32, 199-208.

Vontas, J., David, J.P., Nikou, D., Hemingway, J., Christophides, G.K., Louis, C., Ranson, H. (2007). Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. Insect Mol Biol. 16(3):315-24.

Wang, M., Guerrero, F. D., Pertea, G., and Nene, V. M. (2007). Global comparative analysis of ESTs from the southern cattle tick, *Rhipicephalus (Boophilus) microplus*. BMC Genomics 8, 368.

Wasmuth, J. D., and Blaxter, M. L. (2004). prot4EST: Translating Expressed Sequence Tags from neglected genomes. BMC Bioinformatics 5, 187.

Weed, R. I., Reed, C. F., and Berg, G. (1963). Is hemoglobin an essential structural component of human erythrocyte membranes? J. Clin. Invest. 42 (4): 581–8 42, 581-588.

Willadsen, P. (2001). The molecular revolution in the development of vaccines against ectoparasites. Vet. Parasitol. 101, 353-367.

Willadsen, P. (2006). Tick control: thoughts on a research agenda. Vet. Parasitol. 138, 161-168.

Willadsen, P., Bird, P., Cobon, G. S., and Hungerford, J. (1995). Commercialization of a recombinant vaccine against *Boophilus microplus*. Parasitol. 110, S43-S50.

Willadsen, P., Riding, G. A., McKenna, R. V., Kemp, D. H., Tellam, R. L., Nielsen, J. N., Lahnstein, J., Cobon, G. S., and Gough, J. M. (1989). Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. J. Immunol. 143, 1346-1351.

Zeman, P., and Lynen, G. (2010). Conditions for stable parapatric coexistence between *Boophilus decoloratus* and *B. microplus* ticks: a simulation study using the competitive Lotka-Volterra model. Exp. Appl. Acarol. 52, 409-426.