Characterization of the \textit{Theileria parva} sporozoite proteome

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\textbf{A B S T R A C T}

East Coast fever is a lymphoproliferative disease caused by the tick-borne protozoan parasite \textit{Theileria parva}. The sporozoite stage of this parasite, harboured and released from the salivary glands of the tick \textit{Rhipicephalus appendiculatus} during feeding, invades and establishes infection in bovine lymphocytes. Blocking this initial stage of invasion presents a promising vaccine strategy for control of East Coast fever and can in part be achieved by targeting the major sporozoite surface protein p67. To support research on the biology of \textit{T. parva} and the identification of additional candidate vaccine antigens, we report on the sporozoite proteome as defined by LC–MS/MS analysis. In total, 4780 proteins were identified in an enriched preparation of sporozoites. Of these, 2007 were identified as \textit{T. parva} proteins, representing close to 50% of the total predicted parasite proteome. The remaining 2773 proteins were derived from the tick vector. The identified sporozoite proteins include a set of known \textit{T. parva} antigens targeted by antibodies and cytotoxic T cells from cattle that are immune to East Coast fever. We also identified proteins predicted to be orthologs of \textit{Plasmodium falciparum} sporozoite surface molecules and invasion organelle proteins, and proteins that may contribute to the phenomenon of bovine lymphocyte transformation. Overall, these data establish a protein expression profile of \textit{T. parva} sporozoites as an important starting point for further study of a parasitic species which has considerable agricultural impact.

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\section{1. Introduction}

East Coast fever (ECF), a fatal bovine disease, is caused by the apicomplexan parasite \textit{Theileria parva} (Norval et al., 1992). The disease kills approximately one million cattle every year in Africa and causes significant economic losses to small-hold farmers in eastern, central and southern Africa of approximately $300 million annually (reviewed in Nene et al., 2016). These estimates were derived several years ago and it is highly likely that the current losses due to ECF are much larger. Vaccination against ECF by an infection and treatment method (ITM) is available (Radley et al., 1975). For control of ECF in southern Africa, except South Africa where vaccination is not permitted as it is free of ECF, ITM is performed with a single parasite isolate, and in parts of eastern Africa a composite ITM vaccine, the Muguuga cocktail, is used (Morzaria et al., 2000). Although ITM confers solid and long-lived immunity it has disadvantages in that it is an unstable and potentially lethal product, and animals vaccinated by the ITM protocol remain lifelong asymptomatic carriers of the parasite that pose risks for spread of the disease. It also requires a liquid nitrogen cold chain for storage and oxytetracycline for co-treatment (reviewed in Di Giulio et al., 2009). The vaccine is expensive and laborious to produce, and it requires skilled personnel for delivery (reviewed in Di Giulio et al., 2009). Therefore, development of a subunit vaccine that is easier to produce and with minimal risks is necessary.

Although classified in the phylum Apicomplexa, aspects of the biology of \textit{Theileria} are atypical of such organisms. For example, \textit{Theileria} sporozoites are not motile, they have a less defined apical complex and host cell entry is not orientation-specific (Shaw, 2003). Sporozoites bind and enter bovine lymphocytes by a “zippering” process of the host and the sporozoite cell membranes (Fawcett et al., 1982). After entry into the host cell, rhoptries/microspheres are discharged with a rapid escape of the sporozoite
from the surrounding host cell membrane. Sporozoites differentiate to the schizont stage, which resides in the host cytoplasm surrounded by host cell microtubules that are seemingly nucleated by parasite molecules (reviewed in Shaw, 2003). Schizont-infected cells acquire a cancer-like phenotype and are the cause of disease (Norval et al., 1992). Sporozoites also bind and enter macrophages/afferent lymph veiled cells by a zippering process, but these cells are not susceptible to transformation and sporozoites appear to only differentiate to an early schizont stage (Shaw et al., 1993). Several host cell signalling pathways that contribute to host cell transformation have been studied (Dobbelaere and Kütienzi, 2004; Dessauge et al., 2005). In addition, parasite molecules that are associated with the transformation process have been tentatively identified by taking advantage of comparative genomics and cancer biology (Shiels et al., 2006; Hayashida et al., 2012; Tretina et al., 2015). A parasite-encoded prolyl isomerase has been recently identified as playing a key role in this complex host cell transformation process (Marsolier et al., 2015).

There is good evidence for roles of T. parva sporozoite and schizont antigens as candidate vaccine antigens. Several schizont antigens are targets of cytotoxic T lymphocytes (CTLs) that lyse schizont-infected cells (Graham et al., 2006, 2007, 2008) and two sporozoite antigens are targets of neutralising antibodies (Dobbelaere et al., 1985; Shapiro et al., 1987). One of the latter antigens, p67, has been shown to consistently protect a proportion of immunised cattle against challenge with needle-injected sporozoites and from infected ticks in field trials (reviewed in Nene et al., 2016). The p67-based vaccine might be improved by including additional antigens that can neutralise sporozoite infectivity.

As a first step towards improving our molecular understanding of the biology of T. parva we have undertaken a Multidimensional Protein Identification Technology (MudPIT) LC–MS/MS approach (reviewed in Schirmer et al., 2003) to characterise the proteome of T. parva sporozoites purified by ion-exchange chromatography (Musoke et al., 1992). We have employed bioinformatic tools to analyse 2007 parasite proteins that we were able to detect and identified T. parva orthologs of Plasmodium falciparum invasion organelle proteins, calcium signalling proteins and surface proteins. Some of these may represent novel T. parva vaccine candidates.

2. Materials and methods

2.1. Animals and sporozoite production

The procedure for sporozoites production has been previously described in detail (Patel et al., 2016). Briefly, cattle aged 7–9 months, previously managed under a strict acaricide regime, were allowed to feed on rabbits to induce sporozoite maturation. T. parva sporozoites purified by ion-exchange chromatography ('UNI-FORM' (England), 4–5 ml of homogenizer) and disrupted by gentle up, down and circular motion of the pestle until a uniform cloudy suspension was formed. The suspension was then centrifuged at 1000g at 4 °C for 5 min. The resulting supernatant containing sporozoites was applied to an 8 ml diethylaminoethyl cellulose (DE-52; Sigma–Aldrich (USA) Cat. No. D3764) column (Musoke et al., 1992). Flow-through fractions of 3.5 ml were collected in siliconised 10 ml conical bottomed glass tubes on ice. The sporozoite-rich cloudy fractions (numbers 2–5) were pooled and centrifuged at 12,000g at 4 °C for 5 min. The resulting sporozoite-rich small yellowish pellet was resuspended in SDS–PAGE sample buffer and heated at 100 °C for 5 min prior to fractionation on 10% one-dimensional SDS–PAGE and staining with Coomassie Blue.

2.2. Sporozoite purification

Dissected tick salivary glands were collected in a tube with 2 ml of cold PBS, transferred to a chilled glass hand homogenizer ('UNI-FORM' (England), 4–5 ml of homogenizer) and disrupted by gentle up, down and circular motion of the pestle until a uniform cloudy suspension was formed. The suspension was then centrifuged at 1000g at 4 °C for 5 min. The resulting supernatant containing sporozoites was applied to an 8 ml diethylaminoethyl cellulose (DE-52; Sigma–Aldrich (USA) Cat. No. D3764) column (Musoke et al., 1992). Flow-through fractions of 3.5 ml were collected in siliconised 10 ml conical bottomed glass tubes on ice. The sporozoite-rich cloudy fractions (numbers 2–5) were pooled and centrifuged at 12,000g at 4 °C for 5 min. The resulting sporozoite-rich small yellowish pellet was resuspended in SDS–PAGE sample buffer and heated at 100 °C for 5 min prior to fractionation on 10% one-dimensional SDS–PAGE and staining with Coomassie Blue.

2.3. In-gel trypsin digestion for LC–MS/MS analysis

The gel lane containing sporozoite material was excised and divided into four fractions of different size proteins (>100 kDa, 100–55 kDa, 55–35 kDa and <35 kDa). The gel pieces were detached overnight in 50% methanol, 5% acetic acid in water. After dehydration with acetonitrile, proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate, and subsequently alkylated using 50 mM iodoacetamide in ammonium bicarbonate for 30 min at room temperature. Following washing of the gel pieces in 100 mM ammonium bicarbonate, proteolytic digestion was carried out with 100 ng of trypsin in 50 mM ammonium bicarbonate overnight at 37 °C. Tryptic peptides were extracted from each gel with 50% acetonitrile, 5% acetic acid in water, dried and re-suspended in 20 μl of 2% acetonitrile, 0.1% formic acid in water for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

Peptides from each gel fraction were separated on an Ultimate 3000 RSLC nano System utilising a PepMap C18 column, 2 μm particle size, 75 μm × 50 cm (Thermo Scientific, USA) and subsequently analysed on a QExactive mass spectrometer (Thermo Scientific, USA). Peptides were introduced into the mass spectrometer using an EASY-Spray™ nano source at a flow rate of 250 nl/min at approximately 400–600 bars. The 15 most intense precursors were selected for MS/MS analysis using higher-energy collisional dissociation (HCD) fragmentation and all fragmented precursor ions were actively excluded from repeated MS/MS analysis for 27 s.

2.5. Data analysis

2.5.1. Protein identification and relative abundance

RNAseq data of the schizont stage of T. parva has recently been made available in GenBank (BioSample accession SAMN03981746) and used to improve the annotation of the genome (da Silva, pers. comm.). The format for open reading frame (ORF) locus tags in the re-annotated genome of the Muguga strain has been changed (Non-ribosomal, gene symbols, chromosome number, gene number, or ORF locus tag). This study relied on the re-annotated genome and the new format for T. parva ORF locus tags has been adopted.
Raw MS/MS data files were analysed with Peaks software (Bioinformatics solutions) using a database containing all Uniprot database entries for *R. appendiculatus* and the re-annotated proteome of *T. parva* (a total of 16,969 protein entries with 4084 *T. parva* and 12,884 *R. appendiculatus* protein entries, July 2016). A peptide false discovery rate (FDR) of 1.2% was applied and only proteins with unique peptide identification were included in the final sporozoite proteome list.

In order to rank the identified proteins by their abundance in the sample, an Exponentially Modified Protein Abundance Index (emPAI) was calculated using Mascot (Matrix Science) (Ishihama et al., 2005). EmPAI is the exponential form of protein abundance index (PAI) minus 1 (emPAI = 10^PAI – 1) and is proportional to protein content in a protein mixture (Ishihama et al., 2005). PAI is calculated by dividing the number of observed peptides by the number of observable peptides per protein.

### 2.5.2. Classification of subcellular localization of the identified *T. parva* sporozoite proteins

Proteins were classified according to their putative localization in the sporozoite using TargetP (Emanuelsson et al., 2000). Transmembrane domains (TMDs) were predicted by TMHMM Server v. 2.0 (Krogh et al., 2001), signal peptides (SP) by SignalP 4.1 (Petersen et al., 2011) and glycosylphosphatidylinositol (GPI)-anchor signal by GPI-SOM (Fankhauser and Mäser, 2005).

### 2.5.3. Classification of the identified *T. parva* sporozoite proteins by orthology

We clustered all sporozoite protein coding sequences of *T. parva* with the complete predicted proteome of *Plasmodium falciparum* into putative orthologous groups using the OrthoMCL standalone software Version 2.0.2 (Li et al., 2003). The blast step of OrthoMCL was performed using an E-value cut-off of 1e-10, and minimum 70% sequence identity over 75% sequence coverage. *Plasmodium falciparum* 3D7 release of 26-06-2017 sequence data was downloaded from PlasmoDB (Aurrecoechea et al., 2010).

### 3. Results

#### 3.1. Sporozoite proteome determination and relative quantification of expressed sporozoite proteins

LC–MS/MS was used to identify proteins expressed in a sample of semi-purified *T. parva* sporozoites. A complete list of all the proteins identified in this study is provided in Supplementary Table S1. In total 4780 proteins were identified of which 2007 originated from *T. parva* and 2773 from *R. appendiculatus*. Most proteins (3847) were identified with two or more peptides, while 933 protein identifications were based on a single peptide match, which includes 296 *T. parva* proteins (Supplementary Table S2). Thus, there is higher confidence in the identification of 1711 expressed parasite proteins. Sequence coverage of over 85% was achieved for three proteins, glyceraldehyde 3-phosphate dehydrogenase NAD binding domain, histone H2B.1 and ribosomal protein S21e, all encoded by single copy genes. Protein TpMuguga_03g00067, annotated as a hypothetical protein, was identified by as many as 94 unique peptides. The 2007 *T. parva* sporozoite proteins that were identified represent approximately 50% of the 4084 proteins predicted to be encoded by the re-annotated *T. parva* genome (da Silva, pers. comm.). Of the 2007 proteins, 1287 were further ranked by calculation of relative abundance in the sample using emPAI values (Supplementary Table S3), a parameter that is useful for obtaining an overview of proteome profiles with a wide dynamic range of concentrations (30 fmol to 1.8 pmol/μl) (Ishihama et al., 2005).

The most abundant *T. parva* sporozoite proteins are shown in Table 1. Even within this data set, there is a large range in the abundance of parasite proteins with histones being the most abundant. Other housekeeping proteins, e.g., ribosomal proteins, heat shock protein 70, phosphoglycerate mutase and glyceraldehyde 3-phosphate dehydrogenase, feature in this list.

#### 3.2. Identification of known *T. parva* antigens within the sporozoite proteome

The major circumsporozoite surface protein of *T. parva*, p67, which is a target of sporozoite neutralising antibodies (Dobbelare et al., 1984), was identified among the 20 most abundant proteins (Table 1). Additional antigens that had been previously characterised were also identified in this study, e.g., p104 (Ebel et al., 1999), p150 microneme/rhoptry proteins (Skilton et al., 1998) and p32, a merozoite antigen (Skilton et al., 2000). We searched the sporozoite proteome data (Supplementary Table S1) for characterised schizont CDB T cell antigens (Graham et al., 2006, 2008) and found evidence for expression of all known CTL antigens (reviewed in Nene and Morrison, 2016) in the sporozoite stage. In contrast to CD8 T cell antigens, CD4 T cell antigens still require better characterization. CD4 antigens are presented by intact schizont-infected cells (Brown et al., 1989) as well as cell extracts (Ballinger et al., 2000). Soluble cytosolic parasite antigens stimulatory to CD4 T cell clones with fractions ranging between 10 and 24 kDa (Brown et al., 1995) have been described, but further analysis and identification of the specific constituent proteins is required. However, among the most abundant sporozoite proteins we identified a protein that is homologous to a *Theileria annulata* protein annotated as rhoptry associated protein-1 (RAP-1) (Table 1). RAP-1 of *B. bigemina* has been shown to be a CD4 T cell antigen (Brown et al., 1999), suggesting that the *T. parva* RAP-1 is a potential CD4 T cell antigen.

### Table 1

| ORF locus tag (Da)        | Annotation            | Mass (Da) | emPAI |
|--------------------------|-----------------------|-----------|-------|
| TpMuguga_04g00404         | Histone H2B.1         | 12,940    | 7228.34 |
| TpMuguga_04g00071         | Histone H2B           | 13,682    | 900.39  |
| TpMuguga_04g00675         | Ribosomal protein S19 | 17,150    | 99.38   |
| TpMuguga_01g00555         | Hypothetical protein  | 17,296    | 96.3    |
| TpMuguga_04g00036         | AhpC/TSA family       | 22,120    | 84.68   |
| TpMuguga_01g00541         | Histone H2A           | 13,498    | 60.26   |
| TpMuguga_03g00067         | Hypothetical protein  | 11,572    | 54.06   |
| TpMuguga_01g00726         | elf-Tu GTP binding domain | 49,779    | 52.64  |
| TpMuguga_01g00541         | Hypothetical protein  | 24,796    | 52.36   |
| TpMuguga_04g00383         | GAPDH NAD binding domain | 36,883    | 51.37   |
| TpMuguga_01g00701         | RAP-1                 | 70,441    | 47.73   |
| TpMuguga_03g00067         | Heat shock 70 kDa protein | 71,445    | 42.22   |
| TpMuguga_04g00179         | RanBP1 domain         | 38,992    | 41.34   |
| TpMuguga_03g00700         | Hypothetical protein  | 23,995    | 40.00   |
| TpMuguga_03g00287         | Sporozoite P67 surface antigen | 75,453    | 39.16   |
| TpMuguga_01g00067         | Acyl CoA binding protein | 10,043    | 39.11   |

* Open reading frame (ORF) locus tag refers to a unique *T. parva* gene identifier.
* Hypothetical protein is of unknown function; AhpC/TSA, alkyl hydroperoxide reductase subunit C; thiol specific antioxidant family; elf, elongation factor; RAP-1, homolog of a *Theileria annulata* protein annotated as Rhoptry-associated protein 1; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NAD, Nicotinamide adenine dinucleotide; RanBP1, Ran binding protein 1; CoA, co-enzyme A.
* emPAI, relative protein abundance in sample.
3.3. *Theileria parva* sporozoite proteins associated with apicomplexan invasion of host cells

Sporozoite secretory organelle proteins are typically located in the micronemes, rhoptries and dense granules in *P. falciparum* (reviewed in Lindner et al., 2013). To determine whether these proteins are present in *T. parva* sporozoites, we clustered our identified *T. parva* sporozoite proteins, using OrthoMCL, into orthologous groups with all *P. falciparum* genes. A total of 1105 proteins of the 2007 *T. parva* proteins were defined as *P. falciparum* orthologs (Supplementary Table S4) and we searched this list for apical organelle proteins. We identified *T. parva* orthologs of eight rhoptry proteins, three microneme proteins and a dense granule protein (Table 2). Featured in the list are orthologs of *P. falciparum* apical membrane antigen 1 (AMA-1), a microneme protein essential during host cell invasion (Ttreeck et al., 2009), and cell-traversal protein for ookinetes and sporozoites (CelTOS), a malaria antigen mediating host cell invasion (Kariu et al., 2006). AMA-1 is a leading malaria vaccine candidate (Remarque et al., 2008), and rhoptry proteins, three microneme proteins and a dense granule proteins of the 2007 orthologous groups with all *T. parva* identified proteins.

In *T. parva*, mobilisation of Ca^{2+} is necessary for successful sporozoite invasion of bovine lymphocytes and reagents that prevent Ca^{2+} mobilisation significantly curf zipper and internalisation stages of the entry process (Shaw, 1995). Table 3 shows 13 proteins identified in *T. parva* clustered with the *Plasmodium* proteins that play a role in calcium signalling, including calmodulin, an intracellular target for Ca^{2+} activation that acts on proteins such as guanylyl cyclases, protein kinases and phosphatases to aid in signal transduction.

3.4. Putative *T. parva* surface proteins within the sporozoite proteome

We used two methods to search for novel putative surface proteins. The first method was to use bioinformatics tools to predict the presence of GPI anchors and SP in proteins expressed by sporozoites. Only 12 proteins of the 2007 sporozoite proteins were found in this category (Table 4). The list includes *TmMuguga*_07g00939 (gp34), a protein that undergoes GPI modification when expressed in mammalian cells and a schizont surface protein (*Xue et al., 2010*). Since there is no direct evidence for addition of GPI anchors to parasite expressed proteins, we searched for enzymes of the GPI biosynthesis pathway encoded by the *T. parva* genome. Eight genes are essential for GPI synthesis in *P. falciparum* (Delorenzi et al., 2002). We found homologs of all eight genes in *T. parva* (*TmMuguga*_04g000759, *TmMuguga*_04g000881, *TmMuguga*_04g00525, *TmMuguga*_01g001669, *TmMuguga*_02g00201, *TmMuguga*_03g00397, *TmMuguga*_03g00846 and *TmMuguga*_02g00741) and identified that the latter four are expressed in the sporozoite proteome. This suggests the existence of GPI anchored proteins in *T. parva*, although this remains to be formally proven.

Secondly, we compiled from the literature a list of surface-exposed *P. falciparum* proteins and determined if orthologs exist within the *T. parva* sporozoite proteome. We identified orthologs of AMA-1 (*TmMuguga*_01g00650) and a hexose transporter (*TmMuguga*_01g01069) which were both identified by specific biontinylation of *P. falciparum* and *Plasmodium yoelii* surface proteins (Lindner et al., 2013).

3.5. *Theileria parva* proteins as candidate manipulators of host cell signalling leading to transformation

Lymphocyte transformation characterised by cancer-like cell phenotypes is solely dependent on viable schizonts and requires the schizont proteins to interact with and manipulate pathways regulating lymphocyte apoptosis, proliferation and gene expression (Dobelaere and Heussler, 1999; Tretina et al., 2015). We identified 17 proteins predicted to be manipulators of host cells (Shiels et al., 2006) in the sporozoite stage, suggesting that these proteins may be required immediately upon host cell entry.

### Table 2

Invasion organelle proteins. Proteins detected from whole cell lysates of *Theileria parva* salivary gland sporozoites are aligned by orthology with *Plasmodium falciparum* sporozoite proteins.

| Orthologous group | Organism | ORF locus tag | Gene name | Annotation (reference) |
|-------------------|----------|---------------|-----------|------------------------|
| **Rhoptry proteins** |          |               |           |                        |
| OC5.142870        | *P. falciparum* | PFID7.1452000 | PRON2     | Rhopty neck protein 2 (Cao et al., 2009) |
| OC5.153587        | *T. parva* | TmMuguga_01g00014 | –         | Hypothetical protein |
| OC5.153567        | *P. falciparum* | PFID7.1347500 | RON5      | Conserved Plasmodium protein, unknown function (Curtidor et al., 2011) |
| OC5.153433        | *T. parva* | TmMuguga_01g01161 | –         | Hypothetical protein |
| OC5.128020        | *P. falciparum* | PFID7.0932300 | PM18AAP   | M18 aspartyl aminopeptidase (Lauterbach and Coetzer, 2008) |
| OC5.145111        | *T. parva* | TmMuguga_01g01150 | RNaseI    | RNI-like protein, putative (Sam-Yellowe et al., 2004) |
| OC5.141731        | *P. falciparum* | PFID7.1361800 | GAC       | Conserved Plasmodium protein, unknown function (Sam-Yellowe et al., 2004) |
| OC5.129273        | *T. parva* | TmMuguga_01g00992 | –         | Hypothetical protein |
| OC5.154281        | *P. falciparum* | PFID7.1006200 | ALBA3     | DNA/RNA-binding protein Alba 3 (Lindner et al., 2013) |
| OC5.154281        | *T. parva* | TmMuguga_03g00655 | –         | Hypothetical protein |
| **Microneme proteins** |          |               |           |                        |
| OC5.147452        | *P. falciparum* | PFID7.1133400 | AMA1      | Apical membrane antigen 1, AMA1 (Cowman and Crabb, 2006) |
| OC5.171217        | *T. parva* | TmMuguga_01g00650 | –         | Apical membrane antigen 1 |
| OC5.135185        | *P. falciparum* | PFID7.0129000 | CelTOS    | Cell-traversal protein for ookinetes and sporozoites, putative (Kariu et al., 2006) |
| OC5.126706        | *T. parva* | TmMuguga_02g00627 | –         | Hypothetical protein |
| **Dense granules** |          |               |           |                        |
| OC5.126706        | *P. falciparum* | PFID7.0818200 | –         | 14-3-3 protein, putative (Lindner et al., 2013) |

* Open reading frame (ORF) locus tag refers to a unique *T. parva* or *P. falciparum* gene identifier.
* Gene name is as defined in *P. falciparum.*
Transcripts for the genes encoding all 17 proteins are found in the RNAseq data from schizont-infected cells (see Section 2.5.1), and contain orthologous Group Organism ORF locus tag a Gene name b Annotation (reference)OG5_126800 P. falciparum PF3D7_1434200 CAM CalmodulinOG5_129380 P. falciparum PF3D7_1027700 PICEN1 Centrin-1 (Khan et al., 2005)OG5_152981 P. falciparum PF3D7_1140000 CA Centrin OG5_126600 P. falciparum PF3D7_0717500 PIPCK4 Calcium-dependent protein kinase 4 (Holder et al., 2012)OG5_131887 P. falciparum PF3D7_1213000 CDPK7 Calcium-dependent protein kinase 7 (Lindner et al., 2013)OG5_127599 P. falciparum PF3D7_1436600 PKG cGMP-dependent protein kinase (Lindner et al., 2013)OG5_133188 P. falciparum PF3D7_1138400 GCalpha Guanylyl cyclase (Pradel, 2007)OG5_141757 P. falciparum PF3D7_1246400 MTIP Myosin A tail domain interacting protein (Jones et al., 2006)OG5_126577 P. falciparum PF3D7_1329100 MynC Myosin CTyagi et al., 2009)OG5_126674 P. falciparum PF3D7_1211900 ATPase4 Non-SERCA-type Ca^{2+}-transporting P-ATPase (Budu and Garcia, 2012)OG5_127599 P. falciparum PF3D7_0934800 PKAc cAMP-dependent protein kinase catalytic subunit (Lindner et al., 2013) a Open reading frame (ORF) locus tag refers to a unique T. parva or P. falciparum gene identifier. b Gene name is as defined in P. falciparum.

Table 3
Proteins involved with calcium signalling. Proteins detected from whole cell lysates of Theileria parva salivary gland sporozoites are aligned by orthology with Plasmodium falciparum sporozoite proteins.

| Orthologous Group | Organism | ORF locus tag a | Gene name b | Annotation (reference) |
|-------------------|----------|----------------|-------------|------------------------|
| OG5_126800 | P. falciparum | PF3D7_1434200 | CAM | Calmodulin |
| T. parva | TpMuguga_02g00717 | – | Calmodulin |
| OG5_129380 | P. falciparum | PF3D7_1027700 | PICEN1 | Centrin-1 (Khan et al., 2005) |
| T. parva | TpMuguga_01g00227 | – | Centrin |
| OG5_152981 | P. falciparum | PF3D7_1140000 | CA | Centrin |
| T. parva | TpMuguga_02g00412 | – | Hypothetical protein |
| OG5_126600 | P. falciparum | PF3D7_0717500 | PIPCK4 | Calcium-dependent protein kinase 4 (Holder et al., 2012) |
| T. parva | TpMuguga_01g01073 | – | Calcium-dependent protein kinase |
| OG5_131887 | P. falciparum | PF3D7_1213000 | CDPK7 | Calcium-dependent protein kinase 7 (Lindner et al., 2013) |
| T. parva | TpMuguga_04g00518 | – | Protein tyrosine kinase |
| OG5_127599 | P. falciparum | PF3D7_1436600 | PKG | cGMP-dependent protein kinase (Lindner et al., 2013) |
| T. parva | TpMuguga_03g00511 | – | Protein tyrosine kinase |
| OG5_133188 | P. falciparum | PF3D7_1138400 | GCalpha | Guanylyl cyclase (Pradel, 2007) |
| T. parva | TpMuguga_02g00848 | – | Guanylyl cyclase |
| OG5_141757 | P. falciparum | PF3D7_1246400 | MTIP | Myosin A tail domain interacting protein (Jones et al., 2006) |
| T. parva | TpMuguga_01g00513 | – | Myosin light chain |
| OG5_126577 | P. falciparum | PF3D7_1329100 | MynC | Myosin C(Tyagi et al., 2009) |
| T. parva | TpMuguga_01g00774 | – | Myosin C |
| OG5_126674 | P. falciparum | PF3D7_1211900 | ATPase4 | Non-SERCA-type Ca^{2+}-transporting P-ATPase (Budu and Garcia, 2012) |
| T. parva | TpMuguga_01g00720 | – | Calcium-transporting ATPase |
| OG5_127599 | P. falciparum | PF3D7_0934800 | PKAc | cAMP-dependent protein kinase catalytic subunit (Lindner et al., 2013) |
| T. parva | TpMuguga_02g00378 | – | cAMP-dependent protein kinase |

Table 4
Potential sporozoite surface proteins. Proteins detected from whole cell lysates of Theileria parva salivary gland sporozoites predicted to contain a glycosylphosphatidylinositol anchor and either transmembrane domain or signal peptide.

| ORF locus tag a | Annotation b | Unique peptides c | emPAI d | TMD e | GPI-anchor f | SP g |
|----------------|--------------|------------------|--------|--------|-------------|-----|
| TpMuguga_01g00438 | Hypothetical protein | 34 | 1.01 | 0 | 1 | 1 |
| TpMuguga_01g00939 | Hypothetical protein (gp34) | 12 | 3.65 | 1 | 1 | 1 |
| TpMuguga_01g00939 | Hypothetical protein | 12 | 3.65 | 1 | 1 | 1 |
| TpMuguga_01g00972 | Hypothetical protein | 4 | 0.45 | 1 | 1 | 1 |
| TpMuguga_01g01056 | Merozoite antigen (p32) | 12 | 6.78 | 0 | 1 | 1 |
| TpMuguga_02g00412 | Hypothetical protein | 5 | 0.3 | 0 | 1 | 1 |
| TpMuguga_02g00338 | Hypothetical protein | 31 | 1.34 | 1 | 1 | 1 |
| TpMuguga_02g00553 | Hypothetical protein | 6 | 2.95 | 1 | 1 | 1 |
| TpMuguga_02g00550 | Hypothetical protein | 2 | <0.05 | 1 | 1 | 1 |
| TpMuguga_03g00287 | Sporozoite p67 surface antigen | 53 | 39.16 | 1 | 1 | 1 |
| TpMuguga_04g00437 | 104 kDa microneme/rhoptry antigen (p104) | 60 | 12.35 | 1 | 1 | 1 |
| TpMuguga_04g00615 | Probable N-acetylgalactosaminyl-phosphatidylinositol de-N-acetylase | 2 | <0.05 | 0 | 1 | 1 |

a Open reading frame (ORF) locus tag refers to a unique T. parva gene identifier.  
b Hypothetical protein is of unknown function(s).  
c Unique peptides refers to the total number of peptides identified matching the protein sequence that do not match any other protein in the database searched.  
d emPAI, relative protein abundance in sample.  
e Predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).  
f Predicted using the GPI-SOM (http://gpi.unibe.ch/).  
g Predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/).
host cells (Marsolier et al., 2015). Interestingly, the bulk schizont RNAseq data identified a high level of anti-sense transcription of the TpSCOP gene (Supplementary Table S5), suggesting that expression of this gene in the schizont stage may be under differential control. Resolution of the significance of this observation will require analysis of cell-cycle synchronised samples.

3.6. Sporozoite proteins involved in glycolysis and tricarboxylic acid (TCA) cycles

Proteins of the glycolytic pathway are conserved in most eukaryotic organisms and 10 genes encoding glycolytic enzymes have been found in *Theileria* genomes (Gardner et al., 2005; Pain et al., 2005). We identified expression of all the 10 enzymes of the glycolytic pathway in this study (Fig. 1) including lactate dehydrogenase (LDH), an enzyme that converts pyruvate, the last product of glycolysis, to lactate during low or no oxygen conditions. We also identified all the TCA cycle enzymes by MS (Fig. 1) with the exception that malate dehydrogenase is functionally replaced by malate-quinone oxidoreductase (Gardner et al., 2005), and was identified in the proteome data. Glutamate dehydrogenase was also identified in the proteome and glutamate has been suggested to be a supplementary intermediate for the TCA cycle (Gardner et al., 2005). The only functional link we have found between glycolysis and the TCA cycle is the presence of phosphoenolpyruvate carboxykinase. Neither glycerol kinase nor glycerol 3-phosphate dehydrogenase were identified, although *T. parva* encodes genes for both enzymes. These enzymes were identified with low levels in the schizont stage of *T. annulata* (Witschi et al., 2013).

3.7. Tick proteins

Searching the Uniprot database entries for *R. appendiculatus* with the LC–MS/MS data led to identification of 2773 tick proteins in addition to the *T. parva* proteins. The complete list of all the identified tick proteins is presented in Supplementary Table S1, but a detailed analysis of vector proteins will be reported elsewhere.

4. Discussion

In this study, we employed a DE-52 ion-exchange column purification method to semi-purify *T. parva* sporozoites from infected tick salivary glands. Although tick proteins constituted

![Fig. 1. Most of Theileria parva proteins involved in glycolysis and tricarboxylic acid cycles were identified in this study. The glycolysis pathway is shown on the left and tricarboxylic acid cycle is shown on the right. Colour code for proteins: green, enzymes that were identified by MS; blue, enzymes encoded in the *T. parva* genome but not identified by MS; red, enzymes not encoded by the *T. parva* genome and not identified in this study. Enzymes identified by MS have open reading frame locus tags included in brackets (see below): HK, hexokinase (TpMuguga_01g00043); GPI, glucose 6-phosphate-isomerase (TpMuguga_03g00346); PFK, phosphofructokinase (TpMuguga_02g00577); FBP, fructose bisphosphate; FBA, fructose bisphosphate aldolase (TpMuguga_01g00101); TPI, triosephosphate isomerase (TpMuguga_04g00464); Gly-3PDH, glycerol-3-phosphate dehydrogenase; Gly-K, glycerol kinase; GAPDH, glyceraldehyde phosphohexose dehydrogenase (TpMuguga_02g00858); PGK-1, phosphoglycerate kinase (TpMuguga_01g00965); PGM-1, phosphoglycerate mutase (TpMuguga_04g00060); EVO, enolase (TpMuguga_01g00116); PC, pyruvate carboxylase: ACH, aconitate hydratase-1 (TpMuguga_01g01050); IDH, isocitrate dehydrogenase (TpMuguga_04g00620); OGDH, oxoglutarate dehydrogenase (TpMuguga_01g00226); SCL, succinyl coenzyme A ligase (TpMuguga_04g00600); SDH, succinate dehydrogenase (TpMuguga_01g00210); FH, fumarate hydratase-1 (TpMuguga_03g00078); MDH, malate dehydrogenase; MQR, Malate: quine oxidoreductase (TpMuguga_03g00758); CS, citrate synthase (TpMuguga_02g00666); PEPC, phosphoenolpyruvate carboxylase; PEPCCK, phosphoenolpyruvate carboxykinase (TpMuguga_01g00495). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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was also identified (Table 1). Unexpectedly, the polymorphic antigen load is sufficient. For the purposes of our current analyses we have, however, reported on the entire data set of 2007 proteins. Failure to identify T. parva proteins does not directly imply their absence in the sample. Peptides from proteins with low abundance may fall below the detection limit or are simply not sequenced by the mass spectrometer due to limited dynamic range and/or speed of instrument acquisition. Furthermore, unknown, complex, or amino acid modifications leading to alteration of the peptide mass can only be detected in some cases by the Peaks software. Finally, trypsin digestion of proteins with low or irregular arginine/lysine content can lead to very short and very long tryptic peptides that fall out of the acquired mass range, and may therefore not be detected.

Identification of T. parva sporozoite proteins through MS has allowed us to assess the proteomic component of the mammalian-infective stage of the parasite. We have also calculated the emPAI using Mascot to estimate the relative abundance ranking of proteins in the sample, which revealed a very high range of abundance with histones ranked with an emPAI of 60–7200 for the different histone subunits to an emPAI index of 39 for the major circumsporozoite p67 protein. Subunits of two enzymes within the glycolytic pathway, phosphoglycerate mutase and glyceraldehyde 3-phosphate dehydrogenase, are among the 20 most abundant sporozoite proteins detected (Table 1). A high abundance of histones is not unusual. Lindner et al. (2013) reported histones as the third most abundant protein in Plasmodium sporozoite proteome after the circumsporozoite protein and mitochondrial ATP synthase, subunit beta (Lindner et al., 2013).

A number of known antigens were identified within the sporozoite proteome including p67, a leading vaccine antigen, and previously described components of secretary organelles (e.g., p104 and p150) (Table 4). RAP-1, a rhoptry antigen protective against P. falciparum infection in Saimiri monkeys (Ridley et al., 1990), was also identified (Table 1). Unexpectedly, the polymorphic immuno-dominant molecule (PIM), a well-known antigen (Toye et al., 1991), was not identified in this study even though p150, an antigen that is immunologically cross-reactive with PIM (Skilton et al., 1998), was identified. The PIM protein sequence contains a large repetitive domain rich in glutamine and proline amino acid residues that is devoid of lysine and arginine residues (Toye et al., 1991). An in silico digestion of PIM with trypsin leads to prediction of few detectable tryptic peptides (data not shown). This may explain why we did not detect it. All schizont proteins that have been identified as CTL antigens are also expressed in sporozoites (emPAI range of 0.76–31.69). Some of these antigens are encoded by housekeeping proteins (e.g., hsp90) and translation elongation initiation factor 1A, so expression by sporozoites of some CTL antigens is not unexpected. The finding that all the known CTL antigens are expressed by sporozoites raises the question of whether infection of dendritic cells by sporozoites results in direct presentation of antigen for priming CTL responses in vivo, provided the antigen load is sufficient.

A study on the proteome of T. annulata schizont stage, in which 798 proteins were identified, failed to identify any member of SVSP and TashAT families (Witschi et al., 2013). In contrast, we identified members of both families in the sporozoite stage (Supplementary Table S5). There is increasing evidence pointing to a role of these proteins in host-parasite interaction, for instance SVSP epitope-tagged TpMuguga_03g00882 protein expressed in mammalian cells was found to translocate into the host nucleus, pointing to potential role in transforming host cells (Schmuckli-Maurer et al., 2009). In addition, we identified other predicted host manipulation proteins such as DEAD-box RNA helicases, peptidases and two proteins, namely a peptydyl prolyl-isomerase and TpSCOP, demonstrated to play roles in host manipulation by stabilising c-JUN and activation of NF-kB, respectively (Shiels et al., 2006; Hayashida et al., 2010; Marsolier et al., 2015). Host cell manipulation is known to occur at the schizont stage and the finding of these proteins in the sporozoite stage suggests early expression of these proteins may be required, or multi-functionality of the proteins.

Sporozoite invasion of lymphocytes begins with binding events that trigger the mobilisation of Ca2+ within the sporozoites (Dobbelare and Heussler, 1999). The free intracellular Ca2+ can be utilised by calmodulin to remodel itself structurally so as to act on downstream effectors such as calcium-dependent protein kinases (CDPKs), which have been shown to be useful for parasite infectivity. We detected both calmodulin and CDPKs in this study (Table 3). We also detected other calcium signalling proteins including guanylyl cyclases, cGMP-dependent protein kinases and CAMP-dependent protein kinases, all of which are important for downstream calcium signalling and have been shown to be essential for sporozoite invasion (reviewed in Dobbelare and Heussler, 1999).

In motile apicomplexans such as Plasmodium, free calcium induces microneme secretion by sporozoites to aid in gliding motility and host invasion (Lindner et al., 2013). Although T. parva sporozoites are immotile (Shaw, 2003), we identified orthologs of Plasmodium microneme components, including AMA-1, CetTOS and Leu/Phe-tRNA protein transferase (Cowman and Crabb, 2006; Kariu et al., 2006; Lindner et al., 2013). These proteins may be involved solely in invasion processes, together with the detected rhoptry proteins (PRON2, Conserved Plasmodium protein, PM18AAP and RNB-like protein) and dense granules protein (14-3-3 protein) (Sam-Yellowe et al., 2004; Lauterbach and Coetzer, 2008; Cao et al., 2009; Curtidor et al., 2011; Lindner et al., 2013). Since most of these proteins are still classified as hypothetical in T. parva, they present an opportunity for further analysis in studies aiming to block parasite invasion of host cells.

In conclusion, these data establish the expression profile of T. parva sporozoite proteins, the only such profile available to date.
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might be combined with the major surface protein p67 to induce 

One or more of the newly identified proteins, in particular putative 

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