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Vector-pathogen interactomics: connecting the dots

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Antunes, S., **Couto, J.**, Ferrolho, J., Rodrigues, F., Nobre, J., Santos, A.S., Santos-Silva, M.M., de la Fuente, J. and Domingos, A., 2018. *Rhipicephalus bursa* sialotranscriptomic response to blood feeding and *Babesia ovis* infection: identification of candidate protective antigens. Frontiers in Cellular and Infection Microbiology, 8, p.116. DOI: 10.3389/fcimb.2018.00116.

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Couto, J.*, Villar, M.*, Mateos-Hernández, L., Ferrolho, J., Sanches, G.S., Sofia Santos, A., Santos-Silva, M.M., Nobre, J., Moreira, O., Antunes, S., de la Fuente, J. and Domingos, A., 2020. Quantitative proteomics identifies metabolic pathways affected by *Babesia* infection and blood feeding in the sialoproteome of the vector *Rhipicephalus bursa*. Vaccines, 8(1), p.91. DOI: 10.3390/vaccines8010091 (* shared co-first authorship).

Couto, J., Seixas, G.; Stutzer, C; Olivier, N.; Maritz-Olivier, C; Antunes, S. and Domingos, A., 2021. Probing the *Rhipicephalus bursa* sialomes in potential anti-tick vaccine candidates: a reverse vaccinology approach. Biomedicines, 9(4), p.363. DOI:10.3390/biomedicines9040363.

Oral and poster communications

Antunes, S., Ferrolho, F., Nobre, J., **Couto, J.**, Santos, A.S., Santos-Silva, M.M., de la Fuente, J. and Domingos, A. (2016) *Vitellogenin* and *Lachesin* knockdown in *Rhipicephalus bursa* influence in feeding and pathogen acquisition. AITVM-STVM Conference: april, Berlin, Germany **(Oral communication)**

Couto, J., Antunes, S., de la Fuente, J. and Domingos, A. (2016) Vector-pathogen interactomics: connecting the dots using tick cell lines. 7as. Jornadas Científicas do IHMT: december, Lisbon, Portugal **(Oral communication)**

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Couto, J., Antunes, S., Ferrolho, J., Santos, A.S., Santos-Silva, M.M., de la Fuente, J. and Domingos, A. (2016) An insight into the transcriptome of *Rhipicephalus bursa*: differential gene expression upon feeding and in response to salivary glands infection with *Babesia ovis*. AITVM-STVM Conference: april, Berlin, Germany **(Poster communication, received the Dik Zwart Award)**

Couto, J., Antunes, S., Ferrolho, J., Santos, A.S., Santos-Silva, M.M., de la Fuente, J. and Domingos, A. (2016) Análise do sialotranscriptoma de *Rhipicephalus bursa* em resposta à alimentação e infeção por *Babesia ovis*. II Workshop NOVA INSA de Doenças Transmitidas por Vetores: october, Lisbon, Portugal **(Poster communication)**

Couto, J., Antunes, S., de la Fuente, J. and Domingos, A. (2017) Omics reveal metabolic pathways of *Rhipicephalus bursa* during infection and feeding. 8as. Jornadas Científicas do IHMT: december, Lisbon, Portugal **(Poster communication)**

Couto, J., Mateos-Hernández, L., Villar, M., Santos, A.S., Santos-Silva, M.M., de la Fuente, J., Domingos, A. and Antunes, S. (2018) Sialoproteome of *Rhipicephalus bursa*: unravelling the pathogen-vector interface. Congress of the Federation of European Biochemical Societies (FEBS): july, Prague, Czech Republic **(Poster communication)**

Couto, J., Mateos-Hernández, L., Villar, M., Antunes, S., Santos, A.S., Santos-Silva, M., de la Fuente, J. and Domingos, A. (2018) *Rhipicephalus bursa* inside out: a proteomic approach for the identification of vaccine targets. Encontro Ciência 2018: july, Lisbon, Portugal **(Poster communication)**

Dias, F., **Couto, J.**, Ferrolho, J., Antunes, S. and Domingos, A. (2018) Folate pathway modulation in *Rhipicephalus* ticks and cultured tick cells in response to infection. AITVM-STVM Conference: september, Buenos Aires, Argentina **(Poster communication, received the Best Poster Award)**

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Dias, F., **Couto, J.**, Ferrolho, J., Bell-Sakyi, L., Antunes, S. and Domingos, A. (2018) Tick's folate pathway modulation by infection. TiBE2018 Host-Parasite interactions CIBIO-InBIO - U Porto: december, Porto, Portugal **(Poster communication)**

Couto, J., Villar, M., Mateos-Hernández, L., Ferrolho, J., Sanches, G.S., Santos, A.S., Santos-Silva, M., Nobre, J., Antunes, S., de la Fuente, J. and Domingos, A. (2019) Silencing of a ubiquitin ligase protein: double edged sword? Encontro Ciência 2019: julho, Lisbon, Portugal **(Poster communication)**

Couto, J., Seixas, G., Stutzer, C., Olivier, N., Maritz-Olivier, C., Antunes, S. and Domingos, A. (2020) Immunoinformatics towards vaccine development: sialoproteins as potential anti tick candidates. 10as. Jornadas Científicas do IHMT: december, Lisbon, Portugal **(Poster communication)**

Couto, J., Seixas, G., Stutzer, C., Olivier, N., Maritz-Olivier, C., Antunes, S. and Domingos, A. (2020) Epitope mapping in the direction of anti-tick vaccine development. XI Jornadas Científicas do IHMT: december, Lisbon, Portugal **(Poster communication)**

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Resumo

As carraças e doenças associadas a carraças têm um impacto negativo considerável na saúde humana e animal. *Rhipicephalus bursa* é uma carraça multi-hospedeiro hematófaga e é o principal vetor de *Babesia ovis*, um hemoparasita altamente patogénico em pequenos ruminantes, que pode levar a uma taxa de mortalidade de 30-50% em animais suscetíveis e, indiretamente contribuir para um impacto socioeconómico negativo na sociedade humana. O controlo de carraças e doenças associadas depende principalmente do uso de fármacos, que apresentam grandes desvantagens, como a contaminação de alimentos e ambiente e o aumento da resistência, reforçando assim a necessidade de medidas alternativas, como a vacinação. Com base na premissa de que as glândulas salivares da carraça têm um papel crucial no comportamento hematófago e na transmissão de agentes patogénicos, o objetivo principal deste trabalho é aumentar o conhecimento sobre a interação *R. bursa-B. ovis* neste tecido, de forma a identificar novos candidatos a antigénios protetores para o desenvolvimento de vacinas. Assim sendo, os sialotranscritos e as sialoproteínas de *R. bursa* foram analisados em diferentes condições, para compreender melhor os processos de alimentação e infeção e contribuir para o desenvolvimento de novas vacinas anti-carraça e doenças associadas. A análise comparativa dos transcriptomas e proteomas revelou que a alimentação por sangue induz a produção de moléculas por parte da carraça, o que se traduziu no aumento da expressão genética e da síntese proteica. Além disso, os dados mostram que a combinação de estímulos (alimentação e infeção) influenciou positivamente a expressão genética, mas negativamente a tradução, podendo sugerir a manipulação de *B. ovis* no sialoma de *R. bursa*. Estes resultados aliados a diferentes metodologias como RNA de interferência (*in vitro* e *in vivo*) e vacinologia reversa, permitem explorar a maquinaria celular da carraça e identificar vários alvos como potenciais antigénios para vacinas. Os ensaios de silenciamento revelaram o impacto direto de algumas moléculas na sobrevivência da carraça e a sua fixação ao hospedeiro (como a putativa "Vitelogenin-3" e uma proteína do "cement"), enquanto que outros demonstraram um efeito duplo divergente na sobrevivência do vetor e do agente patogénico (como a "lachesin" e a "UB2N"). A análise imunoinformática dos dados anteriores de sequenciação permitiu a identificação de proteínas/peptídeos capazes de induzir, no hospedeiro vertebrado, uma resposta imunológica forte e robusta contra o vetor e o agente patogénico. Nesta análise, uma proteína membranar (proteína contendo domínios "Marvel") e duas secretórias (uma "Evasin" e uma proteína contendo domínios de "Ricin") foram selecionadas e promissores "immunological kernels" foram encontrados, contendo características ideais de uma vacina baseada em peptídeos, sem causar alergia e toxicidade. Além disso, a integração de diferentes análises ómicas de diferentes espécies de carraças foi usada como uma estratégia para pesquisar e caracterizar vias biológicas conservadas, a fim de selecionar novos alvos capazes de impactar uma ampla gama de espécies de carraças e bloquear a transmissão de vários agentes patogénicos transmitidos por estas. Deste estudo, destacou-se a via de biossíntese de folato, ao observar que durante a infeção da carraça, quer por bactéria quer por protozoário, a expressão de genes relacionados com esta via era aumentada. No entanto, ensaios de silenciamento numa linha celular de carraça mostraram que, a curto prazo, a redução da expressão de um gene relacionado ao folato (*gch-I*), não exorta alterações significativas nas células de carraça ou no

comportamento do agente patogénico em termos de invasão ou multiplicação. Estudos aplicados e ensaios de vacinação precisam ser conduzidos para validar o potencial desses alvos promissores para o desenvolvimento de abordagens anti-carraça e de bloqueio de transmissão de doenças.

Palavras-chave: Carraças e doenças associadas a carraças, transcriptómica, proteómica, RNAi, vacinologia reversa

Abstract

Ticks and tick-borne diseases have a considerable negative impact on human and animal health. *Rhipicephalus bursa* is a hematophagous multi-host tick and the main vector of *Babesia ovis*, a highly pathogenic hemoparasite in small ruminants, which leads to a 30-50 % of mortality rate in susceptible animals and, indirectly, to a negative socioeconomic impact in human society. Tick and disease control rely mainly in the use of chemotherapy and acaracides, which has major drawbacks including food and environment contamination and the increase of resistance, reinforcing the need for alternatives measures, such as vaccination. Based on the premise that tick salivary glands have a crucial role on hematophagous behaviour and on pathogen transmission, the main objective of this research was to increase the understanding on the *Rhipicephalus bursa-Babesia ovis* interaction in this organ, in order to find new protective antigen candidates for vaccine design. Thus, the *R. bursa* sialotranscripts and sialoproteins were screened under different conditions, to better understand the feeding and infection processes and contribute for the development of new anti-tick and tick-borne diseases. The comparative analyses of the transcriptomes and proteomes revealed that blood feeding induces the production of tick molecules, which was translated by the increased gene expression and protein synthesis. Moreover, the data unveiled that the combination of stimuli (feeding and infection) influenced positively gene expression but negatively translation, suggesting that *B. ovis* might manipulate *R. bursa* sialome. These results allied to interference RNA (*in vitro* and *in vivo*) and reverse vaccinology, allowed to explore the tick cellular machinery and pinpointed several targets as potential vaccine antigens. The silencing assays revealed the direct impact of some molecules in tick survival and attachment to the host (such as putative Vitellogenin-3 and a Cement protein), while others demonstrated a divergent dual-effect on both vector and parasite survival (such as Lachesin and UB2N). Immunoinformatic analysis of the previous sequencing data allowed the identification of proteins/peptides capable of elicit, in the vertebrate host, a strong and robust immune response against both vector and pathogen. In this experiment, one membrane-related (Marvel-containing protein) and two secreted (a Evasin and a ricin-containing protein) proteins were selected and promising "immunological kernels" were found to have ideal characteristics for an anti-tick peptide-based vaccine, without causing allergy and toxicity. Furthermore, the integration of different omics analyses from different tick species was used as a strategy to search and characterize conserved biological pathways in order to select new targets able to impact a wide range of tick vectors and block the transmission of several transmitted pathogens. From this study the folate biosynthesis pathway stood out by observing that during tick infection, by either bacteria or protozoan, the expression of genes related to this pathway were increased. However, silencing assays in a tick cell line demonstrate that, in a short term, the reduction of expression of a folate-related gene (*gch-I*), did not lead to significant changes in tick cells or pathogen behaviour of invasion or multiplication. Applied studies and vaccination trials need to be conducted to validate the potencial of these promising targets for the development of anti-tick and transmission blocking approaches.

Keywords: Ticks and tick-borne diseases, transcriptomic, proteomic, RNAi, reverse vaccinology

Table of contents

List of figures

Chapter I: General Introduction

Chapter II-1: Influence of blood feeding and *Babesia ovis* infection on *Rhipicephalus bursa* sialome

List of tables

List of equations

Chapter II-2: Influence of blood feeding and *Babesia ovis* infection on *Rhipicephalus bursa* sialome

List of acronyms

% – Percentage

µg – Microgram

µl – Microliter

µm – Micrometer

µM – Micromolar

16S – Ribossomal Subunit 16

AMP – Antimicrobial peptide

AP – Alkaline Phosphatase

BLAST – Basic Local Alignment Search Tool

BmVDAC – *Boophylus microplus* Voltage-Dependent Anion-selective Channel

BoSPD – *Babesia ovis* Surface Protein D

bp – Base pair

BP – Biological Process

BSA – Bovine Serum Albumin

BUSCO – Benchmarking Universal Single-Copy Orthologue

C – coiled-coil structure

CC – Cellular Component

cDNA – Complementary DNA

CEGs – Coincident Epitopes Groups

CEVDI – Centro de Estudos de Vetores e Doenças Infeciosas

CoA – Coenzyme A

CP – Hemelipoglyco-Carrier protein

Cq – Quantification Cycle

Cys – Cysteine

Da – Dalton

DGAV – Divisão Geral de Alimentação e Veterinária

DMEM – Dulbecco's Modified Eagle Medium

DNA – Desoribonucleic acid

DO – Drop-off

dsb – Disulfide bond formation protein

dsRNA – Double-stranded RNA

dsβ2M – Mouse β-2-microglobulin dsRNA

E – beta sheet structure

E1s – Ubiquitin-activating enzymes

E2s – Ubiquitin-conjugating enzymes

E3s – Ubiquitin-ligase enzymes

EC – Evidence Codes

EFW – Engorged Female Weight

EHR – Egg Hatching Rate

elf – Elongation factor

ELISA – Enzyme-Linked Immunosorbent Assay

EMW – Egg Mass Weight

EPE – Egg Production Efficiency

ESTs – Expressed Sequence Tags

F – Fed ticks

Fi – Fed and *Babesia ovis* infected ticks.

GCH-I – GTP cyclohydrolase I

GO – Gene Ontology

GPI – Glycosylphosphatidylinositol

GRAVY – Grand Average of Hydropathicity

GRPs – Glycine Rich Proteins

H – alpha helix structure

h – Hour

HAT – Hypoxanthine-Aminopterin-Thymidine

IDA – Information-Dependent Acquisition

IF – Infected fed

Ig – Immunoglobulin

IHMT – Instituto de Higiene e Medicina Tropical

Imnp – Immunophilin

INF – Infected unfed

INIAV – Instituto Nacional de Investigação Agrária e Veterinária

INSA – Instituto Nacional de Saúde Doutor Ricardo Jorge

kDa – Kilodaltons

LRR – Leucine-Rich Repeat domain-containing protein

Mbp – Mega base pair

MF – Molecular Function

mg – Miligram

MHC – Major Histocompatibility Complex

min – Minute

mL – Mililiter

mm – Milimeter

mM – Milimolar

mol – Mole

mRNA – Messenger RNA

NCBI – National Center for Biotechnology Information

NFni – Unfed and uninfected ticks

ng – Nanogram

NIF – Uninfected fed

NINF – Uninfected unfed

nL – Nanoliter

ºC – Celsius degree

ORF – Open Reading Frames

Ov18S – Ovine Ribossomal Subunit 18

PBS – Phosphate-buffered saline

PCCA – Propionyl-CoA carboxylase

PCR – Polymerase Chain Reaction

pI – Theoretical Isoelectric Point

PTM – Post-Translational Modification

PTPS – 6-pyrovoyltetrahydropterin

qPCR – Quantitative real-time Polymerase Chain Reaction

RBC – Red Blood Cells

rDNA – Ribossomal DNA

RNA – Ribonucleic acid

RNAi –Interference RNA

RNA-seq – RNA sequencing

ROS – Reactive Oxygen Species

RP-LC-MS/MS – Reverse Phase Liquid Chromatography coupled with Mass Spectrometry

RR – Recovery Rate

rRNA – Ribossomal RNA

RT – Room Temperature

RTT – Replication-Transcription-Translation

RV – Reverse Vaccinology

s – Second

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SG – Salivary Gland

Spi – Kunitz-type Serine protease inhibitor

SRA – Sequence Read Archives

SWATH – Sequential Windowed data independent Acquisition of the Total High-resolution mass spectra

TBD – Tick-borne disease

TBP – Tick-borne pathogen

TBST – Tris-Buffered Saline complemented with 0.05% (v/v) Tween 20

Th – T helper lymphocyte cell

TROSPA – Tick Receptor of the Outer Surface Protein A

TS – Thymidylate Synthase

TSA – Transcriptome Shotgun Assembly

TTBDs – Ticks and Tick-borne diseases

UB2N – Ubiquitin-conjugating enzyme E2 N

V – Volt

v/v – Volume per volume

VBD – Vector-borne disease

Vg – Vitellogenin

VgR – Vitellogenin Receptor

w/v – Weight per volume

x g – Times gravity

Chapter I: General Introduction

1. Tick-borne diseases: the ovine babesiosis case

Vector-borne diseases (VBDs) result from an intrinsic **interplay** between **vector**, **pathogen** and **host**. In this triad, a vector is responsible for the spread of pathogens to hosts. The host is considered infected when it acquires the pathogen, allows its development (amplifier) and enables parasite transmission to a vector, otherwise it will be considered as a carrier or reservoir[1]. This interaction is complex, since it requires an introduced and/or established competent vector population, a pathogen capable of invasion and infection, and susceptible hosts sharing the same suitable environmental conditions across the full cycle of the VBD transmission[2]. Human VBDs, such as malaria, dengue, leishmaniasis, are accountable to one sixth of the illness and disability suffered worldwide, been responsible for more than one billion people infected and more than one million human deaths *per* year[3]. It is estimated that half of the world's population is living in areas exposed to two or more VBDs, being many of those co-endemic[4]. However, VBDs affect not only humans but also domestic animals and wildlife worldwide. Together, human and animal health are mostly affected by diseases transmitted by ticks, *i.e.* **tick-borne diseases (TBDs)**[5].

Ticks rank second after mosquitoes as the main vectors of human VBDs worldwide[6], though, in the United States of America, the most common reportable VBD is transmitted by ticks (Lyme disease)[7,8]. Nevertheless, ticks are the most important vectors of infectious diseases when considering both human and animal health[9]. This remarkable **success as vectors** is due to several features such as their mechanism of blood feeding and digestion, resilience to survive to different environments, hosts, and pathogens, and even their life cycle and propensity to transmit the pathogen through life stages and to their massive progeny. Such behaviour and life cycle may lead to anaemia, tick paralysis, tick toxicosis, injure of host skin tissues, causing irritation, inflammation, hypersensitivity, predisposition to localized dermatitis, secondary bacterial infections and even myiasis[10]. More importantly, due to their hematophagous behaviour, ticks are responsible for the transmission of viruses (*e.g.* tick-borne encephalitis, Thogoto virus), bacteria (*e.g. Rickettsiales*, *Borrelia*, *Francisella*, *Anaplasma*) and protozoa (*Babesia* and *Theileria*) some of which are zoonotic pathogens (*e.g.* Crimean Congo Haemorrhagic Fever virus, *Coxiella burnetti*, *Anaplasma phagocytophilum*)[11,12] affecting human and animal health.

The relevance of ticks and TBDs (TTBDs) dramatically increases due to its impact on the intrinsic relationship between humans and animals[13]. For instance, if animal health is neglected and negatively affected by TTBDs, this jeopardizes a sustainable livestock production (such as of cattle, small ruminants, swine, poultry and equines) which usually provides transportation, draught power, fuel, clothing (fur, leather) as well as food (meat, eggs and milk)[14] to human society. This has more significance in developing countries, since livestock is a pivotal source of income for many small farmers and animal holders[15], and contributes to the country agriculture gross domestic product and economic growth. Basically, if animal health is affected, food/nutrition security and livestock production/trade worldwide are condicioned[14] which contributes for the cycle of poverty, poor nutrition, hunger[16], and even to the emergence and spread of zoonoses[17,18]. The recognition of these interdependencies between human and animal health is essential to achieve global health[13].

Based on this **"One World, One Health"** concept, currently, several studies focusing on TTBDs control on **livestock** are being conducted[19,20]. Cattle are the focal point since they have high economical value, represents the most numerous of the ruminant species and provides the largest quantity of animal-derived resources. Nonetheless, **small ruminant**'s health is getting more attention, due to their high representation of the world ruminant population following catlle[21], its contribution for landscape and ecosystems preservation[22], and also for meat, milk, skin, and wool production in several countries[21], having a crucial socioeconomic role in different continents[23]. For example, in China, it is estimated that the total annual loss of small ruminants due to TBDs (such as ovine babesiosis, anaplasmosis, and theileriosis) is around 70 million United States Dollar[14,24]. These losses are associated to mortality, production impact, diagnostics, veterinary treatment and vector control costs[23].

The ***Rhipicephalus bursa*** tick is the major ectoparasite of small ruminants such as sheep and goats, in the Mediterranean basin with the propensity to transmit hemoparasites, such as *Babesia ovis*, *Anaplasma ovis* and *Theileria ovis*[25–27], being responsible for much of the economic and veterinary burden in livestock in some countries. Besides, it is the main vector of the most relevant haemoparasitic TBD of small ruminants in tropical and subtropical areas of the world[28], **ovine babesiosis**. The etiological agent with more pathogenicity of this TBD is ***Babesia ovis***[29], which is able to

provoke a severe clinical and hematological abnormalities in sheep, causing substantial economic losses in the livestock industry[27,30–33]. Control of ovine babesiosis and *R. bursa* is mostly based on the use of anti-*Babesia* drugs and acaricides, which is a limited approach.

Despite the increasing awareness of TTBDs, the development of novel vector and disease **control** methods is greatly hampered, mostly due to the lack of **knowledge** on tick-pathogen interactions. Therefore, increased investment in **applied research** focusing on those interactions is needed to open avenues for a deeper understanding of TBDs dynamics[34,35], specially on neglected and poorly studied TTBDs such as *R. bursa* and ovine babesiosis. For this, it is necessary to comprehend *R. bursa* tick biology (including evolutionary characteristics, their anatomy and life cycle), *Babesia ovis* life cycle and better understand the dynamics with the host and the *Rhipicephalus* tick. Building knowledge on biology of the vector, parasite, host, and their interactions, will contribute to better control them. Taking all in consideration, this **thesis** will be focusing on ***Rhipicephalus bursa* and *Babesia ovis* interaction and use this model to explore and identify new targets for disease control using different strategies and methodologies.** These approaches can be extrapolated to other TBDs contexts.

1.1. The tick *Rhipicephalus bursa*

In the last decade, ticks have expanded their geographical distribution carrying with them several and different pathogens, which contributed for the doubled incidence of reported TBDs[36]. Like some other tick-borne diseases, ovine babesiosis prevalence is closely related to the activity period and distribution area of the tick vector. Hence, the epidemiology of ovine babesiosis due to *B. ovis* is closely related to the bioecology of its main tick vector, *R. bursa*[31].

Even without updated information about *R. bursa* geographical distribution, this tick species is generally found in the **Mediterranean climatic region** and in the coastal areas from Morocco to Lybia[37]. It is commonly found in grassy areas, but it can also dwell in mountainous slopes or even semi-desert environments[38].

Also known as "brown ear tick", *R. bursa* is recognized as a **hard tick** that belongs **phylogenetically** to the family **Ixodidae** by the virtue of their rigid chitinous dorsal shield[39], which covers the entire dorsal surface of the adult male (defined as conscutum), while a small area in adult female, nymph and larva ticks (appointed as scutum)[14] (see Figure 1).

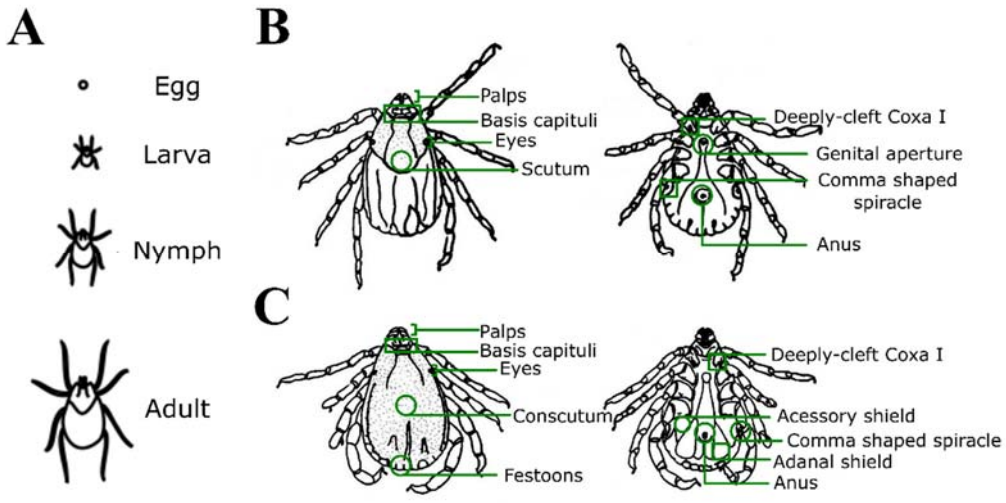

Figure 1. Representation of *Rhipicephalus bursa* development (A) and external morphology of adult ticks (B, C). (A) *R. bursa* go through four life stages: egg, six-legged larva, eight-legged nymph, and adult. After hatching from the eggs, ticks have a blood meal at every stage to survive and molt to the next stage. (*Original from the author*). **(B, C)** Tick morphology consists of two main regions, the mouthparts (capitulum) and the body (idiosoma). The capitulum have three specialized structures: palps, chelicerae and

a hypostome. Palps have a sensory role while the chelicerae allow the penetration of a hollow, tube-like structure such as the hypostome, in the host skin for blood extraction. Those structures are attached to a base named the basis capitula, which is hexagonally in *R. bursa*. The idiosoma includes the eyes, legs, dorsal shield (scutum/conscutum), digestive (anus), respiratory (spiracle) and reproductive (genital aperture, adanal and accessory shields) structures. Dorsal (Left) and ventral (Right) side of *R. bursa* female (B) and male (C) adult ticks is represented. (*Adapted from http://www.bristoluniversitytickid.uk/page/Rhipicephalus+bursa/29/#.X6QpDoj7THo and authorized by Richard Wall*).

R. bursa presents other physiological characteristics typically found in their genus, ***Rhipicephalus***, such as short palps, basis capitulum usually hexagonal dorsally, coxa I deeply cleft, males with adanal shields and usually accessory shields, spiracular plates comma-shaped, and presence of eyes and festoons (Figure 1). ***R. bursa*** normally presents dense interstitial punctations in the scutum/conscutum (Figure 1).

Besides the anatomic complexity, tick development is based in an intertwined life cycle. As **obligate hematophagous ectoparasites**, *R. bursa* ticks need a blood meal to acquire nutrients to complete their life cycle[39,40]. *R. bursa* is a multi-host tick that parasitizes mainly small ruminants, but it can also affect other animals such as cattle, horses, dogs and even humans. It has a **ditropic cycle** (also known as two-host cycle), meaning that hatched larvae remain on the same host to feed, moult into nymphs and feed again before drooping off (Figure 2).

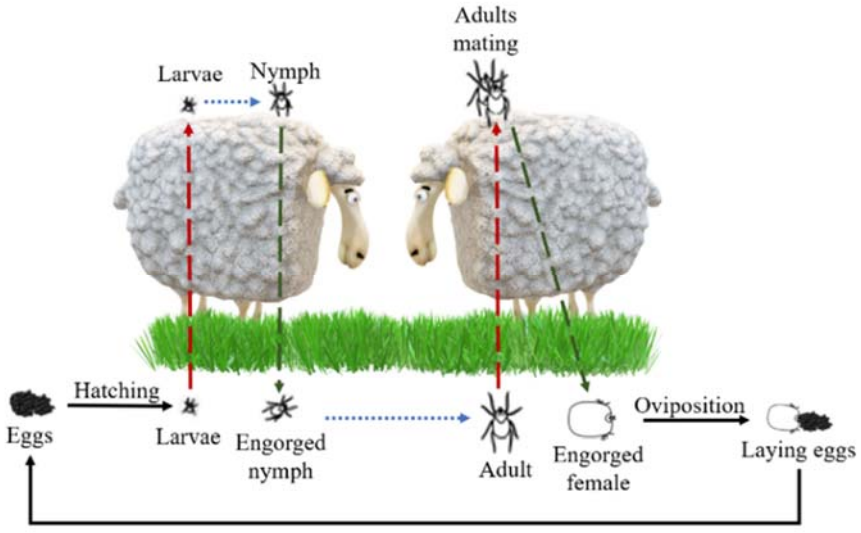

Figure 2. Depiction of the two-host life cycle of *Rhipicephalus bursa*. Red arrow: feeding on the host; Blue arrow: molting; Green arrow: host detachment (drop-off) (*Original from the author*).

Briefly, in the warm periods of the year, ticks surpass diapause (a state of inactivity)[41] and become active, engaging in a pursuit for a host to feed[39]. On vegetation, questing ticks "ambush" the host and crawl over its skin to find a fitting place to attach and feed[39]. This tick exophilic behaviour is regulated by pheromones that are also responsible for other physiological processes such as reproduction[42]. Besides, the tick legs have sensory or tactile hairs and a unique sensory structure located on the 1st pairs of legs, the fore-tarsal Haller's organ, which allows the chemoreception of odors and infrared light emanating from the host[43–45], facilitating its localization. After attaching to the host, larvae feed, engorge and molt into nymphs. Past approximately one-month on host, engorged nymphs detach from the host and on the ground moult into the imago phase (adult). Then, the adult ticks seek for another host to obtain a blood meal to survive and reproduce. At this point, while male ticks take only small blood meals in order to mate, female ticks become engorged as much as twenty-fold to acquire nutrients for egg development and oviposition (they can feed up to 2.0 mL of host blood)[39,46]. After host detachment and before dying, engorged and fertilized female ticks can lay in the soil over 8000 eggs, which in approximately one month, will hatch, and the larvae will emerge perpetuating its life cycle (Figure 2). Transition between stages is through molting (cuticle shedding) after a blood meal.

In controlled laboratorial conditions, this life cycle can be completed in approximately six months, using rabbits or sheep as hosts[40] (Figure 3).

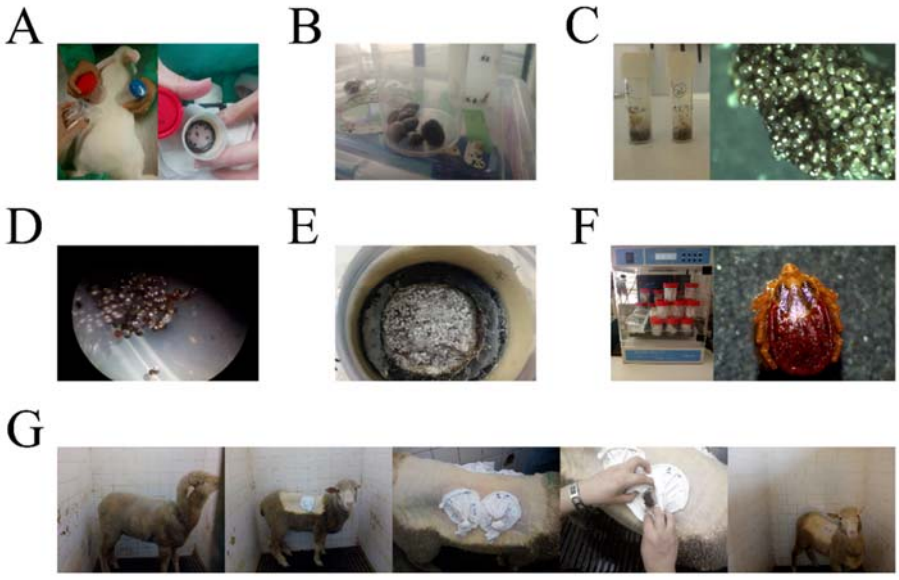

Figure 3. Compilation of images related to *Rhipicephalus bursa* life cycle in laboratory conditions. To perform assays *in vivo* regarding ticks and tick-borne diseases, it is necessary to maintain a colony of

ticks. Since these ectoparasites are obligate hematophagous and their feeding is prolonged in time, rearing ticks is a challenging and complex methodology. Here is presented figures related to *R. bursa* life cycle in rabbits using glued feeding chambers (A-F). Initially, pathogen-free *R. bursa* adult ticks are allowed to feed and mate in rabbits (A). Engorged female adult ticks (B) detach from the host and then are maintained in incubators where temperature and humidity are controlled. The oviposition initiates and several eggs are layed (C). After hatching (D), larvae emerge and can feed in a new host, where they molt and feed again differentiating into engorged nymphs (E). Once detached, these nymphs are maintained in an incubator until molting into the adult stage (F). For experiments requiring *Babesia ovis* infection, infected sheep are used as hosts, in which adult ticks feed in feeding bags and acquire infection (G) (*Original by the author*).

In nature, **host and climatic factors** significantly influence tick life cycle and its prevalence as demonstrated by phenology[47]. For example, even *R. bursa* being a multi-host tick, *i.e.* that has no specificity regarding hosts and for that reason having more probability to find a host, it must share a similar geographical distribution with the hosts and its environment[39]. Vegetation can be an attractive point to hosts facilitating tick encounter but also to tick life cycle. Flora contributes for the formation of microclimates that support the tick life cycle on the ground[39], since humidity and temperature are intrinsically related to tick biology. These factors can actively contribute for the triggering of quiescence (diapause) and affect tick development[14,39].

This **resilience** to survive can be also observed on the host during **blood feeding**. To acquire blood, these arachnids need to pierce the host's epidermis, securely attach, prevent the blood from clotting and dampen down the immunological response in its advance in order to complete the blood meal[48–50]. To penetrate and anchor the host skin, ticks use their mouthparts, being the rest of the mechanism mainly assured by the salivary glands (SGs). In Figure 4, the internal anatomy of *R. bursa* ticks is showed and a closer view on this pivotal tick organ, the SG, is presented.

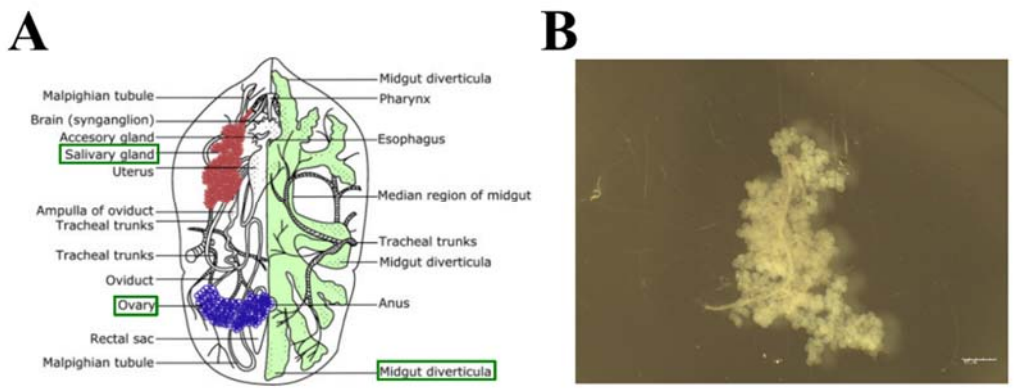

Figure 4. Internal anatomy of *Rhipicephaus bursa* ticks. (A) Overall representation of the internal anatomy of ticks and the highlighted tissues targeted for ticks and tick-borne diseases control (*Retrieved from Nicholson et al. 2019*[51]). **(B)** Salivary gland dissected from a *R. bursa* tick. (*Original from the author*).

Tick SGs produce saliva which is a complex blend of peptidic (*e.g.* variegins, hyalomins, madanins, chitinases, mucins, ixostatins, cystatins, defensins, glycine-rich, hyaluronidases, Kunitz-type proteins, lipocalins, metalloproteases) and non-peptidic (*e.g.* adenosine, prostaglandins, endocannabinoids, microRNAs[52]) tick molecules, in an aqueous solution[50], containing even extracellular vesicles (exosomes[53])[54]. Host proteins (such as immunoglobulins, haptoglobin and transferrin) are also found in tick saliva due to their hemathophagous behavior[54]. The complexity of tick saliva is characterized by their **quantity**, **pluoripotency** and **redundancy**. For example, the hard tick saliva has several hundred, or thousands of polypeptides, while the adult sand fly saliva has less than 50 and the mosquito saliva has near 100 polypeptides[55–57]. Moreover, studies have shown that one tick salivary molecule can target more than one host cell population (pluripotency) and this molecular function can be shared by different molecules (redundancy)[58,59]. This myriad of salivary compounds are documented to be involved in several **processes**: tick attachment by producing the cement cone components (a glue-like structure with allows host attachment)[47], in host 'immunity manipulation' by secreting a cocktail of proteins with analgesic, anticoagulant, platelet aggregation inhibitor[48,49] or anti-inflammatory properties, and in homeostasis by secreting the excess of water and ions derived from the blood meal[47,50,54].

Such potent protein blend ensures tick blood feeding, but it simultaneously undermine human and animal health. During feeding, ticks inject saliva to absorb their blood meal in an alternating pattern[50,60], which **directly fragilizes the host's health** by causing anaemia, skin irritation, inflammation, hypersensitivity, dermatitis and in specific cases tick paralysis[39,61], anaphylaxis[62] and toxicosis[63]. But it also **"opens a gate" that pathogens exploit** to achieve successful transmission. By adapting to tick life cycle as well as manipulating tick salivary molecules[50], pathogens developed strategies to ensure transmission through and within hosts. The tick sialome can influence directly the duration and frequency of blood feeding allowing transmission to occur in a longer-time frame. Furthermore, ticks typically feed close to one another, secreting anti-inflammatory and anti-coagulant salivary molecules in a group on the bite site, in order to form a blood pool easier to ingest. This intense co-feeding behaviour originates a localized repression of the immune responses which promotes pathogen transmission to the host[64]. Once

reached the vertebrate host cells, pathogens encounter the host immune response and if this challenge is overcomed, it will end up causing illness(es) leading to host morbidity and mortality. At this point, infection is established and feeding of naïve ticks will allow the perpetuation of the pathogen life cycle.

All these mechanisms show the exceptional role of ticks as vectors, underscoring the importance to study SGs and saliva due to their assistance in TBDs transmission[50]. Such is particularly important in the case of the *R. bursa* tick since it is able to transmit the zoonotic Crimean Congo Haemorrhagic Fever virus and *Anaplasma phagocytophilum* bacteria[63,65], and has the propensity to transmit infectious pathogens to animals such as *Anaplasma marginale*, *Anaplasma ovis*, *Babesia bigemina* and *Babesia ovis* [63,66–68]. Moreover, *R. bursa* is the **primary vector of *B. ovis***[69] which ultimately causes the most pathogenic and neglected TBD in sheep, ovine babesiosis. A more active surveillance of this tick, due to its vector competence for several tick-borne diseases and zoonosis, is necessary.

1.2. *Babesia ovis* as a tick-borne pathogen

Firstly identified by Victor Babes as a parasitic inclusion in cattle's erythrocytes[70], ***Babesia*** is now recognized as a tick-transmitted haemoparasite that causes **babesiosis**[71]. Ever since this discovery and by the virtue of the advances in microscopy and cell and molecular biology[72], the number of *Babesia* species and their respective susceptible hosts' species increased[73,74]. Schnittger and colleagues reviewed an array of *Babesia* spp. that can cause disease in humans, wildlife and domesticated animals[74].

Taxonomically, all those *Babesia* parasites are unicellular protozoan organisms that belong to the **Apicomplexa** class and the **Piroplasmida** family. Also known as malaria-like parasites, *Babesia* spp. shares several characteristics with other apicomplexan organisms[75] such as *Plasmodium* spp. (the ethiological agent of malaria), by: possessing a specialized organelle for host erythrocytic invasion (apical complex) (Figure 5A), depending on an arthropod vector to be transmitted, and having similar life cycle stages.

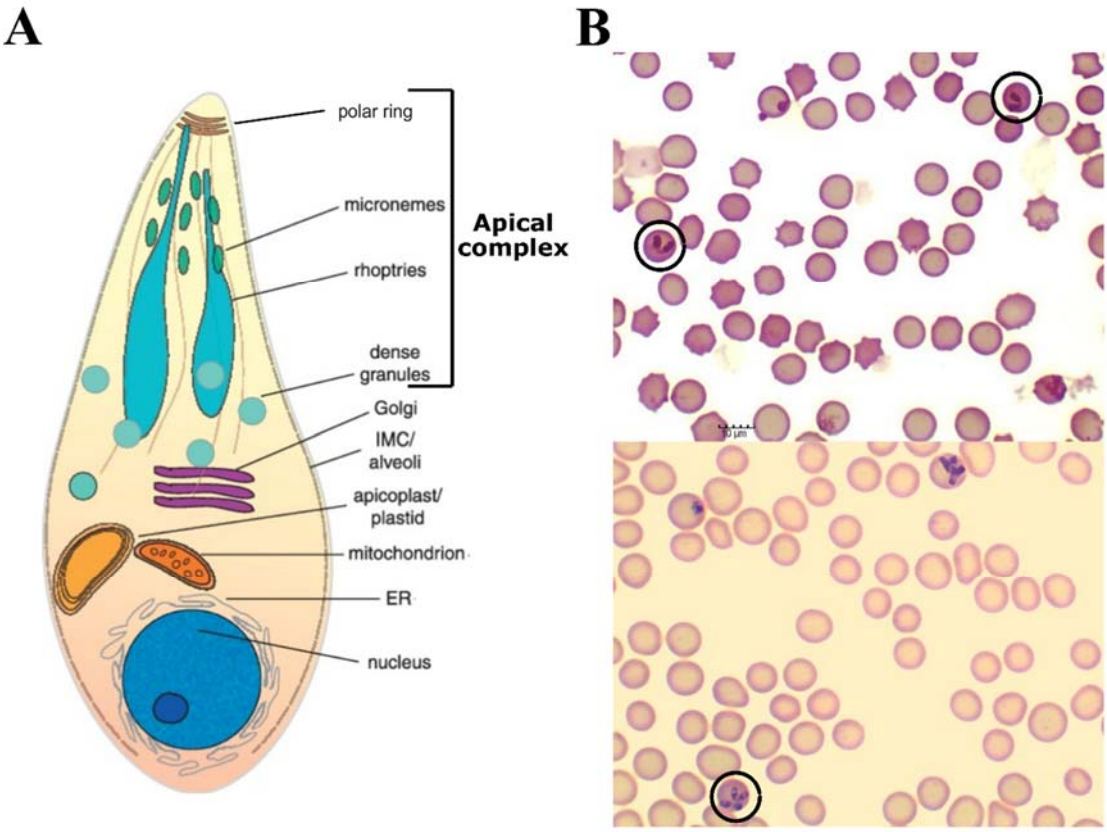

Figure 5. Morphology of *Babesia* spp. parasites. (A) Representation of *Babesia* cellular morphology. As other Alveolata members, the *Babesia* merozoites present an inner membrane complex (IMC) as a homolog of ciliate alveoli which is involved in parasite motility and replication. A secondary endosymbiotic

organelle, an apicoplast, is also present in these parasites harboring metabolic pathways distinct from those of host species. Additionally, as in other Apicomplexans, an assembly of invasion-specialized organelles, the apical complex, is present in *Babesia*. It is composed of a polar ring, micronemes, rhoptries and dense granules (spherical bodies specifically in *Babesia* spp.). The spherical bodies are unique secretory organelles of *Babesia* spp. and homologous to the dense granules present in other apicomplexans[72,76] (*Adapted from Kingler et al., 2013*[76]). **(B)** Giemsa-stained smears of *in vitro* cultered *Babesia ovis* parasites in ovine erythrocytes. Morphologically, *Babesia* parasites are pleomorphic, but in the vertebrate host, it presents as a piriform intra-erythrocyte inclusion. The characteristic Maltese cross is highlighted by the third black circle. Magnification, 400x; bar 10 µm (*Original from the author*).

Babesia spp. can be distinguished from the other apicomplexan protozoan by being nonpigment-forming piroplasmids, *i.e.* lacking the formation of pigment deposits (hemozoin) in the parasitized host cells[27] (Figure 5B). Additionally, piroplasmids, such as *Babesia* spp., *Theileria* spp. and *Cytauxzoon* spp., are piriform hemoparasites (pear-shaped), absent of conoids and flagella in all life stages in the vertebrate host[76,77] (Figure 5B). In the invertebrate host, these organisms are characterized by, their sexual stages associated with the formation of a large axopodium-like structure, and absence in developing oocysts[72]. Moreover, piroplasmids can be separated in different lineages[78], being one of those the ***Babesia sensu stricto* group**, which includes the majority of the **ruminant-infecting *Babesia*** parasites each with host specificity[72]. For instance, *Babesia bovis* and *Babesia bigemina* are recognized to infect cattle, causing bovine babesiosis, while *B. ovis* is the etiological agent of ovine babesiosis in sheep and goats[72,78].

Nonetheless, *B. ovis* shares with other intraerythrocytic protozoan parasites the responsibility on causing ovine babesiosis. Besides *B. ovis*, other parasites, such as *Babesia crassa*, *Babesia motasi*, *Babesia foliate*, *Babesia taylori*, *Babesia* sp. Xinjiang, and *Babesia* sp. BQ1 (*B. motasi*-like) isolates[72], are affecting negatively small ruminants in several countries[72,74,79]. However, among those *Babesia* spp., the **worldwide distributed** and **highly pathogenic species to sheep** is *B. ovis*[72,74], leading to 30-50 % of mortality rate in susceptible sheep and concerning several nations in Africa, Asia and Europe[14,72,79–81].

As observed for other *Babesia* spp.[75], a remarkable feature of *B. ovis* is its **resilience to endure in the host and the vector**[82]. A persistent infection can occur in ovine herds, *i.e.*, after recovery, animals become asymptomatic carriers and disease recrudescence occurs after exposure to stress environments or splenectomy[83]. Besides, in the vector, *B. ovis* possesses the ability to infect successive tick developmental stages (transstadial transmission) and disseminate to the tick offspring (transovarial transmission)[78]. Figure 6

elucidates about a widely accepted general life cycle of *Babesia* parasites, including the *B. ovis* development in the host (sheep) and in the vector (*R. bursa*).

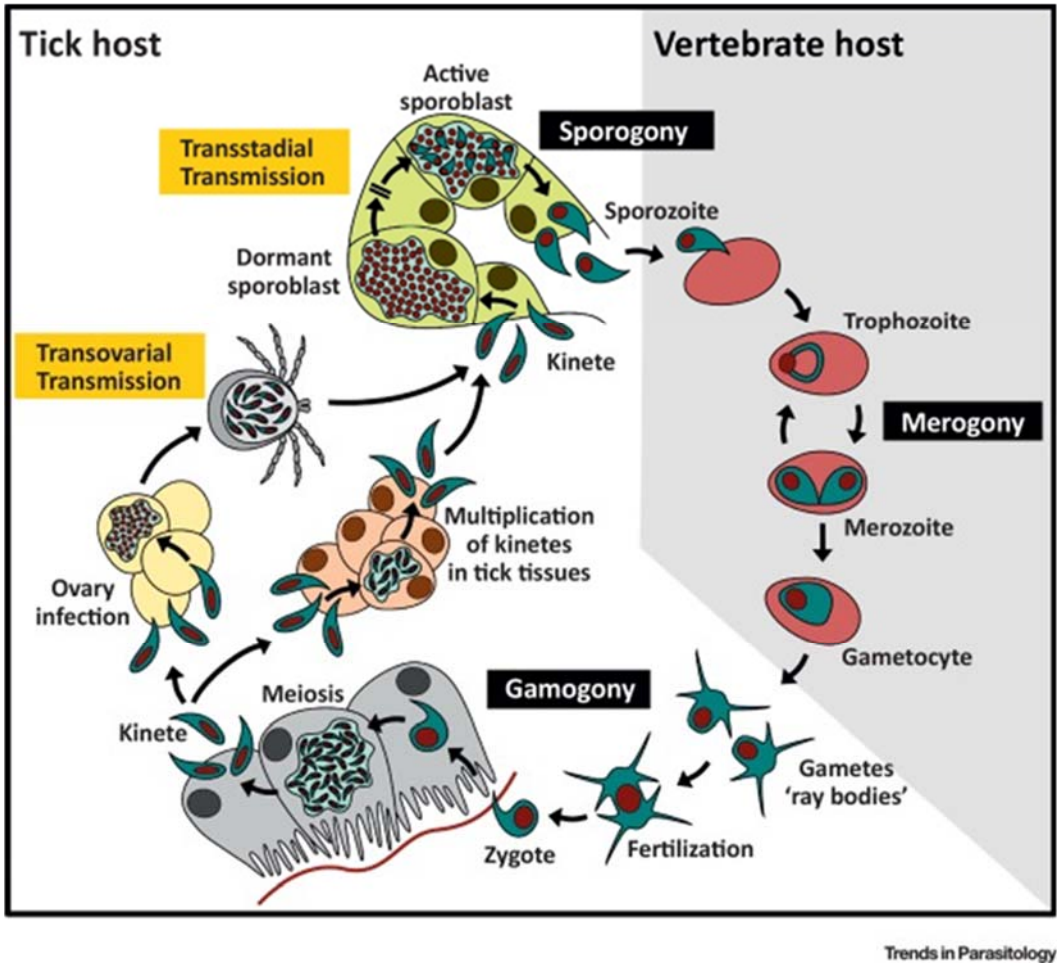

Figure 6. Typical *Babesia ovis* life cycle in both vertebrate (sheep) and invertebrate host (*Rhipicephalus bursa* tick). The *Babesia* life cycle follows three successive phases: merogony, gamogony, and sporogony. Essentially, when an infected tick transmits *Babesia* sporozoites to the vertebrate host, those hemoparasites invade the RBCs and start their asexual reproduction, merogony. Then, those infected RBCs are ingested by a tick during its blood meal, where later occurs sexual reproduction, gamogony. After invasion and differentiation within the tick cells, *Babesia* ultimately undergoes sporogony originating several infectant sporozoites that can be transmitted to the vertebrate host (*Retrieved from Jalovecka et al., 2019*[78]).

Even with notable differences being documented regarding *Babesia* spp. development, a general life cycle of *Babesia* species has been widely accepted (Figure 6). *Babesia* spp. has an obligatory dixenous life cycle[78] (requires the tick vector and the vertebrate host) representing a complex system of interactions[82]. During blood feeding on a susceptible vertebrate host, a *Babesia*-infected tick successfully transmit the hemoparasites, which will be reactivated through tick saliva after a period of 24-48 h of

tick attachment[84]. Once in the blood stream of the vertebrate host, the *Babesia* spp. sporozoites faces one of two fates: either be eliminated by the host's innate and/or adaptative immune response[85], or surpass it and invade the red blood cells (RBCs)[78,86]. This is associated to **clinical symptoms** in adult animals such as fever, anorexia, depression, jaundice, dyspnea, tachycardia, impotence, haemolytic anaemia, thrombocytopenia, icterus and haemoglobinuria[79,87]. Depending on the host age, immune status, co-infections and genetic factors, the severity of infections could increase, leading even to death[79,88].

The aforementioned erytrocytic invasion is achieved by a process denominated "gliding motility", which involves the presence of cytoadherent[82,89] and specialised apical organelle proteins (apical complex)[90,91] (Figure 5A). Once inside, the *Babesia* parasite faces an asynchronized asexual reproduction (binary fission) within terminally-differentiated erythrocytes, which is known as **merogony**[88] (Figure 6). Lacking any *de novo* protein or lipid synthesis and having a limited capacity of metabolism[92,93], RBCs became an ideal host cell that the parasite can manipulate to hide from the immune system and fulfil its needs[92]. As such, the parasite makes use of its own biochemical tools and generates a profile of excreted proteins known as the parasite secretome, to modulate the host RBC, a phenomenon common to other apicomplexan parasites. These piroplasms develop into trophozoites (a "ring" form) and then into merozoites, which ultimately can egress and re-infect naïve RBCs[78] (Figure 6). Merozoites are pyriform organisms which are normally observed in pairs, although Maltese cross structures can occasionally be found[72] (Figure 5B). Particularly, *B. ovis*, is characterized by the lack of a schizont stage, which clearly distinguish it from the Apicomplexan, *Theileria* and *Babesia sensu lato* parasites[72].

Eventually, the merozoites differentiate into gametocytes, which will be ingested by the tick during its blood meal[78]. In the tick's gut, the **gamogony** starts with the differentiation of the gametocytes into gametes, followed by syngamy (fusion of gametes) leading to the formation of a motile zygote that surpass the tick midgut barrier. In contrast to the other piroplasmids, *Babesia* gametes (also known as Strahlenkörper bodies) present a *Babesia*-specific spiky-rayed shape[94], and after syngamy, the zygotes undergo a meiotic division resulting in kinetes instead of oocysts[78]. With high mobility, these kinetes disseminate through the tick haemolymph to peripheral tick tissues, such as salivary

glands and ovaries[95]. In the SGs, **sporogony** occurs and the kinetes give rise to a generation of multiple infective sporozoites[95], which can be maintained within a tick for long periods of time until a new tick blood meal perpetuates this life cycle[64] (Figure 6).

Even with this archetypal developmental cycle of members of the Apicomplexa class, *Babesia* parasites have evolved novel **strategies** to adapt to the feeding and molting processes of their definitive hosts, the ticks[95]. Noteworthy, *B. ovis* is known to be maintained in ticks for several generations without reinfections[96] due to **transstadial and transovarial transmission**[94]. The driving force for such adaptation is the fact that *B. ovis* need to survive through tick molting in order to infect naive hosts during the on-host feeding of the next tick development stage (by using transstadial transmission) and disseminate during the absence of the vertebrate host (by using transovarial transmission)[78]. Curiously, among the *Rhipicephalus* ticks that have been involved in the transmission of *B. ovis* (such as *R. bursa*, *R. sanguineus* and *R. turanicus*), so far, only *R. bursa* is the vector of this parasite in which **transovarial transmission** occurs[97]. In the next subsection, other examples of *Babesia*-vector interactions are highlighted.

This intertwined relationship results from the co-evolution of *Babesia* and both vertebrate and invertebrate hosts, resulting in the adaptation of each other's life cycle by manipulation of parasite and vector biological processes[98].

1.3. "Deciphering *Babesia*-vector interactions"

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1.1.1. Abstract

Understanding host-pathogen-tick interactions remains a vitally important issue that might be better understood by basic research focused on each of the dyad interplays. Pathogens gain access to either the vector or host during tick feeding when ticks are confronted with strong hemostatic, inflammatory and immune responses. A prominent example of this is the *Babesia* spp.—tick—vertebrate host relationship. *Babesia* spp. are intraerythrocytic apicomplexan organisms spread worldwide, with a complex life cycle. The presence of transovarial transmission in almost all the *Babesia* species is the main difference between their life cycle and that of other piroplasmida. With more than 100 species described so far, *Babesia* are the second most commonly found blood parasite of mammals after trypanosomes. The prevalence of *Babesia* spp. infection is increasing worldwide and is currently classified as an emerging zoonosis. *Babesia microti* and *Babesia divergens* are the most frequent etiological agents associated with human babesiosis in North America and Europe, respectively. Although the *Babesia*-tick system has been extensively researched, the currently available prophylactic and control methods are not efficient, and chemotherapeutic treatment is limited. Studying the molecular changes induced by the presence of *Babesia* in the vector will not only elucidate the strategies used by the protozoa to overcome mechanical and immune barriers, but will also contribute toward the discovery of important tick molecules that have a role in vector capacity. This review provides an overview of the identified molecules involved in *Babesia*-tick interactions, with an emphasis on the fundamentally important ones for pathogen acquisition and transmission.

1.1.2. Introduction

Parasites from the genus *Babesia* are responsible for causing an emerging zoonotic disease called babesiosis. Transmission occurs mainly through the bite of a *Babesia*-infected tick and, less commonly, by blood transfusion (Leiby, 2006; Ord and Lobo, 2015).

At least four Ixodidae genus are recognized as *Babesia* vectors: *Rhipicephalus*, *Ixodes*, *Haemaphysalis*, and *Hyalomma* (Sonenshine and Michael Roe, 2014). This disease has a considerable impact on the health and economy of the livestock industry, mainly in tropical and subtropical climates, with *Rhipicephalus microplus* and *Rhipicephalus annulatus* the main vectors of *Babesia bovis* and *Babesia bigemina*, the etiological agents of bovine babesiosis (Bock et al., 2004). In small ruminants, infections can be caused by several *Babesia* species, such as *B. ovis*, transmitted to sheep usually by the tick *R. bursa* (Shayan et al., 2007; Ranjbar-Bahadori et al., 2012; Ferrolho et al., 2016). Dogs are susceptible of infection by *B. canis vogeli* and *B. gibsoni*, primarily transmitted by *R. sanguineus* (Solano-Gallego et al., 2016; Chao et al., 2017). Human babesiosis, caused largely by *Babesia microti* and *Babesia divergens*, is not acknowledged as a tropical neglected disease, but there is a growing concern globally regarding this emerging zoonosis (Ord and Lobo, 2015).

Despite the fact that *Babesia* infections tend to impair tick development, an adaptive tolerance to *Babesia* has been described in *R. microplus* suggesting a balance between tick defense mechanisms and tick-pathogen mutual interaction(s) (Cen-Aguilar et al., 1998; Chauvin et al., 2009; Florin-Christensen and Schnittger, 2009; Lack et al., 2012; Gou et al., 2013; de la Fuente et al., 2016).

The development of improved tick and tick-borne disease control measures are essential to overcome the lack of data regarding which tick molecules are important and how they may be suitable as study targets. Based on this, herein we will discuss the functional roles of several molecules involved during the infection of tick tissues by *Babesia* spp.

1.1.3. Tick Midgut Molecules with a Role in *Babesia* Acquisition

Once ingested *Babesia*-infected red-blood cells reach the tick midgut many parasites will be destroyed or degenerate, but a small number will evolve to gametocytes, essential

for zygote fusion and penetration of the midgut peritrophic membrane (Sonenshine and Hynes, 2008; Chauvin et al., 2009; Maeda et al., 2017). Recently, it was proposed that during the *Babesia* spp. sexual phase, some specific proteins with known functional roles in recognition and adhesion are expressed, including glycosylphosphatidylinositol (GPI) anchored proteins that interact with specific targets in the epithelial cells (Bastos et al., 2013; Alzan et al., 2016).

In the *R. microplus* midgut, proteomic analysis has identified a mitochondrial voltage-dependent anion-selective channel (BmVDAC) polypeptide, also known as mitochondria porin that binds to *B. bigemina* sexual stage proteins (Mosqueda et al., 2004; Rodríguez-Hernández et al., 2012). VDAC was first described as located in the external mitochondrial membrane that regulates the flux of small molecules into the mitochondrial space membrane having a role in cell metabolism and apoptosis (Young et al., 2007). In mosquitoes, VDAC plays a role during *Plasmodium* sp. invasion of the midgut; likewise, the dissemination of *B. burgdorferi* through the tick midgut might be associated with the ability of VDAC to bind a tissue-type plasminogen activator (Coleman et al., 1997; Ghosh et al., 2011). Under *Babesia* invasion this protein was found over-represented in the *R. microplus* midgut (Rodríguez-Hernández et al., 2012).

The tick receptor of the outer surface protein A (TROSPA) was firstly identified in the *I. scapularis* midgut epithelium as a receptor for *B. burgdorferi*, suggesting it has the potential to control bacterial infections in ticks (Pal et al., 2004; Konnai et al., 2012; Urbanowicz et al., 2016). In *R. annulatus*, an orthologue of *trospa* gene was over-expressed during *B. bigemina* infection and gene knockdown significantly reduced *B. bigemina* infection levels by 70 and 83% in *R. microplus* and *R. annulatus*, respectively (Antunes et al., 2012). In addition, *B. bigemina*-infected cattle vaccinated with TROSPA revealed close to an 80% decrease in pathogen transmission to ticks (Merino et al., 2013). In *R. annulatus*, this receptor was found not only in the midgut, but also in the salivary glands (SGs) and ovaries (Antunes et al., 2014).

During protozoal invasion, the tick innate immune response leads to the rapid, synthesis of defensins and tick antimicrobial peptides (AMPs). These constitute an important humoral defense mechanism, which is also active against intracellular bacteria and fungi (Antunes et al., 2012; Hajdusek et al., 2013; Tonk et al., 2015). The midgut defensin-like protein, longicin, was first identified in the tick *Haemaphysalis longicornis*

and has a role in *Theileria equi* proliferation (Tsuji et al., 2007). Merozoite *in vitro* cultures were inhibited in the presence of recombinant longicin while the inoculation of this protein led to a reduction of *B. microti* parasitaemia in infected mice. Also, *longicin* silencing led to an increase in *B. gibsoni* parasitaemia in several tick tissues, including midgut, ovaries and eggs. Accumulated data on the function of this protein indicate that longicin has a babesiacidal effect. Microplusin was the first fully characterized member of a family of cysteine-rich AMPs in *R. microplus* (Fogaça et al., 2004); in *R. annulatus*, was found over represented in response to *B. bigemina* infection (Antunes et al., 2012).

Other molecules present in the midgut that also protect the tick from pathogen invasion are the MD-2-related lipid-recognition (ML)-domain containing proteins related with lipid recognition (Rudenko et al., 2005), proteases and protease inhibitors (Sonenshine and Hynes, 2008; Kopacek et al., 2010; Antunes et al., 2012; Hajdusek et al., 2013). Longipain, a *H. longicornis* midgut cysteine protease, has shown similar effects to longicin. Recombinant longipain was also able to inhibit the proliferation of *T. equi* merozoites, and gene silencing resulted in an increase of protozoa in the midgut lumen, ovaries and hatched larvae (Tsuji et al., 2008). Also in *H. longicornis*, a leucine-rich repeat domain-containing protein (LRR) has been identified as over represented in all tick tissues, with the exception of the ovary, where it is constitutively expressed. *In vitro*, a specific recombinant LRR has demonstrated a growth inhibitory effect on *B. gibsoni* with similar or better results than traditional anti-babesial drugs (Maeda et al., 2015).

Tick Kunitz-type protease inhibitors may restrict pathogen infection, presumably via the inhibition of microbial proteinases (Sasaki and Tanaka, 2008; Antunes et al., 2012). This group of genes was upregulated in response to infection (Antunes et al., 2012; Heekin et al., 2013), but its influence in *Babesia* acquisition was only related to ovary infection (Rachinsky et al., 2007; Bastos et al., 2009).

Bm86 is a glycoprotein, recognized for the first time in *R. microplus*, and present in midgut cells, that is likely to be involved in the endocytosis of the blood ingested by ticks (Gough and Kemp, 1993; Bastos et al., 2010; Rodríguez-Mallon, 2016). Regardless of the efficiency of Bm86 against tick infestation, some studies aimed to evaluate the role of Bm86 in *Babesia* infection (Bastos et al., 2010; Rodríguez-Mallon et al., 2013). RNA interference (RNAi) studies carried out in *R. microplus* females showed that *Bm86*

silencing significantly reduced the number of ticks; by contrast, silencing did not affect the efficiency of transovarial transmission of *B. bovis* (Bastos et al., 2010). In a different study using Gavac®, a vaccine based on the Bm86 antigen, naïve nymphs that co-fed on immunized dogs presented lower levels of *B. canis*, (Rodríguez-Mallon et al., 2013). It is conceivable that the lysis of midgut cells inhibited the entry of zygotes and/or their posterior differentiation into motile ookinetes, compromising *B. canis* acquisition by the nymphs.

Subolesin, firstly identified in *I. scapularis* ticks as an orthologue of akirin in insects and vertebrates (Almazán et al., 2003; Galindo et al., 2009), is a highly conserved protein in eukaryotes, including many tick species (Moreno-Cid et al., 2013; Antunes et al., 2014), suggesting its potential as a candidate antigen for an anti-tick and tick-borne pathogen (TTBP) vaccine. Subolesin family proteins are transcriptional factors, regulating protein expression in cellular pathways involved in the response to pathogen infection (de la Fuente et al., 2013; Sultana et al., 2015). *Subolesin* silencing mediated by RNAi led to a lower *B. bigemina* infection in *R. microplus* (Merino et al., 2011) but, in contrast, in *R. annulatus*, silencing did not lead to a significant decrease in *B. bigemina* levels (Antunes et al., 2012). Vaccination using subolesin and a chimera containing subolesin protective epitopes (Q38) revealed an effect on *B. bigemina* transmission to feeding ticks (Merino et al., 2013). Subolesin expression and subolesin-mediated innate immunity varies according to the pathogen and tissue (Zivkovic et al., 2010), which explains the variation in the results. However, it seems that targeting subolesin by vaccination or its gene by RNAi would result in lower *Babesia* infection levels.

The tick midgut is one of the few major organs that defines vector competence since it is the first obstacle that several pathogens, including *Babesia*, have to cross. Still, our understanding of the interplay between an infective pathogen and the tick midgut continues to be poor and requires further studies to better define this important interaction.

1.1.4. Tick Haemolymph and Ovary Molecules Acting in *Babesia* Dissemination

After the successful invasion of the midgut epithelium, *Babesia* zygotes go through meiosis and differentiate into motile ookinetes that go across the haemocoel, with the help of haemolymph; in the haemocoel, the parasite undergoes asexual reproduction, resulting

in several sporokinetes spread for all tick organs throughout all tick life stages (transstadial transmission) (Schnittger et al., 2012).

When a tick experiences microbial invasion, for example from a protozoa like *Babesia* spp., the hemocytes increase their circulating number to destroy and control the invader, phagocytizing small particles and microbes (Inoue et al., 2001; Villar et al., 2015). Besides phagocytosis, other processes including nodulation and encapsulation, and molecules like AMPs, lysozymes, proteases, protease inhibitors, and lectins, that exist in the haemolymph act directly on the pathogen (Esteves et al., 2008; Kotsyfakis et al., 2015). *B. bigemina* exhibits motility when reaching the haemolymph and adheres to *R. microplus* haemocyte membranes (de Rezende et al., 2015), however there is no information about how *Babesia* spp. invasion is controlled at the haemolymph level.

In female ticks, effective infection of ovaries and the eggs allow transovarial transmission of almost all *Babesia* species, a distinctive characteristic of this genus (Homer et al., 2000; Chauvin et al., 2009) that can be interpreted as an adaptation to efficiently persist in the ecosystem (Chauvin et al., 2009). The first ovarian proteomic profile of *R. microplus* infected with *B. bovis* identified a small number of differentially represented proteins. Among these proteins were calreticulin, glutamine synthetase and a family of Kunitz-type serine protease inhibitors; whereas between the less represented proteins were a tick lysozyme and a group of small proteins that may belong to a family of AMPs (Rachinsky et al., 2007). Ovarian genes involved in the stress response, detoxification and immune responses were found potentially regulated by *B. bovis* infection (Heekin et al., 2013); many of these genes translate into proteases and protease inhibitors that participate in the ovarian immune response. A putative *immunophilin* (*Imnp*) and a putative *Kunitz-type serine protease inhibitor* (*Spi*) genes were found to be up regulated when tick ovaries were infected (Rachinsky et al., 2007) and the *Imnp* knockdown revealed a significant increase of larval infection, suggesting that this molecule might control the protozoan invasion of tick ovaries, and subsequent larval progeny. Immunophilin proteins, also known as cyclophilins, are associated with multiple cellular processes, like protein folding, trafficking and defense mechanisms (Wang and Heitman, 2005), however their role(s) during *Babesia* infection is still unknown.

The *H. longicornis* vitellogenin receptor (VgR) has been associated with the transovarial transmission of *B. gibsoni*. *VgR* silencing results in the absence of B. gibsoni

infection and development of abnormal eggs (Boldbaatar et al., 2008) confirming its influence on oogenesis acting on heme detoxification and egg maturation (Boldbaatar et al., 2010; Perner et al., 2016). These results may suggest that *Babesia* molecules have ligand-binding activity for tick VgR, consequently invading the developing oocyte (Boldbaatar et al., 2008).

Ovarian proteins can affect tick biology by decreasing oogenesis and embryogenesis, which reduce tick reproduction rates and TBP transmission by blocking transovarial transmission, making these molecules promising targets for vaccine development.

1.1.5. Tick Salivary Gland Molecules that Intervene in *Babesia* Transmission

When *Babesia* kinetes reach the SGs they undergo a final step of multiplication to produce sporozoites, the vertebrate host-infective stage. SGs can be considered as the last barrier that parasites must overcome to complete their life cycle in the vector, facing similar obstacles to those of the midgut (Chauvin et al., 2009).

Different SGs transcriptomes, commonly referred to as sialomes, from soft and hard ticks have been published (Francischetti et al., 2008, 2011; Anatriello et al., 2010; Karim et al., 2011; Ribeiro et al., 2011; Garcia et al., 2014; Yu et al., 2015; de Castro et al., 2016), showing genes encoding AMPs, such as defensins, microplusin/hebraein, Kunitz domain-containing proteins, lipocalins, proteases and other molecules related to tick defense mechanisms. Despite their importance for transmission, reports describing the influence of SG molecules on *Babesia* infection are absent.

The sialome of the soft tick *Ornithodoros parkeri* contains a putative serum amyloid A protein, whose orthologue was also found in the *I. scapularis* genome. In vertebrates, this protein is involved in the acute phase of an inflammatory response (Francischetti et al., 2008; Antunes et al., 2012). Vertebrate serum amyloid A protein was found increased in cattle with more resistance to tick infections, suggesting its involvement in the stress response induced by tick infestations (Ferreira et al., 2004). The expression of a putative *serum amyloid A* gene was increased in response to *B. bigemina* infection in *R. annulatus* and gene knockdown resulted in a reduction of 66 and 86% of the infection levels, in *R. microplus* and *R. annulatus*, respectively (Antunes et al., 2012).

Calreticulin, has been identified in tick ovaries, midgut and SGs (Antunes et al., 2012, 2015). The role of this molecule in ticks is still not clear but some studies support its presence in the SGs and saliva is presumably related to a mechanism to avoid vertebrate host defense responses (Jaworski et al., 1995; Ferreira et al., 2004; Antunes et al., 2015) and may lack the anti-thrombotic and complement-inhibiting characteristics that suppress host defense actions (Kim et al., 2015). The gene encoding this protein was found to be over expressed in *R. annulatus* infected with *B. bigemina*. *Calreticulin* knockdown had a significant effect on pathogen infection in *R. microplus*, but not in *R. annulatus* ticks, affecting the body weight in both tick species (Antunes et al., 2012). According to this and to other reports, it is thought that calreticulin acts during blood feeding (Ferreira et al., 2002; Antunes et al., 2012) and may alter calcium metabolism during *Babesia* infection. *Babesia* sp. may need calcium ions to invade tick cells as shown for *T. equi* (previously classified as *B. equi*). A pilot immunization trial in cattle using recombinant calreticulin failed to reduce tick infestation, probably due to the low immunogenicity of the protein (Ferreira et al., 2002). More recently, serum with anti-calreticulin antibodies also failed to promote a significant decrease in *B. bigemina* infection in *R. microplus* (Antunes et al., 2015). In this study, calreticulin immunolocalization assays have shown that this molecule can be found in the tick midgut, ovaries and SGs, suggesting that it might have a role in *Babesia* infection in all these tissues.

Other molecules, such as TROSPA, already discussed, have been also identified in tick SGs, where it may function as a receptor for *Babesia* parasites. Tick SG proteins are of extreme importance during *Babesia*-vector-host interactions and it seems likely that more molecules will emerge as key players in these vector-parasite networks in the near future.

Figure 1, Table 1 summarizes the so far identified tick molecules networking with *Babesia* spp. showing their localization and suggested interaction.

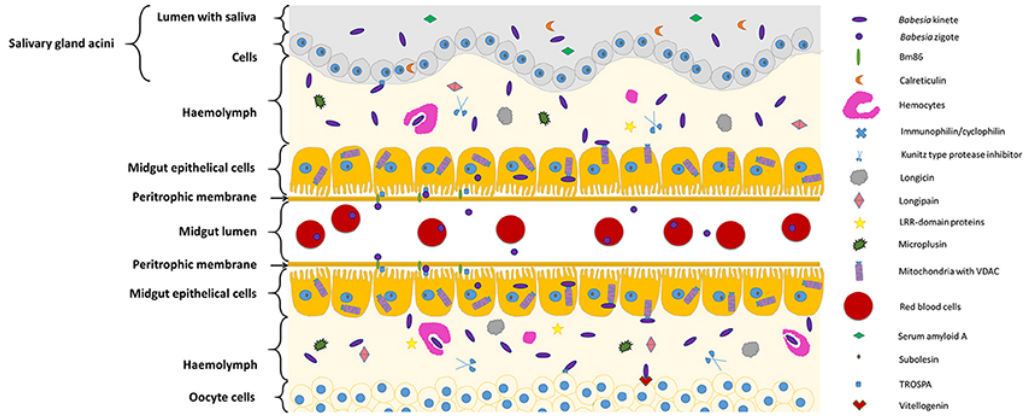

Figure 7. Diagram representing tick molecules implicated in *Babesia* spp. acquisition and transmission by the vector. When ticks feed on *Babesia*-infected animals, parasites within red blood cells reach and penetrate the tick midgut peritrophic membrane to invade the epithelial cells (in the figure center). Once these cells are infected, transcriptional factors, such as subolesin, can regulate protein expression in several cellular pathways, facilitating *Babesia* infection. In the microvilli of the midgut cells, parasite zygotes will probably interact with a tick glycoprotein (Bm86) and a tick receptor of the outer surface protein A (TROSPA). Inside the epithelial cells, mitochondria porins (VDAC) can bind to *Babesia* kinete proteins promoting plasminogen activation in the cell surface, allowing their passage to the haemolymph. Once here, the haemocytes can phagocyte circulating parasites and the tick antimicrobial molecules such as, longicin, micropulsin, longipain, LRR-domain and Kunitz-type protease inhibitors are activated potentially reducing the infection in the vector. If the infectious parasites surpass these barriers of defense, they will be capable to spread across the tissues and invade ovaries (represented in the bottom of the figure) and SGs (represented in the top of the figure). In the ovary, the interaction of *Babesia* molecules with tick vitellogenin and TROSPA receptors may contribute for the occurrence of transovarial transmission; while in the SGs, *Babesia* interacts with TROSPA and calreticulin.

Table 1. Tick molecules interfering with *Babesia* spp. infection.

Protein name	Assigned function	Proteins localization				Described effect in *Babesia* spp. infection	References
		MD	HL	OV	SG		
Mitochondrial voltage-dependent anion-selective channel (BmVDAC)	Cell metabolism and apoptosis regulation	X				Enhance acquisition	Rodríguez-Hernández et al., 2012, 2015
Tick receptor of the outer surface protein A (TROSPA)	Factor for tick gut colonization	X		X	X	Enhance acquisition	Antunes et al., 2012; Merino et al., 2013; Urbanowicz et al., 2016
Longicin	Defensin	X				Control acquisition and transovarial transmission	Tsuji et al., 2007
Microplusin	Antimicrobial peptide	X					Antunes et al., 2012
Longipain	Cysteine protease	X					Tsuji et al., 2008
Leucine-rich repeat domain-containing proteins	Component of the innate immune system	X	X	X	X	Control infection	Maeda et al., 2015
Kunitz-type protease inhibitors	Blood coagulation	X			X	Enhance transovarial transmission	Rachinsky et al., 2007; Bastos et al., 2009; Antunes et al., 2012
Bm86	Blood coagulation and cell growth	X		X		Enhance acquisition	Bastos et al., 2010
Subolesin	Transcriptional factor involved in the immune signaling pathways	X				Enhance acquisition	Merino et al., 2011; de la Fuente et al., 2013
Immunophilin	Protein folding, trafficking and defense		X			Control transovarial transmission	Rachinsky et al., 2007; Wang and Heitman, 2005
Vitellogenin receptor (VgR)	Vitellogenin uptake			X		Enhance transovarial transmission	Boldbaatar et al., 2008, 2010
Serum amyloid A	Response to inflammation	X				Enhance acquisition	Antunes et al., 2012
Calreticulin	Protein folding and signaling	X		X	X	Enhance acquisition.	Antunes et al., 2012

MD, Midgut; HL, Haemolymph; OV, Ovaries; SG, Salivary Glands.

1.1.6. Conclusions

The major critical point for the development of vaccines is the identification of new targets. In this review, our objective was to gather relevant information about the tick molecules involved with *Babesia* parasite infections. During the last decade, several studies using "omics" and systems biology approaches have greatly improved our knowledge of the interactions taking place at the tick-pathogen interface. The *Babesia*-tick interactome is still neglected with scattered information, and only a few tick proteins have been shown to influence the acquisition, dissemination and transmission of the parasite. From this short list, subolesin, having a role in the tick innate immune response, stands out as a potential candidate antigen for a universal anti-vector vaccine. During *Babesia* infection, this molecule produced positive results, making it a candidate antigen for a transmission-blocking vaccine. Other proteins involved in *Babesia* acquisition, including the TROSPA receptor, are also promising candidates for a multi-antigenic vaccine. Some of these datasets were obtained through use of transcriptomic, proteomic, and systems biology approaches. These and future technologies will be fundamental to the improvement and development of new control strategies and more effective vaccines.

1.1.7. References

Almazán, C., Kocan, K. M., Bergman, D. K., Garcia-Garcia, J. C., Blouin, E. F., and de la Fuente, J. (2003). Identification of protective antigens for the control of *Ixodes scapularis* infestations using cDNA expression library immunization. Vaccine 21, 1492–1501. doi: 10.1016/S0264-410X(02)00683-7

Alzan, H. F., Lau, A. O., Knowles, D. P., Herndon, D. R., Ueti, M. W., Scoles, G. A., et al. (2016). Expression of 6-Cys gene superfamily defines *Babesia bovis* sexual stage development within *Rhipicephalus microplus*. PLoS ONE 11:e0163791. doi: 10.1371/journal.pone.0163791

Anatriello, E., Ribeiro, J. M. C., de Miranda-Santos, I. K. F., Brandao, L. G., Anderson, J. M., Valenzuela, J. G., et al. (2010). An insight into the sialotranscriptome of the brown dog tick, *Rhipicephalus sanguineus*. BMC Genomics 11:450. doi: 10.1186/1471-2164-11-450

Antunes, S., Galindo, R. C., Almazan, C., Rudenko, N., Golovchenko, M., Grubhoffer, L., et al. (2012). Functional genomics studies of *Rhipicephalus* (*Boophilus*) *annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. Int. J. Parasitol. 42, 187–195. doi: 10.1016/j.ijpara.2011.12.003

Antunes, S., Merino, O., Lerias, J., Domingues, N., Mosqueda, J., de la Fuente, J., et al. (2015). Artificial feeding of *Rhipicephalus microplus* female ticks with anti calreticulin serum do not influence tick and *Babesia bigemina* acquisition. Ticks Tick Borne Dis. 6, 47–55. doi: 10.1016/j.ttbdis.2014.09.003

Antunes, S., Merino, O., Mosqueda, J., Moreno-Cid, J. A., Bell-Sakyi, L., Fragkoudis, R., et al. (2014). Tick capillary feeding for the study of proteins involved in tick-pathogen interactions as potential antigens for the control of tick infestation and pathogen infection. Parasit. Vectors 7:42. doi: 10.1186/1756-3305-7-42

Bastos, R. G., Suarez, C. E., Laughery, J. M., Johnson, W. C., Ueti, M. W., and Knowles, D. P. (2013). Differential expression of three members of the multidomain adhesion CCp family in *Babesia bigemina*, *Babesia bovis* and *Theileria equi*. PLoS ONE 8:e67765. doi: 10.1371/journal.pone.0067765

Bastos, R. G., Ueti, M. W., Guerrero, F. D., Knowles, D. P., and Scoles, G. A. (2009). Silencing of a putative *immunophilin* gene in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* increases the infection rate of *Babesia bovis* in larval progeny. Parasit. Vectors 2:57. doi: 10.1186/1756-3305-2-57

Bastos, R. G., Ueti, M. W., Knowles, D. P., and Scoles, G. A. (2010). The *Rhipicephalus* (*Boophilus*) *microplus Bm86* gene plays a critical role in the fitness of ticks fed on cattle during acute *Babesia bovis* infection. Parasit. Vectors 3:111. doi: 10.1186/1756-3305-3-111

Bock, R., Jackson, L., De Vos, A., and Jorgensen, W. (2004). Babesiosis of cattle. Parasitology 129, S247–S269. doi: 10.1017/S0031182004005190

Boldbaatar, D., Battsetseg, B., Matsuo, T., Hatta, T., Umemiya-Shirafuji, R., Xuan, X., et al. (2008). Tick vitellogenin receptor reveals critical role in oocyte development

and transovarial transmission of *Babesia* parasite. Biochem. Cell Biol. 86, 331–344. doi: 10.1139/O08-071

Boldbaatar, D., Umemiya-Shirafuji, R., Liao, M., Tanaka, T., Xuan, X. N., and Fujisaki, K. (2010). Multiple vitellogenins from the *Haemaphysalis longicornis* tick are crucial for ovarian development. J. Insect Physiol. 56, 1587–1598. doi: 10.1016/j.jinsphys.2010.05.019

Cen-Aguilar, J. F., Rodriguez-Vivas, R. I., Dominguez-Alpizar, J. L., and Wagner, G. G. (1998). Studies on the effect of infection by *Babesia* sp. on oviposition of *Boophilus microplus* engorged females naturally infected in the Mexican tropics. Vet. Parasitol. 78, 253–257. doi: 10.1016/S0304-4017(98)00148-4

Chao, L. L., Liao, H. T., Ho, T. Y., and Shih, C. M. (2017). First detection and molecular identification of *Babesia gibsoni* from *Rhipicephalus sanguineus* ticks. Acta Trop. 166, 356–362. doi: 10.1016/j.actatropica.2016.09.022

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., and Malandrin, L. (2009). *Babesia* and its hosts: adaptation to long-lasting interactions as a way to achieve efficient transmission. Vet. Res. 40:37. doi: 10.1051/vetres/2009020

Coleman, J. L., Gebbia, J. A., Piesman, J., Degen, J. L., Bugge, T. H., and Benach, J. L. (1997). Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. Cell 89, 1111–1119. doi: 10.1016/S0092-8674(00)80298-6

de Castro, M. H., de Klerk, D., Pienaar, R., Latif, A. A., Rees, D. J. G., and Mans, B. J. (2016). *De novo* assembly and annotation of the salivary gland transcriptome of *Rhipicephalus appendiculatus* male and female ticks during blood feeding. Ticks Tick Borne Dis. 7, 536–548. doi: 10.1016/j.ttbdis.2016.01.014

de la Fuente, J., Moreno-Cid, J. A., Galindo, R. C., Almazan, C., Kocan, K. M., Merino, O., et al. (2013). Subolesin/Akirin vaccines for the control of arthropod vectors and vectorborne pathogens. Transbound. Emerg. Dis. 60, 172–178. doi: 10.1111/tbed.12146

de la Fuente, J., Villar, M., Cabezas-Cruz, A., Estrada-Pena, A., Ayllon, N., and Alberdi, P. (2016). Tick-host-pathogen interactions: conflict and cooperation. PLoS Pathog. 12:e1005488. doi: 10.1371/journal.ppat.1005488

de Rezende, J., Rangel, C. P., McIntosh, D., Silveira, J. A., Cunha, N. C., Ramos, C. A., et al. (2015). *In vitro* cultivation and cryopreservation of *Babesia bigemina* sporokinetes in hemocytes of *Rhipicephalus microplus*. Vet. Parasitol. 212, 400–403. doi: 10.1016/j.vetpar.2015.07.008

Esteves, E., Lara, F. A., Lorenzini, D. M., Costa, G. H., Fukuzawa, A. H., Pressinotti, L. N., et al. (2008). Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem. Mol. Biol. 38, 568–580. doi: 10.1016/j.ibmb.2008.01.006

Ferreira, C. A. S., Vaz, I. D., da Silva, S. S., Haag, K. L., Valenzuela, J. G., and Masuda, A. (2002). Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) calreticulin. Exp. Parasitol. 101, 25–34. doi: 10.1016/S0014-4894(02)00032-2

Ferreira, V., Molina, M. C., Valck, C., Rojas, A., Aguilar, L., Ramirez, G., et al. (2004). Role of calreticulin from parasites in its interaction with vertebrate hosts. Mol. Immunol. 40, 1279–1291. doi: 10.1016/j.molimm.2003.11.018

Ferrolho, J., Antunes, S., Santos, A. S., Velez, R., Padre, L., Cabezas-Cruz, A., et al. (2016). Detection and phylogenetic characterization of *Theileria* spp. and *Anaplasma marginale* in *Rhipicephalus bursa* in Portugal. Ticks Tick Borne Dis. 7, 443–438. doi: 10.1016/j.ttbdis.2016.01.004

Florin-Christensen, M., and Schnittger, L. (2009). Piroplasmids and ticks: a long-lasting intimate relationship. Front. Biosci. 14:3435. doi: 10.2741/3435

Fogaça, A. C., Lorenzini, D. M., Kaku, L. M., Esteves, E., Bulet, P., and Daffre, S. (2004). Cysteine-rich antimicrobial peptides of the cattle tick *Boophilus microplus*: isolation, structural characterization and tissue expression profile. Dev. Comp. Immunol. 28, 191–200. doi: 10.1016/j.dci.2003.08.001

Francischetti, I. M. B., Anderson, J. M., Manoukis, N., Pham, V. M., and Ribeiro, J. M. C. (2011). An insight into the sialotranscriptome and proteome of the coarse bontlegged tick, *Hyalomma marginatum* rufipes. J. Proteomics 74, 2892–2908. doi: 10.1016/j.jprot.2011.07.015

Francischetti, I. M., Mans, B. J., Meng, Z., Gudderra, N., Veenstra, T. D., Pham, V. M., et al. (2008). An insight into the sialome of the soft tick, *Ornithodorus parkeri*. Insect Biochem. Mol. Biol. 38, 1–21. doi: 10.1016/j.ibmb.2007.09.009

Galindo, R. C., Doncel-Perez, E., Zivkovic, Z., Naranjo, V., Gortazar, C., Mangold, A. J., et al. (2009). Tick subolesin is an ortholog of the akirins described in insects and vertebrates. Dev. Comp. Immunol. 33, 612–617. doi: 10.1016/j.dci.2008.11.002

Garcia, G. R., Gardinassi, L. G., Ribeiro, J. M., Anatriello, E., Ferreira, B. R., Moreira, H. N., et al. (2014). The sialotranscriptome of *Amblyomma triste*, *Amblyomma parvum* and *Amblyomma cajennense* ticks, uncovered by 454-based RNA-seq. Parasit. Vectors 7:430. doi: 10.1186/1756-3305-7-430

Ghosh, A. K., Coppens, I., Gårdsvoll, H., Ploug, M., and Jacobs-Lorena, M. (2011). *Plasmodium* ookinetes coopt mammalian plasminogen to invade the mosquito midgut. Proc. Natl. Acad. Sci. U.S.A. 108, 17153–17158. doi: 10.1073/pnas.1103657108

Gou, H., Guan, G., Liu, A., Ma, M., Chen, Z., Liu, Z., et al. (2013). Coevolutionary analyses of the relationships between piroplasmids and their hard tick hosts. Ecol. Evol. 3, 2985–2993. doi: 10.1002/ece3.685

Gough, J. M., and Kemp, D. H. (1993). Localization of a low abundance membrane protein (Bm86) on the gut cells of the cattle tick *Boophilus microplus* by immunogold labeling. J. Parasitol. 79, 900–907. doi: 10.2307/3283728

Hajdusek, O., Sima, R., Ayllon, N., Jalovecka, M., Perner, J., de la Fuente, J., et al. (2013). Interaction of the tick immune system with transmitted pathogens. Front. Cell. Infect. Microbiol. 3:26. doi: 10.3389/fcimb.2013.00026

Heekin, A. M., Guerrero, F. D., Bendele, K. G., Saldivar, L., Scoles, G. A., Dowd, S. E., et al. (2013). Gut transcriptome of replete adult female cattle ticks, *Rhipicephalus*

(*Boophilus*) *microplus*, feeding upon a *Babesia bovis*-infected bovine host. Parasitol. Res. 112, 3075–3090. doi: 10.1007/s00436-013-3482-4

Homer, M. J., Aguilar-Delfin, I., Telford, S. R., Krause, P. J., and Persing, D. H. (2000). Babesiosis. Clin. Microbiol. Rev. 13, 451–469. doi: 10.1128/CMR.13.3.451-469.2000

Inoue, N., Hanada, K., Tsuji, N., Igarashi, I., Nagasawa, H., Mikami, T., et al. (2001). Characterization of phagocytic hemocytes in *Ornithodoros moubata* (Acari: Ixodidae). J. Med. Entomol. 38, 514–519. doi: 10.1603/0022-2585-38.4.514

Jaworski, D. C., Simmen, F. A., Lamoreaux, W., Coons, L. B., Muller, M. T., and Needham, G. R. (1995). A secreted calreticulin protein in ixodid tick (*Amblyomma americanum*) saliva. J. Insect Physiol. 41, 369–375. doi: 10.1016/0022-1910(94)00107-R

Karim, S., Singh, P., and Ribeiro, J. M. C. (2011). A Deep insight into the Sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS ONE 6:e28525. doi: 10.1371/journal.pone.0028525

Kim, T. K., Guaratini Ibelli, A. M., and Mulenga, A. (2015). *Amblyomma americanum* tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade. Ticks Tick Borne Dis. 6, 91–101. doi: 10.1016/j.ttbdis.2014.10.002

Konnai, S., Yamada, S., Imamura, S., Nishikado, H., Githaka, N., Ito, T., et al. (2012). Identification of TROSPA homologue in *Ixodes persulcatus* Schulze, the specific vector for human Lyme borreliosis in Japan. Ticks Tick Borne Dis. 3, 75–77. doi: 10.1016/j.ttbdis.2012.02.001

Kopacek, P., Hajdusek, O., Buresova, V., and Daffre, S. (2010). Tick innate immunity. Invertebr. Immun. 708, 137–162. doi: 10.1007/978-1-4419-8059-5_8

Kotsyfakis, M., Kopácek, P., Franta, Z., Pedra, J. H., and Ribeiro, J. M. (2015). Deep sequencing analysis of the *Ixodes ricinus* haemocytome. PLoS Negl. Trop. Dis. 9:e0003754. doi: 10.1371/journal.pntd.0003754

Lack, J. B., Reichard, M. V., and Van Den Bussche, R. A. (2012). Phylogeny and evolution of the Piroplasmida as inferred from 18S rRNA sequences. Int. J. Parasitol. 42, 353–363. doi: 10.1016/j.ijpara.2012.02.005

Leiby, D. A. (2006). Babesiosis and blood transfusion: flying under the radar. Vox Sang. 90, 157–165. doi: 10.1111/j.1423-0410.2006.00740.x

Maeda, H., Hatta, T., Alim, M. A., Tsubokawa, D., Mikami, F., Kusakisako, K., et al. (2017). Initial development of *Babesia ovata* in the tick midgut. Vet. Parasitol. 233, 39–42. doi: 10.1016/j.vetpar.2016.11.020

Maeda, H., Kurisu, K., Miyata, T., Kusakisako, K., Galay, R. L., Rio, T. M., et al. (2015). Identification of the *Babesia*-responsive leucine-rich repeat domain-containing protein from the hard tick *Haemaphysalis longicornis*. Parasitol. Res. 114, 1793–1802. doi: 10.1007/s00436-015-4365-7

Merino, O., Almazan, C., Canales, M., Villar, M., Moreno-Cid, J. A., Galindo, R. C., et al. (2011). Targeting the tick protective antigen subolesin reduces vector infestations and pathogen infection by *Anaplasma marginale* and *Babesia bigemina*. Vaccine 29, 8575–8579. doi: 10.1016/j.vaccine.2011.09.023

Merino, O., Antunes, S., Mosqueda, J., Moreno-Cid, J. A., Perez de la Lastra, J. M., Rosario-Cruz, R., et al. (2013). Vaccination with proteins involved in tick-pathogen interactions reduces vector infestations and pathogen infection. Vaccine 31, 5889–5896. doi: 10.1016/j.vaccine.2013.09.037

Moreno-Cid, J. A., Perez de la Lastra, J. M., Villar, M., Jimenez, M., Pinal, R., Estrada-Pena, A., et al. (2013). Control of multiple arthropod vector infestations with subolesin/akirin vaccines. Vaccine 31, 1187–1196. doi: 10.1016/j.vaccine.2012.12.073

Mosqueda, J., Falcon, A., Antonio Alvarez, J., Alberto Ramos, J., Oropeza-Hernandez, L. F., and Figueroa, J. V. (2004). *Babesia bigemina* sexual stages are induced *in vitro* and are specifically recognized by antibodies in the midgut of infected *Boophilus microplus* ticks. Int. J. Parasitol. 34, 1229–1236. doi: 10.1016/j.ijpara.2004.07.003

Ord, R. L., and Lobo, C. A. (2015). Human babesiosis: pathogens, prevalence, diagnosis and treatment. Curr. Clin. Microbiol. Rep. 2, 173–181. doi: 10.1007/s40588-015-0025-z

Pal, U., Li, X., Wang, T., Montgomery, R. R., Ramamoorthi, N., deSilva, A. M., et al. (2004). TROSPA, an *Ixodes scapularis* receptor for *Borrelia burgdorferi*. Cell 119, 457–468. doi: 10.1016/j.cell.2004.10.027

Perner, J., Sobotka, R., Sima, R., Konvickova, J., Sojka, D., Oliveira, P. L., et al. (2016). Acquisition of exogenous haem is essential for tick reproduction. Elife 5:e12318. doi: 10.7554/eLife.12318

Rachinsky, A., Guerrero, F. D., and Scoles, G. A. (2007). Differential protein expression in ovaries of uninfected and *Babesia*-infected southern cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem. Mol. Biol. 37, 1291–1308. doi: 10.1016/j.ibmb.2007.08.001

Ranjbar-Bahadori, S., Eckert, B., Omidian, Z., Shirazi, N. S., and Shayan, P. (2012). *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. Parasitol. Res. 110, 1531–1536. doi: 10.1007/s00436-011-2658-z

Ribeiro, J. M., Anderson, J. M., Manoukis, N. C., Meng, Z., and Francischetti, I. M. (2011). A further insight into the sialome of the tropical bont tick, *Amblyomma variegatum*. BMC Genomics 12:136. doi: 10.1186/1471-2164-12-136

Rodríguez-Hernández, E., Mosqueda, J., Alvarez-Sánchez, M. E., Neri, A. F., Mendoza-Hernández, G., and Camacho-Nuez, M. (2012). The identification of a VDAC-like protein involved in the interaction of *Babesia bigemina* sexual stages with *Rhipicephalus microplus* midgut cells. Vet. Parasitol. 187, 538–541. doi: 10.1016/j.vetpar.2012.01.028

Rodríguez-Hernández, E., Mosqueda, J., León-Ávila, G., Castañeda-Ortiz, E. J., Álvarez-Sánchez, M. E., Camacho, A. D., et al. (2015). BmVDAC upregulation in the midgut of *Rhipicephalus microplus*, during infection with *Babesia bigemina*. Vet. Parasitol. 212, 368–374. doi: 10.1016/j.vetpar.2015.06.016

Rodríguez-Mallon, A. (2016). Developing Anti-tick Vaccines. Methods Mol. Biol. 1404, 243–259. doi: 10.1007/978-1-4939-3389-1_17

Rodríguez-Mallon, A., Bechara, G., Zacarias, R., Benavides-Ortiz, E., Soto-Rivas, J., Gómez-Ramírez, A., et al. (2013). Inhibition of *Ehrlichia canis* and *Babesia canis* transmission among ticks fed together on dogs vaccinated with Bm86 antigen. Open J. Anim. Sci. 3:24. doi: 10.4236/ojas.2013.33A004

Rudenko, N., Golovchenko, M., Edwards, M. J., and Grubhoffer, L. (2005). Differential expression of *Ixodes ricinus* tick genes induced by blood feeding or *Borrelia burgdorferi* infection. J. Med. Entomol. 42, 36–41. doi: 10.1093/jmedent/42.1.36

Sasaki, S. D., and Tanaka, A. S. (2008). rBmTI-6, a Kunitz-BPTI domain protease inhibitor from the tick *Boophilus microplus*, its cloning, expression and biochemical characterization. Vet. Parasitol. 155, 133–141. doi: 10.1016/j.vetpar.2008.03.031

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M., and Morrison, D. A. (2012). *Babesia*: a world emerging. Infect. Genet. Evol. 12, 1788–1809. doi: 10.1016/j.meegid.2012.07.004

Shayan, P., Hooshmand, E., Rahbari, S., and Nabian, S. (2007). Determination of *Rhipicephalus* spp. as vectors for *Babesia ovis* in Iran. Parasitol. Res. 101, 1029–1033. doi: 10.1007/s00436-007-0581-0

Solano-Gallego, L., Sainz, Á., Roura, X., Estrada-Peña, A., and Miró, G. (2016). A review of canine babesiosis: the European perspective. Parasit. Vectors 9, 336. doi: 10.1186/s13071-016-1596-0

Sonenshine, D. E., and Hynes, W. L. (2008). Molecular characterization and related aspects of the innate immune response in ticks. Front. Biosci. 13:3209. doi: 10.2741/3209

Sonenshine, D. E., and Michael Roe, R. (2014). Biology of Ticks, 2nd Edn. New York, NY: Oxford University Press.

Sultana, H., Patel, U., Sonenshine, D. E., and Neelakanta, G. (2015). Identification and comparative analysis of subolesin/akirin ortholog from *Ornithodoros turicata* ticks. Parasit. Vectors 8:132. doi: 10.1186/s13071-015-0749-x

Tonk, M., Cabezas-Cruz, A., Valdés, J. J., Rego, R. O., Grubhoffer, L., Estrada-Peña, A., et al. (2015). *Ixodes ricinus* defensins attack distantly-related pathogens. Dev. Comp. Immunol. 53, 358–365. doi: 10.1016/j.dci.2015.08.001

Tsuji, N., Battsetseg, B., Boldbaatar, D., Miyoshi, T., Xuan, X., Oliver, J. H. Jr., et al. (2007). Babesial vector tick defensin against *Babesia* sp. parasites. Infect. Immun. 75, 3633–3640. doi: 10.1128/IAI.00256-07

Tsuji, N., Miyoshi, T., Battsetseg, B., Matsuo, T., Xuan, X., and Fujisaki, K. (2008). A cysteine protease is critical for *Babesia* spp. transmission in *Haemaphysalis* ticks. PLoS Pathog. 4:e1000062. doi: 10.1371/journal.ppat.1000062

Urbanowicz, A., Lewandowski, D., Szpotkowski, K., and Figlerowicz, M. (2016). Tick receptor for outer surface protein A from *Ixodes ricinus*-the first intrinsically disordered protein involved in vector-microbe recognition. Sci. Rep. 6:25205. doi: 10.1038/srep25205

Villar, M., Ayllon, N., Alberdi, P., Moreno, A., Moreno, M., Tobes, R., et al. (2015). Integrated metabolomics, transcriptomics and proteomics identifies metabolic pathways affected by *Anaplasma phagocytophilum* Infection in tick cells. Mol. Cell. Proteomics 14, 3154–3172. doi: 10.1074/mcp.M115.051938

Wang, P., and Heitman, J. (2005). The cyclophilins. Genome Biol. 6:226. doi: 10.1186/gb-2005-6-7-226

Young, M. J., Bay, D. C., Hausner, G., and Court, D. A. (2007). The evolutionary history of mitochondrial porins. BMC Evol. Biol. 7:31. doi: 10.1186/1471-2148-7-31

Yu, X., Gong, H., Zhou, Y., Zhang, H., Cao, J., and Zhou, J. (2015). Differential sialotranscriptomes of unfed and fed *Rhipicephalus haemaphysaloides*, with particular regard to differentially expressed genes of cysteine proteases. Parasit. Vectors 8:597. doi: 10.1186/s13071-015-1213-7

Zivkovic, Z., Torina, A., Mitra, R., Alongi, A., Scimeca, S., Kocan, K. M., et al. (2010). Subolesin expression in response to pathogen infection in ticks. BMC Immunol. 11:7. doi: 10.1186/1471-2172-11-7

2. Disease and vector control

By leveraging the knowledge of **ticks and TBPs biology**, the development of new methods can be improved to **control** tick infestations and disease transmission[39,99]. However, the control and prevention of TTBDs is as complex as the tick-pathogen-host biological interactions, since it can be influenced by epidemiological[13], social[100,101] and politico-economic[102] factors among others. Therefore, as stated by Braks and colleagues, ***"With wicked problems there are no trivial solutions"***[103]. For this, it is necessary a continued investment in TTBD's control[99] (**surveillance, diagnosis, treatment and prevention**) and also an alliance towards the conception of **"One World-One Health"**[104], which incorporates a holistic and multidisciplinary expertise in dealing with human and animal health, vectors, vector-borne diseases and their shared environment[1,13,104].

To improve approaches, an enhanced communication between vector control programs, local environmental/public health departments, veterinarians, and physicians, is fundamental[105,106]. The **community engagement** in disease surveillance and prevention can also be critically important[100,103]. For example, iniciatives such as the Dutch Tick Radar (https://www.tekenradar.nl/) (which enables people bitten by ticks to indicate their location, contact information, and send in the ticks) are synergistic to public health authorities' implementation of community engagement. Additionally, entomological **surveillance and risk assessments** should be conducted as proactive approaches, in order to assess the tick species that are present or can be introduced in a defined area, the pathogen(s) that they may carry and their distribution and abundancy in hosts on specific environments[39,105,107]. However, due to budget constraints, those strategies are usually implemented after an increase of prevalence of ticks and/or TBDs, which allows the expansion of infected vector populations leaving the human and animal community at higher risk of infestation and infection[108,109]. Unfortunately, such lack of surveillance places the diagnosis, treatment and prevention as the central strategy for control of many TTBDs.

Currently, the main protocol for controlling many of the TTBDs is centered on vector and pathogen control. For instance, ovine babesiosis control is based in the prompt

diagnosis and effective treatment of unwell animals, identification and elimination of ticks, and protection of healthy animals[79]; using only chemotherapy and tick control[110].

Following, a review on babesiosis diagnosis, treatment, and prevention, with more emphasis on new strategies for tick control, is discussed.

2.1. Babesiosis diagnosis, treatment, and prophylaxis

The diagnosis of babesiosis is founded on epidemiological studies (*e.g.* in endemic areas, during seasons), medical history, physical examination (clinical signs such as fever, lethargy, and icterus), and laboratory tests (*e.g.* hematological test and *Babesia* detection)[13,111]. The main *Babesia*-confirmatory tests are based on cytology, serology, and molecular biology techniques[112]. In suspected **acute cases** of babesiosis, blood should be collected either from capillaries in the ear/tail tip or from venous blood sampling, depending on *Babesia* spp. and where a higher concentration of those parasites can be found[87,112]. For example, *B. bovis* can be detected in the peripheral blood collected from the ear/tail, whereas *B. ovis*, *B. bigemina* and *B. divergens* are typically found in venous blood samples. Besides, for **cytological examination**, multiple thin and thick **blood smears** should be prepared and stained with either Giemsa or acridine orange[112]. For **subclinical infections** with low levels of parasitemia, **serological** diagnostics[83,113,114], such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT), can be used. However, **molecular diagnostic assays** such as PCR and its variants (*e.g.* qPCR, nested PCR, LAMP), are preferred to detect clinical infections and asymptomatic carriers of babesiosis and/or other TBDs[87,111,112]. Such versatility to detect the origin of such low quantity of pathogen DNA is crucial to assess a quick and assure an appropriate therapeutic response[111].

Acute cases of ovine babesiosis are treated with **chemotherapy** combined with supportive treatment which might include the use of anti-inflammatory drugs, corticosteroids, and fluid therapy containing iron, folate and vitamins[115] (such as B12[87], to stimulate erythropoiesis[116]), aiding recovery. For animals with acute anemic anoxia, blood transfusion is recommended[111,115]. The chemotherapy in small ruminants is based on the application of diminazene aceturate and imidocarb dipropionate[72,87], being this last drug used as an effective prophylactic medicine when applied twice the therapeutic dose[72,113]. The combination of imidocarb dipropionate and oxytetracycline has been proposed as well, since an efficacy above 80 % was observed for sheep and goats in Pakistan[117]. Several mechanisms of action of these drugs have been proposed however, additional studies need to be conducted to validate and complement these suggestions[118,119], as well as the mechanisms of possible resistance events[120].

Chemotherapy for babesiosis has some drawbacks. Besides the cost implied in diagnosis and drug acquisition for a proper treatment, chemotherapy itself may introduce drug residues into the food chain, such as meat and dairy products, posing a public health concern[72,120,121]. Moreover, the chemotherapy based on the extensively used imidocarb dipropionate is shown to be recombinogenic[122], which highlights the need for new safer strategies for the control of ovine and other types of babesiosis, such as vaccination and vector control.

Vaccination should be implemented if the endemic status is instable[79,114]; *i.e.* if the hosts are exposed to a high infection rate, presented clinical symptoms frequently and developed low quantity of antibodies having, consequently, no protective immunity[79,111,114]. Studies focusing on the endemic instability of ovine babesiosis are scarce. So far, such instable endemic status about ovine babesiosis was observed in Turkey[79], which ascertain the necessity for a vaccine against *B. ovis*.

There are several forms of vaccines (live-attenuated, inactivacted, viral vector, subunit- and DNA/RNA-based vaccines[123,124]) however, until now, only live-attenuated blood-stage vaccines are commercially available for bovine babesiosis caused by *B. bovis* and *B. bigemina*[72]. Undesirably, such type of vaccine can occasionally fail due to incorrect handling or storage, administration of chemotherapeutics immediately before or during vaccination, stress, concomitant infections, pathogenicity reversion of the attenuated vaccine strains for a virulent phenotype, and/or changes in the parasite population that can lead to lack of protection[72]. To overcome these constraints associated to the live-attenuated and even inactivated vaccines, several promising subunit antigens have been proposed, showing protective responses against distinct *Babesia* spp. in their hosts[110,125].

Overall, there is a need for safer, efficient and commercially available blood-stage vaccines for babesiosis, aiming for the induction of the host immune system in order to prevent *Babesia* parasites from completing RBC invasion and merogony[78]. Moreover, this absence of commercially available vaccines leaves **tick control** as the safer strategy for babesiosis control specially for neglected diseases such as ovine babesiosis.

2.2. Tick control, towards an integrated approach

Since the late 1800s, vector control has been an approach to reduce the dispersion of many VBDs[99]. With a thorough understanding of vector biology, epidemiology and environmental impact, **vector control** must rely on the integration of different control methods[14] (physical, ecological, biological, genetic and chemical control and vaccination[39]). The combination of two or more control methods is known as **integrated tick management** (ITM), which aim to control the vector in a sustainable, environmentally compatible, and cost-effective way, maintaining adequate levels of animal production[126]. Some ITM strategies were reviewed by Rodriguez-Vivas and colleagues[127].

The **manual removal** of ticks is a direct and physical control of the vector, used mainly by small farms when the levels of infestation are low. This method needs to be executed constantly and its efficacy is considerably conditioned by the number of ticks and their size in the immature phases, number of animals and workers availability[127].

Knowing that ecological factors can influence ticks, **ecological approaches** can be also implemented to control tick population dynamics. For instance, vegetation is a shelter for ticks enabling the accomplishment of their life cycle. Therefore, the removement of vegetation, cropping and soil cultivation can enhance tick control[39]. Burning pasture is another practice to control tick's population. It is widely used in many countries, affecting directly ticks but also the vegetation layer that protects them[127,128]. Still, ecological control has disadvantages such as management difficulties, the cost of fencing and pasture irrigation facilities and the possible adverse effect on pasture quality[14,127].

Biological control has been documented using tick biocontrol agents and biopesticides[129]. While some plants are described to act as attractants of ticks (such as *Stylosanthes scabra* and *Acalypha fruticose*[127]), other animals feed on (birds: *Buphagus* sp., *Crotophagus* sp., various magpies, village fowl; ants: *Solenopsis germinata*, *S. saevissima* and *Ectatomma cuadridens*[39]) or parasite them (chalcid wasps: *Ixodiphagus hookeri*; nematodes: *Heterorhabditis* spp. and *Steinernema* spp.[39]) until their death. Entomopathogenic fungi and *Bacillus thuringiensis* and its derivates have been proposed as biopesticides since they are pathogenic to ticks[39] but, their efficacy is questionable[127].

Genetic control can also be considered, by using hosts or ticks with ideal genetic background. Resistant breeds of livestock are been used for vector control, since those animals are resistant to tick infestations. This hereditary characteristic[39] in the host has been associated to the accumulation of basophils at the tick re-infestation site[98]. Once activated, the basophils release histamine in the animal blood, which affects negatively tick feeding, egg production and its viability[98,130]. However, this resistance to ticks can be diminished by illnesses such TBDs[131], revealing the interference of TBPs in the tick life cycle. While resistant breeds of cattle are widely used for vector control, limited research is conducted on sheep[132], which rules out this option in the control of ovine babesiosis and the primary vector *R. bursa*. On the vector perspective, the release of sterile male hybrids (from mating of *R. annulatus* with *R. microplus*)[133] was proposed in 1982 but is still controversial due to the high cost associated to their production and the ecological risk of an extended range of those ticks in the environment[127].

Chemical acaricides have been used extensively in vector control through dipping vats, spraying, pour-on and parenteral delivery[134], having a repellent/acaricidal effect. The chemical classes reported as acaricides are arsenicals, chlorinated hydrocarbons, organochlorides, organophosphates, pyrethroids, amitraz, carbamates, amidines, macrocyclic lactones (ivermectin, doramectin, moxidectin), insect grown regulators (IGRs), and phenilpirazolons (fipronil), spinosad and fluazuron, permethrin, flumethrin and their used have been documented[14,39,111,135,136]. In the particular case of small ruminants, chemical control of ticks based on dipping or pour-on applications of pyrethroids is the main strategy[11,14].

Alarmingly, the intensive and inadequate application of acaricides is driving the emergence of chemical resistance and ultimately selection of drug-resistant ticks[137]. The processes behind acaricide resistance are reviewed in detail in several reports[127,135,137]. This major constrain is mostly associated with one-host ticks, such as cattle ticks, which regardless of their stage, are constantly exposed to those compounds. Nonetheless, in Iran, resistance to pyrethroids in two-host *R. bursa* tick populations have been demonstrated[138], increasing the exposure of hosts to ticks and transmission of TBDs, including ovine babesiosis. Other disadvantages associated to acaricides use are: drug residues in the food chain, negative impact on the environment[120,137], adverse effects on animal health (carcinogenic effects[14,136]), and its high cost[139].

Some of these drawbacks can be surmounted by understanding the withdrawal time of a specific acaricide[39], use of so-called "green pesticides"[127,136,140,141] (*e.g. Eucalyptus globulus* and *Zingiber officinalis* for *R. bursa*[142]), improvement and development of new drugs (*e.g.* use of nanoparticles[136]). Overall, chemical control needs to be reevaluated in tick control programs, by implementing ITM strategies[143,144] regarding the epidemiology of acaricide susceptibility/resistance[135] and developing novel methods of disease control.

Alternative to acaricides, **passive and active immunity** launched the several transmission-blocking methods that affected not only pathogens but also vectors. Passive immunity, which consists in the transfer of pre-synthethized elements of the immune system, such as sera, from tick-immune animals to naive animals[145], have affected pathogen infection[146] but also tick infestations[146,147] and tick toxicosis[148]. Nevertheless, these findings reflected more the impact of humoral response on tick resistance than the use of the methodology itself to control TTBDs.

Active immunity entails the inoculation of an antigen inducing an immunological protective response in the host. **Vaccination** is an artificial active immunization that could affect both pathogen and vector without promoting drug resistance, being also cost-effective and environmentally friendly[11,149]. Currently, of the two Bm86 tick-stage vaccines commercialized in the 1990s (TickGARD$^{PLUS TM}$ in Australia[150] and Gavac^{TM} in Cuba[151]), only Gavac^{TM} is available[152]. Nonetheless, both products are based on the same antigen, which is a cattle tick *R. microplus* midgut membrane-derived protein, known as Bm86[153]. This protein is a concealed antigen that grants an immunological response capable of reducing the number of engorging females, their weight and reproductivity, lowering tick populations after several generations[150,153]. Investment has been placed on the development of new anti-tick vaccines, and several promising protein/subunit-based antigens derived from ticks are being proposed[152]. However, since the discovery of BM86 in early 90´s no other antigens have matched its potential. The high number of tick species with striking differences, their life cycle and the complex dynamics of tick pathogen interactions have been hurdles extremely difficult to overcome. Besides, it is known that the levels of protection under field conditions are greatly reduced in comparison to those controlled and defined in a laboratory[154]. Consequently, research is constantly needed to increase the panoply of targets that can be used on the development of vaccines[135,155],

either by developing combined tick vaccines or by improving the vaccine delivery systems available in this research area[152].

2.3. Technologies boosting tick control

Before the 1990's, the laborious and time-consuming "isolate-inactive-inject" methodology was the main approach to search for new and limited tick protective antigen candidates in several *in vivo* experiments[149,156]. As in other areas of research, tick control has benefited from the tecnological advances established in the last decades[157–159].

Cutting-edge technologies based on **next-generation sequencing** are been used to assemble a massive profile of compounds involved in the vector-host-pathogen interactome[149,160] to further, evaluate their use in the development of transmission-blocking vaccines or even drug targets[98]. By using high-throughput technologies it is possible to detect, quantify, and identify a myriad of biological molecules (such as DNA with genomics, RNA with transcriptomics, proteins with proteomics) at specific conditions, unraveling the biological activity behind the complex tick-pathogen-host interactions in specific tissues[156,161,162]. Having in mind the involvement of SGs in pathogen-host interface, such technologies can be used to better understand processes like host attachment, blood feeding/digestion and pathogen transmission[149,163–165]. Furthermore, the holistic approach of systems biology can be used, in which all the information from different biological molecules collected from genomics, transcriptomics, proteomics and others can be analysed simultaneously to better describe this dynamic system[166,167]. With this vast information, it is possible to highlight protein families or metabolic pathways that are prone to be associated to specific tick biological processes.

To date, less than 5 % of tick salivary proteins have been functionally validated, stressing the necessity for functional studies to address this knowledge gap on protein function[154,165,168]. Therefore, several techniques (interference RNA (RNAi), Tetracycline-Controlled Transcriptional Activation (TET system), CRISPR gene editing) can be used to modulate gene expression and perform gene editing to elucidate about gene function. **RNAi** emerges as an commonly applicable methodology in TTBD research, either *in vivo* or *in vitro*, to enlighten the tick-host-pathogen network[169–171]. By inoculating triggering molecules of the RNAi pathway (*e.g.* double-stranded RNA (dsRNA)), the disruption of gene expression can be achieved inhibiting its translation to protein[171,172], which could influence tick biology and pathogen transmission. Allied to tools such as **tick cell lines**,

the research on this complex network and discovery of new antigens could be greatly enhanced, by reducing the need for expensive *in vivo* studies involving maintenance of tick colonies and feeding on laboratory animals[164173,174]. Despite the great potential of RNAi, the reduced number of available annotated tick genomes renders important constrains such as in the identification of several tick transcripts without an open reading frame (ORF) which hampers the design of molecules for functional studies[58]. Moreover, a lack on tick genomics research hinder the identification of "off-target effects" from dsRNA treatments, which complicates the interpretation of functional studies results[154].

Besides targeting and understanding the function of tick stimuli-specific proteins, it is possible to comprehend the role of core and **conserved pathways** on tick biology to improve vaccine design[154,175]. By characterizing pathways conserved across vector species, with similar functional motifs and putative structure, it is possible to provide new insights regarding vector-pathogen interface in general and develop versatile methods to manage different TBDs and tick infestations worldwide[145,175]. Even more conserved are the active sites residues of enzymes and the binding specific epitopes occurring in protein-protein interactions. Addressing the impact of such conserved spots could improve the methods of disease control[154].

As a direct approach towards vaccine development, **reverse vaccinology** uses an *in silico* methodology to search for immunogenic targets that elicits the production of protective antibodies on the vertebrate host that could ultimately interfere with TTBDs[154,176]. Knowing the mechanism behind host antibody production facilitates the elaboration of an efficient *in silico* pipeline. Briefly, it begins with the recognition and processing of foreign tick protein antigens by a host antigen presenting cell (*e.g.* macrophage, dendritic cells). Here, protein topology must be considered, since it must be exposed to the immune system. Then, the exposed tick antigen-derived peptides are presented through the host MHC II receptor complex to helper T (Th) cells, that circulate to the secondary lymphoid tissues[177] and activate those cells. These activated Th cells will, in turn, trigger the activation of B cells in the lymph nodes and cause their differentiation and maturation into memory B cells and plasma cells[178]. While the memory B cells confer immunological memory, the plasma cells differentiate into plasmablasts, which will produce antibodies that will confer protection to TTBDs[179].

Computational methods have been combined to predict B and T cell epitopes as well as its topology, hydrophobicity, polarity, solubility and more[180], in order to select promising candidates comprising all the requirements for a suitable vaccine or even for disease diagnosis and disease therapy[181]. There are several publications using RV approaches in either TBPs or tick's omics data, towards vaccine development but also drug discovery and disease diagnosis[182,183]. However, there is insufficient information on tick protein evolution, structure and annotation that would be essential to improve RV approaches in tick research.

The integration of such pioneering methodologies (systems biology, functional genomics through RNAi of pathway-related targets, RV and pathways) allow network-based analyses to better understand the complexity and functionality of tick–pathogen interactions that could be targeted for vaccine development or drug discovery against TTBDs[154,184,185].

3. Thesis and strategies

With the premises that tick SGs have an important role in pathogen transmission and during blood feeding, and that a deeper understanding on tick-pathogen interface in this tissue contributes to the discovery of promising targets that will spur the control of TTBDs, the present thesis has three main strategies that aims to explore:

- Build knowledge on the molecular dynamics behind *B. ovis* infection and blood feeding on the SGs of *R. bursa* ticks, select targets that might interfere with those biological processes and evaluate their function,
- Search for immunogenic targets that can be proposed for vaccine development using *in silico* analysis,
- Screening for conserved biological pathways across *Rhipicephalus* ticks and evaluate their purpose on pathogen-vector interplay.

Using these strategies, studies were conducted and described throughout the following chapters:

Chapter III is dedicated to increase insight on the complex molecular events that occur during *Babesia* infection and tick blood feeding. Therefore, next generation sequencing techniques, such as RNA-seq and SWATH-MS, were used, for the first time, to catalogue and characterize the sialotranscriptome and sialoproteome of *R. bursa* ticks upon those processes. After obtaining those profiles, promising targets were selected, and their function evaluated using the RNAi methodology. By diminishing the gene expression of selected targets, it was possible to assess their impact on tick biology and pathogen infection, elucidating about their potential to be included in an anti-tick and transmission-blocking vaccine.

Chapter IV is centered on RV as an alternative method for vaccine candidate discovery using the previous *R. bursa* omic data. By applying immunoinformatic tools, it was possible to filter antigenic proteins that might be capable of inducing a protective, robust, and long-lasting immune response with no allergenic or toxic effects *in vivo*. Regions containing overlapping CEGs of those targets were screen for their potential as peptide-based therapeutics against tick infestations and *Babesia* transmission.

Chapter V is devoted to understanding a conserved pathway between ticks and their role on tick biology and pathogen survival. To accomplish this, previously obtained transcriptomics and proteomics data regarding the models *R. bursa – B. ovis*, *R. annulatus – B. bigemina* and *R. sanguineus – E. canis* were thoroughly screened, exhibiting the folate biosynthesis pathway in all datasets. With a central biological importance in ticks and pathogens, and absence in higher eukaryotes such as mammals' hosts, this pathway is an attractive target for the development of transmission-blocking approaches. Herein, RNAi in alliance to a tick cell line allowed the characterization of specific folate-related proteins function in vector-pathogen interface and their potential use in controlling ticks and TBDs.

Chapter VI addresses a general discussion about the results obtained in this thesis, as well as concluding remarks with perspectives for future research.

4. References

1. Barker, C. M. & Reisen, W. K. Epidemiology of Vector-Borne Diseases. in *Medical and Veterinary Entomology* 33–49 (Academic Press, 2019). doi:10.1016/B978-0-12-814043-7.00004-2.
2. Randolph, S. E. & Rogers, D. J. The arrival, establishment and spread of exotic diseases: Patterns and predictions. *Nature Reviews Microbiology* vol. 8 361–371 (2010).
3. World Health Organization. *The world health report 2004 - changing history*. (2004).
4. Golding, N. *et al.* Integrating vector control across diseases. *BMC Med.* **13**, 1–6 (2015).
5. Paddock, C. D., Lane, R. S., Staples, J. E. & Labruna, M. B. Changing paradigms for tick-borne diseases in the americas. *Global Health Impacts of Vector-Borne Diseases: Workshop Summary* (2016).
6. de la Fuente, J. Controlling ticks and tick-borne diseases…looking forward. *Ticks and Tick-borne Diseases* vol. 9 1354–1357 (2018).
7. Nathavitharana, R. R. & Mitty, J. A. Diseases from North America: Focus on tick-borne infections. *Clin. Med. J. R. Coll. Physicians London* **15**, 74–77 (2015).
8. Sonenshine, D. Range Expansion of Tick Disease Vectors in North America: Implications for Spread of Tick-Borne Disease. *Int. J. Environ. Res. Public Health* **15**, 478 (2018).
9. Pérez De León, A. A. *et al.* One health approach to identify research needs in bovine and human babesioses: Workshop report. in *Parasites and Vectors* vol. 3 36 (BioMed Central, 2010).
10. Taylor, M., Coop, R. & Wall, R. Facultative ectoparasites and arthropod vectors. in *Veterinary Parasitology* 921–973 (Wiley-Blackwell, 2016).
11. Stuen, S. Haemoparasites in small ruminants in European countries: Challenges and clinical relevance. *Small Rumin. Res.* **142**, 22–27 (2016).
12. Alessandra, T. & Santo, C. Tick-borne diseases in sheep and goats: Clinical and diagnostic aspects. *Small Rumin. Res.* **106**, S6–S11 (2012).
13. Dantas-Torres, F., Chomel, B. B. & Otranto, D. Ticks and tick-borne diseases: a

One Health perspective. *Trends Parasitol.* **28**, 437–46 (2012).

14. Jaime Betancur Hurtado, O. & Giraldo-Ríos, C. Economic and Health Impact of the Ticks in Production Animals. in *Ticks and Tick-Borne Pathogens* (IntechOpen, 2019). doi:10.5772/intechopen.81167.
15. FAO. Animal health. *Food and Agriculture Organization of the United Nations* http://www.fao.org/animal-health/en/ (2020).
16. FAO. *Animal diseases and livestock emergencies*. http://www.fao.org/emergencies/ (2017).
17. FAO. Transboundary animal diseases. *FAO in Emergencies* http://www.fao.org/emergencies/emergency-types/transboundary-animal-diseases/en/ (2020).
18. FAO. *Protecting people and animals from disease threats*. (2019).
19. *Diseases of Cattle in the Tropics - Economic and Zoonotic Relevance*. vol. 6.
20. Perry, B. D. The control of East Coast fever of cattle by live parasite vaccination: A science-to-impact narrative. *One Heal.* **2**, 103–114 (2016).
21. Monteiro, A. L. G. *et al.* The role of small ruminants on global climate change. *Acta Sci. - Anim. Sci.* **40**, (2018).
22. Marino, R. *et al.* Climate change: Production performance, health issues, greenhouse gas emissions and mitigation strategies in sheep and goat farming. *Small Ruminant Research* vol. 135 50–59 (2016).
23. Bilgic, H. B. *et al.* Prevalence of tick-borne haemoparasites in small ruminants in Turkey and diagnostic sensitivity of single-PCR and RLB. *Parasites and Vectors* **10**, 1–13 (2017).
24. Yin, H. & Luo, J. Ticks of small ruminants in China. *Parasitol. Res.* **101**, 187–189 (2007).
25. Altay, K., Aktas, M. & Dumanli, N. Detection of *Babesia ovis* by PCR in *Rhipicephalus bursa* collected from naturally infested sheep and goats. *Res. Vet. Sci.* **85**, 116–119 (2008).
26. Preston, P. M. Theilerioses. in *Encyclopedia of Arthropod-transmitted Infections of Man and Domesticated Animals* (ed. M. W. Service) 487–502 (Springer, 2001). doi:10.1023/A:1021249422392.
27. Uilenberg, G. *Babesia*-A historical overview. *Vet. Parasitol.* **138**, 3–10 (2006).

28. Uilenberg, G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. *Vet. Parasitol.* **57**, 19–41 (1995).
29. Yin, H., Schnittger, L., Luo, J., Seitzer, U. & Ahmed, J. S. Ovine theileriosis in China: A new look at an old story. *Parasitol. Res.* **101**, 191–195 (2007).
30. Uilenberg, G. General review of tick-borne diseases of sheep and goats world-wide. *Parassitologia* **39**, 161–165 (1997).
31. Yeruham, I., Hadani, A. & Galker, F. Some epizootiological and clinical aspects of ovine babesiosis caused by *Babesia ovis* - A review. *Vet. Parasitol.* **74**, 153–163 (1998).
32. De La Fuente, J., Estrada-Peña, A., Venzal, J. M. & Kocan, K. Overview: ticks as vectors of pathogens that cause disease in humans and animals. *Front. Biosci.* **13**, 6938–6946 (2008).
33. Ranjbar-Bahadori, S., Eckert, B., Omidian, Z., Shirazi, N. S. & Shayan, P. *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. *Parasitol. Res.* **110**, 1531–1536 (2012).
34. Wisely, S. & Glass, G. Advancing the Science of Tick and Tick-Borne Disease Surveillance in the United States. *Insects* **10**, 361 (2019).
35. Junker, L. ESA Position Statement on Tick-Borne Diseases. *Ann. Entomol. Soc. Am.* **113**, 62–63 (2020).
36. Obregón Alvarez, D. *et al.* Ticks and Tick-Borne Diseases in Cuba, Half a Century of Scientific Research. *Pathogens* **9**, 616 (2020).
37. Walker, A. R. *Ticks of Domestic Animals in Africa: a guide to identification of species*. (Bioscience Reports, 2003).
38. Bristol University. *Rhipicephalus bursa*. http://www.bristoluniversitytickid.uk/page/Rhipicephalus+bursa/29/#.X5MWvtBKiUm.
39. Asebe, G., Hailu, Y. & Kumar Basu, A. Overview of the Biology, Epidemiology and Control Methods Against Hard Ticks: A Review. *Glob. J. Sci. Front. Res.* **16**, (2016).
40. Yeruham, I., Hadani, A. & Galker, F. The life cycle of *Rhipicephalus bursa* Canestrini and Fanzago, 1877 (Acarina: Ixodidae) under laboratory conditions. *Vet. Parasitol.* **89**, 109–116 (2000).

41. Belozerov, V. N. Diapause and Biological Rhythms in Ticks. in *Physiology of Ticks* 469–500 (Elsevier, 1982). doi:10.1016/b978-0-08-024937-7.50018-4.
42. Sonenshine, D. E. Tick Pheromones And Their Use In Tick Control. *Annu. Rev. Entomol.* **51**, 557–580 (2006).
43. Josek, T., Walden, K. K. O., Allan, B. F., Alleyne, M. & Robertson, H. M. A foreleg transcriptome for *Ixodes scapularis* ticks: Candidates for chemoreceptors and binding proteins that might be expressed in the sensory Haller's organ. *Ticks Tick. Borne. Dis.* **9**, 1317–1327 (2018).
44. Mitchell, R. D. *et al.* Infrared light detection by the haller's organ of adult american dog ticks, *Dermacentor variabilis* (Ixodida: Ixodidae). *Ticks Tick. Borne. Dis.* **8**, 764–771 (2017).
45. Carr, A. L. *et al.* Tick Haller's organ, a new paradigm for arthropod olfaction: How ticks differ from insects. *Int. J. Mol. Sci.* **18**, (2017).
46. Sharifah, N., Heo, C. C., Ehlers, J., Houssaini, J. & Tappe, D. Ticks and tick-borne pathogens in animals and humans in the island nations of Southeast Asia: A review. *Acta Tropica* vol. 209 105527 (2020).
47. Estrada-Peña, A. Ticks as vectors: Taxonomy, biology and ecology. *OIE Rev. Sci. Tech.* **34**, 53–65 (2015).
48. Goddard, J. *Infectious Diseases and Arthropods*. *Infectious Diseases and Arthropods* (Humana Press, 2008). doi:10.1007/978-1-60327-400-5.
49. Mans, B. J., Louw, A. I. & Neitz, A. W. H. Evolution of Hematophagy in Ticks: Common Origins for Blood Coagulation and Platelet Aggregation Inhibitors from Soft Ticks of the Genus Ornithodoros. *Mol. Biol. Evol.* **19**, 1695–1705 (2002).
50. Šimo, L., Kazimirova, M., Richardson, J. & Bonnet, S. I. The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. *Front. Cell. Infect. Microbiol.* **7**, 281 (2017).
51. Nicholson, W. L., Sonenshine, D. E., Noden, B. H. & Brown, R. N. Ticks (ixodida). in *Medical and Veterinary Entomology* 603–672 (Elsevier, 2018). doi:10.1016/B978-0-12-814043-7.00027-3.
52. Hackenberg, M., Langenberger, D., Schwarz, A., Erhart, J. & Kotsyfakis, M. *In silico* target network analysis of *de novo*-discovered, tick saliva-specific microRNAs reveals important combinatorial effects in their interference with

vertebrate host physiology. *RNA* **23**, 1259–1269 (2017).

53. Zhou, W. *et al.* Discovery of Exosomes From Tick Saliva and Salivary Glands Reveals Therapeutic Roles for CXCL12 and IL-8 in Wound Healing at the Tick–Human Skin Interface. *Front. Cell Dev. Biol.* **8**, 554 (2020).
54. Nuttall, P. A. Tick saliva and its role in pathogen transmission. *Wien. Klin. Wochenschr.* 1–12 (2019) doi:10.1007/s00508-019-1500-y.
55. Ribeiro, J. M. C. & Mans, B. J. TickSialoFam (TSFam): A Database That Helps to Classify Tick Salivary Proteins, a Review on Tick Salivary Protein Function and Evolution, With Considerations on the Tick Sialome Switching Phenomenon. *Front. Cell. Infect. Microbiol.* **10**, 374 (2020).
56. Ribeiro, J. M. C., Mans, B. J. & Arcà, B. An insight into the sialome of blood-feeding Nematocera. *Insect Biochem. Mol. Biol.* **40**, 767–784 (2010).
57. Ribeiro, J. M. C. & Arcà, B. From Sialomes to the Sialoverse. An Insight into Salivary Potion of Blood-Feeding Insects. *Advances in Insect Physiology* vol. 37 59–118 (2009).
58. Aounallah, H. *et al.* Tick Salivary Compounds for Targeted Immunomodulatory Therapy. *Front. Immunol.* **11**, 583845 (2020).
59. Chmelař, J. *et al.* Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick-Host Interactions. *Trends Parasitol.* **32**, 242–254 (2016).
60. Werner, E. R., Blau, N. & Thöny, B. Tetrahydrobiopterin: biochemistry and pathophysiology. *Biochem. J.* **438**, 397–414 (2011).
61. Mans, B. J. & Neitz, A. W. H. Biochemical perspectives on paralysis and other forms of toxicoses caused by ticks. *researchgate.net* **129**, S95 (2004).
62. De La Fuente, J., Pacheco, I., Villar, M. & Cabezas-Cruz, A. The alpha-Gal syndrome: New insights into the tick-host conflict and cooperation. *Parasites and Vectors* **12**, 154 (2019).
63. Jongejan, F. & Uilenberg, G. The global importance of ticks. *Parasitology* **129**, (2004).
64. Kahl, O. Hard ticks as vectors—some basic issues. *Wiener Klinische Wochenschrift* vol. 130 479–483 (2018).
65. Piesman, J. & Eisen, L. Prevention of Tick-Borne Diseases. *Annu. Rev. Entomol.* **53**, 323–343 (2008).

66. Labuda, M. & Nuttall, P. A. Tick-borne viruses. *Parasitology* **129**, (2004).
67. Hubálek, Z. Biogeography of Tick-Borne Bhanja Virus (Bunyaviridae) in Europe. *Interdiscip. Perspect. Infect. Dis.* **2009**, 1–11 (2009).
68. Barandika, J. F. *et al.* Prevalence of tick-borne zoonotic bacteria in questing adult ticks from Northern Spain. *Vector-Borne Zoonotic Dis.* **8**, 829–835 (2008).
69. Moltmann, U., Mehlhorn, H. & Friedhoff, K. Electron microscopic study on the development of *Babesia ovis* (Piroplasmia) in the salivary glands of the vector tick *Rhipicephalus bursa*. *Acta Trop.* **39**, 29–40 (1982).
70. Babes, V. Sur lhemoglobinurie bacterienne du boeuf. *Compt. Rend. Acad. Sci. Ser. III Sci. Vie.* **107**, 692–694 (1888).
71. Hunfeld, K.-P., Hildebrandt, A. & Gray, J. S. Babesiosis: recent insights into an ancient disease. *Int. J. Parasitol.* **38**, 1219–37 (2008).
72. Ganzinelli, S., Rodriguez, A., Schnittger, L. & Florin-Christensen, M. *Babesia* in domestic ruminants. in *Parasitic Protozoa of Farm Animals and Pets* 215–239 (Springer International Publishing, 2018). doi:10.1007/978-3-319-70132-5_9.
73. Krause, P. J. Human babesiosis. *Int. J. Parasitol.* **49**, 165–174 (2019).
74. Schnittger, L., Rodriguez, A. E., Florin-Christensen, M. & Morrison, D. A. *Babesia*: A world emerging. *Infect. Genet. Evol.* **12**, 1788–1809 (2012).
75. Bloch, E. M., Kumar, S. & Krause, P. J. Persistence of *Babesia microti* infection in humans. *Pathogens* **8**, 102 (2019).
76. Klinger, C. M., Nisbet, R. E., Ouologuem, D. T., Roos, D. S. & Dacks, J. B. Cryptic organelle homology in apicomplexan parasites: Insights from evolutionary cell biology. *Curr. Opin. Microbiol.* **16**, 424–431 (2013).
77. Blackman, M. J. & Bannister, L. H. Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation. *Mol. Biochem. Parasitol.* **117**, 11–25 (2001).
78. Jalovecka, M., Sojka, D., Ascencio, M. & Schnittger, L. *Babesia* Life Cycle - When Phylogeny Meets Biology. *Trends Parasitol.* **35**, 356–368 (2019).
79. Ceylan, O. & Sevinc, F. Endemic instability of ovine babesiosis in Turkey: A country-wide sero-epidemiological study. *Vet. Parasitol.* **278**, 109034 (2020).
80. Friedhoff, K. T. Tick-borne diseases of sheep and goats caused by *Babesia*, *Theileria* or *Anaplasma spp*. *Parassitologia* **39**, 99–109 (1997).

81. Stuen, S. Haemoparasites—Challenging and Wasting Infections in Small Ruminants: A Review. *Animals* **10**, 2179 (2020).
82. Chauvin, A., Moreau, E., Bonnet, S., Plantard, O. & Malandrin, L. *Babesia* and its hosts: Adaptation to long-lasting interactions as a way to achieve efficient transmission. *Vet. Res.* **40**, 1–18 (2009).
83. Sevinc, F. *et al.* Therapeutic and prophylactic efficacy of imidocarb dipropionate on experimental *Babesia ovis* infection of lambs. *Vet. Parasitol.* **149**, 65–71 (2007).
84. Spielman, A. & Piesman, J. Human Babesiosis on Nantucket Island: Prevalence of *Babesia microti* in Ticks. *Am. J. Trop. Med. Hyg.* **29**, 742–746 (1980).
85. Brown, W. C., Norimine, J., Goff, W. L., Suarez, C. E. & McElwain, T. F. Prospects for recombinant vaccines against *Babesia bovis* and related parasites. *Parasite Immunol.* **28**, 315–327 (2006).
86. Chakraborty, S. *et al.* Potential sabotage of host cell physiology by apicomplexan parasites for their survival benefits. *Front. Immunol.* **8**, 1261 (2017).
87. Sevinc, F. *et al.* Haemoparasitic agents associated with ovine babesiosis: A possible negative interaction between *Babesia ovis* and *Theileria ovis*. *Vet. Parasitol.* **252**, 143–147 (2018).
88. Vannier, E. G., Diuk-Wasser, M. A., Ben Mamoun, C. & Krause, P. J. Babesiosis. *Infect. Dis. Clin. North Am.* **29**, 357–370 (2015).
89. Allred, D. R. Molecular technology and antigenic variation among intraerythrocytic hemoparasites: Do we see reality? *Vet. Parasitol.* **101**, 261–274 (2001).
90. Cooke, B. M., Mohandas, N., Cowman, A. F. & Coppel, R. L. Cellular adhesive phenomena in apicomplexan parasites of red blood cells. *Vet. Parasitol.* **132**, 273–295 (2005).
91. Lobo, C. A., Rodriguez, M. & Cursino-Santos, J. R. *Babesia* and red cell invasion. *Current Opinion in Hematology* vol. 19 170–175 (2012).
92. Suarez, C. E. *et al.* Unravelling the cellular and molecular pathogenesis of bovine babesiosis: is the sky the limit? *Int. J. Parasitol.* **49**, 183–197 (2019).
93. Wadhwa, R. *et al.* Red blood cells as an efficient *in vitro* model for evaluating the efficacy of metallic nanoparticles. *3 Biotech* **9**, 279 (2019).

94. Mehlhorn, H. & Schein, E. The Piroplasms: Life Cycle and Sexual Stages. *Adv. Parasitol.* **23**, 37–103 (1985).
95. Jalovecka, M., Hajdusek, O., Sojka, D., Kopacek, P. & Malandrin, L. The complexity of piroplasms life cycles. *Front. Cell. Infect. Microbiol.* **8**, 248 (2018).
96. Homer, M. J., Aguilar-Delfin, I., Telford, S. R., Krause, P. J. & Persing, D. H. Babesiosis. *Clin. Microbiol. Rev.* **13**, 451–469 (2000).
97. Esmaeilnejad, B. *et al.* PCR-based detection of *Babesia ovis* in *Rhipicephalus bursa* and small ruminants. *J. Parasitol. Res.* (2014) doi:10.1155/2014/294704.
98. Ali, A., Mulenga, A. & Vaz, I. S. Tick and Tick-Borne Pathogens: Molecular and Immune Targets for Control Strategies. *Front. Physiol.* **11**, 744 (2020).
99. Wilson, A. L. *et al.* The importance of vector control for the control and elimination of vector-borne diseases. *PLoS Negl. Trop. Dis.* **14**, 1–31 (2020).
100. Daniel de Vries, A. *et al. Community engagement for public health events caused by communicable disease threats in the EU/EEA*. www.ecdc.europa.eu (2020) doi:10.2900/427139.
101. Barker, C. M. & Reisen, W. K. Epidemiology of vector-borne diseases. in *Medical and Veterinary Entomology* 33–49 (Elsevier, 2018). doi:10.1016/B978-0-12-814043-7.00004-2.
102. de la Fuente, J. & Estrada-Peña, A. Why New Vaccines for the Control of Ectoparasite Vectors Have Not Been Registered and Commercialized? *Vaccines* **7**, 75 (2019).
103. Braks, M., Giglio, G., Tomassone, L., Sprong, H. & Leslie, T. Making Vector-Borne Disease Surveillance Work: New Opportunities From the SDG Perspectives. *Front. Vet. Sci.* **6**, 232 (2019).
104. Destoumieux-Garzón, D. *et al.* The one health concept: 10 years old and a long road ahead. *Front. Vet. Sci.* **5**, 14 (2018).
105. Colwell, D. D., Dantas-Torres, F. & Otranto, D. Vector-borne parasitic zoonoses: Emerging scenarios and new perspectives. *Vet. Parasitol.* **182**, 14–21 (2011).
106. Kilpatrick, A. M. & Randolph, S. E. Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. *Lancet* **380**, 1946–1955 (2012).
107. Semenza, J. C. & Suk, J. E. Vector-borne diseases and climate change: a European perspective. *FEMS Microbiol. Lett.* **365**, 244 (2018).

108. Richards, S. L., Ruiz, M. O., Fryxell, R. T. T. & Vitek, C. J. Disease Vectors. *Environ. Health Insights* **8**, (2020).
109. Del Rosario, K. L., Richards, S. L., Anderson, A. L. & Balanay, J. A. G. Current Status of Mosquito Control Programs in North Carolina: The Need for Cost-Effectiveness Analysis. *J. Environ. Health* **76**, 8–15 (2014).
110. Bilgic, H. B. *et al.* Comparison of protectiveness of recombinant *Babesia ovis* apical membrane antigen 1 and *B. ovis*-infected cell line as vaccines against ovine babesiosis. *Ticks Tick. Borne. Dis.* **11**, 101280 (2020).
111. Dantas-Torres, F., Alves, L. C. & Uilenberg, G. Babesiosis. in *Arthropod Borne Diseases* 347–354 (Springer International Publishing, 2016). doi:10.1007/978-3-319-13884-8_21.
112. Antonio Alvarez, J., Rojas, C. & Figueroa, J. V. Diagnostic tools for the identification of *Babesia* sp. In persistently infected cattle. *Pathogens* **8**, 143 (2019).
113. Sevinc, F. *et al. Babesia ovis* infections: Detailed clinical and laboratory observations in the pre- and post-treatment periods of 97 field cases. *Vet. Parasitol.* **191**, 35–43 (2013).
114. Ekici, O. D., Sevinc, F. & Isik, N. Instability of ovine babesiosis in an endemic area in Turkey. *Vet. Parasitol.* **188**, 372–375 (2012).
115. Alam Tufani, N. *et al.* Cross sectional study on prevalence of ovine babesiosis in different breeds of Kashmir valley. *J. Entomol. Zool. Stud.* **5**, 1492–1496 (2017).
116. Koury, M. J. Red blood cell production and kinetics. in *Rossi's Principles of Transfusion Medicine* 85–96 (John Wiley & Sons, Ltd., 2016). doi:10.1002/9781119013020.ch08.
117. Ijaz, M. *et al.* Clinico-epidemiology and therapeutical trials on babesiosis in sheep and goats in Lahore, Pakistan. *J. Anim. Plant Sci.* **23**, 666–669 (2013).
118. Baneth, G. Antiprotozoal treatment of canine babesiosis. *Vet. Parasitol.* **254**, 58–63 (2018).
119. Vial, H. J. & Gorenflot, A. Chemotherapy against babesiosis. *Vet. Parasitol.* **138**, 147–160 (2006).
120. Mira, A. *et al.* High genetic diversity and differentiation of the *Babesia ovis* population in Turkey. *Transbound. Emerg. Dis.* **67**, 26–35 (2020).

121. Belloli, C. *et al.* Pharmacokinetics and Mammary Elimination of Imidocarb in Sheep and Goats. *J. Dairy Sci.* **89**, 2465–2472 (2006).
122. Santos, P. *et al.* Induced mitotic homologous recombination by the babesicide imidocarb dipropionate in *Aspergillus nidulans* diploid cells. *Genet. Mol. Res.* **11**, 1810–1818 (2012).
123. Vetter, V., Denizer, G., Friedland, L. R., Krishnan, J. & Shapiro, M. Understanding modern-day vaccines: what you need to know. *Ann. Med.* **50**, 110–120 (2018).
124. Plotkin, S. History of vaccination. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 12283–12287 (2014).
125. Rathinasamy, V., Poole, W. A., Bastos, R. G., Suarez, C. E. & Cooke, B. M. Babesiosis Vaccines: Lessons Learned, Challenges Ahead, and Future Glimpses. *Trends Parasitol.* **35**, 622–635 (2019).
126. Willadsen, P. Tick control: Thoughts on a research agenda. *Vet. Parasitol.* **138**, 161–168 (2006).
127. Rodriguez-Vivas, R. I., Jonsson, N. N. & Bhushan, C. Strategies for the control of *Rhipicephalus microplus* ticks in a world of conventional acaricide and macrocyclic lactone resistance. *Parasitology Research* vol. 117 3–29 (2018).
128. Abbas, R. Z., Zaman, M. A., Colwell, D. D., Gilleard, J. & Iqbal, Z. Acaricide resistance in cattle ticks and approaches to its management: The state of play. *Vet. Parasitol.* **203**, 6–20 (2014).
129. Samish, M., Ginsberg, H. & Glazer, I. Biological control of ticks. *Parasitology* **129**, S389–S403 (2004).
130. Wikel, S. K. Host Immunity to Ticks. *Annu. Rev. Entomol.* **41**, 1–22 (1996).
131. Willadsen, P. & Jongejan, F. Immunology of the tick-host interaction and the control of ticks and tick-borne diseases. *Parasitol. Today* **15**, 258–262 (1999).
132. Cloete, J. J. E., Scholtz, A. J., Matthee, S. & Cloete, S. W. P. Genetic parameters and breed differences for ovine tick counts on indigenous and commercial sheep in Autumn and spring. *Proc. Assoc. Advmt. Anim. Breed. Genet* **22**, 35–38 (2017).
133. Osburn, R. L. & Knipling, E. F. The Potential Use of Sterile Hybrid *Boophilus* Ticks (Acari: Ixodidae) as a Supplemental Eradication Technique. *J. Med. Entomol.* **19**, 637–644 (1982).
134. Sonenshine, D. E. & Roe, R. M. *Biology of ticks*. (Oxford University Press, 2014).

135. Almazan, C., Tipacamu, G. A., Rodriguez, S., Mosqueda, J. & Perez de Leon, A. Immunological control of ticks and tick-borne diseases that impact cattle health and production. *Front. Biosci. (Landmark Ed)* **23**, 1535–1551 (2018).
136. Banumathi, B. *et al.* Exploitation of chemical, herbal and nanoformulated acaricides to control the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* – A review. *Vet. Parasitol.* **244**, 102–110 (2017).
137. Ziapour, S. P. *et al.* Susceptibility status of field populations of *Rhipicephalus bursa* (Acari: Ixodidae) to pyrethroid insecticides. *Trop. Biomed.* **33**, 446–461 (2016).
138. Enayati, A. A. *et al.* Pyrethroid insecticide resistance in *Rhipicephalus bursa* (Acari, Ixodidae). *Pestic. Biochem. Physiol.* **97**, 243–248 (2010).
139. Marcondes, C. B. *Arthropod Borne Diseases*. (Springer International Publishing, 2017). doi:10.1007/978-3-319-13884-8.
140. Benelli, G., Pavela, R., Canale, A. & Mehlhorn, H. Tick repellents and acaricides of botanical origin: a green roadmap to control tick-borne diseases? *Parasitol. Res.* **115**, 2545–2560 (2016).
141. Salman, M. *et al.* Repellent and acaricidal activity of essential oils and their components against *Rhipicephalus* ticks in cattle. *Vet. Parasitol.* **283**, 109178 (2020).
142. Madreseh-Ghahfarokhi, S., Dehghani-Samani, A., Pirali, Y. & Dehghani-Samani, A. *Zingiber officinalis* and *Eucalyptus globulus*, Potent Lethal/Repellent Agents against *Rhipicephalus bursa*, Probable Carrier for Zoonosis. *J. Arthropod. Borne. Dis.* **13**, 223 (2019).
143. Zhao, J.-Z., Collins, H. L. & Shelton, A. M. Testing insecticide resistance management strategies: mosaic versus rotations. *Pest Manag. Sci.* **66**, 1101–1105 (2010).
144. Insecticide Resistance Action Committee & (IRAC) Public Health Team. *Prevention and management of insecticide resistance in vectors of public health importance*. https://irac-online.org/content/uploads/VM-Layout-v2.6_LR.pdf (2011).
145. Rego, R. O. M. *et al.* Counterattacking the tick bite: Towards a rational design of anti-tick vaccines targeting pathogen transmission. *Parasites and Vectors* **12**, 1–

20 (2019).

146. Dai, J. *et al.* Tick histamine release factor is critical for *Ixodes scapularis* engorgement and transmission of the Lyme disease agent. *PLoS Pathog.* **6**, e1001205 (2010).
147. Brossard, M. & Wikel, S. K. Tick Immunobiology. *Parasitology* **129**, S161 (2004).
148. Lysyk, T. J., Veira, D. M., Kastelic, J. P. & Majak, W. Inducing Active and Passive Immunity in Sheep to Paralysis Caused by *Dermacentor andersoni*. *J. Med. Entomol.* **46**, 1436–1441 (2009).
149. Bhowmick, B. & Han, Q. Understanding Tick Biology and Its Implications in Anti-tick and Transmission Blocking Vaccines Against Tick-Borne Pathogens. *Front. Vet. Sci.* **7**, 319 (2020).
150. Willadsen, P., Bird, P., Cobon, G. S. & Hungerford, J. Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology* **110**, S43–S50 (1995).
151. Canales, M. *et al.* Large-scale production in *Pichia pastoris* of the recombinant vaccine Gavac$^{(TM)}$ against cattle tick. *Vaccine* **15**, 414–422 (1997).
152. Ndawula, C. & Tabor, A. E. Cocktail anti-tick vaccines: The unforeseen constraints and approaches toward enhanced efficacies. *Vaccines* **8**, 1–23 (2020).
153. Willadsen, P. *et al.* Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*. *J. Immunol.* **143**, 1346–51 (1989).
154. de la Fuente, J., Kopáček, P., Lew-Tabor, A. & Maritz-Olivier, C. Strategies for new and improved vaccines against ticks and tick-borne diseases. *Parasite Immunol.* **38**, 754–769 (2016).
155. Kubinski, M. *et al.* Tick-borne encephalitis virus: A quest for better vaccines against a virus on the rise. *Vaccines* **8**, 1–45 (2020).
156. Kennedy, R. B. & Poland, G. A. The Top Five "Game Changers" in Vaccinology: Toward Rational and Directed Vaccine Development. *Omi. A J. Integr. Biol.* **15**, 533–537 (2011).
157. Liu, C. *et al.* Computational network biology: Data, models, and applications. *Phys. Rep.* **846**, 1–66 (2020).
158. Gasperskaja, E. & Kučinskas, V. The most common technologies and tools for functional genome analysis. *Acta medica Litu.* **24**, 11 (2017).

159. Nierode, G., Kwon, P. S., Dordick, J. S. & Kwon, S. J. Cell-based assay design for high-content screening of drug candidates. *J. Microbiol. Biotechnol.* **26**, 213–225 (2015).
160. Couto, J. *et al.* Solute carriers affect *Anopheles stephensi* survival and *Plasmodium berghei* infection in the salivary glands. *Sci. Rep.* **7**, (2017).
161. Martins, L. A., Bensaoud, C., Kotál, J., Chmelař, J. & Kotsyfakis, M. Tick salivary gland transcriptomics and proteomics. *Parasite Immunol.* (2020) doi:10.1111/pim.12807.
162. Marcelino, I. *et al.* Tick-borne diseases in cattle: Applications of proteomics to develop new generation vaccines. *J. Proteomics* **75**, 4232–4250 (2012).
163. Chmelar, J., Kotál, J., Kovaríková, A. & Kotsyfakis, M. The use of tick salivary proteins as novel therapeutics. *Frontiers in Physiology* vol. 10 812 (2019).
164. de la Fuente, J. *et al.* Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases. *Front. Cell. Infect. Microbiol.* **7**, 114 (2017).
165. Martins, L. A., Kotál, J., Bensaoud, C., Chmelař, J. & Kotsyfakis, M. Small protease inhibitors in tick saliva and salivary glands and their role in tick-host-pathogen interactions. *Biochim. Biophys. Acta - Proteins Proteomics* **1868**, 140336 (2020).
166. Villar, M. *et al.* A Systems Biology Approach to the Characterization of Stress Response in *Dermacentor reticulatus* Tick Unfed Larvae. *PLoS One* **9**, e89564 (2014).
167. Ayllón, N. *et al.* Systems Biology of Tissue-Specific Response to *Anaplasma phagocytophilum* Reveals Differentiated Apoptosis in the Tick Vector *Ixodes scapularis*. *PLOS Genet.* **11**, e1005120 (2015).
168. Nuttall, P. A. Wonders of tick saliva. *Ticks Tick. Borne. Dis.* **10**, 470–481 (2019).
169. Whitten, M. M. Novel RNAi delivery systems in the control of medical and veterinary pests. *Curr. Opin. Insect Sci.* **34**, 1–6 (2019).
170. Barnard, A.-C., Nijhof, A. M., Fick, W., Stutzer, C. & Maritz-Olivier, C. RNAi in Arthropods: Insight into the Machinery and Applications for Understanding the Pathogen-Vector Interface. *Genes (Basel).* **3**, 702–41 (2012).
171. de la Fuente, J., Kocan, K. M., Almazán, C. & Blouin, E. F. RNA interference for

the study and genetic manipulation of ticks. *Trends Parasitol.* **23**, 427–433 (2007).

172. Mello, C. C. & Conte, D. Revealing the world of RNA interference. *Nature* **431**, 338–342 (2004).
173. Al-Rofaai, A. & Bell-Sakyi, L. Tick Cell Lines in Research on Tick Control. *Front. Physiol.* **11**, 152 (2020).
174. Bell-Sakyi, L., Zweygarth, E., Blouin, E. F., Gould, E. A. & Jongejan, F. Tick cell lines: tools for tick and tick-borne disease research. *Trends Parasitol.* **23**, 450–457 (2007).
175. Merino, O., Alberdi, P., Pérez De La Lastra, J. M. & de la Fuente, J. Tick vaccines and the control of tick-borne pathogens. *Front. Cell. Infect. Microbiol.* **3**, 30 (2013).
176. Sharma, M., Krammer, F., García-Sastre, A. & Tripathi, S. Moving from Empirical to Rational Vaccine Design in the 'Omics' Era. *Vaccines* **7**, 89 (2019).
177. Alberts, B. *et al.* T Cells and MHC Proteins. in *Molecular Biology of the Cell* (Garland Science, 2002).
178. Chaplin, D. D. Overview of the immune response. *J. Allergy Clin. Immunol.* **125**, S3-23 (2010).
179. Ullah, M. A., Sarkar, B. & Islam, S. S. Exploiting the reverse vaccinology approach to design novel subunit vaccines against Ebola virus. *Immunobiology* **225**, 151949 (2020).
180. Alvarez, P., Leguizamón, M. S., Buscaglia, C. A., Pitcovsky, T. A. & Campetella, O. Multiple overlapping epitopes in the repetitive unit of the shed acute-phase antigen from *Trypanosoma cruzi* enhance its immunogenic properties. *Infect. Immun.* **69**, 7946–7949 (2001).
181. Sela-Culang, I. *et al.* Using a combined computational-experimental approach to predict antibody-specific B cell epitopes. *Structure* **22**, 646–657 (2014).
182. Elton, C. M., Rodriguez, M., Ben Mamoun, C., Lobo, C. A. & Wright, G. J. A library of recombinant *Babesia microti* cell surface and secreted proteins for diagnostics discovery and reverse vaccinology. *Int. J. Parasitol.* **49**, 115–125 (2019).
183. Lew-Tabor, A. E. & Rodriguez Valle, M. A review of reverse vaccinology approaches for the development of vaccines against ticks and tick borne diseases.

Ticks and Tick-borne Diseases vol. 7 573–585 (2016).

184. Nuttall, P. A. Tick saliva and its role in pathogen transmission. *Wien. Klin. Wochenschr.* 1–12 (2019) doi:10.1007/s00508-019-1500-y.

185. Valle, M. R. Anti-tick vaccines in the omics era. *Front. Biosci.* **10**, 812 (2018).

Chapter II: Influence of blood feeding and *Babesia ovis* infection on *Rhipicephalus bursa* sialome

1. "*Rhipicephalus bursa* Sialotranscriptomic Response to Blood Feeding and *Babesia ovis* Infection: Identification of Candidate Protective Antigens"

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1.1. Abstract

Ticks are among the most prevalent blood-feeding arthropods, and they act as vectors and reservoirs for numerous pathogens. Sialotranscriptomic characterizations of tick responses to blood feeding and pathogen infections can offer new insights into the molecular interplay occurring at the tick-host-pathogen interface. In the present study, we aimed to identify and characterize *Rhipicephalus bursa* salivary gland (SG) genes that were differentially expressed in response to blood feeding and *Babesia ovis* infection. Our experimental approach consisted of RNA sequencing of SG from three different tick samples, fed-infected, fed-uninfected, and unfed-uninfected, for characterization and

inter-comparison. Overall, 7,272 expressed sequence tags (ESTs) were constructed from unfed-uninfected, 13,819 ESTs from fed-uninfected, and 15,292 ESTs from fed-infected ticks. Two catalogs of transcripts that were differentially expressed in response to blood feeding and *B. ovis* infection were produced. Four genes coding for a putative vitellogenin-3, lachesin, a glycine rich protein, and a secreted cement protein were selected for RNA interference functional studies. A reduction of 92, 65, and 51% was observed in vitellogenin-3, secreted cement, and lachesin mRNA levels in SG, respectively. The *vitellogenin-3* knockdown led to increased tick mortality, with 77% of ticks dying post-infestation. The reduction of the secreted cement protein-mRNA levels resulted in 46% of ticks being incapable of correctly attaching to the host and significantly lower female weights post-feeding in comparison to the control group. The *lachesin* knockdown resulted in a 70% reduction of the levels associated with *B. ovis* infection in *R. bursa* SG and 70% mortality. These results improved our understanding of the role of tick SG genes in *Babesia* infection/proliferation and tick feeding. Moreover, lachesin, vitellogenin-3, and secreted cement proteins were validated as candidate protective antigens for the development of novel tick and tick-borne disease control measures.

Keywords: sialotranscriptomics, *Rhipicephalus bursa*, *Babesia* spp., RNA interference, vaccine, vector-pathogen interactions

1.2. Introduction

Ticks are widely distributed obligate hematophagous ectoparasites, which have recognized effects on host species. During blood feeding, ticks secrete varying substances into the host bloodstream acting as remarkable vectors of numerous pathogens, some of which can cause severe diseases in vertebrate hosts, including humans (Jongejan and Uilenberg, 2004; Domingos et al., 2013; Sonenshine and Michael Roe, 2014). Reflecting the progress of feeding, salivary glands (SG) increase ~25-fold in mass and protein content, as the glands are responsible for the production of complex saliva that is capable of quelling host innate and adaptive immune responses (Sauer et al., 2000; Kazimírová and Stibraniova, 2013; Kotál et al., 2015; Šimo et al., 2017). SG play an essential role in tick survival and success as parasites by modulating host haemostasis and complement

systems (Sauer et al., 2000; Francischetti et al., 2009; Kazimírová and Stibraniova, 2013). In addition to being involved with osmoregulation (Kaufman, 2010), this tissue is also responsible for the production of cement, which is an adhesive substance that surrounds the mouthparts and the host skin that ensures tick attachment (Sauer et al., 2000; Francischetti et al., 2009; Kazimírová and Stibraniova, 2013; Šimo et al., 2017). SG are also pivotal in tick pathogen interactions, because pathogens need to cross the physical barrier of SG epithelium and endure the salivary biochemical environment to gain access to the next host. Remarkably, to increase their proliferation and transmission, pathogens adapted to SG in a way that exploits tick salivary molecules (Ramamoorthi et al., 2005; Kaufman, 2010). Therefore, these features make SG an exceptional target for the identification of new candidate protective antigens that are relevant to biological functions associated with tick development, fertility, feeding, and pathogen infection and transmission (Merino et al., 2013; Shahein et al., 2013).

Research that examined tick SG made the characterization of a large number of tick salivary compounds possible, but the function of several of these molecules remains unknown (Francischetti et al., 2009). The sialomes of some tick species have been described (Francischetti et al., 2008, 2011; Anatriello et al., 2010; Karim et al., 2011; Tan et al., 2015; de Castro et al., 2016; Moreira et al., 2017), and this information represents an important data source for functional studies and analyses of gene expression dynamics during tick feeding. Moreover, high-throughput technologies have also enabled researchers to study the effects of sex, physiological stages, and different tick statuses such as the presence of pathogens in tick tissues (Chmelar et al., 2016).

Rhipicephalus bursa is a multi-host tick that is mainly associated with ruminants, but it can occasionally parasitize other animals such as wild ungulates and small mammals (Walker et al., 2000; de la Fuente et al., 2004; Santos-Silva et al., 2011; Mihalca et al., 2012). *R. bursa* is recognized as the primary vector of *Babesia ovis* (Moltmann et al., 1982a), but it transmits other pathogens such as *Rickettsia* spp. and *Anaplasma* spp. (Raele et al., 2015; Dahmani et al., 2016; Ferrolho et al., 2016b), thus demonstrating its importance in animal health, particularly in livestock. *B. ovis*, an intraerythrocytic apicomplexan parasite, is the main etiological agent of ovine babesiosis, which is a tick-borne disease of small ruminants, and its geographical distribution overlaps with that of *R. bursa* (Walker et al., 2000; Ranjbar-Bahadori et al., 2012; Erster et al., 2015; Ferrolho

et al., 2016a). This highly pathogenic organism is characterized by low parasitaemia, and it causes severe infections (Habela et al., 1990; Sevinc et al., 2013; Hurtado et al., 2015). *B. ovis* is extremely well adapted to the vector, and it survives in the tick during several successive generations (Yeruham et al., 2001) using horizontal and vertical transmission (Friedhoff, 1988). Microscopy studies in the 1980's discovered that the *B. ovis* cycle within the tick is similar to other *Babesia* spp. (Moltmann et al., 1982a,b). Briefly, *Babesia* penetrates the tick midgut, undergoes meiosis, and differentiates into motile ookinetes that propagate via haemolymph to reach all tick organs. *B. ovis* kinetes reach SG within 48 h post-infestation, and they undergo a final step of multiplication to produce sporozoites (Moltmann et al., 1982a; Antunes et al., 2017). Adult ticks are the main vector, and both females and males are implicated in the transmission of the hemoparasite. However, females present a higher threat due to transovarial transmission and extended feeding periods (Friedhoff, 1988).

The importance of the *R. bursa*-*B. ovis* system was emphasized in a disease outbreak that resulted in animal morbidity and mortality (Hurtado et al., 2015). Pathogen and vector control methods are limited to the common usage of imidocarb dipropionate (to manage animal disease) and acaricides (McHardy et al., 1986; Belloli et al., 2006; Domingos et al., 2013). Safer and effective alternatives are urgently needed, including the development of vaccines that may reduce tick infestations and block pathogen transmission (Merino et al., 2013; Liu and Bonnet, 2014; Neelakanta and Sultana, 2015). Studies of the molecular interactions associated with the tick-pathogen interface represent a bridge for the identification of antigenic targets to implement vaccination strategy. Information about the *R. bursa* and *B. ovis* interactome is scarce. Thus, in the present study, SG of *R. bursa* adult females were used to assess the transcriptomic response to blood feeding and *B. ovis* infection. Fed-infected, fed-uninfected, and unfed-uninfected female ticks were produced, SG were isolated and used for RNA extraction. RNA-seq and *de novo* transcriptome assembly approaches were used to construct the sialotranscriptome of fed-infected, fed-uninfected, and unfed-uninfected *R. bursa* specimens. These catalogs were analyzed, and four genes were selected for further functional studies, thus allowing the evaluation of encoded proteins for inclusion in anti-tick and tick-borne pathogen vaccines. These data are essential for vaccinomics pipelines,

which could enhance our knowledge of the dynamic processes that occur at the tick-pathogen-host interface.

1.3. Materials and methods

1.3.1. Ethics Statement

Animal experiments were conducted with the approval of the Divisão Geral de Alimentação e Veterinária (DGAV), Portugal, under Art◦ 49, Portaria n◦1005/92 from 23rd October (permit number 0421/2013) and the Council of Ethics of the Instituto de Higiene e Medicina Tropical (IHMT). Animals were maintained and manipulated following protocols compliant with the national and European Animal Welfare legislation, in frame with DL 113/2013 and Directive 2010/63/EU based on the principle of the Three R's, to replace, reduce, and refine the use of animals for scientific purposes.

1.3.2. *Rhipicephalus bursa* Colony

R. bursa colony was established under laboratory conditions and further maintained. For colony initiation, adult ticks were collected either in naturally infested domestic animals or by dragging/flagging the vegetation and kept in a chamber regulated at 25 ± 1ºC, 70 ± 10% relative humidity and a photoperiod of 16:8 (light: dark). During oviposition, the dark period was increased to improve female egg laying. After oviposition, each female and a sample of eggs were tested by conventional PCR for pathogens detection (*Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp.) during two generations using the protocols and primers described elsewhere (Inokuma et al., 2000; de la Fuente et al., 2003; Aktaş et al., 2005; Harrus et al., 2011). Ticks were fed on Hyla breed rabbits at Centro de Estudos de Vetores e Doenças Infeciosas, Instituto Nacional de Saúde Doutor Ricardo Jorge (CEVDI/INSA) in appropriate conditions. Ten lineages of *R. bursa* were selected in order to reduce interbreeding.

1.3.3. *In Vitro Babesia ovis* Cultures

In vitro B. ovis cultures were established at IHMT in biosafety level 2 facilities, following a protocol adapted from Vega et al. (1985). Briefly, cryopreserved *B. ovis*

(Israeli strain) infected red blood cells (RBC) were used to initiate the culture. *B. ovis* merozoites were cultured in lamb erythrocytes maintained in 20% lamb serum-containing medium, in an atmosphere of 5% CO_2/2% O_2/93% N_2 at 37ºC, as described elsewhere (Horta et al., 2014). Half of the medium was replaced daily and cultures monitored for parasitaemia by preparing thin blood smears stained with Hemacolor® Rapid staining of blood smear (EMD Millipore, Darmstadt, Germany). Intraerythrocytic parasites were observed under a 400x original magnification of a Nikon eclipse 80i fluorescence microscope.

1.3.4. Salivary Glands and RNA Samples for RNA-Seq

Fed and Unfed *R. bursa*

Thirty adult female ticks were carefully removed from the rabbits ear 10–12 days post attachment. Equally, thirty unfed adult female ticks were also obtained. Ticks were individually rinsed in distilled water, after in 75% (v/v) ethanol, once more in water and dissected under a stereoscopic microscope at 4x magnification (Motic SMZ-171B, China) using sterile conditions in ice-cold phosphate-buffered saline (PBS). The SG were stored in RNAlater (Ambion, Austin, TX, USA) and afterwards pooled, resulting in two samples for the fed condition and other two for the unfed. Total RNA was extracted from each sample using Tri-reagent (Sigma–Aldrich, St. Louis, MO, USA). RNA quantity was estimated using the ND-1000 Spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, Whaltman, MA, USA).

Fed-*B. ovis* Infected *R. bursa*

A batch of 60 female ticks were inoculated with *B. ovis* in the trochanter—coxae articulation and allowed to feed on rabbits. After drop off, SG were carefully isolated and DNA/RNA extracted has mentioned previously. Genomic DNA was used to amplify a 549 bp fragment of *B. ovis* 18S ribosomal DNA (18S rRNA) using primers and conditions described elsewhere (Aktaş et al., 2005). RNA from positive samples (Supplementary Figure 1) were used for the production of two RNA pools with fifteen samples each. All samples were promptly shipped in dry ice to Parque Cientifico de Madrid for sequencing. The tick infection model and vector competence was evaluated. *B. ovis* inoculated *R. bursa* were allowed to feed in a naïve lamb. The lamb was monitored every two days for

babesiosis clinical symptoms and blood collected for *B. ovis* detection by PCR (Supplementary Figure 1) using the above mentioned conditions. After 8 days, the ticks were recovered for analysis.

1.3.5. RNA-Seq

RNA quality was assessed using an Agilent RNA 6000 bioanalyzer (Agilent Technologies, CA, USA). Libraries preparation was performed with "NEBNext Ultra Directional RNA Library Prep" kit (New England Biolabs, Ipswich, MA, USA) following manufacturer instructions. Briefly, prior to cDNA library construction magnetic beads with oligo (dT) were used to enrich poly (A) mRNA from 1 μg of total-RNA. Next, the purified mRNAs were disrupted into short fragments, and double-stranded cDNAs were immediately synthesized. The cDNAs were subjected to end-repair and adenilation, then connected with sequencing adapters. Suitable fragments, purified by size selection protocol with AMPure XP beads (Beckman Coulter), were selected as templates for PCR amplification. The final library sizes and qualities were evaluated electrophoretically using an Agilent High Sensitivity DNA kit (Agilent Technologies, CA, USA); the mean fragment size was 510 bp. Subsequently, the library was sequenced using a HiSeq 2500 sequencer (Illumina, CA, USA) in rapid run mode. Cluster generation was performed, followed by 2 × 100 cycle sequencing reads separated by a paired-end turnaround. Image analysis was performed using the HiSeq control software version 1.8.4. The raw fastq files were deposited in the Sequence Read Archives (SRA) of the National center for Biotechnology information (NCBI) under the accession numbers SRR4428986, SRR4428987 and SRR4428988, Biosamples SAMN05916213, SAMN05916214, and SAMN05916215, regarding the unfed-uninfected, fed-uninfected and fed-infected populations, respectively, of Bioproject PRJNA348674. The Transcriptome Shotgun Assembly (TSA) projects have been deposited at DDBJ/EMBL/GenBank under the accessions GFZD00000000, GFZJ00000000, and GFZK00000000. The versions described in this paper are the first versions, GFZD01000000, GFZJ01000000, and GFZK01000000.

1.3.6. Transcriptomic Data of Female *R. bursa* Sialome

Assembly and Analysis of Transcripts

This project comprised *de novo* assembly of six transcriptomes. Three conditions and two replicas *per* condition: F, SG from fed ticks; NFni, SG from unfed-uninfected ticks; and Fi, SG from fed-*B. ovis* infected ticks. Subsequently, two comparisons were performed: F vs. NFni (response to blood feeding) and F vs. Fi (response to *B. ovis* infection). Quality analysis of the raw reads was done with Prinseq tool (Schmieder and Edwards, 2011). Pre-processing of reads included: (a) right trimming where quality < Q30; (b) left trimming of the first base; (c) filtering out reads with Ns; (d) quality analysis of the processed data. For each of the four transcriptomes three *de novo* assemblies were made with three different k-values using the *de novo* transcriptome assembler Oases (Velvet, version: 1.2.10) (Schulz et al., 2012). The annotation of each transcript was done based on the Basic Local Alignment Search Tool (BLAST) results comparing the transcript to a database of reference proteins. The set of reference proteins was selected from UniProt database from all the organisms belonging to the taxon "Ixodidae". In total 76, 475 proteins were used as reference proteins. A set of unigenes for each sample was obtained. The assignment of each transcript to a protein was based on BLAST similarity. Rich functional annotation for each unigene extracted from the UniProt protein in which the read clustering process has been centered for this unigene is provided. Afterwards a unigene expression quantification was performed using eXpress. To compare the transcripts from the samples, the transcripts were clustered by protein. The protein driven transcript clusters that were done using UniProt proteins, were furtherly clustered by UniRef90 proteins. The mapping from the UniProt proteins to UniRef90 was done using UniProt retrieval tool. The quantification per UniRef90 cluster was calculated adding the quantification *per* protein included in each UniRef90 cluster. P-value calculation of the Z-test was based on the raw counts (total exon reads per gene). Genes were considered significantly differentially expressed if the P-value was below 0.05. Functional annotation of these genes was manually done by compiling information from UniProt, RefSeq, GO, Panther, KEGG, Pfam, and NCBI databases.

Gene Ontology Assignments

Functional data for each identified protein was obtained using Blast2GO platform version 4.0.7 available at https://www. blast2go.com (Conesa et al., 2005; Götz et al., 2008). Homology to the protein sequences was searched by BLAST against Arthropoda (nr subset) [arthropoda, taxa:6656] from 30.01.2017 as well as against to InterPro protein signature databases, using InterProScan. To retrieve gene ontology (GO) terms, a mapping step was performed gathering GO annotations and evidence codes (EC). Annotation to assign functional terms was performed next. At this step, the most specific and reliable annotation was considered. Finally, to map a set of annotations to high level GO terms, GO slim option was used. GO frequency charts were constructed using the Microsoft Office 2016 Excel tool. The most up and down-regulated genes in response to feeding and infection ($P < 0.1$) were analyzed using the same approach.

1.3.7. Validation of RNA-Seq Data

A total of 18 transcripts with differential regulation and belonging to different functional classes with a potential interference in response to blood feeding and *B. ovis* infection, were chosen for RNA-Seq validation through qPCR using the minimum information for publication of qPCR experiments (Bustin et al., 2009). Ten individual *R. bursa* SG, from each condition studied, were used to extract total RNA using the GRS FullSample Purification kit, GrispTM (Porto, Portugal), which included DNAse treatment and 60 ng/µL of each sample were used to synthesize cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). qPCR reactions of 10 µL were performed in triplicate using IQ™ SYBR® Green Supermix kit (Bio-Rad, CA, USA) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The cycling conditions were as follows: an initial cycle of denaturation at 95ºC for 10min; followed by 45 cycles of 95ºC for 15s and temperature of each primer set for 45s. Fluorescence readings were taken at 62ºC after each cycle and a dissociation curve (60–95ºC) was performed. Negative controls were prepared with water. To determine the reaction efficiency standard curves were constructed with five-fold serial dilutions of cDNA from *R. bursa*. Reactions specificity was assured by the absence of PCR product in control reactions and by the dissociation curves (60–95ºC) run at the end the cycling protocol. The average expression stability (M-value) of the reference genes, *β-tubulin*, *β-actin*, *elongation factor*, and 16S,

was assessed based in geNorm algorithm (Vandesompele et al., 2002) included in the CFX Manager™ Software (Bio- Rad, CA, USA) and gene relative quantification was evaluated using the CFX Manager™ Software including the Pfaff method (Pfaffl, 2001) using the above-mentioned reference genes for normalization. Normalized Cq-values were compared between conditions by Student's t test ($P < 0.05$). Primers were design using Primer3 platform (http://bioinfo.ut.ee/primer3-0.4.0/) and their conditions are summarized in Supplementary Table 1. Pearson's correlation was used to compare the expression values between RNA-Seq and qPCR methods for the 18 selected genes.

1.3.8. RNA Interference Assays

Lamb Infection with *B. ovis*

A six-month old lamb bred and maintained at the Instituto Nacional de Investigação Agrária e Veterinária (INIAV) animal facility was splenectomized and, 45 days after, intravenously inoculated with 1 mL of cryopreserved *B. ovis* culture with 9% parasitemia. The *B. ovis* infection was monitored daily by blood screening. Genomic DNA was extracted from lamb blood using the NZY Blood gDNA Isolation Kit (NZYTech, Lisboa, Portugal) as *per* manufacturer instructions. As previously mentioned, *B. ovis* infection was screened using conventional PCR with primers and conditions described elsewhere (Aktaş et al., 2005). PCRs were performed in 25 µl reactions with Supreme NZYTaq 2× Green Master Mix (NZYTech), 1 µM primers and 5 µl of template DNA. A negative control with water and a positive *B. ovis* (Israeli strain) control were added. The PCR was carried out with a thermal cycling profile of 95ºC for 2min, and 35 cycles of 95ºC for 30 s, 62ºC for 45 s and 72ºC for 45 s, followed by a 72ºC extension for 5min, in a T-100® Thermal Cycler (Bio-Rad, CA, USA). Resulting amplicons were checked on a 0.5X TBE, 1.2% (w/v) agarose gel.

Synthesis of dsRNA

Specific primers containing T7 promoter sequences (5′-TAATACGACTCACTATAGGGTACT-3′) at the 5′- end were manually designed using as template available sequences, in particular, GACK01008016 from *Rhipicephalus pulchellus*, GBBO01000019 from *Rhipicephalus microplus*, GBBR01000108 from *R. microplus*, and GACK01007634 from *R. pulchellus* and synthesized by StabVida

(Lisbon, Portugal) (Supplementary Table 2). *R. bursa* cDNA was synthetized using the iScript cDNA synthesis (Bio-Rad) following the manufacturer instructions and further used as template to amplify fragments of interest by PCR. Amplifications of target DNA fragments were achieved using the iProof High Fidelity PCR kit (Bio-Rad) in a 50 µl of final volume reaction, including 200 mM of each primer. Cycling conditions were for 40 cycles: 30 s at 94ºC, 30 s at specific annealing temperature and 30 s at 72ºC with a final extension step of 7min at 72ºC (Supplementary Table 2). All PCR assays were performed in a T100 thermal cycler (Bio-Rad). Amplification results were analyzed on a 0.5x TBE, 1.2 % (w/v) agarose gel. Amplicons were purified using the NZYGelpure kit (NZYtech) and sent for Sanger sequencing at StabVida (Lisbon, Portugal). The obtained sequences were aligned and compared to reference sequences. After validation of the amplified sequences the MEGAscript RNAi Kit (Ambion, Austin, TX, USA) was used to synthesize dsRNA according to manufacturer's instructions. The resulting dsRNA was purified and analyzed by spectrometry and agarose gel.

Inoculation of dsRNA and Tick Infestation

R. bursa adult female ticks from the established colony at CEVDI/INSA were cleaned and placed ventral side up on double sticky tape, affixed to a plane wood table. Thirty female ticks *per* group were injected in the trochanter-coxae articulation with 69 nL of gene specific dsRNA (1×10^{11} to 1×10^{12} molecules) or unrelated dsRNA as control, using the nanoinjector (Nanoject, Drummond Scientific, PA, USA). The mouse *β-2-microglobulin* dsRNA (dsβ2M) (GenBank: NM_009735) was used as control (Couto et al., 2017). After dsRNA injection, female ticks were held in a humidity chamber for 4 h after which they were allowed to feed on the splenectomized lamb infected with *B. ovis* together with 30 male ticks *per* feeding cell. Tick-feeding cells (450 × 400mm) (cotton fabric) were glued to shaved skin using Pattex® contact glue (Henkel Nederland, Nieuwegein, Netherlands) on the day before infestation. Ticks were monitored daily and allowed to feed in the infected lamb for 8 days. After this period, attached ticks were manually removed.

Analysis of Tick Biological Parameters After Gene Knockdown

Tick mortality was evaluated as the ratio of dead ticks to the total number of initial ticks. To analyze tick mortality, the Chi-square test ($P > 0.05$) was used with the null hypothesis that tick mortality was independent of gene knockdown. The ability to attach to the vertebrate host was also evaluated as the ratio of attached ticks and the total number of live ticks. The Chi-square test ($P > 0.05$) was also used in this analysis. Tick weight was determined in individual female ticks collected after feeding and further compared between ticks injected with test genes dsRNA and control dsRNA by Student's t-test with unequal variance ($P > 0.05$).

Gene Knockdown Assessment and Determination of *B. ovis* Infection by qPCR

To assess gene knockdown efficiency in tick SG ten ticks *per* group were randomly selected and tissues dissected and further used to extract total RNA and DNA and synthetize cDNA, as described previously. Quantity and quality of the RNA samples was estimated using the QIAxcel Advanced system (Qiagen™, Hilden, Germany). qPCR assays were performed under the conditions aforementioned. Gene expression was analyzed by the CFX Manager™ Software (Bio-Rad) as previously referred. Infection levels in tick SG were estimated using qPCR by evaluation of the levels of *B. ovis* 18S ribosomal DNA (18S rRNA) normalized against tick 16S rDNA, as described previously for other *Babesia* spp. (Antunes et al., 2012). The primers used for detection of *B. ovis* were the same used previously for conventional PCR. The cycling conditions are described in the Supplementary Table 1. Normalized Cq-values were compared between ticks injected with dsRNA and control ticks by Student's t-test with unequal variance ($P > 0.05$).

Antigenicity Prediction

Antigenicity of the selected molecules was estimated *in silico* using VaxiJen Server (Doytchinova and Flower, 2007) to allow antigen classification based on the physicochemical properties of proteins without resorting to sequence alignment. Complete sequences of the proteins were retrieved from UniProt in FASTA format and antigenicity estimated using the settings of parasite as target organism and threshold level 0.4.

1.4. Results

1.4.1. Assembly and Annotation of Female *R. bursa* Sialomes

R. bursa female ticks representing the three conditions were produced and used for SG dissections, which were followed by DNA and RNA extractions. RNA qualitative and quantitative analysis are summarized in Supplementary Table 3. Infection of protozoan-exposed group (Fi) was confirmed prior to experimentation, and total RNA was used in RNA-Seq analyses. Data were collected as two sets of matched 100-bp reads and quality analysis and raw read pre-processing were performed. The *de novo* assembly statistics are presented in Table 1.

Table 1. Assembly statistics of the six examined *Rhipicephalus bursa* sialotranscriptomes.

Assembly	NFni (1)	NFni (2)	F (1)	F (2)	Fi (1)	Fi (2)
# contigs (≥0 bp)	9,832	3,433	16,931	14,051	18,801	17,032
# contigs (≥1,000 bp)	1,317	345	3,200	2,143	3,224	2,655
# contigs (≥200 bp)	9,824	3,429	16,911	14,043	18,785	17,003
Total length (≥0 bp)	5,924,670	1,825,230	12,261,455	9,122,113	13,243,635	11,318,332
Total length (≥1,000 bp)	2,478,605	589,891	6,455,940	4,306,138	6,743,456	5,408,930
Total length (≥200 bp)	5,923,273	1,824,505	12,258,149	9,120,798	13,240,893	11,313,392
Largest contig	12,501	9,307	10,194	11,098	14,540	12,991
GC (%)	51.20	55.34	57.58	51.10	50.23	51.15
# N's per 100 kbp	0.02	0.00	0.00	0.00	0.02	0.25

NFni (1) and (2) correspond to the replicates of the pools of SG mRNA collected from unfed-uninfected females; F (1) and (2) correspond to the replicates of the pools of SG mRNA from fed-uninfected females; and Fi (1) and (2) correspond to the replicates of the pools of SG mRNA from fed-Babesia ovis infected female ticks.

A substantial increase in the number of contigs was observed in fed-uninfected samples compared to unfed-uninfected samples. The fed-*B. ovis* infected samples exhibited the highest number of contigs (Table 1). Each transcript was annotated based on BLAST results that compared the transcript to a database of reference proteins. The complete list of results can be accessed in Supplementary Datasheets 1 and 2.

The obtained transcriptomes were analyzed using the Blast2GO tool and a public Arthropoda database (nr subset) (arthropoda, taxa: 6656; from 30.01.2017). Molecular functions (Figure 1A) and biological processes (Figure 1B) of the three transcriptomes were analyzed.

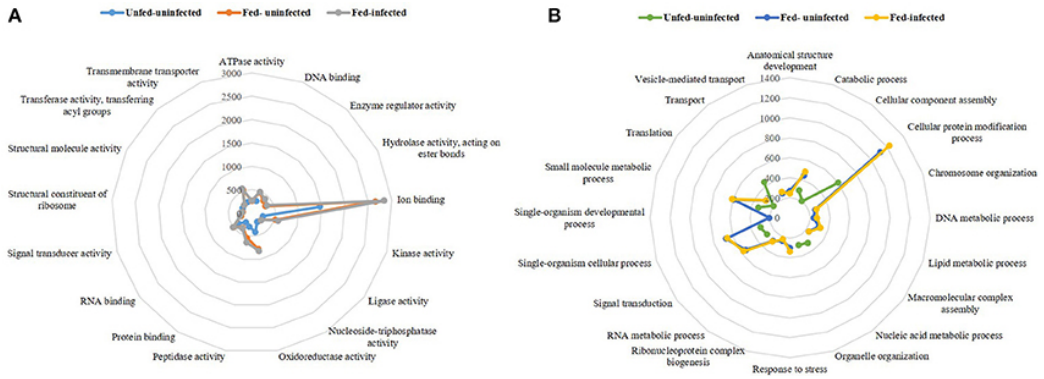

Figure 1. Radar plots of the three transcriptomes *per* represented molecular functions (A) and biological processes (B). The lines represent a pattern of the three transcriptomes unfed-uninfected, fed-uninfected and fed-*Babesia ovis* infected, allowing a visual comparison between conditions.

The molecular functions represented in the three sialotranscriptomes included DNA, RNA, protein, and ion binding properties as well as kinase, oxidoreductase, peptidase, and transmembrane transporter activities (Figure 1A). The remaining functions represented molecular functions that were present in both fed-uninfected and fed-infected catalogs, with the exception of nucleoside-triphosphatase and structural molecule activities that were exclusive to the unfed-uninfected sialotranscriptome. Ion binding was the most represented molecular function in all three datasets (Figure 1A). Biological processes such as catabolic, cellular protein modification, single-organism cellular, small molecule metabolic processes, translation, and signal transduction were also overrepresented in all sialotranscriptomes (Figure 1B). Anatomical structure development, chromosome organization, macromolecular complex assembly, response to stress, ribonucleoprotein complex biogenesis, vesicle-mediated transport, and DNA, RNA, and lipid metabolic processes were represented in the two sialotranscriptomes associated with fed-uninfected and fed-infected conditions (Figure 1B). Single-organism development is a feeding-exclusive process, while cellular component assembly, organelle organization, transport, and nucleic acid metabolic processes were exclusive to the unfed-uninfected samples.

1.4.2. Profile of SG Transcriptomic Dynamics in Response to Tick Feeding and *B. ovis* Infection

To clarify the response of *R. bursa* sialotranscriptomes to *Babesia* infection and blood feeding, an analysis that focused on the most ($P < 0.1$) up-regulated and down-regulated transcripts (Supplementary Figure 2) and as well as significantly differentially expressed ($P < 0.05$) genes (Figures 2, 3) was conducted.

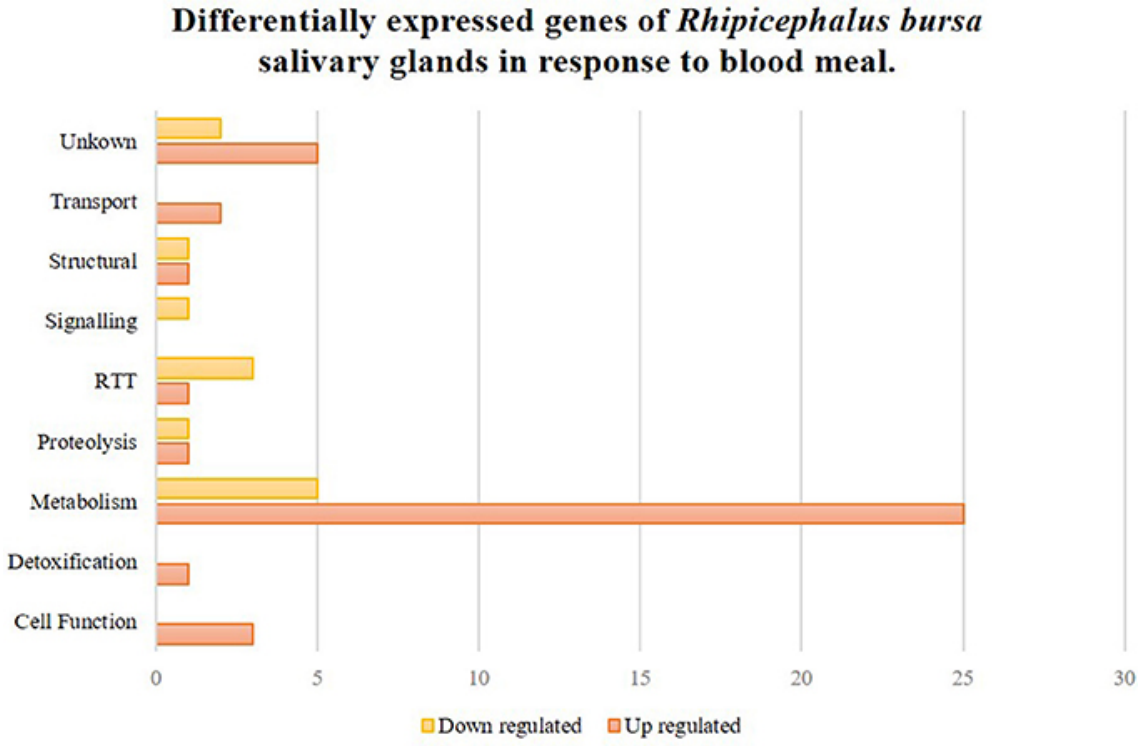

Figure 2. *Rhipicephalus bursa* SG transcriptional response to blood meal based on Gene Ontology functional classes assignments of encoded proteins. Yellow bars represent down regulated genes, orange bars represent up regulated genes with statistical significance ($P < 0.05$).

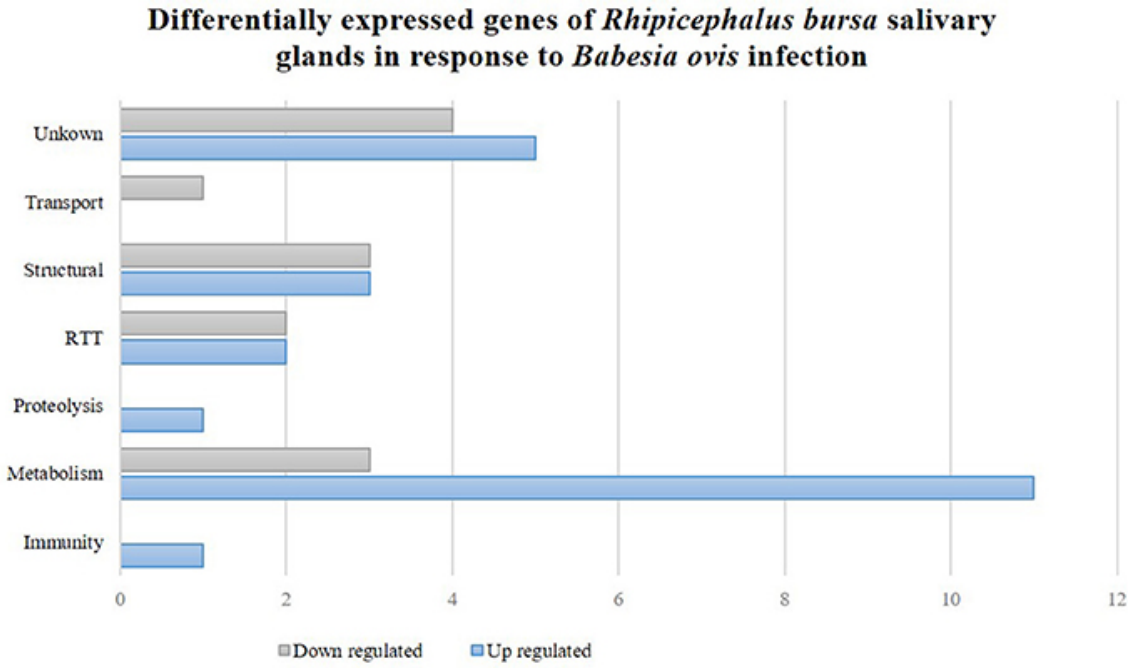

Figure 3. *Rhipicephalus bursa* SG transcriptional response to *Babesia ovis* infection based on Gene Ontology functional classes assignments of encoded proteins. Gray bars represent down regulated genes and blue bars represent up regulated genes with statistical significance ($P < 0.05$).

In total, 7,272 and 13,819 different expressed sequence tags (ESTs) were obtained from the SG of unfed and fed ticks, respectively. From these, 5,188 were found in both conditions, 2,884 were exclusive to the unfed population, and 8,631 were only present in the SG of fed *R. bursa* females. The sialotranscriptome associated with the fed-uninfected condition was compared to the fed-infected one. The results of RNA-Seq analyses indicated that 13,819 ESTs were obtained from the sialotranscriptome of the fed sample, and 15,292 ESTs were obtained from the fed-infected sample. Of these, 9,722 ESTs were present in both samples. A total of 4,097 ESTs were exclusive to the fed-uninfected ticks, and 5,570 ESTs were only present in the SG of the fed *R. bursa* females.

Analysis of the most up-regulated and down-regulated transcripts ($P < 0.1$) (Supplementary Figure 2) indicated that 500 and 216 ESTs were differentially regulated upon feeding and infection, respectively. The diversity of molecular functions and biological processes was higher in response to blood feeding compared to infection conditions. Regarding molecular functions, hydrolase activity was the only *Babesia* infection exclusive function, and it was completely down-regulated. The blood-feeding

exclusive functions were anion, metal ion, heterocyclic, and organic cyclic compound, and protein binding activities, and these functions were only associated with up-regulated transcripts. Regarding biological processes, *B. ovis* infection resulted in the induction of biosynthetic processes, cellular protein metabolic processes, gene expression, macromolecular complex assembly, organelle organization, and symbiosis (encompassing mutualism through parasitism). However, infection was also associated with the down-regulation of catabolic processes, cellular component organization, lipid metabolic and single-organism cellular process, and transmembrane transport. *R. bursa* blood meals predominantly induced biological processes such as oxidation-reduction, organic substance biosynthetic, and cellular biosynthetic processes, and cellular amino acid metabolic process and signal transduction were down-regulated.

1.4.3. SG Gene Differential Expression in Response to Blood Feeding

Fifty-two genes were considered significantly differentially expressed ($P < 0.05$), and these were classified based on GO for biological process and molecular functions (Figure 2). Seventy-five percent of these genes were up-regulated, and metabolism was the most up-regulated functional class in response to blood feeding. Functional classes such as transport, detoxification, and cell functions were only up-regulated, while signaling was down-regulated. Transcripts from structural, RTT (replication-transcription-translation), proteolysis, and metabolism functional classes were also differentially regulated during blood meals.

1.4.4. SG Gene Differential Expression in Response to *B. ovis* Infection

Thirty-six genes were considered differentially expressed ($P < 0.05$) and classified by functional classes as previously described (Figure 3). Further analyses revealed that 64 and 36% of the differentially expressed genes were up-regulated and down-regulated, respectively. Metabolism was a highly represented functional class that was associated with both up- and down-regulated genes. Structural and RTT functional classes were also affected in the *R. bursa* sialome by *Babesia* infection. Proteolysis and immunity were exclusively up-regulated, while transport was down-regulated.

1.4.5. Validation of RNA-Seq Results

Sixteen genes identified as differentially expressed in response to infection and blood feeding in RNA-Seq were selected for data validation by qPCR analysis. From the RNA-Seq catalog derived from the comparison of fed vs. unfed populations, nine transcripts that encoded the following proteins were selected: annexin (UniProt ID: A0A023FX57), aspartic protease (UniProt ID: Q2WFX6), yolk cathepsin (UniProt ID: Q56CZ1), a putative hydroxysteroid 17-beta dehydrogenase (UniProt ID L7M196), hirudin-like (UniProt ID: F0JA28), lachesin (UniProt ID: L7M018), lipocalin 9 (UniProt ID: A0A034WWJ8), a putative scinderin-like (UniProt ID: L7MCZ6), and vitellogenin- 3 (UniProt ID: A0A034WWF8) (Figure 4).

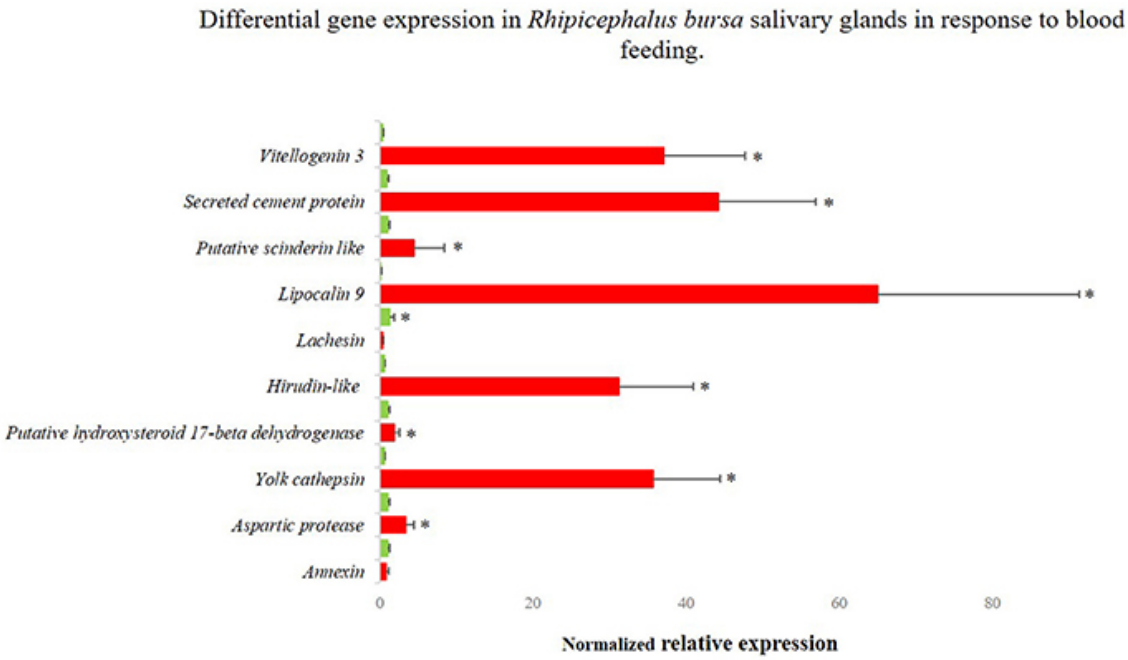

Figure 4. Differentially gene expression of *Rhipicephalus bursa* SG in response to blood feeding evaluated by qPCR. Red bars represent SG from fed *R. bursa* ticks and green bars represent the SG from unfed *R. bursa* ticks. *P < 0.05.

Regarding the RNA-Seq data obtained from the comparison of infected and uninfected SG, eight genes encoding the following proteins were selected: a putative chondroitin sulfate synthase 1-like (UniProt ID: V5H7Q8), lachesin (UniProt ID: L7M018), laminin receptor (UniProt ID: E2J6W6), a putative glycine rich protein

(UniProt ID:L7M1K6), a mucin-like protein (UniProt ID: C9W1L9), a putative ornithine decarboxylase antizyme (UniProt ID: A0A023FCB3), a secreted cement protein (UniProt ID: A0A034WWS7), and a putative yurt (UniProt ID: V5HE08) (Figure 5).

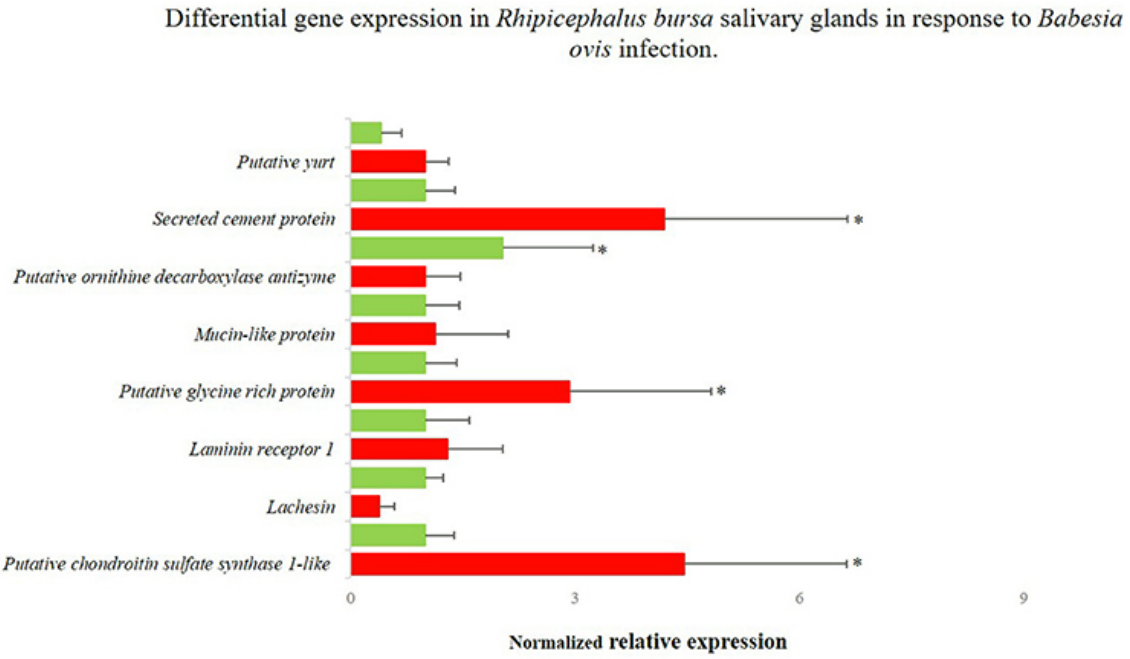

Figure 5. Differentially gene expression of *Rhipicephalus bursa* SG in response to *Babesia ovis* infection evaluation by qPCR. Red bars represent the *B. ovis* infected SG and green bars represent the SG from uninfected *R. bursa* ticks. *P < 0.05.

A moderate positive correlation between the mRNA levels by both RNA-Seq and qPCR methods was obtained (Pearson's correlation coefficient 0.5394, P = 0.025).

1.4.6. Selection of Genes for RNA Interference Studies

Genes for RNAi functional studies were selected based on their potential role in the condition studied and fold change of expression. The gene encoding a putative vitellogenin-3 (Vg-3) was identified herein as up-regulated in response to feeding in both RNA-Seq (fold-change 17.51, P = 0.025) and qPCR (foldchange 98.05, P < 0.001) evaluations. The GO analysis assigned the encoded protein to a lipid transporter activity function (molecular function), belonging to the lipid transport biological process. Lachesin, which was also selected for functional analysis, was found to be up-regulated in the RNA-Seq analysis (foldchange = 15.14, P = 0.045) in response to blood feeding,

while it was down-regulated based on the qPCR analysis (foldchange = −3.83, P < 0.001). This gene was also identified in the transcriptomic response to infection (fold-change = −0.80, P = 0.857), so its expression during *B. ovis* infection was also verified by qPCR (fold-change = −2.6427, P = 0.955). Lachesin belongs to the UniRef90_A0A1E1X7K6 cluster that is related to neural cell adhesion molecules. The gene designated as secreted cement encodes a component that is potentially involved in cement cone formation and tick attachment, and it was upregulated in response to infection based on both RNA-Seq (foldchange = 15.73, P = 0.0298) and qPCR (fold-change = 4.197, P =0.007) results. The expression of this secreted cement protein was also characterized by qPCR in response to blood feeding, indicating high up-regulation (fold-change = 47.4, P < 0.0001) in accordance with its role in the feeding process. Lastly, an uncharacterized gene designated as glycine rich that encodes a putative glycine rich protein was selected from the catalog associated with infection response, and it was up-regulated based on the results of both RNA-Seq (fold-change = 14.76, P = 0.0382) and qPCR (fold-change = 2.931, P = 0.016) analyses.

1.4.7. Functional Analyses of Differentially Expressed Tick Genes in Response to Feeding and *B. ovis* Infection

Tick Attachment, Weight and Survival Rate after RNAi

After dsRNA injection, biological parameters such as tick mortality, attachment, and weight were determined and statistically analyzed (Table 2).

Table 2. Evaluation of tick mortality, attachment, and weight after dsRNA injection in *Rhipicephalus bursa* ticks.

Group	% of mortality rate (N)	% of the ticks completing feeding (N)	*R. bursa* weight (mean ± s.d.; mg/tick)
vitellogenin-3	76.67 (23)*	57.14(4)[b]	40 ± 19[b]
lachesin	70.00 (21)*	88.89(8)	149 ± 108
glycine-rich	36.37 (11)	57.90(11)[a]	73 ± 72[a]
secreted cement	20.00 (6)	54.20(13)*	52 ± 46*
Control	16.67 (5)	88.00(22)	136 ± 163

*Thirty female ticks per group were injected with dsRNA or unrelated dsRNA. Ticks were allowed to feed in three separated patches on a lamb experimentally infected with Babesia ovis. All attached ticks were removed after seven days of feeding, weighed, and held in a humidity chamber for four days to allow ticks to digest the blood meal. Tick mortality was evaluated as the ratio of dead ticks to the total number of ticks placed on the lamb using Chi-square test. (*P < 0.05). Female tick weight after feeding was compared between dsRNA and unrelated dsRNA ticks by Student's t-test. ds, double-stranded. [a]No statistical analysis was performed due to no gene knockdown. [b]No statistical analysis was performed because of the insufficient number of samples.*

RNAi assays indicated that tick survival was significantly affected in dsRNA-injected ticks, in both ds*vitellogenin* (7/30; Chi-square, $P < 0.001$) and ds*lachesin* (9/30; Chi-square, $P < 0.001$) groups compared to controls (25/30), suggesting that these genes may play an important role in tick survival. The ds*vitellogenin* group was most affected with the highest mortality rate (76.67%). As represented in Table 2, the ds*cement* group was the most significantly affected by RNAi ($P = 0.008$), as 45.8% of the ticks were not able to correctly attach to the vertebrate host to complete blood meal. The ds*lachesin* injected population mimicked the control group's ability to attach to the host and feed. The average body weight was also measured, and it was significantly higher in the control group (133 ± 119mg) than the Vg-3-silenced group (40 ± 19mg); however, no statistical study was conducted because of the low number of ticks (N = 4). *Lachesin* knockdown did not affect tick weight (149 ± 108mg) ($P > 0.05$). The knockdown of the gene encoding the cement protein significantly reduced female weight (52 ± 46mg, $P = 0.021$) and only 13 ticks were able to attach to the host.

Gene Silencing Efficiency and *Babesia* Infection Evaluation

Under the studied conditions, dsRNA-mediated gene knockdown efficiency and its effect on *B. ovis* infection was assessed (Table 3).

Table 3. Efficiency of gene knockdown by RNA interference and its influence on *B. ovis* infection levels in *Rhipicephalus bursa* ticks SG.

Group	Gene silencing (% Ave ± *S.D.*)	*B. ovis* infection levels (Ave ± *S.D.*)	Infection rate (Ave ± *S.D.*) (N)
vitellogenin-3	92 ± 2*	$4.67e^{-04} \pm 3.05e^{-04}$	314 ± 255.17(4)[a]
lachesin	51 ± 9*	$4.48e^{-07} \pm 1.20e^{-07}$	0.30 ± 0.09(8)*
glycine-rich	ND	–	–
secreted cement	65± 11**	$2.97e^{-06} \pm 2.68e^{-06}$	1.99 ± 2.18
Control	–	$1.49e^{-06} \pm 1.09e^{-06}$	–

*Thirty female ticks per group were injected with dsRNA or unrelated dsRNA. Ticks were allowed to feed in six separated patches on a lamb experimentally infected with Babesia ovis. All attached ticks (n = 4–22) were removed after seven days of feeding and held in a humidity chamber for four days to allow ticks to digest the blood meal. Gene knockdown was analyzed by qPCR by comparing mRNA levels between specific dsRNA-injected and control ticks using the CFX Manager™ Software by means of the Pfaff method, *P < 0.05. The B. ovis infection levels were determined by qPCR of the pathogen 18S rRNA gene and normalized against tick 16S rRNA using the ddCq method ($2^{-Cq\ target - Cq\ control}$). Infection rate was calculated by the ratio of silenced per control groups. The mRNA levels and B. ovis infection in ticks were compared between specific dsRNA injected and control ticks by a Student's t-test (*P < 0.05; **P < 0.01). ds, double-stranded; ND, not demonstrated.*
[a]*No statistical analysis was made due to the insufficient number of samples.*

The injection of dsRNA molecules in *R. bursa* ticks led to a significant reduction of *vitellogenin*, *lachesin*, and *secreted cement* mRNA levels in SG by 92% (P = 0.040), 51% (P = 0.047), and 65% (P = 0.018), respectively. Regarding the levels of infection acquired after feeding on an experimentally *B. ovis*-infected lamb, the results indicated that the knockdown of *lachesin* significantly reduced *B. ovis* infection levels by 70% (P = 0.00251) in *R. bursa* SG(Table 3). The remaining groups exhibited increased infection levels.

Antigenicity of vitellogenin-3, lachesin, and secreted cement proteins were predicted by VaxiJen tool selecting parasite as the target organism. The three proteins showed to be probable antigens.

1.5. Discussion

Babesiosis is one of the most important diseases transmitted by ticks that affect a wide range of vertebrates, considered an emerging zoonose (Hunfeld et al., 2008; Ord and Lobo, 2015; Antunes et al., 2017). *B. ovis* is a potentially lethal pathogen that is normally found in small ruminants, and it is primarily transmitted by *R. bursa*, a tick species that is widely distributed in the Mediterranean region (Walker et al., 2000; Ferrolho et al., 2016a). Despite the importance of the *R. bursa*-*B. ovis*-vertebrate host interactome, no studies have examined these molecular relationships. Although it is recognized that transcripts and protein levels in ticks do not always correlate because of post-transcriptional and post-translational modifications (Ayllón et al., 2015; Villar et al., 2015), transcriptomic analysis is essential for a proper understanding of the molecular constituents of cells and tissues and the interactions and relationship between parasites and disease development (Li and Biggin, 2015; Rokyta et al., 2015). The integration of different omics analyses have allowed the detailed characterization of tick-pathogen molecular pathways (Ayllón et al., 2015; Cabezas-Cruz et al., 2017a,b). Herein, to elucidate the cellular mechanisms behind blood feeding and *Babesia* infection, three sialotranscriptomes of *R. bursa* females were analyzed and SG genes were selected for further characterization with RNAi to assess their potential as tick protective antigens.

1.5.1. Overall Characteristics of the *R. bursa* Sialome in Response to Blood Feeding and *Babesia* Infection

A strong transcriptional response was induced after tick feeding and during *B. ovis* infection, since a higher and more diverse number of transcripts were detected in the fed-uninfected sample, and even more diverse transcripts were detected in the fed-infected samples (Table 1 and Figure 1A) in comparison with the unfed-uninfected SG samples. This type of response was previously described in other systems (Heekin et al., 2013; Tirloni et al., 2014; Ayllón et al., 2015; Villar et al., 2015; de Castro et al., 2016, 2017;

Kim et al., 2016; Perner et al., 2016; Valdés et al., 2016; Schroeder et al., 2017), thus indicating that different tick biological processes or statuses stimulate different gene expression regulatory strategies.

Functional annotation indicated that in all transcriptomes, ion binding molecular function was the most represented category, and its representation nearly doubled in response to feeding (Figure 1A). Being obligatory hematophagous ectoparasites, ticks must deal with the iron and heme resulting from blood catabolism. Ticks are known to express iron and heme binding proteins that sequester excess iron or heme, preventing cell damage for physiologically normal cells (Galay et al., 2015; Kim et al., 2016).

Structural molecule activity is the only class more represented in the unfed-uninfected SG transcriptome, while other molecular function categories such as structural constituent of ribosome or enzyme regulator activity are exclusive to the fed-uninfected and fed-infected populations that exhibit high cellular activity (Villar et al., 2014).

The most represented biological process in all sialomes was the cellular protein modification. The transcript abundance of transcripts belonging to this biological process doubled in the fed-uninfected and fed-infected SG samples in comparison to the unfed-uninfected ones. The anatomical structure development process was only represented in the fed populations, and this possibly reflected SG enlargement during feeding as the majority of acinar cells undergo marked hypertrophy in Ixodid females (Šimo et al., 2017). Furthermore, some pathogens induce cytoskeletal rearrangement by affecting the regulation of specific mRNAs (Ayllón et al., 2013, 2015; Ireton, 2013; Cotté et al., 2014; de la Fuente et al., 2017). As expected, metabolism-related processes were markedly represented in the transcriptomes of fed samples. The response to stress was only identified in the fed-uninfected and fed-infected SG samples, and this was in accordance with previous studies that indicated high regulation of such pathways in ticks and cells infected with *Anaplasma* spp. (Villar et al., 2010, 2014) and during feeding (oxidative stress response) (Kim et al., 2016). The unfed sialotranscriptome profile revealed the maintenance of basal cellular metabolism (Figure 1B). Lipid metabolic processes were exclusively represented in the fed-uninfected and fed-infected samples, thus correlating with higher cellular energy requirements and saliva production (Denardi et al., 2011). Being a cellular energy source, lipids in tick SG are implied in cement cone formation, thus explaining the high representation of such metabolic activity (Denardi et al., 2011).

A comparable result was obtained in *Ixodes ricinus* and *Rhipicephalus appendiculatus* SG after feeding (Kotsyfakis et al., 2015; de Castro et al., 2016). Salivary lipid interacting proteins were up-regulated in *I. ricinus* infected with *Borrelia burgdorferi* (Cotté et al., 2014) suggesting that certain pathogens can manipulate vector lipid metabolism to facilitate infection and multiplication (Perera et al., 2012; Grabowski et al., 2017).

1.5.2. Specific *R. bursa* Sialome Response to Blood Feeding

Few studies have focused on the sialotranscriptomic response to tick feeding (McNally et al., 2012; Kotsyfakis et al., 2015; Yu et al., 2015; de Castro et al., 2016, 2017; Maruyama et al., 2017), but all demonstrated that transcription was highly affected in SG. Kotsyfakis et al. (2015) showed that fed *I. ricinus*, SG exhibit 10 times more overexpression compared to the midgut. Herein, genes that were highly differentially expressed in response to blood meals indicated up-regulation at rates of 75.0% ($P < 0.05$) to 83.8% ($P < 0.1$). GO analyses revealed that expression of secreted proteins was induced during tick feeding, including 14 lipocalins, four metalloproteases, two glycine rich proteins, and three microplusins (Supplementary Datasheet 1, Supplementary Figure 2). Such transcriptional regulation differs throughout tick feeding, thus reflecting the necessity of the tick to first attach to the host, evade and modulate host immune defenses, and maintain this status during the prolonged feeding period (McNally et al., 2012; Kotsyfakis et al., 2015; Chmelar et al., 2016; de Castro et al., 2017). Furthermore, fatty-acid related transcripts were highly represented in the up-regulated SG genes, suggesting a significant investment in carbohydrate metabolism. After tick attachment, SG differentiate and convert from an inactive to a metabolically active status with intense biosynthesis of molecules and ion transport, which increase cell energy requirements (McNally et al., 2012). The most upregulated transcripts identified herein using RNA-Seq analyses encoded a fatty acid synthase (fold-change = 17.67), followed by vitellogenin-3 (fold-change = 17.51) and a glycine-rich cell wall structural protein (fold-change = 17.51). Two uncharacterized proteins (fold-changes = −17.66 and −16.63) and two glycine rich proteins (fold-changes = −16.63 and −15.71) encoded transcripts were highly down-regulated (Supplementary Datasheet 1). These results suggested that in the late stage of feeding, female ticks switch the regulation of specific proteins related to the

production of cement cone, thus driving drop-off in accordance with previous reports (McNally et al., 2012; Kotsyfakis et al., 2015; de Castro et al., 2017).

1.5.3. Specific *R. bursa* Sialome Response to *B. ovis* Infection

The sialotranscriptomes of fed-infected and fed-uninfected female *R. bursa* were compared to characterize SG transcriptional regulation in response to pathogen infection. As all of the SG samples belonged to fed ticks, the effect of the feeding process can be annulled. Some studies aimed to understand the effects of pathogens on tick SG at transcriptomic, proteomic, and metabolomic levels (Nene et al., 2004; Zivkovic et al., 2010; Mercado-Curiel et al., 2011; McNally et al., 2012; Cotté et al., 2014; Ayllón et al., 2015; Villar et al., 2015; Valdés et al., 2016). Because of their medical importance, many of these studies were dedicated to *Anaplasma* spp./*Borrelia* spp.-*Ixodes* spp. interactions This is the first study that specifically focused on the *Rhipicephalus* SG transcriptomic response to *Babesia* infection. Pathogens highly adapted to the vector such as *Anaplasma*-*R. microplus* do not induce great effects on SG, while pathogens that pose a higher threat to vector fitness would lead to a greater gene modulation (Cen-Aguilar et al., 1998; Zivkovic et al., 2010; Mercado-Curiel et al., 2011; Chmelar et al., 2016; de la Fuente et al., 2016; Šimo et al., 2017). In *Babesia* infections, tick development tends to be impaired, but adaptive parasite tolerance has been described in *R. microplus* (Cen-Aguilar et al., 1998; Antunes et al., 2017). Furthermore, a small number of genes were considered differentially expressed (36 genes at $P < 0.05$ and 260 genes at $P < 0.1$), suggesting the long co-evolution of *R. bursa* and *B. ovis*. In both analyses an up-regulation of 63–64% of the genes occurred. Our results showed that during *Babesia* invasion, cellular metabolism tended to increase, whereas biosynthesis and protein processing were the most represented categories (Supplementary Datasheet 2, Supplementary Figure 2). This metabolism induction was previously demonstrated in other vector-pathogen systems (Mercado-Curiel et al., 2011; Heekin et al., 2012; Ayllón et al., 2015; Villar et al., 2015). The most up-regulated genes found were related to glycine rich proteins (GRPs), including uncharacterized protein (foldchange = 17.53), glycine rich proteins (fold-change = 16.45 and 15.65), and secreted cement protein (fold-change = 15.73). Glycine rich proteins have been identified as upregulated in response to infection and cement proteins (Nene et al., 2004; Zivkovic et al., 2010). With rare exceptions, the role of such

proteins during pathogen infection/dissemination have not been investigated (Trimnell et al., 2002). Lipocalins and defensins were identified as up-regulated in our dataset, showing an investment of the tick in the immune response, as expected. To validate the RNA-Seq results, qPCR was employed targeting putatively down-regulated *ornithine decarboxylase antizyme*, *lachesin*, and *chondroitin sulfate synthase* genes and putative upregulated *laminin receptor*, *yurt*, *glycine rich*, *secreted cement*, and *mucin*. *Chondroitin sulfate synthase and lachesin* expression trends were not confirmed, indicating up-regulation in infected SG. Chondroitin's are known to be involved in *Plasmodium* spp. adhesion to cells (Dinglasan et al., 2007; Couto et al., 2017), so the up-regulation of related molecules in infected tick SG suggests that *Babesia* spp. (considered a *Plasmodium*-like parasite) may use similar strategies to invade cells.

1.5.4. Functional Studies for the Identification of Tick Protective Antigens

Vitellogenin-3

Multiple vitellogenins (Vgs) have been described in ticks (Thompson et al., 2007; Boldbaatar et al., 2010; Khalil et al., 2011; Taheri et al., 2014; Rodriguez et al., 2016), and they are involved in detoxification and oxidative molecular processes (Galay et al., 2015). In the sialotranscriptome obtained in response to blood feeding, the translation of one of the assembled transcripts showed high similarity to *R. microplus* putative Vg-3 protein (UniProt ID: A0A034WWF8). An up-regulation of the expression of the correspondent gene in the SG of fed *R. bursa* was demonstrated by both RNA-Seq and qPCR (RNA-Seq: foldchange = 17.509, $P = 0.025$; and qPCR fold-change = 98.05, $P < 0.001$), and the results were in accordance with those of previous studies (Horigane et al., 2010; Yang et al., 2015). Vgs are thought to be absent from SG, whereas heme transport and storage are thought to be dependent of the hemelipoglyco-carrier protein (CP) (Donohue et al., 2009). In ticks, both Vg proteins and CP bind heme (Logullo et al., 2002), which is a functional component of many hemoproteins, but it is cytotoxic in larger amounts (Ferrolho et al., 2016b; Hajdusek et al., 2016). The similarities between CPs and Vgs in ticks, as well as their common evolutionary origin, greatly complicate their differentiation and function assignments (Gudderra et al., 2002; Donohue et al., 2009; Boldbaatar et al., 2010). The present study showed that *R. bursa* possesses a gene very

similar to Vgs in SG, and it shares several molecular features with CPs. Further studies are necessary to clarify Vgs classification in ticks as well as the function and localization of Vg-3 in *R. bursa* species as these Vgs are expressed in a tissue-specific manner in ticks (Rodriguez et al., 2016). Vg-3 knockdown experiments resulted in increased tick mortality. No statistical analyses were performed regarding feeding behaviors, body weight and *Babesia* infection, because of the low number of samples; however, decreased blood-uptake and increased *Babesia* infection was observed. Based on the principal functions associated to this type of molecule, we can suggest that a decrease in the expression of putative Vg-3 reduces heme and lipid binding and storage (Figure 6A).

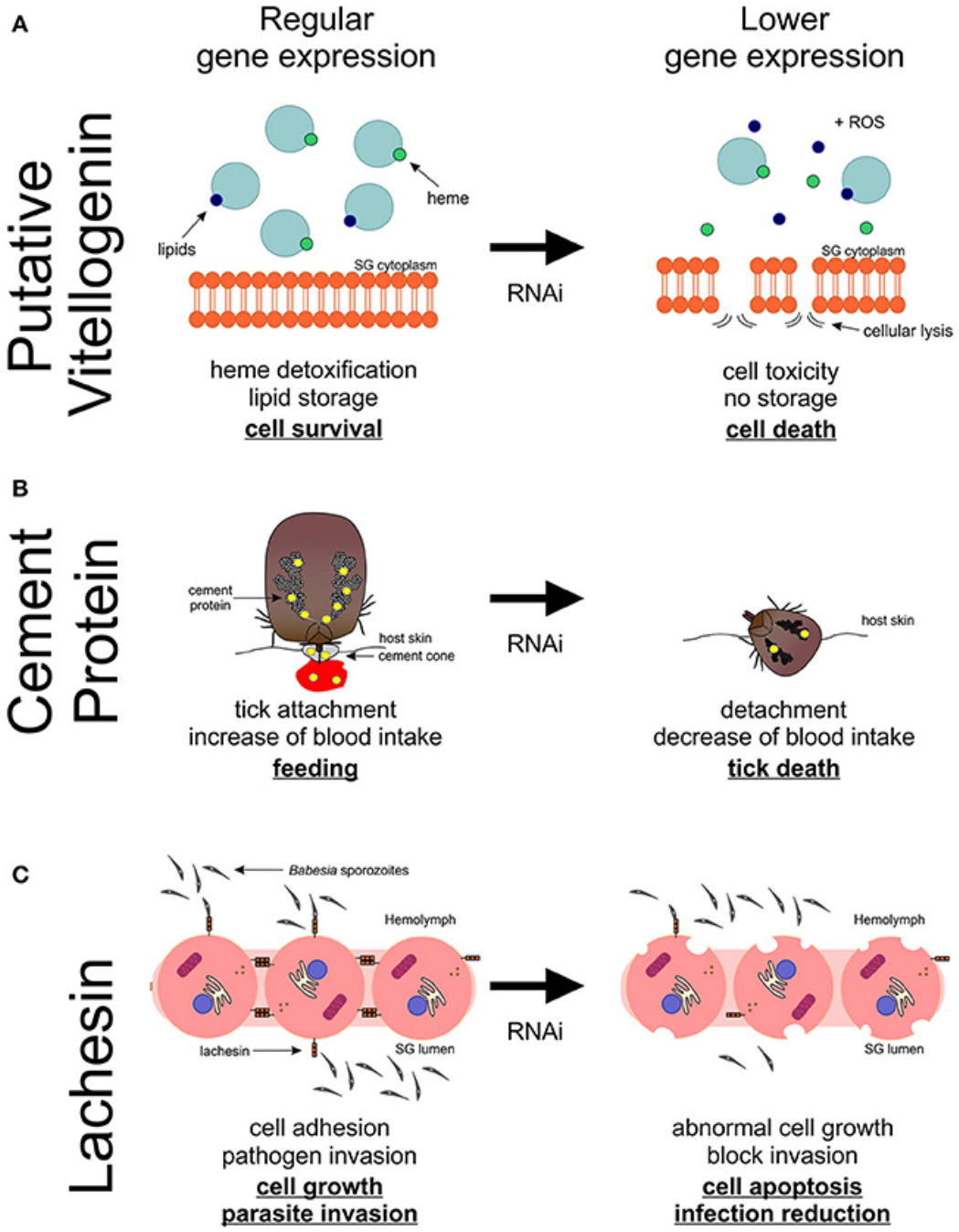

Figure 6. Proposed model of putative vitellogenin-3, cement protein and lachesin functions and its impact on *Rhipicephalus bursa* SG during feeding and *Babesia ovis* infection. (A) Vitellogenin-3 described function relates to heme detoxification and lipid storage contributing for cell survival. A decrease of the expression of putative vitellogenin-3 leads to deficient heme seizure, increasing the formation of reactive oxygen species (ROS) as well as cellular toxicity. Lipid storage is also compromised leading to an unbalance in the production of energy. **(B)** Putative cement protein is a component of the cement cone, which facilitates the tick attachment and feed on the host. An impact in the production of cement proteins leads to an incapacity of ticks to correctly attach and subsequently feed on the host, resulting in tick death and reduced blood ingestion. **(C)** Lachesin is a cell surface protein that as a potential role in cell adhesion,

maintaining apical-basal polarity, vesicle trafficking, cell growth and survival, as well as parasite invasion. A negative manipulation of the expression of lachesin results in an abnormal cell growth and ultimately cell apoptosis, and also a decrease of *Babesia* spp. infection.

A deficient heme seizure may increase cellular toxicity, thus contributing for the formation of reactive oxygen species (ROS). Also, the role of Vgs on lipid transport is compromised, and this may unbalance normal energy production. Vgs have been consistently discovered has highly immunogenic molecules in *Rhipicephalus* ticks (Boldbaatar et al., 2008, 2010; Smith and Kaufman, 2013; Taheri et al., 2014; Rodriguez et al., 2016), and the results of the present study stimulates future research.

Putative Secreted Cement Protein and Glycine-Rich Protein

The genes encoding putative cement protein and GRP were found to be significantly up-regulated in response to *B. ovis* infection in *R. bursa* SG, in accordance with a previous study (Nene et al., 2004). The cement cone is composed of several molecules that are embedded in a proteinaceous matrix, presenting several GRPs (Bishop et al., 2002; Trimnell et al., 2005; Maruyama et al., 2010). Different species of ticks rely on different types and amounts of GRPs in order to attach and feed on their hosts. Briefly, ticks with short mouthparts need higher amounts of GRPs than those with long mouthparts. Moreover, one-host ticks present a greater variety of these proteins than ticks that feed on several hosts (Maruyama et al., 2010). A successfully knockdown was observed in *cement*-silenced ticks, but no silencing was demonstrated in *glycine-rich* dsRNA-injected ticks, suggesting that a higher concentration may be needed to reduce the expression of this gene. The *cement*-silenced ticks significantly affected tick attachment, feeding, and body weight (Figure 6B). The ds*glycine-rich* RNA inoculated group exhibited a slight decrease in these two parameters, reflecting its potential in tick feeding capacity and attachment to the host. Curiously, in both dsRNA-injected groups, an increase of *Babesia* levels was detected. Previous studies concerning cement cone proteins showed that immunization with these proteins significantly affected tick attachment to the host (Trimnell et al., 2005) and it reduced pathogen transmission (Labuda et al., 2006). Therefore, these two proteins are attractive targets for vaccine development.

Lachesin

Lachesin is a cell surface protein of the immunoglobulin superfamily (Karlstrom et al., 1993; Llimargas et al., 2004) that regulates organ size by influencing cell length and cell detachments, suggesting a role in cell adhesion and connection (Llimargas et al., 2004). In ticks, the gene encoding lachesin was first identified in the genome of *Ixodes scapularis* (Gulia- Nuss et al., 2016) and more recently in the sialotranscriptome of *Amblyomma cajennense* (Garcia et al., 2014), *R. pulchellus* (Tan et al., 2015), and *R. appendiculatus* (de Castro et al., 2016). However, no studies that focus on this molecule in ticks have been performed. In the present study, an assembled transcript translated to a protein highly similar to lachesin (UniProt ID: L7M018). A highly dynamic expression profile of *lachesin* in response to infection and feeding was found in the present study, and this observation aligned to its presumed role on cell-adhesion led to its selection for RNAi studies. Tick inoculation with ds*lachesin* resulted in 51% gene knockdown that led to a significantly high tick mortality. Lachesin accumulates in specific invertebrate cell junctions, and it is responsible for establishing and/or maintaining cell polarity, cell adhesion, and cell-cell interactions (Tepass et al., 2001). Apical-basal polarity is subjected to tight regulation, as it is crucial during tissue formation, including vesicle trafficking machinery, morphogenesis, and modulation of epithelial cell growth and survival (Bonazzi and Cossart, 2011). Moreover, adhesive contacts between cells and the extracellular matrix appear as important landmarks for polarity. Therefore, manipulating the expression of genes involved in this processes can induce abnormal cell growth and cell apoptosis (Tepass, 2012). In addition, the *lachesin* knockdown resulted in lower pathogen infection in the SG. No statistical effect was demonstrated in the other biological parameters studied. Despite the tight organization of the epithelium barrier and its interactions with cellular factors that are crucial to cell-pathogen defense, a large number of pathogens have developed strategies to target host proteins involved in cell adhesion, to colonize epithelia, invade host cells, or even disrupt host barriers to facilitate access to other tissues (Bonazzi and Cossart, 2011). Thus, our results suggest that lachesin plays an important role in tick survival and also that *B. ovis* may require this molecule for tissue invasion (Figure 6C). This molecule appears to be good candidate for future vaccination assays, as it demonstrates a dual-effect targeting both tick and pathogen.

1.6. Conclusions

Tick and tick-borne diseases constitute a growing burden for human and animal health, stressing the urgency in the development of new effective tools to control this global threat. Due to the important role of tick SG in tick biology and pathogen transmission, the main objective of the present study was the identification and functional characterization of *R. bursa* SG genes involved in tick feeding and *B. ovis* infection. Quantitative transcriptome analysis showed *lachesin* and putative *vitellogenin-3* has highly upregulated in response to blood meal and the genes encoding for a putative secreted cement and GRPs highly upregulated in response to *B. ovis* infection. RNAi studies suggest that lachesin and putative vitellogenin-3 affect tick survival while the putative cement protein has an impact in tick attachment to the host and tick weight after feeding. Moreover, *B. ovis* infection levels in tick SG were reduced, subsequently to *lachesin* knockdown. Overall the results of the present study endorse the inclusion of these proteins in vaccination trials.

1.7. References

Aktaş, M., Altay, K., and Dumanli, N. (2005). Development of a polymerase chain reaction method for diagnosis of *Babesia ovis* infection in sheep and goats. Vet. Parasitol. 133, 277–281. doi: 10.1016/j.vetpar.2005.05.057

Anatriello, E., Ribeiro, J. M. C., de Miranda-Santos, I. K. F., Brandao, L. G., Anderson, J. M., Valenzuela, J. G., et al. (2010). An insight into the sialotranscriptome of the brown dog tick, *Rhipicephalus sanguineus*. BMC Genomics 11:450. doi: 10.1186/1471-2164-11-450

Antunes, S., Galindo, R. C., Almazan, C., Rudenko, N., Golovchenko, M., Grubhoffer, L., et al. (2012). Functional genomics studies of *Rhipicephalus* (*Boophilus*) *annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. Int. J. Parasitol. 42, 187–195. doi: 10.1016/j.ijpara.2011.12.003

Antunes, S., Rosa, C., Couto, J., Ferrolho, J., and Domingos, A. (2017). Deciphering *Babesia*-vector interactions. Front. Cell. Infect. Microbiol. 7:429. doi: 10.3389/fcimb.2017.00429

Ayllón, N., Villar, M., Busby, A. T., Kocan, K. M., Blouin, E. F., Bonzon-Kulichenko, E., et al. (2013). *Anaplasma phagocytophilum* inhibits apoptosis and promotes cytoskeleton rearrangement for infection of tick cells. Infect. Immun. 81, 2415–2425. doi: 10.1128/IAI.00194-13

Ayllón, N., Villar, M., Galindo, R. C., Kocan, K. M., Sima, R., Lopez, J. A., et al. (2015). Systems biology of tissue-specific response to *Anaplasma phagocytophilum* reveals differentiated apoptosis in the tick vector *Ixodes scapularis*. PLoS Genet. 11:e1005120. doi: 10.1371/journal.pgen.1005120

Belloli, C., Lai, O. R., Ormas, P., Zizzadoro, C., Sasso, G., and Crescenzo, G. (2006). Pharmacokinetics and mammary elimination of imidocarb in sheep and goats. J. Dairy Sci. 89, 2465–2472. doi: 10.3168/jds.S0022-0302(06)72320-7

Bishop, R., Lambson, B., Wells, C., Pandit, P., Osaso, J., Nkonge, C., et al. (2002). A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 32, 833–842. doi: 10.1016/S0020-7519(02)00027-9

Boldbaatar, D., Battsetseg, B., Matsuo, T., Hatta, T., Umemiya-Shirafuji, R., Xuan, X., et al. (2008). Tick vitellogenin receptor reveals critical role in oocyte development and transovarial transmission of *Babesia* parasite. Biochem. Cell Biol. 86, 331–344. doi: 10.1139/O08-071

Boldbaatar, D., Umemiya-Shirafuji, R., Liao, M., Tanaka, T., Xuan, X. N., and Fujisaki, K. (2010). Multiple vitellogenins from the *Haemaphysalis longicornis* tick are crucial for ovarian development. J. Insect Physiol. 56, 1587–1598. doi: 10.1016/j.jinsphys.2010.05.019

Bonazzi, M., and Cossart, P. (2011). Impenetrable barriers or entry portals? The role of cell-cell adhesion during infection. J. Cell Biol. 195, 349–358. doi: 10.1083/jcb.201106011

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. doi: 10.1373/clinchem.2008.112797

Cabezas-Cruz, A., Alberdi, P., Valdés, J. J., Villar, M., and de la Fuente, J. (2017a). *Anaplasma phagocytophilum* infection subverts carbohydrate metabolic pathways in the

tick vector, *Ixodes scapularis*. Front. Cell. Infect. Microbiol. 7:23. doi: 10.3389/fcimb.2017.00023

Cabezas-Cruz, A., Espinosa, P. J., Obregón, D. A., Alberdi, P., and de la Fuente, J. (2017b). *Ixodes scapularis* tick cells control *Anaplasma phagocytophilum* infection by increasing the synthesis of phosphoenolpyruvate from tyrosine. Front. Cell. Infect. Microbiol. 7:375. doi: 10.3389/fcimb.2017.00375

Cen-Aguilar, J. F., Rodriguez-Vivas, R. I., Dominguez-Alpizar, J. L., and Wagner, G. G. (1998). Studies on the effect of infection by *Babesia* sp. on oviposition of *Boophilus microplus* engorged females naturally infected in the Mexican tropics. Vet. Parasitol. 78, 253–257.

Chmelar, J., Kotal, J., Karim, S., Kopacek, P., Francischetti, I. M. B., Pedra, J. H. F., et al. (2016). Sialomes and mialomes: a systems-biology view of tick tissues and tick-host interactions. Trends Parasitol. 32, 242–254. doi: 10.1016/j.pt.2015.10.002

Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674–3676. doi: 10.1093/bioinformatics/bti610

Cotté, V., Sabatier, L., Schnell, G., Carmi-Leroy, A., Rousselle, J. C., Arsène-Ploetze, F., et al. (2014). Differential expression of *Ixodes ricinus* salivary gland proteins in the presence of the *Borrelia burgdorferi* sensu lato complex. J. Proteomics 96, 29–43. doi: 10.1016/j.jprot.2013.10.033

Couto, J., Antunes, S., Pinheiro-Silva, R., do Rosário, V., de la Fuente, J., and Domingos, A. (2017). Solute carriers affect *Anopheles stephensi* survival and *Plasmodium berghei* infection in the salivary glands. Sci. Rep. 7:6141. doi: 10.1038/s41598-017-06317-6

Dahmani,M., Davoust, B., Rousseau, F., Raoult, D., Fenollar, F., and Mediannikov, O. (2016). Natural anaplasmataceae infection in *Rhipicephalus bursa* ticks collected from sheep in the French Basque Country. Ticks Tick Borne Dis. 8, 18–24. doi: 10.1016/j.ttbdis.2016.09.009

de Castro,M. H., de Klerk, D., Pienaar, R., Latif, A. A., Rees, D. J. G., and Mans, B. J. (2016). *De novo* assembly and annotation of the salivary gland transcriptome of *Rhipicephalus appendiculatus* male and female ticks during blood feeding. Ticks Tick Borne Dis. 7, 536–548. doi: 10.1016/j.ttbdis.2016.01.014

de Castro, M. H., de Klerk, D., Pienaar, R., Rees, D. J. G., and Mans, B. J. (2017). Sialotranscriptomics of *Rhipicephalus zambeziensis* reveals intricate expression profiles of secretory proteins and suggests tight temporal transcriptional regulation during blood-feeding. Parasit. Vec. 10:384. doi: 10.1186/s13071-017-2312-4

de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A. G., Estrada-Pena, A., et al. (2017). Tick-Pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front. Cell. Infect. Microbiol. 7:114. doi: 10.3389/fcimb.2017.00114

de la Fuente, J., Van Den Bussche, R. A., Prado, T. M., and Kocan, K. M. (2003). *Anaplasma marginale msp1* genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 41, 1609–1616. doi: 10.1128/JCM.41.4.1609-1616.2003

de la Fuente, J., Vicente, J., Hofle, U., Ruiz-Fons, F., de Mera, I. G. F., Van Den Bussche, R. A., et al. (2004). *Anaplasma* infection in free-ranging Iberian red deer in the region of Castilla-La Mancha, Spain. Vet. Microbiol. 100, 163–173. doi: 10.1016/j.vetmic.2004.02.007

de la Fuente, J., Villar, M., Cabezas-Cruz, A., Estrada-Pena, A., Ayllon, N., and Alberdi, P. (2016). Tick-Host-Pathogen interactions: conflict and cooperation. PLoS Pathog. 12, 5488–5488. doi: 10.1371/journal.ppat.1005488

Denardi, S. E., Furquim, K. C., Bechara, G. H., and Camargo-Mathias, M. I. (2011). Salivary glands of *Amblyomma cajennense* (Acari: Ixodidae): a histological and an ultrastructural overview. Exp. Appl. Acarol. 54, 177–189. doi: 10.1007/s10493-010-9421-5

Dinglasan, R. R., Alaganan, A., Ghosh, A. K., Saito, A., van Kuppevelt, T. H., and Jacobs-Lorena, M. (2007). *Plasmodium falciparum* ookinetes require mosquito midgut chondroitin sulfate proteoglycans for cell invasion. Proc. Natl. Acad. Sci. U.S.A. 104, 15882–15887. doi: 10.1073/pnas.0706340104

Domingos, A., Antunes, S., Borges, L., and Rosario, V. E. (2013). Approaches towards tick and tick-borne diseases control. Rev. Soc. Bras. Med. Trop. 46, 265–269. doi: 10.1590/0037-8682-0014-2012

Donohue, K. V., Khalil, S. M. S., Sonenshine, D. E., and Roe, R. M. (2009). Heme binding storage proteins in the Chelicerata. J. Insect Physiol. 55, 287–296. doi: 10.1016/j.jinsphys.2009.01.002

Doytchinova, I. A., and Flower, D. R. (2007). VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics 8:4. doi: 10.1186/1471-2105-8-4

Erster, O., Roth, A., Wolkomirsky, R., Leibovich, B., Savitzky, I., Zamir, S., et al. (2015). Molecular detection of *Babesia ovis* in sheep and ticks using the gene encoding *B. ovis* surface protein D (BoSPD). Vet. Parasitol. 214, 282–288. doi: 10.1016/j.vetpar.2015.09.013

Ferrolho, J., Antunes, S., Sanches, G. S., Couto, J., Evora, P. M., Rosa, C., et al. (2016a). *Ferritin 1* silencing effect in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) during experimental infection with *Ehrlichia canis*. Ticks Tick Borne Dis. 8, 174–184. doi: 10.1016/j.ttbdis.2016.10.015

Ferrolho, J., Antunes, S., Santos, A. S., Velez, R., Padre, L., Cabezas-Cruz, A., et al. (2016b). Detection and phylogenetic characterization of *Theileria* spp. and *Anaplasma marginale* in *Rhipicephalus bursa* in Portugal. Ticks Tick Borne Dis. 7, 443–448. doi: 10.1016/j.ttbdis.2016.01.004

Francischetti, I. M., Anderson, J. M., Manoukis, N., Pham, V. M., and Ribeiro, J. M. C. (2011). An insight into the sialotranscriptome and proteome of the coarse bontlegged tick, *Hyalomma marginatum* rufipes. J. Proteomics 74, 2892–2908. doi: 10.1016/j.jprot.2011.07.015

Francischetti, I. M., Mans, B. J., Meng, Z., Gudderra, N., Veenstra, T. D., Pham, V. M., et al. (2008). An insight into the sialome of the soft tick, *Ornithodorus parkeri*. Insect Biochem. Mol. Biol. 38, 1–21. doi: 10.1016/j.ibmb.2007.09.009

Francischetti, I. M., Sa-Nunes, A., Mans, B. J., Santos, I. M., and Ribeiro, J. M. C. (2009). The role of saliva in tick feeding. Front. Biosci. 14, 2051–2088. doi: 10.2741/3363

Friedhoff, K. T. (1988). “Chapter 2: Transmission of *Babesia*,” in Babesiosis of Domestic Animals and Man, ed M. Ristic (Boca Raton, FL: CRC Press), 23–52.

Galay, R. L., Umemiya-Shirafuji, R., Mochizuki, M., Fujisaki, K., and Tanaka, T. (2015). Iron metabolism in hard ticks (Acari: Ixodidae): the antidote to their toxic diet. Parasitol. Int. 64, 182–189. doi: 10.1016/j.parint.2014.12.005

Garcia, G. R., Gardinassi, L. G., Ribeiro, J. M., Anatriello, E., Ferreira, B. R., Moreira, H. N., et al. (2014). The sialotranscriptome of *Amblyomma triste*, *Amblyomma parvum* and *Amblyomma cajennense* ticks, uncovered by 454-based RNA-seq. Parasit. Vec. 7:430. doi: 10.1186/1756-3305-7-430

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36, 3420–3435. doi: 10.1093/nar/gkn176

Grabowski, J.M., Gulia-Nuss, M., Kuhn, R. J., and Hill, C. A. (2017). RNAi reveals proteins for metabolism and protein processing associated with Langat virus infection in *Ixodes scapularis* (black-legged tick) ISE6 cells. Parasit. Vec. 10:24. doi: 10.1186/s13071-016-1944-0

Gudderra, N. P., Sonenshine, D. E., Apperson, C. S., and Roe, R. M. (2002). Tissue distribution and characterization of predominant hemolymph carrier proteins from *Dermacentor variabilis* and *Ornithodoros parkeri*. J. Insect Physiol. 48, 161–170. doi: 10.1016/S0022-1910(01)00160-3

Gulia-Nuss, M., Nuss, A. B., Meyer, J. M., Sonenshine, D. E., Roe, R. M., Waterhouse, R. M., et al. (2016). Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease. Nat. Commun. 7:10507. doi: 10.1038/ncomms10507

Habela, M., Reina, D., Nieto, C., and Navarrete, I. (1990). Antibody response and duration of latent infection in sheep following experimental infection with *Babesia ovis*. Vet. Parasitol. 35, 1–10. doi: 10.1016/0304-4017(90)90111-N

Hajdusek, O., Sima, R., Perner, J., Loosova, G., Harcubova, A., and Kopacek, P. (2016). Tick iron and heme metabolism - new target for an anti-tick intervention. Ticks Tick Borne Dis. 7, 565–572. doi: 10.1016/j.ttbdis.2016.01.006

Harrus, S., Perlman-Avrahami, A., Mumcuoglu, K. Y., Morick, D., Eyal, O., and Baneth, G. (2011). Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. Clin. Microbiol. Infect. 17, 459–463. doi: 10.1111/j.1469-0691.2010.03316.x

Heekin, A. M., Guerrero, F. D., Bendele, K. G., Saldivar, L., Scoles, G. A., Dowd, S. E., et al. (2013). The ovarian transcriptome of the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*, feeding upon a bovine host infected with *Babesia bovis*. Parasit. Vec. 6:276. doi: 10.1186/1756-3305-6-276

Heekin, A. M., Guerrero, F. D., Bendele, K. G., Saldivar, L., Scoles, G. A., Gondro, C., et al. (2012). Analysis of *Babesia bovis* infection-induced gene expression changes in larvae from the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. Parasit. Vectors 5:162. doi: 10.1186/1756-3305-5-162

Horigane, M., Shinoda, T., Honda, H., and Taylor, D. (2010). Characterization of a *vitellogenin* gene reveals two phase regulation of vitellogenesis by engorgement and mating in the soft tick *Ornithodoros moubata* (Acari: Argasidae). Insect Mol. Biol. 19, 501–515. doi: 10.1111/j.1365-2583.2010.01007.x

Horta, S., Barreto, M. C., Pepe, A., Campos, J., and Oliva, A. (2014). Highly sensitive method for diagnosis of subclinical *B. ovis* infection. Ticks Tick Borne Dis. 5, 902–906. doi: 10.1016/j.ttbdis.2014.07.005

Hunfeld, K. P., Hildebrandt, A., and Gray, J. S. (2008). Babesiosis: recent insights into an ancient disease. Int. J. Parasitol. 38, 1219–1237. doi: 10.1016/j.ijpara.2008.03.001

Hurtado, A., Barandika, J. F., Oporto, B., Minguijón, E., Povedano, I., and García-Pérez, A. L. (2015). Risks of suffering tick-borne diseases in sheep translocated to a tick infested area: a laboratory approach for the investigation of an outbreak. Ticks Tick Borne Dis. 6, 31–37. doi: 10.1016/j.ttbdis.2014.09.001

Inokuma, H., Raoult,D., and Brouqui, P. (2000).Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. J. Clin. Microbiol. 38, 4219–4221. doi: 10.1016/S0304-4017(02)00289-3

Ireton, K. (2013). Molecular mechanisms of cell-cell spread of intracellular bacterial pathogens. Open Biol. 3:130079. doi: 10.1098/rsob.130079

Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. Parasitology 129, S3–S14. doi: 10.1017/S0031182004005967

Karim, S., Singh, P., and Ribeiro, J. M. C. (2011). A deep insight into the sialotranscriptome of the gulf coast tick, *Amblyomma maculatum*. PLoS ONE 6:e28525. doi: 10.1371/journal.pone.0028525

Karlstrom, R. O., Wilder, L. P., and Bastiani, M. J. (1993). Lachesin: an immunoglobulin superfamily protein whose expression correlates with neurogenesis in grasshopper embryos. Development 118, 509–522.

Kaufman, W. R. (2010). Ticks physiological aspects with implications for pathogen transmission. Ticks Tick Borne Dis. 1, 11–22. doi: 10.1016/j.ttbdis.2009.12.001

Kazimírová, M., and Stibraniova, I. (2013). Tick salivary compounds: their role in modulation of host defences and pathogen transmission. Front. Cell. Infect. Microbiol. 3:43. doi: 10.3389/fcimb.2013.00043

Khalil, S. M. S., Donohue, K. V., Thompson, D. M., Jeffers, L. A., Ananthapadmanaban, U., Sonenshine, D. E., et al. (2011). Full-length sequence, regulation and developmental studies of a second *vitellogenin* gene from the American dog tick, *Dermacentor variabilis*. J. Insect Physiol. 57, 400–408. doi: 10.1016/j.jinsphys.2010.12.008

Kim, T. K., Tirloni, L., Pinto, A. F., Moresco, J., Yates, J. R., da Silva Vaz, I., et al. (2016). *Ixodes scapularis* tick saliva proteins sequentially secreted every 24 h during blood feeding. PLoS Negl. Trop. Dis. 10:e0004323. doi: 10.1371/journal.pntd.0004323

Kotál, J., Langhansova, H., Lieskovska, J., Andersen, J. F., Francischetti, I. M. B., Chavakis, T., et al. (2015). Modulation of host immunity by tick saliva. J. Proteomics 128, 58–68. doi: 10.1016/j.jprot.2015.07.005

Kotsyfakis, M., Schwarz, A., Erhart, J., and Ribeiro, J. M. (2015). Tissue- and time-dependent transcription in *Ixodes ricinus* salivary glands and midguts when blood feeding on the vertebrate host. Sci. Rep. 5:9103. doi: 10.1038/srep09103

Labuda, M., Trimnell, A. R., Lickova, M., Kazimirova, M., Davies, G. M., Lissina, O., et al. (2006). An antivector vaccine protects against a lethal vector-borne pathogen. PLoS Pathog. 2:e27. doi: 10.1371/journal.ppat.0020027

Li, J. J., and Biggin, M. D. (2015). Gene expression. Statistics requantitates the central dogma. Science 347, 1066–1067. doi: 10.1126/science.aaa8332

Liu, X. Y., and Bonnet, S. I. (2014). Hard tick factors implicated in pathogen transmission. PLoS Negl. Trop. Dis. 8:e2566. doi: 10.1371/journal.pntd.0002566

Llimargas, M., Strigini, M., Katidou, M., Karagogeos, D., and Casanova, J. (2004). Lachesin is a component of a septate junction-based mechanism that controls tube size

and epithelial integrity in the *Drosophila* tracheal system. Development 131, 181–190. doi: 10.1242/dev.00917

Logullo, C., Moraes, J., Dansa-Petretski, M., Vaz, I. S., Masuda, A., Sorgine, M. H. F., et al. (2002). Binding and storage of heme by vitellin from the cattle tick, *Boophilus microplus*. Insect Biochem. Mol. Biol. 32, 1805–1811. doi: 10.1016/S0965-1748(02)00162-5

Maruyama, S. R., Anatriello, E., Anderson, J. M., Ribeiro, J. M., Brandão, L. G., Valenzuela, J. G., et al. (2010). The expression of genes coding for distinct types of glycine-rich proteins varies according to the biology of three metastriate ticks, *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus sanguineus* and *Amblyomma cajennense*. BMC Genomics 11:363. doi: 10.1186/1471-2164-11-363

Maruyama, S. R., Garcia, G. R., Teixeira, F. R., Brandão, L. G., Anderson, J. M., Ribeiro, J. M. C., et al. (2017). Mining a differential sialotranscriptome of *Rhipicephalus microplus* guides antigen discovery to formulate a vaccine that reduces tick infestations. Parasit. Vec. 10:206. doi: 10.1186/s13071-017-2136-2

McHardy, N., Woollon, R. M., Clampitt, R. B., James, J. A., and Crawley, R. J. (1986). Efficacy, toxicity and metabolism of imidocarb dipropionate in the treatment of *Babesia ovis* infection in sheep. Res. Vet. Sci. 41, 14–20.

McNally, K. L., Mitzel, D. N., Anderson, J. M., Ribeiro, J. M., Valenzuela, J. G., Myers, T. G., et al. (2012). Differential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. Ticks Tick Borne Dis. 3, 18–26. doi: 10.1016/j.ttbdis.2011.09.003

Mercado-Curiel, R. F., Palmer, G. H., Guerrero, F. D., and Brayton, K. A. (2011). Temporal characterisation of the organ-specific *Rhipicephalus microplus* transcriptional response to *Anaplasma marginale* infection. Int. J. Parasitol. 41, 851–860. doi: 10.1016/j.ijpara.2011.03.003

Merino, O., Antunes, S., Mosqueda, J., Moreno-Cid, J. A., Perez de la Lastra, J. M., Rosario-Cruz, R., et al. (2013). Vaccination with proteins involved in tick-pathogen interactions reduces vector infestations and pathogen infection. Vaccine 31, 5889–5896. doi: 10.1016/j.vaccine.2013.09.037

Mihalca, A. D., Dumitrache, M. O., Magdas, C., Gherman, C., Domsa, C., Mircean, V., et al. (2012). Synopsis of the hard ticks (Acari: Ixodidae) of Romania with update on

host associations and geographical distribution. Exp. Appl. Acarol. 58, 183–206. doi: 10.1007/s10493-012-9566-5

Moltmann, U. G., Mehlhorn, H., and Friedhoff, K. T. (1982a). Electron-microscopic study on the development of *Babesia ovis* (piroplasmia) in the salivary glands of the vector tick *Rhipicephalus bursa*. Acta Trop. 39, 29–40.

Moltmann, U. G., Mehlhorn, H., and Friedhoff, K. T. (1982b). Ultrastructural study of the development of *Babesia ovis* (Piroplasmia) in the ovary of the vector tick *Rhipicephalus bursa*. J. Protozool. 29, 30–38. doi: 10.1111/j.1550-7408.1982.tb02877.x

Moreira, H N, Barcelos, R. M., Vidigal, P. M., Klein, R. C., Montandon, C. E., Maciel, T. E., et al. (2017). A deep insight into the whole transcriptome of midguts, ovaries and salivary glands of the *Amblyomma sculptum* tick. Parasitol. Int. 66, 64–73. doi: 10.1016/j.parint.2016.10.011

Neelakanta, G., and Sultana, H. (2015). Transmission-blocking vaccines: focus on anti-vector vaccines against tick-borne diseases. Arch. Immunol. Ther. Exp. 63, 169–179. doi: 10.1007/s00005-014-0324-8

Nene, V., Lee, D., Kang'a, S, Skilton, R., Shah, T., de Villiers, E., et al. (2004). Genes transcribed in the salivary glands of female *Rhipicephalus appendiculatus* ticks infected with *Theileria parva*. Insect Biochem. Mol. Biol. 34, 1117–1128. doi: 10.1016/j.ibmb.2004.07.002

Ord, R. L., and Lobo, C. A. (2015). Human babesiosis: pathogens, prevalence, diagnosis and treatment. Curr. Clin. Microbiol. Rep. 2, 173–181. doi: 10.1007/s40588-015-0025-z

Perera, R., Riley, C., Isaac, G., Hopf-Jannasch, A. S.,Moore, R. J.,Weitz, K.W., et al. (2012). Dengue virus infection perturbs lipid homeostasis in infected mosquito cells. PLoS Pathog. 8:e1002584. doi: 10.1371/journal.ppat.1002584

Perner, J., Provazník, J., Schrenková, J., Urbanová, V., Ribeiro, J.M., and Kopáček, P. (2016). RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks. Sci. Rep. 6:36695. doi: 10.1038/srep36695

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45. doi: 10.1093/nar/29.9.e45

Raele, D. A., Galante, D., Pugliese, N., De Simone, E., and Cafiero, M. A. (2015). *Coxiella*-like endosymbiont associated to the "Anatolian brown tick" *Rhipicephalus bursa* in Southern Italy. Microbes Infect. 17, 799–805. doi: 10.1016/j.micinf.2015.09.011

Ramamoorthi, N., Narasimhan, S., Pal, U., Bao, F., Yang, X. F., Fish, D., et al. (2005). The Lyme disease agent exploits a tick protein to infect the mammalian host. Nature 436, 573–577. doi: 10.1038/nature03812

Ranjbar-Bahadori, S., Eckert, B., Omidian, Z., Shirazi, N. S., and Shayan, P. (2012). *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. Parasitol Res. 110, 1531–1536. doi: 10.1007/s00436-011-2658-z

Rodriguez, P. B. R., Cruz, R. R., Garcia, D. I. D., Gutierrez, R. H., Quintanilla, R. E. L., Sahagun, D. O., et al. (2016). Identification of immunogenic proteins from ovarian tissue and recognized in larval extracts of *Rhipicephalus* (*Boophilus*) *microplus*, through an immunoproteomic approach. Exp. Parasitol. 170, 227–235. doi: 10.1016/j.exppara.2016.10.005

Rokyta, D. R., Margres, M. J., and Calvin, K. (2015). Post-transcriptional mechanisms contribute little to phenotypic variation in snake venoms. G3 5, 2375–2382. doi: 10.1534/g3.115.020578

Santos-Silva, M. M., Beati, L., Santos, A. S., De Sousa, R., Nuncio, M. S., Melo, P., et al. (2011). The hard-tick fauna of mainland Portugal (Acari: Ixodidae): an update on geographical distribution and known associations with hosts and pathogens. Exp. Appl. Acarol. 55, 85–121. doi: 10.1007/s10493-011-9440-x

Sauer, J. R., Essenberg, R. C., and Bowman, A. S. (2000). Salivary glands in ixodid ticks: control and mechanism of secretion. J. Insect Physiol. 46, 1069–1078. doi: 10.1016/S0022-1910(99)00210-3

Schmieder, R., and Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863–864. doi: 10.1093/bioinformatics/btr026

Schroeder, C. L. C., Narra, H. P., Sahni, A., Khanipov, K., Patel, J., Fofanov, Y., et al. (2017). Transcriptional profiling of *Rickettsia prowazekii* coding and non-coding transcripts during in vitro host-pathogen and vector-pathogen interactions. Ticks Tick Borne Dis. 8, 827–836. doi: 10.1016/j.ttbdis.2017.06.008

Schulz, M. H., Zerbino, D. R., Vingron, M., and Birney, E. (2012). Oases: robust *de novo* RNA-seq assembly across the dynamic range of expression levels. Bioinformatics. 28, 1086–1092. doi: 10.1093/bioinformatics/bts094

Sevinc, F., Guler, L., Sevinc, M., Ekici, O. D., and Isik, N. (2013). Determination of immunoreactive proteins of *Babesia ovis*. Vet. Parasitol. 198, 391–395. doi: 10.1016/j.vetpar.2013.09.012

Shahein, Y. E., Abouelella, A. M., Hussein, N. A., Hamed, R. R., El-Hakim, A. E., Abdel-Shafy, S., et al. (2013). Identification of four novel *Rhipicephalus annulatus* upregulated salivary gland proteins as candidate vaccines. Protein J. 32, 392–398. doi: 10.1007/s10930-013-9498-x

Šimo, L., Kazimirova, M., and Richardson, J., Bonnet, S. I. (2017). The essential role of tick salivary glands and saliva in tick feeding and pathogen transmission. Front. Cell Infect. Microbiol. 7:281. doi: 10.3389/fcimb.2017.00281

Smith, A. D., and Kaufman, W. R. (2013). Molecular characterization of the vitellogenin receptor from the tick, *Amblyomma hebraeum* (Acari: Ixodidae). Insect Biochem. Mol. Biol. 43, 1133–1141. doi: 10.1016/j.ibmb.2013.10.002

Sonenshine, D. E., and Michael Roe, R. (2014). Biology of Ticks. New York, NY: Oxford University Press.

Taheri,M., Nabian, S., Ranjbar, M., Fard, R.M. N., Sadeghian, A. G., and Sazmand, A. (2014). Study of vitellogenin in *Boophilus annulatus* tick larvae and its immunological aspects. Trop. Biomed. 31, 398–405.

Tan, A. W. L., Francischetti, I. M. B., Slovak, M., Kini, R. M., and Ribeiro, J. M. C. (2015). Sexual differences in the sialomes of the zebra tick, *Rhipicephalus pulchellus*. J. Proteomics 117, 120–144. doi: 10.1016/j.jprot.2014.12.014

Tepass, U. (2012). The apical polarity protein network in *Drosophila* epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annu. Rev. Cell Dev. Biol. 28, 655–685. doi: 10.1146/annurev-cellbio-092910-154033

Tepass, U., Tanentzapf, G., Ward, R., and Fehon, R. (2001). Epithelial cell polarity and cell junctions in *Drosophila*. Annu. Rev. Genet. 35, 747–784. doi: 10.1146/annurev.genet.35.102401.091415

Thompson, D. M., Khalil, S. M. S., Jeffers, L. A., Sonenshine, D. E., Mitchell, R. D., Osgood, C. J., et al. (2007). Sequence and the developmental and tissue specific

regulation of the first complete *vitellogenin* messenger RNA from ticks responsible for heme sequestration. Insect Biochem. Mol. Biol. 37, 363–374. doi: 10.1016/j.ibmb.2007.01.004

Tirloni, L., Reck, J., Terra, R.M. S., Martins, J. R., Mulenga, A., Sherman, N. E., et al. (2014). Proteomic analysis of cattle tick *Rhipicephalus* (*Boophilus*) *microplus* saliva: a comparison between partially and fully engorged females. PLoS ONE 9:e94831. doi: 10.1371/journal.pone.0094831

Trimnell, A. R., Davies, G. M., Lissina, O., Hails, R. S., and Nuttall, P. A. (2005). A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine. Vaccine 23, 4329–4341. doi: 10.1016/j.vaccine.2005.03.041

Trimnell, A. R., Hails, R. S., and Nuttall, P. A. (2002). Dual action ectoparasite vaccine targeting 'exposed' and 'concealed' antigens. Vaccine 20, 3560–3568. doi: 10.1016/S0264-410X(02)00334-1

Valdés, J. J., Cabezas-Cruz, A., Sima, R., Butterill, P. T., RuŽek, D, and Nuttall, P. A. (2016). Substrate prediction of *Ixodes ricinus* salivary lipocalins differentially expressed during *Borrelia afzelii* infection. Sci. Rep. 6:32372. doi: 10.1038/srep32372

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:research0034. doi: 10.1186/gb-2002-3-7-research0034

Vega, C. A., Buening, G. M., Green, T. J., and Carson, C. A. (1985). *In vitro* cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46, 416–420.

Villar, M., Ayllon, N., Alberdi, P., Moreno, A., Moreno, M., Tobes, R., et al. (2015). Integrated metabolomics, transcriptomics and proteomics identifies metabolic pathways affected by *Anaplasma phagocytophilum* infection in tick cells. Mol. Cell Proteomics 14, 3154–3172. doi: 10.1074/mcp.M115.051938

Villar, M., Ayllon, N., Busby, A. T., Galindo, R. C., Blouin, E. F., Kocan, K.M., et al. (2010). Expression of heat shock and other stress response proteins in tcks and cultured tick cells in response to *Anaplasma* spp. infection and heat shock. Int. J. Proteomics 2010:657261. doi: 10.1155/2010/657261

Villar, M., Popara, M., Ayllon, N., Fernandez de Mera, I. G., Mateos-Hernandez, L., Galindo, R. C., et al. (2014). A systems biology approach to the characterization of stress

response in *Dermacentor reticulatus* tick unfed larvae. PLoS ONE 9:e89564. doi: 10.1371/journal.pone.0089564

Walker, J. B., Keirans, J. E., and Horak, I. G. (2000). The genus *Rhipicephalus* (Acari, Ixodidae): a guide to the brown ticks of the World. Trop. Anim. Health Prod. 32, 417–418. doi: 10.1017/CBO9780511661754

Yang, X., Yu, Z., He, Y., Xu, X., Gao, Z., Wang, H., et al. (2015). Purification of vitellin and dynamics of vitellogenesis in the parthenogenetic tick *Haemaphysalis longicornis* (Acari: Ixodidae). Exp. Appl. Acarol. 65, 377–388. doi: 10.1007/s10493-014-9866-z

Yeruham, I., Hadani, A., and Galker, F. (2001). The effect of the ovine host parasitaemia on the development of *Babesia ovis* (Babes, 1892) in the tick *Rhipicephalus bursa* (Canestrini and Fanzago, 1877). Vet. Parasitol. 96, 195–202. doi: 10.1016/S0304-4017(00)00433-7

Yu, X., Gong, H., Zhou, Y., Zhang, H., Cao, J., and Zhou, J. (2015). Differential sialotranscriptomes of unfed and fed *Rhipicephalus haemaphysaloides*, with particular regard to differentially expressed genes of cysteine proteases. Parasit. Vec. 8:597. doi: 10.1186/s13071-015-1213-7

Zivkovic, Z., Esteves, E., Almazan, C., Daffre, S., Nijhof, A. M., Kocan, K. M., et al. (2010). Differential expression of genes in salivary glands of male *Rhipicephalus* (*Boophilus*) *microplus* in response to infection with *Anaplasma marginale*. BMC Genomics 11:12. doi: 10.1186/1471-2164-11-186

2. "Quantitative Proteomics Identifies Metabolic Pathways Affected by *Babesia* Infection and Blood Feeding in the Sialoproteome of the Vector *Rhipicephalus bursa*"

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2.1. Abstract

The negative impact of ticks and tick-borne diseases on animals and human health is driving research to discover novel targets affecting both vectors and pathogens. The salivary glands are involved in feeding and pathogen transmission, thus are considered as a compelling target to focus research. In this study, proteomics approach was used to characterize *Rhipicephalus bursa* sialoproteome in response to *Babesia ovis* infection and blood feeding. Two potential tick protective antigens were identified and its influence in tick biological parameters and pathogen infection was evaluated. Results demonstrate that the *R. bursa* sialoproteome is highly affected by feeding but infection is well tolerated by tick cells. The combination of both stimuli shifts the previous scenario and a more evident pathogen manipulation can be suggested. Knockdown of *ub2n* led to a significative

increase of infection in tick salivary glands but a brusque decrease in the progeny, revealing its importance in the cellular response to pathogen infection, which is worth pursuing in future studies. Additionally, an impact in the recovery rate of adults (62%), the egg production efficiency (45.75%), and the hatching rate (88.57 %) was detected. Building knowledge on vector and/or pathogen interplay bridges the identification of protective antigens and the development of novel control strategies.

Keywords: ticks; *Babesia*; proteomic; RNAi; UB2N; PCCA

2.2. Introduction

Ticks have a significant negative impact on host species through their feeding behavior, causing direct skin and sub-cutaneous tissue damage and blood depletion, and also acting as vectors of different pathogens such as viruses, bacteria, and protozoa [1,2]. Belonging to the Ixodidae family, *Rhipicephalus bursa* is a multi-host tick widely distributed in the Mediterranean region having cattle, sheep, and goats as its primary hosts but can occasionally be found in wild ungulates, small mammals, or even humans [3,4,5]. This tick species is the main vector of the etiological agent of ovine babesiosis, *Babesia ovis*. This tick-borne disease affects small ruminants and is prevalent in Eastern Asia, Southern Europe (Mediterranean basin), Middle East, and Northern Africa, overlapping *R. bursa* geographical distribution [6,7]. Ovine babesiosis is an acute disease whose onset is characterized by high fever, that can progress to other clinical symptoms such as hemolytic anaemia, hemoglobinuria, icterus, and in severe cases, pancytopenia. Untreated cases usually lead to death and even upon treatment the animal may die as the result of a heavy infection or suffer disease relapse after the withdrawal of therapy [8,9,10]. Despite an established enzootic situation in countries such as Iran, fatal disease outbreaks have been reported in Spain and particularly in Turkey, demonstrating the deleterious effect of *B. ovis* in naïve sheep transferred from a tick-free region to a *R. bursa*-infested region with endemic babesiosis [11,12,13]. As in other babesiosis, disease control relies on chemotherapy with imidocarb dipropionate to manage clinical symptoms, and on vector control using acaricides [14,15]. Both these strategies have major drawbacks in the host, such as safety issues concerning animal-derived food products as milk contamination but

also the potential carcinogenicity of imidocarb [16,17]. Furthermore, acaricide resistance and its detrimental impact in the environment [14,15,18] supports the need for safer alternatives for disease control. A deeper understanding of tick biology and tick-pathogen interactions is fundamental to identify candidate protective antigens that can be targeted to reduce vector competence and ultimately control babesiosis.

Pathogens have co-evolved and adapted to survive within the tick vector cells by regulating host processes such as the acquisition of nutrients, modification of the host environment, and meddling with immune responses [18,19,20,21,22,23] that could be targeted for the identification of protective antigens [24,25]. After entering the vector, pathogens need to disseminate through tick tissues, infect and multiply within salivary gland (SG) cells to be successfully transmitted to susceptible hosts during tick blood meal [23]. Tick SGs are morphologically complex organs with multifunctional roles in different biological processes such as osmoregulation, feeding, and pathogen transmission [1,26,27,28,29,30,31,32,33,34]. Tick salivary compounds, or sialome, include a plethora of molecules essential to counteract host immune reaction to tick attachment and feeding, including anti-platelet aggregator compounds, anticoagulants, and vasodilators that will be released to host bloodstream via saliva [1,34]. SGs are also responsible for the production of cement cone related proteins which are not only accountable for an efficient attaching but also show antimicrobial properties and act against the host immune system [35,36].

Previous studies focused on tick sialome, aiming to characterize the transcriptome and proteome for different tick species and recently the sialotranscriptome response of adult *R. bursa* to *B. ovis* infection has been investigated [37]. As in other tick species, results confirmed the complexity of the SG transcriptomics response to different conditions such as pathogen infection and feeding [37,38] leading to the synthesis of a wide range of proteins [26,37].

Thus, proteomics approach was used in the present study to obtain first, information regarding the SG protein composition and second, to evaluate the sialoproteome in response to blood feeding and pathogen infection. The present study constitutes the first *R. bursa* sialoproteome report, demonstrating the dynamic changes occurring in the tick-pathogen interface. Understanding the SG molecular dynamics is a key for the discovery

of pharmacologically active compounds of clinical interest such as protective antigens for anti-tick and pathogen transmission blocking vaccines.

2.3. Materials and Methods

2.3.1. Ethics Statement

This study was carried out with the approval of the Divisão Geral de Alimentação e Veterinária (DGAV), Portugal, (under Art° 49, Portaria n°1005/92 from 23rd October, permit number 0421/2013) and the Council of Ethics of the Instituto de Higiene e Medicina Tropical (IHMT). Animals experiments were conducted in accordance with the national and European Animal Welfare legislation (in frame with DL 113/2013 and Directive 2010/63/EU) and the principle of the Three R's, to replace, reduce, and refine the use for scientific purposes.

2.3.2. *Rhipicephalus bursa* Tick Colony

According to the described protocol [39], established *R. bursa* colony was fed in white rabbits (strain Hyla) and for moulting kept in a chamber regulated at 25 ± 1 °C, 70 ± 10% relative humidity, and a photoperiod of 16:8 (light:dark) at Instituto Nacional de Saúde Doutor Ricardo Jorge. After oviposition and during the two generations, eggs and ticks were tested for pathogens (*Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp.) by PCR, using the protocols and primers described elsewhere [40,41,42,43]. Pathogen-free progeny was then used to establish the tick colony.

2.3.3. *Babesia ovis* Culture

Babesia ovis (Israeli strain) were maintained *in vitro* at the Institute of Hygiene and Tropical Medicine (IHMT) as previously described by Antunes et al. 2018 [37].

2.3.4. Infection and Feeding of *Rhipicephalus bursa* Ticks

The experimental design concerning the production of female *R. bursa* ticks is described in Figure 1.

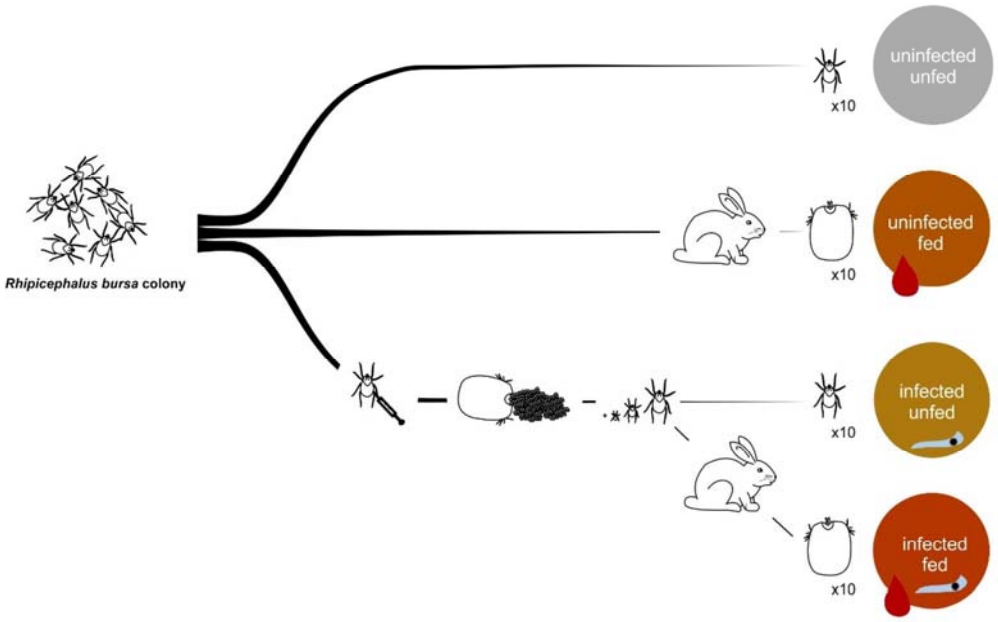


Figure 1. Experimental design for production of ticks at different feeding and infection conditions.

From a pathogen-free colony, four groups of 10 *R. bursa* females were generated in triplicate: uninfected unfed (grey circle), uninfected fed (orange circle), infected unfed (yellow circle), and infected fed (red circle). The uninfected unfed ticks were obtained directly from the colony; and the uninfected fed ticks were carefully detached from the rabbit ear after blood meal. Infection of groups were achieved by inoculation of *B. ovis* in female ticks. After egg laying and hatching, infected batch of larvae developed to the adult phase. To obtain the infected unfed group, ticks were directly used, and to obtain infected fed groups, ticks were allowed to feed. Finally, tick salivary glands were dissected to perform the proteomics analysis of the *R. bursa* sialoproteome.

Briefly, four groups of ticks were generated: uninfected unfed (NINF), uninfected fed (NIF), infected unfed (INF), and infected fed (IF). Uninfected unfed ticks from the colony were used to obtain NINF group and adult female ticks feeding on rabbits were carefully removed from the rabbit ear 6-8 days post attachment to produce the NIF group. To produce *B. ovis* infected ticks, female adult ticks were directly inoculated in the first leg articulation of trochanter-coxae with *B. ovis* from a 15–20% infected blood culture and allowed to feed in rabbits. After drop-off, females were kept under the rearing conditions described above. Progenies were tested for *B. ovis* as described elsewhere [44].

Infected larvae were allowed to feed in order to obtain adults. A part of infected batch of female ticks were fed to obtain the IF group and the remaining ticks were used to produce the INF group.

2.3.5. Tick Dissection, DNA Extraction, and *B. ovis* Infection

First, ticks were rinsed individually in distilled water and 75% (v/v) ethanol. Salivary glands (SG) of *R. bursa* females were dissected in ice-cold phosphate-buffered saline (PBS) under a stereomicroscope and stored in RNA later (Ambion, Austin, TX, USA) at −20 °C. DNA was extracted using TRI-Reagent® (Sigma–Aldrich, MO, USA). SG infection was evaluated using the above referred protocol [44].

2.3.6. Protein Extraction and Trypsin Digestion

SG were homogenized with a 20 gauge needle in lysis buffer (7 M Urea, 2 M Thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, CHAPS). Samples were sonicated for 1 min in an ultrasonic cooled bath and vortexed for 10 s. After three cycles of sonication-vortex, the homogenates were centrifuged at 200× g for 5 min to remove cellular debris. The supernatants were collected, and protein concentration was determined using the RC-DC protein assay (Bio-Rad, CA, USA) with BSA as standard.

Protein extracts (200 µg) were precipitated and digested as performed by Artigas-Jerónimo and colleagues [45], until the peptides were finally desalted onto OMIX Pipette tips C18 (Agilent Technologies, CA, USA), dried-down and stored at −20 °C until downstream applications.

2.3.7. Proteome Analysis by SWATH-MS

The desalted protein digests were resuspended in 2% acetonitrile with 5% acetic acid and analyzed by reverse phase liquid chromatography coupled online with mass spectrometry (RP-LC-MS/MS) using an Ekspert nLC 415 system combined to a 6600 TripleTOF® mass spectrometer (AB SCIEX®, MA, USA) through information-dependent acquisition (IDA) followed by sequential windowed data independent acquisition of the total high-resolution mass spectra (SWATH). Approximately 5 µg of each protein digest from each replicate sample were pooled together as a mixed sample from each group (uninfected unfed, uninfected fed, infected unfed, and infected fed).

Pooled mixed samples were then used for the generation of the reference spectral ion library as part of SWATH-MS analysis. For details, see Supplementary Material and Methods S1.

2.3.8. Library Generation/Protein Identification, Data Processing and Relative Quantitation

A spectral library of all the detectable peptides in the samples and relative quantitation was performed according to Estrada-Peña [46], with the exception of spectra identification which was performed by searching against a compiled database containing all sequences from Ixodidae and *Babesia* taxonomies and rabbit (*Oryctolagus cuniculus*) proteome (135,071, 19,087 and 21,178 Uniprot entries, respectively, in September, 2017) with the following parameters: iodoacetamide cysteine alkylation, trypsin digestion, identification focus on biological modification, and thorough ID as search effort. The MS raw proteomics data have been deposited at the PeptideAtlas repository (http://www.peptideatlas.org/) with the dataset identifier PASS01362. All the identified proteins and quantitation data are represented in the Supplementary spreadsheet.

2.3.9. Gene ontology

Gene ontology was obtained using Blast2GO software (version 3.0.11, available at http://www.blast2go.org) [47,48]. Homology to the protein identification (UniprotID) was searched by blast against Arthropoda (nr subset) [arthropoda, taxa:6656] from 30.01.2017 as well as a mapping and annotation steps to assign functional terms at level 3. GO terms were also assigned manually based on UniProt-associated databases. GO frequency and protein regulation charts were constructed for each condition using the Microsoft Office 2016 Excel tool. To elucidate about the GO and the differentially representation of proteins in response to infection, feeding, or both, chord diagrams were generated using the GOplot R package in RStudio (Version 1.1.453) [49].

2.3.10. *In Silico* Analysis of Proteins, Selection of Targets and Recombinant Protein, and Peptide Production

Proteins commonly represented in the four conditions were further characterized using the software STRING 10.5 (Search Tool for the Retrieval of Interacting Genes/Proteins available at http://string-db.org) in order to identify known/novel protein–protein interactions in the *Ixodes scapularis* database. Briefly, the program generates the network images based on a spring model. The selection of targets for further analysis was based on three main criteria: (1) Proteins present in all the datasets from proteomics; (2) proteins that may have a pivotal role in tick-parasite interplay; and (3) proteins that may be potential protective antigens resulting in a vaccine candidate. The amino acid sequences of L7M1X7 and L7MAU7 were analyzed *in silico* in order to predict the protein localization (CELLO v.2.5 [50]), transmembrane domains (TMHMM v.2.0, based on a hidden Markov model [51,52]), signal peptides (SignalP v.5.0 [53]), antigenic determinants (using the method developed by Kolaskar and Tongaonkar [54] available at http://imed.med.ucm.es/Tools/antigenic.pl), solubility (PROSOII [55]), and crystallizability (SECRET [56]). With a purity higher than 75%, a recombinant protein (based on L7M1X7, UB2N, 151 a.a.) and a peptide (VKTPEECVKIAQSIGYPVMIKASAGGGGKGMRIAWND based on L7MAU7, PCCA, 37 a.a.) were selected to be synthetically produced by GenScript Corporation (Piscataway, NJ, USA) for the polyclonal antibodies production and immunoassays. To increase peptide immunogenicity, an Imject™ Blue Carrier™ Protein (highly soluble, mollusc-derived hemocyanin) (Pierce Biotechnology Inc., IL, USA) was conjugated with the peptide using one step glutaraldehyde conjugation [57]. Protein concentration was assessed by spectrophotometry using a NanoDrop ND-1000 (Thermo Scientific, MA, USA) and samples were analyzed by SDS-PAGE. Briefly, 10 µg of peptide or Imject™ Blue Carrier™ Protein or recombinant protein and 50 µg of protein extracts from each condition were re-suspended in Laemmli buffer (Bio-Rad, CA, USA) containing 5% (v/v) of 2-β-mercaptoethanol, separated on a 12.5% or 4–20% discontinuous SDS-PAGE gels.

2.3.11. Hybridoma and Polyclonal Antibody Production

Polyclonal antibodies were obtained by immunization of 3–4 weeks-old CD1 male mice (reared in IHMT) with the recombinant protein or conjugated peptide. For each target, three CD1 male mice were primed and boosted intraperitoneally every 2–3 weeks with 20 µg of protein or conjugated peptide emulsified with incomplete Freund's adjuvant (Sigma–Aldrich, MO, USA) in a 1:1 proportion. Pre-immune serum was collected prior to immunization and serum from mandibular vein blood was collected before each inoculation to monitor anti-target antibodies titers by indirect ELISA. The mouse with higher antibody titer was selected and 3 days before euthanasia a final boost was given. Spleens from the selected animals were collected, as well as total blood, to further obtain spleen cells and antiserum, respectively. Spleen cells were used to fuse with Sp2/0 myeloma cells (ATCC) (previously cultured in DMEM media, supplemented with 10% of fetal bovine serum (Gibco, Invitrogen Corporation, Paisley, UK)) at a ratio of 1:1 in the presence of polyethylene glycol (PEG, Sigma–Aldrich, MO, USA). Hybridoma cells were selected in DMEM media, 10% (v/v) fetal calf serum (Biowest, MO, USA) supplemented with hypoxanthine-aminopterin-thymidine HAT (Sigma–Aldrich, MO, USA) and subsequently cloned by limiting dilution technique. Cell cultures were maintained at 37 °C in a 5 % CO2 incubator. Clones producing the highest titers of specific antibodies, as assessed in the indirect ELISA and Western blot, were selected for further use.

2.3.12. Indirect ELISA and Western Blot

To determinate the antibody titer of mice serum and cell supernatant, the ELISA protocol described by Couto et al. (2017) [58] was employed with minor modifications: a high binding 96-well ELISA plate (Corning® Costar®, MA, USA) was coated with 0.1 µg of peptide or protein diluted in PBS. Mice serum (diluted 1:200 in PBS) or cell supernatant (without dilution) was incubated for one hour at 37 °C. Antibody capacity to recognize specific targets was assessed by Western blot. After SDS-PAGE, the proteins were transferred overnight at a constant 25 V to a nitrocellulose membrane, with a pore size of 0.2 µm (Bio-Rad, CA, USA), using the Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, CA, USA). Membranes were stained with Ponceau S (Figure S5) and

polyacrylamide gels with BlueSafe (NZYTech, Lisbon, PT) to validate the transfer process. Later, membranes were blocked with 5% (w/v) non-fat dry milk (Bio-Rad, CA, USA) in PBS containing 0.05% (v/v) Tween 20 (PBS-T) at room temperature (RT) for one hour. After washing with Tris-buffered saline complemented with 0.05% (v/v) Tween 20 (TBST), membranes were incubated with mouse serum (1:200) or a hybridoma supernatant (without dilution), for 2 h at RT. After three 15-min washes with TBST, membranes were incubated for 1 h with alkaline phosphatase-conjugated anti-mouse polyvalent immunoglobulins (G, A, M) (1:3000; Sigma–Aldrich, MO, USA) or a goat anti-mouse IgG (H+L)-horseradish peroxidase conjugated secondary antibody (1:2000; Bio-Rad, CA, USA). The antigen–antibody complexes were detected using the alkaline phosphatase (AP) conjugate substrate kit (Bio-Rad, CA, USA) or ECL Western blotting detection reagent (ECL, GE Healthcare Life Sciences, PA, USA) exposed for 10 s and 10 min on a Hyperfilm (GE Healthcare Life Sciences, PA, USA). Validation of differential protein representation on SG protein extracts used for proteomic analysis was performed by Western blot using polyclonal serum and hybridomas produced. Protein band intensities were estimated using ImageJ Software (version 1.51K). Additionally, the *pcca* and *ub2n* expression was also assessed using qPCR in infected and naïve SGs of fed R. bursa ticks, following the protocol described below.

2.3.13. Synthesis of dsRNA and RNAi Assays

RNA obtained from *R. bursa* females was used to synthesize cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA), and the cDNA was subsequently used to amplify a region of interest of each mRNA sequence from unrelated mouse *beta-2-microglobulin* (*β2m*), *pcca* and *ub2n* genes using specific primers containing T7 promoter sequences at the 5′- end (Supplementary Table S2) [59,60] according to previous studies. Females ticks were injected with 138 nL of dsRNA (with 1.38×10^{11} molecules) following the protocol described in other studies [37,61].

Gene silencing assays were performed to evaluate the effect of the genes that code for L7M1X7 and L7MAU7 proteins on tick biological parameters and *Babesia* infection. The conditions of this assays were previously described in Antunes et al. 2018 [37]. Briefly, a splenectomised, six-month-old lamb was maintained and fed *ad libitum* at the Instituto Nacional de Investigação Agrária e Veterinária animal facility. The lamb was

intravenously inoculated with 3 mL of cryopreserved *B. ovis* culture with 6% of parasitemia and infection was monitored using qPCR described in the following section. In parallel, a *R. bursa* colony reared and maintained in IHMT was used to obtain adult ticks. Four groups were generated: two control groups (unrelated mouse *beta-2-microglobulin* dsRNA, *dsβ2M*, and non-inoculated), a group targeting UB2N and another targeting PCCA. Each group included fifty female ticks that were previously injected with gene-specific dsRNA and fifty male ticks to allow mating to further analyze reproductive-related parameters. Infestation was performed during infection peak and ticks were allowed to feed for 9 days in a specific tick-feeding cell. Ticks were monitored daily and dropped ticks were collected. After 9 days, attached ticks were manually removed. Ticks were randomly selected for two purposes: ten ticks were dissected to further evaluate gene knockdown efficiency and infection rate in the SG, and the remaining ticks were maintained under controlled conditions to evaluate the biological parameters of the progeny and transovarial transmission of *Babesia*.

2.3.14. Gene Expression and Knockdown Assessment

To evaluate gene expression and knockdown efficiency through qPCR, ten female ticks were randomly selected *per* group and its SG dissected as previously described. Total RNA and DNA were extracted from each sample using TRI-Reagent®. RNA quantity was determined using the ND-1000 Spectrophotometer (NanoDrop ND1000) and its integrity was evaluated using the Qubit™ RNA IQ Assay Kit in the Qubit™ 4 Fluorometer (Thermo Scientific, MA, USA). RNA concentrations of 1 µg/µL were used for cDNA synthesis with iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) in a T100 Thermal Cycler (Bio-Rad, CA, USA). Using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA), *ub2n* and *pcca* expression was assessed. The following conditions were used: initial cycle of denaturation at 95 °C for 10 min; followed by 45 cycles of 95 °C for 15 s and temperature of each primer set for 30 s (Supplementary Table S3) [59,60]; and finally a dissociation curve (55–95 °C, 0.5 °C/s). Negative controls and standard curves (constructed with ten-fold serial dilutions) were included in each qPCR to validate reaction specificity and determine the PCR efficiency. The average expression stability (M-value) of the reference genes (Supplementary Table S3) [62] and gene relative quantification were assessed based in the geNorm algorithm [63] and the

Pfaff method [64], respectively, included in the CFX Manager™ Software (Bio-Rad, CA, USA).

2.3.15. *Babesia ovis* Quantification

In order to evaluate the infection in the host blood as well as in tick SG after feeding and in progeny, absolute quantification of *B. ovis* was assessed by qPCR. Genomic DNA was extracted from 200 µL of blood collected at day 32, 34, 35, 37, 38, 41, 44, and 46 from the lamb; and from ticks SG and larvae, using TRI-Reagent® as described above. qPCR reactions of 10 µL were performed in triplicate using SYBR Green Supermix kit (Bio-Rad, CA, USA) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The following conditions were used: Initial cycle of denaturation at 95 °C for 5 min; followed by 49 cycles of 95 °C for 10 s and temperature of each primer set for 30 s (Supplementary Table S4) [59,60]; and finally a dissociation curve (55–95 °C, 0.5 °C/s). Reaction specificity was validated by including negative controls using RNase-free water as template. To determine the PCR efficiency and gene copy number, synthetized gBlocks® Gene Fragments (Integrated DNA Technologies, Leuven, BE) (Supplementary Table S4) [65] were used to produce standard curves with ten-fold serial dilutions. Based on Dandasena et al. (2018) [66], copy number was calculated using the Equation (1):

$$number\ of\ copies\ (molecules) = \frac{a\ ng\ \times 6.0221x10^{23}}{b\ g/mol\ \times 1x10^{9}\ ng/g}$$

Equation 1. Gene copy number equation.

a: mean of quantity obtain from qPCR, b: target molar mass.

Babesia infection in the host was evaluated as the ratio of copy number of *BoSPD*/Ov18S genes, whereas in the vector it was evaluated by *BoSPD*/16S genes. *Babesia* infection was compared between dsRNA-inoculated groups using the non-parametric Mann-Whitney test (SPSS v24.0) [67]. Logarithm (10 based) was applied to evaluated the percentage of increase or reduction of infection.

2.3.16. Tick Biological Parameters

Recovery rate (RR, as the percentage of the ratio between live ticks and the total number of female ticks), drop-off (DO, as the percentage of the ratio between ticks that

dropped-off and the total number of female ticks), and engorged female weight (EFW) were determined in order to elucidate about tick fitness, while reproductive parameters such as egg mass weight (EMW), egg production efficiency (EPE, as percentage of the ratio between EMW and EFW), and egg hatching rate (EHR, as the mean value of visual evaluation performed by five technicians separately) were analyzed after dropped-off females laid the eggs. The data are expressed as mean ± standard deviation, and statistical significance was determined using SPSS v24.0 [67] (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Chi-square and Phi and Cramer's V tests were used to evaluate the level of association of RR and DO between dsRNA-inoculated groups. Normality and homogeneity of variance were first checked using Shapiro-Wilk's and Levene's tests, respectively. Mann-Whitney and t-test were used as non-parametric and parametric tests.

2.4. Results and Discussion

2.4.1. *R. bursa* Sialoproteome

The main objective of this study was the identification of candidate tick protective antigens based on the characterization of the *R. bursa* sialoproteome in response to blood feeding and *B. ovis* infection. Four conditions with 10 *R. bursa* females each were produced in triplicate, to understand the processes of infection when ticks are unfed or fed, as well as the effect of feeding when ticks are uninfected and infected (Figure 1).

SG were dissected from all groups for DNA and protein extraction, to perform respectively, *B. ovis* detection and proteomics analysis. The PCR detected *Babesia* DNA in all samples from infected groups, confirming the infection of these ticks (Supplementary Figure S1). Proteomics analysis of tick SGs were carried out resulting in the identification of a total of 1617 proteins, in which a high percentage of proteins (98.08 %) corresponded to the tick vector. After excluding the host and parasite-related proteins, from the 1586 tick proteins identified, 585 differentially represented proteins were found ($p < 0.05$) in response to blood feeding or parasite infection and used for further characterization (Figure 2).

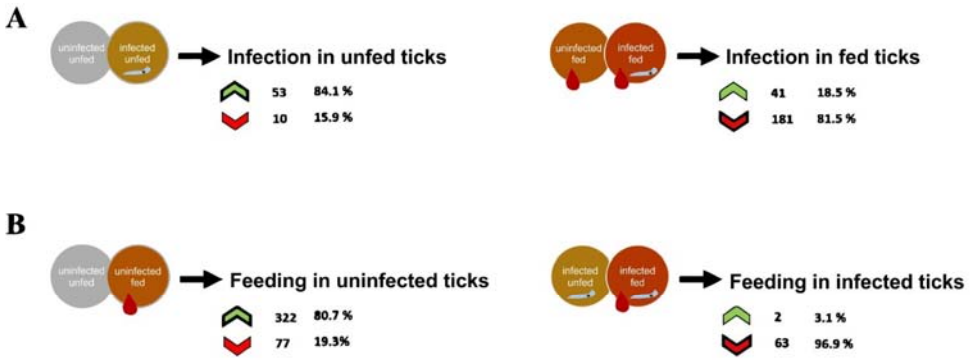


Figure 2. Differentially represented proteins from the *R. bursa* sialoproteome after infection (A) and blood feeding (B). For each condition it is represented the process of infection (parasite) and feeding (blood drop), as well as the number and percentage of proteins differentially over (green arrow) and under (red arrow) represented in *R. bursa* salivary glands. Grey circle + no symbols = uninfected and unfed ticks, yellow circle + parasite = infected and unfed ticks, orange circle + blood drop = uninfected and fed ticks, red circle + parasite + blood drop = infected and fed ticks.

The results showed that while infection in unfed ticks resulted in a higher number of over-represented than under-represented proteins, in fed ticks the number of under-represented proteins increased and was higher than the number of over-represented proteins (Figure 2A). In uninfected ticks, blood feeding resulted in a higher number of over-represented proteins but in infected ticks blood feeding reduced the levels of a larger number of proteins (Figure 2B). These results suggested that the response to *Babesia* infection and blood feeding leads to an increase in tick vector protein levels when acting independently, but the combination of both stimuli overcomes this effect by reducing protein levels in response to feeding and infection.

Gene ontology (GO) annotation was assessed for each UniProt ID obtained from the *R. bursa* proteome using Blat2GO and UniProt-related databases. Of the 1586 identified-proteins, only 97 proteins were classified as "unknown" due to the absence of GO and domain function. The remaining 1489 annotated proteins were classified according to the GO terms molecular function (MF), biological process (BP), and cellular component (CC) at level 3. Focusing on the 585 differentially represented proteins, the representation of GO terms in each condition is shown in Figure 3 and Figure 4 and Supplementary Figures S2 and S3.

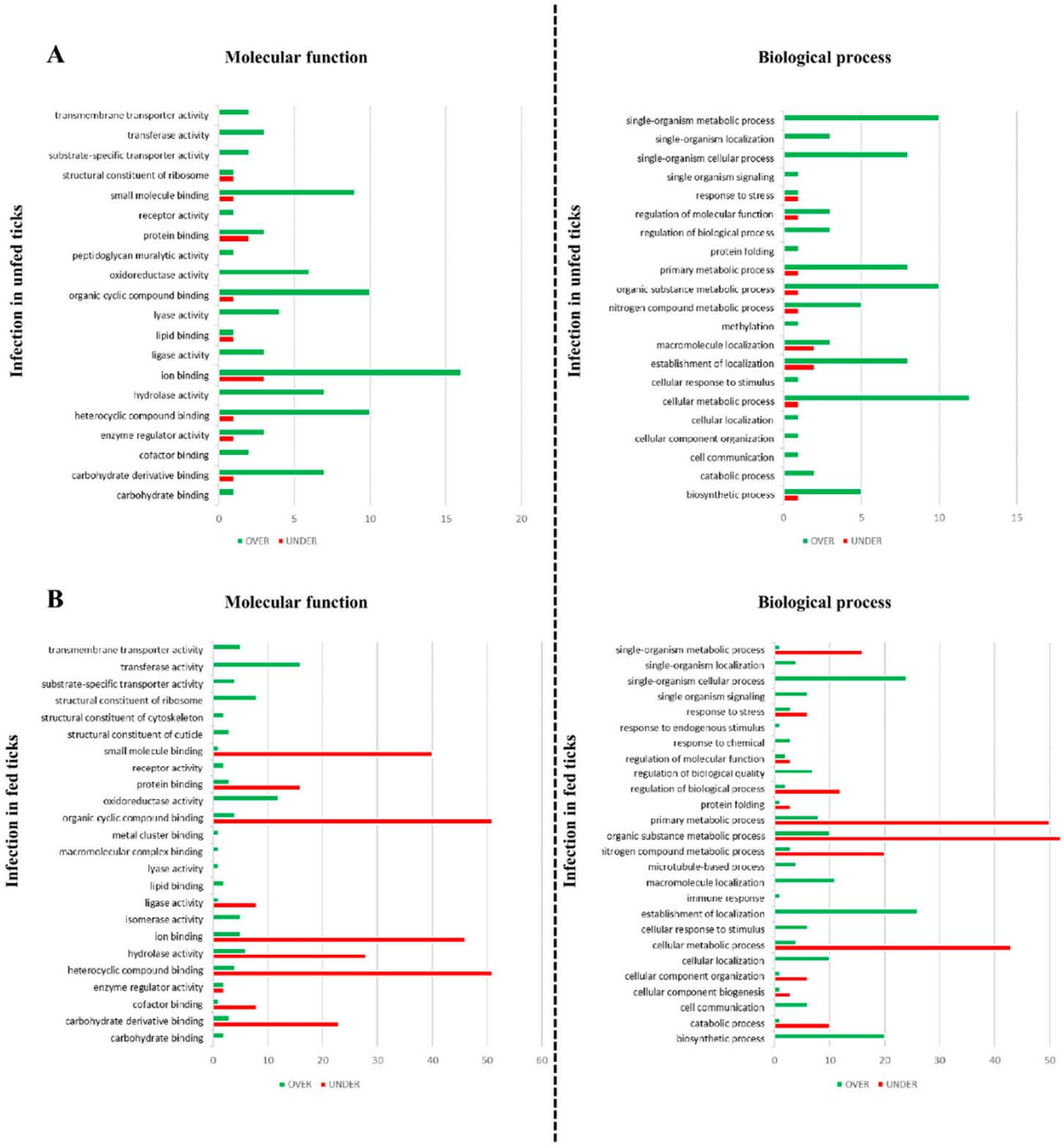


Figure 3. Gene ontology of the effect of *B. ovis* infection in unfed (A) and fed (B) *R. bursa* ticks. Functional terms at level 3 were assigned based on UniProt and associated databases. Green bars = over-represented proteins, red bars = under-represented proteins.

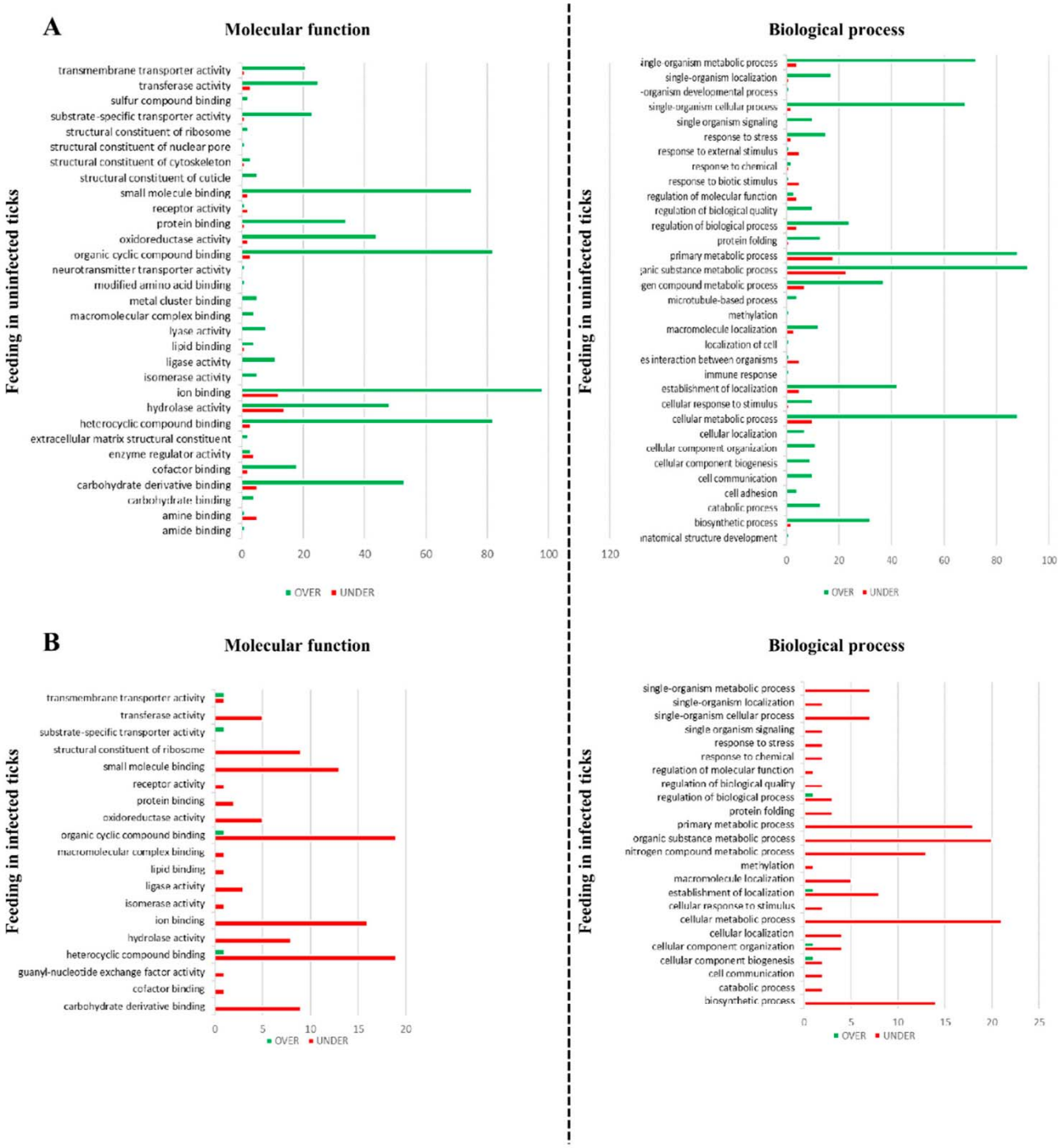


Figure 4. Gene ontology of the effect of feeding in uninfected (A) and *B. ovis* infected (B) *R. bursa* ticks. Functional terms at level 3 were assigned based on UniProt and associated databases. Green bars = over-represented proteins, red bars = under-represented proteins.

2.4.2. Effect of *Babesia* Infection on *R. bursa* Sialoproteome

To characterize the effect of *B. ovis* infection on *R. bursa* SG, proteomics data from uninfected unfed ticks was compared to the infected unfed group (Figure 2A and Figure 3A and Supplementary Figure S2A).

In Figure 2A, a set of 63 proteins differentially represented were obtained, where 84.1% and 15.9% were significantly over- and under-represented proteins, respectively. Such lower number of differently represented proteins suggest that *Babesia* might influence *R. bursa* SGs translation but at a lower rate because of its parasitic relationship [18]. Focusing on the GO, the sialoproteome during infection presented more proteins linked to cell and membrane part, membrane-bounded organelle (CC) (Supplementary Figure S2A), heterocyclic and organic cyclic compound binding, ion binding, small molecule binding (MF) (Figure 3A), cellular, organic substance, and single-organism metabolic processes but also with establishment of localization (BP) (Figure 3A). Additionally, some GO terms were exclusively constituted by over-represented proteins and none exclusively under-represented (Figure 3A). Peptidoglycan muralytic activity was a GO term exclusive of this process of infection in unfed ticks, being constituted by an over-represented protein (UniProt ID: L7M9R2), presenting domains that are found among peptidoglycan recognition proteins being associated to innate immunity and conserved between insects and mammals [68]. By recognizing microbial particles and activating antimicrobial defense systems such as prophenoloxidase and Toll receptor cascade, it is possible to induce the production of antimicrobial peptides [68] that have a negative effect on parasite multiplication and survival [69]. Together with the presence of only a GO term (GO term: “response to stress”) associated to cellular response (N = 2), as well as the absence of proteins related to “regulation of biological quality” and “response to chemical,” this cellular response suggest that *Babesia* is recognized by *R. bursa* SG cells in a moderate manner, producing lower levels of such proteins because of its evolutionary relationship [70,71].

2.4.3. Effect of Blood Feeding on *R. bursa* Sialoproteome

A perspective of feeding process was obtained with the comparison of SG proteome from uninfected unfed and uninfected fed groups (Figure 2B and Figure 4A and Supplementary Figure S3A).

In this set, from the 399 differently represented proteins, 80.7% and 19.3% were found to be over and under-represented, respectively (Figure 2B), belonging to a wide range of functional classes (Figure 4A and Supplementary Figure S3A). In our previous work concerning *R. bursa* sialotranscriptome, the cellular machinery was highly activated when uninfected ticks undergo blood feeding demonstrated that during *R. bursa* feeding SG shifts from an inactive to a metabolically active status with intense gene transcription prevailing gene over expression (75% up regulated) [37]. Overall, both studies clearly demonstrate that blood ingestion requires a high production of cellular molecules, which is reflected by the high expression of genes and its subsequent translation. In this set of proteins, multiple GO terms are exclusively over-represented. Moreover several proteins were exclusively found in this dataset, being associated to amide and amine binding, extracellular matrix structural constituent, modified amino acid binding, neurotransmitter transporter activity, structural constituent of nuclear pore, and sulphur compound binding (MF), as well as anatomical structural development, cell adhesion, interspecies interaction between organisms, localization of cells, response to biotic and external stimulus, and finally, single-organism development process (BP). Besides that, the GO terms related to "structural constituent of cuticle," "cell-cell junction," and "anatomical structural development" confirms the investment of blood feeding in tick engorgement and development [72].

2.4.4. Effect of Infection in the Sialoproteome of Fed *R. bursa*

To evaluate the process of infection in fed ticks, the infected fed and uninfected fed sialoproteomes were compared (Figure 2A and Figure 3B and Supplementary Figure S2B).

A high number of proteins (N = 222) were found significantly represented, with 81.5% being under-represented (Figure 2A). Tick feeding is a process that demands the synthesis of a high number of molecules [37,73] in which transcription and translation

appears to be correlated resulting in an over expression and over representation of both transcripts and proteins. In contrast, in the presence of parasite infection this does not seems to occur. While, sialotranscripts of fed *R. bursa* during *B. ovis* infection that were analyzed in Antunes study [37] demonstrated that its majority is up regulated (64%), in the present study the majority of the differentially represented proteins are under-represented suggesting that infection in fed ticks may influence translation and ultimately protein production. Exposure to infection in fed ticks resulted in the production of proteins linked to diverse GO terms (Figure 3B and Supplementary Figure S2B). The most represented GO terms of CC and MF categories are the same when ticks are fed or unfed, being more under-represented when tick have a blood meal. At BP level, cellular, organic substance and single-organism metabolic processes are the most represented GO terms (Figure 3B), constituted by more proteins under-represented. Some GO terms are entirely over-represented, being only six CC GO terms associated exclusively to under-represented proteins (protein, ribonucleoprotein and transporter complex, supramolecular polymer, virion part and whole membrane). "Response to endogenous stimulus" was a GO term only present in this condition of infection in fed ticks. Besides, other two GO term related to stress response were identified ("response to stress" and "response to chemical"). This suggests that the production of proteins that mediate cellular response to stimuli is being stimulated only when blood meal occurs.

2.4.5. Effect of Feeding in the Sialoproteome of *B. ovis* Infected *R. bursa*

By comparing the infected fed and infected unfed sialoproteomes, a feeding process is analyzed when ticks are subjected to infection (Figure 2B and Figure 4B and Supplementary Figure S3B).

In this context, from a total of 65 proteins, 96.9% proteins were under-represented (Figure 2B). The most represented GO terms are represented in Figure 4A and Supplementary Figure S3A, with more proteins under-represented than in the uninfected dataset. The majority of GO terms includes exclusive under-represented proteins, except DNA packing and protein-DNA complex, outer and whole membrane (CC), and finally substrate-specific transporter activity (MF) that are exclusively over-represented. Interestingly, when ticks are exposed to both *Babesia* infection and blood meal (infection

in fed ticks and feeding in infected ticks), over-represented proteins with DNA packaging and protein-DNA complex properties are commonly presented. Such proteins influence the transcription and translation mechanism resulting in a decrease in the production of proteins as shown before.

2.4.6. *R. bursa* Cellular Machinery in Response to Feeding and Infection

To build knowledge about the response of *R. bursa* SG, the datasets were analyzed to identify proteins commonly represented at the different conditions (Supplementary Table S1). Figure 5 summarizes the proteins involved in infection, feeding, or both.

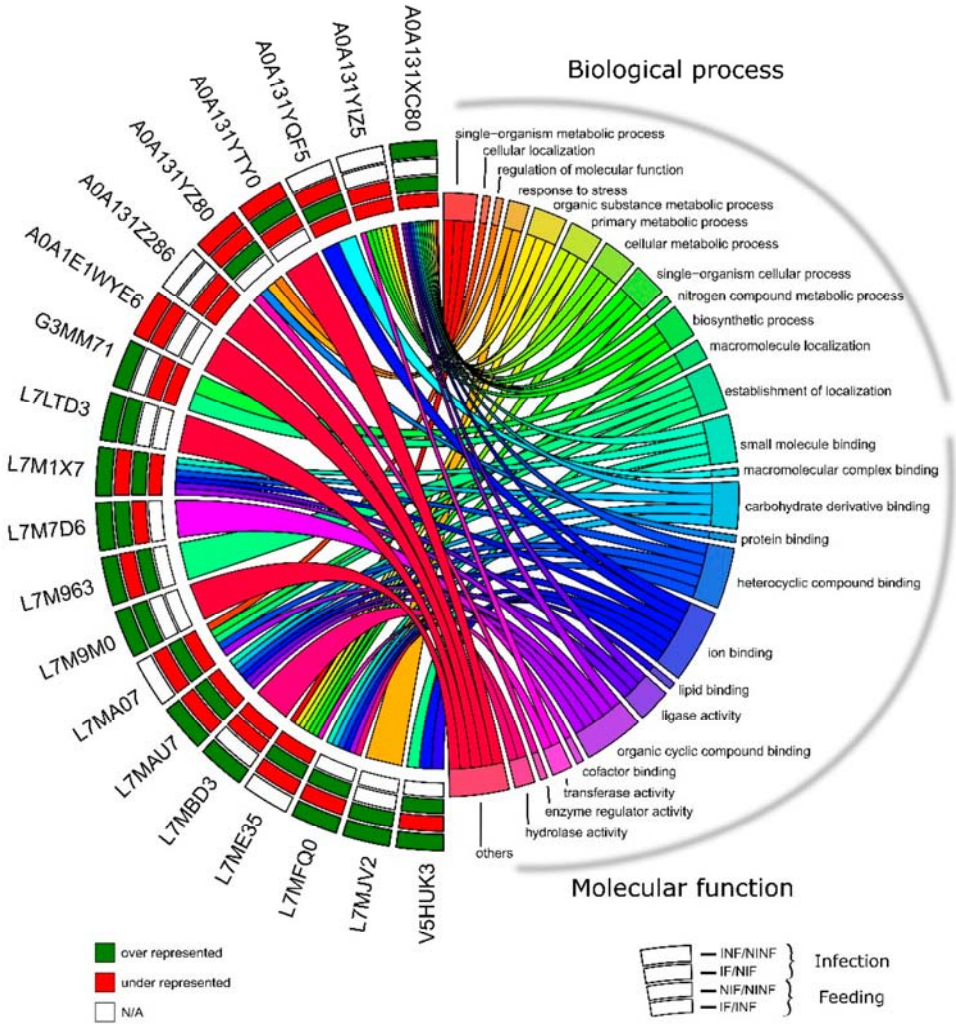


Figure 5. Chord diagram presenting gene ontology of the differentially represented proteins in *R. bursa* sialoproteome in response to infection and/or feeding. Each protein found in each comparison is shown on the left alongside with UniProt ID, while the GO clusters are shown on the right. Outer annulus

to inner annulus: infection in unfed ticks (INF/NINF), infection in fed ticks (IF/NIF), feeding in uninfected ticks (NIF/NINF), and feeding in infected ticks (IF/INF). Green square: over-represented, red square: under-represented and white square: not applicable.

The process of infection (when ticks are fed or unfed) modulate the representation of twelve proteins of *R. bursa* SGs, while blood meal (when ticks are infected or uninfected) influence ten proteins. From these, two proteins, a putative ubiquitin-protein ligase (UniProt ID: L7M1X7, UB2N) and an uncharacterized protein (UniProtID: L7MAU7, PCCA), were found in all comparisons, its representation being modulated positively when ticks are exposed to a single stimulus (INF/NINF and NIF/NINF) and negatively when ticks are exposed to both stimuli (IF/NIF and IF/INF) (Figure 5, Supplementary Table S1). According to the defined criteria for target selection for RNA interference studies, *in silico* analysis were performed revealing characteristics such as the putative function, subcellular localization, and immunogenicity of these proteins. STRING analysis showed that those targets and their network are linked to ubiquitination and metabolic pathways (Supplementary Figure S4), essential for tick fitness [21,74] and described as drug targets in other contexts [75,76]. CELLO, TMHMM, and SignalP servers predicted the cytoplasmatic localization and absence of transmembrane helices or signal peptides in both proteins, that alongside with their antigenic propensity (UB2N: 1.0357, with 8 antigenic determinants; PCCA: 1.0357, with 28 antigenic determinants) indicate that those proteins could be tested to evaluate their potential as protective antigens.

2.4.7. The Role of a Putative Ubiquitin-Protein Ligase in *R. bursa* and *B. ovis* Interface

Ubiquitination is a biological process that affects proteins by adding to them ubiquitin moieties [77]. This process could influence proteins by altering their cellular location, activity, and interaction with other molecules, being involved in pleiotropic roles such as protein degradation [78], cell–cell communication [79], pathogen invasion [80], and innate immune system [80]. Ubiquitin addition involves the sequential action of three main groups of enzymes: ubiquitin-activating (E1s), ubiquitin-conjugating (E2s), and ubiquitin-ligase (E3s) enzymes [79]. An E1 enzyme interacts with an E2 that

subsequently coupled with a specific E3 leading to ubiquitin incorporation in a protein sequence. The putative ubiquitin-protein ligase, UB2N (UniProt ID: L7M1X7) possesses several domains that are common among E2s (CDD: cd00195, InterPro: IPR023313 and IPR000608) reflecting its function as an ubiquitin-conjugating enzyme. This 17.1 kDa protein was found and validated as positively regulated in response to feeding or infection stimulus alone (Proteomics: INF/NINF = 0.57, NIF/NINF = 0.49; Western blot: NIF/NINF = 78347.143/ND), while occurrence of both processes, feeding and infection, lead to its negative regulation (Proteomics: IF/NIF = −1.07, IF/INF = −1.15; Western blot: IF/NIF = ND/78347.143) (Supplementary Table S1, Figure 6A).

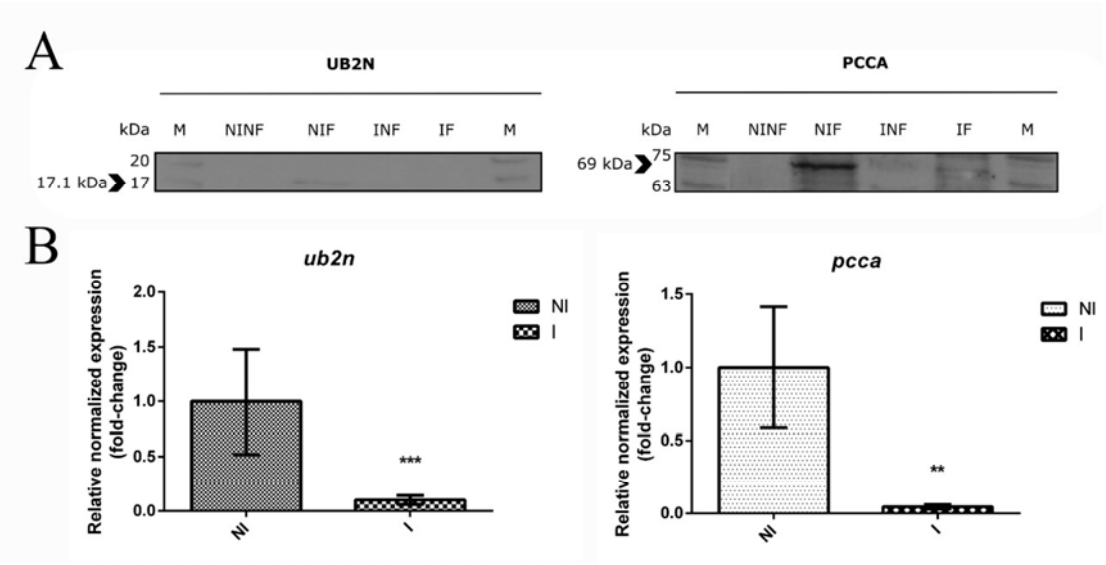
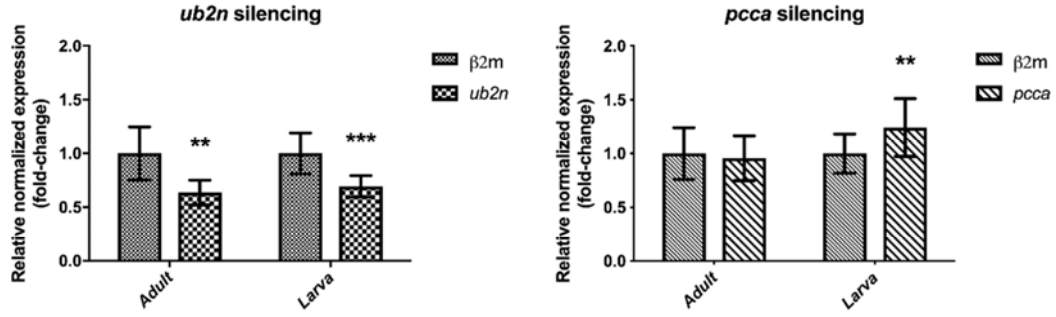


Figure 6. Protein representation and gene expression of selected targets. (A) Western blot of UB2N and PCCA. Protein extracts from salivary glands exposed to different conditions were used to validate the protein representation of UB2N and PCCA by using mouse serum (1:200) and a hybridoma supernatant (without dilution), respectively. Arrows indicate the molecular size of the target. M: molecular weight, NZYColour Protein Marker II, NZYTech. NINF: uninfected unfed. NIF: uninfected fed. INF: infected unfed. IF: infected fed. Exposure and contrast parameters were not modified. The full-length blots are displayed in Supplementary Figure S5. **(B)** Relative expressions of *ub2n* and *pcca* were evaluated in fed uninfected and fed *B. ovis* infected salivary glands of *R. bursa* using qPCR. Data was normalized using 16S rRNA, *elongation factor*, and *β-tubulin* reference genes. The expression of fed uninfected group (control) is set to 1 for a better interpretation. NI: fed uninfected group. I: fed infected group. Statistical analysis were conducted using the Pfaff method. Significance is represented by ** $p < 0.01$, *** $p < 0.001$.

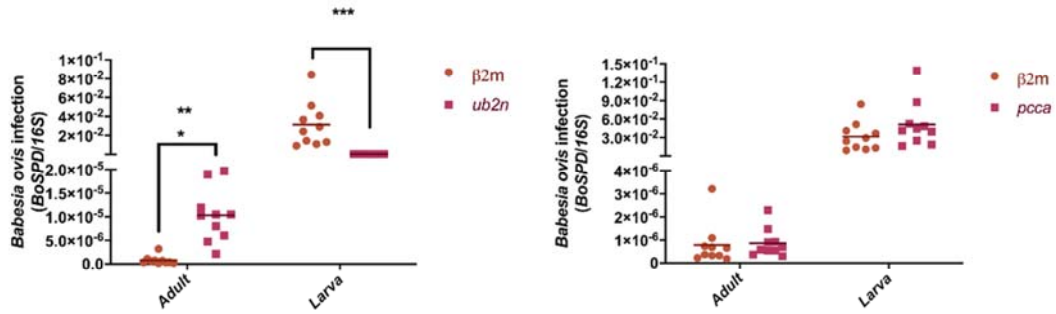
Also, qPCR results demonstrates that the *ub2n* transcript was down regulated (qPCR: 0.126, p value < 0.001, Figure 6B) during infection when ticks are fed, suggesting no

impact of the translation process [81] in protein levels. Such directional regulation of UB2N and pivotal role in the cell machinery and pathogen colonization reflects its potential as a protective vaccine candidate, as described in other studies for other ubiquitination-related proteins [74]. This regulation could be a way of SG cells to overcome a specific event such as feeding or infection by stimulating the ubiquitination pathway in order to achieve homeostasis and cellular protection [79,80]. However, when dealing with various extracellular threats, cells become sensitive to several stimuli [82] and could be influenced by manipulative organisms [80] such as *Babesia*. Considering this, RNAi assays were conducted in order to evaluate the impact of *ub2n* knockdown in *B. ovis* infection (in SGs and progeny) and tick fitness (Figure 7).

A



B



C

	RR[a] (%)	DO[a] (%)	EFW[b] (g, Ave ± SD)	EMW[c] (g, Ave ± SD)	EPE[b] (%)	EHR[b] (%)
β2m	80 % (40/50)	48 % (24/50)	0.3454 ± 0.1027	0.1808 ± 0.1075	48.59 ± 3.27	94.38 ± 4.82
ub2n	62 % (31/50)*	36 % (18/50)	0.2916 ± 0.0779	0.1359 ± 0.0703	45.75 ± 2.98**	88.57 ± 5.34*
pcca	58 % (29/50)*	40 % (20/50)	0.2941 ± 0.1139	0.1388 ± 0.0538	39.58 ± 14.06	66.34 ± 27.23*

[a] *Chi-square test,* [b] *Mann-Whitney test,* [c] *Unpaired t test.*

Figure 7. Effect of dsRNA-inoculation in *R. bursa* during *B. ovis* infection. (A) Gene knockdown assessment by measuring *ub2n* and *pcca* relative expressions in dsRNA-inoculated ticks. Data were normalized using 16S rRNA, *elongation factor*, and *β-tubulin* reference genes. The expression of *β2m*-inoculated group (control) is set to 1 for a better interpretation. Statistical analysis were conducted using

the Pfaff method. **(B)** *B. ovis* infection in salivary glands (Adult) and progeny (Larvae) of dsRNA-inoculated female *R. bursa* ticks. A ratio between copy number of *BoSPD* and 16SrRNA for each sample in each condition is represented. Statistical analysis were conducted using the Mann-Whitney test. **(C)** Evaluation of tick biological parameters after dsRNA inoculation. Data are represented as percentage, ratio, means, and standard deviation. Statistical analysis were conducted using the Chi-squared, Mann-Whitney, and Student's t tests. RR: recovery rate, DO: drop-off, EFW: engorged female weight, EMW: egg mass weight, EPE: egg production efficiency, EHR: egg hatching rate. Significance is represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Regarding the adult ticks, ds*ub2n* inoculation resulted in a significant reduction of *ub2n* mRNA levels (0.636; $p = 0.010$) in SG with a silencing efficiency of 36.4% (Figure 7A). Moreover, *ub2n* silencing lead to a significant increase in *B. ovis* infection in *R. bursa* SGs (18.32 %, $p < 0.001$) (Figure 7B), suggesting the UB2N as a protective molecule against *Babesia*. Shaw and colleagues also demonstrated that the reduction of ubiquitin-related enzymes gene expression hampered protection against *Anaplasma phagocytophilum* infection leading to an increase of bacteria load [74]. Regarding tick biological parameters (Figure 7C), results show that *ub2n* silencing did not influence female engorgement (EFW, p value = 0.104) and drop-off (DO, X2 = 1.478, p value = 0.224). This absence of impact in tick feeding was previously demonstrated after silencing an *ubiquitin-ligase enzyme* (XIAP) [74]. The mortality rate increased significantly (RR, p value = 0.047, Phi and Cramer's V: weak/moderate) suggesting an essential role of UB2N in tick survival. Additionally, results show that *ub2n* silencing did not influence significantly egg weight (EMW, p value = 0.102) (Figure 7C). However, a negative influence in egg production (EPE, p value = 0.001) and its viability (EHR, p value = 0.045) was observed in ds*ub2n*-inoculated group. Assessment of gene knockdown in the progeny of dsRNA inoculated ticks showed a significant reduction of *ub2n* expression (0.693; $p < 0.001$) in larvae with a silencing efficiency of 30.7% (Figure 7A) confirming that gene silencing can be perpetuated through future generations. While *Babesia* infection increased in SGs, the opposite occurred in larvae suggesting an impact in *Babesia* transovarial transmission since the parasite load decreased abruptly (−138.53%, $p < 0.001$) (Figure 7B). The hypothesis of *ub2n* expression stabilization that could stimulate the IMD signalling pathway promoting antimicrobial peptides production [83] capable to control apicomplexan infection [69] is discarded in this context since the silencing efficiency was similar to the adult phase. Such decrease of infection could be

explained by a putative effect of *ub2n* silencing in ovaries, *i.e.*, could have blocked the invasion of *B. ovis* in the ovaries and consequently through the progeny.

The results of the present study indicate that even with a silencing efficiency of about 30%, ticks and parasite were significantly affected in both stages. Overall, UB2N demonstrated to be a key molecule in tick biology with an important role on the cellular response to pathogen infection worth to pursue in future studies, specially evaluate its effect in ovary development.

2.4.8. New Insights about an Uncharacterized Protein

Biotin-dependent carboxylases, which includes a major group that uses as substrate coenzyme A (CoA), *e.g.*, acetyl-CoA carboxylase (ACCA), propionyl-CoA carboxylase (PCCA), and 3-methylcrotonyl-CoA carboxylase (MCCA) [84], are key molecules in several metabolic pathways influenced during tick-pathogen interplay including fatty acid, amino acid, and carbohydrate metabolisms [21,22]. Such enzymes are considered attractive targets for drug discovery against several diseases, including bacterial and fungal infections [85,86]. The previously mentioned uncharacterized protein (UniProtID: L7MAU7, PCCA) has similarities to the sequence and domains of a propionyl-CoA carboxylase alpha chain protein from *Rhipicephalus appendiculatus* (E-value: 0.0, Identity: 96.1%), thus its function as a PCCA enzyme can be assumed. In most organisms, this carboxylase catalyzes the conversion of a glucose precursor propionyl-CoA to D-methylmalonyl-CoA in the mitochondrial matrix, playing a role in the catabolism of β-branched amino acids, cholesterol side chain and fatty acids [84]. During feeding, catabolism of molecules allows the use of smaller elements in anabolic reactions required for tick development. In our proteomics analysis, this 69.5 kDa protein was found over-represented in response to feeding stimulus alone (Proteomics: NIF/NINF = 0.30; Western blot: NIF/NINF = 99915.597/ND) and also in response to infection (Proteomics: INF/NINF = 0.38; Western blot: INF/NINF = 9234.421/ND) (Supplementary Table S1, Figure 6A). This positive regulation suggests a role of PCCA enzyme as a key for energy supply, maybe through the formation of building blocks or nutrients that ultimately contributes to tick and parasite growth [21]. Moreover, previous studies reported that bacteria and fungi metabolize and detoxify propionyl-CoA by the 2-methylcitrate cycle [87] to overcome its toxicity and growth inhibition properties [88]. In ticks such

detoxification and cell growth maintenance could be achieved by PCCA since this carboxylase catalyzes the propionyl-CoA. Interestingly, when feeding and infection processes are combined, the protein levels of PCCA in tick SGs (Proteomics: IF/NIF = −0.48, IF/INF = −0.55; Western blot: IF/NIF = 22503.948/99915.597) (Supplementary Table S1, Figure 6A) as well as the gene expression decrease (qPCR: 0.058, p value < 0.001, Figure 6B). We hypothesize that such reduction of PCCA culminate in a toxic environment to tick cells as well as growth inhibition, facilitating *Babesia* dissemination since this apicomplexan parasite could use an alternative way such as the 2-methylcitrate cycle to surpass toxic environments and cell growth inhibition in order to pursue infection dissemination. Based on this and considering the potential of carboxylases as versatile targets for drug discovery against apicomplexan infections [85,89,90,91], silencing assays were conducted in order to evaluate the influence of *pcca* mRNA reduction on *Babesia* infection and tick biological parameters (Figure 7). In the conditions undertaken in the present study, *pcca* gene knockdown was not achieved (0.957; p = 0.285) (Figure 7A) with only 4.3 % reduction of mRNA levels. Also, the progeny of *pcca* dsRNA inoculated ticks revealed that *pcca* expression increased significantly (1.242; p = 0.010). Further studies are required to clarify if PCCA has a role in the metabolic pathways related to tick development that could be influenced by infection.

2.5. Conclusions

The numerous proteins detected in the *R. bursa* SG highlight the complexity of the processes in this issue. The dynamic response of *R. bursa* SG to feeding, infection, and to both stimuli was characterized, pinpointing the potential tick antigens involved in relevant tick biological functions. RNAi assays place UB2N as an important protein in the cellular response to pathogen infection in *R. bursa*, which should be further explored. The putative role of PCCA in the evaluated tick parameters and infection is not disclosed herein; however future experiments using different conditions should be performed to characterize it.

2.6. References

1. Šimo, L.; Kazimirova, M.; Richardson, J.; Bonnet, S.I. The Essential Role of Tick Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. Front. Cell. Infect. Microbiol. 2017, 7, 281.

2. Dantas-Torres, F.; Chomel, B.B.; Otranto, D. Ticks and tick-borne diseases: A One Health perspective. Trends Parasitol. 2012, 28, 437–446.

3. Ferrolho, J.; Antunes, S.; Santos, A.S.; Velez, R.; Padre, L.; Cabezas-Cruz, A.; Santos-Silva, M.M.; Domingos, A. Detection and phylogenetic characterization of *Theileria* spp. and *Anaplasma marginale* in *Rhipicephalus bursa* in Portugal. Ticks Tick. Borne. Dis. 2016, 7, 443–448.

4. Santos-Silva, M.M.; Beati, L.; Santos, A.S.; De Sousa, R.; Núncio, M.S.; Melo, P.; Santos-Reis, M.; Fonseca, C.; Formosinho, P.; Vilela, C.; et al. The hard-tick fauna of mainland Portugal (Acari: Ixodidae): An update on geographical distribution and known associations with hosts and pathogens. Exp. Appl. Acarol. 2011, 55, 85–121.

5. Mihalca, A.D.; Dumitrache, M.O.; Magda¸s, C.; Gherman, C.M.; Dom¸sa, C.; Mircean, V.; Ghira, I.V.; Pocora, V.; Ionescu, D.T.; Sikó Barabási, S.; et al. Synopsis of the hard ticks (Acari: Ixodidae) of Romania with update on host associations and geographical distribution. Exp. Appl. Acarol. 2012, 58, 183–206.

6. Ranjbar-Bahadori, S.; Eckert, B.; Omidian, Z.; Shirazi, N.S.; Shayan, P. *Babesia ovis* as the main causative agent of sheep babesiosis in Iran. Parasitol. Res. 2012, 110, 1531–1536.

7. Rjeibi, M.R.; Gharbi, M.; Mhadhbi, M.; Mabrouk, W.; Ayari, B.; Nasfi, I.; Jedidi, M.; Sassi, L.; Rekik, M.; Darghouth, M.A. Prevalence of piroplasms in small ruminants in North-West Tunisia and the first genetic characterisation of *Babesia ovis* in Africa. Parasite 2014, 21, 23.

8. Esmaeilnejad, B.; Tavassoli, M.; Asri-Rezaei, S. Investigation of hematological and biochemical parameters in small ruminants naturally infected with *Babesia ovis*. Vet. Res. Forum. 2012, 3, 31–36.

9. Sevinc, F.; Guler, L.; Sevinc, M.; Ekici, O.D.; Isika, N. Determination of immunoreactive proteins of *Babesia ovis*. Vet. Parasitol. 2013, 198, 391–395.

10. Erster, O.; Roth, A.;Wolkomirsky, R.; Leibovich, B.; Savitzky, I.; Shkap, V. Transmission of *Babesia ovis* by different *Rhipicephalus bursa* developmental stages and infected blood injection. Ticks Tick. Borne. Dis. 2016, 7, 13–19.

11. Hurtado, A.; Barandika, J.; Oporto, B.; Minguijón, E.; Povedano, I.; García-Pérez, A.L. Risks of suffering tick-borne diseases in sheep translocated to a tick infested area: A laboratory approach for the investigation of an outbreak. Ticks Tick. Borne. Dis. 2015, 6, 31–37.

12. Sevinc, F.; Zhou, M.; Cao, S.; Ceylan, O.; Aydin, M.F.; Sevinc, M.; Xuan, X. Haemoparasitic agents associated with ovine babesiosis: A possible negative interaction between *Babesia ovis* and *Theileria ovis*. Vet. Parasitol. 2018, 252, 143–147.

13. Haghi, M.M.; Etemadifar, F.; Fakhar, M.; Teshnizi, S.H.; Soosaraei, M.; Shokri, A.; Hajihasani, A.; Mashhadi, H. Status of babesiosis among domestic herbivores in Iran: A systematic review and meta-analysis. Parasitol. Res. 2017, 116, 1101–1109.

14. Domingos, A.; Antunes, S.; Borges, L.; Rosário, V.E. do Approaches towards tick and tick-borne diseases control. Rev. Soc. Bras. Med. Trop. 2013, 46, 265–269.

15. Vudriko, P.; Okwee-Acai, J.; Tayebwa, D.S.; Byaruhanga, J.; Kakooza, S.; Wampande, E.; Omara, R.; Muhindo, J.B.; Tweyongyere, R.; Owiny, D.O.; et al. Emergence of multi-acaricide resistant *Rhipicephalus* ticks and its implication on chemical tick control in Uganda. Parasit. Vectors 2016, 9, 4.

16. Belloli, C.; Lai, O.R.; Ormas, P.; Zizzadoro, C.; Sasso, G.; Crescenzo, G. Pharmacokinetics and Mammary Elimination of Imidocarb in Sheep and Goats. J. Dairy Sci. 2006, 89, 2465–2472.

17. McHardy, N.;Woollon, R.M.; Clampitt, R.B.; James, J.A.; Crawley, R.J. Efficacy, toxicity and metabolism of imidocarb dipropionate in the treatment of *Babesia ovis* infection in sheep. Res. Vet. Sci. 1986, 41, 14–20.

18. de la Fuente, J.; Antunes, S.; Bonnet, S.; Cabezas-Cruz, A.; Domingos, A.G.; Estrada-Peña, A.; Johnson, N.; Kocan, K.M.; Mansfield, K.L.; Nijhof, A.M.; et al. Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases. Front. Cell. Infect. Microbiol. 2017, 7, 114.

19. Ayllón, N.; Villar, M.; Busby, A.T.; Kocan, K.M.; Blouin, E.F.; Bonzón-Kulichenko, E.; Galindo, R.C.; Mangold, A.J.; Alberdi, P.; Pérez de la Lastra, J.M.; et al.

Anaplasma phagocytophilum inhibits apoptosis and promotes cytoskeleton rearrangement for infection of tick cells. Infect. Immun. 2013, 81, 2415–2425.

20. Ayllón, N.; Villar, M.; Galindo, R.C.; Kocan, K.M.; Šíma, R.; López, J.A.; Vázquez, J.; Alberdi, P.; Cabezas-Cruz, A.; Kopáˇcek, P.; et al. Systems Biology of Tissue-Specific Response to *Anaplasma phagocytophilum* Reveals Differentiated Apoptosis in the Tick Vector *Ixodes scapularis*. PLoS Genet. 2015, 11, e1005120.

21. Cabezas-Cruz, A.; Espinosa, P.J.; Obregón, D.A.; Alberdi, P.; de la Fuente, J. *Ixodes scapularis* Tick Cells Control *Anaplasma phagocytophilum* Infection by Increasing the Synthesis of Phosphoenolpyruvate from Tyrosine. Front. Cell. Infect. Microbiol. 2017, 7, 375.

22. Cabezas-Cruz, A.; Alberdi, P.; Valdés, J.J.; Villar, M.; de la Fuente, J. *Anaplasma phagocytophilum* Infection Subverts Carbohydrate Metabolic Pathways in the Tick Vector, *Ixodes scapularis*. Front. Cell. Infect. Microbiol. 2017, 7, 23.

23. Antunes, S.; Rosa, C.; Couto, J.; Ferrolho, J.; Domingos, A. Deciphering *Babesia*-Vector Interactions. Front. Cell. Infect. Microbiol. 2017, 7, 429.

24. de la Fuente, J.; Kopáˇcek, P.; Lew-Tabor, A.; Maritz-Olivier, C. Strategies for new and improved vaccines against ticks and tick-borne diseases. Parasite Immunol. 2016, 38, 754–769.

25. de la Fuente, J.; Contreras, M.; Estrada-Peña, A.; Cabezas-Cruz, A. Targeting a global health problem: Vaccine design and challenges for the control of tick-borne diseases. Vaccine 2017, 35, 5089–5094.

26. Sauer, J.; Essenberg, R.; Bowman, A. Salivary glands in ixodid ticks: Control and mechanism of secretion. J. Insect Physiol. 2000, 46, 1069–1078.

27. Kazimírová, M.; Štibrániová, I. Tick salivary compounds: Their role in modulation of host defences and pathogen transmission. Front. Cell. Infect. Microbiol. 2013, 3, 43.

28. de Castro, M.H.; de Klerk, D.; Pienaar, R.; Latif, A.A.; Rees, D.J.G.; Mans, B.J. *De novo* assembly and annotation of the salivary gland transcriptome of *Rhipicephalus appendiculatus* male and female ticks during blood feeding. Ticks Tick. Borne. Dis. 2016, 7, 536–548.

29. Moreira, H.N.S.; Barcelos, R.M.; Vidigal, P.M.P.; Klein, R.C.; Montandon, C.E.; Maciel, T.E.F.; Carrizo, J.F.A.; Costa de Lima, P.H.; Soares, A.C.; Martins, M.M.; et al.

A deep insight into the whole transcriptome of midguts, ovaries and salivary glands of the *Amblyomma sculptum* tick. Parasitol. Int. 2017, 66, 64–73.

30. Zivkovic, Z.; Esteves, E.; Almazán, C.; Da re, S.; Nijhof, A.M.; Kocan, K.M.; Jongejan, F.; de la Fuente, J. Diferential expression of genes in salivary glands of male *Rhipicephalus* (*Boophilus*) *microplus* in response to infection with *Anaplasma marginale*. BMC Genom. 2010, 11, 186.

31. McNally, K.L.; Mitzel, D.N.; Anderson, J.M.; Ribeiro, J.M.C.; Valenzuela, J.G.; Myers, T.G.; Godinez, A.; Wolfinbarger, J.B.; Best, S.M.; Bloom, M.E. Diferential salivary gland transcript expression profile in *Ixodes scapularis* nymphs upon feeding or flavivirus infection. Ticks Tick. Borne. Dis. 2012, 3, 18–26.

32. Yu, X.; Gong, H.; Zhou, Y.; Zhang, H.; Cao, J.; Zhou, J. Differential sialotranscriptomes of unfed and fed *Rhipicephalus haemaphysaloides*, with particular regard to differentially expressed genes of cysteine proteases. Parasit. Vectors 2015, 8, 597.

33. L'Amoreaux, W.J.; Junaid, L.; Trevidi, S. Morphological evidence that salivary gland degeneration in the American dog tick, *Dermacentor variabilis* (Say), involves programmed cell death. Tissue Cell 2003, 35, 95–99.

34. Francischetti, I.M.B.; Sa-Nunes, A.; Mans, B.J.; Santos, I.M.; Ribeiro, J.M.C. The role of saliva in tick feeding. Front. Biosci. (Landmark Ed.) 2009, 14, 2051–2088.

35. Bullard, R.; Allen, P.; Chao, C.-C.; Douglas, J.; Das, P.; Morgan, S.E.; Ching, W.-M.; Karim, S. Structural characterization of tick cement cones collected from *in vivo* and artificial membrane blood-fed Lone Star ticks (*Amblyomma americanum*). Ticks Tick. Borne. Dis. 2016, 7, 880–892.

36. Bishop, R.; Lambson, B.; Wells, C.; Pandit, P.; Osaso, J.; Nkonge, C.; Morzaria, S.; Musoke, A.; Nene, V. A cement protein of the tick *Rhipicephalus appendiculatus*, located in the secretory e cell granules of the type III salivary gland acini, induces strong antibody responses in cattle. Int. J. Parasitol. 2002, 32, 833–842.

37. Antunes, S.; Couto, J.; Ferrolho, J.; Rodrigues, F.; Nobre, J.; Santos, A.S.; Margarida Santos-Silva, M.; de la Fuente, J.; Domingos, A. *Rhipicephalus bursa* sialotranscriptomic response to blood feeding and *Babesia ovis* infection: Identification of candidate protective antigens. Front. Cell. Infect. Microbiol. 2018, 8, 1–17.

38. Perner, J.; Kropáˇcková, S.; Kopáˇcek, P.; Ribeiro, J.M.C. Sialome diversity of ticks revealed by RNAseq of single tick salivary glands. PLoS Negl. Trop. Dis. 2018, 12, e0006410.

39. Santos-Silva, M.; Filipe, A. Ciclos biológicos de ixodídeos (Ixodoidea: Ixodidae) em condições de laboratório. Rev. Port. Ciências Veterinárias 1998, 527, 143–148.

40. Akta¸s, M.; Altay, K.; Dumanlı, N. Development of a polymerase chain reaction method for diagnosis of *Babesia ovis* infection in sheep and goats. Vet. Parasitol. 2005, 133, 277–281.

41. de la Fuente, J.; Van Den Bussche, R.A.; Prado, T.M.; Kocan, K.M. *Anaplasma marginale msp1alpha* genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 2003, 41, 1609–1616.

42. Harrus, S.; Perlman-Avrahami, A.; Mumcuoglu, K.Y.; Morick, D.; Eyal, O.; Baneth, G. Molecular detection of *Ehrlichia canis*, *Anaplasma bovis*, *Anaplasma platys*, *Candidatus Midichloria mitochondrii* and *Babesia canis vogeli* in ticks from Israel. Clin. Microbiol. Infect. 2011, 17, 459–463.

43. Inokuma, H.; Raoult, D.; Brouqui, P. Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. J. Clin. Microbiol. 2000, 38, 4219–4221.

44. Gubbels, J.M.; de Vos, A.P.; van derWeide, M.; Viseras, J.; Schouls, L.M.; de Vries, E.; Jongejan, F. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 1999, 37, 1782–1789.

45. Artigas-Jerónimo, S.; Alberdi, P.; Villar Rayo, M.; Cabezas-Cruz, A.; Prados, P.J.E.; Mateos-Hernández, L.; de la Fuente, J. *Anaplasma phagocytophilum* modifies tick cell microRNA expression and upregulates *isc-mir-79* to facilitate infection by targeting the Roundabout protein 2 pathway. Sci. Rep. 2019, 9, 9073.

46. Estrada-Peña, A.; Villar, M.; Artigas-Jerónimo, S.; López, V.; Alberdi, P.; Cabezas-Cruz, A.; de la Fuente, J. Use of Graph Theory to Characterize Human and Arthropod Vector Cell Protein Response to Infection With *Anaplasma phagocytophilum*. Front. Cell. Infect. Microbiol. 2018, 8, 265.

47. Conesa, A.; Götz, S.; García-Gómez, J.M.; Terol, J.; Talón, M.; Robles, M. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 2005, 21, 3674–3676.

48. Götz, S.; García-Gómez, J.M.; Terol, J.; Williams, T.D.; Nagaraj, S.H.; Nueda, M.J.; Robles, M.; Talón, M.; Dopazo, J.; Conesa, A. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 2008, 36, 3420–3435.

49. Walter, W.; Sánchez-Cabo, F.; Ricote, M. GOplot: An R package for visually combining expression data with functional analysis: Figure 1. Bioinformatics 2015, 31, 2912–2914.

50. Yu, C.-S.; Chen, Y.-C.; Lu, C.-H.; Hwang, J.-K. Prediction of protein subcellular localization. Proteins Struct. Funct. Bioinform. 2006, 64, 643–651.

51. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J. Mol. Biol. 2001, 305, 567–580.

52. Sonnhammer, E.L.; von Heijne, G.; Krogh, A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc. Int. Conf. Intell. Syst. Mol. Biol. 1998, 6, 175–182.

53. Almagro Armenteros, J.J.; Tsirigos, K.D.; Sønderby, C.K.; Petersen, T.N.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 2019, 37, 420–423.

54. Kolaskar, A.; Tongaonkar, P. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990, 276, 172–174.

55. Smialowski, P.; Martin-Galiano, A.J.; Mikolajka, A.; Girschick, T.; Holak, T.A.; Frishman, D. Protein solubility: Sequence based prediction and experimental verification. Bioinformatics 2006, 23, 2536–2542.

56. Smialowski, P.; Schmidt, T.; Cox, J.; Kirschner, A.; Frishman, D.Will my protein crystallize? A sequence-based predictor. Proteins Struct. Funct. Bioinform. 2005, 62, 343–355.

57. Wold, S.; Jonsson, J.; Sjörström, M.; Sandberg, M.; Rännar, S. DNA and peptide sequences and chemical processes multivariately modelled by principal component analysis and partial least-squares projections to latent structures. Anal. Chim. Acta 1993, 277, 239–253.

58. Couto, J.; Antunes, S.; Ferrolho, J.; De La Fuente, J.; Domingos, A. Reduction of Mosquito Survival in Mice Vaccinated with *Anopheles stephensi* Glucose Transporter. Biomed Res. Int. 2017, 2017, 3428186.

59. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012, 40, e115.

60. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinform. 2012, 13, 134.

61. Ferrolho, J.; Antunes, S.; Sanches, G.S.; Couto, J.; Évora, P.M.; Rosa, C.; André, M.R.; Machado, R.Z.; Bechara, G.H.; Domingos, A. *Ferritin 1* silencing effect in *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) during experimental infection with *Ehrlichia canis*. Ticks Tick. Borne. Dis. 2017, 8, 174–184.

62. Nijhof, A.M.; Balk, J.A.; Postigo, M.; Jongejan, F. 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. 2009, 10, 112.

63. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002, 3, research0034.1–research0034.11.

64. Pfaff, M.W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001, 29, e45.

65. Taylor, D.L.; Zhong, L.; Begg, D.J.; de Silva, K.; Whittington, R.J. *Toll-like receptor* genes are differentially expressed at the sites of infection during the progression of Johne's disease in outbred sheep. Vet. Immunol. Immunopathol. 2008, 124, 132–151.

66. Dandasena, D.; Bhandari, V.; Sreenivasamurthy, G.S.; Murthy, S.; Roy, S.; Bhanot, V.; Arora, J.S.; Singh, S.; Sharma, P. A Real-Time PCR based assay for determining parasite to host ratio and parasitaemia in the clinical samples of Bovine Theileriosis. Sci. Rep. 2018, 8, 15441.

67. IBM Corporation. IBM SPSS Statistics for Windows; IBM: Armonk, NY, USA, 2016.

68. Irazoki, O.; Hernandez, S.B.; Cava, F. Peptidoglycan Muropeptides: Release, Perception, and Functions as Signaling Molecules. Front. Microbiol. 2019, 10, 500.

69. Couto, J.; Tonk, M.; Ferrolho, J.; Antunes, S.; Vilcinskas, A.; de la Fuente, J.; Domingos, A.; Cabezas-Cruz, A. Antiplasmodial activity of tick defensins in a mouse model of malaria. Ticks Tick. Borne. Dis. 2018, 9, 844–849.

70. Schnittger, L.; Rodriguez, A.E.; Florin-Christensen, M.; Morrison, D.A. *Babesia*: A world emerging. Infect. Genet. Evol. 2012, 12, 1788–1809.

71. Jalovecka, M.; Sojka, D.; Ascencio, M.; Schnittger, L. *Babesia* Life Cycle—When Phylogeny Meets Biology. Trends Parasitol. 2019, 35, 356–368.

72. Mans, B.J.; Neitz, A.W. Adaptation of ticks to a blood-feeding environment: Evolution from a functional perspective. Insect Biochem. Mol. Biol. 2004, 34, 1–17.

73. Antunes, S.; Couto, J.; Ferrolho, J.; Sanches, G.S.; Merino Charrez, J.O.; De la Cruz Hernández, N.; Mazuz, M.; Villar, M.; Shkap, V.; de la Fuente, J.; et al. Transcriptome and Proteome Response of *Rhipicephalus annulatus* Tick Vector to *Babesia bigemina* Infection. Front. Physiol. 2019, 10, 318.

74. Severo, M.S.; Choy, A.; Stephens, K.D.; Sakhon, O.S.; Chen, G.; Chung, D.-W.D.; Le Roch, K.G.; Blaha, G.; Pedra, J.H.F. The E3 Ubiquitin Ligase XIAP Restricts *Anaplasma phagocytophilum* Colonization of *Ixodes scapularis* Ticks. J. Infect. Dis. 2013, 208, 1830–1840.

75. Bedford, L.; Lowe, J.; Dick, L.R.; Mayer, R.J.; Brownell, J.E. Ubiquitin-like protein conjugation and the ubiquitin–proteasome system as drug targets. Nat. Rev. Drug Discov. 2011, 10, 29–46.

76. Tong, L. Acetyl-coenzyme A carboxylase: Crucial metabolic enzyme and attractive target for drug discovery. Cell. Mol. Life Sci. 2005, 62, 1784–1803.

77. Sun, L.; Chen, Z.J. The novel functions of ubiquitination in signaling. Curr. Opin. Cell Biol. 2004, 16, 119–126.

78. Finley, D.; Chau, V. Ubiquitination. Annu. Rev. Cell Biol. 1991, 7, 25–69.

79. Katzmann, D.J.; Stefan, C.J.; Babst, M.; Emr, S.D. Vps27 recruits ESCRT machinery to endosomes during MVB sorting. J. Cell Biol. 2003, 162, 413–423.

80. Walker, D.H. Rickettsiae and Rickettsial Infections: The Current State of Knowledge. Clin. Infect. Dis. 2007, 45, S39–S44.

81. Liu, Y.; Beyer, A.; Aebersold, R. On the Dependency of Cellular Protein Levels on mRNA Abundance. Cell 2016, 165, 535–550.

82. Finley, D.; Ozkaynak, E.; Varshavsky, A. The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation, and other stresses. Cell 1987, 48, 1035–1046.

83. Shaw, D.K.; Wang, X.; Brown, L.J.; Chávez, A.S.O.; Reif, K.E.; Smith, A.A.; Scott, A.J.; McClure, E.E.; Boradia, V.M.; Hammond, H.L.; et al. Infection-derived lipids elicit an immune deficiency circuit in arthropods. Nat. Commun. 2017, 8, 14401.

84. Tong, L. Structure and function of biotin-dependent carboxylases. Cell. Mol. Life Sci. 2013, 70, 863–891.

85. Tong, L.; Harwood, H.J. Acetyl-coenzyme A carboxylases: Versatile targets for drug discovery. J. Cell. Biochem. 2006, 99, 1476–1488.

86. Campbell, J.W.; Cronan, J.E. Bacterial Fatty Acid Biosynthesis: Targets for Antibacterial Drug Discovery. Annu. Rev. Microbiol. 2001, 55, 305–332.

87. Brock, M. Generation and Phenotypic Characterization of *Aspergillus nidulans* Methylisocitrate Lyase Deletion Mutants: Methylisocitrate Inhibits Growth and Conidiation. Appl. Environ. Microbiol. 2005, 71, 5465–5475.

88. Seeber, F.; Limenitakis, J.; Soldati-Favre, D. Apicomplexan mitochondrial metabolism: A story of gains, losses and retentions. Trends Parasitol. 2008, 24, 468–478.

89. Lizundia, R.; Werling, D.; Langsley, G.; Ralph, S.A. *Theileria* apicoplast as a target for chemotherapy. Antimicrob. Agents Chemother. 2009, 53, 1213–1217.

90. Bork, S.; Yokoyama, N.; Matsuo, T.; Claveria, F.G.; Fujisaki, K.; Igarashi, I. Clotrimazole, Ketoconazole, and Clodinafop-Propargyl as Potent Growth Inhibitors of Equine *Babesia* Parasites during *In vitro* Culture. J. Parasitol. 2013, 89, 604–606.

91. Bork, S.; Yokoyama, N.; Matsuo, T.; Claveria, F.G.; Fujisaki, K.; Igarashi, I. Clotrimazole, ketoconazole, and clodinafop-propargyl inhibit the *in vitro* growth of *Babesia bigemina* and *Babesia bovis* (Phylum Apicomplexa). Parasitology 2003, 127, S0031182003003895.

Chapter III: Reverse vaccinology using the *Rhipicephalus bursa* sialome

1. "Probing the *Rhipicephalus bursa* sialomes in potential anti-tick vaccine candidates: a reverse vaccinology approach"

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1.1. Abstract

In the wake of the 'omics' explosion of data, reverse vaccinology approaches are being applied more readily as an alternative for the discovery of candidates for next generation diagnostics and vaccines. Promising protective antigens for the control of ticks and tick-borne diseases can be discovered by mining available omics data for immunogenic epitopes. The present study aims to explore the previously obtained *Rhipicephalus bursa* sialotranscriptome during both feeding and *Babesia* infection, to select antigenic targets that are either membrane-associated or a secreted protein, as well as unique to the ectoparasite and not present in the mammalian host. Further, they should be capable of stimulating T and B cells for a potential robust immune response, and be non-allergenic or toxic to the host. From the *R. bursa* transcriptome, 5706 and 3025 proteins were identified as belonging to the surfaceome and secretome, respectively. Following a reverse genetics immunoinformatics pipeline, nine preferred candidates,

consisting of one transmembranerelated and eight secreted proteins, were identified. These candidates showed a higher predicted antigenicity than the Bm86 antigen, with no homology to mammalian hosts and exposed regions. Only four were functionally annotated and selected for further *in silico* analysis, which examined their protein structure, surface accessibility, flexibility, hydrophobicity, and putative linear B and T-cell epitopes. Regions with overlapping coincident epitopes groups (CEGs) were evaluated to select peptides that were further analyzed for their physicochemical characteristics, potential allergenicity, toxicity, solubility, and potential propensity for crystallization. Following these procedures, a set of three peptides from the three *R. bursa* proteins were selected. *In silico* results indicate that the designed epitopes could stimulate a protective and long-lasting immune response against those tick proteins, reflecting its potential as anti-tick vaccines. The immunogenicity of these peptides will be evaluated in a pilot immunization study followed by tick feeding to evaluate its impact on tick behavior and pathogen transmission. Combining *in silico* methods with *in vivo* immunogenicity evaluation enabled the screening of vaccine candidates prior to expensive infestation studies on the definitive ovine host animals.

1.2. Introduction

Tick and tick-borne diseases are an increasing threat for both human and animal health [1]. The multi-host hard tick species, *Rhipicephalus bursa*, has a wide distribution throughout the Mediterranean basin and transmits several pathogens of economic importance in ungulates (*i.e.*, cattle, sheep, and goats) from several genera, including *Babesia*, *Anaplasma*, *Theileria*, *Rickettsia*, and *Coxiella* [2]. Recently, *R. bursa* has been implicated in the transmission of several zoonotic pathogenic agents highlighting its impact in human health [3]. This tick is the primary vector of *Babesia ovis*, a highly pathogenic hemoparasite in small ruminants, recognized for having an important socioeconomic impact, primarily in low income countries, related with production losses and costs of the animal treatment [4]. Moreover, *B. ovis* is present in all developmental stages of *R. bursa* species since it has the capacity for transovarial and transstadial transmission [5].

The (re)emergence of ticks and tick-borne diseases and the lack of safer and more effective control strategies have reinvigorated research efforts by the scientific community to explore ways to control ticks and, subsequently, their associated diseases. Biological control [6], acaricides [7,8], resistant production breeds [9], and vaccines [10–12] are being proposed and readily tested for tick population control. Vaccines are one of the most environmentally friendly pharmaceutical products [13], as well as effective prophylactic treatment [14], used in infectious disease control. In tick research, the development and commercialization of novel vaccines [15] have been hindered by different factors [16,17] such as the lack of knowledge regarding tick-host interactions and tick biology. Particularly, transmission-blocking vaccines are considered very attractive tools for vector-borne disease control since they can affect the vector's biology and behavior, thereby interfering with its capacity to transmit diseases [18–20]. Traditionally, vaccines confer protection by stimulating a humoral response mediated by antibodies [21]. These antibodies are involved in recognition and binding of the foreign antigen resulting in neutralization, agglutination, precipitation, as well as complement activation using chemoattractants to facilitate inflammation [22].

To date only one subunit anti-tick vaccine has been commercialized, based on a surface exposed 89 kDa glycosylphosphatidylinositol (GPI)-linked glycoprotein, Bm86, originally found in *Rhipicephalus microplus* midgut tissues [23,24]. Vaccination with this antigen can induce a protective immune response mediated mainly by host humoral response and the complement system, damaging the tick midgut wall and leading to a decrease in tick survival and diminished capacity to produce viable progeny [25–27]; however, with an efficacy depending on the tick species and strains [11].

Thus, a panoply of targets must be studied and tested to increase the current antigen repertoire for use in novel anti-tick vaccines and improve their efficiency. Immunoinformatics-based approaches have been recently applied to catalogue potential protective antigens, reducing cost and time in anti-tick vaccine development [25,28–30]. Reverse vaccinology (RV) is an approach exploring available omics data and *in silico* tools to select a great amount of predicted antigenic proteins potentially capable of inducing a protective immune response *in vivo* [21,31–34]. A combination of such techniques is steadily being implemented to develop novel and effective vaccines against several infectious diseases, including parasitosis [35–40]. These approaches have been

already used on tissue-specific tick omics data in the pursuit of potential protective candidates [26,30,41–43]. Immunoinformatics focusing tick sialomics data are of particular interest since tick secreted salivary proteins can closely influence the host immune response at the vector feeding site, as well as enable pathogen dissemination and multiplication inside the host [44–48]. Even though functional redundancy is expected in tick salivary gland proteins [49], transcriptomic and proteomic studies of this tissue represent a collection of pharmaco-active molecules with therapeutic exploitation potential [48]. Moreover, peptide and multi-epitope constructs can be designed to synergize the impact of recognizing multiple antigens [50] and overcoming functional redundancy, as well as minimizing the side-effects caused by the immunization of an entire protein [51].

Regarding topology, proteins that contain extracellularly exposed portions on the cell outer membranes (surfaceome) or that are secreted into the extracellular space (secretome) are considered suitable antigens for vaccine development due to better accessibility to the immune system [26,32], as opposed to cytoplasmic proteins that can rather be considered for small molecule drug development [26,32,52]. Targeting a membrane-related protein may also interfere with the tick capacity to transmit or acquire pathogens if produced antibodies directly block the parasite from crossing the midgut or salivary glands barriers, as observed in other vectors, such as mosquitoes [18,53]. The tick secretome represents a chemical pool, critical for tick feeding and life cycle, as well as pathogen transmission. Thus, tick salivary secretions could be the core for the development of novel therapeutics for host disorders [48,54] or anti-tick and transmission blocking vaccines [55] as in the case of Salp15 and *Borrelia burgdorferi* transmission by *Ixodes* ticks [56].

Therefore, this study aims to scrutinize available high-throughput omics data, using a RV approach, focusing on the *Rhipicephalus bursa-Babesia ovis* (vector-pathogen) interface in order to identify antigenic peptides from tick sialoproteins that could be promising candidates for future vaccination trials. For this, computational methods have been combined to predict B and T cell epitopes, as well as its topology, hydrophobicity, polarity, solubility, and other physicochemical aspects [57], in order to select candidates comprising all the requirements for a suitable vaccine or even for disease diagnosis and

disease therapy [58]. Such exposed antigens could become targets for peptide-based therapeutics if they present high antigenicity and no toxicity to the host.

1.3. Material and Methods

1.3.1. *Rhipicephalus bursa* sialotranscriptomes: new assembly

The data analyzed was obtained previously by Antunes et al. [59]. Briefly, *R. bursa* female ticks were obtained under different conditions: uninfected-unfed ticks, uninfectedfed ticks, and *B. ovis* infected-fed ticks. Salivary glands were isolated, RNA extracted, and two replicates *per* condition were used for RNA sequencing in a HiSeq 2500 sequencer (Illumina, CA, USA) after quality assessment and library construction. Cluster generation was performed, followed by 2 × 100 cycle sequencing reads separated by a paired-end turnaround. The raw fastq files deposited at the National Center for Biotechnology Information (NCBI) under the accession numbers SRR4428986, SRR4428987 and SRR4428988 [59] were re-analyzed under the present study. For this, sequence reads were quality filtered and trimmed using Trimmomatic [60] and the transcriptomes reassembled using Trinity [61]. To evaluate the completeness of the assemblies, BUSCO [62] analyses were performed using the Arthropoda dataset as a reference.

1.3.2. *In silico* characterization of *Rhipicephalus bursa* protein coding sequences

A filtering process was performed using various bioinformatics tools to identify protein coding open-reading frames, topological features, antigenic regions and annotate potential candidates. See Figure 1, for experimental outline.

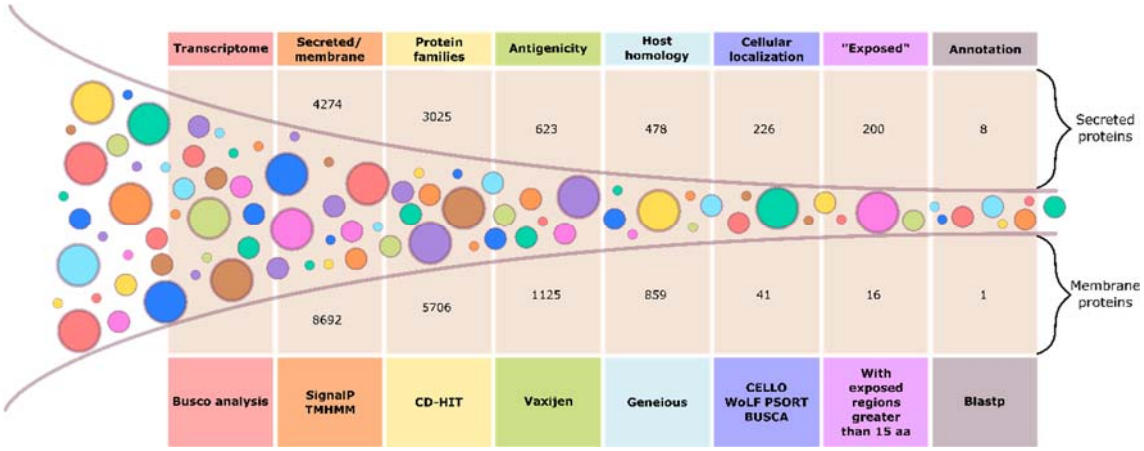

Figure 1. Graphical depiction of the RV-based methodology used for antigen mining. From the "Fed and infected" *R. bursa* sialotranscriptome dataset, the secretome (Secreted proteins) and the surfaceome (Membrane proteins) was differentiated using SignalP and TMHMM online servers, respectively. Several filters were applied using different programs as indicated, to filter promising targets.

Open reading frames (ORF) in transcriptome contigs were predicted using TransDecoder [62]. InterProScan [63] was used to classify the predicted protein sequences in terms of signal peptide and transmembrane regions with SignalP (v. 4.0) [64] and TMHMM (v. 2.0) [65,66], respectively. Redundant (or highly similar; identity >90%) and closely related protein families were analyzed with CD-HIT v4.8.1[67], and only the representative sequences were used in subsequent analyses.

The online server Vaxijen (v. 2.0) was used to select the antigenic proteins [68]. Based on the immunogenicity of the only commercially available anti-tick vaccine, Bm86, a threshold of 0.7 was applied [30].

Then, to select antigens that differ from possible vertebrates used in future vaccination trials, the Geneious R8.1 software was used to search for homology between the *R. bursa* dataset and the mammalian hosts amino acid databases (*Mus musculus*, *Oryctolagus cuniculus* and *Ovis aries*).

Next, CELLO (v. 2.5) [69], WoLF PSORT [70], and BUSCA [70] online servers were used to select the surfaceome and secretome using the names "plasma membrane" and "extracellular" as filters. SignalP (v. 5.0) [71], big-PI [72], GPI-SOM [73], Phobius [74], TMHMM (v. 2.0), CCTOP (v. 1.0) [75], and SACS TMHMM [75] programs were used for the selection of membrane-related antigens with exposed regions ("outside" or "non-

cytoplasmatic", with regions greater than 15 a.a.) without signal peptide neither glycosylphosphatidylinositol (GPI)-anchor in all the *in silico* results.

For the selection of secreted antigens, the same approach was used including proteins with signal peptide but no GPI-anchor in all the *in silico* results.

Finally, the selected *R. bursa* proteins were functionally annotated by BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins, accessed on 30 October 2019) against the NCBI non-redundant (nr) and Arthropoda (6656) databases using the PAM70 matrix (E value < 1×10^{-1}, coverage: 50–100%, identity: 50–100%). Only the proteins functionally annotated were further used in the ensuing analysis.

1.3.3. ORFs and proteomics

The occurrence of the predicted ORFs from the transcriptomes in the previously published *R. bursa* sialoproteomic data was assessed. The proteomic data was obtained previously by Couto et al. [76]. Briefly, four groups of ticks were generated considering the conditions of uninfected unfed, uninfected fed, infected unfed, and infected fed, and salivary glands from each were dissected for protein extraction. Protein extracts were precipitated and digested, until the peptides were desalted; samples were analyzed *via* reverse phase liquid chromatography coupled online with mass spectrometry (RP-LC-MS/MS) using an Ekspert nLC 415 system combined to a 6600 TripleTOF® mass spectrometer (AB SCIEX®, MA, USA) through information-dependent acquisition (IDA) followed by sequential windowed data independent acquisition of the total high-resolution mass spectra (SWATH). The BLASTP was used to perform a local analysis using the predicted Transdecode ORFs from the sialotrancriptome as protein database and the respective peptides from proteomic analysis as a query (E value cutoff of 0.0001, word-size of 7 for a shorter input sequence).

1.3.4. Protein structure and epitope exposure

The presence of coiled-coil (C), alpha helix (H), and beta sheet (E) was predicted using the NetSurfP-2.0 [75] and BepiPred 2.0 (structural frame) [77] programs. The Chou and Fasman prediction method [78] (from IEDB Analysis Resource, v. 2.22, http://tools.iedb.org/bcell/, accessed on 30 October 2019) was used to predict beta turns

within the amino acid sequence, considering probable turn regions those with values higher than 1.

The surface accessibility of the amino acids was evaluated using the Emini prediction method [78] (from IEDB Analysis Resource, v. 2.22, threshold of 1, http://tools.iedb.org/bcell/, accessed on 30 October 2019) and BepiPred 2.0 (surface frame) program. Features such as flexibility and hydrophobicity were evaluated using the Karplus–Schultz [79] and Parker [80] methods (from IEDB Analysis Resource, v. 2.22, threshold of 1, http://tools.iedb.org/bcell/, accessed on 30 October 2019), respectively.

1.3.5. Prediction of B and T cell epitopes

Potential immunogenetic epitopes were predicted using linear B-cell epitope predictors: the Kolaskar and Tongaonkar method [81] (from IEDB Analysis Resource, v. 2.22, threshold of 1, http://tools.iedb.org/bcell/, accessed on 30 October 2019), the predicting antigenic peptides online server (from Immunomedicine Group, http://imed.med.ucm.es/Tools/antigenic.pl, accessed on 30 October 2019), BepiPred 1.0 [80] (from DTU Bioinformatics, http://www.cbs.dtu.dk/services/BepiPred-1.0/, accessed on 30 October 2019), and BepiPred 2.0 (epitope frame) program.

T-cell epitopes were also predicted using all prediction method versions. MHC-I Binding Predictions program (from IEDB Analysis Resource, v. 2.22, http://tools.iedb.org/mhci/, accessed on 30 October 2019) was used to predict epitopes with high affinity to human, mouse, and rat MHC-I. Prediction of MHC-II binding epitopes was obtained using the MHC-II Binding Predictions (from IEDB Analysis Resource, v. 2.22, using all method versions, selecting 12-18-mer peptides, http://tools.iedb.org/mhcii/, accessed on 30 October 2019) and the MHC2Pred (http://crdd.osdd.net/raghava/mhc2pred/, accessed on 30 October 2019) programs for human and mouse MHC-II databases. Predicted T-cell epitopes containing a percentile rank lower or equal to one were selected to identify the representative epitopes using the Epitope Cluster Analysis tools (from IEDB Analysis Resource, v. 2.22, http://tools.iedb.org/cluster/, accessed on 30 October 2019). NetChop (v. 3.1) [82] and PCPS (http://imed.med.ucm.es/Tools/pcps/index.html, accessed on 30 October 2019) programs were used to explore if, after proteasomal processing, the epitope generated could be an MHC binder, which means that could be presented in the host immune

system, processed, and ultimately induced in the host humoral and cellular immune pathway.

1.3.6. Peptide properties

Amino acid sequence of selected peptides was used to predict it physicochemical characteristics including molecular weight (Da), theoretical isoelectric point (pI), instability index, grand average of hydropathicity (GRAVY), and aliphatic index, using Expasy ProtParam server (http://expasy.org/cgi-bin/protpraram, 30/11/2019). The allergenicity of the epitopes was predicted by the online servers AllergenFP (v. 1.0) [83], AllerTop (v. 2.0) [84], and AllerCatPro (v. 1.7) [85]. Protein-Sol was used to predict the peptide solubility (>0.8 values indicate a soluble molecule) [86] and CRYSTALP2 for crystallization propensity [87]. Post-translational modification sites in the peptides were predicted using ModPred [88] and PROSITE [89], regarding its impact on protein/peptide production, structure and function [90]. The hemolytic, anti-angiogenic or toxic properties of the selected peptides were analyzed using HemoPI (all SVM methods were used; SVM scores ranges between 0 and 1, *i.e.*, 1 very likely to be hemolytic, 0 very unlikely to be hemolytic) [91], AntiAngioPred (NT15 AAC and whole peptide AAC prediction methods were used; threshold -0.2,) [92], and ToxinPred (all SVM methods were used; E value 10; threshold 0.0) [93], respectively. For comparison, all these analysis were performed for the published synthetic multi-epitope peptide SBm7462® [94]), which has demonstrated to be a protective candidate for a next generation anti-tick vaccine [94,95].

1.4. Results and Discussion

Before searching for promising antigens, the previously published Sequence Read Archives regarding uninfected-unfed, uninfected-fed, and *B. ovis* infected-fed *R. bursa* salivary glands RNA sequencing [59] were reassembled and assessed for completeness using BUSCO analysis [62] and a reference database of 1066 conserved arthropod genes (see Table 1).

Table 1. BUSCO statistics for each *Rhipicephalus bursa* sialotranscriptome assembly against an Arthropod database. Conserved BUSCO genes were assigned to four classes of genes: missing, fragmented, duplicated and complete.

Dataset	*Babesia ovis* infected and fed	Uninfected and fed	Uninfected and unfed
SCAFFOLDS (#)	70535	63942	58670
ASSEMBLY SIZE (Mbp)	64.5	67.6	47.3
N50 (bp)	1856	2266	1522
Number of conserved arthropod genes in BUSCO reference set		1066	
Complete and single-copy	683 (64.1%)	690 (64,7%)	692 (64.9%)
Complete and duplicated	273 (25.6%)	312 (29.3%)	200 (18.8%)
Fragmented	78 (7.3%)	36 (3.4%)	125 (11.7%)
Missing	32 (3%)	28 (2.6%)	49 (4.6%)

The assembly of the sequencing reads of the salivary glands of *B. ovis*-infected-fed *R. bursa* ticks yielded a transcriptome with 70,535 scaffolds, a total assembly size of 64.5 Mbp, and a scaffold N50 length of 1856 bp. The BUSCO completeness report of the assembly indicated that 89.7% complete BUSCOs were obtained for this assembly. Specifically, there were 956 complete (683 complete and single-copy; 273 duplicated), 78 fragmented, and 32 missing BUSCOs. Similarly, a final percentage of complete orthologous genes of 94.0% (uninfected-fed) and 83.7% (uninfected-unfed), respectively, were determined for the remaining uninfected *R. bursa* sialotranscriptomes. It is unlikely to produce a complete BUSCO transcriptome and it is accepted for non-model organisms, such as ticks, to obtain complete scores ranging from 50% to 90% [96]. This is an indicator of a proper transcriptome assembly; thus, these are acceptable ranges for percentage of completeness relative to other RNAseq assemblies in the field [43,97]. Reassembled and complete transcriptomes were considered for the next phase.

1.4.1. Feeding and pathogen transmission: selection of targets

The systematic workflow of an RV approach must focus on filtering ideal antigens that provide a robust, long-lasting, and deliverable immune response, such as the humoral response, which ultimately interferes with the host-vector-pathogen triad [20]. Thus, the features of an ideal antigen for anti-tick vaccines includes: being a pivotal molecule on tick/pathogen biology, not being homologous to the mammalian host, encoded by a single gene, expressed across life stages and tick tissues, and capable of inducing B and T cells

to incite an immunological response without allergenic, hemolytic, and toxic effects [14,98]. Such humoral response is linked to topological features, such as extracellular or intramembrane location, and the presence of coincident epitope groups (CEGs) (also known as "immunological kernels"), are accessible protein regions containing overlapped B and T cell epitopes with ideal chemo-physical properties [52,99,100].

Therefore, the dataset from fed-infected tick salivary glands were analyzed. They were found to correspond to proteins involved in blood feeding and parasite transmission. These processes are intrinsically related to vector survival and competence [101]. Moreover, we performed *in silico* screening for the antigenic surfaceome and secretome using different filters (Figure 1).

Transcripts containing membrane-related regions were filtered and analyzed regarding its protein antigenicity, homology to vertebrate hosts, cell localization, and annotated function (Supplementary material—Spreadsheet S1). From the transcriptomic selected dataset, 8692 sequences were predicted to be membrane-related proteins that were associated with 5706 different protein family clusters. Each representative of every protein family was investigated for its predicted antigenicity and 1125 proteins were found to be probable antigens in comparison to the Bm86 tick vaccine antigen (Vaxijen score ≥ 0.7). From these predicted antigenic proteins, 859 presented no homology to the vertebrate hosts, warranting the probability of inducing a target tick-specific immune response in the host animal and not leading to an auto-immune phenomenon [25,30,52]. In total, 16 proteins were predicted to be localized in the cell plasma membrane, as a transmembrane protein without signal peptide or GPI anchor (i.e., secreted or anchored). Following this analysis, a putative lipid raft-associated protein containing a MARVEL domain (M_MARVEL, DN25304, EEC06674.1) was identified.

Transcripts containing signal peptides were identified in a similar way as previously described for the transmembrane-related proteins (Supplementary material—Spreadsheet S2). From the current dataset, 4274 sequences contained signal peptides clustered into 3025 different protein families. Predicted antigenicity *via* Vaxijen identified 623 proteins that presented higher probability of being antigenic relative to Bm86. Alignment and homology analysis indicated that 478 proteins to be tick-specific and non-related to vertebrate hosts (Figure 1). About 200 transcripts were predicted to be extracellular with no predicted membrane-spanning regions following the signal peptide region. Finally,

seven putative proteins were identified consisting of two glycine-rich proteins (DN21364, DN28608), an evasin (S_EVASIN, DN20966, AST14849.1), a ricin (S_RICIN, DN33470, EEC03321.1), an antimicrobial peptide (DN7637), and two proteins related to heterodimerization interface (DN16497) and coagulation (DN45898).

1.4.2. *In silico* characterization of selected candidates

In this study, one membrane-related (MARVEL) and two secreted (EVASIN, RICIN) proteins were selected to proceed for specific immunoinformatic analysis, since their putative function and occurrence in previously published proteomic data highlighted them as promising targets for anti-tick or disease transmission blocking vaccine development. While MARVEL can be found in the infected-fed, uninfected-fed, and uninfected-unfed conditions of *R. bursa* sialoproteomic data, EVASIN and RICIN are only found in the infected-fed state. This suggests the persistence of MARVEL in the tick cellular machinery as a static membrane protein, while EVASIN and RICIN could be strongly or exclusively linked to infection and feeding.

For each target, prediction methods were used to assess protein structure (Table 2) and putative epitope exposure for a better identification of exposed and immunogenic regions, including B and T (Supplementary material—Spreadsheet S3, S4 and S5) cell epitopes.

Table 2. Topology and structure properties of the selected targets. Several bioinformatic tools were used to obtain this data. (Prosite (*), VectorBase (**), Phobius (a), TMHMM (b), CCTOP (c), SACS TMHMM (d) and SignalP (e)).

Protein name	Length (aa)	MW (Da)	pI	Functional Domains	Transmembrane Domains	Extracellular Domains	SP
MARVEL	155	16508.53	9.03	Contains: leucine zipper domain (pos. 24-52) * and Marvel domain (pos. 29-157 from ISCW003585) **	Pos. 29-52, 64-84, 96-117, 129-150 a Pos. 28-50, 63-85, 98-120, 127-149 b Pos. 29-52, 62-85, 95-117, 127-149 c Pos. 28-50, 63-85, 98-120, 127-149 d	Pos. 1-28, 85-95, 151-155 a Pos. 1-27, 86-97, 150-155 b Pos. 1-28, 86-94, 150-155 c Pos. 1-27, 86-97, 150-155 d	No
EVASIN	164	17681.84	4.20	Homology to an evasin protein (AST14849)	None	Pos. 28-164 a Pos. 1-164 b Pos. 29-164 c	Yes Pos. 1-27 a Pos. 1-26 e
RICIN	133	14401.49	8.19	Homology to a hypothetical protein which contains a Ricin-type beta-trefoil lectin domain (EEC03321)	None	Pos. 1-133 a Pos. 1-133 b Pos. 40-133 c	Yes Pos. 1-39 c Pos. 1-37 e

Since very limited information on MHC alleles from sheep and other host vertebrates of *R. bursa* ticks is currently available, “pan-computational methods” predictions were

used, as described before [102]. The available allelic datasets from different but wellknown hosts, such as humans, mice, and rats, were used to extrapolate the vertebrate host with unknown alleles such as sheep.

Regions with overlapping CEGs were thoroughly examined to screen ideal features for efficient production, using bioinformatic tools to evaluate physicochemical characteristics, post-translational modification sites, propensity for solubility and crystallization, allergenicity, and toxicity (Table 3).

Table 3. Physicochemical properties of the selected overlapping coincident epitopes groups (CEGs) from MARVEL, EVASIN and RICIN. "+" represents high probability, "-" represents low probability.

Protein name	CEG Length (aa)	Molecular weight (Da)	pI	Instability index	GRAVY	Aliphatic index	Allergenicity (AllerFP/AllerTop /AllerCatPro)	Solubility and crystallization propensity (Protein-Sol, CRYSTALP2)	Hemolytic Potency (HemoPI)	Anti-angiogenic property (AntiAngioPred)	Toxici predicti (ToxinP
MARVEL	27	2698.98	9.5	74.70	0.167	71.85	no/yes/no	0.669, none	0.01, 0.49, 0.44, 0.00, 0.49	yes, yes	no, no, nc
EVASIN	43	4835.13	3.69	66.52	-0.856	74.88	no/no/no	0.662, none	0.00, 0.47, 0.33, 0.00, 0.47	no, no	no, no, nc
RICIN	41	4243.91	6.43	57.47	0.285	97.56	no/yes/no	0.659, none	0.00, 0.48, 0.47, 0.00, 0.48	no, no	no, no, nc
SBm7462®	45	5056.78	6.87	49.21	-0.300	47.78	no/no/no	0.484, none	0.45, 0.35, 0.37, 0.00, 0.35	yes, yes	yes, no, ye

Depending on these characteristics, the production and solubilization of these peptides or proteins are different. Finally, as far as possible, the potential negative effects on the host should be predicted a priori to testing a potential vaccine [103]. Many highly reactive proteins identified from various parasites (including mites and helminths) are prone to cause allergic reactions in the host [14,104]. Therefore, predicting allergenicity and anti-angiogenic or toxic properties of the selected proteins/peptides are also required when screening for a promising candidate [13]. Taking all the aforementioned components into account, the three chosen candidates are discussed in the following sections.

Putative MARVEL domain-containing protein

MARVEL domain-containing proteins generally present an M-shaped topology (four transmembrane-helix region architecture with cytoplasmic N- and C-terminal regions) and function in cholesterol-rich membrane apposition events, such as biogenesis of vesicular transport carriers or tight junction regulation [105]. The putative MARVEL protein identified in this study is 155 amino acids long, thus lacking a signal peptide or GPI-anchor and containing four transmembrane and three extracellular domains (Table 2, Figure 2).

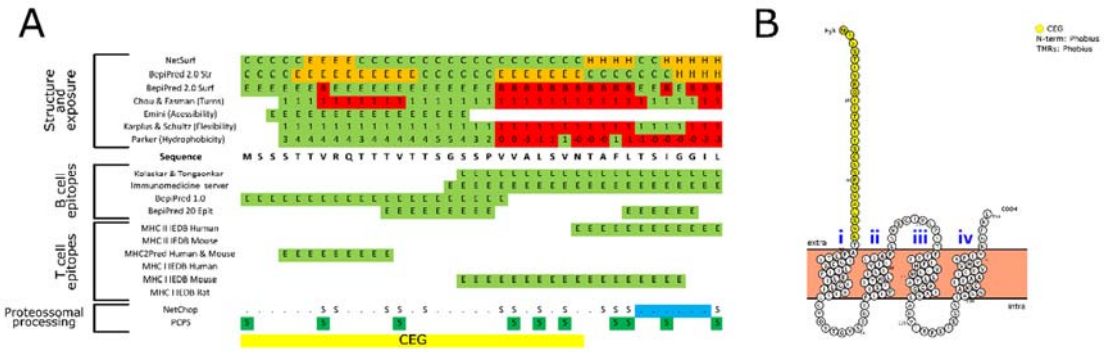

Figure 2. *In silico* analysis of the transmembrane MARVEL protein. (A) Identification of overlapping coincident epitopes groups (CEG) using different immunoinformatic approaches. **(B)** Topology prediction (based on Phobious) and localization of the CEG region (yellow) in the protein structure. For detail information see Supplementary material – Spreadsheet S4 and S5.

From the predicted extracellular domains, only an N-terminal region containing 27 amino acids was predicted to be majorly exposed, with a low structural complexity and a single predicted post-translational modification (PTMs) and glycosylation (Figure 2, Supplementary material—Spreadsheet S3). Such PTMs are associated with protein structure, stability, activity, trafficking, and protein–protein interactions [90]. All of these targeted characteristics are being considered for a potential vaccine candidate. The propensity of this protein to induce the humoral pathway was evaluated by predicting *in silico* the B and T cell epitopes (Figure 2, Supplementary material—Spreadsheet S3). The three B cell epitope predictors showed that this segment of the MARVEL protein could be presented directly to B cells and induce a humoral response. Additionally, this protein

portion has predicted protease cut sites (pos. 32-37) that could originate peptides that would enable presentation through MHC I (pos. 18-27) and II (pos. 4-12) (Figure 2, Supplementary material—Spreadsheet S3).

The predicted coincident epitope (MSSSTTVRQTTTVTTSGSSPVVALSVN) possesses flexibility and hydrophobicity which makes this fragment a promising candidate for synthetic production (Figure 2, Supplementary material—Spreadsheet S3). Other predictions were performed (Table 3), which indicated that the peptide alone is alkaline with a high probability to be unstable and hydrophobic. Nevertheless, peptide bioengineering by selection of a compatible carrier protein or linkage to other promising targets may contribute towards alleviating such drawbacks.

This peptide has more thermostability and solubility than Bm86-derived peptide. It also has low probability in causing allergic host reactions and hemolysis. Further, it can be anti-angiogenic or toxic. These are all characteristics that improve the use of this antigen for vaccine administration.

Putative Evasin

Other studies have mentioned that evasins are a secreted salivary glycoprotein that enables the endurance of tick feeding by suppressing the host immune response [106]. During blood feeding, such molecules are injected into the tick bite site and bind to the host chemokines to inhibit its function, resulting in a prevention of chemotaxis of leukocytes and subverting the host anti-inflammatory immune response associated to this phenomenon [106]. Besides, evasins can be ubiquitously expressed by a wide variety of tick species, constituting a promising target as an anti-tick vaccine that needs to be explored [48,106].

The putative evasin identified in this study contained an N-terminal signal peptide and no transmembrane helices (Table 2), suggesting that it might be a secretory protein. Moreover, this sequence has high homology to an evasin protein from *Rhipicephalus microplus* (AST14849) and possess several characteristics from the evasin protein family [106], such as nine Cys residues and N-linked glycosylation, as well as putative tyrosine sulfation sites (Figure 3A, Supplementary material—Spreadsheet S4).

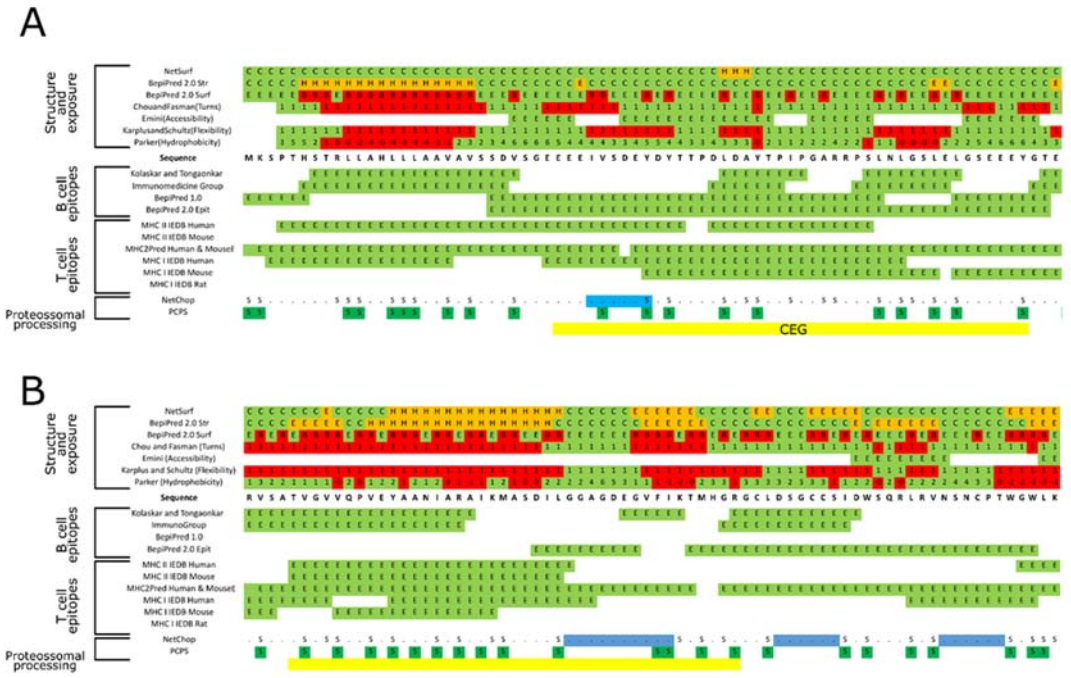

Figure 3. *In silico* analysis of the two signal proteins, EVASIN and RICIN. Identification of overlapping coincident epitopes groups (CEGs) within EVASIN **(A)** and RICIN **(B)** aminoacids sequences. For detail information see Supplementary material – Spreadsheet S4 and S5.

A region following the predicted signal peptide (between pos. 29 and 70) has a low complexity structure with several exposed residues (Figure 3A, Supplementary material—Spreadsheet S4), which could facilitate epitope presentation. Predictors indicates that the protein could be potentially cleaved in some positions, *e.g.*, pos. 26 to 36 and 98 to 103, leaving a peptide portion (37–97) to be potentially processed and presented by the MHCs. Within this secretory region, many putative epitopes can be detected and processed by B and T cells containing a few putative PTMs (*i.e.*, phosphorylation and sulfation) (Supplementary material— Spreadsheet S4). In this predicted highly immunogenic region, a peptide (EEEIVSDEYDYTTPDLDAYTPIPGARRPSLNLGSLELGSEEEY, pos. 29 to 71) was selected to be further evaluated *in silico* (Table 3). Predictions revealed that this peptide is acidic and unstable upon synthesis but such can be surmounted as previously referenced, in order to benefit on the other properties, such as hydrophilicity, solubility, and having no negative impacts on the host.

Putative Ricin

The most characteristic though not completely conserved sequence feature of ricin B lectin domains is the presence of a Q-W repeats containing an omega loop but no major segments of a helix or beta sheet throughout the sequence [107,108]. The primary structure of ricin proteins has shown the presence of a similar domain in many carbohydrate-recognition proteins like plant, fungi, and bacteria AB-toxins, glycosidases, or proteases [107– 109]. Proteins containing such domains are linked to cytotoxicity [110,111], cytoadhering [112], and possess immunomodulatory properties [113–115]. From the dataset, one sequence shows similarity to a ricin B lectin domain (Table 2), but no Q-W repeats were found. The 133 amino acid sequence contains a signal peptide and no transmembrane helices (Table 2), indicating that it might be secreted. Myristoylation and phosphorylation PTMs were predicted in this sequence (Supplementary material—Spreadsheet S5). Even with a complex structure with alpha helixes and beta sheets that reduces the exposure of epitopes, this sequence has regions that are likely to be recognized by B cell receptors, as well as MHC I and MHC II receptors of different organisms (Figure 3B, Supplementary material—Spreadsheet S5). We identified an N-terminal peptide region (TVGVVQPVEYAANIARAIKMASDILGGAGDEGVFIKTMHGR) that possesses more predicted B and T cell epitopes than the remaining sequence and flanked by an enzymatic cleavage site. Most of the predictors indicated that even with some undesirable characteristics (such as instability index and GRAVY), this peptide has a pI closer to seven as the SBm7462® peptide, could be thermostable, soluble, and present no harmful properties to the host (Table 3).

1.5. Conclusions and Future Perspectives

Transmission-blocking vaccines are considered essential tools for interrupting disease transmission. An immunized host produces inhibitory antibodies against pathogen/vector antigens that are ingested by the vector during blood feeding, interfering ultimately with vector competence and disease transmission [18]. The cellular pathway is compromised since the antibodies alter the activity or signal transduction of proteins through a physical block [116,117]. The discovery of new antigens is a prerequisite in developing new diagnostics and vaccines for disease surveillance and control. Reverse vaccinology is a

preferable approach to overcome the time and resources required to obtain promising candidates. However, there is an urgent need to develop a pipeline to run multiple algorithms in a single platform focused on tick research, including information on tick omics data, vertebrate hosts immune databases (from livestock, domestic animals, humans, including information about the epitope repertoire, and broad population coverage), and proteins from transmitted pathogens.

This study combines the power of several bioinformatics tools to establish a rational pipeline for vaccine antigen discovery. Focusing on peptide design will greatly reduce the cost of a putative vaccine and enhance its accessibility to the community, since smaller biomolecules are easier to synthetize and store [118]. Thus, three peptides that showed the desired characteristics were identified for further testing as next-generation vaccine targets.

These promising antigens will be tested in a follow-up study, yet prior to vaccination trials, a thorough investigation should be conducted to survey the humoral immune response by animals from tick endemic regions to these peptides. A preexistent humoral response to the antigens identified here, within an endemic area, will demonstrate that such molecules do not protect the host from tick infestation. Alternatively, an absence of an established and natural humoral response to these peptides might lend weight their use in a new protective strategy or even as diagnostic markers. Interestingly, the evaluation of the expression of such targets in cells, different tissues/fluids, and developmental stages of *R. bursa* tick species, could elucidate their applicability as broad-spectrum tick antigens. Several approaches to elucidate or validate the *in vivo* cellular localization (e.g., immunofluorescence and western-blot assays), protein structure (e.g., crystallography), and protein–protein interactions (e.g., yeast two-hybrid, etc.) could be conducted in future for top selected predicted targets. Pilot vaccination trials are needed to *in vivo* validate the immunogenicity of peptides where different aspects should be taken in account, such as antigen design/production (peptide, native protein, synthetic, polymers, type of host expression system, recombination with other promising antigens, linkers, etc.), its administration (route/system, dose, adjuvant), the host response (humoral and cellular immune response, physiological and clinical responses), and the influence on tick behavior and physiological features.

1.6. References

1. Swei, A.; Couper, L.I.; Coffey, L.L.; Kapan, D.; Bennett, S.; Patterns, Drivers, and Challenges of Vector-Borne Disease Emergence. Vector-Borne Zoonotic Dis. 2020, 20, 159–170, doi:10.1089/vbz.2018.2432.

2. Jaime Betancur Hurtado, O.; Giraldo-Ríos, C. Economic and Health Impact of the Ticks in Production Animals. Ticks Tick-Borne Pathog. 2019, doi:10.5772/intechopen.81167.

3. Madreseh-Ghahfarokhi, S.; Dehghani-Samani, A.; Pirali, Y.; Dehghani-Samani, A. *Zingiber officinalis* and *Eucalyptus globulus*, Potent Lethal/Repellent Agents against *Rhipicephalus bursa*, Probable Carrier for Zoonosis. J. Arthropod-Borne Dis. 2019, 13, 223.

4. Sevinc, F.; Sevinc, M.; Ekici, O.D.; Yildiz, R.; Isik, N.; Aydogdu, U. *Babesia ovis* infections: Detailed clinical and laboratory observations in the pre- and post-treatment periods of 97 field cases. Vet. Parasitol. 2013, 191, 35–43, doi:10.1016/j.vetpar.2012.07.025.

5. Erster, O.; Roth, A.; Wolkomirsky, R.; Leibovich, B.; Savitzky, I.; Shkap, V. Transmission of *Babesia ovis* by different *Rhipicephalus bursa* developmental stages and infected blood injection. Ticks Tick-Borne Dis. 2016, 7, 13–19, doi:10.1016/J.TTBDIS.2015.07.017.

6. Lonc, E.; Guz-Regner, K.; Kiewra, D.; Szczepanska, A. Insight into Tick Biocontrol with Special Regard to Fungi. Ann Parasitol. 2014, 60, 169–177.

7. Banumathi, B.; Vaseeharan, B.; Rajasekar, P.; Prabhu, N.M.; Ramasamy, P.; Murugan, K.; Canale, A.; Benelli, G. Exploitation of chemical, herbal and nanoformulated acaricides to control the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*—A review. Vet. Parasitol. 2017, 244, 102–110, doi:10.1016/J.VETPAR.2017.07.021.

8. Benelli, G.; Pavela, R.; Canale, A.; Mehlhorn, H. Tick repellents and acaricides of botanical origin: A green roadmap to control tick-borne diseases? Parasitol. Res. 2016, 115, 2545–2560, doi:10.1007/s00436-016-5095-1.

9. Otto, P.I.; Guimarães, S.E.F.; Verardo, L.L.; Azevedo, A.L.S.; Vandenplas, J.; Soares, A.C.C.; Sevillano, C.A.; Veroneze, R.; de Fatima, A.; Pires, M.; et al. Genome-

wide association studies for tick resistance in *Bos taurus* × *Bos indicus* crossbred cattle: A deeper look into this intricate mechanism. J. Dairy Sci. 2018, 101, 11020–11032, doi:10.3168/JDS.2017-14223.

10. Shrivastava, N.; Verma, A.; Dash, P.K. Identification of functional epitopes of structural proteins and *in-silico* designing of dual acting multiepitope anti-tick vaccine against emerging Crimean-Congo hemorrhagic fever virus. Eur. J. Pharm. Sci. 2020, 151, 105396, doi:10.1016/J.EJPS.2020.105396.

11. Rodríguez Mallón, A.; Javier González, L.; Encinosa Guzmán, P.E.; Bechara, G.H.; Sanches, G.S.; Pousa, S.; Cabrera, G.; Cabrales, A.; Garay, H.; Mejías, R.; et al. Functional and Mass Spectrometric Evaluation of an Anti-Tick Antigen Based on the P0 Peptide Conjugated to Bm86 Protein. Pathogens 2020, 9, 513, doi:10.3390/pathogens9060513.

12. Kasaija, P.D.; Contreras, M.; Kabi, F.; Mugerwa, S.; de la Fuente, J. de la Vaccination with Recombinant Subolesin Antigens Provides Cross-Tick Species Protection in *Bos indicus* and Crossbred Cattle in Uganda. Vaccines 2020, 8, 319, doi:10.3390/vaccines8020319.

13. Ullah, M.A.; Sarkar, B.; Islam, S.S. Exploiting the reverse vaccinology approach to design novel subunit vaccines against Ebola virus. Immunobiology 2020, 225, 151949, doi:10.1016/j.imbio.2020.151949.

14. Stutzer, C.; Richards, S.A.; Ferreira, M.; Baron, S.; Maritz-Olivier, C. Metazoan Parasite Vaccines: Present Status and Future Prospects. Front. Cell. Infect. Microbiol. 2018, 8, 67, doi:10.3389/fcimb.2018.00067.

15. Obregón Alvarez, D.; Corona-González, B.; Rodríguez-Mallón, A.; Rodríguez Gonzalez, I.; Alfonso, P.; Noda Ramos, A.A.; DíazSánchez, A.A.; González Navarrete, M.; Rodríguez Fernández, R.; Méndez Mellor, L.; et al. Ticks and Tick-Borne Diseases in Cuba, Half a Century of Scientific Research. Pathogens 2020, 9, 616, doi:10.3390/pathogens9080616.

16. de la Fuente, J.; Estrada-Peña, A. Why New Vaccines for the Control of Ectoparasite Vectors Have Not Been Registered and Commercialized? Vaccines 2019, 7, 75, doi:10.3390/vaccines7030075.

17. de la Fuente, J.; Estrada-Peña, A.; Contreras, M. Modeling tick vaccines: A key tool to improve protection efficacy. Expert Rev. Vaccines 2020, 19, 217–225, doi:10.1080/14760584.2020.1745635.

18. Armistead, J.S.; Morlais, I.; Mathias, D.K.; Jardim, J.G.; Joy, J.; Fridman, A.; Finnefrock, A.C.; Bagchi, A.; Plebanski, M.; Scorpio, D.G.; et al. Antibodies to a single, conserved epitope in *Anopheles* APN1 inhibit universal transmission of *Plasmodium falciparum* and *Plasmodium vivax* malaria. Infect. Immun. 2014, 82, 818–829, doi:10.1128/IAI.01222-13.

19. Bhowmick, B.; Han, Q. Understanding Tick Biology and Its Implications in Anti-tick and Transmission Blocking Vaccines Against Tick-Borne Pathogens. Front. Vet. Sci. 2020, 7, 319, doi:10.3389/fvets.2020.00319.

20. Lavazec, C.; Bourgouin, C. Mosquito-based transmission blocking vaccines for interrupting *Plasmodium* development. Microbes Infect. 2008, 10, 845–849.

21. Rappuoli, R.; Bottomley, M.J.; D'Oro, U.; Finco, O.; De Gregorio, E. Reverse vaccinology 2.0: Human immunology instructs vaccine antigen design. J. Exp. Med. 2016, 213, 469–481, doi:10.1084/JEM.20151960.

22. Forthal, D.N. Functions of antibodies. Microbiol. Spectrum. 2014, 2, doi:10.1128/microbiolspec.

23. Rand, K.N.; Moore, T.; Sriskantha, A.; Spring, K.; Tellam, R.; Willadsen, P.; Cobon, G.S. Cloning and expression of a protective antigen from the cattle tick *Boophilus microplus*. Proc. Natl. Acad. Sci. USA 1989, 86, 9657–9661, doi:10.1073/pnas.86.24.9657.

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

25. Lew-Tabor, A.E.; Rodriguez Valle, M. A review of reverse vaccinology approaches for the development of vaccines against ticks and tick borne diseases. Ticks Tick-Borne Dis. 2016, 7, 573–585.

26. Obolo-Mvoulouga, P.; Oleaga, A.; Manzano-Román, R.; Pérez-Sánchez, R. Evaluation of the protective efficacy of *Ornithodoros moubata* midgut membrane

antigens selected using omics and *in silico* prediction algorithms. Ticks Tick-Borne Dis. 2018, 9, 1158– 1172, doi:10.1016/j.ttbdis.2018.04.015.

27. de la Fuente, J.; Almazán, C.; Canales, M.; de la Lastra, J.; Kocan, K.; Willadsen, P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim. Health Res. Rev. 2007, 8, 23, doi:10.1017/S1466252307001193.

28. María, R.R.; Arturo, C.J.; Alicia, J.A.; Paulina, M.G.; Gerardo, A.O. The Impact of Bioinformatics on Vaccine Design and Development. Vaccines 2017, doi:10.5772/intechopen.69273.

29. Hossain, M.U.; Keya, C.A.; Das, K.C.; Hashem, A.; Omar, T.M.; Khan, M.A.; Rakib-Uz-Zaman, S.M.; Salimullah, M. An immunopharmacoinformatics approach in development of vaccine and drug candidates for West Nile Virus. Front. Chem. 2018, 6, doi:10.3389/fchem.2018.00246.

30. Maritz-Olivier, C.; van Zyl, W.; Stutzer, C. A systematic, functional genomics, and reverse vaccinology approach to the identification of vaccine candidates in the cattle tick, *Rhipicephalus microplus*. Ticks Tick-Borne Dis. 2012, 3, 179–187, doi:10.1016/j.ttbdis.2012.01.003.

31. Bidmos, F.A.; Siris, S.; Gladstone, C.A.; Langford, P.R. Bacterial vaccine antigen discovery in the reverse vaccinology 2.0 Era: Progress and challenges. Front. Immunol. 2018, 9, 2315.

32. Rappuoli, R.; Bagnoli, F. Vaccine Design: Innovative Approaches and Novel Strategies; Horizon Scientific Press: Norfolk, UK, 2011; doi:10.1093/cid/cir415.

33. Flower, D.R. Bioinformatics for Vaccinology; John Wiley & Sons: Hoboken, NJ, USA, 2008; ISBN 9780470027110.

34. Moriel, D.G.; Scarselli, M.; Serino, L.; Mora, M.; Rappuoli, R.; Masignani, V. Genome-based vaccine development: A short cut for the future. Hum. Vaccin. 2008, 4, 184–188, 184–188, doi:10.4161/hv.4.3.6313.

35. Dadgar Pakdel, J.; Zakeri, S.; Raz, A.; Dinparast Djadid, N. Identification, molecular characterization and expression of aminopeptidase N-1 (APN-1) from *Anopheles stephensi* in SF9 cell line as a candidate molecule for developing a vaccine that interrupt malaria transmission. Malar. J. 2020, 19, 1–19, doi:10.1186/s12936-020-03154-3.

36. Elton, C.M.; Rodriguez, M.; Ben Mamoun, C.; Lobo, C.A.; Wright, G.J. A library of recombinant *Babesia microti* cell surface and secreted proteins for diagnostics discovery and reverse vaccinology. Int. J. Parasitol. 2019, 49, 115–125, doi:10.1016/j.ijpara.2018.10.003.

37. Kar, P.P.; Srivastava, A. Immuno-informatics Analysis to Identify Novel Vaccine Candidates and Design of a Multi-Epitope Based Vaccine Candidate Against *Theileria* parasites. Front. Immunol. 2018, 9, 2213, doi:10.3389/fimmu.2018.02213.

38. Pritam, M.; Singh, G.; Swaroop, S.; Singh, A.K.; Singh, S.P. Exploitation of reverse vaccinology and immunoinformatics as promising platform for genome-wide screening of new effective vaccine candidates against *Plasmodium falciparum*. BMC Bioinform. 2019, 19, 219–230, doi:10.1186/s12859-018-2482-x.

39. e Silva, R. de F.; Ferreira, L.F.G.R.; Hernandes, M.Z.; de Brito, M.E.F.; de Oliveira, B.C.; da Silva, A.A.; de-Melo-Neto, O.P.; Rezende, A.M.; Pereira, V.R.A. Combination of *in silico* methods in the search for potential CD4+ and CD8+ T cell epitopes in the proteome of *Leishmania braziliensis*. Front. Immunol. 2016, 7, 327, doi:10.3389/fimmu.2016.00327.

40. Oli, A.N.; Obialor, W.O.; Ifeanyichukwu, M.O.; Odimegwu, D.C.; Okoyeh, J.N.; Emechebe, G.O.; Adejumo, S.A.; Ibeanu, G.C. Immunoinformatics and Vaccine Development: An Overview. ImmunoTargets Ther. 2020, 9, 13–30, doi:10.2147/itt.s241064.

41. Pérez-Sánchez, R.; Manzano-Román, R.; Obolo-Mvoulouga, P.; Oleaga, A. Function-guided selection of midgut antigens from *Ornithodoros erraticus* ticks and an evaluation of their protective efficacy in rabbits. Vet. Parasitol. 2019, 272, 1–12, doi:10.1016/j.vetpar.2019.06.016.

42. Xu, Z.; Yan, Y.; Cao, J.; Zhou, Y.; Zhang, H.; Xu, Q.; Zhou, J. A family of serine protease inhibitors (serpins) and its expression profiles in the ovaries of *Rhipicephalus haemaphysaloides*. Infect. Genet. Evol. 2020, 84, doi:10.1016/j.meegid.2020.104346.

43. Maruyama, S.R.; Garcia, G.R.; Teixeira, F.R.; Brandão, L.G.; Anderson, J.M.; Ribeiro, J.M.C.; Valenzuela, J.G.; Horackova, J.; Veríssimo, C.J.; Katiki, L.M.; et al. Mining a differential sialotranscriptome of *Rhipicephalus microplus* guides antigen discovery to formulate a vaccine that reduces tick infestations. Parasites Vectors 2017, 10, 206, doi:10.1186/s13071-017-2136-2.

44. Kotál, J.; Langhansová, H.; Lieskovská, J.; Andersen, J.F.; Francischetti, I.M.B.; Chavakis, T.; Kopecký, J.; Pedra, J.H.F.; Kotsyfakis, M.; Chmelař, J. Modulation of host immunity by tick saliva. J. Proteom. 2015, 128, 58–68.

45. Francischetti, I.M.B.; Sa-Nunes, A.; Mans, B.J.; Santos, I.M.; Ribeiro, J.M.C. The role of saliva in tick feeding. Front. Biosci. 2009, 14, 2051–2088, doi:10.2741/3363.

46. Wikel, S. Ticks and tick-borne pathogens at the cutaneous interface: Host defenses, tick countermeasures, and a suitable environment for pathogen establishment. Front. Microbiol. 2013, 4, doi:10.3389/fmicb.2013.00337.

47. Neelakanta, G.; Sultana, H. Transmission-Blocking Vaccines: Focus on Anti-Vector Vaccines against Tick-Borne Diseases. Arch. Immunol. Ther. Exp. 2015, 63, 169–179.

48. Chmelar, J.; Kotál, J.; Kovaríková, A.; Kotsyfakis, M. The use of tick salivary proteins as novel therapeutics. Front. Physiol. 2019, 10, 812.

49. Chmelař, J.; Kotál, J.; Kopecký, J.; Pedra, J.H.F.; Kotsyfakis, M. All For One and One For All on the Tick-Host Battlefield. Trends Parasitol. 2016, 32, 368–377.

50. Richards, S.A.; Stutzer, C.; Bosman, A.M.; Maritz-Olivier, C. Transmembrane proteins—Mining the cattle tick transcriptome. Ticks Tick-Borne Dis. 2015, 6, 695–710, doi:10.1016/j.ttbdis.2015.06.002.

51. Nosrati, M.; Behbahani, M.; Mohabatkar, H. Towards the first multi-epitope recombinant vaccine against Crimean-Congo hemorrhagic fever virus: A computer-aided vaccine design approach. J. Biomed. Inform. 2019, 93, 103160, doi:10.1016/j.jbi.2019.103160.

52. Solanki, V.; Tiwari, V. Subtractive proteomics to identify novel drug targets and reverse vaccinology for the development of chimeric vaccine against *Acinetobacter baumannii*. Sci. Rep. 2018, 8, 9044, doi:10.1038/s41598-018-26689-7.

53. Dinglasan, R.R.; Kalume, D.E.; Kanzok, S.M.; Ghosh, A.K.; Muratova, O.; Pandey, A.; Jacobs-Lorena, M. Disruption of *Plasmodium falciparum* development by antibodies against a conserved mosquito midgut antigen. Proc. Natl. Acad. Sci. USA 2007, 104, 13461–13466, doi:10.1073/pnas.0702239104.

54. Couto, J.; Tonk, M.; Ferrolho, J.; Antunes, S.; Vilcinskas, A.; de la Fuente, J.; Domingos, A.; Cabezas-Cruz, A. Antiplasmodial activity of tick defensins in a mouse model of malaria. Ticks Tick-Borne Dis. 2018, doi:10.1016/j.ttbdis.2018.03.011.

55. Hajdusek, O.; Almazán, C.; Loosova, G.; Villar, M.; Canales, M.; Grubhoffer, L.; Kopacek, P.; de la Fuente, J. Characterization of ferritin 2 for the control of tick infestations. Vaccine 2010, 28, 2993–2998, doi:10.1016/j.vaccine.2010.02.008.

56. Dai, J.; Wang, P.; Adusumilli, S.; Booth, C.J.; Narasimhan, S.; Anguita, J.; Fikrig, E. Antibodies against a Tick Protein, Salp15, Protect Mice from the Lyme Disease Agent. Cell Host Microbe 2009, 6, 482–492, doi:10.1016/j.chom.2009.10.006.

57. Alvarez, P.; Leguizamón, M.S.; Buscaglia, C.A.; Pitcovsky, T.A.; Campetella, O. Multiple overlapping epitopes in the repetitive unit of the shed acute-phase antigen from *Trypanosoma cruzi* enhance its immunogenic properties. Infect. Immun. 2001, 69, 7946–7949, doi:10.1128/IAI.69.12.7946-7949.2001.

58. Sela-Culang, I.; Benhnia, M.R.E.I.; Matho, M.H.; Kaever, T.; Maybeno, M.; Schlossman, A.; Nimrod, G.; Li, S.; Xiang, Y.; Zajonc, D.; et al. Using a combined computational-experimental approach to predict antibody-specific B cell epitopes. Structure 2014, 22, 646–657, doi:10.1016/j.str.2014.02.003.

59. Antunes, S.; Couto, J.; Ferrolho, J.; Rodrigues, F.; Nobre, J.; Santos, A.S.; Margarida Santos-Silva, M.; de la Fuente, J.; Domingos, A. *Rhipicephalus bursa* sialotranscriptomic response to blood feeding and *Babesia ovis* infection: Identification of candidate protective antigens. Front. Cell. Infect. Microbiol. 2018, 8, 1–17, doi:10.3389/fcimb.2018.00116.

60. Bolger, A.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 2014, 30, 2114–2120.

61. Grabherr, M.G.; Haas, B.J.; Yassour, M.; Levin, J.Z.; Thompson, D.A.; Amit, I.; Adiconis, X.; Fan, L.; Raychowdhury, R.; Zeng, Q.; et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 2011, 29, 644–652, doi:10.1038/nbt.1883.

62. Simão, F.; Waterhouse, R.; Ioannidis, P.; Kriventseva, E.; Zdobnov, E. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 2015, 31, 3210–3212.

63. Jones, P.; Binns, D.; Chang, H.; Fraser, M.; Li, W.; McAnulla, C.; McWilliam, H.; Maslen, J.; Mitchell, A.; Nuka, G.; et al. InterProScan 5: Genome-scale protein function classification. Bioinformatics 2014, 30, 1236–1240.

64. Petersen, T.; Brunak, S.; Heijne, G. von; Nielsen, H. SignalP 4.0: Discriminating signal peptides from transmembrane regions. Nat. Methods 2011, 8, 785–786.

65. Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J. Mol. Biol. 2001, 305, 567–580, 567–580, doi:10.1006/jmbi.2000.4315.

66. Sonnhammer, E.L.; von Heijne, G.; Krogh, A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc. Int. Conf. Intell. Syst. Mol. Biol. 1998, 6, 175–182.

67. Fu, L.; Niu, B.; Zhu, Z.; Wu, S.; Li, W. CD-HIT: Accelerated for clustering the next-generation sequencing data. Bioinformatics 2012, 28, 3150–3152.

68. Doytchinova, I.A.; Flower, D.R. VaxiJen: A server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinform. 2007, 8, 4, doi:10.1186/1471-2105-8-4.

69. Yu, C.-S.; Chen, Y.-C.; Lu, C.-H.; Hwang, J.-K. Prediction of protein subcellular localization. Proteins Struct. Funct. Bioinform. 2006, 64, 643–651, doi:10.1002/prot.21018.

70. Horton, P.; Park, K.-J.; Obayashi, T.; Fujita, N.; Harada, H.; Adams-Collier, C.J.; Nakai, K. WoLF PSORT: Protein localization predictor. Nucleic Acids Res. 2007, 35, W585-7, doi:10.1093/nar/gkm259.

71. Almagro Armenteros, J.J.; Tsirigos, K.D.; Sønderby, C.K.; Petersen, T.N.; Winther, O.; Brunak, S.; von Heijne, G.; Nielsen, H. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 2019, 37, 420–423, doi:10.1038/s41587-019-0036-z.

72. Eisenhaber, B.; Bork, P.; Eisenhaber, F. Sequence properties of GPI-anchored proteins near the omega-site: Constraints for the polypeptide binding site of the putative transamidase. Protein Eng. Des. Sel. 1998, 11, 1155–1161, doi:10.1093/protein/11.12.1155.

73. Fankhauser, N.; Maser, P. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. Bioinformatics 2005, 21, 1846–1852, doi:10.1093/bioinformatics/bti299.

74. Madeira, F.; Park, Y.M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A.R.N.; Potter, S.C.; Finn, R.D.; et al. The EMBL-EBI search and sequence

analysis tools APIs in 2019. Nucleic Acids Res. 2019, 47, W636–W641, doi:10.1093/nar/gkz268.

75. Dobson, L.; Reményi, I.; Tusnády, G.E. CCTOP: A Consensus Constrained TOPology prediction web server. Nucleic Acids Res. 2015, 43, W408–W412, doi:10.1093/nar/gkv451.

76. Couto, J.; Villar, M.; Mateos-Hernández, L.; Ferrolho, J.; Sanches, G.; Sofia Santos, A.; Santos-Silva, M.; Nobre, J.; Moreira, O.; Antunes, S.; et al. Quantitative Proteomics Identifies Metabolic Pathways Affected by *Babesia* Infection and Blood Feeding in the Sialoproteome of the Vector *Rhipicephalus bursa*. Vaccines 2020, 8, 91, doi:10.3390/vaccines8010091.

77. Jespersen, M.C.; Peters, B.; Nielsen, M.; Marcatili, P. BepiPred-2.0: Improving sequence-based B-cell epitope prediction using conformational epitopes. Nucleic Acids Res. 2017, 45, W24–W29, doi:10.1093/nar/gkx346.

78. Chou, P.Y.; Fasman, G.D. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. Relat. Areas Mol. Biol. 1978, 47, 45–148, doi:10.1002/9780470122921.ch2.

79. Karplus, P.A.; Schulz, G.E. Prediction of chain flexibility in proteins. Naturwissenschaften 1985, 72, 212–213, doi:10.1007/BF01195768.

80. Parker, J.M.; Guo, D.; Hodges, R.S. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: Correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. Biochemistry 1986, 25, 5425–5432, doi:10.1021/bi00367a013.

81. Kolaskar, A.; Tongaonkar, P. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett. 1990, 276, 172–174.

82. Nielsen, M.; Lundegaard, C.; Lund, O.; Keşmir, C. The role of the proteasome in generating cytotoxic T-cell epitopes: Insights obtained from improved predictions of proteasomal cleavage. Immunogenetics 2005, 57, 33–41, doi:10.1007/s00251-005-0781-7.

83. Dimitrov, I.; Naneva, L.; Doytchinova, I.; Bangov, I. AllergenFP: Allergenicity prediction by descriptor fingerprints. Bioinformatics 2014, 30, 846–851.

84. Dimitrov, I.; Bangov, I.; Flower, D.R.; Doytchinova, I. AllerTOP v.2—a server for *in silico* prediction of allergens. J. Mol. Model. 2014, 20, 2278, doi:10.1007/s00894-014-2278-5.

85. Maurer-Stroh, S.; Krutz, N.; Kern, P.; Gunalan, V.; Nguyen, M.; Limviphuvadh, V.; Eisenhaber, F.; Gerberick, G. AllerCatPro— prediction of protein allergenicity potential from the protein sequence. Bioinformatics 2019, 35, 3020–3027.

86. Hebditch, M.; Carballo-Amador, M.A.; Charonis, S.; Curtis, R.; Warwicker, J. Protein-Sol: A web tool for predicting protein solubility from sequence. Bioinformatics 2017, 33, 3098–3100, doi:10.1093/bioinformatics/btx345.

87. Kurgan, L.; Razib, A.A.; Aghakhani, S.; Dick, S.; Mizianty, M.; Jahandideh, S. Crystalp2: Sequence-based protein crystallization propensity prediction. BMC Struct. Biol. 2009, 9, 50, doi:10.1186/1472-6807-9-50.

88. Pejaver, V.; Hsu, W.L.; Xin, F.; Dunker, A.K.; Uversky, V.N.; Radivojac, P. The structural and functional signatures of proteins that undergo multiple events of post-translational modification. Protein Sci. 2014, 23, 1077–1093, doi:10.1002/pro.2494.

89. Sigrist, C.; De Castro, E.; Cerutti, L.; Cuche, B.; Hulo, N.; Bridge, A.; Bougueleret, L.; Xenarios, I. New and continuing developments at PROSITE. Nucleic Acids Res. 2012, 41, D344–D347.

90. Audagnotto, M.; Dal Peraro, M. Protein post-translational modifications: *In silico* prediction tools and molecular modeling. Comput. Struct. Biotechnol. J. 2017, 15, 307–319.

91. Chaudhary, K.; Kumar, R.; Singh, S.; Tuknait, A.; Gautam, A.; Mathur, D.; Anand, P.; Varshney, G.C.; Raghava, G.P.S. A web server and mobile app for computing hemolytic potency of peptides. Sci. Rep. 2016, 6, 1–13, doi:10.1038/srep22843.

92. Ettayapuram Ramaprasad, A.S.; Singh, S.; Gajendra, P.S.R.; Venkatesan, S. AntiAngioPred: A Server for Prediction of AntiAngiogenic Peptides. PLoS ONE 2015, 10, e0136990, doi:10.1371/journal.pone.0136990.

93. Gupta, S.; Kapoor, P.; Chaudhary, K.; Gautam, A.; Kumar, R.; Raghava, G.P.S. *In Silico* Approach for Predicting Toxicity of Peptides and Proteins. PLoS ONE 2013, 8, e73957, doi:10.1371/journal.pone.0073957.

94. Patarroyo, J.H.; Portela, R.W.; De Castro, R.O.; Couto Pimentel, J.; Guzman, F.; Patarroyo, M.E.; Vargas, M.I.; Prates, A.A.; Dias Mendes, M.A. Immunization of cattle

with synthetic peptides derived from the *Boophilus microplus* gut protein (Bm86). Vet. Immunol. Immunopathol. 2002, 88, 163–172, doi:10.1016/S0165-2427(02)00154-X.

95. Patarroyo, S.J.H.; de Sousa Neves, E.; Fidelis, C.F.; Tafur-Gómez, G.A.; de Araujo, L.; Vargas, M.I.; Sossai, S.; Prates-Patarroyo, P.A. Bovine immunisation with a recombinant peptide derived from synthetic SBm7462® (Bm86 epitope construct) immunogen for *Rhipicephalus microplus* control. Ticks Tick-Borne Dis. 2020, 11, 101461, doi:10.1016/j.ttbdis.2020.101461.

96. Seppey, M.; Manni, M.; Zdobnov, E.M. BUSCO: Assessing genome assembly and annotation completeness. In Methods in Molecular Biology; Humana Press Inc: New York, NY, USA, 2019; Volume 1962, pp. 227–245, doi:10.1007/978-1-4939-9173-0_14.

97. Baron, S.; Barrero, R.A.; Black, M.; Bellgard, M.I.; van Dalen, E.; Maritz-Olivier, C. Differentially expressed genes in response to amitraz treatment suggests a proposed model of resistance to amitraz in *R. decoloratus* ticks. Int. J. Parasitol. Drugs Drug Resist. 2018, 8, 361–371, doi:10.1016/j.ijpddr.2018.06.005.

98. Usmani, S.S.; Kumar, R.; Bhalla, S.; Kumar, V.; Raghava, G.P.S. *In Silico* Tools and Databases for Designing Peptide-Based Vaccine and Drugs. In Advances in Protein Chemistry and Structural Biology; Elsevier: Amsterdam, The Netherlands, 2018; Volume 112, pp. 221–263.

99. Zhao, L.; Wu, S.; Jiang, J.; Li, W.; Luo, J.; Li, J. Novel overlapping subgraph clustering for the detection of antigen epitopes. Bioinformatics 2018, 34, 2061–2068, doi:10.1093/bioinformatics/bty051.

100. Bremel, R.D.; Homan, E.J. Recognition of Higher Order Patterns in Proteins: Immunologic Kernels. PLoS ONE 2013, 8, e70115, doi:10.1371/journal.pone.0070115.

101. de la Fuente, J.; Antunes, S.; Bonnet, S.; Cabezas-Cruz, A.; Domingos, A.G.; Estrada-Peña, A.; Johnson, N.; Kocan, K.M.; Mansfield, K.L.; Nijhof, A.M.; et al. Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases. Front. Cell. Infect. Microbiol. 2017, 7, 114, doi:10.3389/fcimb.2017.00114.

102. Farrell, D.; Jones, G.; Pirson, C.; Malone, K.; Rue-Albrecht, K.; Chubb, A.J.; Vordermeier, M.; Gordon, S.V. Integrated computational prediction and experimental validation identifies promiscuous T cell epitopes in the proteome of *Mycobacterium bovis*. Microb. Genom. 2016, 2, e000071, doi:10.1099/mgen.0.000071.

103. Kardani, K.; Bolhassani, A.; Namvar, A. An overview of *in silico* vaccine design against different pathogens and cancer. Expert Rev. Vaccines 2020, 1–28, doi:10.1080/14760584.2020.1794832.

104. Caraballo, L.; Coronado, S. Parasite allergens. Mol. Immunol. 2018, 100, 113–119, doi:10.1016/j.molimm.2018.03.014.

105. Sánchez-Pulido, L.; Martín-Belmonte, F.; Valencia, A.; Alonso, M.A. MARVEL: A conserved domain involved in membrane apposition events. Trends Biochem. Sci. 2002, 27, 599–601, doi:10.1016/S0968-0004(02)02229-6.

106. Hayward, J.; Sanchez, J.; Perry, A.; Huang, C.; Rodriguez Valle, M.; Canals, M.; Payne, R.J.; Stone, M.J. Ticks from diverse genera encode chemokine-inhibitory evasin proteins. J. Biol. Chem. 2017, 292, 15670–15680, doi:10.1074/jbc.M117.807255.

107. Rutenber, E.; Ready, M.; Robertus, J.D. Structure and evolution of ricin B chain. Nature 1987, 326, 624–626, doi:10.1038/326624a0.

108. Hazes, B.; Read, R.J. A mosquitocidal toxin with a ricin-like cell-binding domain. Nat. Struct. Biol. 1995, 2, 358–359.

109. Hirabayashi, J.; Dutta, S.K.; Kasai, K.I. Novel galactose-binding proteins in Annelida: Characterization of 29- kDa tandem repeat-type lectins from the earthworm *Lumbricus terrestris*. J. Biol. Chem. 1998, 273, 14450–14460, doi:10.1074/jbc.273.23.14450.

110. Hutson, R.A.; Zhou, Y.; Collins, M.D.; Johnson, E.A.; Hatheway, C.L.; Sugiyama, H. Genetic characterization of *Clostridium botulinum* type A containing silent type B neurotoxin gene sequences. J. Biol. Chem. 1996, 271, 10786–10792, doi:10.1074/jbc.271.18.10786.

111. Eyigor, A.; Dawson, K.A.; Langlois, B.E.; Pickett, C.L. Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter coli* isolates: Detection and analysis by PCR. J. Clin. Microbiol. 1999, 37, 1646–1650, doi:10.1128/jcm.37.5.1646-1650.1999.

112. Bastos, R.G.; Suarez, C.E.; Laughery, J.M.; Johnson, W.C.; Ueti, M.W.; Knowles, D.P. Differential Expression of Three Members of the Multidomain Adhesion CCp Family in *Babesia bigemina*, *Babesia bovis* and *Theileria equi*. PLoS ONE 2013, 8, e67765, doi:10.1371/journal.pone.0067765.

113. Babenko, V.V.; Podgorny, O.V.; Manuvera, V.A.; Kasianov, A.S.; Manolov, A.I.; Grafskaia, E.N.; Shirokov, D.A.; Kurdyumov, A.S.; Vinogradov, D.V.; Nikitina, A.S.; et al. Draft genome sequences of *Hirudo medicinalis* and salivary transcriptome of three closely related medicinal leeches. BMC Genom. 2020, 21, 1–16, doi:10.1186/s12864-020-6748-0.

114. Gupta, G.S.; Gupta, R.K.; Gupta, G.S. R-Type Lectin Families. In Animal Lectins: Form, Function and Clinical Applications; Springer: Vienna, Austria, 2012; pp. 313–330.

115. Pohleven, J.; Obermajer, N.; Sabotič, J.; Anžlovar, S.; Sepčić, K.; Kos, J.; Kralj, B.; Štrukelj, B.; Brzin, J. Purification, characterization and cloning of a ricin B-like lectin from mushroom *Clitocybe nebularis* with antiproliferative activity against human leukemic T cells. Biochim. Biophys. Acta Gen. Subj. 2009, 1790, 173–181, doi:10.1016/j.bbagen.2008.11.006.

116. Adams, G.P.; Weiner, L.M. Monoclonal antibody therapy of cancer. Nat. Biotechnol. 2005, 23, 1147–1157.

117. Slastnikova, T.A.; Ulasov, A.V.; Rosenkranz, A.A.; Sobolev, A.S. Targeted intracellular delivery of antibodies: The state of the art. Front. Pharmacol. 2018, 9, doi:10.3389/fphar.2018.01208.

118. Palanisamy, N.; Lennerstrand, J. Computational Prediction of Usutu Virus E Protein B Cell and T Cell Epitopes for Potential Vaccine Development. Scand. J. Immunol. 2017, 85, 350–364, doi:10.1111/sji.12544.

Chapter IV: Conserved pathways between *Rhipicephalus* tick species

1. "Folate pathway modulation in *Rhipicephalus* ticks in response to infection"

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1.1. Abstract

Folate pathways components were demonstrated to be present in RNA-sequencing data obtained from uninfected and pathogen-infected *Rhipicephalus* ticks. Here, PCR and qPCR allowed the identification of folate-related genes in *Rhipicephalus* spp. ticks and in the tick cell line IDE8. Genes coding for GTP cyclohydrolase I (gch-I), thymidylate synthase (ts) and 6-pyrovoyltetrahydropterin (ptps) were identified. Differential gene expression was evaluated by qPCR between uninfected and infected samples of four biological systems, showing significant upregulation and largest fold-change for the *gch-I* gene in the majority of the biological systems, supporting the selection for functional analysis by RNAi silencing. Efficient knockdown of the *gch-I* gene in uninfected and *Ehrlichia canis*-infected IDE8 cells showed no detectable impact on the capacity of the bacteria to invade or replicate in the tick cells. Overall, this work demonstrated an increase in the expression of some folate-related genes, though not always statistically significantly, in the presence of infection, suggesting gene expression modulation of these pathways, either as a tick response to an invader or manipulation of the tick cell

machinery by the pathogens to their advantage. This discovery points to folate pathways as interesting targets for further studies.

Keywords: folate, RNAi, tick cell line, tick-borne diseases, vector-pathogen interface

1.2. Introduction

Tick-borne diseases (TBDs) are responsible for a great burden on human and animal health worldwide (Jongejan & Uilenberg, 2004). With the increase in emerging TBDs observed in recent decades (Wikel, 2018), there is an urgent need for the development of cost-effective and environmentally-friendly strategies for tick control and transmission-blocking alternatives (Mapholi et al., 2014). The development of transmission-blocking strategies with the capacity to affect several pathogens across multiple tick species is economically and technically attractive. Such an accomplishment could only be attained by pinpointing key vector pathways. However, the key step for the design of such approaches relies on the selection of promising targets with important biological roles, which can be hampered by the lack of tick genomic resources. RNA-sequencing projects are a useful resource for the selection of targets in "non-model" organisms (Oppenheim, Baker, Simon, & DeSalle, 2015).

Folate pathway components were present in RNA-sequencing data obtained from *Rhipicephalus* spp. ticks (*Rhipicephalus bursa* – Antunes et al., 2018, *Rhipicephalus annulatus* – Antunes et al., 2019 and *Rhipicephalus sanguineus* – BioProject: PRJNA362595) that are important vectors of causative agents of diseases of farm animals and pets such as *Babesia ovis*, *Babesia bigemina* and *Ehrlichia canis* (Sonenshine & Roe, 2014). Folate-related compounds and enzymes are essential in a vast panoply of physiological processes, having a broad impact on cell growth and in the normal development of organisms (Ducker & Rabinowitz, 2017). This study aims to identify and evaluate the expression profile of folate-related genes, and to further assess by gene knockdown the role of a selected target in cell survival and infection. Here, we applied PCR and qPCR for the identification and assessment of expression patterns of three genes from these pathways, coding for GTP cyclohydrolase I (GCH-I), thymidylate synthase (TS) and 6-pyruvoyltetrahydropterin synthase (PTPS), in *Rhipicephalus* ticks and in the tick cell line IDE8. Genes *gch-I* and *ptps* code for the enzymes of the first two steps of

production of tetrahydrobiopterin (BH4), an essential cofactor for the production of nitric oxide (NO) and amine neurotransmissors (Werner, Blau, & Thöny, 2011). TS is responsible for the production of thymidine (dTMP) and therefore involved in DNA replication and cell multiplication (Ackland, Clarke, Beale, & Peters, 2006). Differential expression of these genes during infection was analyzed in four biological systems: *R. annulatus – B. bigemina*; *R. bursa – B. ovis*; *R. sanguineus – E. canis*; IDE8 cells – *E. canis*, allowing the selection of candidate genes for further functional analysis by RNA interference (RNAi) *in vitro* (Barry et al., 2013). Studies focusing on folate-related pathways will contribute to a deeper understanding of their role in the vector-host interface.

1.3. Materials and methods

1.3.1. Samples

RNA from individual salivary glands (SGs) was obtained from: seven uninfected and seven *B. ovis*-infected *R. bursa* ticks, as described by Antunes et al. (2018); 10 uninfected and 10 *B. bigemina*-infected *R. annulatus* ticks, as described by Antunes et al. (2012); and three uninfected and three-*E. canis* infected pools containing ten pairs of SGs each from the tropical lineage of *R. sanguineus* ticks, as described by Ferrolho et al. (2017). Only female ticks were used. All ticks were fully engorged except *R. sanguineus* which were freshly-molted adults. The *Ixodes scapularis* embryo-derived cell line IDE8 (Munderloh, Liu, Wang, Chen, & Kurtti, 1994) was maintained in two conditions: uninfected and infected with semipurified *E. canis*, Spain 105 strain (Zweygarth et al., 2014) following the protocol described by Ferrolho, Simpson, Hawes, Zweygarth, and Bell-Sakyi (2016) except that 0.1% NaHCO3 and 10 mM HEPES were not added to the culture medium. RNA was extracted using Tri-Reagent (Sigma–Aldrich), the quality and integrity of all RNA samples was evaluated using the QIAxcel equipment and kit (Qiagen) according to the manufacturer's instructions, and concentrations were estimated by ND-1000 Spectrophotometer (NanoDrop ND1000; Thermo Fisher Scientific). RNA concentrations of 500 ng/µl for *R. annulatus* and IDE8, 250 ng/µl for *R. sanguineus* and 150 ng/µl for *R. bursa* were used for cDNA synthesis with iScript™ cDNA Synthesis Kit (Bio-Rad) in a T100 Thermal Cycler (Bio-Rad).

1.3.2. Gene identification

PCR was performed with NZYTaq II 2× Green Master Mix (NZYTech) in a total reaction volume of 25 µl following the manufacturer's protocol in a T100 Thermal Cycler (Bio-Rad). qPCR was performed in triplicate using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) and a total reaction volume of 10 µl in a CFX96 Touch Real-time PCR (Bio-Rad). For qPCR a standard curve with serial dilutions was included to determine amplification efficiency through the standard curve slope. Primer sequences and conditions are listed in Table S1. Products were purified with NZYGelpure kit (NZYTech) and sequenced by the Sanger method (StabVida). InterPro (available at www.ebi.ac.uk/interpro/) was used to check the presence in the identified sequences of conserved domains including active sites.

1.3.3. Differential expression analysis

Differential gene expression between uninfected and infected samples was carried out by qPCR. The expression of four candidate reference genes, 16S rDNA (Ferrolho et al., 2017), *β-tubulin*, *β-actin* and *elf* (Nijhof, Balk, Postigo, & Jongejan, 2009) was evaluated in each biological system using the geNorm algorithm (Vandesompele et al., 2002) incorporated in the CFX Manager™ Software (Bio-Rad). Data normalization was performed using the reference genes that showed the lowest variation: 16S rDNA, *β-tubulin*, *β-actin* and *elf* for *R. annulatus*; 16S rDNA, *β-tubulin* and *elf* for *R. bursa*; *β-actin* and *elf* for *R. sanguineus*; 16S rDNA, *β-tubulin* and *β-actin* for IDE8 cells. Relative gene expression after normalization was assessed using the above-mentioned software by the ΔΔCq (Livak & Schmittgen, 2001) and Pfaff (Pfaff, 2001) methods. Outliers were singled out by the Tukey method (Tukey, 1977) and Cq-values were compared between conditions by Student's t test. A statistically-significant difference was considered when the *p*-value was <0.05.

1.3.4. RNA interference

Specific primers containing a T7 promoter sequence in the 5′ end (Fw: 5′-ACGACGAGATGGTCATTGTG-3′ and Rv: 5′-AGCGTCGTGTCCCACTCTT-3′) were used to amplify by PCR a fragment of 461 bp of the *gch-I* gene with iProof™ High

Fidelity DNA Polymerase (Bio-Rad). This product was used for double-stranded RNA (dsRNA) synthesis using the MEGAscript RNAi Kit (Ambion). For the *in vitro* silencing assay, cells were seeded in 24-well plates and 24 hr later *gch-I* dsRNA or dsRNA for an unrelated control gene, mouse *beta-2 microglobulin* (*β2m*) (Couto et al., 2017), was added at a concentration of 5×10^{10} molecules/µl. The assay included three groups: Group A – uninfected IDE8 cells; Group B – uninfected IDE8 cells that were inoculated with *E. canis* 24 hr after dsRNA addition (to evaluate the effect on bacterial invasion); Group C– IDE8 cells with a 7-day pre-established *E. canis* infection (to evaluate the effect on bacterial multiplication). Three time points were evaluated: 24 hr (T1), 96 hr (T2) and 144 hr (T3) after dsRNA addition. Giemsa-stained cytocentrifuge smears (Ferrolho et al., 2016) were also performed for morphological analysis. Five replicates were collected for RNA extraction and 250 ng/µl were used for cDNA synthesis. qPCR analysis of *gch-I* expression was performed as described above and data was normalized with 16S rDNA, *β-actin*, and *r13a* (Weisheit et al., 2015). qPCR was also applied for relative quantification of *E. canis* with the ehrlichial *dsb* gene (Doyle et al., 2005), using cDNA as template, and data was normalized against *β-actin* and *r13a*. Percentage of gene silencing was calculated as the ratio of *gch-I* expression between the treated group (exposed to *gch-I* dsRNA) and the control group (exposed to *β2m* dsRNA).

1.4. Results and discussion

PCR and qPCR allowed the amplification of three genes: *gch-I*, *ts* and *ptps* in *R. annulatus*, *R. bursa*, *R. sanguineus* and the IDE8 cell line, that showed identities between 71% and 99% with the mRNA sequences originally retrieved from different ixodid species (Table S1) and, as such, were considered to correspond to folate pathway-related genes. Conserved domains containing active sites were identified in these sequences. The gch-I sequences presented the two conserved active sites from GTP cyclohydrolase I (IPR018234), while ptps sequences showed the cysteine (IPR022470) and the histidine (IPR022469) active site from the 6-pyruvoyl tetrahydropterin synthase. The ts sequences from *Ixodes* spp. exhibited the active site from thymidylate synthase (IPR020940). Differential expression of those genes, after infection, was evaluated in four biological

systems: *R. annulatus – B. bigemina*; *R. bursa – B. ovis*; *R. sanguineus – E. canis*; IDE8 – *E. canis* (Figure 1).

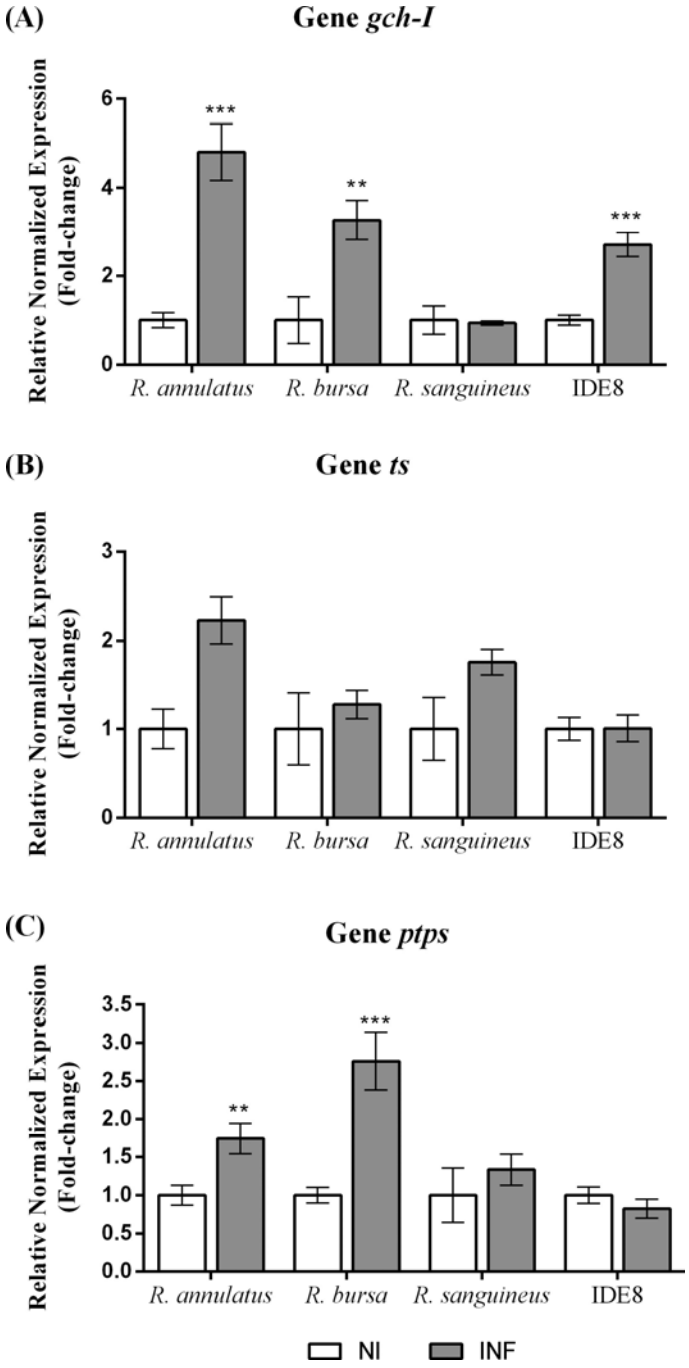

Figure 1. Differential gene expression in four different tick-pathogen biological systems. Relative expression of *gch-I* **(A)**, *ts* **(B)** and *ptps* **(C)** genes for the infected (INF) samples compared with the non-infected (NI) controls in the four biological systems: *Rhipicephalus annulatus – Babesia bigemina*, *Rhipicephalus bursa – Babesia ovis*, *Rhipicephalus sanguineus – Ehrlichia canis*; IDE8 – *E. canis*. The

graphs represent the mean ± SEM with statistically- significant differences indicated with $p < 0.01$ (**), $p < 0.001$ (***)

The non-vector tick cell line IDE8 was used because it supports continuous growth of *E. canis*, in contrast to cell lines derived from the vector *R. sanguineus* (Ferrolho et al., 2016). For the *gch-I* gene, statistically-significant up-regulation ($p < 0.05$) was observed for infected samples of *R. annulatus* ($p < 0.001$; 4.8-fold change), *R. bursa* ($p = 0.002$; 3.3-fold change) and in IDE8 cells ($p < 0.001$; 2.7-fold change). In *R. sanguineus*, however, there was no difference in gene expression between uninfected and *E. canis*-infected ticks. For the *ts* gene, samples from *Rhipicephalus* spp. ticks showed an increase in expression when the pathogen was present, being 2.2-fold change for *R. annulatus* ($p = 0.129$), 1.3-fold change for *R. bursa* ($p = 0.072$) and 1.6-fold change for *R. sanguineus* ($p = 0.428$), although these changes were not significant; for the IDE8 cell line there was no difference in expression between uninfected and *E. canis*-infected samples ($p = 0.634$). For the *ptps* gene, statistically-significant up-regulation was observed in *R. annulatus* ($p = 0.007$; 1.7-fold change) and *R. bursa* ($p < 0.001$; 1.3-fold change) when *Babesia* was present. *R. sanguineus* showed an increase in expression ($p = 0.158$; 1.7-fold change) and the IDE8 cell line had a slight decrease ($p = 0.237$; 0.8-fold change) in the presence of *E. canis*. The proteins encoded by the *gch-I* and *ptps* genes are responsible for *de novo* biosynthesis of BH4, an essential cofactor for the synthesis of NO. In mice, treatment with lipopolysaccharides was proven to stimulate the production of NO by increasing BH4 levels, while treatment with 4-Diamino-6-hydroxypyrimidine, a GCH-I inhibitor, led to the reduction of NO levels (Gross & Levi, 1992). As such, exposure to infectious pathogens may be responsible for the increased expression of these genes, as an immune defense mechanism by the tick. The *ts* gene is involved in the production of nucleotides having an important role in cell replication events, and is a target in cancer therapy (Chu, Callender, Farrell, & Schmitz, 2003); however its role in the tick-pathogen interface is unclear. Overall, we observed a tendency for up-regulation of these genes in the presence of the pathogens suggesting gene expression modulation, either as an auto-protective tick reaction to the invader microorganisms or as subversion of the vector machinery by the pathogens to their advantage in a similar manner to that observed in *Anaplasma phagocytophilum*. This pathogen has been shown to manipulate expression of proteins such as spectrin alpha chain and mitochondrial porins, involved in cytoskeleton

rearrangement and mitochondrial induced apoptosis respectively, to subvert host cell defense (Ayllón et al., 2013).

Gene *gch-I* was selected for an *in vitro* silencing assay, with silencing efficiency ranging from 83.2% to 100% between experimental groups. A significant increase in *dsb* gene expression was observed between time points for Groups B and C, demonstrating typical *E. canis* multiplication within cells. Relative levels of *E. canis dsb* expression (Figure 2) were not significantly different between the infected IDE8 cell groups exposed to *gch-I* and *β2m* dsRNA for any of the conditions ($p > 0.05$).

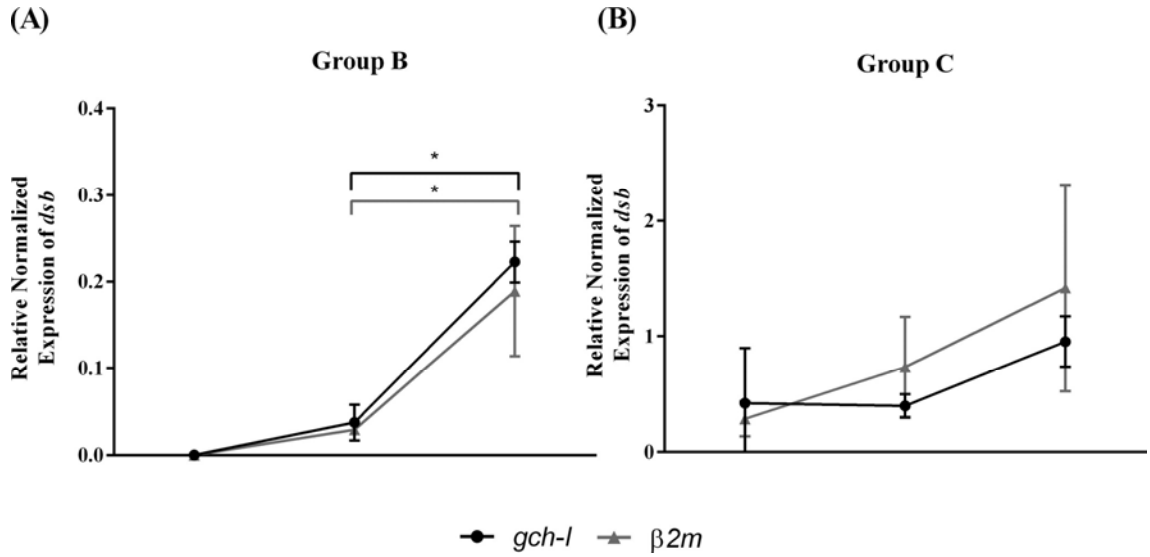

Figure 2. Relative normalized expression of *Ehrlichia canis dsb* over time in infected IDE8 cells. Relative expression of the *dsb* gene for samples of group B – uninfected IDE8 cells inoculated with *E. canis* 24 hr after addition of the dsRNA **(A)**, and group C – IDE8 cells already infected with *E. canis* **(B)**. Samples were exposed to: dsRNA for *β2m* (grey triangles), dsRNA for *gch-I* (black spheres) or medium alone (grey squares). Analysis was carried out at three time points: 24 hr (T1), 96 hr (T2) and 144 hr (T3). Points in the graph represent the means (n = 5), and error bars represent the corresponding standard deviation. Statistically-significant differences ($p < 0.05$) between time points calculated with the Mann-Whitney test for each treatment are indicated with asterisks (*) above the black bar for *gch-I* samples and the grey bar for control *β2m* samples.

Examination of the Giemsa-stained cytocentrifuge smears did not reveal striking differences in morphological characteristics of the tick cell line or the bacteria in the presence of *gch-I* dsRNA. Silencing of the *gch-I* gene did not affect the capacity of *E. canis* to infect and replicate in the tick cells. However, further studies, such as validation of protein under-representation and enzyme inhibition assays, are needed to clarify the

silencing results and to explore the function of the encoded protein. Also, since BH4 can be acquired by salvage pathway, new studies are needed in order to evaluate the role of dihydrofolate reductase in the replenishment of BH4 pools in ticks, through inhibition of the biopterin salvage pathway, a mechanism well described in vertebrates (Crabtree, Tatham, Hale, Alp, & Channon, 2009). The tick microbiome may also play a role in the bioavailability of BH4. A study on GCH-I deficient mice showed that some endosymbiotic bacteria, belonging to the phylum Actinobacteria, have the capacity to produce this compound (Belik et al., 2017). This phylum could also supply BH4 in ticks since those bacteria were shown to be the second most represented in microbiomes of *Amblyomma maculatum* (Varela-Stokes et al., 2018) and *Ixodes ricinus* (Carpi et al., 2011). Moreover, BH4 could also be provided through carrier proteins in cell membranes present in ticks (Perner et al., 2016) which are responsible for the uptake of folate derivates, due to their shared biopterin ring structure (Frye, 2013). Therefore, the effects of *gch-I* knockdown might be undetectable in a short-time frame, hiding potential effects on tick cell fitness and in the interaction with *E. canis*. The performance of assays with an extended time frame would help to understand the importance of this enzyme in the tick-pathogen interaction.

1.5. Conclusions

Here we observed an overall overexpression of three genes from the folate pathways in ticks, *gch-I*, *ts* and *ptps*, which although not always statistically significant in infected ticks and/or cells, suggests gene modulation caused by the presence of the parasite. Although silencing of the *gch-I* gene did not influence the capacity of *E. canis* to infect and replicate in the IDE8 cell line over a short time-frame, this study showed that genes from the folate pathways are interesting targets for further studies on the vector-pathogen interface. *In vitro* assays with folate analogs capable of enzymatic inhibition, taking into consideration both vector and pathogen enzymatic machinery, would help elucidate their role in tick cells and in interaction with pathogens.

1.6. References

Ackland, S. P., Clarke, S. J., Beale, P., & Peters, G. J. (2006). Thymidylate synthase inhibitors. Update on Cancer Therapeutics, 1(4), 403– 427. https://doi.org/10.1016/J.UCT.2006.09.001

Antunes, S., Couto, J., Ferrolho, J., Rodrigues, F., Nobre, J., Santos, A. S., … Domingos, A. (2018). *Rhipicephalus bursa* sialotranscriptomic response to blood feeding and *Babesia ovis* infection: Identification of candidate protective antigens. Frontiers in Cellular and Infection Microbiology, 8(116), 1– 17. https://doi.org/10.3389/fcimb.2018.00116;

Antunes, S., Couto, J., Ferrolho, J., Sanches, G. S., Merino Charrez, J. O., De la Cruz Hernández, N., … Domingos, A. (2019). Transcriptome and proteome response of *Rhipicephalus annulatus* tick vector to *Babesia bigemina* infection. Frontiers in Physiology, 10, 318. https://doi.org/10.3389/fphys.2019.00318;

Antunes, S., Galindo, R. C., Almazán, C., Rudenko, N., Golovchenko, M., Grubhoffer, L., … Domingos, A. (2012). Functional genomics studies of *Rhipicephalus* (*Boophilus*) *annulatus* ticks in response to infection with the cattle protozoan parasite, *Babesia bigemina*. International Journal for Parasitology, 42(2), 187– 195. https://doi.org/10.1016/J.IJPARA.2011.12.003

Ayllón, N., Villar, M., Busby, A. T., Kocan, K. M., Blouin, E. F., Bonzón-Kulichenko, E., … de la Fuente, J. (2013). *Anaplasma phagocytophilum* inhibits apoptosis and promotes cytoskeleton rearrangement for infection of tick cells. Infection and Immunity, 81(7), 2415– 2425. https://doi.org/10.1128/IAI.00194-13

Barry, G., Alberdi, P., Schnettler, E., Weisheit, S., Kohl, A., Fazakerley, J. K., & Bell-Sakyi, L. (2013). Gene silencing in tick cell lines using small interfering or long double-stranded RNA. Experimental & Applied Acarology, 59(3), 319– 338. https://doi.org/10.1007/s10493-012-9598-x

Belik, J., Shifrin, Y., Arning, E., Bottiglieri, T., Pan, J., Daigneault, M. C., & Allen-Vercoe, E. (2017). Intestinal microbiota as a tetrahydrobiopterin exogenous source in hph-1 mice. Scientific Reports, 7, 39854. https://doi.org/10.1038/srep39854

Carpi, G., Cagnacci, F., Wittekindt, N. E., Zhao, F., Qi, J., Tomsho, L. P., … Schuster, S. C. (2011). Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. PLoS One, 6(10), e25604. https://doi.org/10.1371/journal.pone.0025604

Chu, E., Callender, M. A., Farrell, M. P., & Schmitz, J. C. (2003). Thymidylate synthase inhibitors as anticancer agents: From bench to bedside. Cancer Chemotherapy and Pharmacology, 52, 80– 89. https://doi.org/10.1007/s00280-003-0625-9

Couto, J., Antunes, S., Pinheiro-Silva, R., Do Rosário, V., De La Fuente, J., & Domingos, A. (2017). Solute carriers affect *Anopheles stephensi* survival and *Plasmodium berghei* infection in the salivary glands. Scientific Reports, 7, 6141. https://doi.org/10.1038/s41598-017-06317-6

Crabtree, M. J., Tatham, A. L., Hale, A. B., Alp, N. J., & Channon, K. M. (2009). Critical role for tetrahydrobiopterin recycling by dihydrofolate reductase in regulation of endothelial nitric-oxide synthase coupling. Journal of Biological Chemistry, 284(41), 28128– 28136. https://doi.org/10.1074/jbc.M109.041483

Doyle, C. K., Labruna, M. B., Breitschwerdt, E. B., Tang, Y.-W., Corstvet, R. E., Hegarty, B. C., … McBride, J. W. (2005). Detection of medically important *Ehrlichia* by quantitative multicolor TaqMan real-time polymerase chain reaction of the *dsb* gene. The Journal of Molecular Diagnostics, 7(4), 504– 510. https://doi.org/10.1016/S1525-1578(10)60581-8

Ducker, G. S., & Rabinowitz, J. D. (2017). One-carbon metabolism in health and disease. Cell Metabolism, 25(1), 27– 42. https://doi.org/10.1016/j.cmet.2016.08.009

Ferrolho, J., Antunes, S., Sanches, G. S., Couto, J., Évora, P. M., Rosa, C., … Domingos, A. (2017). *Ferritin 1* silencing effect in *Rhipicephalus sanguineus sensu lato* (Acari: Ixodidae) during experimental infection with *Ehrlichia canis*. Ticks and Tick-Borne Diseases, 8(1), 174– 184. https://doi.org/10.1016/j.ttbdis.2016.10.015

Ferrolho, J., Simpson, J., Hawes, P., Zweygarth, E., & Bell-Sakyi, L. (2016). Growth of *Ehrlichia canis*, the causative agent of canine monocytic ehrlichiosis, in vector and non-vector ixodid tick cell lines. Ticks and Tick-Borne Diseases, 7(4), 631– 637. https://doi.org/10.1016/j.ttbdis.2016.01.013

Frye, R. E. (2013). Tetrahydrobiopterin may be transported into the central nervous system by the folate receptor α. North American Journal of Medicine and Science, 6(3), 117– 120. https://doi.org/10.7156/najms.2013.0603117

Gross, S. S., & Levi, R. (1992). Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. Journal of Biological Chemistry, 267(36), 25722– 25729.

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. Parasitology, 129(Suppl), S3– S14.

Livak, K., & Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25(4), 402– 408. https://doi.org/10.1006/meth.2001.1262

Mapholi, N. O., Marufu, M. C., Maiwashe, A., Banga, C. B., Muchenje, V., MacNeil, M. D., … Dzama, K. (2014). Towards a genomics approach to tick (Acari: Ixodidae) control in cattle: A review. Ticks and Tick-Borne Diseases, 5(5), 475– 483. https://doi.org/10.1016/j.ttbdis.2014.04.006

Munderloh, U. G., Liu, Y., Wang, M., Chen, C., & Kurtti, T. J. (1994). Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. The Journal of Parasitology, 80(4), 533– 543. https://doi.org/10.2307/3283188

Nijhof, A. M., Balk, J. A., Postigo, M., & 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 Molecular Biology, 10(1), 112. https://doi.org/10.1186/1471-2199-10-112

Oppenheim, S. J., Baker, R. H., Simon, S., & DeSalle, R. (2015). We can't all be supermodels: The value of comparative transcriptomics to the study of non-model insects. Insect Molecular Biology, 24(2), 139– 154. https://doi.org/10.1111/imb.12154;

Perner, J., Provazník, J., Schrenková, J., Urbanová, V., Ribeiro, J. M. C., & Kopáček, P. (2016). RNA-seq analyses of the midgut from blood- and serum-fed *Ixodes ricinus* ticks. Scientific Reports, 6(June), 1– 18. https://doi.org/10.1038/srep36695

Pfaff, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research, 29(9), e45. https://doi.org/10.1093/NAR/29.9.E45

Sonenshine, D. E., & Roe, R. M. (2014). Biology of ticks (2nd ed.). New York, NY: Oxford University Press.

Tukey, J. (1977). Exploratory data analysis. Retrieved from http://theta.edu.pl/wp-content/uploads/2012/10/exploratorydataanalysis_tukey.pdf

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3(7), research0034.1– research0034.11.

Varela-Stokes, A. S., Park, S. H., Stokes, J. V., Gavron, N. A., Lee, S. I., Moraru, G. M., & Ricke, S. C. (2018). Tick microbial communities within enriched extracts of *Amblyomma maculatum*. Ticks and Tick-Borne Diseases, 9(4), 798– 805. https://doi.org/10.1016/j.ttbdis.2018.02.022

Weisheit, S., Villar, M., Tykalová, H., Popara, M., Loecherbach, J., Watson, M., … Bell-Sakyi, L. (2015). *Ixodes scapularis* and *Ixodes ricinus* tick cell lines respond to infection with tick-borne encephalitis virus: Transcriptomic and proteomic analysis. Parasites & Vectors, 8(1), 599. https://doi.org/10.1186/s13071-015-1210-x

Werner, E. R., Blau, N., & Thöny, B. (2011). Tetrahydrobiopterin: Biochemistry and pathophysiology. Biochemical Journal, 438(3), 397– 414. https://doi.org/10.1042/BJ20110293;

Wikel, S. (2018). Ticks and tick-borne infections: Complex ecology, agents, and host interactions. Veterinary Sciences, 5(2), 60. https://doi.org/10.3390/vetsci5020060

Zweygarth, E., Cabezas-Cruz, A., Josemans, A. I., Oosthuizen, M. C., Matjila, P. T., Lis, K., … Passos, L. M. F. (2014). *In vitro* culture and structural differences in the major immunoreactive protein gp36 of geographically distant *Ehrlichia canis* isolates. Ticks and Tick-Borne Diseases, 5(4), 423– 431. https://doi.org/10.1016/j.ttbdis.2014.01.011

Chapter V: General Discussion and Conclusions

Ovine babesiosis is a neglected tick-borne disease that negatively affects livestock and consequently human society. To treat and control this disease, limited options are available, which motivates the scientific community to explore new strategies to overcome this issue. This thesis is driven by this idea.

Building knowledge on the complex molecular interaction between vector, pathogen and host, has been one of the foundations to discover new targets for tick and tick-borne disease control. One of "main arenas" where this intrinsic interaction occurs is in the SGs, underlining the need to focus on the tick sialoverse under specific biological processes such as feeding and parasite infection.

Regarding this, the *R. bursa* sialoverse was scrutinized using next-generation sequencing technologies, such as transcriptomics and proteomics. As a result, several datasets regarding *R. bursa* blood feeding and *Babesia* infection were obtained revealing specific biological mechanisms behind each process. For instance, omics data revealed that blood feeding is a biological process that alone demands a high production of tick salivary molecules, by increasing the gene expression and protein synthesis. This type of biological response also occurs during infection alone by stimulating transcription and translation. However, the combination of both stimuli, of feeding and infection, influenced positively gene expression but negatively translation.

This repertoire of salivary proteins from *R. bursa* emerges as the groundwork to further concentrate into *R. bursa-B. ovis* interactions and apply new methods to identify new targets to control TTBDs. Thus, four different approaches were applied to search for targets that could imperil the intricate tick-pathogen-host interaction. Figure 1 summarizes the approaches used and the targets investigated.

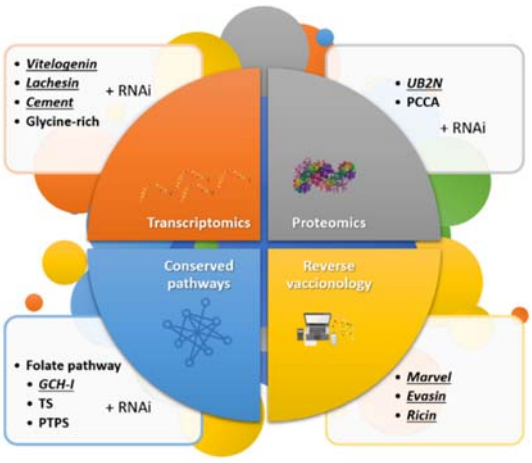

Figure 1. Summary of methodologies and approaches used to find specific targets.

In general, among the sialotranscripts and sialoproteins, certain molecules were selected using different approaches and their functional role in tick fitness and pathogen persistence was addressed through RNAi. This methodology allowed the characterization of the role of putative vitellogenin, lachesin, secreted cement and ubiquitin-related enzyme proteins in tick and parasite biology, but in the cases of the putative glycine-rich, PCCA and GCH-I proteins it failed to produce conclusive results.

The putative **vitellogenin** and secreted **cement** proteins were found to be more related to **vector fitness**, since their gene knockdown influenced negatively tick survival and attachment to the host, respectively. While in the *vitellogenin* gene knockdown experiments led to 77% of the tick mortality after blood feeding, silencing assays of the secreted cement encoding gene contributed to approximately 46 % of the failure in tick attachment to the host and consequently completeness of blood feeding, presenting lower body weights.

Lachesin and **UB2N** were recognised to be implicated in both ***R. bursa*** **and** ***B. ovis*** life cycle in different ways. A "dual effect" of lachesin was observed during RNAi assays since it led to a 70 % decrease on both *R. bursa* tick population and *B. ovis* infection rate. However, in the case of UB2N, the silencing assays suggested the role of this protein as a "double-edged sword" against TTBDs, with potential to jeopardize vector life cycle but facilitate *Babesia* dissemination. Briefly, the knockdown of *ub2n* expression impaired tick life cycle by increasing tick mortality rate (by 40 %), decreasing egg production efficiency (46 %) and hatching rate (89 %), but also increased *Babesia* infection (by 18%).

Furthermore, reverse vaccinology strategy allowed the identification of three promising immunogenic targets. Using immunoinformatic tools, the previous published omics data were explored and proteins predicted to elicit a robust host immune response against both *Rhipicephalus* ticks and *Babesia* hemoparasites were indicated. One membrane-related (**MARVEL**) and two secreted (**EVASIN**, **RICIN**) proteins were selected as putative **immunogenic** proteins containing "immunological kernels" with ideal characteristics for an anti-tick peptide-based vaccine.

Overall, with this research, *R. bursa* sialotranscriptomic and sialoproteomic data during feeding and *Babesia* infection were made available, as a source for the scientific community to scrutinize even further tick-parasite biology. Moreover, the strategies

implemented permitted the identification of several promising targets which can be used in other biological contexts to boost the scientific community to obtain alternatives for TTBDs control.

Nevertheless, "***science begins by asking questions and then seeking answers***"[1]. Tick-pathogen interactions incited the elaboration of the present thesis, and now, with the obtained results and methodology limitations, new questions arise on the path to future discoveries on vector-pathogen interplay and TTBD control.

For instance:

- Can the obtained omic data be refined and further explored?

The *R. bursa* sialotranscriptome obtained here is based on a paired-end sequencing methodology[2] of coding polyadenylated mRNA sequences, which allowed to generate high-quality and alignable sequence data. From this data, it is also possible to explore tick alternative splicing events by discovering isoforms, reflecting the sheer diversity of tick proteins[3]. As for the proteomics, by using a RP-LC-MS/MS followed by a SWATH/DIA analysis, it was possible to identify and quantify several proteins in a complex mixture of proteins with high reproductibility and sensivity. Since the acquisition is data independent, the files can be mapped again on updated databases as tick research continues.

From these big omic data, more information can be extracted. For example, transcripts and proteins from *Babesia* can be selected to better understand the complexity of the tick-pathogen interplay. Moreover, sequences from endosymbionts can elucidate about the enrollment of these organisms on tick biology and *Babesia* infection.

- Could new sequencing data improve the knowledge on tick-pathogen interactions?

Once explored the coding RNA using RNA-seq, it will be interesting to investigate non-coding RNAs (such as transfer RNAs, ribosomal RNAs and small RNAs such as microRNAs, siRNAs) in the *R. bursa-B. ovis* interplay. Other techniques that enrich RNAs based on their size or localization in the cell must be carried out to obtain

information about non-coding RNA[4] and elucidate about their role on tick-pathogen interactions[3].

Additionally, specific PTMs that affect profoundly protein function can be confirmed by proteomics. However precise methods such as phosphoproteomics and glycoproteomics would be more suitable to study in detail those alterations[3], since mapping of PTMs in proteomics is a challenging task. Basically, most PTMs are of low abundance and some are labile (readily open to change) during MS and MS/MS. For these reasons, it is often useful to consider several approaches for enrichment of those modified proteins[5,6].

Besides transcriptomics and proteomics, other "omic" approaches could be implemented to expand and improve our understanding of tick genetic background, tissue-specific and temporal expression patterns, different half-life's and concentration levels of different molecules. Nonetheless, the sequencing and characterization of *R. bursa* genome should be prioritized, since it will allow an accurate assembly and annotation of omics data in general[7]. Such information will uncover the remaining information about gene annotation and evolutionary events such as gene duplication[3,8,9]. Also, it will increase knowledge on the *R. bursa* unique parasitic pathways and advance technologies that depend on genomic data (such as RNAi, CRISPR, genome editing).

Overall, it will be important to study the complexity of the tick biological processes of blood feeding and *Babesia* infection holistically, by combining multi-omics data such as epigenetics, genomics, structural genomics, transcriptomics, proteomics, metabolomics, interactomics of *R. bursa* in different tick tissues and life stages. Ideally, all of this should also be applied to *B. ovis* and even to vertebrate hosts to obtain "the big picture" of this intricate interaction[10]. This will highlight the dynamics of molecular parasitic pathways of *R. bursa* ticks and *B. ovis* pathogen and their interaction with the host. Nevertheless, there is a need for investment on artificial intelligence, specifically machine learning algorithms, for interpretation of "big"-sized complex data in TTBD research.

- <u>Are we missing some bioactive tick saliva molecules?</u>

The sialome of tick is a complex cocktail of biomolecules, some of which, with no functional annotation assigned. Such molecules denominated as "unknown unknowns"

or "orphan genes" are responsible for the unique tick salivary repertoire[9], since their sequences are conserved between tick species and not homologous to mammalians. Those characteristics are appealing for anti-tick vaccine development since such type of antigens could induce a specific immunological response against several tick species without cross-reacting with host mammalian proteins[11]. However, due to the lack of information, those molecules pose a challenge in designing and orientating follow-up functional assays, being left behind. Knowing their sequence and ultimately their structure would facilitate the identification of active sites and putative domains, shedding a light on their putative function. Nevertheless, technologies focusing on those targets must be conducted to search their role based on their domains and structural characteristics. Through functional characterization, new promising protective antigens against TTBDs may arise.

Besides, nucleic acids and proteins, other biomolecules, such lipids and carbohydrates should be considered as targets to explore tick-pathogen interplay and ultimately develop a method to control TTBDs. For instance, pathogens have been shown to scavenge and manipulate host lipids for structural support, metabolism, replication and immune evasion, in order to complete their life cycles in the hosts[12,13]. As described for *Plasmodium*[12], *B. ovis* can also make use of the tick's lipids to survive and propagate. Moreover, tick lipids are components of the cement cone which assists blood feeding on the vertebrate host. Such involvement on tick-pathogen interplay can be targeted to control TTBDs. Additionally, the glycobiology behind the *R. bursa-B. ovis* as well as the tick-host interface should also be evaluated, since carbohydrates and glycans are described to be involved in tick-host-pathogen interactions[14,15] as in other VBD contexts[16,17].

- <u>How to overcome the limitations found in tick-pathogen research?</u>

One of the most important milestones achieved during the execution of these studies was the establishment of *R. bursa* colonies (uninfected and *B. ovis* infected). Ticks are more challenging to work within a laboratory setting than other arthropod vectors (such as mosquitoes), requiring animals such as rabbits and sheep to acquire several and extended blood meals to complete their long-life cycle[18]. An artificial feeding system exclusively defined for the two-host tick *R. bursa* could significantly improve research on *R. bursa* biology as well as its interactions with TBPs. Moreover, attaining a well-

established *R. bursa* tick cell line as well as its infection with previously well stablished *B. ovis* parasite cell culture could facilitate studies towards tick-pathogen interactions.

Regarding the targets selected in this study, their role on *R. bursa* tick biology and *B. ovis* infection is homology-based, *i.e.* putative, and for that reason additional studies are needed to better understand their complete biological function. For instance, even knowing that the putative biological function of cement-like and glycine-rich proteins is related to tick attachment, few studies comprehend their specific role on the triad tick-pathogen-host in detail[19]. This is due to their extensive expression and sequence diversity which hinder protein functional assessment and depreciate their use in TTBD control methods[19]. Therefore, additional research that allows the identification of unique residues of each protein family, such as phylogenetics and crystallography, will allow the assessment the molecular differences of each protein. In contrast, to potentiate the use of those molecules in TTBD control, new experiments focusing on targeting common features are pivotal to maximize the impact on those differentiated protein families.

Targeting conserved proteins or even conserved pathways is an attractive methodology to enlarge the impact on *R. bursa-B. ovis* interplay to several ticks and consequently numerous TBDs[9]. However, several other pathways must be considered during experiment designing. For example, following this strategy, a folate biosynthesis pathway was identified across infected *Rhipicephalus* ticks and RNAi assays were conducted to assess the role of a conserved folate-related enzyme (GCH-I). Still, no biological differences were observed in the tick cells or pathogen behaviour of invasion or multiplication during silencing assays, revealing that such pathway is complex and mechanisms of compensation may occur. Therefore, in this case, hologenomics might be a starting point to explore it, since it will build knowledge on the capacity of either ticks, pathogens or even tick microbiome to possess the salvage pathways that could supply the folate pathway essential product, BH4. Moreover, it is necessary to verify if a possible mechanism of transport of BH4 pools through transmembrane proteins may occur. Then, after considering all this data, inhibition assays should be conducted to fully understand the role of the folate biosynthesis pathway in tick-pathogen relationship.

Protein structure and its distribution inside tick tissues must be investigated to clarify the cellular interactions within the tick and with the pathogen. Therefore, protein crystallography, *in situ* immunofluorescence and scanning confocal electron microscopy

should be conducted. For instance, in the case of Vg and CP proteins, which share many features, such methodologies should be employed to distinguish their functions and localizations. The same approach can also be applied to lachesin and UB2N in order to evaluate their interactions in *Babesia* invasion in different tick tissue cells.

Inevitably, once the functional role of those targets is fully comprehended, their immunogenicity must be evaluated *in silico* and *in vivo* to validate their potential as protective antigens and understand the relevance of those promising targets on the complex triad vector-pathogen-host interaction and consequently TTBD control. The immunogenicity could be predicted *in silico* in the same way performed for MARVEL, EVASIN and RICIN, and then, proven by immunization assays, by analysing humoral and cellular immune response to specific targets (ELISA, flow cytometry). After that, the impact of this immunological response on the vector and pathogen could be addressed by exposing the immunized host the vector and the pathogen. Nonetheless, vaccine design must be thoroughly reviewed[10,20,21] in order to obtain an efficacious, safe, cost-effective, easy-to-use, stable and reproducicle specially in field conditions.

Studies focusing on DNA and RNA-based vaccines should be conducted to evaluate their potential to control TTBDs, as well as alternative vaccine delivery systems such as immune-stimulating complexes, liposomes and nanoparticles[20].

- Which other technologies could be used to update the tick-pathogen research?

Every tick bite represents a complexity of molecular events within the host, pathogen and tick that must be studied in order to control it. The host immunological response to *R. bursa* tick bite and *B. ovis* infections can be dissected through single-cell sequencing and flow cytometry, to better understand the mechanisms behind disease pathogenesis and tick meddling. Antigen fingerprinting should also be considered[22,23], in order to recognize which tick/pathogen-specific proteins confer an immunological response in the host. This should be performed in rabbits and sheep (the natural hosts).

Gene manipulation technologies (CRISPR, cell transfection, gene knockout, gene induction) need to be implemented in parasite and tick cell culture[24–26], to better understand the dynamics and regulation of invasion and evasion processes of *B. ovis* and functional role of tick proteins.

Protein-protein or DNA-protein interactions must be also explored using pull down and chromatin immunoprecipitation assays, or even yeast two-hybrid screening[27] (split-ubiquitin yeast two hybrid assay for membrane proteins[28]) and affinity purification coupled to mass spectrometry. Additionally, the *B. ovis* and the *R. bursa* salivary exosomes can be explored to give more insight on the tick-pathogen-host interactions[3,29]. Based on the results obtained from the ubiquitin-related protein, it would be valuable to explore the tick ubiquitinome during blood feeding and *Babesia* infection, in order to understand which proteins are being marked for degradation (*via* the proteasome) and to promote or prevent protein interactions, in order to pinpoint more promising targetable tick proteins.

Only after deep basic research, the scientific community should promote translational research in order to apply efficient TTBD control measures and perform clinical research.

References

1. Vale, R. D. The value of asking questions. Mol. Biol. Cell 24, 680–682 (2013).

2. Wang, Z., Gerstein, M. & Snyder, M. RNA-Seq: A revolutionary tool for transcriptomics. Nat. Rev. Genet. 10, 57–63 (2009).

3. Mans, B. J. Quantitative Visions of Reality at the Tick-Host Interface: Biochemistry, Genomics, Proteomics, and Transcriptomics as Measures of Complete Inventories of the Tick Sialoverse. Front. Cell. Infect. Microbiol. 10, 574405 (2020).

4. Boivin, V. et al. Simultaneous sequencing of coding and noncoding RNA reveals a human transcriptome dominated by a small number of highly expressed noncoding genes. RNA 24, 950–965 (2018).

5. Larsen, M. R., Trelle, M. B., Thingholm, T. E. & Jensen, O. N. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. Biotechniques 40, 790–798 (2006).

6. Pieroni, L. et al. Enrichments of post-translational modifications in proteomic studies. J. Sep. Sci. 43, 313–336 (2020).

7. Ribeiro, J. M. C. & Mans, B. J. TickSialoFam (TSFam): A Database That Helps to Classify Tick Salivary Proteins, a Review on Tick Salivary Protein Function and Evolution, With Considerations on the Tick Sialome Switching Phenomenon. Front. Cell. Infect. Microbiol. 10, 374 (2020).

8. de la Fuente, J., Kopáček, P., Lew-Tabor, A. & Maritz-Olivier, C. Strategies for new and improved vaccines against ticks and tick-borne diseases. Parasite Immunol. 38, 754–769 (2016).

9. Nuttall, P. A. Wonders of tick saliva. Ticks Tick. Borne. Dis. 10, 470–481 (2019).

10. Almazan, C., Tipacamu, G. A., Rodriguez, S., Mosqueda, J. & Perez de Leon, A. Immunological control of ticks and tick-borne diseases that impact cattle health and production. Front. Biosci. (Landmark Ed) 23, 1535–1551 (2018).

11. Mulenga, A., Kim, T. K. & Ibelli, A. M. G. Deorphanization and target validation of cross-tick species conserved novel *Amblyomma americanum* tick saliva protein. Int. J. Parasitol. 43, 439–451 (2013).

12. O'neal, A. J., Butler, L. R., Rolandelli, A., Gilk, S. D. & Pedra, J. H. F. Lipid hijacking: A unifying theme in vector-borne diseases. Elife 9, 1–31 (2020).

13. Chotiwan, N. et al. Dynamic remodeling of lipids coincides with dengue virus replication in the midgut of *Aedes aegypti* mosquitoes. PLoS Pathog. 14, e1006853 (2018).

14. Vechtova, P. et al. A bite so sweet: The glycobiology interface of tick-host-pathogen interactions 11 Medical and Health Sciences 1108 Medical Microbiology. Parasites and Vectors 11, 1–27 (2018).

15. Cabezas-Cruz, A., Alberdi, P., Valdés, J. J., Villar, M. & de la Fuente, J. *Anaplasma phagocytophilum* Infection Subverts Carbohydrate Metabolic Pathways in the Tick Vector, *Ixodes scapularis*. Front. Cell. Infect. Microbiol. 7, 23 (2017).

16. Kojin, B. B. & Adelman, Z. N. The sporozoite's journey through the mosquito: A critical examination of host and parasite factors required for salivary gland invasion. Front. Ecol. Evol. 7, 284 (2019).

17. Basseri, H. R. et al. Competency of *Anopheles stephensi* mysorensis strain for *Plasmodium vivax* and the role of inhibitory carbohydrates to block its sporogonic cycle. Malar. J. 7, 1–8 (2008).

18. Hart, C. E. & Thangamani, S. Tick-virus interactions: Current understanding and future perspectives. Parasite Immunol. e12815 (2021) doi:10.1111/pim.12815.

19. Maruyama, S. R. et al. The expression of genes coding for distinct types of glycine-rich proteins varies according to the biology of three metastriate ticks, *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus sanguineus* and *Amblyomma cajennense*. BMC Genomics 11, 363 (2010).

20. Ndawula, C. & Tabor, A. E. Cocktail anti-tick vaccines: The unforeseen constraints and approaches toward enhanced efficacies. Vaccines 8, 1–23 (2020).

21. Kennedy, R. B., Ovsyannikova, I. G., Palese, P. & Poland, G. A. Current Challenges in Vaccinology. Front. Immunol. 11, 1181 (2020).

22. Garcia, G. R. et al. Immune recognition of salivary proteins from the cattle tick *Rhipicephalus microplus* differs according to the genotype of the bovine host. Parasites and Vectors 10, 1–18 (2017).

23. Prudencio, C. R., Rodrigues, A. A. R., Cardoso, R., Szabó, M. P. J. & Goulart, L. R. Antigen fingerprinting of polyclonal antibodies raised in immunized chickens with tick

total proteins: A reservoir for the discovery of novel antigens. J. Biomol. Screen. 16, 1027–1036 (2011).

24. de la Fuente, J. Controlling ticks and tick-borne diseases…looking forward. Ticks and Tick-borne Diseases vol. 9 1354–1357 (2018).

25. Al-Rofaai, A. & Bell-Sakyi, L. Tick Cell Lines in Research on Tick Control. Front. Physiol. 11, 152 (2020).

26. Rosa, C. et al. Transient transfection of *Babesia ovis* using heterologous promoters. Ticks Tick. Borne. Dis. 10, 101279 (2019).

27. Rana, V. S. et al. *Aedes aegypti* lachesin protein binds to the domain III of envelop protein of Dengue virus-2 and inhibits viral replication. Cell. Microbiol. 22, e13200 (2020).

28. Chen, J. et al. Uncovering *Arabidopsis* Membrane Protein Interactome Enriched in Transporters Using Mating-Based Split Ubiquitin Assays and Classification Models. Front. Plant Sci. 3, 124 (2012).

29. Zhou, W. et al. Discovery of Exosomes From Tick Saliva and Salivary Glands Reveals Therapeutic Roles for CXCL12 and IL-8 in Wound Healing at the Tick–Human Skin Interface. Front. Cell Dev. Biol. 8, 554 (2020).